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Original Research

Metformin and insulin impact on clinical outcome in patients with advanced hepatocellular carcinoma receiving sorafenib: Validation study and biological rationale

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KEYWORDS
Hepatocellular carcinoma;
Type II diabetes mellitus;
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Liver cancer

Abstract ***Purpose:*** In 2015, we published a study on a small series of patients with hepatocellular carcinoma (HCC) treated chronically with metformin for type II diabetes mellitus (DM2) who showed a poorer response to sorafenib. The aim of the present study was to validate the prognostic significance of metformin in HCC patients treated with sorafenib, providing a biological rationale for the mechanism of resistance to sorafenib in patients on chronic metformin therapy, and to clarify the role of sirtuin-3 (SIRT-3), a protein involved in metabolic diseases and acknowledged as a tumour suppressor in HCC, in this resistance.
Patients and methods: We analysed 279 patients consecutively treated with sorafenib for the clinical analysis. Of the 86 (30%) patients with DM2, 52 (19%) were on chronic treatment with metformin and 34 (12%) with insulin. We included 43 patients with HCC for the biological study: 19 (44.1%) were diabetic and 14 (73.7%) of these received metformin for DM2. SIRT-3 expression was investigated by immunohistochemistry (IHC) in formalin-fixed and paraffin-embedded (FFPE) samples.
Results: In HCC patients undergoing chronic treatment with metformin, the use of sorafenib was associated with poor progression-free survival (PFS) and overall survival (OS) (1.9 and 6.6 months, respectively) compared to 3.7 months and 10.8 months, respectively, for patients without DM2 and 8.4 months and 16.6 months, respectively, for patients on insulin ($P < .0001$). We also observed that SIRT-3 protein expression was significantly higher in patients treated with metformin than in those not taking this medication (65% versus 25%, respectively) ($P = .013$).
Conclusions: Our findings could be attributed to increased tumour aggressiveness and resistance to sorafenib caused by chronic treatment with metformin.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide in men and the second most frequent cause of cancer-related deaths [1–3]. Each year it is diagnosed in more than 500,000 people worldwide. The decrease in virus-associated HCC observed in Italy in recent years has been offset by an increase in HCC caused by non-alcoholic fatty liver disease (NAFLD) [4,5]. It has been seen that the use of sorafenib increases median overall survival of HCC patients (10.7 months for sorafenib group versus 7.9 months for placebo group), representing a 31% decrease in the relative risk of death [6]. However, there is still no validated biological or clinical marker that predicts response to treatment in these patients [7–12].

In 2015, we published a study on a series of HCC patients who showed a poorer response to sorafenib as a result of chronic treatment with metformin for type II diabetes mellitus (DM2) [13]. The patients who developed HCC whilst undergoing chronic therapy with metformin showed a median progression-free survival (PFS) of 2.6 months compared to 5.0 months for those not taking this medication. Overall survival (OS) was 10.4 months and 15.1 months, respectively.

Sirtuin-3 (SIRT-3), one of the evolutionarily conserved mammalian orthologues of the silent information regulator 2 (Sir2) is a nicotinamide adenine dinucleotide $(NAD)^+$-dependent deacetylase involved in regulating mitochondrial metabolism [14]. Its regulatory effects and involvement in metabolic diseases are believed to have a strong impact on the development and treatment of HCC. Although the reported evidences suggest a putative bridge role of SIRT-3 between metabolic disorders and HCC, further studies are necessary to demonstrate such interconnection [15,16]. The aim of the present study was to validate the prognostic significance of metformin and insulin in HCC patients treated with sorafenib, and to establish a biological rationale for the mechanism involved in resistance to sorafenib in those undergoing chronic metformin therapy.

2. Patients and methods

2.1. Patient population for the clinical study

The present study was performed using the medical records from the databases of Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS (Meldola); Department of Medical Oncology, University of Cagliari; Università Campus Bio-Medico (Rome); National Cancer Institute 'Giovanni Paolo II' (Bari), University of Bari Medical School; and Sant'Orsola-Malpighi Hospital, University of Bologna. Data were entered into electronic data files by co-investigators from each centre taking part and checked at the data management centre for missing information and internal consistency. The study protocol was reviewed and approved by the local Ethics Committee. All patients gave their written informed consent.

We enrolled patients receiving sorafenib (400 mg twice daily) for advanced or intermediate-stage HCC (either histologically proven or diagnosed according to the AASLD [American Association for the Study of Liver Diseases 2005] guidelines) that was refractory or no longer amenable to locoregional therapies. Eligibility criteria were the same as those of Llovet's pivotal study on sorafenib in HCC [5]. Dose reductions were applied when clinically indicated. Follow-up consisted of a computed tomography (CT)/magnetic resonance imaging (MRI) scan every 8 weeks or as clinically indicated. Tumour response was evaluated in accordance with modified Response Evaluation Criteria in Solid Tumours (mRECIST) [17]. Treatment with sorafenib was continued until disease progression, unacceptable toxicity or death.

We defined 'patients with diabetes and treated with metformin' as those who had been taking metformin for at least 5 years at the time of the first diagnosis of HCC. 'Patients with diabetes and treated with insulin' were defined as those who had been taking insulin for at least 5 years when HCC was first diagnosed.

The primary objective of this study was to compare PFS in patients taking metformin or insulin or no antidiabetic medication at the time of the first diagnosis of HCC. The second objective was to compare OS in the same patients.

2.2. Patient population for SIRT-3 evaluation

The study was performed on biopsies obtained from 46 patients with early stage HCC treated with curative hepatic resection in the Departments of General Surgery, Morgagni-Pierantoni Hospital (AUSL Romagna, Forli, Italy) and Università Campus Bio-Medico (Rome, Italy). Eligibility criteria were: Child Pugh A, Barcelona clinic liver cancer (BCLC) stage 0 or BCLC A; The BCLC staging classification links the stage of the disease to a specific treatment strategy. We excluded the patients with incomplete clinical data.

SIRT-3 expression was evaluated by immunohistochemistry (IHC) in formalin-fixed paraffin-embedded (FFPE) samples using a rabbit monoclonal antibody directed against SIRT-3 (Abcam: Clone C73E3) and the Ventana Optiview DAB IHC detection kit on a Ventana Benchmark XT automated system. Marker expression was recorded as the percentage of positive tumour cells in relation to the overall neoplastic population.

2.3. Statistical analysis of the clinical study

The patient population was divided into three groups for the clinical study on the basis of the presence of diabetes and the type of antidiabetic treatment received (no diabetes, diabetes with metformin, diabetes with insulin). PFS was calculated from the day of the start of treatment until the day of disease progression or last follow-up. OS was calculated from the day of the start of treatment until the day of death or last follow-up. Patients lost to follow-up were censored at the time of the last contact. Descriptive data were reported as median with range for continuous variables, and absolute and relative frequencies for categorical variables. The association among the categorical variables was analysed by the chi-squared test. Survival distribution was estimated by the Kaplan–Meier method. Significant differences in probability of relapsing between the strata were evaluated by the log-rank test. Cox multiple regression analysis was used to assess the role of variables that proved significant in univariate analysis. A P value $< .05$ was considered statistically significant in all analyses.

Tested variables included gender (male versus female), age ($\leq$68 years versus >68 years), cirrhosis aetiology (hepatitis C infection versus hepatitis B infection versus alcoholic, metabolic or cryptogenic), Eastern Cooperative Oncology Group (ECOG) performance status (ECOG PS: 0 versus 1 versus 2), Barcelona Clinic Liver Cancer (BCLC) stage (B versus C), Child-Pugh class (A versus B), extrahepatic spread (yes versus no), portal vein thrombosis (yes versus no), median alpha-fetoprotein (AFP) serum level ($\leq$37 ng/ml versus > 37 ng/ml), previous treatments received (no other treatments versus surgery alone versus interventional procedures alone versus surgery and interventional procedures). MedCalc package (MedCalc® version 16.8.4) was used for all statistical analysis.

2.4. Statistical analysis of the SIRT-3 evaluation

Descriptive statistics (median and range of variation were used to analyse SIRT-3), considered as a continuous variable. A comparison of median values of SIRT-3, depending on the different clinical features, was performed using the non-parametric Wilcoxon test. All P values were obtained from two-tailed tests, and statistical analyses were performed with SAS statistical software, version 9.4 (SAS Institute, Cary, NC, United States of America).

3. Results

3.1. Patient characteristics

Two hundred and seventy-nine patients (237 males and 42 females) with HCC, consecutively treated with sorafenib from May 2007 to September 2015, were included in the clinical study. Median age was 69 years (range 28–88 years). Two hundred and sixty-two patients had Child-Pugh A HCC, and 17 had Child-Pugh B HCC. Seventy-seven patients had BCLC-B, and 202 patients had BCLC-C. The most common aetiologies of liver disease were hepatitis C (58%), alcohol-derived

liver disease (11%), metabolic liver disease (9%), hepatitis B (13%) and cryptogenic aetiology (7%). Eighty-six (30%) patients had DM2, of whom 52 (19%) were on treatment with metformin and 34 (12%) with insulin.

With the exception of the cirrhosis aetiology, the three groups of patients were comparable for all major clinical characteristics investigated (Table 1). Metabolic liver disease was more frequent in patients with DM2 undergoing treatment with either metformin or insulin than in non-diabetic patients in whom, conversely, viral infection was predominant (metabolic aetiology: DM2 receiving insulin treatment, 44%; DM2 receiving metformin treatment, 27%; no DM2, 1%).

Forty-three patients (36 males and 7 females) with HCC, consecutively treated with surgery during the period from April 2001 to May 2015, were included in the biological study. Median age was 70 years (range 45–87 years). All patients had Child-Pugh A and BCLC-A. The most common aetiologies of liver disease were hepatitis C (46.5%), alcoholic liver disease (16.2%) and metabolic liver disease (37%). Nineteen (44.1%) patients were diabetic, and 14 (73.7%) of these were undergoing treatment with metformin (Table 2).

Table 2
Patient characteristics in the biological study.

Patients	No. (%)
Median age, years [range]	70 [45–87]
Gender	
Male	36 (83.7)
Female	7 (16.3)
Etiology	
Hepatitis C	20 (46.5)
Metabolic liver disease	16 (37)
Alcoholic liver disease	7 (16.2)
BCLC-A	43 (100)
Child-Pugh A	43 (100)
Diabetes mellitus	
Yes	19 (44.1)
No	24 (55.9)
Taking metformin	
Yes	14 (32.5)
No	29 (67.5)

BCLC, Barcelona Clinic Liver Cancer.

3.2. Treatment with metformin or insulin and clinical outcome

The median PFS of all patients was 3.6 months (95% CI: 3.1–4.4) and median OS was 10.7 months (95% CI:

Table 1
Patient characteristics in the clinical study population.

Patients	Diabetes mellitus type II treated with metformin		No diabetes		Diabetes mellitus type II treated with insulin		*P*
	No.	%	No.	%	No.	%	
Gender							
Male	46	88	160	83	31	91	.339
Female	6	12	33	17	3	9	
Median age, years (range)							
>69	29	56	90	47	19	56	.367
≤69	23	44	103	53	15	44	
Cirrhosis aetiology							
HCV	23	44	132	68	8	24	<.0001
HBV	2	4	34	18	1	3	
Alcohol	8	15	16	8	7	20	
Cryptogenic	5	10	10	5	3	9	
Metabolic	14	27	1	1	15	44	
ECOG PS							
0	24	46	117	61	25	74	.103
1–2	28	54	76	39	9	26	
BCLC stage							
B	14	27	47	24	15	44	.058
C	38	73	146	76	19	56	
Extrahepatic metastasis							
Yes	21	40	93	48	11	32	.179
No	31	60	100	52	23	68	
Portal thrombosis							
Yes	17	33	69	36	7	21	.223
No	35	67	124	64	27	79	
Median AFP level							
>37	22	42	100	52	12	35	.135
≤37	30	58	93	48	22	65	
Total no.	52		193		34		

HCV, hepatitis C virus; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein.

9.1–12.8). The use of sorafenib in patients undergoing chronic treatment with metformin was associated with a median PFS of 1.9 months (95% CI 1.8–2.3) compared to 3.7 months (95% CI 3.1–4.6) for non-diabetic patients and 8.5 months (95% CI 5.3–11.4) for those on insulin ($P < .0001$) (Fig. 1A).

Metformin-treated patients showed a median OS of 6.6 months (95% CI 4.6–8.7) compared to 10.8 months (95% CI 9.0–13.1) for non-diabetic patients 16.6 months (95% CI 14.5–25.5) for the insulin group ($P = .0001$) (Fig. 1B).

Conversely, no differences in survival were observed between the overall DM2 population and the non-diabetic group. PFS ($P = .471$): DM2 (3.2 months) versus no DM2 (3.7 months). OS ($P = .734$): DM2 (10.7 months) versus no DM2 (10.8 months).

Of the clinical variables tested in univariate analysis, ECOG PS ($P = .0034$) and BCLC stage ($P = .0336$) were predictive of PFS. ECOG PS ($P < .0001$) and median AFP serum levels ($P = .0145$) were predictive of OS. In particular for PFS: ECOG PS 0 (4.4 months, 95% CI 3.5–5.2) versus 1–2 (2.9 months, 95% CI 2.6–3.7); and BCLC stage B (5.7 months, 95% CI 4.5–6.7) versus C (3.1 months, 95% CI 2.7–3.7). For OS: ECOG PS 0 (14.3 months, 95% CI 11.9–15.5) versus 1–2 (7.9 months, 95% CI 6.8–9.1); and median AFP serum level $\leq$ 37 ng/ml (10.9 months, 95% CI 9.4–15.0) versus >37 ng/ml (10.2 months, 95% CI 8.1–13.1). In multivariate analysis the DM2 profile (DM2 metformin versus no DM2 versus DM2 insulin) maintained an independent prognostic value for PFS ($P < .0001$). Conversely, ECOG PS, median AFP serum level and DM2 profile maintained an independent prognostic value for OS ($P < .0001$). All of the other clinical variables failed to show any correlation with patient outcome.

The effect of metformin on clinical outcome was also investigated in relation to the objective response rate (ORR). Patients treated chronically with metformin showed a higher percentage of progression at the first CT re-evaluation than those treated with insulin or the non-diabetic group (75.8% versus 14.7% versus 38.8%, respectively).

Considering the overall population, the probability of progression was higher in DM2 patients taking metformin than in the non-diabetic group (hazard ratio, HR = 1.91, 95% CI 1.28–2.8). Similar results were observed for survival (HR = 1.70, 95% CI 1.14–2.55). The risk of progression was lower in DM2 patients taking insulin than in non-diabetic patients (HR = .65, 95% CI 0.48–.89). Similar results were observed for survival (HR = .62, 95% CI 0.44–.87).

With regard to diabetic patients, the risk of progression was higher in those taking metformin than in the insulin group (HR: 2.91; 95% CI: 1.84–4.6). Similar results were observed for survival (HR: 2.74; 95% CI: 1.69–4.43).

Significant differences in the toxicity profile were found in the three patient groups (Supplementary Table S1).

3.3. Immunohistochemical expression of SIRT-3 in HCC patients

We studied the functional status of SIRT-3 enzyme in HCC samples by determining the immunohistochemical expression of its short isoform, relocated in the mitochondria. A representative case of SIRT-3 staining is shown in Fig. 2. SIRT-3 was expressed in the majority of cases, with a range of positivity in the neoplastic population varying from 0% to 90% and a median value of 40% of malignant cells (Table 3).

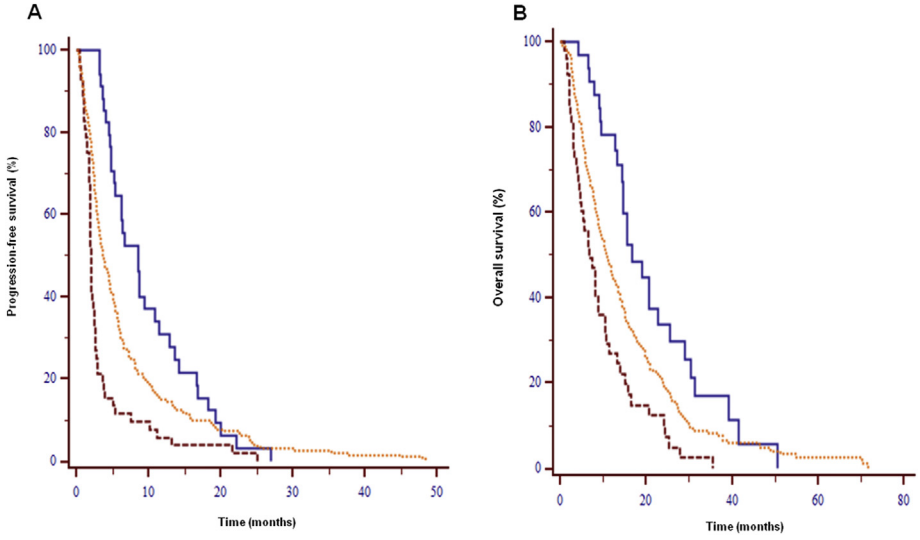

Fig. 1. Kaplan–Meier curves. **A**) Progression-free survival (PFS) and **B**) overall survival (OS). Solid line, diabetes insulin; dotted line, no diabetes; dashed line, diabetes metformin.

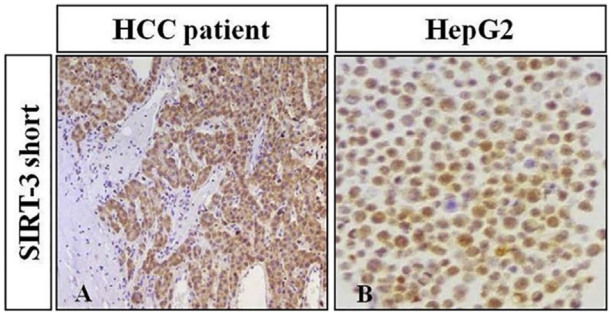

Fig. 2. Immunohistochemical staining for SIRT-3. **A**) SIRT-3 expression in patients with early stage HCC after surgery and **B**) SIRT-3 expression in HepG2 cell line used as positive control.

Table 3
Comparison between median values of SIRT-3 short expression and clinical characteristics.

	Median percentage expression of short form of SIRT-3 (range)	*P*
Etiology		
Metabolic syndrome	60 (10–90)	
Other	30 (0–90)	.044
Diabetes mellitus		
Yes	55 (10–90)	
No	25 (0–90)	.013
Taking metformin		
Yes	65 (10–90)	
No	25 (0–90)	.013

Of the 43 HCC cases, 16 had metabolic syndrome. This subgroup showed a significantly higher expression of SIRT-3 than patients with different aetiology (median 60% versus 30%, respectively). The higher expression of the protein correlated significantly with the presence of DM2 (median value 55% in diabetic patients versus 25% in non-diabetic patients) ($P = .013$) (Table 3). Interestingly, SIRT-3 protein expression was also higher in patients treated with metformin than in those taking insulin (65% versus 25%, respectively) ($P = .013$) (Table 3).

4. Discussion

In the present study, we validated the association between metformin, insulin and sorafenib in HCC patients. Our findings highlighted a lower response to sorafenib in those who developed HCC whilst undergoing chronic therapy with metformin. In contrast to our first study [13], HCC patients on chronic insulin therapy showed a better response to treatment and longer survival. We hypothesised that the discrepancy in reported results may be justified by the different number of patients enrolled in the two studies. Indeed, in the first, we analysed few cases and the data were not significant; on the contrary in this actual study, we enrolled the greater number of patients obtaining promising results.

The population analysed was homogeneous for age, sex, ECOG Performance status, BCLC stage, presence of extrahepatic metastases, portal thrombosis, previous treatments and baseline levels of α-fetoprotein, but not for aetiology. This aetiological non-homogeneity can be attributed to the expected greater frequency of diabetes in patients with metabolic liver disease. In addition, we also reported an increased toxicity in patients treated with insulin, probably related to the most significant response in patients with increased sorafenib toxicity [12,18].

Metformin has been shown to inhibit tumour growth *in vitro* and *in vivo* by inducing apoptosis in various cancers [19–21]. Retrospective studies also suggest that metformin prevents HCC development in individuals with diabetes and in diabetic patients with chronic liver disease [22–26]. A large population-based study by Chen *et al.* [22] demonstrated a dose-dependent decrease in the risk of HCC among diabetic patients. A recent meta-analysis confirmed a 50% decrease in HCC incidence among diabetics on metformin but also revealed a significant increase in the risk of HCC in insulin-treated patients [27]. A possible explanation for these contradictory results is that tumours developing during chronic treatment with metformin have intrinsic mechanisms of resistance to metformin, which may also lead to resistance to sorafenib.

Recently, Di Costanzo *et al.* reported an increase in time to progression and OS in HCC patients with diabetes compared to those without diabetes [28]. However, the authors did not distinguish between different hypoglycaemic therapies used. Conversely, we did not observe any significant difference in the aforementioned parameters between diabetic and non-diabetic patients. Our results could be potentially attributed to increased tumour aggressiveness and resistance to sorafenib in patients treated with metformin compared to insulin or, from a biological point of view, to different molecular mechanisms of the antidiabetic drugs.

It has been demonstrated that metformin activates liver kinase B1 (LKB1) and AMP-activated protein kinase (AMPK), leading to mammalian or mechanistic target of rapamycin (mTOR) inhibition and cell growth arrest [29]. The inhibition of mTOR can also be induced directly by hypoxia-induced factor-1α (HIF-1α) repression via AMPK [30,31] or indirectly by SIRT-3-mediated HIF-1α inhibition [32].

Starting from the evidence that SIRT-3 represents a critical effector in AMPK/HIF-1α/mTOR pathway and in line with the elucidated mechanism, we focussed our attention on SIRT-3 in HCC patients in chronic treatment with metformin. Interestingly, we observed that its expression significantly increased in this setting of patients and this rise correlated with the presence of metabolic syndrome and DM2, suggesting an important role of SIRT-3 in metabolic disorders.

Metformin can also bypass AMPK, directly inhibiting mTOR signalling and inducing cell cycle arrest by

cyclin D1 downregulation via p53 [33,34]. Conversely, insulin exerts a proliferative effect directly through the insulin receptor, leading to the activation of phosphoinositide 3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) pathways, and indirectly through an increase in circulating levels of insulin growth factor-1 (IGF-1) [35]. When blood glucose levels go up, insulin metabolic activity decreases, leading to the overactivation of mTOR, which in turn downregulates insulin signal-related metabolic pathways. In contrast, insulin induces the MAPK pathway, enhancing cell survival [36]. As shown in Fig. 3, metformin suppresses PI-3K and MAPK signal cascades (both of which are targets of sorafenib), directly via IGF or mTOR signalling inhibition or indirectly via AMPK pathway. We hypothesise that patients on chronic treatment with metformin develop resistance to sorafenib because the above pathways are already blocked.

One strength of our study lies in the detailed information it provides on patient characteristics and follow up. However, there are also some limitations, i.e. despite being a retrospective evaluation, cases were selected consecutively to minimise bias. Regarding the biological part, we are aware that the only evaluation of SIRT-3 is not exhaustive to justify the putative mechanism of resistance to sorafenib in patients treated chronically with metformin. However, supported by the literature, our explanation can represent a start point to expand our understanding on SIRT-3 bridge role among metabolic dysfunctions, metformin and HCC.

Overall, our results confirmed a resistance to sorafenib in patients who develop HCC during treatment with metformin. Conversely, insulin-treated patients showed a better response and longer survival. Our findings also reveal different tumour biology between the various aetiologies of HCC and we hypothesise that SIRT-3 could play a fundamental role in the development of resistance to sorafenib. Future research should focus on identifying personalized treatments based on aetiology and different tumour biology.

Author contributions

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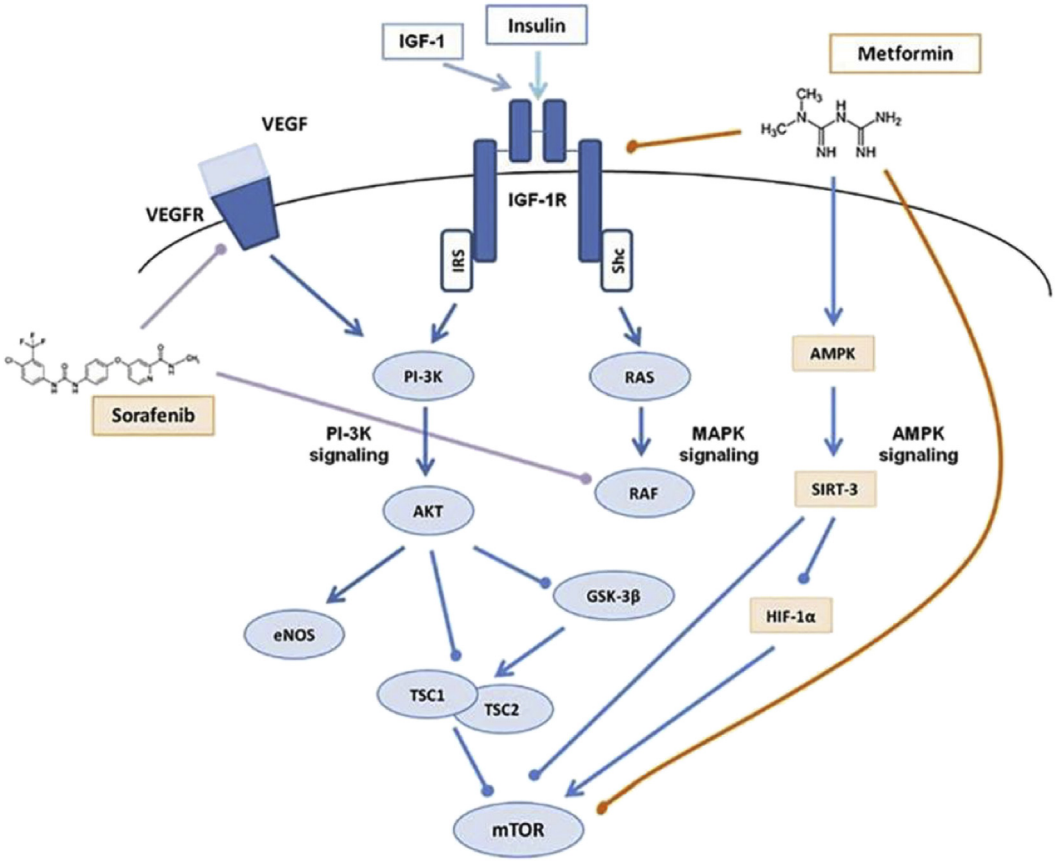

Fig. 3. Mechanisms of action of sorafenib and metformin along different pathways with major downstream effects.

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Manuscript writing: All authors.

Final approval of manuscript: All authors.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejca.2017.09.003.

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