RAP1GAP is a novel marker of trabecular pattern and poor sorafenib treatment response in hepatocellular carcinoma

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Research

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Abstract

Background

The trabecular pattern is one of the most common features of hepatocellular carcinoma (HCC). In this study, we aimed to identify the molecular mechanisms underlying different trabecular patterns in HCC, and their interaction with current therapies.

Methods

To screen potential biomarkers of different trabecular patterns, we first linked gene expression data to haematoxylin and eosin (H&E) images from The Cancer Genome Atlas (TCGA). The Gene Expression Omnibus (GEO) database was used to explore potential targets of sorafenib treatment. Selected candidate biomarkers were further verified by immunohistochemistry, and their relationship with sorafenib efficacy was evaluated in 107 HCC samples with trabecular patterns.

Results

Analysis of RNA sequencing data from TCGA showed that the increasing number of cells in the trabecular structure correlated with increase in the expression of related signalling pathways—Ras, Rap1, IL17, TNF, AGE-RAGE, oestrogen, toll-like receptor signalling, and ubiquitin-mediated proteolysis—and genes related to response to oxygen levels and neoangiogenesis. In contrast, the expression of bile acid and carton, tryptophan, butanoate, and lipid metabolism-related pathways was reduced. The GEO database showed that RAP1GAP, TOB1, ACO2, and SCNN1D expression levels were selectively up-regulated in sorafenib non-responders. Based on the combined analysis of the two datasets and our previous studies, two candidate biomarkers, RAP1GAP and HIF1 α , were selected. Immunohistochemical staining showed that RAP1GAP and HIF1 α were expressed in the tumour tissues. Interestingly, RAP1GAP was also expressed in the tumour sinusoids. Overexpression of RAP1GAP in sinusoids were associated with trabecular patterns. Multivariate analysis also showed that RAP1GAP expression in the sinusoid was an independent predictor of progressive free survival (PFS) and overall survival (OS) in response to sorafenib treatment.

Conclusions

RAP1GAP is an essential microenvironment marker in the trabecular structure of HCC and exhibits an adverse association with outcome in sorafenib treatment.

Background

Liver cancer is the sixth most common malignancy and the fourth leading cause of mortality in patients with cancer worldwide. Hepatocellular carcinoma (HCC) accounts for the majority of primary liver tumours [1]. HCC comprises a group of heterogeneous neoplasms with various morphologies that can be observed at the pathological level [2–4]. HCCs are classified by the World Health Organization (WHO) according to their principal architectural patterns (trabecular, pseudoglandular, solid, and macrotrabecular), morphological variants (steatohepatic, clear cell, macrotrabecular massive, scirrhous, chromophobe, fibrolamellar, neutrophil-rich, and lymphocyte-rich)[5]. However, histopathological classification of tumours has aroused little or no interest in physicians, due to the lack of significant impact on patient outcomes or therapeutic strategies. The trabecular pattern is one of the most common growth patterns of HCC that mimics normal hepatic cord plates. Of note, compared to micro trabecular pattern (MiT), the macro trabecular (MaT) pattern shows early recurrence within one year and multiple intrahepatic metastases or distant metastases [6]. Tumours trabeculae thicker than six cells that occupy more than 50% of the entire tumour area, are defined as macro trabecular massive HCC (MTM-HCC) subtype, according to Calderaro et al. This novel subtype is associated with particular biological and molecular traits, such as high AFP serum levels and microvascular invasion (MVI); and G3 transcriptomic subgroup mutations, TP53 mutations, and FGF19 amplifications, respectively [2]. In addition, it is associated with early and overall recurrences[7]. Necrosis of trabecular structure is seen in the centre of MTM-HCC, which in the present study was defined as MaTN. We divided MTM-HCCs into MaTM (MTM-HCC without necrosis) and MaTN (MTM-HCC with necrosis). The reduced oxygen concentration that characterises larger and highly proliferating tumours is the main trigger for hypoxia-related factor expression [8]. HIF1 α is an important hypoxia-inducible gene that is mainly expressed in neoplastic tissues. Our previous studies revealed that USP22 promotes hypoxia-induced HCC stemness through a HIF1 α /USP22 positive feedback loop in TP53 inactivation [9].

Adjuvant treatment has been the standard treatment for advanced HCC, to prevent HCC recurrence and metastasis. Sorafenib, an oral multi kinase inhibitor, is the most recommended first-line systemic therapy for advanced HCC worldwide [10]. Evidence is available that sorafenib prolonged overall survival (OS) in patients with advanced HCC in an Asian-Pacific study [11]. Nevertheless, the median overall survival (OS) time of sorafenib-treated patients with unresectable HCC is only approximately 3 months longer than that of the placebo patients (10.7 vs. 7.9 months, respectively). The primary endpoint of recurrence-free survival (RFS) is also not significantly different between the sorafenib and placebo groups. (33.3 vs. 33.7 months) [12]. Therefore, it is urgent to identify tumour biomarkers, based on histopathological classification, that have can help in improving patient outcomes or therapeutic strategies.

Clinically, approximately 50 % of resected HCCs present mixed patterns, which usually include trabecular patterns plus one or two others[5]. In this study, we explored the molecular mechanism that regulates different trabeculations of MiT, MaT, MaTM, and MaTN. Using RNA sequencing data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO), we pooled selected candidate markers associated with sorafenib response and genes of interest from our previous study [9]. Selected candidate biomarkers were then analysed by immunohistochemistry, and the relationship between these proteins and sorafenib efficacy was evaluated.

Materials And Methods

TCGA and BIOSFORM cohorts in discovery stage

A total of 379 tumours with available histological slides and RNA sequencing data from the TCGA HCC cohort were included in the analysis. Morphologically, combined HCC-cholangiocarcinoma, cholangiocarcinoma, and normal liver tissue were excluded. The remaining 360 cases were kept.

The BIOSFORM cohort was a subgroup of the STORM clinical trial (NCT00692770) consisting of patients with HCC resected between 2008 and 2010. In this cohort, 83 patients were treated with sorafenib, and 105 were treated with placebo [13]. In the present study, we used transcriptomic data from 140 HCC samples for whole-genome analysis.

Analysis of RNA sequencing data from TCGA and the GEO database

Tumours were classified as MaTM if neoplastic cells arranged in a macro trabecular-massive architectural pattern (trabeculae > 6 cells thick) could be identified. If necrosis occurred in the tumour tissue, we defined it as MaTN. We included 360 tumours (MiT, n = 111; MaT, n = 41; MaTM, n = 29; MaTN, n = 23; non-trabecular structure, n = 156) with available histological slides and RNA sequencing data from the TCGA public database. We obtained transcriptomic data for 140 HCC samples treated with sorafenib derived from GEO under the accession number GSE109211. In the original study, 67 samples were treated with sorafenib (Sor) and 73 were treated with placebo (Plac). A total of 59 and 87 genes were found to lead to a good or poor prognosis, respectively, using a Cox proportional hazard model. R packages limma (3.36.5), pheatmap (1.0.12), survival (3.1-8), clusterProfiler (3.12), and VennDiagram (1.6.20) were used to screen the genes of interest.

Validation cohort

Patients who had received sorafenib treatment between January 2010 and December 2017 were identified from our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China, n = 239). Informed consent was obtained from all the patients, and the study was approved by the ethics committee of the First Affiliated Hospital of Zhejiang University. The inclusion criteria for the sorafenib group were as follows: First, those patients who were available for the clinicopathological variables: gender, age, hepatitis B Viruses (HBV) infection, serum AFP level > or $\leq$ 400 ng/ml, Child-Pugh classification (0–4 or 5–7), BCLC staging classification (A-B or C), tumour size that differed by less than 5 cm, tumour number that differed by less than 3 cm, MVI, satellite nodules, cirrhosis (Y, N), Edmondson – Steiner grade (ES grade, $\leq$ vs. $>$), histological classification (at least 50 % of the tumour area): MiT, MaT, MaTM and MaTN. Second, the tumour tissue sections were used for immunohistochemical staining. Third, recurrent and/or metastatic HCC was confirmed using imaging technology. Patients who received sorafenib before recurrence and/or metastasis were excluded from the present study. Based on these criteria, 123 patients who received sorafenib therapy were selected. Cases with a histological

appearance suggestive of a non-trabecular structure were excluded. Among them, 107 had trabecular structure, 18 cases were MiT, 55 cases were MaT, 13 cases were MaTM, and 21 cases were MaTN. Investigators retrospectively assessed treatment response at 4–6 weeks using the modified Response Evaluation Criteria in Solid Tumours (RECIST) (14) and classified these patients as having progressive disease (PD) or non-progressive disease (non-PD). The definition of progressive free survival (PFS) was calculated from the date of the first sorafenib treatment to the date of PD. OS was defined as the time interval between the date of hepatectomy and the date of death from any cause or the date of the last follow-up.

Immunohistochemistry staining

Samples were collected from HCC patients who had received sorafenib treatment after surgery, and a tissue microarray was created from 107 HCC samples. After histopathological examination, each sample was excised in one core with a 2.0 mm diameter on one tissue array. Immunohistochemical (IHC) staining was then carried out following the manufacturer's protocol for the Histostain TM-Plus kit. Antibodies used for IHC staining and dilutions of the antibodies were as follows: rabbit polyclonal against human RAP1GAP antibody (#19174-1-AP, Proteintech, USA; 1:200) and rabbit monoclonal against human HIF1 α antibody (#51608, Abcam, USA; 1:200). To evaluate the staining results, a dichotomous scoring system, positive or negative for RAP1GAP status, was used. Finally, samples were grouped as negative expression (scores ≤ 200) or positive expression (> 200). If HIF1 α was positive in the tumour, we defined it as HIF1 α positive regardless of its percentage.

Statistical analysis

The SPSS 22.0 statistical software and GraphPad Prism software version 6.0 (GraphPad Software, Inc.) were used for statistical analysis. The t-test was used to identify differentially expressed genes between the sorafenib and placebo groups. Categorical variables were compared using the χ^2 test or Fisher's exact test. The Kaplan-Meier method was employed to analyse PFS and OS, and a p value < 0.05 was considered statistically significant. Univariate Cox proportional hazards regression analysis was used to identify relevant factors associated with a response to sorafenib, in which variables with $p < 0.05$ were subsequently included in the multivariate analysis.

Results

Analysis Process of This Study

The analysis process used in this study is shown in Fig.1. To explore the specific mechanism between different trabecular patterns, we first analysed gene expression profiling data from the TCGA study according to histopathological classification, and then explored the gene expression underlying the different survival benefits of sorafenib treatment. Intersection analysis was performed using trabecular pattern-related genes in the TCGA study, the most significant genes obtained from sorafenib treatment analysis, and our genes of interest (USP22 and HIF1 α). We focused on RAP1GAP and HIF1 α for the

subsequent series of analyses, including survival and correlation of clinicopathological characteristics in HCC patients who were treated with sorafenib.

Flowchart of trabecular pattern related genes screening in TCGA cohort

Based on histopathological classification, we divided HCC into four subtypes: MiT, MaT, MaTM, and MaTN (Fig. 2A). RNA-sequencing data of 204 tumours (MiT n=111, MaT n=41, MaTM n=29, and MaTN n=23) were available and downloaded. In brief, our results showed (i) up-regulation of pathways indicative of poor prognosis, such as Ras, Rap1, IL17, TNF, AGE-RAGE, oestrogen, and toll-like receptor signalling; (ii) amino sugar and nucleotide sugar metabolism; (iii) association with immune-related processes, such as the PD1 and PDL1 pathways; (iv) ubiquitin-mediated proteolysis; (v) angiogenesis activation; and (vi) down-regulation of bile acid and carton, tryptophan, butanoate, and lipid metabolism-related pathways (Fig.2B). These gene functions were associated with (i) neutrophil activation, degranulation, and immune response, (ii) response to oxygen levels and hypoxia, (iii) sister chromatid segregation, and (iv) antigen processing and presentation (Fig.2C). Most of these functions were located in the mitochondria, respiratory chain complex, and Golgi (Fig.2D). Owing to their close association with tumour development, pathways, such as Ras signalling, tumour proliferation, angiogenesis, and microenvironment, are of greatest concern in the following steps.

Refinement of sorafenib response related genes in BIOSTORM cohort

We obtained transcriptomic data for 140 HCC samples derived from the GSE109211 dataset. In the original study, 67 patients were treated with sorafenib, and 73 were treated with placebo. A total of 59 and 87 genes were found to lead to good or poor prognosis, respectively, using a Cox proportional hazard model. Of the differentially expressed genes (fold change > mean + SD, p value <0.05), the top 100 genes are shown in a heatmap (Fig.3A). Venn plots showed that four genes were common to the non-responder sorafenib and poor prognosis groups, but not in the non-responder placebo group (Fig.3B). The volcano plot showed the detailed distribution of the four selected genes (ACO2, RAP1GAP, TOB1, and SCNN1D, Fig.3C). These four genes were included in further validation for their potential effects in response to sorafenib treatment.

Validation of sorafenib response related genes in TCGA

In addition to four sorafenib response-related genes from the BIOSTORM cohort, two others genes, HIF1 α and USP22, were included in the validation. Of the six genes (ACO2, RAP1GAP, TOB1, SCNN1D, HIF1 α , and USP22), RAP1GAP, HIF1 α , and USP22 exhibited trabecular pattern formation susceptibility.

The overall survival of patients in the RAP1GAP-, HIF1 α -, and USP22-positive groups was significantly worse than that in the negative group (Fig.4A, p =0.027, p =0.003, p =0.021, respectively). As shown in Fig.4B, we found a strict increasing trend for RAP1GAP expression in four different trabecular pattern formations, while a slight exception existed for HIF1 α and USP22. As shown in Fig.4C, we included the MVI to analyse its interaction with gene expression. In the MVI group, we found that RAP1GAP showed a

stronger association with trabecular pattern formation than in the non-MVI group, while HIF1 α showed the opposite. However, USP22 did not exhibit a clear trend in either subgroup.

Therefore, we further selected two of these candidate biomarkers, RAP1GAP and HIF1 α , for the subsequent series of analyses, including survival and correlation study of the clinicopathological characteristics and sorafenib treatment.

RAP1GAP was related with treatment response in hepatocellular carcinoma

A total of 107 trabecular-pattern HCC patients who were treated with sorafenib were included in this study. Based on histopathological classification, we divided HCCs into four subtypes: MiT (n=18,16.8 %), MaT (n=55,51.4 %), MaTM (n=13,12.1 %), and MaTN (n=21,19.6 %) (Figure 5A). RAP1GAP is one of the 63 up-regulated genes that encodes a negative regulator of the small GTPase Rap1 [15]. It is considered a tumour suppressor gene and is expressed at low levels in neoplastic tissues [16,17]. IHC staining confirmed that RAP1GAP and HIF1 α were present in the tumour tissue. Interestingly, RAP1GAP staining was also positive in the tumour sinusoid, which was defined as RAP1GAP-EC (Fig. 5B). Tumours were classified as RAP1GAP and HIF1 α positive in 20.6 % (22/107) and 12 % (9/75) of the cases, respectively. RAP1GAP-EC expression was found in 29/107 (27.1 %). As described in Table 1, we compared different subtypes with clinicopathological features, including age, gender, HBV infection, AFP, Child-Pugh classification (0-4 or 5-7), BCLC staging classification (A-B or C), tumour size, tumour number, MVI, satellite nodules, cirrhosis, tumour differentiation, and histological classification in 107 HCC cases. Patients treated with sorafenib were mainly men (91.5 %, 98/107). The main risk factor for liver disease was hepatitis B viral infection (78.5 %, 84/107). The BCLC staging classification was intermediate or advanced in 36 % of patients (85.9 %, 92/107). Elevated AFP levels were detected in 30.8 % of cases (33/107). Notably, histological classification was significantly correlated with MVI and AFP ($p<0.05$). As described in Fig.5C, histological classification was significantly correlated with MVI ($p<0.05$), especially in the MaTN subtype. RAP1GAP positive staining was found in 16.6 % (3/18) MiT, 20% (11/55) MaT, 30.7% (4/13) MaTM, and 19 % (4/21) MaTN. RAP1GAP-EC positive was seen in 11.1 % (2/18) MiT, 14.5 % (8/55) MaT, 30.7 % (4/13) MaTM, and 71.4 % (15/21) MaTN. HIF1 α positive staining was found in 8.3 % (1/12) MiT, 13.2 % (5/38) MaT, 11.1 % (1/9) MaTM, and 12.5 % (2/16) MaTN. RAP1GAP-EC expression was associated with histological classification, but not with RAP1GAP and HIF1 α expression ($p>0.05$, Fig. 5D). Furthermore, survival analysis showed that PFS and OS of patients with MaTN were significantly shorter than those of the other groups ($p=0.001$, for PFS, $p=0.003$ for OS Fig. 6 A, D). RAP1GAP expression was not associated with PFS and OS ($p=0.128$ for PFS, $p=0.153$ for OS; Fig.6 B, E), but RAP1GAP-EC was associated with PFS and OS ($p<0.001$ for PFS, $p<0.001$ for OS; Fig. 6 C, F). Variables associated with tumour progression in univariate analysis were BCLC staging classification (HR= 2.577, $p=0.003$), tumour number (HR=1.631, $p=0.033$), MVI (HR=1.894, $p=0.003$), histological classification (HR=1.305, $p=0.018$), and RAP1GAP-EC expression (HR=2.105, $p=0.001$) (Table 2). Multivariate analysis showed that the BCLC staging classification (HR=1.964, $p=0.041$) and RAP1GAP-EC expression (HR=1.886, $p=0.010$) were independent predictors of PFS. Subsequent univariate analyses confirmed that RAP1GAP-EC expression was associated with OS (HR=2.148, $p=0.001$), and variables associated with

tumour progression in univariate analysis were AFP (HR= 1.563, p=0.043), BCLC staging classification (HR= 2.029, p=0.029), tumour size (HR= 1.930, p=0.002), MVI (HR= 1.951, p=0.002), and histological classification (HR=1.349, p=0.010). Multivariate analysis showed that RAP1GAP-EC expression (HR= 1.845, p=0.009), tumour size (HR= 1.656, p=0.021) and MVI (HR= 1.652, p=0.023) were independent predictors of OS (Table 2).

Discussion

Microscopically, the WHO recognises HCC architectural variants as trabecular, pseudoglandular, solid, and macrotrabecular [5]. Trabecular architecture can be categorized as MiT, MaT, or MaTM. In practice, necrosis is frequently found in the MaTM subtype. Therefore, in this study, we divided MTM-HCC into MaTM (MTM-HCC without necrosis) and MaTN (MTM-HCC with necrosis). Additionally, we defined a new group, MaTN, which is the combination of MaTM and necrosis, in contrast to naïve MaTM without necrosis. In the present study, we attempted to identify genes related to different trabecular structures and their consequent influence on therapy in patients with HCC. The MaT subtype has recently been shown to exhibit higher AFP levels, early recurrence, and worse overall survival than the MiT subtype [6]. MTM-HCC is characterised by a predominant > 50 % MaT (trabeculae of more than 6 cells thick) architectural pattern and is associated with an aggressive phenotype and particular biology (high AFP in serum levels and strong angiogenesis activation) [7, 8]. Microscopically, we linked gene expression data to H&E images from TCGA. According to this analysis, some gene signatures identified in driver genes were associated with the formation of trabecular structures (such as RAS and Rap1 signalling pathways, ubiquitin-mediated proteolysis, and angiogenesis activation). These functions were associated with response to oxygen levels, and sister chromatid segregation. To further study whether these genes were related to chemotherapy response, we used the GEO database to analyse the relationship between RAP1GAP and sorafenib treatment. In the past few years, sorafenib has been widely used as a systemic drug for advanced HCC because of its inhibition of tumour cell proliferation and angiogenesis, and proven efficacy in advanced HCC [12]. Although some researchers have tried to identify biomarkers for predicting the clinical benefit of sorafenib treatment in HCC [18], none have been applied clinically yet. Using a molecular-driven selection of biomarkers, we identified genes associated with sorafenib response. RAP1GAP, TOB1, ACO2, and SCNN1D expression was selectively up-regulated in tumour tissues from the sorafenib non-responder group.

Along with the promising genes obtained from the sorafenib treatment analysis, extra genes of interest, HIF1 α and USP22, were investigated in the TCGA cohort. Interestingly, we found that the negative regulatory gene Rap1 (RAP1GAP) can positively regulate different trabecular patterns. We found that the expression of RAP1GAP gradually increased from the MiT to MaTN pattern. Moreover, the overall survival of patients in the high RAP1GAP, HIF1 α , and USP22 expression groups was significantly worse than that of patients in the low expression groups. In our previous studies, we found that USP22 was significantly associated with MVI and unfavourable HCC progression [19]. It directly interacts with SIRT1 and promotes 5-FU drug resistance by activating the SIRT1/AKT/MRP1 pathway [20]. USP22 promotes hypoxia-induced HCC stemness via a HIF1 α /USP22 positive feedback loop in TP53 inactivation [9].

However, our results show that USP22 expression did not increase from the MiT to MaTN pattern, but was strongly associated with the MaTM structure.

To further clarify whether RAP1GAP and HIF1 α were related to the trabecular structure and efficacy of sorafenib, we analysed the samples by using immunohistochemistry. IHC staining confirmed that the tumour tissue and sinusoids were positive for RAP1GAP. Our results showed that RAP1GAP-EC was associated with the trabecular pattern. However, RAP1GAP and HIF1 α expression levels were not related to the trabecular pattern. In addition, sorafenib-treated patients with positive endothelial-RAP1GAP staining tended to have worse outcomes. Moreover, multivariate analysis showed that EC-RAP1GAP expression was an independent predictor of PFS and OS, indicating that RAP1GAP is associated with sorafenib treatment. Interestingly, various studies suggest that RAP1GAP may function as a tumour suppressor because it is frequently lost in several tumour types, such as colorectal cancer [21], endometrial cancer [16], and gastric cancer [17]. Li et al. also found that the effects of RAP1GAP on human umbilical cord vein endothelial cells inhibited proliferation via the ERK/MAPK signalling pathway [22]. A different study showed that depressing Rap1 activity by expressing RAP1GAP led to the disassembly of these junctions and increased the permeability of endothelial cells [23]. Increased Rap1a activity specifically inhibits choroidal neovascularization [24].

We hypothesised that RAP1GAP is associated with tumour resistance in the following ways: first because tumour blood vessels carry nutrients and oxygen to tumours and serve as pathways for metastasis, they play a key role in cancer progression. RAP1GAP promotes angiogenesis, which contributes to tumour growth or metastasis and leads to chemotherapy resistance. Second, Rap1 is involved in the formation and stabilisation of E-cadherin-based cell-cell adhesion in epithelial cells [25]. RAP1GAP is a negative regulator of Rap1, and might be involved in the formation of vessels encapsulating tumour clusters (VETCs). The VETCs are characterised by the presence of CD34 + vessels completely encapsulating tumour clusters. They are associated with worse survival and were significantly enriched in MTM-HCC [26]. Contrary to the findings of Fang et al., who found that sorafenib therapy was more effective in VETC-HCC [27], our data shows that the survival was worse in the MaTN group after sorafenib treatment. This might be because VETC was not observed in endothelial cells, tumour cells, or heterogeneity of blood vessels. RAP1GAP is involved in the formation of VETC to promote tumour metastasis, which is an important factor in sorafenib resistance.

Conclusions

In this study, we observed that RAP1GAP as a reliable microenvironment immunohistochemical marker of the trabecular structure and sorafenib treatment response. These results represent a step towards the implementation of morpho-molecular HCC subtyping into clinical therapy. The mechanism underlying the potential relationship between drugs and the trabecular structure is of value in future studies.

Abbreviations

HCC: hepatocellular carcinoma, TCGA; The Cancer Genome Atlas, GEO: Gene Expression Omnibus, PD: progressive disease, PFS :progressive free survival, OS: overall survival, WHO: World Health Organization, MiT: Micro trabecular, MaT: Macro trabecular, MTM-HCC: macro trabecular massive HCC, MaTM: MTM-HCC without necrosis, MaTN: MTM-HCC with necrosis, MVI: microvascular invasion, AFP: Alphafoeto protein, HBV: hepatitis B virus, BCLC: Barcelona Clinic of Liver Cancer, ES grade: Edmondson-Steiner grade, ERCIST: evaluation criteria in solid tumours, VETC: vessels encapsulating tumour clusters

Declarations

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Author contributions

XX conceived and coordinated the project. XYW, SSZ and XX designed the research study. XW, KKS,YFY, WWQ, ZKL and BX performed experiments and acquired data. KW and BHP analysed and interpreted data. XW, SBL and DL wrote the paper and critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The experiment was approved by the ethics committee of the First Affiliated Hospital of Zhejiang University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, table 1, 2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Analysis workflow of this study

Figure 2

Related pathways associated with the formation of trabecular pattern. A: Histopathological classification: MiT, MaT, MaTM and MaTN. The number of cells was increased from MiT to MaTN. B: The pathway

enrichment. C: The molecular function of these genes. These gene functions were associated with neutrophil activation, degranulation, and immune response, response to oxygen levels and hypoxia, sister chromatid segregation and antigen processing and presentation. D: Location of these genes. Most of these functions were located in the mitochondria, respiratory chain complex and Golgi.

Figure 3

Up-regulated and down-regulated genes characteristics of 'sorafenib RFS responders' and 'non-responders'. A: Heatmap of dis-regulated genes of 'sorafenib RFS responders' and 'non-responders'. B: Venn plots showed that 4 genes were included in further assays for their potential in process of fail to respond to sorafenib or lead to poor prognosis. C: Heat map showed that the distribution of four genes (ACO2, RAP1GAP, TOB1 and SCNN1D).

Figure 4

Validation of related genes in TCGA. A: Survival analysis showed that overall survival of patients with high expression of RAP1GAP, HIF1 α and USP22 was significantly shorter than overall survival of those with lower expression. B: RAP1GAP, HIF1 α and USP22 expression of different trabecular patterns. RAP1GAP gradually increased in the four subtypes, HIF1 α expression was slightly down-expression in MaTM subtype, and expression of USP22 was the highest in MaTN subtype. C: RAP1GAP, HIF1 α and USP22 expression in non MVI group and MVI group in different trabecular pattern. According to the MVI group, we found that RAP1GAP gradually increased in MVI and four subtypes, indicating that RAP1GAP is closely related to MVI, but we did not find this rule in HIF1 α and USP22 group.

Figure 5

Morphological features, MVI, RAP1GAP and HIF1α immunostaining of HCC in different trabecular pattern. A: Histopathological features of different trabecular pattern: MiT, MaT, MaTM and MaTN. The number of cells was increased from MiT to MaTN. B: RAP1GAP and HIF1α immunostaining of different trabecular pattern in tumour tissues and sinusoid. C: The relationship between MVI and different trabecular pattern. Histological classification was significantly correlated with MVI, especially in the MaTN subtype. D: The proportion of RAP1GAP, EC-RAP1GAP, HIF1α expression in different trabecular pattern. RAP1GAP-EC expression was associated with histological classification, but not with RAP1GAP and HIF1α

expression.* $p<0.05$, MaTN vs. MaTM; # $p<0.05$, MiT vs. MaTN; ** $p<0.05$, MaTN vs. MaT; NS, not statistically significant.

Figure 6

Kaplan–Meier survival curves of postoperative HCC patients stratified by histopathological classification, RAP1GAP and RAP1GAP-EC expression. A, B, C: PFS for HCC patients with regard to histopathological classification, RAP1GAP expression, and RAP1GAP-EC expression. Survival analysis showed that PFS of patients in MaTN was significantly shorter than that in other groups. RAP1GAP-EC expression was associated with PFS, but RAP1GAP expression was not related to PFS. D, E, F: Overall survival curves for HCC patients with regard to histopathological classification, RAP1GAP-EC expression but not related to RAP1GAP expression.

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