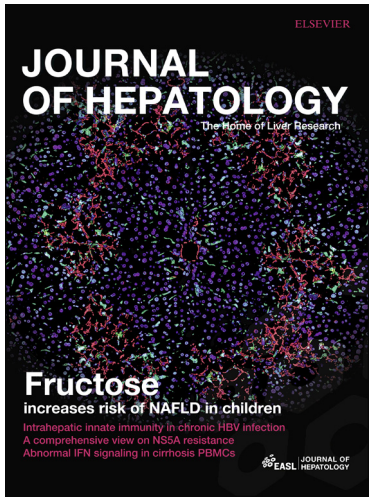

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Efficacy of annexin A3 blockade in sensitizing hepatocellular carcinoma to sorafenib and regorafenib

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and K.M. consented the patients, provided the clinical samples and patient information. J.D. provided HCC tissue microarray and patient information. T.K.L. provided critical scientific input, sorafenib resistance profiling data, samples and reagents. M.T. and S.M. wrote the manuscript. S.M. analyzed and interpreted the data, supervised the project and provided funding for this study.

ABSTRACT

Background & Aims: Advanced hepatocellular carcinoma (HCC) is a lethal malignancy with limited treatment options. Sorafenib is the only FDA approved first-line targeted drug for advanced HCC, but its effect on patients' survival gain is limited. Further, patients ultimately present disease progression. A better understanding of causes of sorafenib resistance, enhancing the efficacy of sorafenib and finding a reliable predictive biomarker are crucial to achieve efficient control of HCC.

Methods: Functional effects of ANXA3 in conferring sorafenib resistance in HCC cells were analyzed in apoptotic and tumorigenicity assays. Role of ANXA3/PKCδ-mediated p38 signaling thus subsequently altering autophagic and apoptotic events was assessed by immunoprecipitation, immunoblotting, immunofluorescence and transmission electron microscopy assays. Prognostic value of ANXA3 in predicting response to sorafenib was evaluated by immunohistochemistry. Therapeutic value of targeting ANXA3 to combat HCC with anti-ANXA3 monoclonal antibody (mAb) alone or in combination with sorafenib/regorafenib, were investigated *ex vivo* and *in vivo*.

Results: ANXA3 conferred ability of HCC cells to resist sorafenib. ANXA3 was found enriched in sorafenib-resistant HCC cells and patient-derived xenografts. Mechanistically, overexpression of ANXA3 in sorafenib-resistant HCC cells suppressed PKCδ/p38 associated apoptosis and activated autophagy for cell survival. Clinically, ANXA3 expression correlated positively with the autophagic marker LC3B in HCC and was associated with a worst overall survival in patients who went on to receive sorafenib treatment. Anti-ANXA3 mAb therapy combined with sorafenib/regorafenib impaired tumor growth *in vivo* and significantly increased survival.

Conclusion: Anti-ANXA3 therapy in combination with sorafenib/regorafenib represents a novel therapeutic strategy for HCC treatment. ANXA3 represents a useful predictive biomarker to stratify HCC patients for sorafenib treatment.

LAY SUMMARY

This study represents the most extensive pre-clinical characterization of anti-ANXA3 mAb for HCC treatment to date and supports its clinical trial development in combination with sorafenib/regorafenib. Further studies will optimize patient target selection as well as the best treatment combinations.

HIGHLIGHTS

- ANXA3 confers ability of HCC cells to resist sorafenib
- ANXA3 is enriched in sorafenib-resistant HCC
- ANXA3 activates autophagy and attenuates PKCδ/p38 dependent apoptosis
- ANXA3 is a useful predictive biomarker to stratify patients for sorafenib treatment
- Anti-ANXA3 therapy with sorafenib/regorafenib as a new treatment strategy for HCC

INTRODUCTION

Hepatocellular carcinoma (HCC) is a very aggressive disease and represents the third leading cause of cancer-related mortality worldwide (1). Although treatment of HCC has greatly improved over the last decade, most HCC patients are presented with an underlying chronic liver disease and diagnosed only at advanced stages, when they are no longer eligible for curative therapies such as liver resection or transplantation (2). In these cases, the multi-target tyrosine kinase inhibitor sorafenib is the only FDA-approved first-line systematic therapy, expanding patient median survival from 7.9 to 10.7 months (3-5). Despite initial response, only rarely do sorafenib-treated tumors regress completely, and the therapeutic effects of the drug are often temporary. Unfortunately, most patients develop disease progression, and in the case of HCC, radiological progression under sorafenib occurs after 4-5 months of treatment (4). Regorafenib has recently been approved by the FDA as a second-line treatment for advanced HCC patients, where regorafenib has provided some survival benefit in HCC patients progressing on sorafenib treatment (6). As sorafenib targets several signaling pathways, acquisition of resistance might involve different mechanisms, including the activation of compensatory signaling cascades, rather than specific DNA aberrations, as was described with BCR-ABL and imatinib (7) and BRAF mutations in melanoma resistant to vemurafenib (8). Further, a targeted anti-cancer drug should best have a biomarker to predict its clinical response. Single kinase inhibitors, such as tarceva (EGFR inhibitor) or crizotinib (ALK inhibitor), have as predictive markers EGFR mutation and ALK translocation, respectively (9-10). However, such markers are still not available for sorafenib because it targets multiple kinases, complicating the mechanism of action. As the precise molecular mechanisms underlying resistance to sorafenib are still barely understood (11), there is an urgent need to characterize drivers of resistance to identify new biomarkers that can predict sorafenib treatment outcome and therapeutic strategy that can improve the efficacy of sorafenib.

Our group has previously reported on the critical role of endogenous and secretory annexin A3 (ANXA3) in promoting aggressive cancer and stem cell-like properties in HCC (12). Clinically, ANXA3 expression in HCC patient sera and tissues closely associated with aggressive clinical features and when used either alone or in combination with alpha-fetoprotein (AFP), was found to represent a more specific and sensitive biomarker for HCC detection as compared to AFP alone (12). Consistent with our findings, ANXA3 was also reported to be overexpressed in 5-fluorouracil resistant HCC cells

(13), to play a role in promoting resistance to chemotherapy in HCC (14), as well as a possible target for immunotherapy of liver cancer stem-like cells (15). In light of these interesting findings, our group has subsequently went on to develop a monoclonal antibody specific against ANXA3 (anti-ANXA3 mAb) and showed *in vivo* that the use of this antibody alone or in combination with cisplatin could efficiently lead to a reduced ability of HCC cells to initiate tumor growth and self-renewal, concomitant with a decrease in liver cancer stem cell proportions including those of CD133, CD24 and EpCAM (12). A number of reports have revealed that $EpCAM^{+}$ and label-retaining HCC cells to be more resistant to sorafenib treatment (16-17); and that sorafenib-resistant HCC cells display enhanced cancer stemness features (18-19). These results, together with our previous findings where a link between ANXA3 and cancer stem cells is demonstrated, led us to hypothesize whether ANXA3 may also contribute to sorafenib resistance in HCC.

Herein, we identified a previously unappreciated role of ANXA3 in regulating sorafenib resistance in HCC. The presence of ANXA3 conferred the ability of HCC cells to resist sorafenib; while ANXA3 expression was also found enriched in sorafenib-resistant HCC cells and patient-derived xenografts. Clinically, high ANXA3 levels in human HCCs predicted a worst response of patients to sorafenib treatment, suggesting that ANXA3 may serve as a biomarker for HCC personalized therapy. Mechanistic studies revealed the involvement of ANXA3 mediated activation of autophagy and attenuation of PKCδ/p38 dependent apoptotic signaling to contribute to the development of this resistance in HCC. We further established that anti-ANXA3 mAb behaved as efficiently as sorafenib and that the combinatorial treatment of sorafenib and anti-ANXA3 mAb shows a significant effect in inhibiting tumor growth in both organotypic *ex vivo* human HCC samples as well as HCC patient-derived xenografts *in vivo*. In addition, using sorafenib-resistant HCC xenografts as a model system, we also demonstrated anti-ANXA3 mAb combined with sorafenib, impaired tumor growth *in vivo* and significantly increased survival, with inhibitory effects further enhanced upon treatment with a combination of anti-ANXA3 mAb and regorafenib. These observations was further confirmed in an immune-competent genetically engineered mouse model of liver cancer. Collectively, we found ANXA3 inhibition to sensitize HCC cells to sorafenib/regorafenib treatment via suppressing autophagy and activating apoptosis. Our study suggests ANXA3 to not only represent a useful predictive biomarker to stratify HCC patients for sorafenib treatment, but also provides pre-clinical evidence

supporting clinical trials to evaluate the potential of anti-ANXA3 mAb in combination with sorafenib and/or regorafenib for HCC treatment.

MATERIALS AND METHODS

Cell lines and organoid cultures. HepG2 was purchased from American Type Culture Collection (ATCC). PLC8024 was obtained from Institute of Virology of the Chinese Academy of Sciences, Beijing, China. Huh7 was provided by H. Nakabayashi, Hokkaido University School of Medicine, Japan. Thyroid cancer cell line K1 was obtained from European Collection of Authenticated Cell Cultures (ECACC). 293T was purchased from ATCC and 293FT was purchased from Invitrogen. Cell lines used in this study were regularly authenticated by morphological observation and tested for absence of mycoplasma contamination. For *ex vivo* organoid culture, HCC tissues were obtained from patients undergoing hepatectomy at Queen Mary Hospital, Hong Kong. Samples were collected from patients who had not received any previous local or systemic treatment prior to operation. Informed consent was obtained from all patients before the collection of liver specimens and the study was approved by the Ethics Committee of the University of Hong Kong. For organoid cultures, cells were isolated and cultured according to published protocol (20). Sorafenib-resistant clones were established by subjecting HepG2 and Huh7 cells to continuous administration of gradually increasing sorafenib concentrations and trained up to 5.5 μM sorafenib.

Animal experiments. All study protocols were approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at The University of Hong Kong and the Animals (Control of Experiments) Ordinance of Hong Kong. For subcutaneous tumor models, $1x10^6$ HCC cell lines (HepG2 or Huh7), sorafenib-resistant HepG2 cells or thyroid cancer cell line (K1) were resuspended in 100μL PBS and injected subcutaneously into either flank of 4-5 week old male BALB/C nude mice. Tumor volumes were measured using an electronic caliper and calculated using the following formula: volume (cm^3) = L x W^2 x 0.5 with L and W representing the largest and smallest diameters, respectively. Drug administration began when the tumors reached 5mm X 5mm (L X W) in size, at which point mice were randomized for treatment with vehicle (DMSO), IgG antibody (200 μg/2 days, *i.p.*), ANXA3 antibody (200 μg/2 days, *i.p.*), sorafenib (30 mg/kg/day, *p.o.*) or regorafenib (10 mg/kg/day, *p.o.*) (12, 19, 21). For the patient-derived tumor xenograft model, $5x10^5$ cells from an HCC patient (PDX#1) were resuspended in 100% Matrigel and injected into the left lobes of the livers in 6-7 week old male BALB/C nude mice. Drug administration began 3 weeks after inoculation. At 7 weeks post-inoculation, tumor-bearing mice were sacrificed. Livers were harvested for histological

analysis. For hydrodynamic injection model, 6-8 week old male wild-type C57BL/6J mice were used and performed as previously described (22-23). In brief, 10µg of plasmids encoding human AKT1 (myristylated AKT1 or myr-AKT1) and human neuroblastoma Ras viral oncogene homolog (N-RasV12) along with sleeping beauty transposase in a ratio of 25:1 were diluted in 2mL saline (0.9% NaCl), filtered through 0.22µm filter and injected into the lateral tail vein of C57BL/6J mice in 5-7 seconds. The constructs used in this study showed long term expression of genes via hydrodynamic injection (23). C57BL/6J mice received the same dosages of treatment as nude mice.

RESULTS

ANXA3 confers sorafenib resistance in HCC and its expression is associated with patients' response to sorafenib treatment. Our hypothesis for a link between ANXA3 and sorafenib resistance in HCC was initially confirmed when we observed HCC cells with ANXA3 suppressed to display an attenuated ability to resist sorafenib in PLC8024 cells; while overexpression of ANXA3 in HepG2 cells would potentiate its resistance (**Fig. 1A-B**). To study acquired resistance to sorafenib, we then generated *in vitro* sorafenib-resistant HCC cells HepG2 and Huh7. Establishment of these sorafenib-resistant clones was accomplished by subjecting the cells to continuous administration of gradually increasing sorafenib concentration over 6 months. Both HepG2 and Huh7 cells were trained to up to 5.5 μM sorafenib; and upon constant incubation with sorafenib at this concentration for 6 months, we successfully established sorafenib-resistant cells derived from HepG2 and Huh7 cells. By phase-contrast microscopy, sorafenib-resistant cells exhibited morphological changes that resulted in elongated spindle shape, as compared to the rounded appearance in mock control cells (**Fig. S1A and S2A**). By Annexin V apoptosis assay, sorafenib-resistant cells also displayed a significantly greater ability to resist sorafenib-induced cell death (**Fig. S1B and S2B**) and initiate tumor formation *in vivo* (**Fig. S1C**). Importantly, we also observed a marked elevation of both genomic and proteomic expression of ANXA3 in sorafenib-resistant HepG2 and Huh7 cells (**Fig. 1C-D and S2C**), as well as in sorafenib-resistant patient-derived HCC xenografts (PDX#1 and PDX#2) (**Fig. 1E**), that was previously established by our group (19). Considering the functional importance of ANXA3 in conferring resistance to sorafenib treatment in HCC, we further investigated the significance of ANXA3 in patient response to sorafenib. HCC patients with low ANXA3 levels showed a significantly better prognosis after sorafenib treatment as compared to patients with high ANXA3 expression ($p<0.05$; 16.25 versus 10 months) (**Fig. 1F and S1D**), suggesting that ANXA3 can serve as a biomarker for patient response/stratification, which is worthy of extended clinical investigation. To further substantiate the role of ANXA3 in mediating sorafenib resistance in HCC, we also knocked down ANXA3 expression in sorafenib-resistant HepG2 and sorafenib-resistant Huh7 cells and showed that this was able to revert the functional abilities of sorafenib-resistant cells to resist sorafenib-induced cell death (**Fig. 1G and S2D**) and initiate tumors *in vivo* (**Fig. 1H and S2E**).

ANXA3 regulates sorafenib resistance in HCC through p38 MAPK mediated autophagic aberrations. In hope to determine the major downstream mediator of ANXA3, we then performed transcriptome sequencing analysis to compare the gene expression profiles of sorafenib-sensitive HepG2, sorafenib-resistant HepG2 and sorafenib-resistant HepG2 cells with or without ANXA3 stably repressed (shNTC vs. shANXA3). Using a fold-change cut-off of >1.5, 398 genes were found to be commonly de-regulated between sorafenib-sensitive/sorafenib-resistant with shANXA3 (i.e. ANXA3 low) versus sorafenib-resistant/sorafenib-resistant with shNTC (i.e. ANXA3 high) (**Fig. 2A**). Analysis by DAVID and PANTHER identified commonly enriched transcription factors that could be universally regulated by the p38 MAPK signaling pathway (**Fig. 2B**). Western blot analysis confirmed inhibition of p-p38 in sorafenib-resistant HepG2 cells with expression that can be rescued upon ANXA3 suppression, concomitant with rescued expression of cleaved caspase 3 and cleaved PARP, suggesting sorafenib-resistant cells to promote survival via ANXA3-mediated attenuation of p38 MAPK/caspase-dependent apoptosis (**Fig. 2C**). Previous studies unraveled that sorafenib alone or in combination with other drugs could induce autophagy in HCC, but the cellular response to drug treatment and the cell fate could be diverse (24-27). We further examined on the effects of ANXA3 signaling on sorafenib-mediated autophagy. Interestingly, sorafenib treatment in sorafenib-resistant HepG2 cells significantly induced LC3B-II accumulation (**Fig. 2D**) and led to a marked increase in the numbers of LC3 punctae per cell (**Fig. 2E**). Transmission electron microscopy further confirmed the abundant presence of vesicular structures which morphologically resemble the characteristics of autophagosomes and autolysosomes in sorafenib treated sorafenib-resistance HepG2 cells (**Fig. S3A**). Consistently, knockdown of ANXA3 in these sorafenib-resistant HepG2 fully reversed these autophagic phenotypes (**Fig. 2D-E and Fig. S3A**).

Next, to confirm that the apoptotic and autophagic properties caused by ANXA3 was due to altered p38 MAPK signaling, similar assays were then carried out in the absence or presence of a specific p38 inhibitor SB202190. In the presence of sorafenib, addition of SB202190 in sorafenib-resistant HepG2 cells (25 μg/ml) with ANXA3 suppressed exhibited rescued abilities to attenuate apoptosis and induce autophagy, as evidenced by immunoblotting for cleaved caspase 3, cleaved PARP and LC3B-II expression, immunofluorescence for LC3B punctae and transmission electron microscopy of autophagic vesicles (**Fig. 2F-G and Fig. S3B**). Interestingly, we also observed an increase in ANXA3

expression in ANXA3 depleted cells upon administration of SB202190, suggesting a potential reciprocal regulation between p38 MAPK signaling and ANXA3 (**Fig. 2F and Fig. S3F-G**). A concomitant increase of ANXA3 expression and increased resistance to sorafenib treatment were also observed in sorafenib-sensitive HepG2 cells upon p38 blockade (**Fig. S3D-E**), further suggesting the role of p38 in mediating ANXA3-driven sorafenib resistance. Consistently, we also found high LC3B expression to be closely associated with high ANXA3 expression in HCC clinical samples, providing evidence that this phenomenon is also frequently observed in a clinical setting (n=97; ***p<0.001) (**Fig. 2H**).

ANXA3 binds and interacts with PKCδ which acts as an upstream regulator of p38 MAPK mediated apoptotic and autophagic events in HCC. Members of the annexin family have been shown to act as scaffolds and interact with the protein kinase C (PKC) family of serine/threonine protein kinases to modulate their localization and activity (28). Among the PKC isozymes, PKCdelta (PKCδ) is primarily known to be pro-apoptotic and was reported to induce apoptosis by regulating p38 MAPK signaling (29-30). In light of the intrinsic property of annexin family to interact with PKC and the role of PKCδ/p38 MAPK signaling in mediating apoptosis, we hypothesized that ANXA3 could interact with PKCδ which in turn regulates p38 MAPK/caspase-dependent apoptosis. The interaction and co-localization of ANXA3 and PKCδ were confirmed by immunoprecipitation and dual-color immunofluorescence assays (**Fig. 3A-B**). Results of ANXA3 pull-down assay in sorafenib resistant HepG2 cells showed that PKCδ could be co-immunoprecipitated, suggesting ANXA3 to be a potential binding protein of PKCδ (**Fig. 3A**). Notably, immunofluorescence images showed a distinct localization of PKCδ proteins in sorafenib-resistant HepG2 cells compared with dispersed cytoplasmic localization in sorafenib-sensitive cells (**Fig. 3B**). To substantiate the role of ANXA3 in regulating the localization and activation of PKCδ, cellular sub-fractionation was performed to evaluate the distribution of total PKCδ and its active tyrosine-phosphorylation forms. In sorafenib-resistant cells, PKCδ is mainly localized in the cytosol where ANXA3 is highly expressed. In sorafenib-sensitive cells and sorafenib-resistant cells with ANXA3 stably suppressed, PKCδ is primarily localized in the particulate/membrane organelle fractions where it is activated (**Fig. 3C**). Involvement of PKCδ in regulating ANXA3-mediated apoptotic and autophagic properties in sorafenib-resistant cells was further evaluated in sorafenib-resistant HepG2 cells with ANXA3 suppressed in the absence or presence of PKCδ knockdown (shNTC vs. shPKCδ clones 888 and 992). Knockdown of PKCδ resulted in a marked decrease in p-p38 and

apoptotic proteins including cleaved caspase 3, cleaved PARP, accompanied by an increase in LC3B-II expression as well as the number of LC3 puncta and autophagic vesicles (**Fig. 3D-E and Fig. S3C**).

Targeting ANXA3 is effective in suppressing tumor growth and sensitizes cells to sorafenib treatment as demonstrated in both organotypic *ex vivo* human HCC clinical samples and HCC patient-derived xenografts in mice. In light of the functional and clinical significance of ANXA3 in mediating sorafenib resistance in HCC, we extended our study to test the effects of anti-ANXA3 mAb alone or in combination with sorafenib in a more physiological setting, in organotypic *ex vivo* culture of HCC tumor tissues, where pathophysiology of the original tumor is better preserved than as compared to cell lines. Note these HCC patient-derived organoids have been thoroughly characterized at both molecular and phenotypic levels, with comparison made against the original tissue samples (see Review Only Supplemental Information). These two HCC organoids were also tested positive for ANXA3 expression by immunohistochemistry (**Fig. 4A**). Treatment efficacy of sorafenib and anti-ANXA3 mAb was evaluated in these *ex vivo* culture of HCC patient-derived organoids treated with either sorafenib or anti-ANXA3 mAb alone or the combination of both. Combination treatment resulted in most significant reduction in tumor growth; where ANXA3 treatment was found to sensitize HCC cells to sorafenib (**Fig. 4B**). This is accompanied by a change in localization of PKCδ, increase in p-p38 expression and decrease in LC3B punctae expression (**Fig. 4C and Fig. S6A**).

Additional analyses were further performed to discern the *in vivo* benefit of their combinatorial effect in HCC. We next evaluated the therapeutic role of targeting anti-ANXA3 alone or in combination with sorafenib in a HCC patient-derived xenograft mouse model (HCC PDX#1). HCC PDX#1 was orthotopically implanted into the livers of immunosuppressive mice and the efficacy of anti-ANXA3 antibody and sorafenib treatment was evaluated. We observed that anti-ANXA3 mAb suppressed tumor volumes similar to the effect of sorafenib. Combined anti-ANXA3 mAb and sorafenib resulted in maximal suppression of tumors, when compared to the control groups or single treatment groups (**Fig. 4D**). Note in this and all our subsequent animal models, the single or combined treatments were well tolerated, with no obvious clinical signs of distress and/or significant changes in body weight. Immunohistochemical staining revealed a marked reduction in ANXA3 and LC3B punctae expression

only in xenografts treated with anti-ANXA3 mAb alone or in combination with sorafenib (**Fig. 4E and Fig. S6B**). To further examine whether sorafenib sensitization was a result of increased apoptosis, we performed TUNEL assay to assess the percentage of apoptotic nuclei present in tumors of the different treatment groups. A higher percentage of apoptotic nuclei was observed in xenografts treated with sorafenib only, anti-ANXA3 mAb only and sorafenib+anti-ANXA3 mAb combination treatment, as compared to vehicle and IgG controls (**Fig. 4E**).

Anti-ANXA3 therapy reverts resistance and enhances the efficacy of sorafenib in sorafenib-acquired resistant HCC xenografts. In addition to patient-derived HCC xenografts, we also examined the effects of anti-ANXA3 mAb alone or in combination with sorafenib in sorafenib-resistant cells and xenografts. Addition of anti-ANXA3 mAb in sorafenib resistant HepG2 and Huh7 cells resulted in enhanced apoptosis, as compared to IgG controls (**Fig. 5A and S2F**). We also extended our studies *in vivo*, where mice harboring sorafenib-resistant HepG2 xenografts were randomly assigned to receive treatment with (i) vehicle+IgG control, (ii) anti-ANXA3 mAb (vehicle+mAb), (iii) continue on sorafenib with IgG (sorafenib+IgG) or (iv) continue on sorafenib with anti-ANXA3 mAb (sorafenib+mAb) combination. Largest tumor development was manifested for mice harboring sorafenib-resistant HepG2 cells treated with vehicle+IgG control. Treatment with sorafenib or anti-ANXA3 mAb alone significantly delayed tumor growth. Combination of sorafenib and anti-ANXA3 mAb was even more effective, with a significant and pronounced reduction in tumor growth and tumor weight. The combination was also significantly more efficacious than sorafenib, the standard of care for HCC patients (**Fig. 5B-C**). No significant treatment-related side effects were observed. In parallel with the *in vivo* efficacy tests, resected xenografts were also harvested for mechanistic analyses to test whether anti-tumor activity correlated with altered autophagic and apoptotic events. Treatment of tumors with anti-ANXA3 mAb, alone or in combination with sorafenib, resulted in a marked decrease in ANXA3 that is accompanied by a similar decrease in LC3B punctae expression but increase in apoptotic cells as evident by TUNEL staining (**Fig. 5D and Fig. S6C**). Transmission electron microscopy analysis further validated a decrease in autophagic vesicles in sorafenib-resistant HCC tumors treated with either anti-ANXA3 mAb alone or in combination with sorafenib (**Fig. 5E**).

Combination of ANXA3 inhibition and regorafenib resulted in maximal tumor suppression in a sorafenib-acquired resistant HCC xenografts. Recently, FDA approved regorafenib as a second-line treatment for advanced HCC patients who progress on sorafenib treatment (6). We compared the anti-tumor effect of regorafenib alone or regorafenib+anti-ANXA3 mAb combination in sorafenib-resistant HepG2 xenografts. Tumor volumes were largest in mice treated with vehicle control only. Treatment with regorafenib alone or in combination with IgG control similarly led to a significant decrease in tumor growth. Combination of regorafenib and anti-ANXA3 mAb was most effective, with no further tumor growth upon treatment initiation. The combination was also significantly more efficacious than regorafenib (**Fig. 6A-B**) or sorafenib+anti-ANXA3 mAb. A marked decrease in ANXA3 that is accompanied by a similar decrease in LC3B punctae expression, autophagosome formation but increase in apoptotic cells was only observed in mice treated with regorafenib+anti-ANXA3 mAb, but not in the other three treatment groups (**Fig. 6C-D and Fig. S6D**).

Inhibition of ANXA3 sensitized HCC cells to both sorafenib and regorafenib treatment in an immune-competent mouse model. In addition to HCC and sorafenib-resistant patient-derived xenografts established in an immune-deficient mouse background, we also investigated the potential of sorafenib, regorafenib, anti-ANXA3 mAb or their combinations for liver cancer treatment in a genetically engineered immune-competent mouse model of HCC (23). Given the frequent activation of both RAS/MAPK and PI3K/AKT/mTOR pathways in almost 50% of HCC patients (31), we performed hydrodynamic tail vein transfection of activation forms of myr-AKT and N-RasV12 proto-oncogenes, which are stably integrated into the genome of hepatocytes following transient expression of sleeping beauty transposase, for HCC induction (**Fig. 7A**). HCC tumor nodules, as evidenced by presence of AFP staining and histological confirmation by a pathologist, started to develop after 2-3 weeks post-injection, at which point vehicle control or sorafenib administration was initiated (**Fig. 7A-B**). Treatment with sorafenib did not result in any survival advantage compared with vehicle control and the mice started to become very weak and die after 2.5 weeks post-treatment of either vehicle or sorafenib (**Fig. 7C**). Therefore, we defined the tumor as sorafenib non-responsive at 2.5 weeks post-treatment of sorafenib. This resembles the clinical situation where sorafenib non-responsive HCC patients progress after sorafenib treatment. At this point, second-line therapy regorafenib was administered in mice bearing sorafenib non-responsive tumors (**Fig. 7B**). Surprisingly, in this model

system, regorafenib treatment did not improve survival of mice nor suppress tumor development (**Fig. 7E**). We further investigated if administration of anti-ANXA3 mAb alone or in combination with sorafenib or regorafenib would improve treatment response (**Fig. 7B**). We found anti-ANXA3 mAb treatment in combination with sorafenib (**Fig. 7D**) or regorafenib (**Fig. 7E**) led to a significantly improved survival as well as a maximal suppression in tumor growth as indicated by liver weight over body weight ratio, indicating that anti-ANXA3 mAb treatment can sensitize sorafenib and regorafenib treatment and be effective against liver tumors *in vivo*. Consistently, reduced ANXA3 and LC3B punctae expression and autophagosome formation, accompanied with increased apoptosis were observed in mice treated with either anti-ANXA3 mAb alone or in combination with sorafenib or regorafenib (**Fig. S4A-B**). In summary, our results revealed that blocking ANXA3 with the neutralizing antibody sensitized and enhanced efficacy of sorafenib and regorafenib treatment. These results provide persuasive evidence that targeting ANXA3 signaling could enhance apoptosis-driven programmed cell death while simultaneously inhibit autophagy-driven cell survival; suggesting an efficient strategy to enhance the anti-tumor efficacy of sorafenib/regorafenib.

CONCLUSIONS

HCC is one of the most lethal malignancies, in part due to the lack of curative treatments. The introduction of molecular targeted therapy has potentiated the efficacy of cancer treatment regimen compared with conventional chemo/radio-therapies. However, the treatment sensitivities and survival benefit vary considerably among patients (32). Sorafenib is the standard of care for patients with advanced HCC, although most patients eventually undergo disease progression (4). Regorafenib is a recently FDA-approved drug used for second-like therapy for advanced HCC patients who progress on sorafenib (6). The limited efficacy of sorafenib is believed to be a result of drug resistance (4-5); as cancer cells are well known to be able to develop ways to compensate for stressful conditions. If we exploit these attributes, it will be possible to increase the vulnerability of cancer to anti-cancer drugs. This prompted us to explore the mechanism by which sorafenib resistance is acquired and to develop biomarkers to determine which patients might have a beneficial or an adverse response to this therapy. To date, the lack of reliable and robust predictive biomarkers of sorafenib sensitivity and treatment response has hindered the development of personalized therapy in HCC. Pre-treatment biomarkers to guide the personalized use of sorafenib are much warranted.

A number of recent studies have revealed that $EpCAM^+$ and label-retaining liver cancer stem-like cells to be more resistant to sorafenib treatment (16-17); and that sorafenib-resistant HCC cells display enhanced cancer stemness features (18-19). Our recent study found ANXA3 to play a crucial role in cancer survival and progression, in particular through contributing to the maintenance of a liver cancer stem cell subset (12). We found ANXA3 to mediate resistance to cisplatin. Clinically, expression of ANXA3 was increased in HCC compared to non-tumor tissue and high ANXA3 expression level in HCC tissue adversely affected clinical outcomes in HCC patients. The results of this past study (12), in combination with previous studies where a link between the presence of cancer stem cells and sorafenib resistance has been suggested, led us to hypothesize that ANXA3 may also exert an important role in resistance to sorafenib. Interestingly, ANXA3 expression was found to be consistently up-regulated in our established sorafenib-resistant HCC cell lines and PDXs, suggesting that sorafenib resistance is driven by the enrichment of ANXA3. Mechanistic studies found ANXA3 to suppress PKCδ/p38-associated apoptosis and activate autophagy for cell survival in sorafenib-resistant HCC cells. This observation is in line with the cancer stemness promoting role of ANXA3 and

reinforce the critical roles of ANXA3 in promoting HCC development and progression. Previous study reported an activation of MAPK14 (p38α)/MEK/ERK signaling in contributing to sorafenib resistance in mouse HCC (33). Contrasting result from a more recent study demonstrated that sorafenib treatment results in attenuation of p38 MAPK activity but a sustained increase in ERK activation in human HCC (34). The roles of p38 MAPK in regulating cell death and survival could be dependent on cellular context and/or its upstream regulators. Here we showed, for the first time, ANXA3 as an upstream regulator in suppressing apoptosis and promoting autophagy responses through PKCδ/p38 MAPK signaling. Our previous finding showed ANXA3 to promote cancer stemness properties through JNK activation (12). JNK and p38 are both important mediators in controlling the balance of cell survival and cell death in response to stressful conditions. JNK and p38 signaling crosstalk under ANXA3/PKCδ regulation could further suggest a holistic view of the control of balance between apoptosis and autophagy in sorafenib resistant HCC. Further clinical investigations also revealed that HCC patients with low ANXA3 expression exhibited superior response to sorafenib treatment following surgical resection, but patients with high ANXA3 levels showed poor response. Although we did not evaluate the ANXA3 levels in serum samples of HCC patients before sorafenib treatment due to the unavailable resources in our affiliated tissue bank, the clinical significance of endogenous ANXA3 for predicting patient prognosis after sorafenib treatment indicates that ANXA3 could potentially serve as serum biomarker for predicting sorafenib sensitivity and treatment response and pave the way for personalized therapy in HCC.

We also provide in this study a comprehensive pre-clinical evaluation of an anti-ANXA3 mAb, in HCC to date, and build the case for its clinical trial development as first- or second-line therapy, potentially in combination with sorafenib and/or regorafenib. We showed that administration of an anti-ANXA3 mAb promoted cell death *in vitro* and suppressed cell proliferation and increased sensitivity to sorafenib treatment in *ex vivo* culture of HCC patient-derived organoids. Thus, targeting ANXA3 with an anti-ANXA3 mAb in combination with sorafenib seems to be a promising novel therapeutic strategy for improving treatment efficacy. In our *in vivo* mouse models of both immune-competent and immune-deficient backgrounds, combination of anti-ANXA3 mAb with sorafenib or regorafenib showed significant tumor suppressive effect. Strikingly, sorafenib and regorafenib treatment did not result in improved survival in our genetically modified mosaic mouse model of HCC. This suggests a

potential limited treatment efficacy of these drugs in a subset of patients carrying aberrant activation of NRAS, AKT and ANXA3 signaling, and further substantiate the therapeutic importance of targeting ANXA3 to achieve sensitization of these drugs to combat HCC.

We noted that our sorafenib treatment in our sorafenib-resistant HepG2 xenografts would result in growth inhibition. We rationalized a number of possibilities for this observation. First, it is possible that the discrepancy of growth inhibition/apoptosis induction observed *in vitro* and *in vivo* upon sorafenib administration may be attributed by the non-cell autonomous effects in the recipient mice including the influence by the microenvironment, like endothelial cells, that can possibly affect the efficacy of sorafenib. Second, the dosages of sorafenib used *in vitro* and *in vivo* are not directly comparable given that the number of cells and the culture condition/mouse *in vivo* environment are different. Third, sorafenib resistant cells are cultured with 5.5μM sorafenib, and although we did observe that these cells do indeed show resistance to sorafenib, they may not be completely resistant.

The experimental study reported herein points to ANXA3 signaling as a relevant activated axis partially responsible of acquired resistance to sorafenib therapy in HCC. Further understanding of all these sorafenib resistance driven mechanisms will allow the design of clinical trial studies using biomarker-based trial enrichment where samples can be obtained before and after treatment. Collecting tissue samples in all HCC clinical trial has emerged as a critical recommendation in the recent Guidelines of Management of HCC (35). This approach would allow us to further understand the mechanisms of sorafenib resistance and also guide trial enrichment and personal/stratified oncotherapeutics. The identification of additive or synergistic partners for anti-ANXA3 in HCC will also allow stronger effects by minimizing undesirable toxicity.

The use of sorafenib in treating cancer patients is not limited to HCC, but is also approved as first-line treatment in primary advanced renal cell carcinoma and more recently in advanced and progressive differentiated thyroid cancer. Disappointingly, the development of sorafenib resistance in most patients who showed partial response to sorafenib treatment could hardly be avoided. As a proof of the therapeutic efficacy of anti-ANXA3 mAb in combating autophagy promoting sorafenib-resistant

tumors in other cancer types, we developed sorafenib-resistant thyroid cancer cells and evaluated the therapeutic potential of targeting ANXA3 to overcome sorafenib resistance (**Fig. S5A**). Our study showed that blockade of ANXA3 with neutralizing antibody alone could achieve similar or slightly better tumor suppressive effect as sorafenib treatment alone; while the combination of both could maximize the treatment response (**Fig. S5B-C**). As in the HCC model system, inhibition of tumor development by either anti-ANXA3 mAb alone or in combination with sorafenib was accompanied by reduced ANXA3 and LC3B punctae expression and autophagosome formation and increased apoptosis (**Fig. S5D-E**). These data proved that ANXA3 driven sorafenib resistance is not limited to the context of HCC, and that targeting ANXA3 with neutralizing antibody could be more widely used in combating different sorafenib-resistant tumors.

In conclusion, ANXA3 is an effective target for overcoming resistance to sorafenib treatment and can also be a predictive marker to predict sorafenib responsiveness and clinical outcomes. Our findings support further clinical evaluation of anti-ANXA3 mAb, in combination with sorafenib/regorafenib, in patients with HCC. Of note, anti-ANXA3 mAb is well tolerated and lacks associated liver toxicity, further reinforcing its potential for liver cancer treatment. ANXA3 signature could be used as a predictor of resistance to sorafenib. The assessment of additional response predictors in these clinical trials may help to refine the patient subgroup most likely to benefit from treatment with anti-ANXA3 and sorafenib, in hope to achieve the most optimal risk-to-benefit ratio and maximize the treatment efficacy for each patient.

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FIGURE LEGENDS

Fig. 1. ANXA3 confers sorafenib resistance in HCC and its expression is associated with patients' response to sorafenib treatment. Representative flow cytometry analysis of Annexin V-PI staining (top) and quantification of net apoptosis (bottom) in (**A**) ANXA3 silenced PLC8024 cells treated with 7.5μM sorafenib and (**B**) ANXA3 overexpressed HepG2 cells treated with 10μM sorafenib. (**C-D**) Genomic and proteomic (endogenous and secretory) ANXA3 expression in sorafenib-sensitive and sorafenib-resistant HepG2 cells. (**E**) Genomic ANXA3 expression in sorafenib-sensitive and sorafenib-resistant HCC patient-derived xenografts (PDX#1 and PDX#2) in different tumor passages. (**F**) Representative images of ANXA3 immunohistochemical staining at different staining intensity levels (1 to 4 representative of progressive low to high staining) in resected HCC patient samples who went on to receive sorafenib treatment. Scale bar, 100μm. Kaplan-Meier survival analysis comparing the cumulative survival rate of HCC patients who have undergone sorafenib treatment, with different ANXA3 expression levels (*n*=97 cases). (**G**) Representative flow cytometry analysis of Annexin V-PI staining (top) and quantification of net apoptosis (bottom) in HepG2 sorafenib-resistant cells with or without ANXA3 silenced, in the presence of 10μM sorafenib. (**H**) Subcutaneous injection of HepG2 sorafenib-resistant cells with or without ANXA3 silenced in nude mice and *ex vivo* images of resected tumors. *n*=5 per group. Representative images of H&E and ANXA3 immunohistochemical staining of resected tumor sections. Scale bar, 100μm.

Fig. 2. ANXA3 regulates sorafenib resistance in HCC through p38 MAPK mediated autophagic aberrations. (**A**) Hierarchical cluster heatmap analysis of gene expression profiles in HepG2 sorafenib-sensitive, sorafenib-resistant and sorafenib-resistant cells with or without ANXA3 silenced. Each cell in the matrix represents a particular expression level, where red and blue indicates high and low gene expression, respectively. (**B**) Pathway analysis identified enriched p38 MAPK signaling and its downstream transcriptional control over differentially expressed genes. (**C**) Western blot analysis for expression of ANXA3, p-p38, total p38, cleaved caspase 3, total and cleaved PARP in the indicated HepG2 cells. (**D**) Western blot analysis for expression of LC3B in the indicated HepG2 cells treated with DMSO control or sorafenib (20μM). (**E**) Immunofluorescence staining of LC3B in the indicated HepG2 cells treated with DMSO control or sorafenib (20μM) and quantification of LC3B puncta at high power field. Scale bar, 10μm. (**F**) Western blot analysis for expression of ANXA3, cleaved caspase

3, total and cleaved PARP and LC3B in the indicated HepG2 cells treated with DMSO control or sorafenib (20μM), in the absence or presence of a p38 inhibitor SB202190 (25μM). (**G**) Immunofluorescence staining of LC3B in the indicated HepG2 cells treated with DMSO control or sorafenib (20μM) and in the absence of presence of SB202190; and quantification of LC3B puncta at high power field. Scale bar, 10μm. (**H**) Representative images of ANXA3 and LC3B immunohistochemical staining at high and low staining intensities in HCC patient samples who went on to receive sorafenib treatment. Scale bar, 100μm. Bar chart summary of the distribution of ANXA3 expression levels in low/high LC3B expressing samples (*n*=97 cases).

Fig. 3. ANXA3 binds and interacts with PKCδ which acts as an upstream regulator of p38 MAPK mediated apoptotic and autophagic events in HCC. (**A**) Co-immunoprecipitation analysis for PKCδ as an ANXA3 interacting protein partner in HepG2 sorafenib-resistant cells. (**B**) Dual-color immunofluorescence staining of ANXA3 and PKCδ in sorafenib-sensitive and sorafenib-resistant HepG2 cells. Scale bar, 10μm. (**C**) Western blot analysis for expression of ANXA3 and PKCδ in total lysates, as well as ANXA3, PKCδ, p-tyrosine in cytosol and particulate subfractions of indicated HepG2 cells. Calnexin and α-tubulin as loading controls for particulate and cytosol fractions, respectively. (**D**) Western blot analysis for expression of PKCδ, p-p38, total p38, cleaved caspase 3, total and cleaved PARP and LC3B in ANXA3 silenced HepG2 sorafenib-resistant cells with or without PKCδ repressed. (**E**) Immunofluorescence staining of LC3B in ANXA3 silenced HepG2 sorafenib-resistant cells with or without PKCδ repressed and quantification of LC3B puncta at high power field. Scale bar, 10μm.

Fig. 4. Targeting ANXA3 is effective in suppressing tumor growth and sensitizes cells to sorafenib treatment as demonstrated in both organotypic *ex vivo* human HCC clinical samples and HCC PDX in mice. (**A**) Images of bright-field, H&E and immunohistochemical analysis of ANXA3 in two HCC patient-derived organoids (#1 and #2). Scale bar, 100μm. (**B**) Percentage of growth in HCC patient-derived organoids #1 and #2 treated with vehicle, sorafenib (2μM), IgG (50μg/ml), ANXA3 mAb (50μg/ml) or combination of sorafenib+anti-ANXA3 mAb. (**C**) Immunofluorescence staining of ANXA3, PKCδ, p-p38 and LC3B in HCC organoids #1 post-treatment. Scale bar, 10μm. (**D**) *Ex vivo* images of resected livers with orthotopically implanted HCC patient-derived xenograft (PDX#1) post-treatment (top) and quantification of tumor volumes (bottom). *n*=8 per group. Scale bar, 1cm. (**E**) H&E and

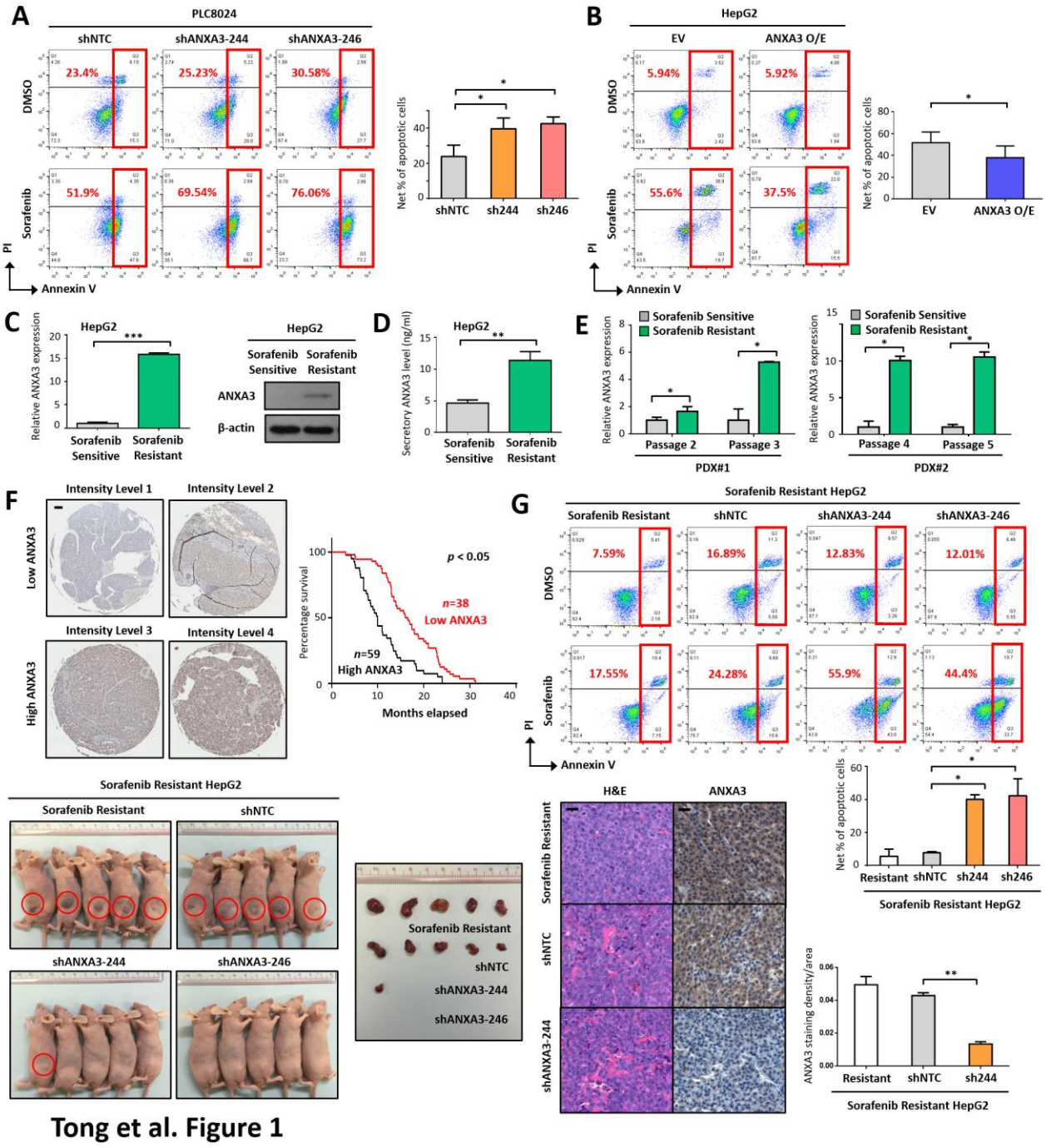
immunohistochemical images of ANXA3 and LC3B and TUNEL staining for apoptotic cells on liver tissues harvested from the resected xenografts. Scale bar, 100 μm; inset scale bar, 25μm.

Fig. 5. Anti-ANXA3 therapy reverts resistance and enhances the efficacy of sorafenib in sorafenib-acquired resistant HCC xenografts. (**A**) Representative flow cytometry analysis of Annexin V-PI staining (left) and quantification of net apoptosis (right) in HepG2 sorafenib-resistant cells treated with 50μg of IgG or ANXA3 mAb. (**B**) Growth curve of HepG2 sorafenib-resistant cells derived xenografts in nude mice under vehicle+IgG, sorafenib+IgG, vehicle+anti-ANXA3 mAb or sorafenib+anti-ANXA3 mAb treatment. (**C**) Representative xenograft tumors at endpoint. Graph showing the weight of tumors generated in each group. *n*=5 per group. Scale bar, 1cm. (**D**) H&E and immunohistochemical images of ANXA3 and LC3B and TUNEL staining for apoptotic cells on resected xenografts. Scale bar, 100μm; inset scale bar, 25μm. (**E**) Representative electron micrographs of autophagic vesicles in HepG2 sorafenib-resistant cells derived xenografts with different treatment combinations. Scale bar, 2μm.

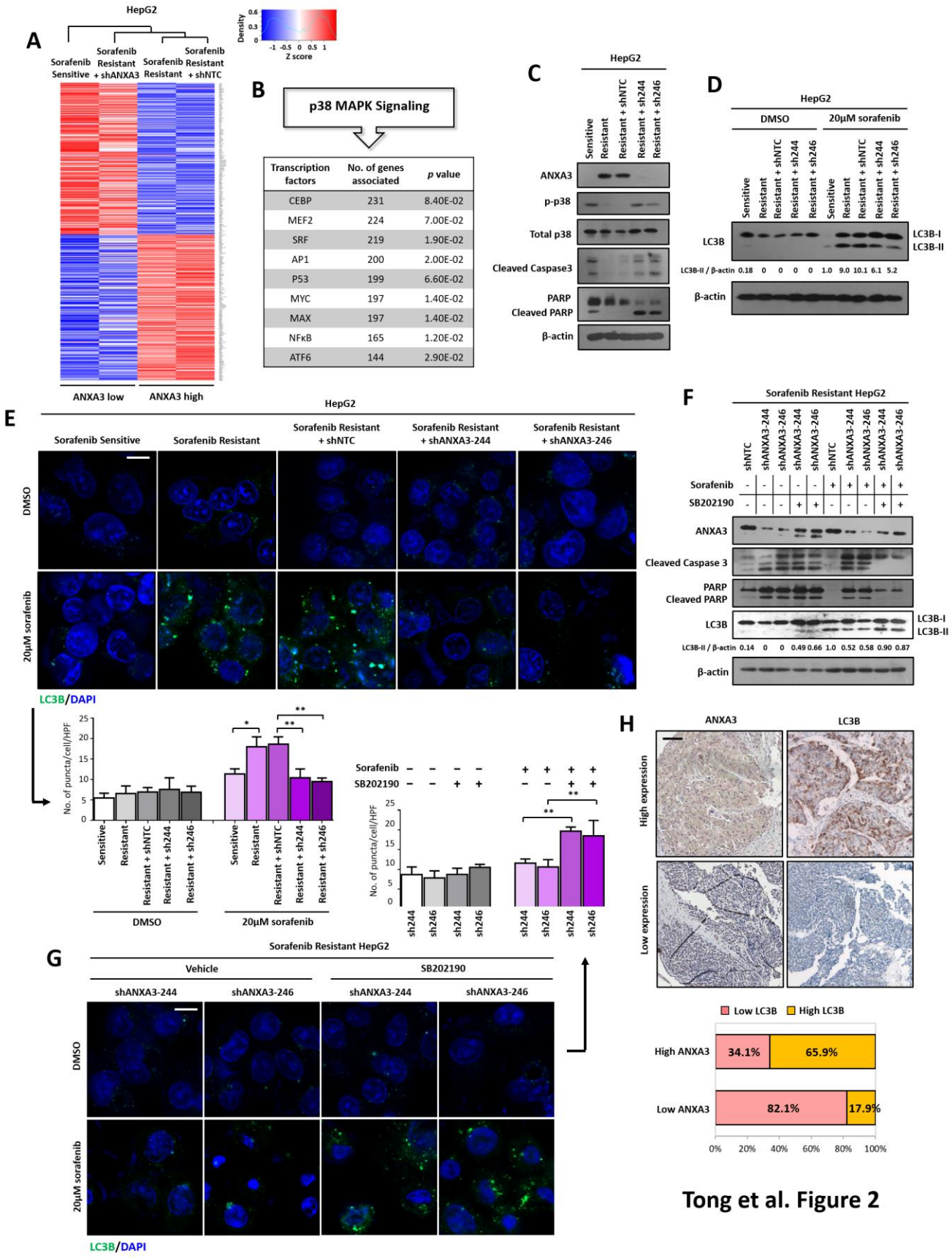
Fig. 6. Combination of ANXA3 inhibition and regorafenib resulted in maximal tumor suppression in a sorafenib-acquired resistant HCC xenografts. (**A**) Growth curve of HepG2 sorafenib-resistant cells derived xenografts in nude mice under vehicle (veh), regorafenib, regorafenib+IgG or regorafenib+anti-ANXA3 mAb treatment. (**B**) Representative xenograft tumors at endpoint. Graph showing the weight of tumors generated in each group. *n*=5 per group. Scale bar, 1cm. (**C**) H&E and immunohistochemical images of ANXA3 and LC3B and TUNEL staining for apoptotic cells on resected xenografts. Scale bar, 100μm; inset scale bar, 25μm. (**D**) Representative electron micrographs of autophagic vesicles in HepG2 sorafenib-resistant cells derived xenografts that has undergone different treatments. Scale bar, 2μm.

Fig. 7. Inhibition of ANXA3 sensitized HCC cells to both sorafenib and regorafenib treatment in an immune-competent mouse model. (**A**) Representative gross and H&E images of HCC tumors formed at week 7 post-hydrodynamic injection of N-RASV12, myr-AKT and sleeping beauty transposase in mice. AFP used as hepatocellular marker. Immunohistochemical analysis of ANXA3 expression. Scale bar, 1cm. (**B**) Schematic diagram of the treatment regimen with sorafenib, regorafenib, anti-ANXA3

mAb alone or the combination of sorafenib/regorafenib with anti-ANXA3 mAb. (**C-E**) (Top) Kaplan-Meier survival curves showing percentage of tumor-free survival of each annotated groups. *n*=10 per group. (Bottom) Graphs showing the liver/body weight ratio generated from mice that died in each treatment group.



Tong et al. Figure 1



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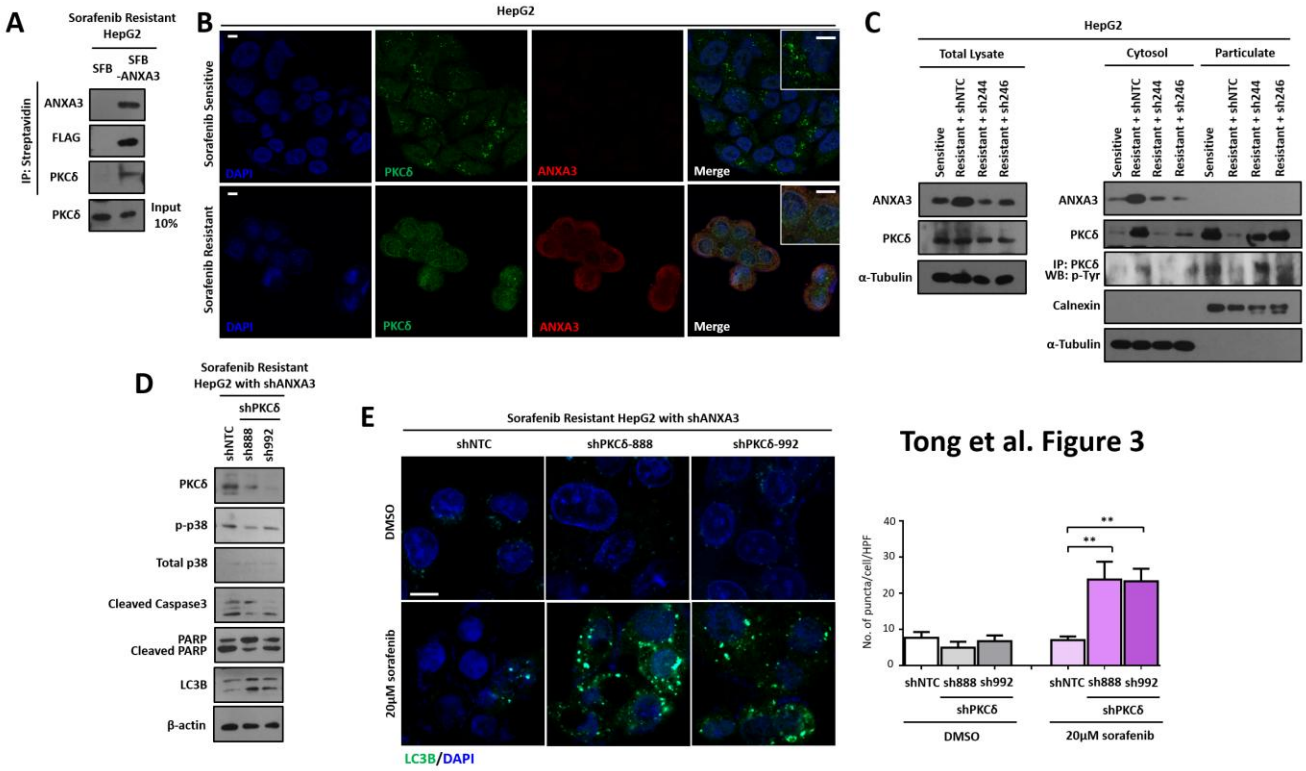
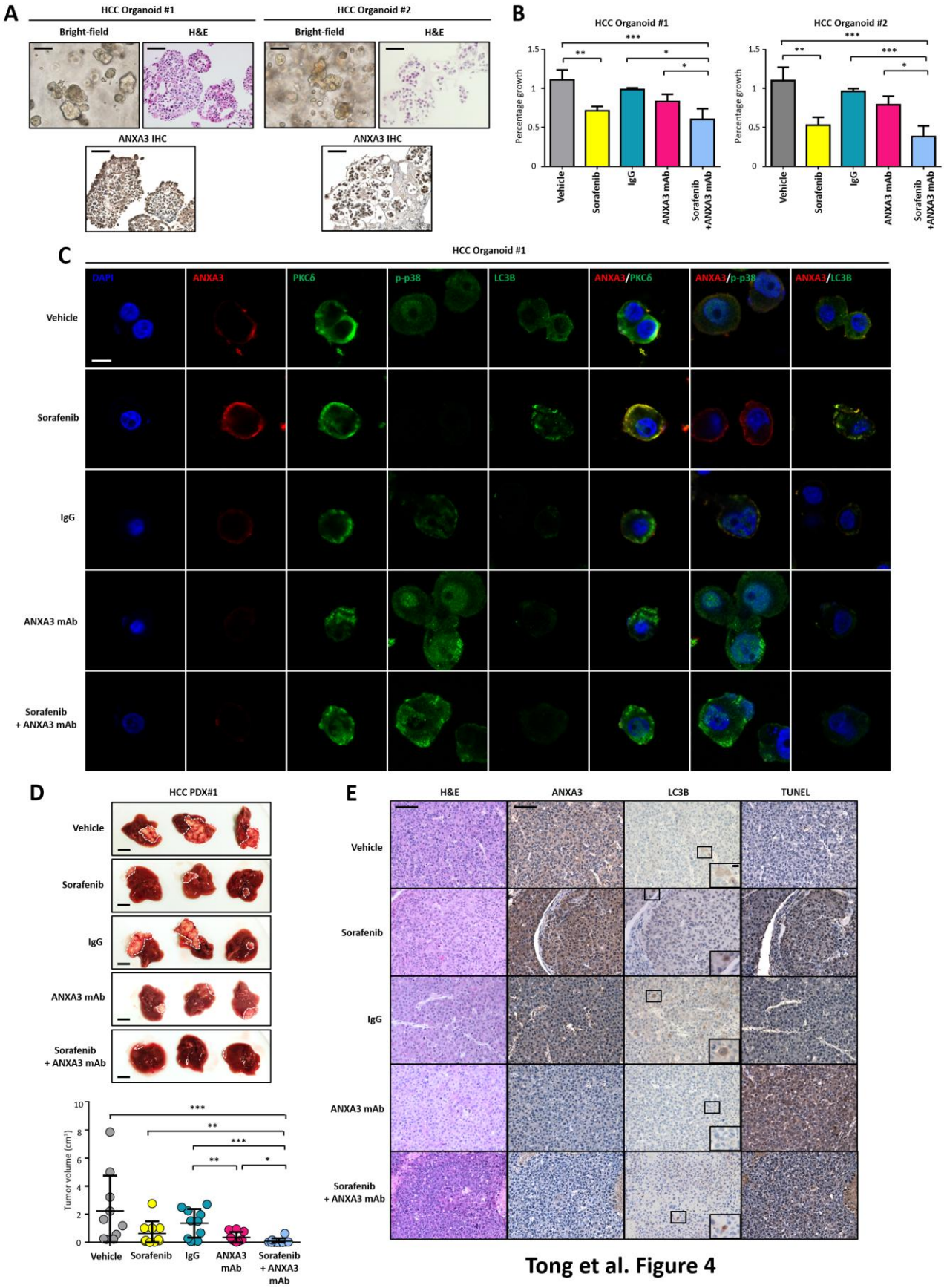


Tong et al. Figure 3



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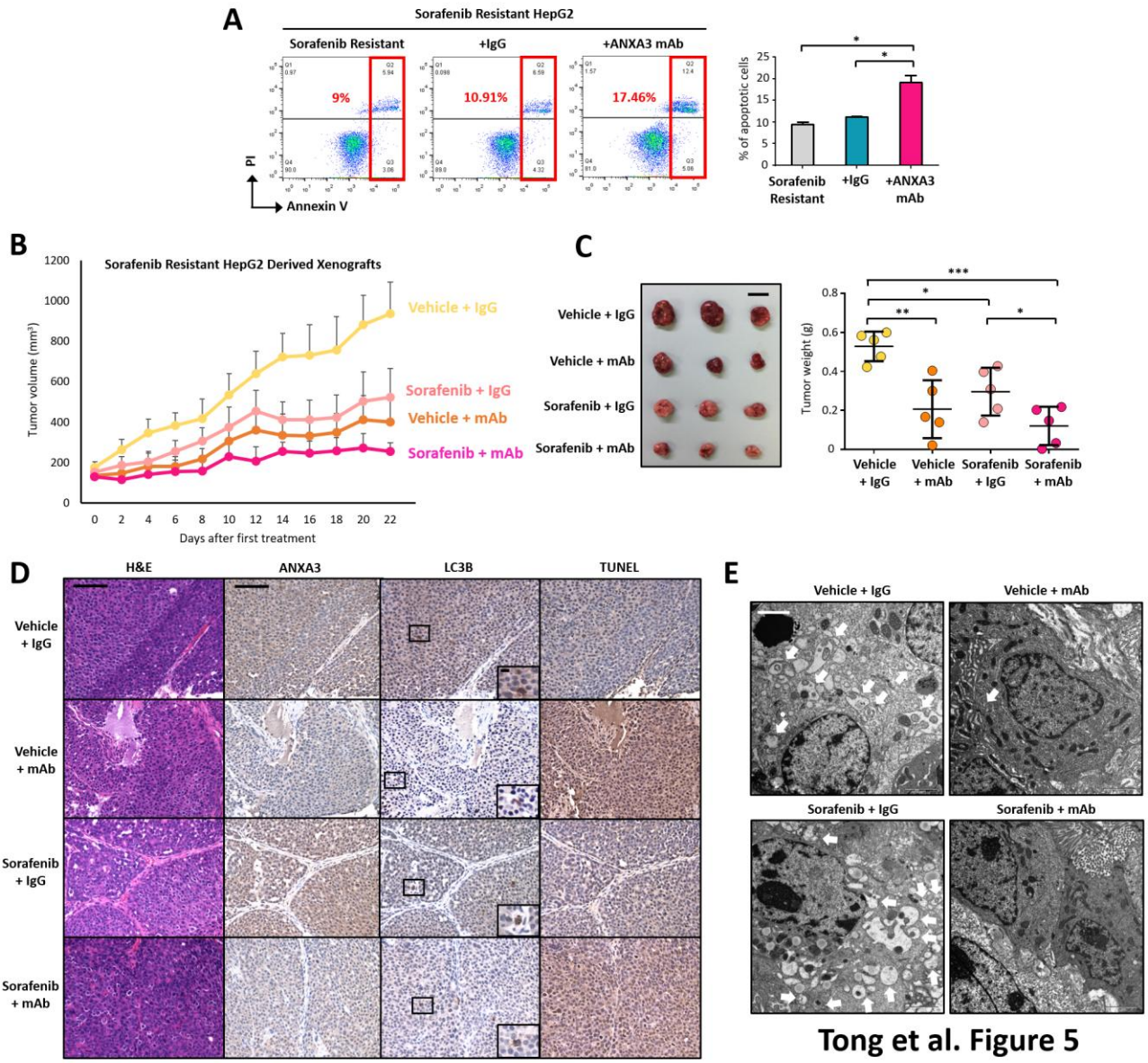

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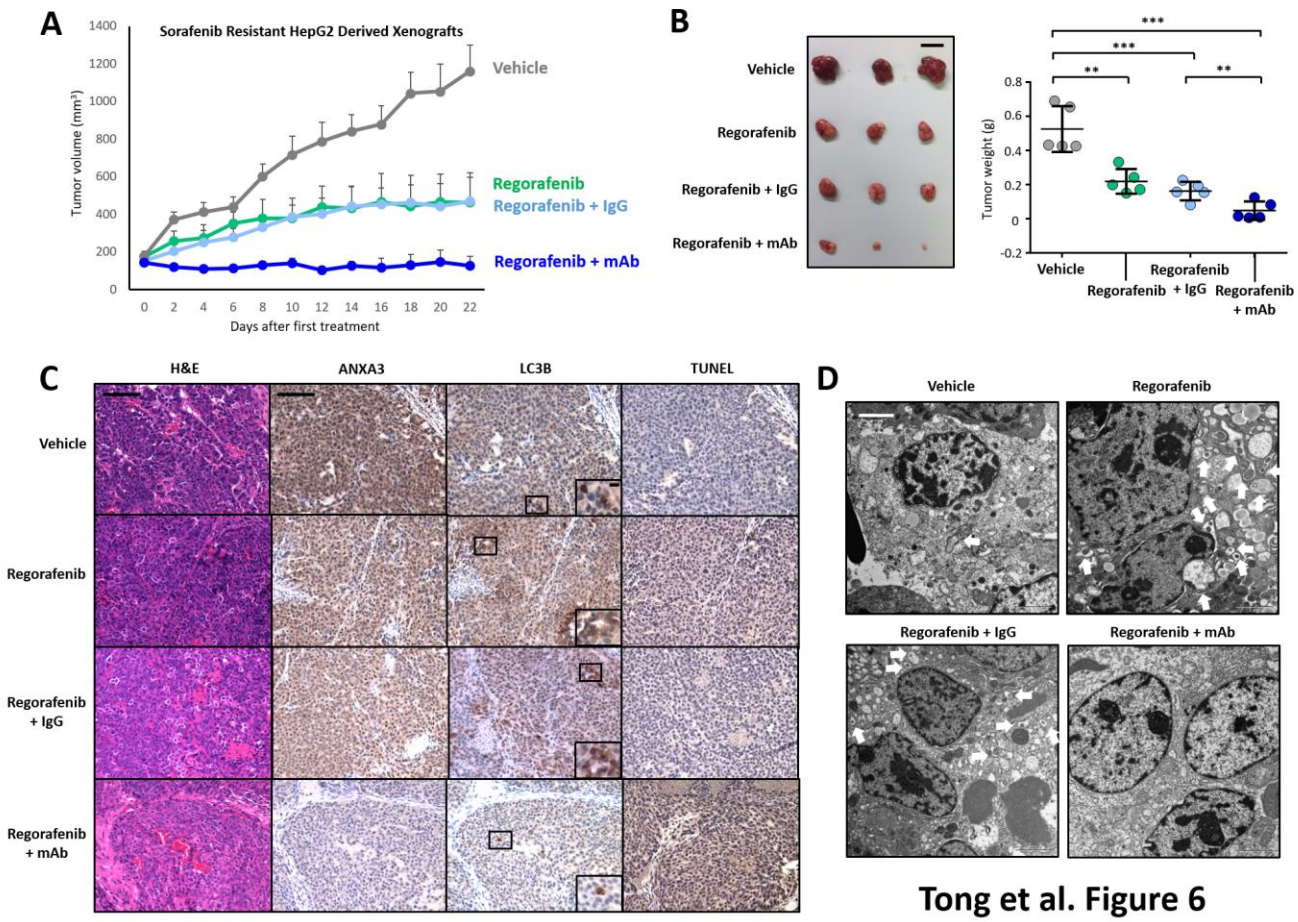
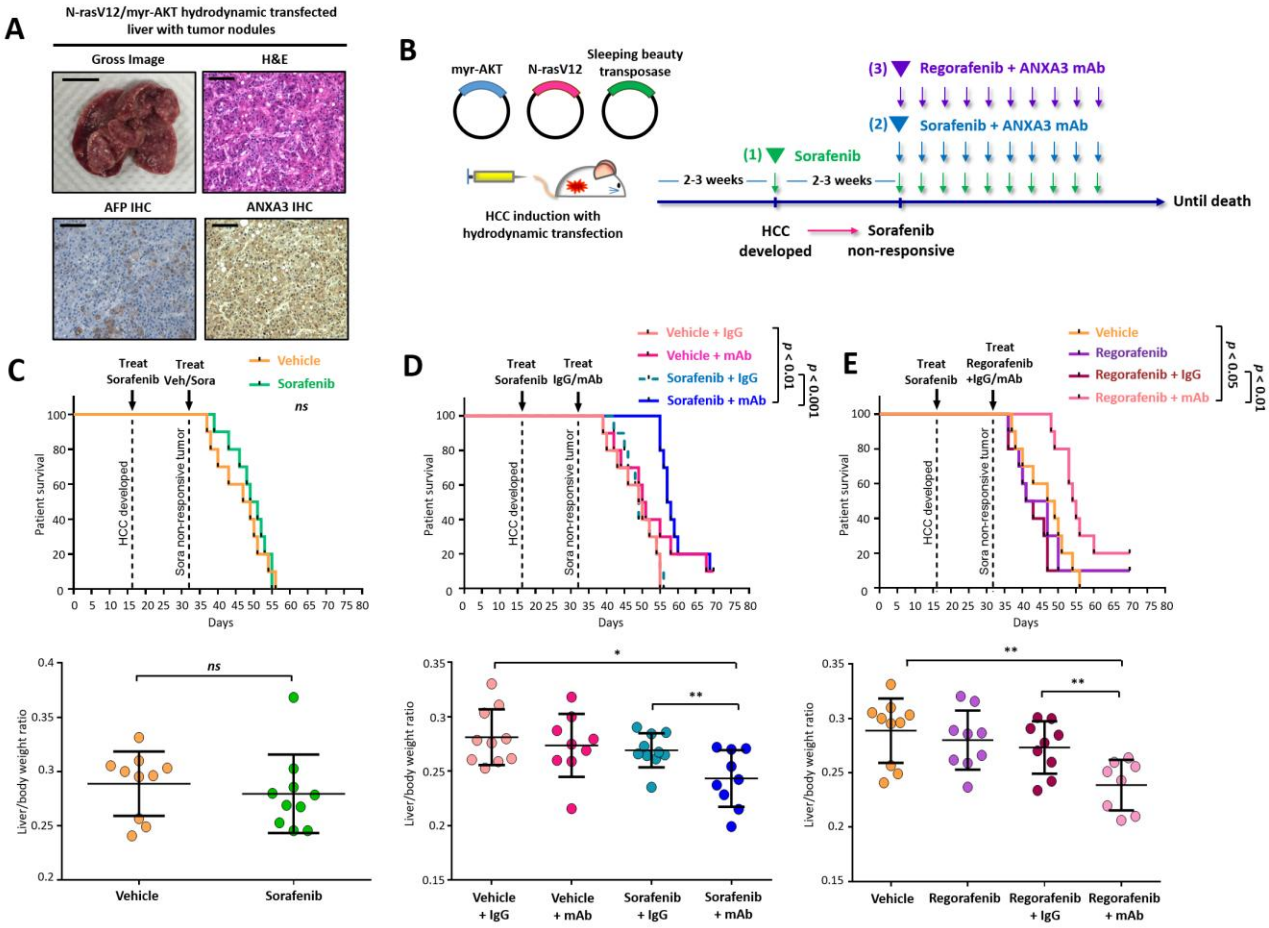


Tong et al. Figure 6



Tong et al. Figure 7

HIGHLIGHTS

- ANXA3 confers ability of HCC cells to resist sorafenib
- ANXA3 is enriched in sorafenib-resistant HCC
- ANXA3 activates autophagy and attenuates PKCδ/p38 dependent apoptosis
- ANXA3 is a useful predictive biomarker to stratify patients for sorafenib treatment
- Anti-ANXA3 therapy with sorafenib/regorafenib as a new treatment strategy for HCC