

# Comprehensive Investigation of a Tyrosine Kinase Inhibitor-Resistant Gene *Zeste White 10* in Hepatocellular Carcinoma

Qing Ling Huang<sup>a, d</sup>, Guan Lan Zhang<sup>b, c, d</sup>, Qin Yan Su<sup>a</sup>, Bang Teng Chi<sup>a</sup>, Qian Lin<sup>a</sup>,  
Shi De Li<sup>a</sup>, Han He<sup>a</sup>, Yu Lu Tang<sup>a</sup>, Yi Wu Dang<sup>a</sup>, Gang Chen<sup>a, e</sup>,  
Rong Quan He<sup>c, e</sup>

## Abstract

**Background:** Tyrosine kinase inhibitors (TKIs) are first-line therapies for hepatocellular carcinoma (HCC), but the drug resistance restricts the long-term clinical outcomes. This study aimed to investigate the expression patterns and possible clinical significance of a TKI-resistant gene *zeste white 10* (*ZW10*) in HCC.

**Methods:** Clustered regularly interspaced short palindromic repeats (CRISPR) screening was conducted to obtain TKI-resistant genes. Pan-cancer analysis was employed to analyze the expression landscape of the critical TKI-resistant gene *ZW10*. Transcriptional expression data for *ZW10* were obtained from 76 centers, including 3,312 HCC samples and 2,703 noncancerous tissues. A summary receiver operating characteristic (SROC) curve was built to evaluate *ZW10* expression characteristics in HCC. Unpaired two-sample Wilcoxon method was conducted to analyze *ZW10* expression levels in HCC of various etiologies. Univariate Cox method was employed to assess the prognostic value of *ZW10*. Moreover, the gene function within HCC cell lines, the TKI treatment responses, key pathways, and tumor microenvironment of *ZW10* were bioinformatically investigated. Drug prediction and molecular docking techniques were used to explore the potency of *ZW10* as a novel therapeutic target.

**Results:** The abundance of small guide RNA (sgRNA) corresponding

to *ZW10* gene was decreased in the whole genome CRISPR knock-out library (LogFC = -1.19), indicating that *ZW10* may participate in TKI resistance. The differential expression landscape of *ZW10* was found in various malignancies including HCC, which was associated with poorer prognosis. Pooled standardized mean difference (SMD) of *ZW10* mRNA expression was 0.47 (95% confidence interval (CI): 0.32 - 0.63), the area under SROC was 0.76 (95% CI: 0.72 - 0.79), the sensitivity was 0.63 (95% CI: 0.53 - 0.72), and the specificity was 0.77 (95% CI: 0.67 - 0.84). *ZW10* was investigated significant for the growth of HCC cells. Nucleocytoplasmic transport was the possible pathway that *ZW10* involved. High level of *ZW10* was reversely associated with TKI responses and the abundance of immune cell infiltration. Mocetinostat and capecitabine were predicted to be the potential inhibitors targeting *ZW10* with a minimum binding energy of -8.2 and -7.1 kcal/mol, respectively.

**Conclusions:** *ZW10* is considered a TKI-resistant and tumor-supportive gene, which is also a promising novel prognostic biomarker for HCC or a therapeutic target for overcoming TKI resistance.

**Keywords:** Hepatocellular carcinoma; *Zeste white 10*; Tyrosine kinase inhibitor; Resistance; Nucleocytoplasmic transport

## Introduction

Systemic antitumor therapy is the preferred treatment for advanced hepatocellular carcinoma (HCC) [1]. Tyrosine kinase inhibitors (TKIs) play an important role in first-line and second-line treatment for HCC [2]. However, intrinsic and acquired drug resistance limits the survival benefit of TKIs, extending patient survival by only a few months and restricting long-term clinical outcomes [2, 3]. Therefore, identifying effective biomarkers for guiding TKI-stratified therapy and targeting TKI resistance is crucial for improving patient prognosis.

Clustered regularly interspaced short palindromic repeats (CRISPR) is a powerful genome-wide tool that utilizes small guide RNAs (sgRNAs) to modify genes across the entire genome [4]. Within the CRISPR knockout library, knocking out a drug-resistant gene prevents cell survival under drug treatment, causing lower sgRNA abundance, as detected via DNA

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<sup>a</sup>Department of Pathology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

<sup>b</sup>Department of Gastroenterology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

<sup>c</sup>Department of Medical Oncology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

<sup>d</sup>These authors contributed equally to this work.

<sup>e</sup>Corresponding Author: Gang Chen and Rong Quan He, Department of Pathology and Department of Medical Oncology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China. Email: chengang@gxmu.edu.cn and herongquan@gxmu.edu.cn

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sequencing. This study employed CRISPR screening to uncover TKI-sensitive and resistant genes, underscoring its importance in drug resistance research. Notably, *zeste white 10* (*ZW10*) was identified as a critical TKI-resistant gene and a potential therapeutic target for overcoming TKI resistance.

*ZW10* plays a crucial role in spindle assembly checkpoint signaling during mitotic chromosome segregation, guiding spindle assembly. Phosphorylation of *ZW10* by PLK1 ensures proper chromosome segregation and contributes to the inactivation of the spindle checkpoint, preventing premature mitotic exit [5, 6]. Despite its pivotal function, *ZW10* remains underexplored in the field of cancer research, with limited reports indicating its upregulation in colorectal cancer and showing that *ZW10* deficiency inhibits cell proliferation [7]. Additionally, loss of *ZW10* promotes melanoma resistance to alkylating agents [8]. However, no studies have yet investigated the relationship between *ZW10* and HCC, presenting an intriguing avenue for future research.

This research was designed to ascertain the TKI-resistant genes through an in-house CRISPR screen experiment, while also evaluating the expression characteristics in HCC along with the prospective clinical value of the critical TKI-resistant gene *ZW10* in HCC. We have collected multicenter mRNA datasets to integrate the expression status of *ZW10* gene in HCC tissue. The expression level of *ZW10* was analyzed in HCC patients with different etiology backgrounds. Prognostic analyses and biological mechanism analysis were used to further investigate the clinical value of *ZW10*, while molecular docking techniques were used to explore the possibility of *ZW10* as an innovative therapeutic target.

## Materials and Methods

### Genome-wide CRISPR knockout screen

For the materials used in this experiment, Huh7 cell line was sourced from the Cell Bank of the Chinese Academy of Sciences. The human genome-wide CRISPR/Cas9 knockout library v2 (GeCKO v2) covering 65,386 specific sgRNAs (Addgene company, number: 1000000048) and lentiCRISPRv2 vectors carrying Cas9 and puromycin resistance (Addgene company, number: 1000000048) was purchased to construct gene-deficient cell models. During the experiment, to ensure every cell was infested by only one specific sgRNA, the multiplicity of infection was set to less than 0.3. After lentivirus transfection, puromycin treatment was performed to screen the whole genome knockout HCC cells. Subsequently, the HCC cells infected with the lentivirus were treated with anlotinib, a TKI drug produced by Jiangsu Zhengda Tianqing Company, for 21 days. The DNAs of the survived HCC cells were extracted and sequenced by Beijing Noa Zhiyuan Technology Company. To ensure the reliability of the final data, joint data, unknown data, and low-quality data were eliminated and quality control was carried out. Ultimately, the MAGeCK algorithm was utilized for quality assessment and gene expression profiling in the CRISPR/Cas9 genome-wide knockout analysis. Based on the negative screening strategy, the genes with lower sgRNA

abundance were considered to be TKI-resistant. The research received Institutional Review Board Approval from the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University and Institutional Animal Care and Use Committee Approval from Guangxi Medical University.

### Pan-cancer analysis

To broaden the biological context for investigating *ZW10* and to validate its significance across diverse cancers, the transcriptional data and matched clinical information for 33 solid tumors were sourced from The Cancer Genome Atlas (TCGA) database [9]. The differential expression of *ZW10* mRNA and its correlation with survival prognosis among various tumors were examined. UALCAN [10] database and The Human Protein Atlas (THPA) database [11] were utilized to demonstrate the *ZW10* protein levels within solid tumors.

### Gene chip or RNA-sequencing dataset collection

Given the distinct biological nature of HCC compared to other cancer types, this study required further investigation into the impact of *ZW10* in HCC to validate the findings observed in the pan-cancer analysis. The Gene Expression Omnibus (GEO) database [12], Sequence Read Archive (SRA) database [13], ArrayExpress database [14], Oncomine database [15], TCGA and the Genotype-Tissue Expression (GTEx) database [16] were retrieved to collect mRNA datasets. The search strategy was as follows: liver neoplasm OR hepatic neoplasm OR hepatocellular cancer OR hepatocellular carcinoma OR hepatic cancer OR hepatic carcinoma OR liver cancer OR cancer of liver. Datasets would be included if the following criteria were met: 1) *Homo sapiens*; 2) The datasets were mRNA sequencing data in human tissue, containing HCC tissue group and non-cancer hepatic tissue group; 3) The number of HCC samples and non-cancer hepatic samples in each dataset was not less than 3. Datasets would be eliminated for the following conditions: 1) Samples were originated from cell line; 2) Samples were intervened by heat treatment, gene editing or drug treatment; 3) Samples belonged to other subtypes of liver cancer such as cholangiocarcinoma, mixed liver cancer or heterozygous liver cancer; 4) The data could not be obtained or calculated. After data screening, the datasets from the same sequencing platform were merged and batch effects were removed. Besides,  $\log_2(x + 1)$  normalization was performed for the datasets with large data fluctuation ranges.

### Pooled analysis

The standardized mean difference (SMD) was integrated to reflect the mRNA level of *ZW10* in HCC through STATA version 17. The overall heterogeneity was estimated using the  $I^2$  statistic and Q test. If  $I^2$  is greater than 50% and Q test has a P value lower than 0.10, the overall heterogeneity is deemed obvious, requiring the use of a random effect model

for SMD combination. A summary receiver operating characteristic (SROC) curve was pictured, along with the computation of the area under the curve (AUC) to further verify *ZW10* expression in HCC. Egger's test was utilized to detect publication bias.

### Etiology stratification analysis

To explore the relationship between *ZW10* and HCC of different etiologies, statistical analyses were conducted to assess the expression levels of *ZW10* mRNA across various HCC subgroups. These subgroups included alcohol consumption, hepatitis (B or C), hepatitis B, hepatitis C, the combination of alcohol consumption and hepatitis (B or C) and non-alcoholic fatty liver disease. The expression data of *ZW10* and clinical information of HCC patients used were downloaded from TCGA database. Unpaired two-sample Wilcoxon method was employed for these analyses.

### Clinical significance and prognosis

TCGA database provided the prognostic data for HCC patients, where samples lacking complete clinical data were deleted, and parameters containing excessive unknown information were excluded from the analysis. Univariate Cox regression method was utilized to compute the hazard ratios (HRs) of *ZW10* transcriptional expression value, age, gender, race, primary tumor stage (pT stage) as well as the American Joint Committee on Cancer (AJCC) stage, and assess the risk of the above factors for HCC death. If an HR is greater than 1, it indicates that the corresponding factor is a risk factor for the occurrence of the event, otherwise is protective. Wilcoxon method was employed to analyze the link between *ZW10* mRNA levels with the clinicopathologic features including age, gender, race, pT stage, and AJCC stage. The package "survminer" in R software was employed to compute the optimum cutoff, thereby categorizing all the samples into *ZW10*-high and *ZW10*-low level groups based on the expression value. The Kaplan-Meier curves combined with the log-rank test were employed to investigate the association between *ZW10* transcriptional level with the overall survival time. When the Kaplan-Meier curves cross, the log-rank test will be unsuitable and replaced with a two-stage test [17].

### Gene interference effect

Gene dependence refers to the necessity of genes for cell colony and proliferation. The DepMap team used genome-wide RNAi or CRISPR knockout techniques to analyze the impacts of different gene knockouts on the growth of cancerous cell lines. This study downloaded the "CRISPR (DepMap 22Q2 Public+Score Chronos)" data, extracted the gene effect value of *ZW10*-deficiency on 20 HCC cell lines and visualized the result by a scatter plot. A gene effect value less than 0 indicates that the effect of gene knockout is to inhibit cell viability.

### Investigation of *ZW10* expression and TKIs responses

To comprehensively understand *ZW10*'s potential contribution to resistance against multiple TKI drugs, the authors utilized the "pRRophetic" package to explore the relationship between *ZW10* transcription levels and the half-maximal inhibitory concentration (IC50) of various TKI components. The cohort of *ZW10* sequencing data was standardized using the transcripts per million (TPM) method based on the LIHC-TCGA dataset. Dependent upon the average of *ZW10* transcriptional level, the samples were categorized into *ZW10*-high and *ZW10*-low level groups, and the relationships between *ZW10* and the effectiveness of TKI drugs were explored using independent samples *t*-test and Pearson analysis.

### Genes with differential expressions and the corresponding biological pathways

The differentially upregulated genes of 371 HCC tissues compared with 50 non-HCC tissues were calculated by "limma" package, with logFC > 1 as the reference standard. Secondly, Pearson correlation coefficients between *ZW10* gene expression and other gene expression were calculated, and the genes exhibiting a Pearson correlation coefficient greater than 0.75 and a P value lower than 0.05 were identified as strongly positive correlation genes of *ZW10*. The intersepective genes between differentially upregulated genes and *ZW10* strongly positive correlation genes were input into DAVID database [18] for biological mechanism analysis [19].

### Tumor microenvironment exploration

The "xCell" is a tool that can predict immune cell abundance in an individual sample through single-sample Gene Set Enrichment Analysis (ssGSEA), founded upon gene expression cohort. Sixty-four cell types, including innate and adaptive immune-associated cells, hematopoietic progenitor cells, epithelial cells, and stromal cells that secrete various factors to regulate the tumor microenvironment, can be predicted [20]. In this study, the "xCell" package was utilized to estimate the tumor microenvironment characteristics of 371 HCC samples. Moreover, the differences in tumor microenvironment characteristics under different *ZW10* expression levels were compared with the Wilcoxon test, and the correlations between the *ZW10* expression level and the tumor microenvironment cells were investigated using the Spearman method.

### Drug prediction and molecular docking simulation

Connectivity Map Linked User (CLUE) database [21] records the gene expression profile of various cell lines after dosing with compounds. The *ZW10* strongly positive correlation genes were input into the "Query" option to identify compounds that make the tumor cell expression profiles resemble the input gene profile. A similarity score was used to show the similarity between

the effect of small molecule compounds and the input gene set. A positive score indicates that small molecule compounds have a positive effect on the input gene set, otherwise the opposite effect. The compound exhibiting the highest negative score was screened as a candidate inhibitor for *ZW10*, and the molecular docking simulation between the compound and *ZW10* protein structure was performed through Autodock Tools 1.5.6 and Vina tool to analyze the affinity. Although first- and second-line treatments for HCC primarily rely on TKIs, clinical trials have demonstrated that capecitabine shows potential in second- and third-line treatments for HCC. Based on this, molecular docking was further conducted to investigate the binding interaction between *ZW10* and capecitabine (PubChem CID: 60953) [22].

### Statistical analyses

The statistical software utilized in this study included R (released version 4.1.1), STATA (released version 17), as well as GraphPad Prism (released version 8.0.2). Wilcoxon test and independent samples *t*-test were employed in the difference analyses between the two groups. Univariate Cox regression method was utilized to compute the HR values and investigate the dangerous factors. The Kaplan-Meier curve, the log-rank test or the two-stage test were conducted for survival prognosis analyses. The Pearson and Spearman methods were employed for correlation analyses. Statistical significance is noted if a *P* value is less than 0.05.

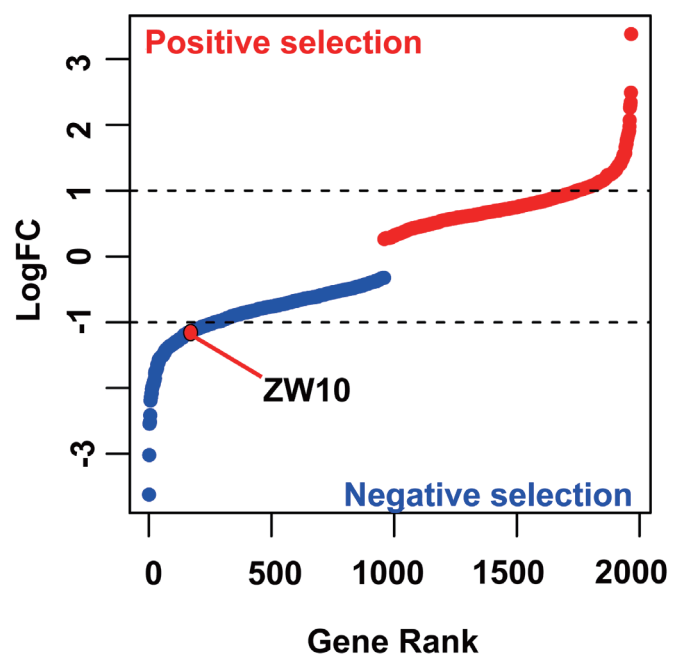
## Results

### Positive and negative CRISPR screen

DNA sequencing and biological analysis were performed on the survived cells. In the gene profile of the TKI treatment group, the abundance of sgRNA corresponding to *ZW10* was decreased (LogFC = -1.19, *P* = 0.01, Fig. 1), indicating that the HCC cells with *ZW10*-defect were more sensitive to the TKI and likely to die.

### *ZW10* expression landscape in various malignancies

As exhibited in Figure 2a, the transcriptional level of *ZW10* was differentially elevated in BLCA, CESC, CHOL, COAD, ESCA, GBM, HNSC, LIHC, LUAD, LUSC, READ, STAD, and UCEC, and differentially decreased in KIRC, KIRP, and THCA. For the protein expression, *ZW10* was highly detected in BRCA, COAD, UCEC, LUAD, HNSC, GBM, and LIHC, and weakly detected in KIRC and PAAD (Fig. 2b). Microscopically, immunohistochemical (IHC) pictures showed that the *ZW10* protein was located in the cytoplasm. Compared with the normal tissue, the cells in HCC tissue had enlarged nuclei and higher staining density (Fig. 2c). According to the outcomes of univariate Cox regression analyses, *ZW10* was related to the poorer overall survival of ACC, BLCA, KICH, LGG, LIHC, PAAD, PRAD, and SARC (Fig. 3a), poorer disease-



**Figure 1.** The positive and negative screen of CRISPR screen experiment. CRISPR: clustered regularly interspaced short palindromic repeats.

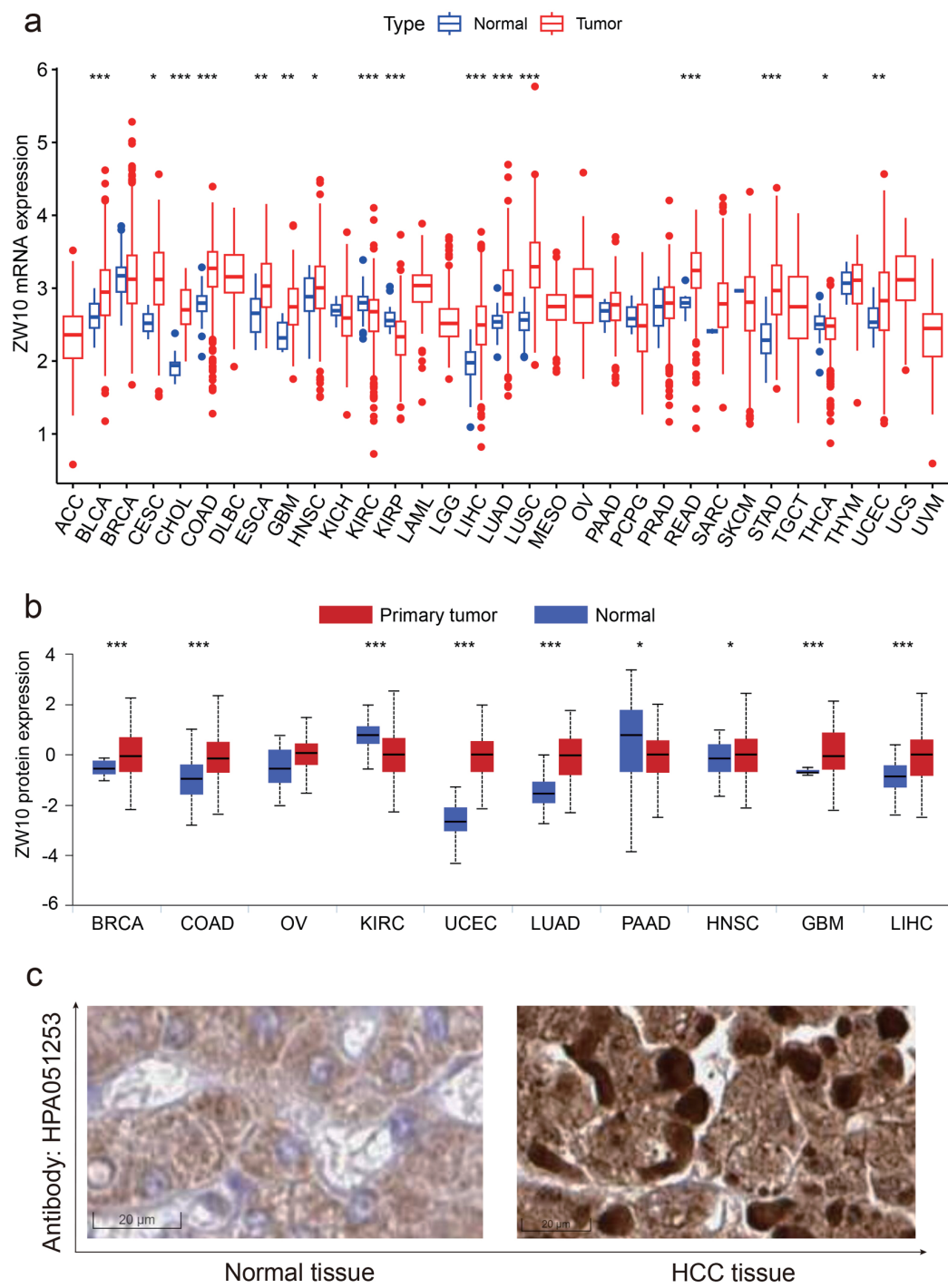
specific survival of ACC, BLCA, KICH, LGG, LIHC, PAAD, and SARC (Fig. 3b), poorer disease-free interval of HNSC and PAAD (Fig. 3c), and progression-free interval of ACC, BLCA, HNSC, KICH, LGG, LIHC, and PAAD (Fig. 3d).

### *ZW10* expression and discrimination efficacy

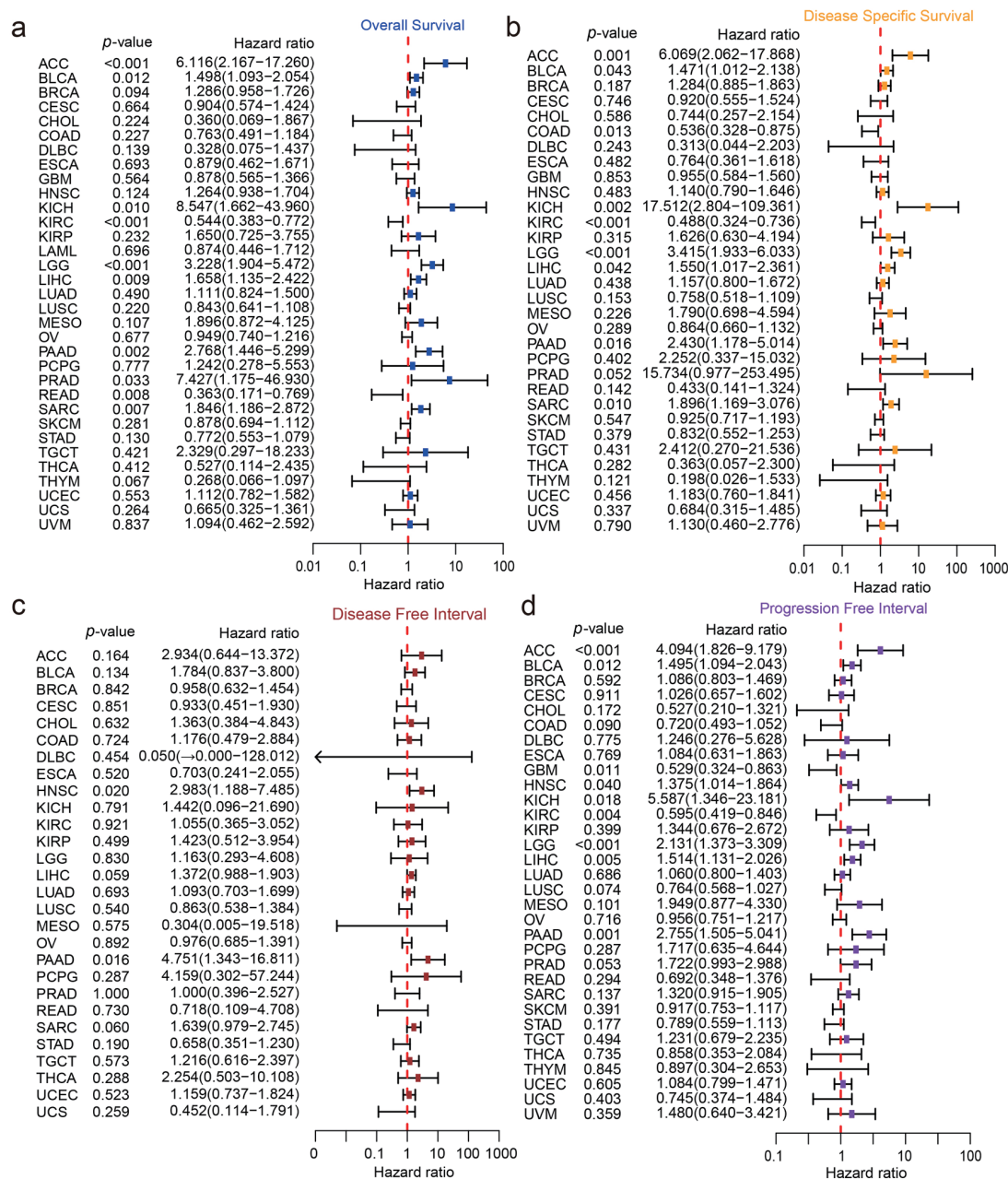
Totally 38 datasets containing 3,312 HCC samples and 2,703 non-HCC samples were included from 76 research centers. Based on the results of  $I^2$  statistics value and the Q test (Fig. 4a), the overall heterogeneity of the integrated analysis was significant ( $I^2 = 82.9\%$ ,  $P < 0.01$ ) and the random effect was adopted to combine the SMD. The mean value and standard deviation were severally used to describe the central tendency and dispersion of the *ZW10* expression level in HCC group compared to non-HCC group (Supplementary Material 1, [wjon.elmerpub.com](http://wjon.elmerpub.com)). The expression level of *ZW10* gene exhibited an obvious elevation in HCC group (SMD = 0.47, 95% CI: 0.32 - 0.63). The AUC was 0.76 (95% CI: 0.72 - 0.79). The sensitivity and specificity of *ZW10* in differentiating HCC were correspondingly 0.63 (95% CI: 0.53 - 0.72) and 0.77 (95% CI: 0.67 - 0.84, Fig. 4b). The included datasets had no obvious publication bias ( $P = 0.115$ , Fig. 4c). All of the aforementioned results indicated an upregulation of *ZW10* expression in HCC tissues.

### Differential expression of *ZW10* in different etiological backgrounds of HCC

Compared to the group without causal risk factors, subgroups



**Figure 2.** The expression level of *ZW10* within primary malignancies. (a) mRNA expression status. (b) Protein expression status. (c) Immunohistochemical staining picture. ACC: adrenocortical carcinoma; BLCA: bladder urothelial carcinoma; BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: cholangiocarcinoma; COAD: colon adenocarcinoma; DLBC: lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: esophageal carcinoma; GBM: glioblastoma multiforme; HNSC: head and neck squamous cell carcinoma; KICH: kidney chromophobe; KIRC: kidney renal clear cell carcinoma; KIRP: kidney renal papillary cell carcinoma; LAML: acute myeloid leukemia; LGG: brain lower grade glioma; LIHC: liver hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; MESO: mesothelioma; OV: ovarian serous cystadenocarcinoma; PAAD: pancreatic adenocarcinoma; PCPG: pheochromocytoma and paraganglioma; PRAD: prostate adenocarcinoma; READ: rectum adenocarcinoma; SARC: sarcoma; SKCM: skin cutaneous melanoma; STAD: stomach adenocarcinoma; TGCT: testicular germ cell tumors; THCA: thyroid carcinoma; THYM: thymoma; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma; UVM: uveal melanoma.



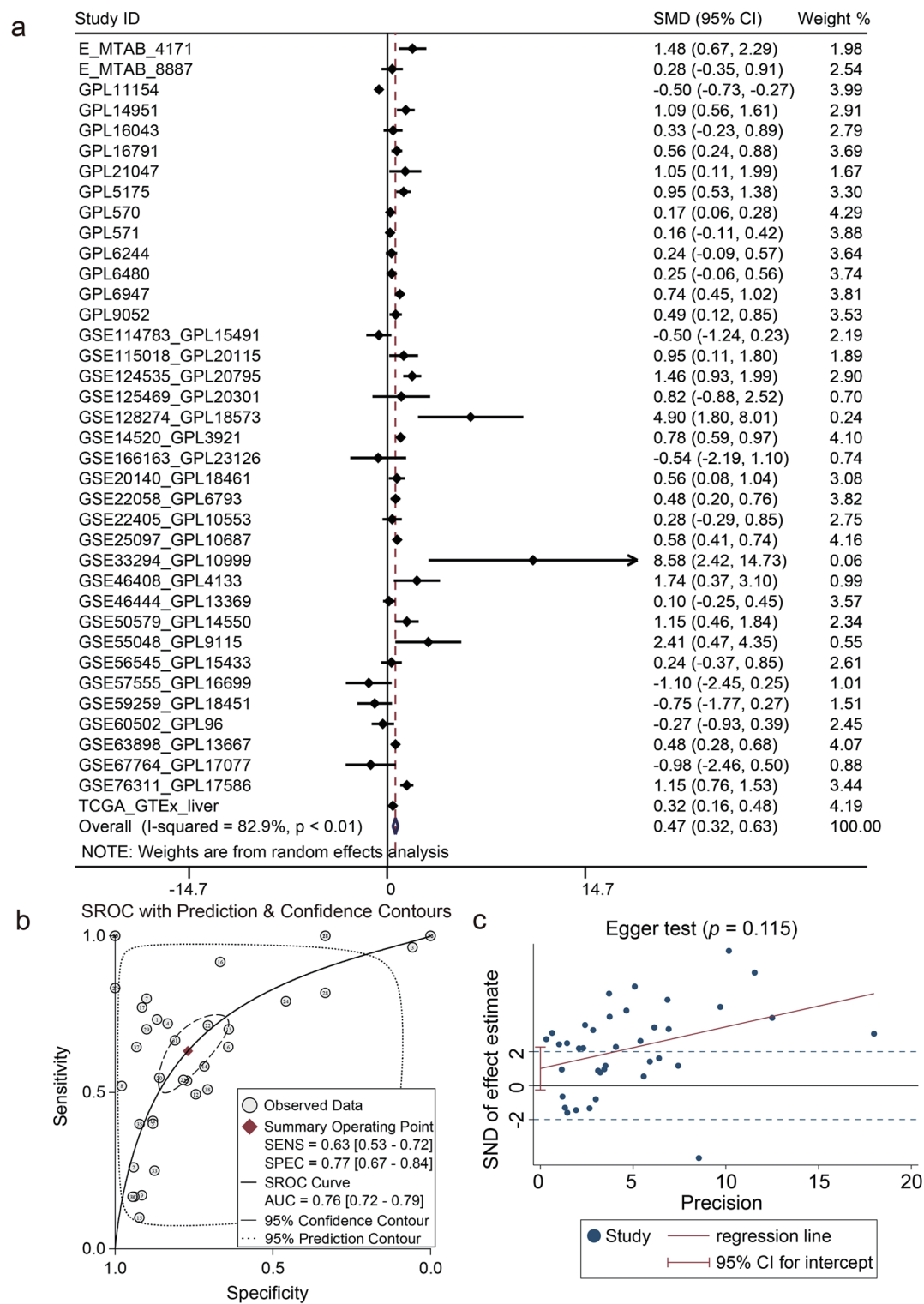
**Figure 3.** The survival analysis of *ZW10* across various cancers. (a) Overall survival analysis. (b) Disease-specific survival analysis. (c) Disease-free interval analysis. (d) Progression-free interval analysis.

with alcohol consumption, hepatitis (B or C), hepatitis B, hepatitis C, the combination of alcohol consumption and hepatitis (B or C), non-alcoholic fatty liver disease and paraneoplastic hepatitis all exhibited significantly higher *ZW10* expression in HCC (Fig. 5a-g). However, there was no significant difference in the magnitude of this increased expression among these subgroups.

Prognosis value

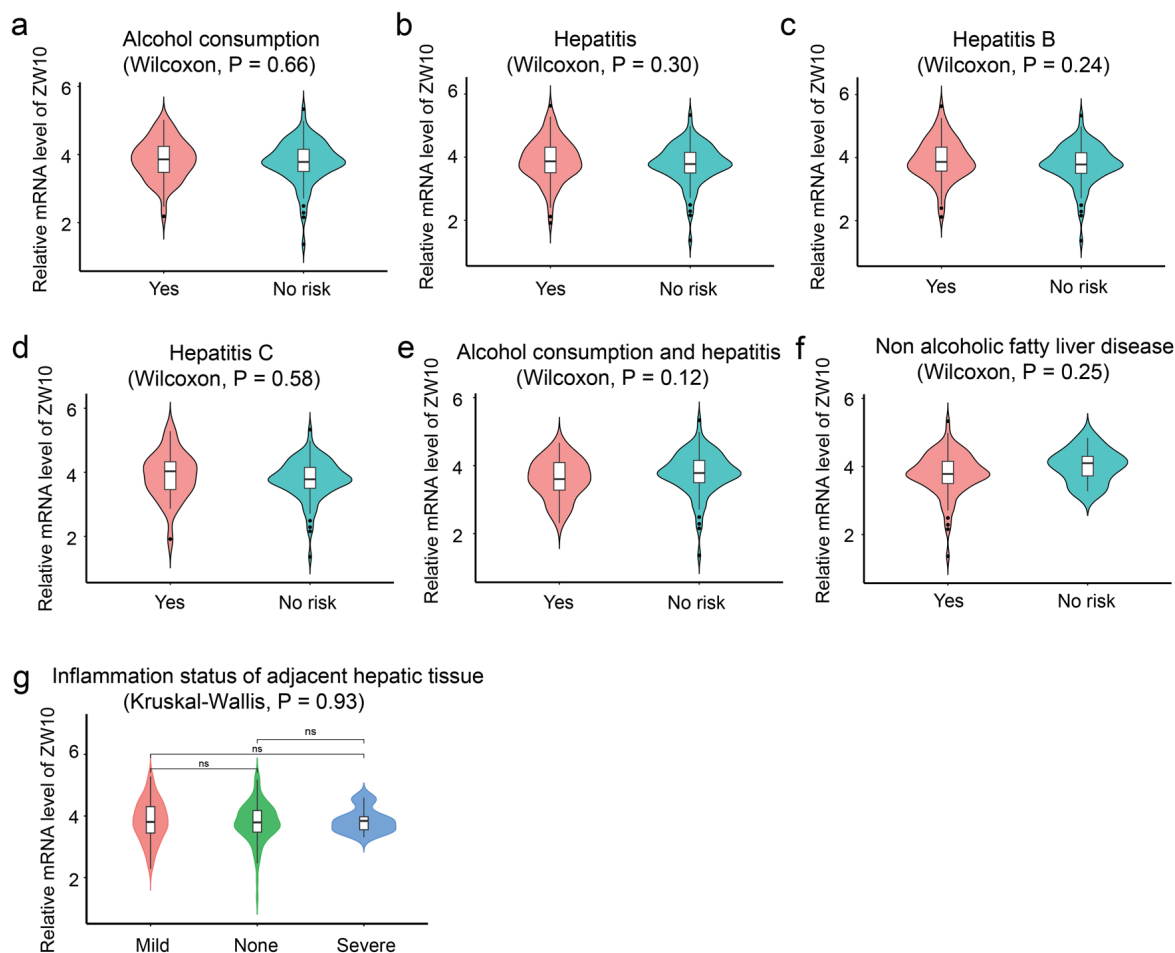
Univariate Cox regression was performed in the transcrip-

tional level of *ZW10* and the clinicopathological parameters including age, gender, race, pT stage as well as AJCC stage. Individuals with high expression of *ZW10* gene had an increased risk of HCC mortality (HR = 1.05, 95% CI: 1.02 - 1.07, P < 0.01, Table 1). Patients in AJCC stage III + IV had an increased *ZW10* expression level compared to those in stage I + II (P < 0.01, Fig. 6e) and had a higher risk of mortality (HR = 2.45, 95% CI: 1.67 - 3.58, P < 0.01, Table 1). In comparison to the patients diagnosed with pT1 + pT2, the patients diagnosed with pT3 + pT4 had an elevated *ZW10* expression level (P < 0.01, Fig. 6d) and a risk of mortality



**Figure 4.** Integrated analysis of *ZW10* mRNA expression level. (a) Forest plot of standardized mean difference. (b) Summary receiver operating characteristic curve. (c) Egger funnel plot ( $P = 0.115$ ).

(HR = 2.47, 95% CI: 1.68 - 3.61,  $P < 0.01$ , Table 1). Other factors cannot be considered as dangerous factors for mortality in patients with HCC ( $P > 0.05$ , Fig. 6a-c). Besides, the *ZW10* high-level group was linked to the overall survival time of patients diagnosed with HCC (two-stage test,  $P < 0.01$ , Fig. 6f).



**Figure 5.** Etiology stratification analysis in different subgroups. (a) Alcohol consumption. (b) Hepatitis (B or C). (c) Hepatitis B. (d) Hepatitis C. (e) The combination of alcohol consumption and hepatitis (B or C). (f) Non-alcoholic fatty liver disease. (g) Inflammation status of adjacent hepatic tissue.

## Biological function

The gene effect scores of *ZW10* knockout for 20 HCC cell lines (PLCPRF5, JHH7, SNU886, HLF, SNU449, SNU475, SKHEP1, JHH6, SNU398, JHH5, HEPG2, JHH1, SNU423, SNU182, HUH7, SNU387, JHH4, HUH1, SNU761, and JHH2)

**Table 1.** Risk Factors Analysis for HCC Based on the TCGA-LIHC Dataset

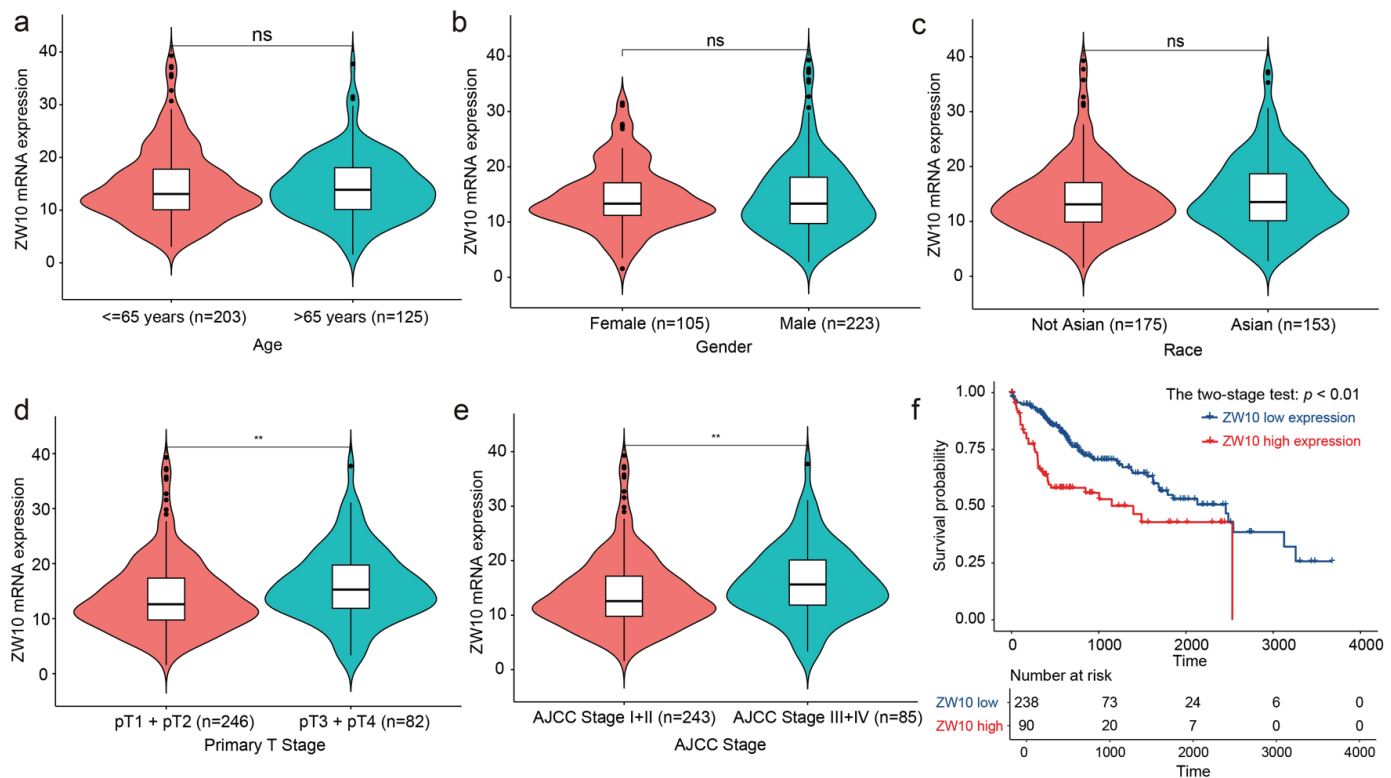
| Variables                        | HR (95% CI)        | P-value |
|----------------------------------|--------------------|---------|
| <i>ZW10</i> (continuous)         | 1.05 (1.02 - 1.07) | < 0.01  |
| Age (> 65 vs. ≤ 65)              | 1.14 (0.78 - 1.67) | 0.49    |
| Gender (male vs. female)         | 0.76 (0.52 - 1.12) | 0.17    |
| Race (Asian vs. not-Asian)       | 0.81 (0.55 - 1.20) | 0.30    |
| pT (pT3 + pT4 vs. pT1 + pT2)     | 2.47 (1.68 - 3.61) | < 0.01  |
| AJCC stage (III + IV vs. I + II) | 2.45 (1.67 - 3.58) | < 0.01  |

AJCC: American Joint Committee on Cancer; CI: confidence interval; HCC: hepatocellular carcinoma; HR: hazard ratio.

were less than 0, demonstrating that *ZW10*-defect could inhibit the growth of HCC cells (Fig. 7).

## Sensitivity of TKIs under varying *ZW10* expressions

This research predicted the sensitivity of 371 HCC samples (including 157 samples in *ZW10*-high expression group and 214 samples in *ZW10*-low expression group) to 10 TKI drugs. Compared to the *ZW10*-low level group, the *ZW10*-high level group showed higher IC<sub>50</sub> values for axitinib, bosutinib, erlotinib, lapatinib, and sunitinib ( $P < 0.05$ , Fig. 8a-e), but no statistical differences were found between the two groups for dasatinib, gefitinib, imatinib, nilotinib, and sorafenib ( $P > 0.05$ , Fig. 8f-j). The transcriptional expression of *ZW10* exhibited a positive relationship with the IC<sub>50</sub> values of axitinib ( $r = 0.21$ ,  $P < 0.01$ ), bosutinib ( $r = 0.21$ ,  $P < 0.01$ ), lapatinib ( $r = 0.20$ ,  $P < 0.01$ ), and sunitinib ( $r = 0.17$ ,  $P < 0.01$ ), indicating that the increase in *ZW10* expression showed an association with a decrease in response rates to TKIs (Supplementary Material 2, [wjon.elmerpub.com](http://wjon.elmerpub.com)).



**Figure 6.** The association between ZW10 mRNA expression status and clinical parameters. (a) Age. (b) Gender. (c) Race. (d) Primary stage. (e) AJCC stage. (f) Overall survival. \*\*P < 0.01, nsP > 0.05. AJCC: American Joint Committee on Cancer.

### Pathway enrichment analysis

Totally 42 ZW10 differentially and strongly related genes presented a notable enrichment in the nucleocytoplasmic transport pathway of biological process, the nucleoplasm, kinetochore, nuclear envelope, and nuclear pore pathways of cellular component, and the protein binding pathway of molecular function (Fig. 9, Supplementary Material 3, wjon.elmerpub.com). The nucleocytoplasmic transport pathway was the only enriched mechanism in KEGG analysis (Supplementary Material 3, wjon.elmerpub.com).

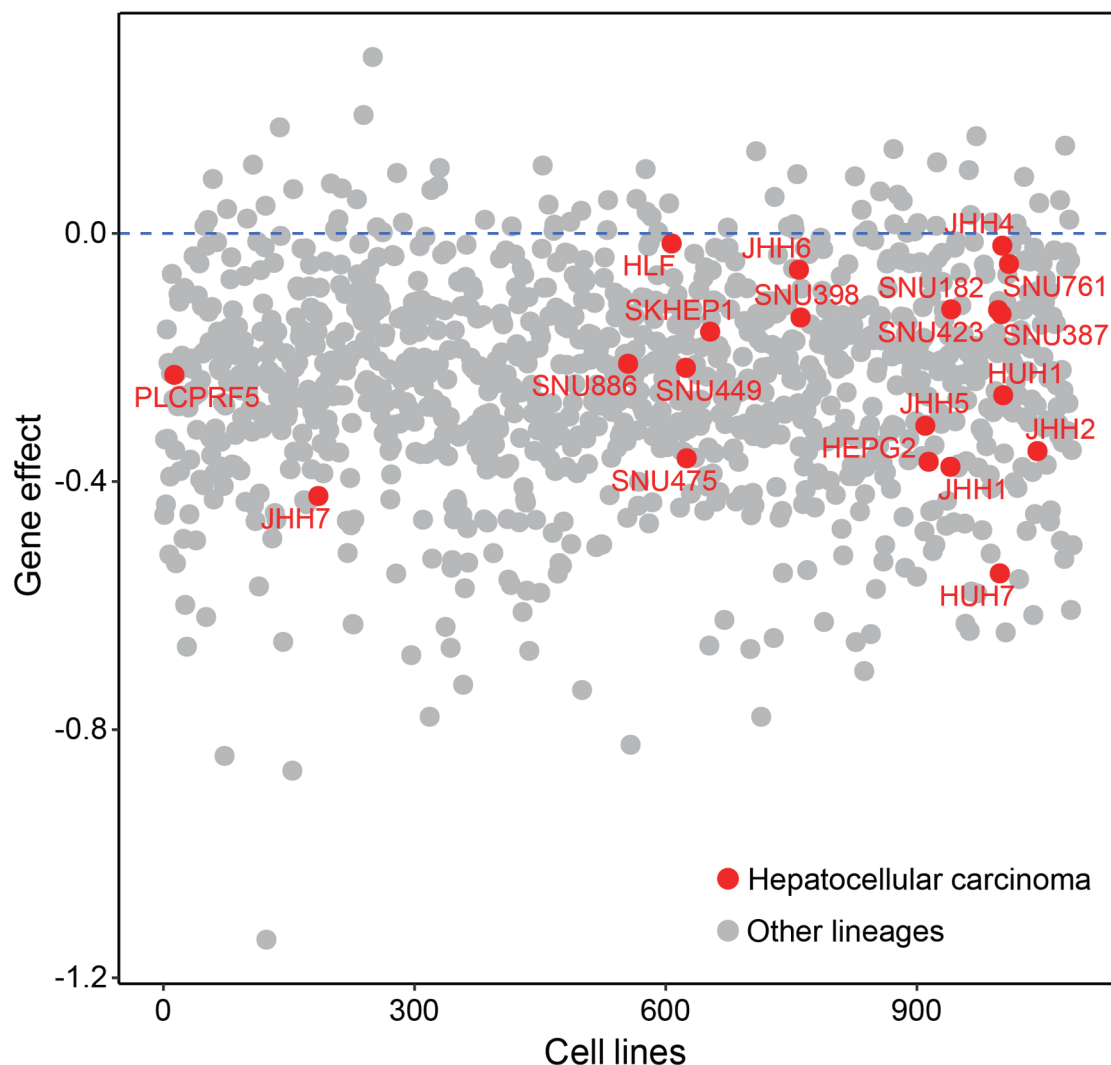
### Characteristics in HCC microenvironment

All of the 371 HCC samples were classified into ZW10 highly expressed and ZW10 low expressed groups, according to the average level of ZW10 gene. In the differential analyses, lower stroma score, microenvironment score, CD4<sup>+</sup> effect memory T cells, CD8<sup>+</sup> naive T cells, conventional dendritic cells, immature dendritic cells, macrophages, macrophages M2, mesenchymal stem cells, adipocytes, endothelial cells, lymphatic endothelial cells, microvascular endothelial cells, and hepatocytes were observed in ZW10 elevated expression status (P < 0.05, Fig. 10). Conversely, the infiltrations of CD4<sup>+</sup> memory T cells, T-helper 2 cells, common lymphoid progenitor, and mast cells were more abundant in ZW10 high expression group (P

< 0.05, Fig. 10). In the correlation analyses, the ZW10 expression value was negatively correlated with the stroma score, microenvironment score, microvascular endothelial cells, macrophages M2, lymphatic endothelial cells, immature dendritic cells, mesenchymal stem cells, hepatocytes, endothelial cells, macrophages, adipocytes, CD8<sup>+</sup> naive T cells, conventional dendritic cells, CD4<sup>+</sup> effect memory T cells, natural killing T cells, T-helper 1 cells, plasmacytoid dendritic cells, dendritic cells, as well as CD4<sup>+</sup> central memory T cells, and exhibited positive correlations with the T-helper 2 cells, mast cells, CD4<sup>+</sup> memory T cells, common lymphoid progenitor, and class switched memory B cells (Supplementary Material 4, wjon.elmerpub.com).

### Compounds opposing ZW10 protein

A total of 42 ZW10 differentially and strongly related genes were used as input dataset to predict small molecule compounds, and mocetinostat (-99.93) (Fig. 11a), lypressin (-99.89) (Fig. 11b), and indatraline (-99.89) (Fig. 11c) were the top three compounds with negative score (Table 2). For the combination affinities to ZW10 protein (PDB ID: 7QPG), mocetinostat needed the minimum binding energy (-8.2 kcal/mol), followed by lypressin (-7.6 kcal/mol) and indatraline (-7.0 kcal/mol). The combination between mocetinostat and ZW10 protein was simulated and is exhibited in Figure 12a. Mocetinostat formed two hydrogen bonds with TYR and GLU



**Figure 7.** Gene effect of *ZW10* for 20 HCC cell lines based on the DepMap database. HCC: hepatocellular carcinoma.

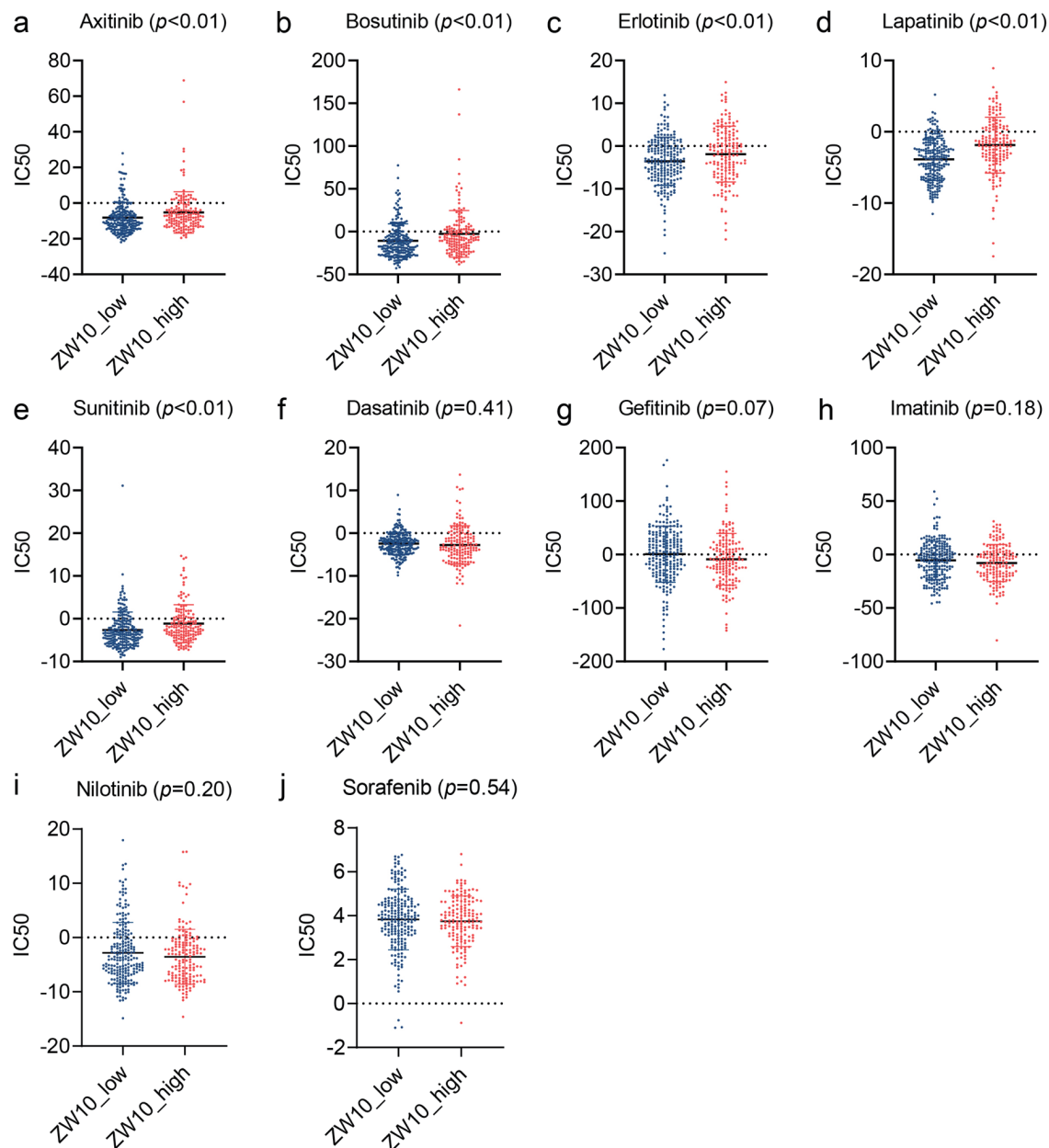
amino acid residues, four hydrophobic bonds with TYR, PHE, ARG, and LEU amino acid residues, and two electrostatic interactions with GLU and ARG amino acid residues of *ZW10* protein. The combination between capecitabine and *ZW10* protein was simulated and is exhibited in Figure 12b. The minimum binding energy of the molecular docking between *ZW10* protein and capecitabine was -7.1 kcal/mol, with the interaction involving three hydrogen bonds, two hydrophobic interactions, and one halogen bond.

## Discussion

TKIs have become the cornerstone in the first-line or second-line treatment of HCC since 2007. Nevertheless, the response rate to TKIs remains suboptimal in advanced HCC due to the heterogeneity of systemic therapies and the emergence of drug resistance [23]. It is urgent to find effective genetic markers to assist clinical TKI stratification therapy. To solve this prob-

lem, this study combined CRISPR screening gene knockout with continuous TKI drug treatment to eliminate the effects of the drug's transient effects while ensuring the accuracy and comprehensiveness of gene screening. *ZW10* was ultimately identified as a potential gene involved in resistance to TKI in HCC [24].

TKI resistance remains a challenge in HCC treatment. Therefore, exploring the expression profile of *ZW10* and its prognostic significance in tumors, particularly in HCC, could further elucidate its potential as a biomarker for drug resistance. Pan-cancer analysis confirmed the upregulation of *ZW10* across various tumor types, which correlated with poor patient prognosis. Interestingly, *ZW10* expression at both mRNA and protein levels was significantly elevated in HCC, and high *ZW10* expression was associated with unfavorable outcomes in HCC patients. Notably, while no direct studies have reported a relationship between *ZW10* and HCC, a study indicated that *ZW10* is involved in KNTC1-induced PI3K/Akt signaling regulation, where silencing KNTC1 inhibits PI3K/Akt signal-

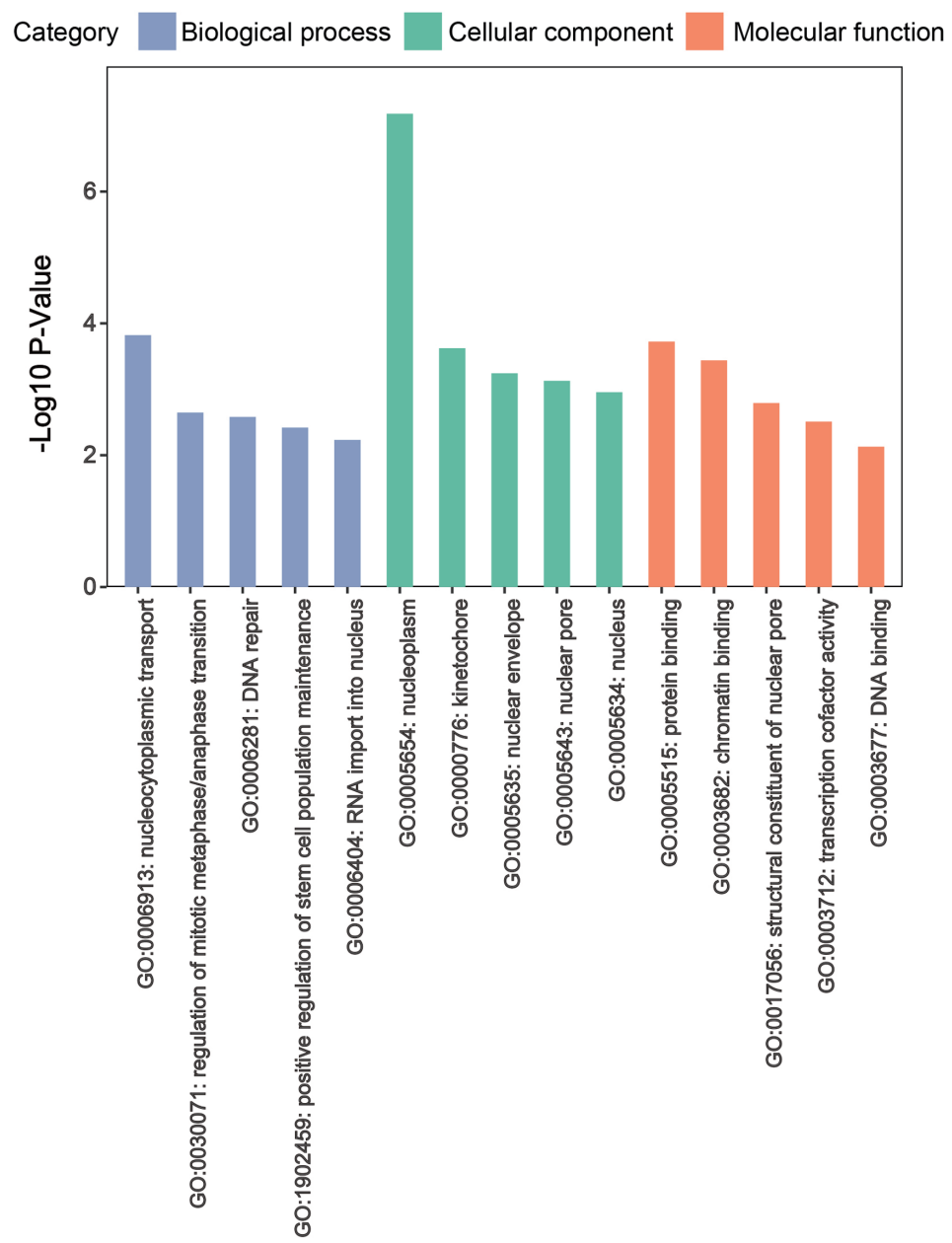


**Figure 8.** The differences of the predicted half-maximal inhibitory concentration of TKIs in *ZW10*-high expression and *ZW10*-low expression groups. (a) Axitinib. (b) Bosutinib. (c) Erlotinib. (d) Lapatinib. (e) Sunitinib. (f) Dasatinib. (g) Gefitinib. (h) Imatinib. (i) Nilotinib. (j) Sorafenib. IC50: half-maximal inhibitory concentration; TKIs: tyrosine kinase inhibitors.

ing and thereby restrains HCC progression [25]. Pan-cancer evidence supports the critical contribution of *ZW10* to HCC, highlighting its promise as a therapeutic strategy to overcome TKI resistance.

Building upon the pan-cancer findings, which may overlook the unique characteristics, heterogeneity, and clinical relevance specific to individual malignancies, multi-center HCC samples were further integrated to investigate the expression landscape of *ZW10*. In a cohort of 3,312 HCC tissue samples, elevated *ZW10* mRNA levels were observed. These elevated levels correlated with poor prognosis. Additionally,

*ZW10* deficiency suppressed HCC cell growth, thus validating the reliability of pan-cancer results. HCC exhibits significant heterogeneity, largely due to the differing impacts of chronic liver diseases [26]. Therefore, a detailed analysis of the specific etiology is crucial. The results of the etiology stratification analysis showed that *ZW10* was highly expressed in HCC caused by various etiologies but no significant differences, indicating that high expression of *ZW10* may not lead to HCC by affecting a single etiology. Instead, it likely exerts a broad influence across various etiologies that contribute to the development of HCC. Moreover, response



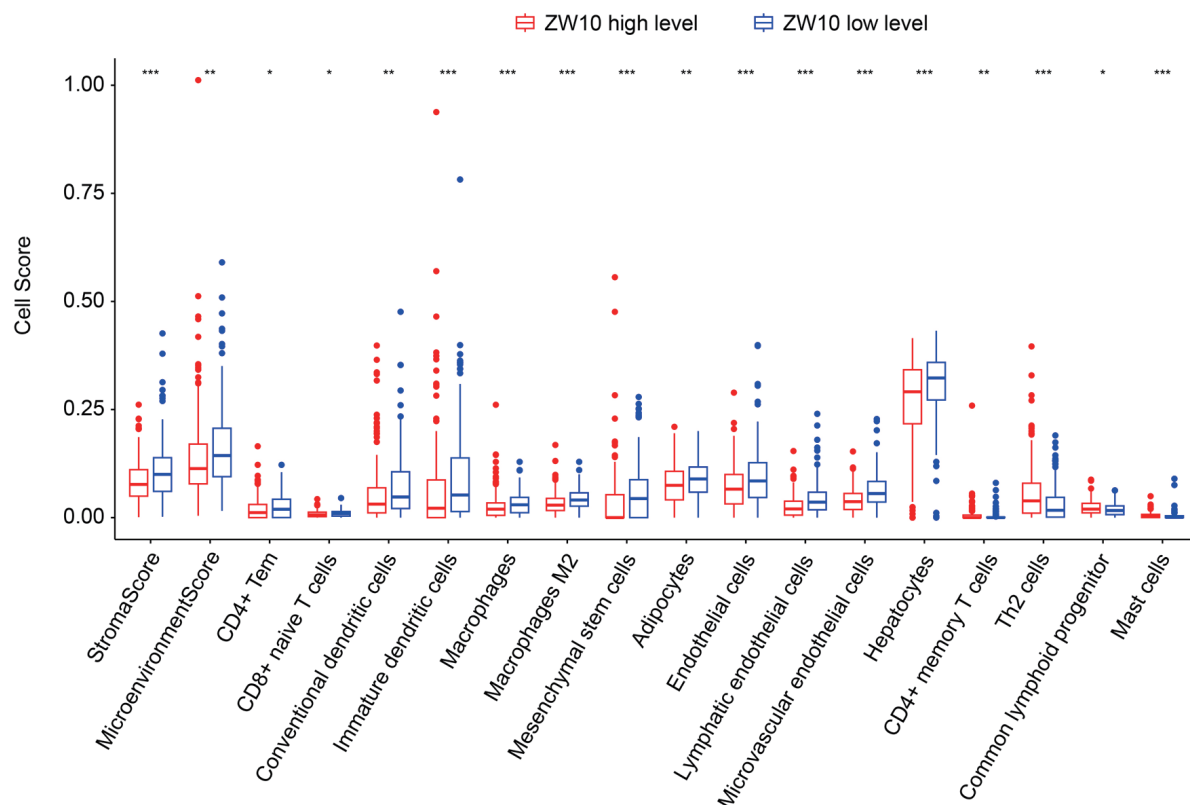
**Figure 9.** Functional enrichment analysis for *ZW10* strongly related and differentially expressed genes.

analysis to TKIs revealed that lower *ZW10* expression was related to superior TKI treatment responses of axitinib, bosutinib, lapatinib, and sunitinib in this research. Taken together, these findings suggest that *ZW10* may function as both an oncogene and a key mediator of TKI.

Pathway analysis showed significant enrichment of the nucleocytoplasmic transport pathway in both GO and KEGG pathways. The nucleocytoplasmic transport of oncogenes and proteins plays a pivotal role in driving tumorigenesis, enhancing tumor invasiveness, and promoting the advancement of drug resistance, particularly to TKI therapies [27-30]. Pathway analysis results further indicated that *ZW10* may be a promis-

ing cancer-promoting and drug-resistant target.

HCC is a tumor characterized by chronic inflammation, and increasing evidence suggests that the tumor microenvironment plays a pivotal role in promoting HCC invasiveness, adverse prognosis, and the development of TKI resistance [26, 31, 32]. In this research, elevated *ZW10* status was associated with lower stroma score, microenvironment score, and lower infiltration of various immune cells. Previous studies have demonstrated that high levels of immune infiltration are associated with improved prognosis. Immune cell infiltration into the tumor microenvironment enhances immune surveillance and clearance of tumor cells, thereby improving prognosis in



**Figure 10.** Differential analyses between *ZW10* expression and the microenvironment characteristics. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

HCC patients. In contrast, lower immune and stroma scores, along with higher tumor purity, are linked to poorer prognosis in HCC [33]. Overall, *ZW10* overexpression likely induces an immunosuppressive state in HCC, which could be reversed by drugs targeting *ZW10* or immune-related treatments, potentially enhancing antitumor effects and improving prognosis [34, 35].

Based on the potential of *ZW10* as a target for TKI resistance and its high expression possibly contributing to immune suppression in HCC, this study further utilized small molecule compounds prediction and molecular docking to predict drugs targeting *ZW10*. The results showed that both mocetinostat and capecitabine had good docking efficiency with *ZW10* protein. Mocetinostat is a class I histone deacetylase inhibitor with antitumor effects [36-39]. Notably, except direct anti-neoplastic properties, mocetinostat can also enhance the therapeutic effectiveness of immune checkpoint inhibitors and be conducive to overcome the problem of immunotherapy resistance, suggesting its potential for improving the immune microenvironment [37, 39, 40]. Capecitabine has recently achieved very satisfactory efficacy and safety in the second-line treatment of HCC [22]. Existing studies have shown that the combination of mocetinostat and capecitabine in cancer treatment can reduce tumor metastasis and demonstrate enhanced tumor-suppressive effects [38, 41]. It is therefore proposed that mocetinostat and capecitabine may reverse TKI resistance by targeting *ZW10* protein.

## Conclusion

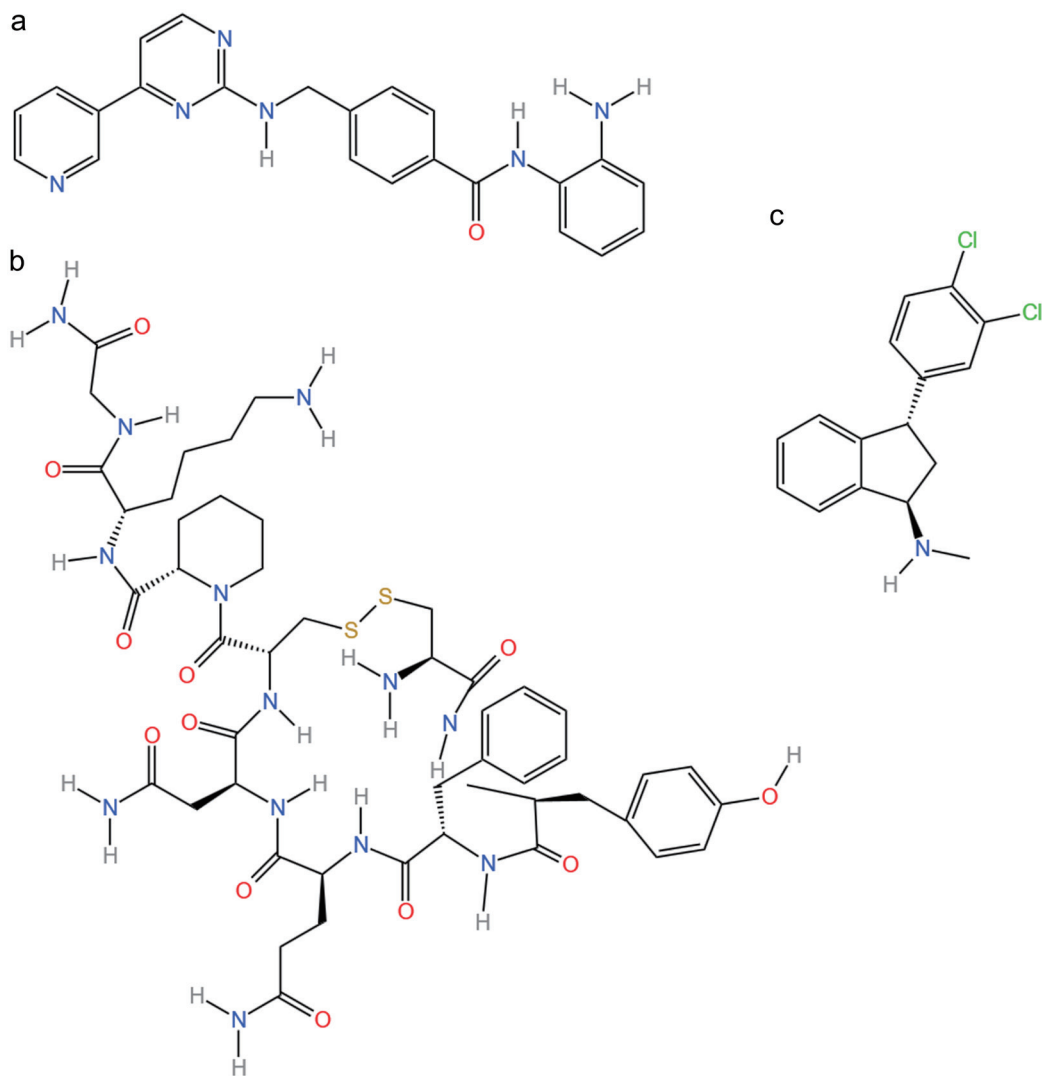
In summary, this study demonstrated that *ZW10* may be associated with TKI resistance. The transcriptional expression status of *ZW10* was elevated in HCC tissue, and the upregulation of *ZW10* was related to poor prognosis. Additionally, *ZW10* could promote the growth of HCC cells, and potentially become a therapeutic target to reverse TKI resistance. The functional mechanism of *ZW10* in HCC needs further experimental verification.

## Supplementary Material

**Suppl 1.** The statistical descriptions, true positive values, false positive values, true negative values, and false negative values of *ZW10* transcriptional expression levels.

**Suppl 2.** The Pearson analyses between the predicted half-maximal inhibitory concentration of TKIs and the expression level of *ZW10*. (a) Axitinib. (b) Bosutinib. (c) Lapatinib. (d) Sunitinib. (e) Dasatinib. (f) Erlotinib. (g) Gefitinib. (h) Imatinib. (i) Nilotinib. (j) Sorafenib. IC50: half-maximal inhibitory concentration; TPM: transcripts per million.

**Suppl 3.** Details of the Gene Oncology and the Kyoto Encyclopedia of Genes and Genomes analysis. BP: biological process, CC: cellular component, MF: molecular function.



**Figure 11.** Structure of mocetinostat (a), lypressin (b), and indatraline (c). The structures of the three compounds were downloaded from the PubChem database.

**Suppl 4.** Spearman analyses between *ZW10* expression and the microenvironment characteristics. CD4<sup>+</sup> tem: CD4<sup>+</sup> effect memory T cells; CD4<sup>+</sup> tcm: CD4<sup>+</sup> central memory T cells.

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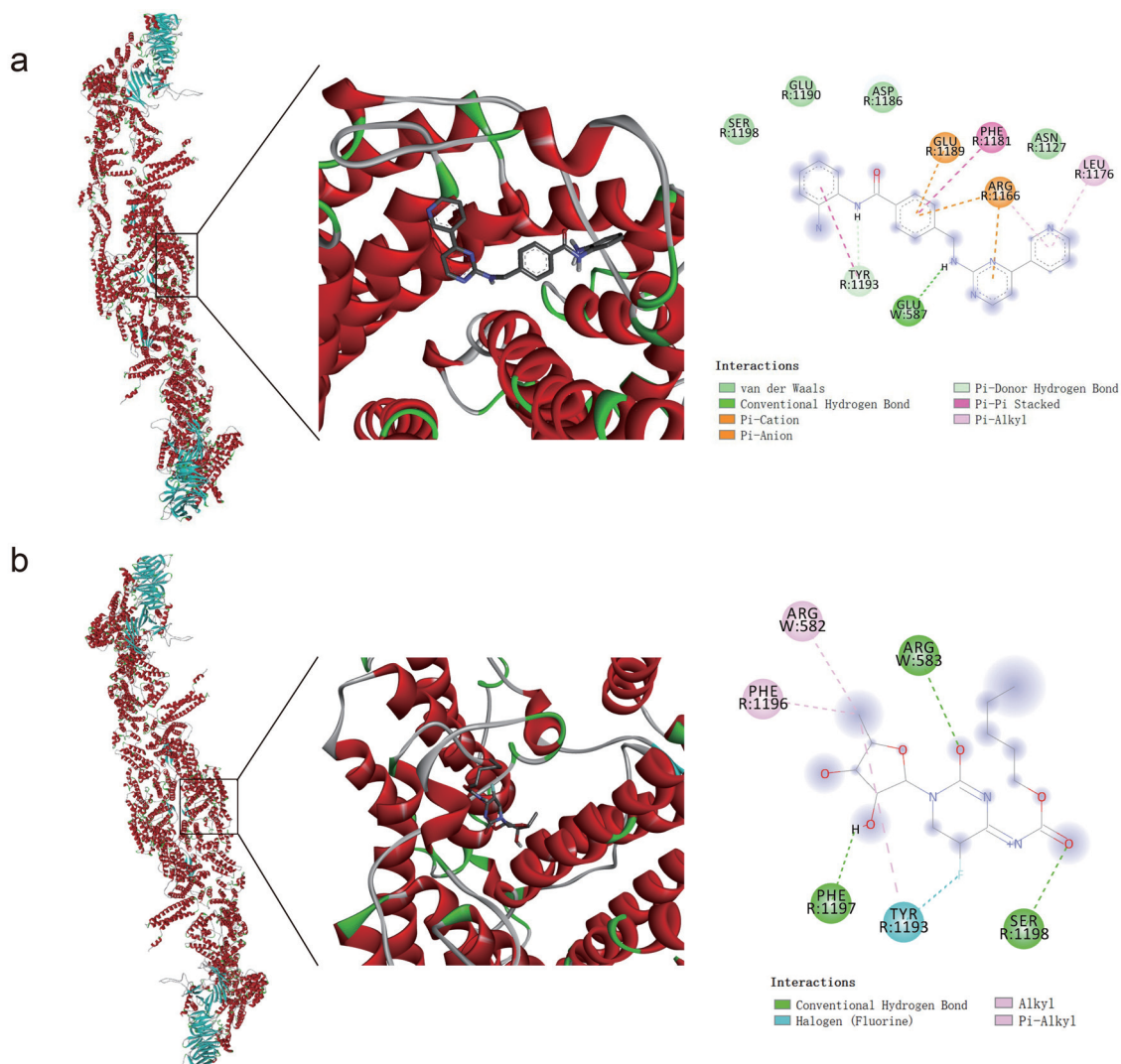
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**Table 2.** The Top Three Compounds Opposing *ZW10* and Their Affinity to *ZW10* Protein

| Compound     | Description                          | Score  | PubChem CID | Minimum binding energy with <i>ZW10</i> |
|--------------|--------------------------------------|--------|-------------|-----------------------------------------|
| Mocetinostat | HDAC inhibitor                       | -99.93 | 9865515     | -8.2 kcal/mol                           |
| Lypressin    | Vasopressin receptor agonist         | -99.89 | 644076      | -7.6 kcal/mol                           |
| Indatraline  | Norepinephrine transporter inhibitor | -99.89 | 126280      | -7.0 kcal/mol                           |

HDAC: histone deacetylase.



**Figure 12.** Three-dimensional and two-dimensional interaction networks of mocetinostat, capecitabine, and ZW10 protein. (a) Mocetinostat binding to ZW10 protein. (b) Capecitabine binding to ZW10 protein.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## Informed Consent

This study was not involved in human samples, so there is no need to obtain informed consent.

## Author Contributions

Qing Ling Huang, Gang Chen and Rong Quan He designed the study; Qing Ling Huang, Guan Lan Zhang and Qin Yan Su participated in the CRISPR screening experiment; Bang Teng Chi and Qian Lin collected and screened the public datasets; Guan Lan Zhang, Qin Yan Su, Shi De Li and Han He analyzed the data and presented the results; all the authors involved in writing the draft; Yu Lu Tang, Yi Wu Dang, Gang Chen and Rong Quan He revised the manuscript; all the authors have reviewed and approved the final manuscript.

## Data Availability

The data supporting this study’s findings are available from the corresponding authors upon reasonable request.

## Abbreviations

TKI: tyrosine kinase inhibitor; ZW10: zeste white 10; CRISPR: clustered regularly interspaced short palindromic repeats; SROC: summary receiver operating characteristic; sgRNA: small guide RNA; SMD: standardized mean difference; CI: confidence interval; HCC: hepatocellular carcinoma; TCGA: The Cancer Genome Atlas; THPA: The Human Protein Atlas; GEO: Gene Expression Omnibus; SRA: Sequence Read Archive; GTEx: Genotype-Tissue Expression; AUC: area under the curve; HR: hazard ratio; pT stage: primary tumor stage; AJCC: American Joint Committee on Cancer; IC50: half-maximal inhibitory concentration; TPM: transcripts per million; ssGSEA: single-sample Gene Set Enrichment Analysis; CLUE: Connectivity Map Linked User; IHC: immunohistochemical; ACC: adrenocortical carcinoma; BLCA: bladder urothelial carcinoma; BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: cholangiocarcinoma; COAD: colon adenocarcinoma; DLBC: lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: esophageal carcinoma; GBM: glioblastoma multiforme; HNSC: head and neck squamous cell carcinoma; KICH: kidney chromophobe; KIRC: kidney renal clear cell carcinoma; KIRP: kidney renal papillary cell carcinoma; LAML: acute myeloid leukemia; LGG: brain lower grade glioma; LIHC: liver hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; MESO: mesothelioma; OV: ovarian serous cystadenocarcinoma; PAAD: pancreatic adenocarcinoma; PCPG: pheochromocytoma and paraganglioma; PRAD: prostate adenocarcinoma; READ: rectum adenocarcinoma; SARC: sarcoma; SKCM: skin cutaneous melanoma; STAD: stomach adenocarcinoma; TGCT: testicular germ cell tumors; THCA: thyroid carcinoma; THYM: thymoma; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma; UVM: uveal melanoma; HDAC: histone deacetylase; CD4<sup>+</sup> tem: CD4<sup>+</sup> effect memory T cells; CD4<sup>+</sup> tcm: CD4<sup>+</sup> central memory T cells; BP: biological process; CC: cellular component; MF: molecular function

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