

RESEARCH PAPER

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## Upregulation of oxidative stress-responsive 1(OXSR1) predicts poor prognosis and promotes hepatocellular carcinoma progression

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### ABSTRACT

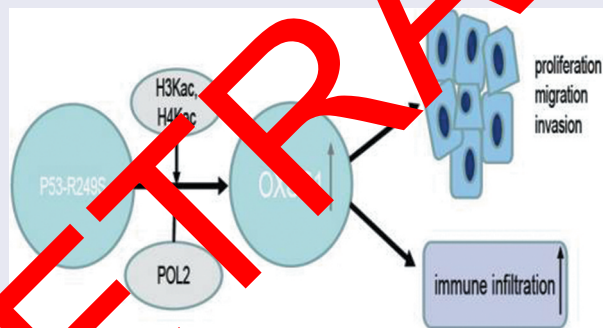
Many studies reported that Oxidative stress-responsive 1(OXSR1) is closely related to the malignant progression of several malignancies. Nevertheless, the expression pattern and function of OXSR1 in HCC remains unknown. In present research, it was observed that the expression of OXSR1 was abnormally elevated in HCC. Upregulated OXSR1 was associated with TNM stage, and grade and was confirmed as an independent prognostic factor in HCC patients. The downregulation of OXSR1 expression effectively repressed the proliferation, migration and invasion of HCC in vivo and in vitro experiments. Western blot and qRT-PCR analysis demonstrated that mutant p53-R249S was critical for regulating the aberrant elevation of OXSR1 in HCC. Chip assay indicated that p53-R249S abrogated the binding of p53 to the OXSR1 promoter region and increased the level of POL2, H3Kac and H4Kac in the promoter region of the OXSR1, thus promoting the transcriptional expression of OXSR1. GSEA revealed that numerous cancer-related pathways were enriched in the high OXSR1 expression group. Furthermore, the expression level of OXSR1 was positively correlated with the infiltration levels of tumor infiltrating immune cells (TIICs) and PD-L1 expression in HCC by TIMER platform. In summary, our study revealed that upregulated OXSR1 was a determinant of prognosis and immune infiltration in HCC. The expression of OXSR1 was released by p53-R249S mutant, and upregulated OXSR1 in HCC promoted proliferation, migration and invasion.

### ARTICLE HISTORY

Received 29 June 2020  
Revised 20 August 2020  
Accepted 11 August 2020

### KEYWORDS

Oxidative stress-responsive 1; hepatocellular carcinoma; p53-R249S; immune infiltration



### Introduction

The most common histological subtype of liver cancer is hepatocellular carcinoma (HCC), which accounting for approximately 90% of primary liver cancer [1]. According to statistical results in recent years, HCC ranks fourth among the most common causes of cancer death in the world, and the number of deaths

from HCC is approximately 780,000 per year [2]. In addition, 80% of HCC cases occur in developing regions and countries [3,4], including sub-Saharan Africa and eastern Asia. In the past few decades, the treatment of HCC has made great progress, the 5-year survival rate of patients with early HCC after surgical resection can reach over 60% [5]. However, due to the late diagnosis, distant metastasis and recurrence,

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This article has been retracted. Please see Retraction (<https://doi.org/10.1080/21655979.2021.1926173>).

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the overall prognosis of HCC is still poor. At present, some biomarkers have been used as screening methods for early HCC, but their effectiveness is still not satisfactory. For example, alpha-fetoprotein (AFP) is a reliable biomarker and has been widely used in the screening of HCC, yet its sensitivity in early HCC patients is only 40–50% [6]. Hence, it is pressing to find novel biomarkers for increasing the detection rate of early HCC patients and improving the clinical outcome of the disease.

Mammalian STE20-like kinases (MST) were identified as homologs of the yeast Sterile-20 protein kinase. According to structure and function, MST are further divided into p21-activated kinase (PAK) and germinal center kinase (GCK) families [7,8]. GCKs are involved in the regulation of various signaling pathways related to cell function, including apoptosis, cell growth, stress responses, migration, polarity and immune regulation. Therefore, a slight imbalance in the level or activity of GCKs results in human diseases, such as cancer, virus infection and immunological disorders [9]. In cancer, abnormal expression of many GCKs affects progression of HCC in multiple cellular pathways. Among these GCKs, MST4 is upregulated in HCC and is linked with the metastasis and poor prognosis of HCC [10]. And the deletion of MST1 and MST2 gene promotes the development of HCC [11].

Oxidative stress-responsive (OXSR1), a 58-kD protein of 527 amino acids, encoded by OXSR1 gene, is widely expressed in most tissues [12]. OXSR1 belongs to the GCK subfamily and is composed of an N-terminal catalytic domain and two regulatory regions [13]. Previous studies identified that the activity of OXSR1 is regulated by with no lysine [K] (WNKs) kinases and MO25, and the function of OXSR1 is to regulate ion homeostasis and cell volume [14,15]. Increasing studies have reported that OXSR1 participates in multiple cell events, including cell apoptosis, migration and autophagy [16,17]. Recent evidence suggested that OXSR1 is closely related to the malignant progression of several tumors [18–20]. For instance, elevated OXSR1 predicates poor clinical outcome and lymph node metastasis in breast cancer [20]. And targeted inhibition of WNK1/OXSR1/NKCC1 pathway can significantly decreased the migration of glioma cells [19]. Nonetheless, the prognostic value and function of OXSR1 in HCC are still unknown.

In this research, we determined the relationship between P53-R249S and OXSR1 expression in HCC, and analyzed the function of OXSR1 in the progression of HCC. Finally, it was found that OXSR1 is a novel biomarker and promising target for treatment of HCC.

## Methods

### Data acquisition and analyses

The expression of OXSR1 in HCC was analyzed using transcriptome data extracted from the Oncomine (<https://www.oncomine.org>), The Cancer Genome Atlas (TCGA, <http://portal.gdc.cancer.gov/>) and Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/info/linking.html>) databases. We obtained clinical information of HCC patients from TCGA. In the subsequent analysis, cases with unknown or missing clinical characteristics and overall survival time were excluded. The relationship between OXSR1 expression level and tumor infiltrating immune cells (TIC) infiltration level was detected by using the TIMER platform [21] (<https://cistrome.shinyapps.io/timer/>). Gene set enrichment analysis [22] (GSEA) was performed to explore the signaling pathways related to OXSR1 expression in HCC.

### Cell culture

The human HCC cell lines Hep3B, SK-hep1, PLC/PRF/5, and HepG2 were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All these cells grew in Dulbecco's modified Eagle's medium (DMEM; Gibco; USA) with 10% fetal bovine serum, and placed at 37°C in a cell culture incubator with 5% CO<sub>2</sub>.

### Patient samples

Twelve pairs of tissues were obtained from HCC patients diagnosed by pathological examination at The First Affiliated Hospital of Wenzhou Medical University. The Ethics Committee of Wenzhou Medical University approved our research. All patients signed informed consent forms before the start of the research.

### Immunohistochemistry

The tissue sections were dewaxed using xylene, gradient alcohol, and distilled water, then boiled in 10 mM sodium citrate buffer (pH 6.0) at 95–100°C for 30 min. After blocking with 10% goat serum for 1 h, primary antibody was added overnight. The primary antibodies used were against OXSR1 (15,611-1-AP; Proteintech; China) and Ki67 (19,972-1-AP; Proteintech; China). Then, peroxidase rabbit IgG (ABC kit; Vectorlabs; USA) was added and incubate for 1 h. The immunological reaction was visualized by 3,3'-diaminobenzidine (DAB; CWBIO; China). Finally, hematoxylin was used to stain the nuclei. Quantitative analysis was performed by using IHC Profiler of Imagej software.

### Plasmid construction and transfection

The pGIPZ shNT construct was generated with the control oligonucleotide 5'-GCTTCTAACACCGGAGGTCTT-3'; and pGIPZ OXSR1-shRNA was generated with 5'-CCAATCAGTCTAGTACTAAGA-3' oligonucleotide. Transfection was carried out in accordance with the experiment manual. Five hundred microliter of serum-free medium containing 10 µl LipoFiter™ (Hanbio; China) and 1 µg DNA mixture (1.6 µg plasmid vector, 1.2 µg psPAX2, and 1.2 µg pMD2G) were added to a 25 cm<sup>2</sup> culture dish with 293 T cells. After 8 hours of incubation, the medium was changed to DMEM containing 10% FBS, and filtered virus supernatant were collected at 48 and 72 hours. Cultured tumor cells were infected with medium-diluted virus supernatant for 24 h. After adding 2 µg/mL puromycin to kill the cells that have not been transduced with the lentiviral vector, the stably transfected cells were successfully constructed.

### Western blot assay

The protein samples were obtained by lysing the collected cells with RIPA lysis buffer. All samples were put into the SDS-PAGE and transferred to nitrocellulose blotting membranes (Ge Healthcare Life Sciences; Germany). Then, after incubating with the primary antibodies overnight at 4°, the membranes were immersed in the secondary antibodies (goat anti-rabbit IgG; Thermo; USA) at room temperature for 1–2 hours. The primary antibodies included anti-

OXSR1 (15,611-1-AP; Proteintech; China), and anti-p53 (A0263; Abclonal; China). The image was developed and exposed using an ECL chemiluminescence kit (Thermo; USA).

### Quantitative real-time PCR

Total RNA was extracted from cells or tissues by adding 1 mL of Trizol (Invitrogen; USA). cDNA was synthesized according to the corresponding experiment manual. The primers were as follows: p53 (F:5'-CCCAAGCAATGGATGATTGGA-3', R:5'-ATGAGGGTGCTGTCTTTGTG-3'); OXSR1 (F:5'-GTGGCAATCAAACCGATACC-3', R:5'-TGATGGCATTGACTCTGGCT-3').

### Cell migration and invasion assay

A total of  $1 \times 10^5$  cells were seeded in the upper chamber of a Transwell filter containing 8-µm pores (Falcon; USA) with 300 µl serum-free medium, and the bottom chamber was filled with 500 µl medium containing 10% FBS. The invasion experiments were conducted similar to the migration experiments, except that the bottom of the chamber was precoated with Matrigel (BD Biosciences; USA). After incubating for 24 hours in a 37°C incubator, the infiltrating cells were stained with 0.1% crystal violet, followed by observation and counting with a microscope. Three individual migration and invasion experiments were performed.

### Cell proliferation assay

Cell Counting Kit-8 (CCK-8 Cell Counting Kit; Vazyme; China) assay was conducted to observe cells proliferation. The cells were added to 96-well plates ( $2 \times 10^3$  cells per well) then grew for 1–6 days. After growing for a while, 10 µl of CCK8 reagent was added to each well, and the plates were placed back into the 37°C incubator and incubated for 2–4 h. Then, the plates were measured at 450 nm.

### Chromatin-immunoprecipitation (CHIP) assay

Hep3B cells were fixed in 1% formaldehyde solution, sonicated and pretreated with protein A/G-immunomagnetic beads, followed by the addition of a H3Kac (pan-acetyl) antibody (Catalog No:

39,040; Active Motif; USA), H4Kac (Lys5/8/12/16) antibody (3HH4-4C10; Sigma; USA), RNA pol II antibody (Catalog No: 39,497; Active Motif; USA), IgG antibody (ab171870; Abcam; UK) and p53 antibody (ab1101; Abcam; UK). The purified DNA fragments obtained after purification of the eluted product were used as a template for qPCR amplification using an LC96 real-time quantitative PCR instrument (LightCycler96; Roche; Switzerland). The primers were 5'-TGGGCCCAAGCTTCT-3' (forward) and 5'-ACCAATCGTCTCGGAACAGC-3' (reverse).

### Animal model

Nude mice (4 to 5 weeks old, male) were randomly grouped (n = 6 per group). Hep3B cells (transfected with shOXSR1 or shNT) were resuspended in 100  $\mu$ L of PBS, and injected subcutaneously into the backs of the nude mice. After 24 days, the nude mice with tumors were put to death and the tumors were taken out, weighed, used for HE and immunohistochemistry staining.

### Statistical analyses

SPSS 21.0, GraphPad Prism 7 and R v3.6.1 software were applied to all statistical analyses. The survival curve was drawn by the Kaplan–Meier method and log-rank test. In general,  $P < 0.05$  indicates a statistically significant difference.

## Results

### OXSR1 is overexpressed in HCC

We first performed a pan-cancer analysis via the TCGA database and revealed the expression of OXSR1 gene in 33 types of malignant tumors (Figure 1a). The result showed that OXSR1 was notably upregulated in liver hepatocellular carcinoma (LIHC) compared to normal tissue. Additionally, expression level of OXSR1 was also notably elevated in several other cancers, including glioblastoma multiforme (GBM), esophageal carcinoma (ESCA), stomach adenocarcinoma (STAD) and cholangiocarcinoma (CHOL). Then, we mined multiple HCC-related datasets (GSE14520, GSE121248, GSE45436, Was Liver) from the GEO and Oncomine databases to further

determine the upregulated expression of OXSR1 in HCC (Figure 1b–e).

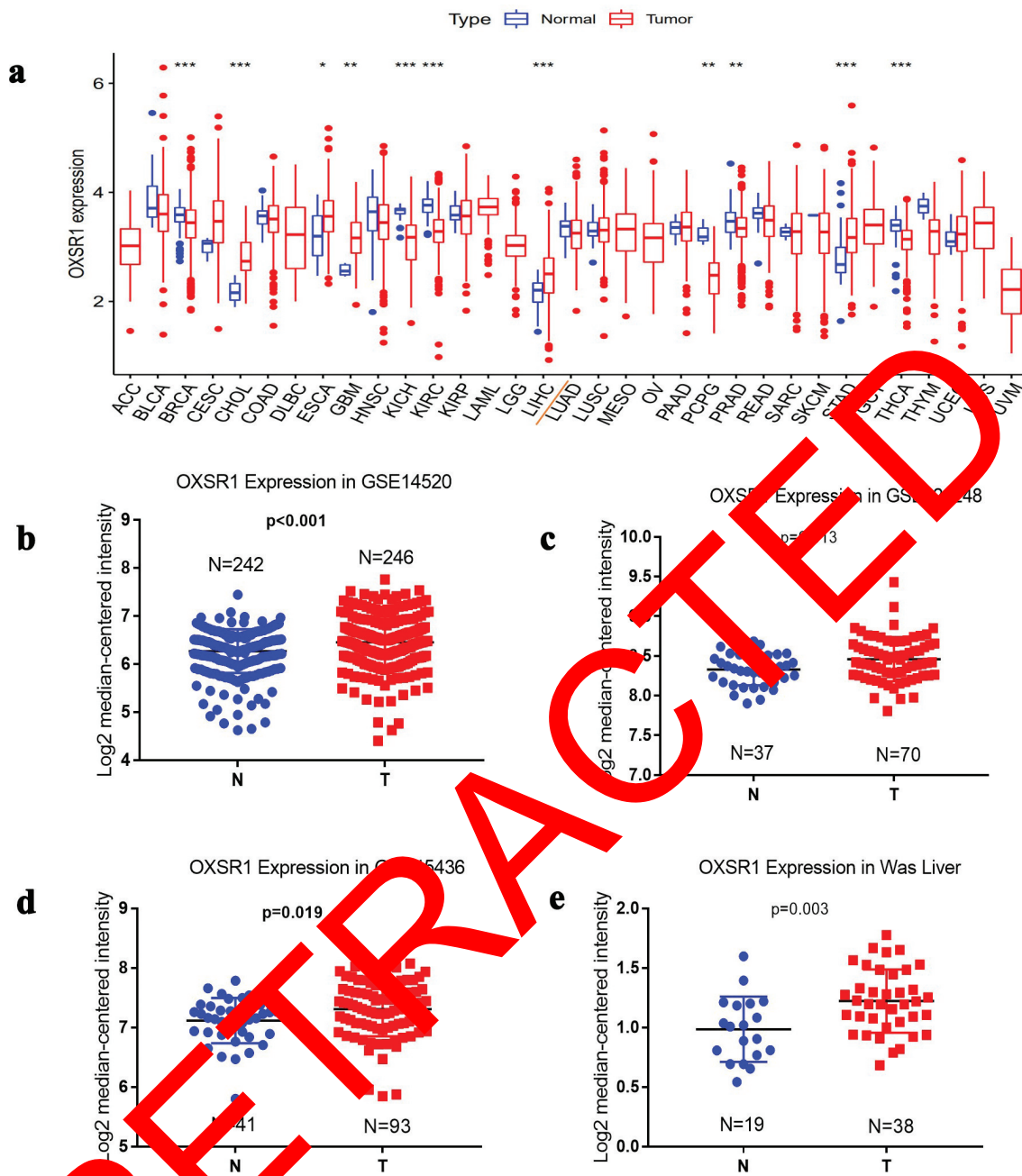
To further verify the upregulation of OXSR1 in HCC, we carried out qRT-PCR to assess the mRNA expression level of OXSR1 in 12 paired HCC tissues and adjacent normal tissues. In addition, we randomly selected six paired samples to observe the protein expression level of OXSR1 using western blot. These data indicated that the protein and mRNA levels of OXSR1 significantly elevated in HCC tumors (Figure 2a and b). Immunohistochemical analysis further showed that OXSR1 had higher expression in HCC tumors and mainly expressed in the cytoplasm (Figure 2c).

### OXSR1 is related to the prognosis of HCC patients

Survival curve revealed that elevated OXSR1 predicted shorter overall survival time in HCC patients based on the TCGA LIHC (Figure 2d). And the high expression of OXSR1 in HCC was positively associated with TNM stage ( $p = 0.011$ ) and histopathological grade ( $p < 0.001$ ) (Figure 2e, and f). These results showed that upregulation of OXSR1 had poor prognostic value for poorly differentiated and advanced patients. As shown in Table 1, univariate analysis revealed that T stage ( $p < 0.001$ ), TNM stage ( $p < 0.001$ ) and OXSR1 expression ( $p = 0.002$ ) were significantly associated with the prognosis of HCC patients. In multivariate Cox analysis, OXSR1 (HR = 1.14, 95%CI = 1.03–1.27,  $p = 0.012$ ) was identified as an independent prognostic factor.

### OXSR1 promotes the malignant progression of HCC in vitro

To further analyze the biological function of OXSR1 in HCC, we first performed western blot to investigate the expression of OXSR1 in four HCC cell lines namely Hep3B, SK-hep1, PLC/PRF/5 and HepG2 cells (Figure 3a). Among them, PLC/PRF/5 and Hep3B cells had higher OXSR1 expression, and were chosen for loss-of-function experiment. These two cell lines were transfected with OXSR1 shRNA to construct cells that stably knockdown OXSR1 expression. The effect of knock-down was determined by using western blot (Figure 3b). Then, the impacts of OXSR1 downregulation on tumor cell proliferation, migration and invasion were revealed by performing CCK8 and



**Figure 1.** OXSR1 was downregulated in hepatocellular carcinoma.

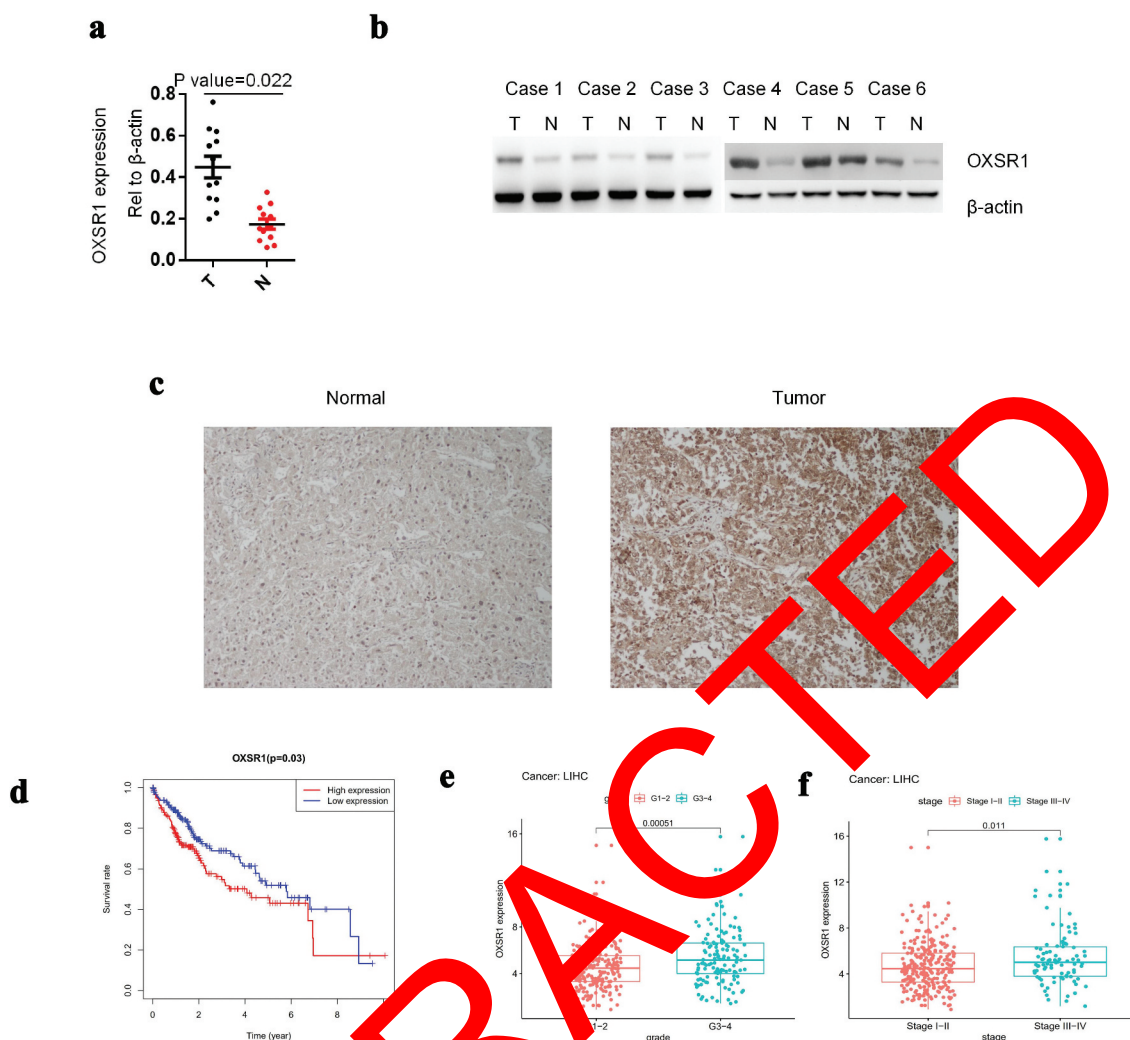
(a) Overview of OXSR1 mRNA expression in different tumor tissues and adjacent normal tissues via TCGA datasets (\*\* $p < 0.001$ ). (b–e) Relative expression of OXSR1 mRNA in HCC tissues and adjacent normal tissues in GSE14520, GSE121248, GSE45436 and Was liver datasets.

Transwell migration and invasion assays. Downregulation of OXSR1 notably suppressed the proliferation, migration and invasion ability of HCC cells (Figure 3c–e).

### ***OXSR1 contributes to HCC progression in vivo***

To determine the impact of OXSR1 on tumor proliferation in vivo experiment, the stable shOXSR1 or

shNT transfected Hep3B cells were injected subcutaneously into the backs of the nude mice and observed its proliferation for 24 days. Compared with the shNT control group, downregulation of OXSR1 significantly inhibited tumor proliferation in shOXSR1 group (Figure 4a–c). HE staining showed the formation of solid tumors, and immunohistochemical analysis indicated that OXSR1 and Ki67 expression was decreased in shOXSR1 group,



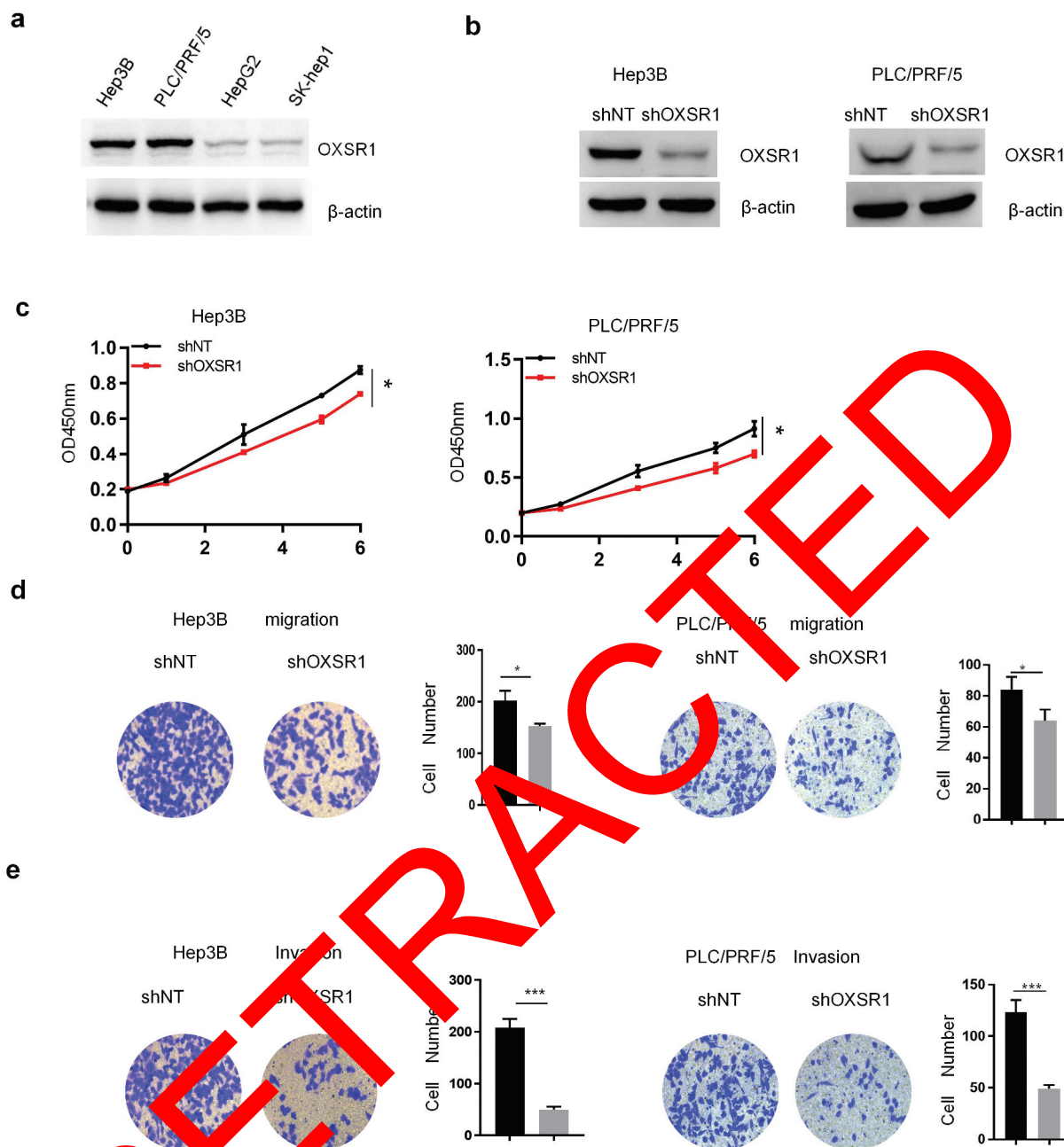
**Figure 2.** Elevated OXSR1 predicted poor clinical outcome of HCC patients.

(a, b) The expression level of OXSR1 identified by Western blot and RT-qPCR. (c) Immunohistochemistry for OXSR1 expression in Hepatocellular carcinoma tissue and adjacent normal tissue. Magnification, 400  $\times$ . (d) Survival curve showing the difference in survival between patients with different OXSR1 expression based on TCGA-LIHC cohort. (e, f) The correlations between OXSR1 expression and the clinicopathological characteristics in TCGA-LIHC cohort.

**Table 1.** Univariate and multivariate Cox regression of OXSR1 expression for overall survival in TCGA liver Hepatocellular carcinoma patients.

Parameter	Univariate Cox analysis			Multivariate Cox analysis		
	HR	95%CI	p-value	HR	95%CI	p-value
Age	1.18	0.75–1.86	0.48			
Gender	1.28	0.80–2.05	0.30			
Grade	1.08	0.68–1.71	0.74			
Stage	3.02	1.92–4.77	<b>&lt;0.001</b>	1.02	0.13–7.82	0.99
T	3.05	1.93–4.81	<b>&lt;0.001</b>	2.61	0.34–19.77	0.35
M	3.85	1.21–12.3	<b>0.02</b>	2.64	0.78–8.91	0.12
N	2.02	0.49–8.28	0.33			
OXSR1	1.17	1.06–1.29	<b>0.002</b>	1.14	1.03–1.27	<b>0.012</b>

Bold values indicate  $P < 0.05$ ; HR, hazard ratio; CI, confidence interval.



**Figure 3.** OXSR1 promotes malignant progression of HCC in vitro.

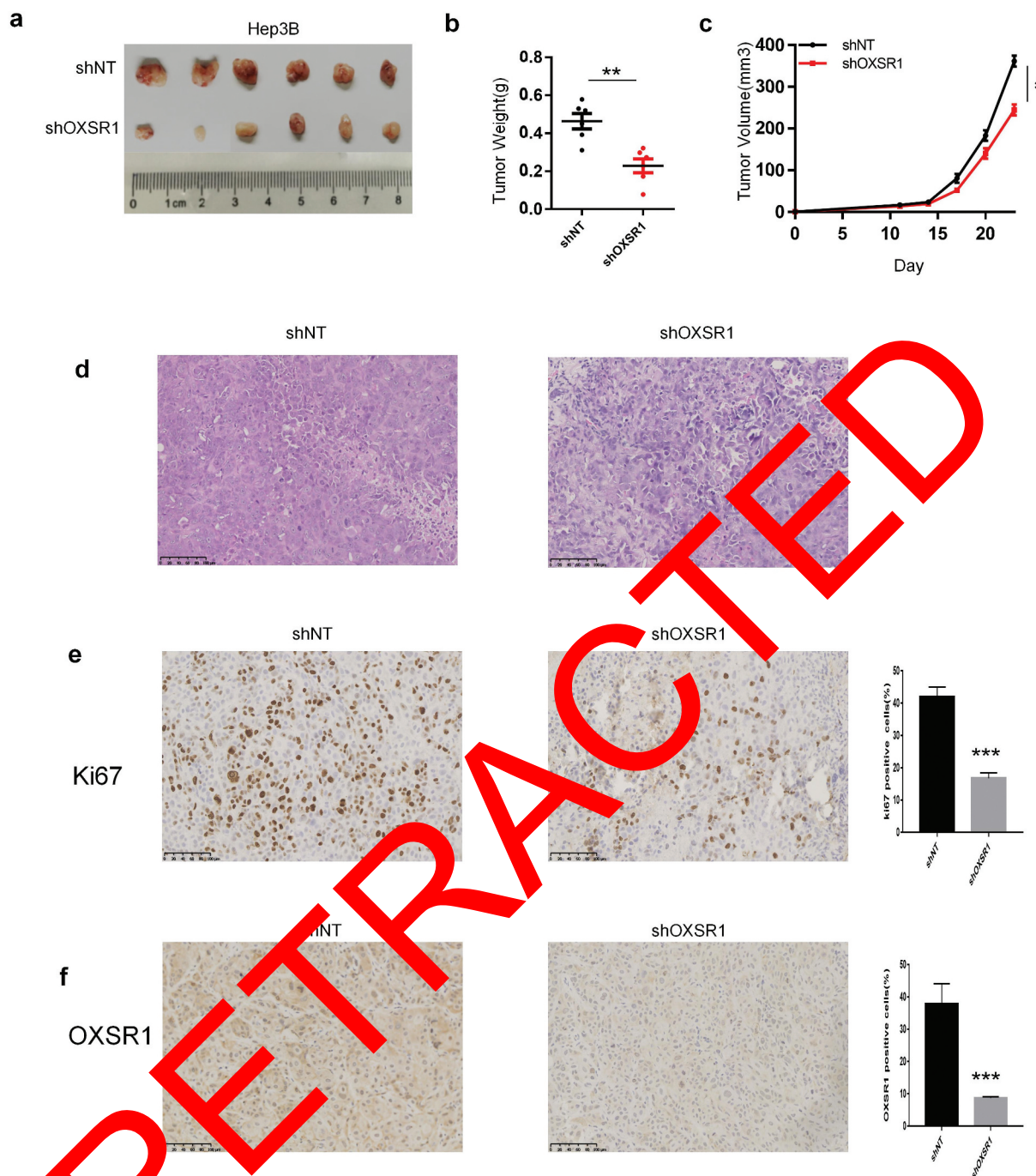
(a) The protein level of OXSR1 in four HCC cell lines determined by western blot. (b) The knockdown efficiency of OXSR1-shRNA in Hep3B and PLC/PRF/5 cells was detected by western blot. (c) Proliferation of Hep3B and PLC/PRF/5 cells was evaluated using CCK8 assay after silencing OXSR1. (d, e) Migratory and invasive abilities of Hep3B and PLC/PRF/5 cells were measured using the Transwell assay after silencing OXSR1. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

which further determined upregulated OXSR1 promoted the proliferation of HCC (Figure 4d–f).

#### **P53 mutants release OXSR1 expression**

Data mining of the TCGA-LIHC cohort showed that OXSR1 had a higher expression when TP53 had

a high mutation frequency. A permutation test of OXSR1 expression between non-silent mutation and no mutation samples were performed (Figure 5a). Based on the results of TCGA data analysis, we next expressed wild-type p53 and p53R249S in p53 null Hep3B cells. Both western blot and qPCR analysis revealed that the R249S mutation of p53 can release



**Figure 4.** OXSR1 promotes tumor proliferation in vivo.

(a) Nude mice were subcutaneously in the back injected with Hep3B cells ( $n = 6$  per group) and tumors were removed. (b, c) Tumor weight and volume were measured at indicated. (d) Representative images of HE staining in tumor tissue section. Scale bar = 100  $\mu\text{m}$ . (e, f) Representative images of immunohistochemistry staining of Ki67 and OXSR1 in tumor tissue section. Scale bar = 100  $\mu\text{m}$ .

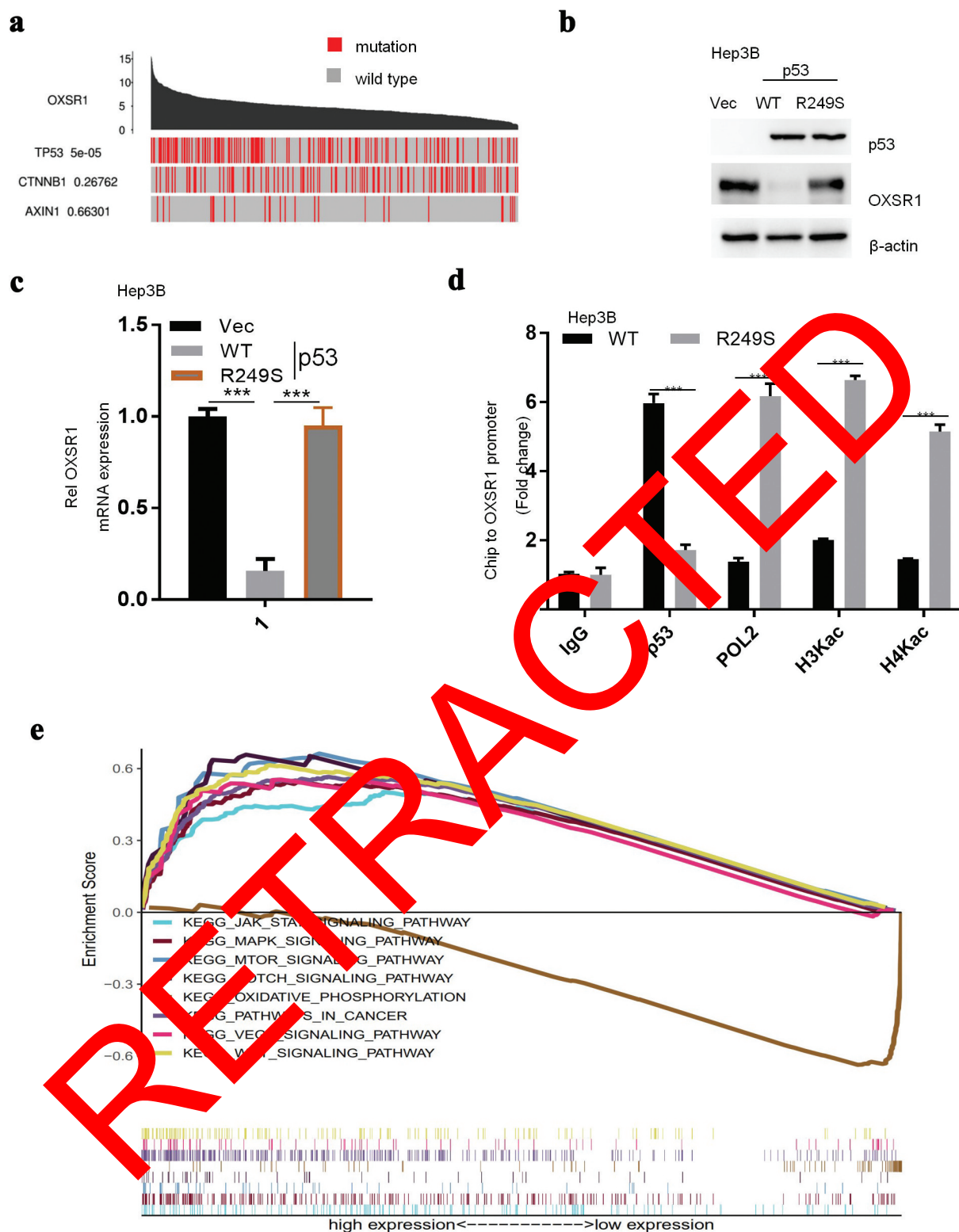
the expression of OXSR1 (Figure 5b and c). To explore whether the levels of histones acetylation and POL2 were involved in the transcriptional regulation of the OXSR1 gene by p53-R249S. ChIP assay was used to study the changes of POL2, H3Kac and H4Kac on the OXSR1 gene promoter. The results showed that p53-R249S significantly

increased the levels of POL2, H3Kac and H4Kac in the promoter of the OXSR1 gene (Figure 5d).

### GSEA

To further explore the downstream molecular mechanism of OXSR1 in HCC, we compared the





**Figure 5.** OXSR1 expression was regulated by p53-R249S.

(a) Heatmap showing OXSR1 expression level and corresponding mutant genes in TCGA-LIHC cohort. The number next to the gene names represents p-value,  $p < 0.05$  was considered to have a significant correlation between the mutation of this gene and the expression of the target gene. (b, c) The expression efficiency of wild-type p53 and p53R249S in p53 null Hep3B cells was detected by western blot and RT-qPCR. (d) ChIP analysis with anti-p53, anti-RNA poll II, anti-H3Kac and anti-H4kac antibodies was performed in Hep3B cells. (e) GSEA results showed differential enrichment of genes in OXSR1 high and low expression phenotype.

high ( $\geq$ mean) and low ( $<$ mean) OXSR1 expression in the Molecular Signatures Database via GSEA for identifying differentially activated signaling pathways in HCC. In general, gene sets with FDR  $<$  0.05 were considered to be significantly enriched. The results demonstrated that the group of high OXSR1 expression in HCC had significant correlation with multiple cancer-related pathways, including JAK, MTOR, NOTCH, MAPK, WNT, and VEGF signaling pathways (Figure 5e, Table 2).

### Relationship between OXSR1 expression and immune infiltration in HCC

Increasing evidence suggested that TIICs are related to the progression of malignancies and the prognosis of patients [23,24]. Therefore, we used the CIBERSORT algorithm to explore the role of immune-related biological functions of OXSR1 in HCC. The CIBERSORT algorithm was used to determine the concentrations of 22 kinds of TIIC in each downloaded sample. The results revealed that resting memory CD4 T cells, T regulatory cells (Tregs), activated NK cells and neutrophils were the primary TIICs affected by OXSR1 expression (Figure 6a). Resting memory CD4 T cells ( $p = 0.045$ ), T regulatory cells (Tregs) ( $p = 0.013$ ) and activated NK cells ( $p = 0.049$ ) showed a higher proportion in the low OXSR1 expression group. Conversely, neutrophils ( $p = 0.014$ ) had a higher proportion in the high OXSR1 expression group. In addition, we used TIMER to examine the link of OXSR1 expression with immune infiltrating levels. As shown in Figure 6b, OXSR1 was positively correlated with tumor purity ( $r = 0.109$ ,  $p < 0.05$ ) and the infiltrating levels of B cells ( $r = 0.23$ ,

$p < 0.001$ ), CD4 + T cells ( $r = 0.345$ ,  $p < 0.001$ ), CD8 + T cells ( $r = 0.2$ ,  $p < 0.001$ ), neutrophils ( $r = 0.441$ ,  $p < 0.001$ ), dendritic cells ( $r = 0.337$ ,  $p < 0.001$ ) and macrophages ( $r = 0.381$ ,  $p < 0.001$ ).

### Correlation between OXSR1 and immune checkpoints in HCC

Immune checkpoint blockade (ICB) therapy has shown excellent effects in the clinical treatment of cancer. Given the above results, we attempted to determine whether the expression of OXSR1 had a special role in cancer immunotherapy. As shown in Figure 6c, OXSR1 expression was positively correlated with the expression of PD-L1 ( $r = 0.43$ ,  $p < 0.001$ ), TIGIT ( $r = 0.166$ ,  $p < 0.001$ ), and TIM-3 ( $r = 0.219$ ,  $p < 0.001$ ). However, there was no obvious correlation between CTLA4 expression and OXSR1 expression.

## Discussion

In this study, our findings determined that the OXSR1 expression is abnormally elevated in HCC, which is associated with higher histopathological grade and TNM stage. Furthermore, we confirmed that upregulated OXSR1 is an independent prognostic factor to predict the worse clinical outcome of HCC patients. These results implied that OXSR1 is a potential biomarker for HCC.

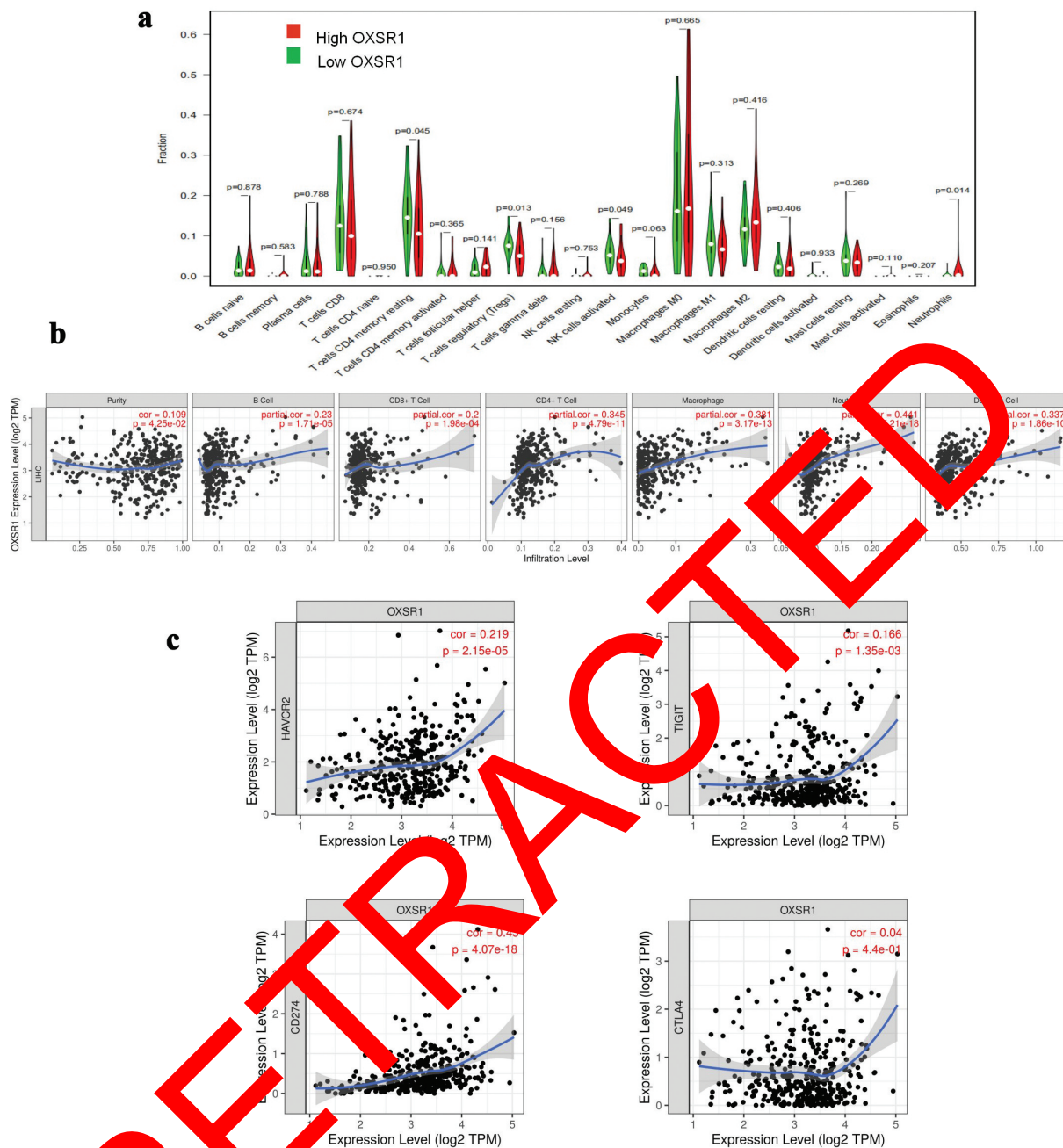
GCKs are related to multiple cellular functions including cell migration, growth and immune regulation, and many members of GCKs family participate in the progression of malignant tumors [25–27]. For instance, SPAK, a member of GCK-IV subfamily as well as OXSR1, was found to promote KCC3-mediated aggressiveness of cervical cancer through

**Table 2.** GSEA results of pathways significantly correlated with OXSR1 expression in TCGA-LIHC cohort.

Gene set name	NES	NOM p-val	FDR q-val
KEGG_WNT_SIGNALING_PATHWAY	2.0498774	0	0.002978303
KEGG_MTOR_SIGNALING_PATHWAY	2.0096757	0	0.003161641
KEGG_MAPK_SIGNALING_PATHWAY	1.9267097	0	0.004907752
KEGG_PATHWAYS_IN_CANCER	1.9140106	0	0.00544316
KEGG_NOTCH_SIGNALING_PATHWAY	1.8701198	0.00210084	0.007637583
KEGG_VEGF_SIGNALING_PATHWAY	1.8185564	0	0.011931653
KEGG_JAK_STAT_SIGNALING_PATHWAY	1.6403431	0.010162601	0.044632148
KEGG_OXIDATIVE_PHOSPHORYLATION	-1.8785712	0.005952381	0.047589604

NES: normalized enrichment score; NOM: nominal; FDR: false discovery rate.

Gene sets with NOM p-value  $<$  0.05 and FDR q-value  $<$  0.05 are considered as significant.



**Figure 6.** OXSR1 was related to immune infiltration in HCC patients based on the TCGA-LIHC cohort. (a) Violin plots showing the proportion of 22 TIICs in high OXSR1 and low OXSR1 groups. (b) Scatterplots displaying the correlation between OXSR1 expression and TIICs infiltration levels. (c) Correlation between OXSR1 expression and immune checkpoint expression (PD-L1, CD274), CTLA-4, TIGIT, and TIM-3 (HAVCR2)).

the NF- $\kappa$ B/p38 MAPK/MMP2 axis [27]. Previous studies identified that OXSR1 is activated by WNK1 and acts as a significant part of the regulation of ion homeostasis and cell volume [15]. However, the latest research showed that WNK1-OXSR1 signaling is involved in angiogenesis, of which OXSR1 is an indispensable part and is related to the invasion of endothelial cells [17]. In addition, recent studies

demonstrated that targeting OXSR1 could improve the anti-cancer effect of chemotherapy and inhibit tumors progression. For example, activation of the WNK1-OXSR1-NKCC1 pathway may reduce the anti-neoplastic effect of temozolomide in the treatment of glioma [19]. And a recent funding indicated that high expression of OXSR1 can independently predict poor prognosis and lymph node metastasis

in breast cancer [20]. In our study, the results revealed that upregulated OXSRI significantly promotes the growth and invasion of HCC. Therefore, OXSRI may be a potential target in HCC treatment.

The acetylation level of histones acts as a vital role in regulating transcription of gene. Histone acetyltransferases (HATs) can catalyze the acetylation of histone, resulting in a relaxed chromatin state and the promotion of gene transcription [28]. Whereas histone deacetylation induced by histone deacetylase (HDAC) is related to gene silencing [29]. As a tumor suppressor gene, p53 has been determined to inhibit the transcription of certain genes [30]. Previous studies reported that p53 can recognize and bind to the specific DNA sequences of the target gene and recruit HDAC or other chromatin modifying factors to inhibit its transcription [31,32]. However, mutations in almost all domains of p53 may abolish its inhibitory ability. For instance, p53 recruited HDAC2 to inhibit the transcription of p21, but DNA damage abolished this inhibitory ability and increased histone Lys acetylation to promote p21 transcription [33]. p53 is the most frequently mutated gene in the progression of malignancies, and its mutations are mainly missense mutations that occur in the sequence-specific DNA binding (SSDB) domain [34]. The few residues that mutate with exceptionally high frequency in the DNA binding region are defined as hotspot mutations [35]. p53-R249S is the sole hotspot in HCC, and is highly correlated with aflatoxin B infection. In the R249S mutation, the third base G of codon 249 of p53 is replaced by a T, resulting in the substitution of arginine with threonine. This mutation accounts for 30% of all HCC with p53 mutations and drives HCC proliferation and tumorigenesis [36]. RNA polymerase II (POL2) is widely used to assess transcription initiation and events that occur at the transcription start site [37]. Our results first showed that p53 can bind to the promoter region of OXSRI and repress its transcription. The mechanism may be that p53 binds to DNA and recruits HDAC to cause histone hypoacetylation, thereby inhibiting transcription. However, p53-R249S abolishes this inhibitory ability and increases the level of POL2, H3Kac and H4Kac in the promoter region of the OXSRI gene, thus promoting the transcriptional expression of OXSRI.

GCK-VI kinases including OXSRI and SPAK contribute to the regulation of T-cell activation [38]. Our findings indicated that OXSRI expression is positively correlated with the infiltration levels of CD4 + T cells and CD8 + T cells in HCC. This is consistent with the previous results [9]. Although the specific mechanism is unclear, it is likely that OXSRI regulates T cell activation by altering osmotic pressure. Programmed death ligand-1 (PD-L1) is currently important targets for clinical immunotherapy, and have shown great anticancer effects in multiple cancers [39]. In HCC, elevated PD-L1 is closely related to poor prognosis and tumor aggressiveness [40,41]. TIM3 is an emerging immune checkpoint molecule and current research has proven that TIM3 blockade combined with anti-PD-1 and/or anti-CTLA-4 Abs can obtain better therapeutic effects [42]. Our results revealed that there is a significantly positive relationship between the expression level of OXSRI and the expression levels of PD-L1, TIM-3 and TIGIT in HCC. These findings indicate that targeting OXSRI can be an alternative strategy for immunotherapy against HCC.

## Conclusions

In summary, our research explored the expression pattern of OXSRI in HCC, and determined that elevated OXSRI is an independent prognostic factor in HCC patients. In addition, we revealed the mechanism of OXSRI expression in HCC regulated by p53-R249S and the latent impact of OXSRI in the immunotherapy of HCC.

## Disclosure statement

The authors have declared that no competing interest exists.

## Funding

This work was supported by grants from the National Natural Science Foundation of China, Grant/Award Number: 81700568.

## Availability of data and materials

The data supporting the results of this research are available within this article and the supplementary materials.

## Highlights

- (1) OXSR1 was abnormally upregulated in HCC and associated with poor clinical outcome.
- (2) Targeting OXSR1 significantly suppress the proliferation, migration and invasion of HCC.
- (3) p53-R249S release the OXSR1 expression by increasing the level of POL2, H3Kac and H4Kac in the promoter region of the OXSR1 gene.
- (4) The expression level of OXSR1 was positively correlated with the infiltration levels of TIICs and PD-L1 expression in HCC.

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