Identification of Transmembrane Protein 98 as a Novel Chemoresistance-Conferring Gene in Hepatocellular Carcinoma

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Abstract

Chemoresistance is one of the major obstacles in systemic chemotherapy and targeted therapy for patients with advanced hepatocellular carcinoma. To identify novel chemoresistance-associated targets in hepatocellular carcinoma, chemoresistant hepatocellular carcinoma cell lines were established. By comparing the global gene expression profiles between chemoresistant and chemosensitive cell lines, eight novel chemoresistance-associated genes were identified to be significantly associated with the commonly augmented chemoresistance of hepatocellular carcinoma cells. One upregulated candidate named transmembrane protein 98 (TMEM98) was found to be overexpressed in 80 of 118 (67.80%) of patients with hepatocellular carcinoma. TMEM98 mRNA in tumor tissues was significantly higher than non-tumor tissues of patients with hepatocellular carcinoma (P < 0.0001). Upregulation of TMEM98 was significantly correlated with advanced tumor stage (P = 0.048), high incidence of early tumor recurrence (P = 0.005), poor overall survival (P = 0.029), and poor disease-free survival (P = 0.011) of patients with hepatocellular carcinoma after hepatectomy. Importantly, upregulation of TMEM98 mRNA in patients with hepatocellular carcinoma who received transarterial chemoembolization (TACE) treatment was significantly higher than in patients without TACE treatment (P = 0.046). Moreover, patients with poor response to TACE treatment had higher degree of TMEM98 upregulation than the responsive patients. In vitro and in vivo studies showed that suppression of TMEM98 in chemoresistant hepatocellular carcinoma cells restored their chemosensitivity, while forced overexpression of TMEM98 enhanced their chemoresistance. The mechanism of TMEM98 in conferring chemoresistance of hepatocellular carcinoma might be possibly through activation of the AKT pathway and deactivation of p53. In conclusion, we identified a panel of novel common chemoresistance-associated genes and demonstrated that TMEM98 is a chemoresistance-conferring gene in hepatocellular carcinoma. Mol Cancer Ther; 13(5); 1–13. ©2014 AACR.

Introduction

Liver cancer is the sixth most common cancer and the third most common cause of cancer-related deaths worldwide (1). Hepatocellular carcinoma represents 70% to 85% of the total liver cancer (1). Up to 70% of patients with hepatocellular carcinoma have been suffering from limited treatment options because of late diagnosis and/or advanced stage of the disease when, however, surgical treatments including liver transplantation and hepatectomy as well as regional therapy are not feasible. Currently, there is no proven effective conventional systemic chemotherapy for patients with advanced hepatocellular carcinoma because of the inherent chemoresistant nature of hepatocellular carcinoma and with intolerable cytotoxicity, resulting in the dismal prognosis of these patients (2–4). Hepatocellular carcinoma is a heterogeneous disease in terms of etiology, molecular, and carcinogenic mechanisms as well as biologic behaviors, which can collectively contribute to diverse mechanisms of chemoresistance among patients with hepatocellular carcinoma (5). Recent success of clinical trial of single-agent sorafenib in treating advanced patients with hepatocellular carcinoma (6). However, most of the targeted agents have demonstrated a very low response rate, including sorafenib (3, 7), leaving the problem of chemoresistance to be solved. Therefore,
identification of novel molecular targets must be very important for understanding the underlying mechanisms of chemoresistance of hepatocellular carcinoma and eventually for developing more effective therapeutic regimens.

The development of chemoresistance in hepatocellular carcinoma could be either intrinsic and/or acquired. There have been several molecular targets associated with chemoresistance of hepatocellular carcinoma. Chemoresistance of hepatocellular carcinoma can be achieved by upregulation of the drug transporter family known as the adenosine triphosphate-binding cassette (ABC) transporters such as ABCB1, ABCC1, ABCC2, and ABCC3 (8–11), leading to increment of drugs efflux system to remove drugs out of cells. High levels of MRP2, MRP3, MRP4, and MRP5 genes have been found in an acquired cisplatin-resistant hepatocellular carcinoma cell line (12). Moreover, recent findings have suggested that liver cancer stem cells contribute to chemoresistance of hepatocellular carcinoma. For instance, upregulation of Octamer 4 gene, a transcriptional factor of pluripotent cells, can significantly augment chemoresistance of hepatocellular carcinoma cells via the Oct4–AKT–ABCG2 pathway (13). CD133+ hepatocellular carcinoma cancer stem cells can also contribute to chemoresistance through activation of the Akt/PKB pathway (14). Overexpression of granulin epithelin precursor and ABCB5 in liver cancer stem cells can lead to significant increment of chemoresistance (15). Besides, a study from acquired doxorubicin-resistant hepatocellular carcinoma cell lines has identified a panel of differentially overexpressed genes and subsequently characterized that upregulation of TOP2A gene is one of the contributors of acquired doxorubicin-resistance in hepatocellular carcinoma (16). Yet, the molecular information governing chemoresistance in hepatocellular carcinoma so far is far away from achieving effective therapeutic regimens.

To search for novel targets, we started from establishing chemoresistant sublines from a human metastatic hepatocellular carcinoma cell line and further identified a panel of common differential genes linking to the development of acquired chemoresistance of hepatocellular carcinoma cells. Moreover, we investigated one of the upregulated candidates, transmembrane protein 98 (TMEM98), to unveil its clinical significance and roles in chemoresistance of hepatocellular carcinoma.

Materials and Methods

Patients

One hundred and eighteen patients with hepatocellular carcinoma who underwent liver resection between December 1999 and May 2009 were recruited from Department of Surgery, Queen Mary Hospital, the University of Hong Kong. Twelve normal liver tissues were obtained from patients with signed consent. The study was approved by the Ethics Committee of the University of Hong Kong.

Cell lines

Human hepatocellular carcinoma cell lines, MHCC97L (metastatic), PLC (nonmetastatic), and Hep3B (nonmetastatic), were cultured at Dulbecco’s modified Eagle medium (DMEM) medium with 10% FBS (Invitrogen). To establish chemoresistant cells, MHCC97L/CisR, and MHCC97L/DoxR, MHCC97L was chronically incubated with increased concentrations of cisplatin (Pharmachemie BV) or doxorubicin (Sigma-Aldrich) for 12 months, starting from concentration of 100 ng/mL of cisplatin or 20 ng/mL of doxorubicin. On average, the concentration of cisplatin or doxorubicin was increased for every 2 to 3 weeks. Proliferation rates of the chemoresistant sublines were examined for each month. Before cDNA microarray analysis, the chemoresistant sublines were cultured in DMEM medium without drug for 1 month. MHCC97L/CisR2 and MHCC97L/DoxR2 were sublines under exposure of cisplatin and doxorubicin, respectively, for additional of 2 months.

Cloning and transfection

Full length of human TMEM98 cDNA was cloned into pcDNA3.1(+) vector (Invitrogen). The primers for cloning included forward primer: 5′-GTACAGGATCCAGC ATGGAGACTGTGGTGATTGTT-3′; reverse primer: 5′-CTCGAGTCAGCTGA TAAAAATGGCAGACTGCTCTGCA-3′. Transfection of plasmids to cells was performed by Lipofectamine-2000 (Invitrogen). Stable transfectants were selected from G418-containing medium for 2 weeks. siRNA of targeting human TMEM98 mRNA and negative control siRNA were purchased from Invitrogen. siRNAs were transfected to cells by using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer’s instruction.

Proliferation assays

MTT (Invitrogen) assay and colony formation assay were performed as previously described (18). Cells were treated with drugs for 72 hours and 2 weeks for MTT assay and colony formation assay, respectively. Each experiment consisted of 3 replications and at least 3 individual experiments were carried out.
cDNA microarray analysis
cDNA microarray profiles of MHCC97L, MHCC97L/CisR, and MHCC97L/DoxR were conducted by gene chip system Human U133 Plus 2.0 (Affymetrix Inc.), by Genome Research Centre, The University of Hong Kong (18). Microarray data (GEO accession number: GSE54175) were analyzed by GeneSpring Version 10 (Agilent Technologies). A 3-fold difference was used to select differential genes.

Quantitative reverse transcription PCR
Total RNA from cells and liver tissue samples were purified by Trizol regent (Invitrogen). Method of quantitative reverse transcription PCR (qRT-PCR) analysis was described as in previous study (19). The expression of 18S ribosomal RNA was used as internal control. Primers used in this study were listed in Supplementary Table S1.

Western blot analysis
Method of Western blot analysis was described in previous study (18). TMEM98 antibody was purchased from Sigma-Aldrich Corporation. Antibodies, including AKT, phospho-AKT(Ser473), BCL-XL, phospho-ASK-3β(Ser9), p21, p53, phospho-p53(Ser6), phospho-p53(Ser9), phospho-p53(Ser15), phospho-p53(Ser20), and phospho-p53(Ser392), were purchased from Cell Signaling Technology. β-Actin antibody was purchased from Santa Cruz Biotechnology.

Apoptosis assay
Cells (3 × 10^5) were seeded onto 6-well plate for 24 hours. The cells were harvested and stained with Annexin-V Fluor Staining Kit (Roche) according to manufacturer’s instruction and analysis by flow cytometer. Early apoptosis was defined as Annexin V–positive and propidium iodide (PI)-negative cells. Late apoptosis was defined as Annexin V–positive and PI-positive cells. The number of apoptotic cells included early and late apoptosis. Each experiment was analyzed in triplicate and at least 3 independent experiments were performed.

Animal model
 Xenograft ectopic liver tumor model in nude mice was adopted (18). For drug treatment, single dose of cisplatin (5 mg/kg) or doxorubicin (5 mg/kg) was administrated to nude mice at day 5 after subcutaneous injection of cells (5 × 10^6 cells/100 μL). Tumor volume was calculated as the following equation: tumor volume (cm^3) = length × width × thickness. At least 6 mice were performed for each experimental group. Animal study was specifically approved by Animal (Control of Experiments) Ordinance Chapter 340, the Department of Health, Hong Kong Special Administrative Region [Ref.: (12–63) in DH/HA&P/8/2/3 Pt. 37].

Statistical analysis
TMEM98 mRNA of clinical samples was analyzed by Prism Version 5.01 (Graphpad). The difference of TMEM98 mRNA between tumor and nontumor tissues of each patient with hepatocellular carcinoma was determined as: ΔΔCt(TMEM98) = ΔΔCt(tumor) − ΔΔCt(non-tumor). Statistical analysis of clinical parameters was carried out using SPSS 16 for Windows (SPSS Inc.). Receiver operating characteristic (ROC) curve was generated to analyze the sensitivity and 1-specificity of ΔΔCt(TMEM98) value to predict overall survival of patient with hepatocellular carcinoma after hepatectomy. Youden index was used to determine high deregulation (High group) and low deregulation (Low group) of patient with hepatocellular carcinoma. The association of TMEM98 deregulation and clinicopathologic parameters was analyzed by a χ² test. The prognostic value of TMEM98 mRNA for predicting overall and disease-free survival of patient with hepatocellular carcinoma after hepatic resection was calculated by Kaplan–Meier analysis with the log-rank test. For disease-free survival analysis, patient with hepatocellular carcinoma under the category of hospital mortality were excluded. Cox proportional hazard regression model was performed to test factors that were significantly associated with the overall survival or disease-free survival of the patient with hepatocellular carcinoma. P value < 0.05 was considered to be statistically significant.

Results
Establishment of chemoresistant hepatocellular carcinoma sublines
After continuous incubation of increasing concentrations of cisplatin or doxorubicin to a human metastatic hepatocellular carcinoma cell line named MHCC97L for 12 months, 2 chemoresistant sublines, MHCC97L/CisR and MHCC97L/DoxR, were established. The morphology of MHCC97L/CisR and MHCC97L/DoxR sublines was similar to MHCC97L. In a nondrug culture medium, these sublines grew slightly faster than MHCC97L (Supplementary Fig. S1). The in vivo growth rate of these sublines without drug was similar to MHCC97L (Supplementary Fig. S2).

The in vitro chemoresistance of MHCC97L/CisR and MHCC97L/DoxR sublines was significantly increased compared with MHCC97L. MTT assay showed that chemoresistance of MHCC97L/CisR to cisplatin (IC₅₀ = 20 μg/mL) was nearly 10-folds higher than MHCC97L (IC₅₀ = 2.2 μg/mL). MHCC97L/DoxR exhibited more than 25-fold increase of resistance to doxorubicin compared with MHCC97L (IC₅₀ of doxorubicin: 40 μg/mL vs. 1.5 μg/mL; Fig. 1A). The colony-forming ability of MHCC97L/CisR and MHCC97L/DoxR retained similar colony-forming ability at 1,000 ng/mL of cisplatin (Fig. 1B) and 200 ng/mL of doxorubicin (Fig. 1C), respectively, whereas MHCC97L was dramatically suppressed in >500 ng/mL of cisplatin or >50 ng/mL of doxorubicin (Fig. 1B and C). The IC₅₀ of MHCC97L/CisR2 and MHCC97L/DoxR2 was approximately 1.3- and 1.2-fold of the MHCC97L/CisR and MHCC97L/DoxR.
A MTT Assay

B Cisplatin (ng/mL)

Colony formation assay

C Doxorubicin (ng/mL)

Colony formation assay

D E

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respectively (data not shown). The in vivo chemoresistance of MHCC97L/CisR and MHCC97L/DoxR was increased compared with MHCC97L under cisplatin and doxorubicin treatment, respectively (Fig. 1D and E).

Identification of common chemoresistance-associated genes
The experimental procedures to identify common chemoresistance candidates were illustrated in Supplementary Fig. S3. To avoid overestimation of differential genes from cDNA microarray analysis, 3-fold difference was used as filter condition rather than 2-fold difference. Comparing to cDNA microarray profile of MHCC97L, 537 and 678 differential genes were identified in MHCC97L/CisR and MHCC97L/DoxR respectively, in which 235 genes were commonly differentially expressed in both sublines. Among them, 164 genes were commonly upregulated (Supplementary Table S2) and 71 genes were commonly downregulated (Supplementary Table S3). By applying 10-fold difference as secondary selection criterion, 36 candidate genes were identified to be commonly differentially expressed. After validation by qRT-PCR analysis, 19 common differential genes, 7 upregulated and 12 downregulated, were identified (Supplementary Table S4).

To examine whether the degree of differential expression is correlated to the degree of chemoresistance of hepatocellular carcinoma cells, the expression of 19 differential genes were further verified in MHCC97L, MHCC97L/CisR, MHCC97L/CisR2, MHCC97L/DoxR, and MHCC97L/DoxR2 cells. Among the upregulated genes, the expression levels of APOB, RUNDC3B, SYK, TMEM47, and TMEM98 were significantly increased along with the increased degrees of chemoresistance of cells, reaching the highest level in both MHCC97L/CisR2 and MHCC97L/DoxR2 compared with MHCC97L/CisR and MHCC97L/DoxR, respectively (Supplementary Fig. S4A). Among the downregulated genes, the expression levels of FGB, ZNF284, and BTB11 were significantly lowered along with increased degrees of chemoresistance of cells, reaching the lowest level in both MHCC97L/CisR2 and MHCC97L/DoxR2 compared with MHCC97L/CisR and MHCC97L/DoxR, respectively (Supplementary Fig. S4B). Totally, 8 chemoresistance-associated genes were identified to be significantly associated with common chemoresistance of hepatocellular carcinoma cells.

To identify hepatocellular carcinoma-associated candidate genes, mRNA expression levels of APOB, RUNDC3B, SYK, TMEM47, and TMEM98 genes were preliminarily investigated in 37 pairs of tumor and adjacent nontumor liver tissues of patients with hepatocellular carcinoma. One of them named TMEM98 was found to be overexpressed in approximately 65% of the patients with hepatocellular carcinoma (Supplementary Table S5). Owing to chemoresistance- and hepatocellular carcinoma–associated features of TMEM98 gene, it was selected for further characterization.

Deregulation of TMEM98 predicts poor prognosis of patients with hepatocellular carcinoma
Among 118 patients with hepatocellular carcinoma, 80 patients (67.80%) were found to differentially overexpress TMEM98 mRNA (ΔΔCt(TMEM98) ≥ 1). The average relative ΔΔCt(TMEM98) value among tumor liver tissues, nontumor liver tissues, and healthy donor liver tissues were 7.00, 5.34, and 4.29, respectively (Fig. 2A). The expression level of TMEM98 mRNA in tumor liver tissue was significantly higher than in nontumor liver tissues (unpaired 2-tailed t test, P < 0.0001; paired 2-tailed t test, P < 0.0001) and healthy donor liver tissues (unpaired 2-tailed t test, P = 0.0002). Agreed with the result from qRT-PCR analysis, Western blot analysis showed that TMEM98 protein was overexpressed in tumor tissue of patients with hepatocellular carcinoma compared with nontumor tissues and healthy donor tissues (Fig. 2B).

Fifty-one (43.22%) and 67 (56.78%) patients with hepatocellular carcinoma were defined as TMEM98 high deregulation group (High group) and low deregulation group (Low group) respectively according to Youden index analysis. High deregulation of TMEM98 mRNA was significantly correlated with the presence of advanced New AJCC stage (P = 0.001) and recurrence of tumor within first year (P = 0.005; Table 1). Moreover, low deregulation of TMEM98 mRNA was significantly correlated with early pathologic tumor–node–metastasis (pTNM) stage (P = 0.048).

Kaplan–Meier analysis illustrated that patients with high deregulation of TMEM98 mRNA were significantly associated with poor overall survival (log-rank = 4.741, P = 0.029) and poor disease-free survival (log-rank = 6.543, P = 0.011; Fig. 2C). The mean periods of overall and disease-free survival for the High group were 66.6 and 38.8 months, whereas for the Low group were 85.8 and 62.8 months.

Cox proportional hazard regression analysis was used to find out the independent predictors for predicting overall survival and disease-free survival of patients with hepatocellular carcinoma after hepatectomy among selected 5 factors, including TMEM98 mRNA, pTNM stage, venous infiltration, New AJCC stage, and serum a-fetoprotein (AFP) level, which were significantly associated with the overall survival of patients with hepatocellular carcinoma by Kaplan–Meier analysis (Supplementary Table S6). Univariable Cox proportional hazard regression analysis showed that TMEM98 mRNA was a significant...
Figure 2. Clinical significance of TMEM98 in hepatocellular carcinoma. A, TMEM98 mRNA in liver tissues of patients with hepatocellular carcinoma and healthy donors. B, expression of TMEM98 protein in 5 pairs of tumor and nontumor tissues of patients with hepatocellular carcinoma. C, Kaplan–Meier analysis of overall and disease-free survival of patients with hepatocellular carcinoma. D, Western blot analysis of TMEM98 protein between non-TACE and TACE treatment groups. E, comparison of tumor to nontumor ratio (T/NT) of TMEM98 mRNA between patients with and without TACE treatment. F, comparison of tumor to nontumor ratio (T/NT) of TMEM98 mRNA between patients with hepatocellular carcinoma with poorly responsive and responsive effects in the TACE treatment group. G, TMEM98 mRNA in different hepatocellular carcinoma cells in responding to cisplatin or doxorubicin treatment. *P < 0.05; **P < 0.01.
predictor for both of overall survival (HR = 1.818; 95% confidence interval (CI), 1.05–3.14; P = 0.032) and disease-free survival (HR = 1.816; 95% CI, 1.14–2.89; P = 0.012) of patients with hepatocellular carcinoma after hepatectomy (Supplementary Table S6). Multivariable Cox proportional hazard regression analysis revealed that TMEM98 was not an independent factor for overall or disease-free survival of patients with hepatocellular carcinoma.

To determine whether the deregulation of TMEM98 is correlated to increased chemoresistance of patients with hepatocellular carcinoma, the expression levels of TMEM98 mRNA and protein patients with hepatocellular carcinoma received TACE treatment (TACE group) was examined. The expression level of TMEM98 protein in the TACE group was found to be higher than in the non-TACE group (Fig. 2D). Eighty percent of patients with hepatocellular carcinoma were found to overexpress TMEM98 mRNA in tumor tissues after TACE treatment. The average tumor to nontumor (T/NT) ratio of TMEM98 mRNA in the TACE group was significantly higher than patients without TACE treatment (P = 0.046; Fig. 2E). Moreover, in the TACE group, patients with poor response to TACE treatment showed a higher degree of T/NT ratio of TMEM98 mRNA [ΔΔCt(TMEM98) = 2.92] compared with patients responded to TACE treatment [ΔΔCt (TMEM98) = 1.42; Fig. 2F].

### Table 1. Correlation analysis of TMEM98 mRNA and clinicopathologic features of patients with hepatocellular carcinoma

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^aTotal number less than 118 because of missing data.
^bFour patients were excluded because of their death within the first year without recurrence.
^c, P < 0.05.
MTT assay of MHCC97L/CisR subline

MTT assay of MHCC97L/DoxR subline

Cisplatin concentration (ng/mL)

Doxorubicin concentration (ng/mL)

Cisplatin concentration (μg/mL)

Doxorubicin concentration (μg/mL)

Apoptosis assay on MHCC97L/CisR

Apoptosis assay on MHCC97L/DoxR

MTT assay of MHCC97L-derived transfectants in cisplatin

MTT assay of MHCC97L-derived transfectants in doxorubicin

Apoptosis assay on MHCC97L/CisR

In vivo xenograft model under cisplatin treatment

In vivo xenograft model under doxorubicin treatment
TMEM98 is a novel chemoresistance-conferring target in hepatocellular carcinoma

Treatment with low dosages of cisplatin or doxorubicin in short period could upregulate TMEM98 mRNA in both MHCC97L and PLC cells in a dose-dependent manner, while Hep3B gained upregulation of TMEM98 in cisplatin treatment but not in doxorubicin treatment (Fig. 2G). Upregulation of TMEM98 mRNA after cisplatin or doxorubicin treatment could be persisted after removal of drug (Supplementary Fig. S5).

TMEM98 siRNA could significantly suppress the expression of TMEM98 mRNA to 96 hours after transfection in MHCC97L/CisR and MHCC97L/DoxR (Supplementary Fig. S6). Suppression of TMEM98 expression resulted in significant decrement of the chemoresistance of MHCC97L/CisR to cisplatin (si-Neg: IC50 = 15 μg/mL; si-TMEM98: IC50 = 5 μg/mL; Fig. 3A) and MHCC97L/DoxR to doxorubicin (si-Neg: IC50 > 30 μg/mL; si-TMEM98: IC50 = 11 μg/mL; Fig. 3A). Suppression of TMEM98 expression in MHCC97L/CisR or MHCC97L/DoxR cells by siRNA could significantly enhance their apoptosis under cisplatin (Fig. 3B) or doxorubicin (Fig. 3C) treatment compared with control cells. Moreover, overexpression of TMEM98 in MHCC97L cells could significantly increase their chemoresistance to both cisplatin (MHCC97L-pcDNA3.1: IC50 = 4.0 μg/mL; MHCC97L-TMEM98-1: IC50 = 5.5 μg/mL; Fig. 3D) and doxorubicin (MHCC97L-pcDNA3.1: IC50 = 4.2 μg/mL; MHCC97L-TMEM98-1: IC50 = 10 μg/mL; Fig. 3D). Over-expression of TMEM98 in PLC cell line also increased its chemoresistance (Supplementary Fig. S7). Most importantly, overexpression of TMEM98 in MHCC97L cell line significantly enhanced its in vivo resistance to both cisplatin (Fig. 3E) and doxorubicin (Fig. 3F). However, overexpression of TMEM98 in MHCC97L cell line could not increase sorafenib chemoresistance (Supplementary Fig. S8).

TMEM98 modulates chemoresistance of hepatocellular carcinoma through AKT and p53 pathways

To understand the molecular mechanisms of TMEM98 on chemoresistance of hepatocellular carcinoma, 2 important signaling pathways, including the AKT and p53 pathways, were investigated. Activation of the AKT [i.e., phospho-AKT(Ser473)] pathway was found in MHCC97L/CisR and MHCC97L/DoxR compared with MHCC97L (Fig. 4A). Suppression of TMEM98 by siRNA resulted in repression of activation of AKT (Fig. 4B). Moreover, suppression of TMEM98 in MHCC97L/CisR and MHCC97L/DoxR could repress EGF- and IGF2-induced activation of AKT and its downstream targets, including BCL-XL and phospho-GSK-3β(Ser9) (Fig. 4C). Furthermore, suppression of TMEM98 in MHCC97L/CisR and MHCC97L/DoxR could inhibit the activation of the AKT pathway under drug environment in a dose-dependent manner (Fig. 4D). In addition, forced overexpression of TMEM98 in MHCC97L led to elevation of AKT activation under cisplatin or doxorubicin treatment (Fig. 4E).

Deactivation of p53, illustrated by deactivations of phospho-p53(Ser15) and phospho-p53(Ser392), was found in MHCC97L/CisR and MHCC97L/DoxR, while other forms of activated p53, including phospho-p53 (Ser20), phospho-p53(Ser6), and phospho-p53(Ser9), could not be detected (Fig. 5A). Suppression of TMEM98 in chemoresistant sublines restored the activation of phospho-p53(Ser15) and phospho-p53(Ser392) (Fig. 5B). The degree of activation of the p53 signaling pathway in MHCC97L/CisR and MHCC97L/DoxR was elevated under drug environment after suppression of TMEM98 (Fig. 5C). Meanwhile, forced-overexpression of TMEM98 in MHCC97L could repress p53 activation under cisplatin or doxorubicin treatment (Fig. 5D).

Discussion

Systemic chemotherapy has been adopted to treat advanced patients with hepatocellular carcinoma for more than 30 years, but the survival outcome of these patients remained unsatisfactory (3, 4). The emergence of targeted therapy, encouraged by improved survival benefits from sorafenib, will become a major strategy for treatment of advanced patients with hepatocellular carcinoma. However, inadequate knowledge on the molecular mechanisms of chemoresistance of hepatocellular carcinoma from intrinsic and acquired pathways hinders the development of effective targeted therapy on eradicating cancer cells (7, 20). Therefore, identification of novel targets becomes an important task not only to understand molecular mechanism of chemoresistance of hepatocellular carcinoma but to eventually develop new effective therapeutic strategies for advanced patients with hepatocellular carcinoma (21).

We applied a step-by-step approach to identify novel chemoresistance-associated genes. The in vitro and in vivo growth rates of the chemoresistant sublines without drug treatment were similar to parental MHCC97L cell line, indicating that the selection process did not alter the proliferation and tumorigenesis of the sublines. The in vitro and in vivo chemoresistance of the chemoresistant sublines significantly higher than MHCC97L indicated that the acquired molecular changes of these sublines under drug selection, therefore, may be mainly prone to develop their chemoresistance. There were 235 commonly
differential genes, resting more than 300 and 400 genes were distinctly differential in MHCC97L/CisR and MHCC97L/DoxR, respectively. These data indicated that the acquired chemoresistance of hepatocellular carcinoma is contributed by acquired genetic or epigenetic changes of a variety of genes, which were either or both drug-specific and nonspecific. Hepatocellular carcinoma can resist to different chemotherapeutic and targeting agents (2, 20). Identification of common differential genes is indispensable for understanding the molecular mechanism of chemoresistance of hepatocellular carcinoma on different drugs.

The first question we would like to ask was whether change in expression of these genes was correlated to the changes of chemoresistance in hepatocellular carcinoma. Among 19 highly differential genes, 5 genes were found to have significantly increasing upregulation and 3 genes were found to have significantly increasing downregulation in both MHCC97L/CisR2 and MHCC97L/DoxR2 comparing to MHCC97L/CisR and MHCC97L/DoxR, respectively, indicating that the increasing differential expressions of these genes are significantly correlated to the acquired chemoresistance in hepatocellular carcinoma. So far, the roles of these genes in chemoresistance of...
hepatocellular carcinoma is not yet clear, suggesting that they may be novel chemoresistance-associated candidates.

Genes involved in tumorigenesis have been also found to contribute to chemoresistance of hepatocellular carcinoma (13, 15, 22, 23). Identification of hepatocellular carcinoma–associated targets is thus critical not only for understanding the molecular mechanisms of both intrinsic and acquired chemoresistance but also for developing hepatocellular carcinoma–associated targeted therapy. From preliminary examination of clinical samples, one of the differential genes, TMEM98, was found to be overexpressed in most of the hepatocellular carcinoma tissue. Human TMEM98 protein, composed of 226 amino acid residues, is a potential single-pass transmembrane protein located in endoplasmic reticulum. TMEM98 has been found to be overexpressed in adenocarcinoma subtype of adenocarcinoma whose patients have relative poor prognosis than other subtypes (24). TMEM98 mRNA has been included into adenocarcinoma-like expression signature, which is associated with an epithelial mesenchymal transition and activated β-catenin pathway (24). So far the function of TMEM98 is unclear. In our study, TMEM98 mRNA was found to be overexpressed in early stages of chemoresistance. 

Figure 5. The roles of TMEM98 in the p53 pathway. A, Western blot analysis of p53 and different phosphorylated p53 proteins in MHCC97L and chemoresistant sublines. B, Western blot analysis of p53 and different phosphorylated p53 proteins in chemoresistant sublines after suppression of TMEM98. C, overexpression of TMEM98 enhanced p53 activation in chemoresistant sublines under drug treatments. D, overexpression of TMEM98 in MHCC97L cell line suppressed p53 activation under drug treatments. –, si-Ctr; +, si-TMEM98.
70% of tumor tissues of patients with hepatocellular carcinoma, indicating that this gene is hepatocellular carcinoma-associated and its deregulation in hepatocellular carcinoma may also possibly contribute to intrinsic chemoresistance of hepatocellular carcinoma. Furthermore, high deregulation of TMEM98 in hepatocellular carcinoma was found to be significantly associated with advanced New AJCC stage. Low deregulation of TMEM98 in hepatocellular carcinoma was significantly associated with early pTNM stage and nearly significantly associated with absence of venous infiltration. These demonstrated a positive correlation between TMEM98 deregulation and progressive phenotype of hepatocellular carcinoma. Moreover, high deregulation of TMEM98 in hepatocellular carcinoma was found to be significantly correlated with higher incidence of early tumor recurrence and poor overall and disease-free survivals of patients with hepatocellular carcinoma after hepatectomy, suggesting that TMEM98 mRNA may be a potential prognostic marker for patients with hepatocellular carcinoma. The diagnostic value of TMEM98 protein in patients with hepatocellular carcinoma is also valuable for further characterization.

TACE has been widely used to improve the survival of patients with unresectable hepatocellular carcinoma (25). Although a recent study demonstrated improved survival rates and low mortality rate using TACE with lipiodol in 8,510 patients with unresectable hepatocellular carcinoma (26), the survival rate of advanced hepatocellular carcinoma from TACE treatment thus far has been unsatisfactory (25, 27) suggesting a pressing need to identify molecular mechanism linking to acquired chemoresistance during TACE treatment. Our result showed that a significantly increasing upregulation of TMEM98 mRNA in tumor was found in patients with hepatocellular carcinoma received TACE treatment compared with patients with hepatocellular carcinoma without TACE treatment, indicating that hepatocellular carcinoma tumor cells acquired higher changes of TMEM98 expression than nontumor cells after chemotherapy. In addition, the degree of deregulation of TMEM98 in patients who were poorly responsive to TACE treatment was higher than that in TACE-responsive patients, implying that acquired overexpression of TMEM98 in hepatocellular carcinoma may be linking to the acquired chemoresistance of patients with hepatocellular carcinoma after chemotherapy. The in vitro study indicated that TMEM98 is responsive to chemotherapeutic agents. Altogether, the above data suggested a possible association of acquired TMEM98 expression in development of chemoresistance in hepatocellular carcinoma. However, the sample size of patients received TACE treatment in this study was insufficient to reach solid conclusion because of the lack of patients with hepatocellular carcinoma who were subjected for TACE as the first treatment option before surgical treatment.

In our functional study, suppression of TMEM98 expression could trim down chemoresistance of chemoresistant sublines, while forced overexpression of TMEM98 could increase chemoresistance of different hepatocellular carcinoma cells. These data indicated that TMEM98 may plays important roles in the development and maintenance of chemoresistance in hepatocellular carcinoma and targeting suppression of TMEM98 in hepatocellular carcinoma may be a potential strategy to overcome chemoresistance of hepatocellular carcinoma.

Several lines of evidence illustrated that activation of AKT and deactivation of p53 play important roles on chemoresistance of cancers and hepatocellular carcinoma leading to important targets for treating advanced patients with hepatocellular carcinoma (28–32). In our study, the chemoresistant sublines exhibited higher level of activated AKT and lower levels of activated forms of p53 than parental MHCC97L, indicating that AKT and p53 may be involved in the development of chemoresistance. Our data demonstrated that TMEM98 could modulate chemoresistance of hepatocellular carcinoma cells through modulating the status of the AKT and p53 pathways.

In conclusion, our clinical and experimental evidences suggested that TMEM98 is not only a prognostic marker for patients with hepatocellular carcinoma but also a novel molecular target associated with intrinsic and acquired chemoresistance of hepatocellular carcinoma. Furthermore, TMEM98 may confer chemoresistance of hepatocellular carcinoma by activation of the AKT signaling pathway and deactivation of p53. These findings should provide important information for developing effective strategy in the future to overcome chemoresistance of hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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