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Overexpression of DLX2 is associated with poor prognosis and sorafenib resistance in hepatocellular carcinoma

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ABSTRACT

The mechanism underlying poor prognosis and sorafenib resistance in patients with hepatocellular carcinoma (HCC) is unknown and, to date, no useful predictive biomarkers of sorafenib resistance have been identified. Distal-less homeobox 2 (DLX2) is a transcription factor involved in cell cycle regulation that is closely correlated with cancer prognosis. In this study, we showed that DLX2 is overexpressed in HCC tissues and cell lines and that the level of DLX2 overexpression is positively correlated with histological grade, metastasis and Ki67 expression, which are indicators of poor prognosis. We also found that DLX2 accumulates in proliferating HCC cells, where it is associated with the expression of proliferating cell nuclear antigen (PCNA), Cyclin D1 and Cyclin A. Flow cytometry and cell counting kit-8 (CCK-8) assays indicated that DLX2 depletion causes cell cycle arrest at the G1 phase and hinders cell proliferation. Moreover, the sensitivity of HCC cells to sorafenib is restored when the DLX2 gene is knocked down using a short interfering RNA. We demonstrated that DLX2 facilitates sorafenib resistance by promoting the expression of markers of epithelial–mesenchymal transition and by activating the extracellular signal-regulated protein kinase pathway. Our findings reveal that DLX2 plays a regulatory role in HCC cell proliferation and suggests that targeting DLX2 represents a novel strategy to increase sorafenib efficacy in the management of HCC. In conclusion, DLX2 is a novel marker of poor prognosis and sorafenib resistance in patients with HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common tumour and third leading cause of cancer-related death worldwide (Torre et al., 2015). The poor prognosis of patients with HCC is mainly due to delayed diagnosis and the high prevalence of metastasis (Malek et al., 2014). When patients are diagnosed at an advanced stage, conventional therapies such as surgical resection and percutaneous ablation are ineffective (Llovet and Bruix, 2003). Sorafenib is a unique molecular targeted agent that retards disease progression and improves overall survival in patients with advanced HCC (Kudo, 2012). According to previous studies, few patients show tumour regression after sorafenib treatment, suggesting that its primary effect is the induction of dormancy (Llovet et al., 2008; Kudo, 2014). However, a considerable number of patients are resistant to sorafenib and face an unfavorable prognosis and reduced survival time. Nevertheless, the molecular mechanism underlying acquired chemo-resistance to sorafenib remains unclear.

The distal-less homeobox (DLX) gene family, a homologue of the Drosophila distal-less gene, consists of six DLX genes in humans that exist as the bi-gene clusters DLX1/DLX2, DLX3/DLX4 and DLX5/DLX6 (Shao et al., 2013). DLX genes are involved in physiological and pathological events such as tissue homeostasis, embryonic development, cell proliferation and apoptosis (Suh et al., 2009; Panganiban and Rubenstein, 2002; Kraus and Lufkin, 2006; Sunwoo et al., 2008). DLX2 plays a crucial role in the progression of human cancers such as acute leukaemia, glioma and melanoma and breast, lung, prostate, ovarian and colon cancers (Yilmaz et al., 2011; Ferrari et al., 2003; Lee et al., 2011; Starkova et al., 2011). DLX2 activates the epidermal growth factor receptor (EGFR) signalling pathway by directly inducing the expression

Abbreviations: HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen; DLX2, distal-less homeobox 2; TGF-β, transforming growth factor-β; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated protein kinase; EMT, epithelial–mesenchymal transition; HBsAg, hepatitis B antigen; ALB, serum albumin; PT, prothrombine time; TBIL, total bilirubin; PVDF, polyvinylidine difluoride; PBS, phosphate-buffered saline; FBS, foetal bovine serum; TBST, Tris-buffered saline with Tween 20; PI, propidium iodide; CCK-8, cell counting kit-8.

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of its ligand betacellulin (Galli et al., 2004). In addition, DLX2 functions in switching the transforming growth factor-β (TGF-β) pathway from tumour-suppressive to tumour-promoting activity in melanoma (Yilmaz et al., 2011). Moreover, DLX2 reportedly phosphorylates extracellular signal-regulated protein kinase (ERK) pathways (Sivertsen et al., 2007). Activation of both the TGF-β and ERK pathways is associated with epithelial–mesenchymal transition (EMT) and chemo-resistance in cancer cells (Shen et al., 2014; Li et al., 2015). However, the precise role of DLX2 in HCC prognosis and sorafenib resistance is poorly understood.

In this study, we investigated the expression of DLX2 in HCC tissues using Western blotting and immunohistochemistry. Then, we examined the role of DLX2 in cell proliferation and sorafenib resistance in HCC. Our results indicated that DLX2 accelerates cell proliferation and upregulates the expression of EMT markers, as well as ERK1/2, processes that contribute to sorafenib resistance. Our findings show that DLX2 represents a novel prognostic marker for HCC that may function in sorafenib resistance.

2. Materials and methods

2.1. Participants and tissue samples

We enrolled 124 patients with HCC who underwent curative hepatic resection at the Surgery Department of the Affiliated Hospital of Nantong University, Nantong, Jiangsu, China, between 2006 and 2009. All patients were fully informed about our experiment protocols, which were approved by the ethics committee at our hospital, and provided written informed consent to participate. Eligible patients had not received chemotherapy or radiotherapy before surgery and had complete clinical and pathological data, as shown in Table 1. The 124 patients comprised 101 males and 23 females with a median age of 48.23 years. Of them, 75.81% were positive for hepatitis B antigen (HBsAg) and 79.03% had cirrhosis. Patients were classified into the well-differentiated group, which comprised 22 patients, the moderately differentiated group, which comprised 55 patients and the poorly differentiated group, which comprised 47 patients. Survival data were integrated by periodic interviews with their relatives and were determined for each patient on 30 April, 2015. Patients were closely followed up for a period of 1–92 months (median: 32.64 months). Overall survival time was defined as the interval between surgery and death or the last observation taken. Cancerous and noncancerous tissue samples for histological comparison were fixed in 10% formalin and embedded in paraffin immediately after surgical removal. Three pathologists with extensive experience in the identification of HCC performed the histological grading. Eight pairs of tissue samples were randomly collected from 124 patients for Western blotting, immediately (≤15 min) snap-frozen in liquid nitrogen and stored at −80 °C.

Table 1
Association of DLX2 expression with clinicopathological parameters in 124 HCC specimens.

Parameters	Total	DLX2 expression		*P*	χ^2
		Low	High		
Age (years)					
≤45	42	18	24	0.912	0.012
>45	82	36	46		
Gender					
Female	23	13	10	0.164	1.933
Male	101	41	60		
Histological grade					
Well	22	14	8	<0.001*	13.254
Mod	55	29	26		
Poor	47	11	36		
Metastasis					
Negative	58	11	47	<0.001*	26.787
Positive	66	43	23		
Tumour size (cm)					
≤5	54	20	34	0.199	1.650
>5	70	34	36		
Microvascular invasion					
Absence	96	44	52	0.342	0.903
Presence	28	10	18		
Tumour number					
Single	86	37	49	0.859	0.032
Multiple	38	17	21		
Capsular formation					
Absence	47	17	30	0.195	1.676
Presence	77	37	40		
HBsAg					
Negative	30	17	13	0.096	2.770
Positive	94	37	57		
Cirrhosis					
Negative	26	11	15	0.886	0.021
Positive	98	43	55		
ALB (g/L)					
<35	26	10	16	0.556	0.346
≥35	98	44	54		
PT (s)					
≤14	108	45	63	0.272	1.206
>14	16	9	7		
TBIL (μmol/L)					
<34.2	92	36	56	0.092	2.830
≥34.2	32	18	14		
Child–Pugh score					
A	106	43	63	0.104	2.642
B	18	11	7		
AFP (ng/mL)					
≤50	47	15	32	0.051	4.167
>50	77	39	38		
Ki-67					
Low	59	32	27	0.022*	5.231
High	65	22	43		
Survival					
Died	75	20	55	<0.001*	22.003
Alive	49	34	15		

Statistical analyses were performed by the Pearson χ^2 test.
* $P < 0.05$ was considered significant.

2.2. Antibodies

The antibodies used for Western blotting were as follows: anti-DLX2 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-PCNA (1:1000; Santa Cruz Biotechnology, Inc.); anti-Cyclin D1 (1:500; Santa Cruz Biotechnology, Inc.); anti-Cyclin A (1:500; Santa Cruz Biotechnology, Inc.); anti-ERK1/2 (1:500; Santa Cruz Biotechnology, Inc.); anti-pERK1/2 (1:500; Santa Cruz Biotechnology, Inc.); anti-E-cadherin (1:1000; Santa Cruz Biotechnology, Inc.); anti-Vimentin (1:1000; Abcam, Cambridge, UK); anti-N-cadherin (1:100; Santa Cruz Biotechnology, Inc.); and anti-glyceraldehyde-3-phosphate dehydrogenase (1:1000; Sigma-Aldrich Corp., St. Louis, MO, USA). The antibodies used for immunohistochemistry were as follows: anti-DLX2 (1:200; Sigma-Aldrich Corp.); and anti-Ki67 (1:500; EMD Millipore Corp., Billerica, MA, USA).

2.3. Immunohistochemical staining

Paraffin-embedded tissue slices were grilled, dewaxed, hydrated and placed in sodium citrate buffer for antigen retrieval using 3% hydrogen peroxide to block endogenous peroxidase and 4% skimmed-milk and blocked for 30 min. Anti-DLX2 and anti-Ki67 antibodies were incubated at room temperature for 2 h, then washed with phosphate-buffered saline (PBS). Negative control slides were incubated in parallel using the nonspecific Immunoglobulin G (Sigma-Aldrich Corp.) at the same concentration as the primary antibody. Slides were processed using the peroxidase–anti-peroxidase method (Dako Deutschland GmbH, Hamburg, Germany); they were then thoroughly washed with