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ORIGINAL ARTICLE

# ALDH3A1 is overexpressed in a subset of hepatocellular carcinoma characterised by activation of the Wnt/ß-catenin pathway

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**Abstract** Aldehyde dehydrogenase isoforms, ALDH1A1 and ALDH3A1, are associated with poor clinical outcome and resistance to chemotherapy in a wide variety of human malignancies. So far, their expression and prognostic significance in hepatocellular carcinoma (HCC) remains unknown. The aim of our study was to investigate their expression in HCC, and to correlate this to clinical, pathological and molecular features. *ALDH1A1* and *ALDH3A1* expression was first evaluated by microarray analysis in a series of 60 HCCs and five tumour-free liver tissue samples. Our findings related to ALDH3A1 were further validated by immunohistochemistry in a series of 81 HCCs and 23 hepatocellular adenomas (HCA). Microarray analysis showed no difference in *ALDH1A1* expression between HCCs and tumour-free liver tissue. In contrast, ALDH3A1 was strongly upregulated in a subset of HCCs characterised by activation of the Wnt/ß-catenin pathway and *CTNNB1* mutations. Using immunohistochemistry, we confirmed that high *ALDH3A1* expression is associated with nuclear staining for ß-catenin and strong homogeneous staining for glutamine synthetase, two classical Wnt/ß-catenin pathway activation markers. Consistent with this finding, in tumour-free liver tissue, *ALDH3A1* expression was observed in centrilobular hepatocytes, in which the Wnt/ß-catenin pathway is known to be physiologically activated. We also observed higher *ALDH3A1* expression in *CTNNB1*-mutated HCA when compared with other subtypes. No correlation between *ALDH3A1* expression and patient survival or tumour recurrence was observed.

In conclusion, ALDH3A1 is a marker of activation of the Wnt/ß-catenin pathway in HCC, HCA and tumour-free liver tissue. Further studies may help to elucidate the potential role of ALDH3A1 in HCC development and resistance to chemotherapy.

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**Keywords** Hepatocellular carcinoma · ALDH3A1 · Wnt/beta catenin · CTNNB1

## Introduction

Aldehyde dehydrogenase superfamily is a group of nicotinamide-adenine dinucleotide phosphate (NADPH+)-dependent enzymes that catalyse the oxidation of endogenous and exogenous aldehyde substrates to their corresponding carboxylic acids [1–3]. Endogenous aldehydes are generated during metabolism of amino acids, alcohol, lipids or vitamins, whilst exogenous aldehydes, whether intermediates or products, are derived from the metabolism of a wide variety of environmental agents or drugs (cigarette smoke, vehicle exhaust fumes, cytotoxic drugs) [1].

Expression of two ALDH isoforms, ALDH1A1 and ALDH3A1, and/or ALDH overall enzymatic activity has also been reported to represent cancer stem cell markers in various malignancies, including breast, lung, upper urinary tract and endometrioid adenocarcinoma [4–7]. Luo et al. [6] have demonstrated that *ALDH1A1* silencing by siRNA or shRNA in melanoma cell lines leads to cell cycle arrest, apoptosis, and reduced tumourigenesis in vivo. Downregulation of expression of *ALDH3A1* has been shown to affect cell growth, proliferation and motility in lung and hepatocellular carcinoma (HCC) cell lines [1, 8–10]. *ALDH1A1* and/or *ALDH3A1* expression also confers resistance to a wide variety of cytotoxic drugs, including gemcitabine, temozolomide and cyclophosphamide [1, 11–14].

Expression of *ALDH1A1*, which has been reported in numerous human tumours, is associated with poor clinical outcome in breast and lung adenocarcinoma [4, 5, 13]. So far, very few studies have investigated the expression of *ALDH1A1* and *ALDH3A1* in HCC [15], a malignancy associated with strong resistance to chemotherapy and adverse prognosis.

HCC is a heterogeneous disease for which we have formerly established a robust genomic and transcriptomic classification linked to different clinical and molecular features [16–18]. HCC groups G1 to G3 are characterised by overexpression of genes involved in cell and cycle proliferation, high rates of chromosomal instability, *TP53* mutations (G2–G3) and *HBV* infection (G1–G2) [20]. HCC groups G4 to G6 are characterised by chromosomal stability. Groups G5 and G6 are strongly related to activating mutations of *CTNNB1*, encoding ß-catenin which is the most frequently mutated gene in HCC [17–19]. These mutations result in strong Wnt/ß-catenin pathway activation, which plays a critical role in hepatic carcinogenesis [17, 19].

We investigated *ALDH1A1* and *ALDH3A1* expression by gene expression microarray analysis in a series of 60 HCCs ('microarray series') and validated our results related to *ALDH3A1* by immunohistochemistry in an additional series of 81 HCCs with available paraffin-embedded tissue ('validation series'). *ALDH1A1* and *ALDH3A1* expression were correlated to HCC transcriptomic subgroups and *CTNNB1*-activating mutations. In the validation series, we compared immunohistochemical expression of *ALDH3A1* to that of the Wnt/ß-catenin pathway activation markers nuclear ß-catenin staining and diffuse strong staining for glutamine synthetase (GS). Finally, we investigated *ALDH3A1* expression in tumour-free liver tissue and in a series of 23 hepatocellular adenomas (HCA), and correlated this to patient prognosis and tumour reccurence.

## Material and methods

### Patients and samples

For the microarray series, 60 frozen tumour samples and 5 nontumour liver samples were collected from patients surgically treated in three French departments of surgery from 1992 to 1999, as previously described [16].

For the validation series, 81 HCCs cases treated by surgical resection were retrieved from the archives of the Departments of Pathology of Henri Mondor (Créteil, France) and Bordeaux (France) University Hospitals. As required by French law, informed consent was obtained from all patients. The following items were systematically recorded: age (< or ≥60 years), sex, liver disease etiology, follow-up ($n$ = 78, mean 33 months), tumour size (< or ≥50 mm), Edmondson grade and vascular invasion.

Main clinical and pathological features are listed in Table 1.

The 23 HCA cases included six CTNNB1 mutated, ten inflammatory, five HNF1A-inactivated and two unclassified HCAs, according to molecular classification [20–23] (Table 2).

### Immunohistochemistry

All immunostainings were performed on whole sections. For ALDH3A1, after deparaffinisation and rehydratation, the sections were placed into a boiling target retrieval solution (DiaPath Citrate Buffer pH 6) for 40 min, and left to cool down without cover for 20 min. Slides were then processed on an automated immunostainer (Dako AutoStainer). Endogenous peroxidase was blocked with H2O2 (10 min) and nonspecific background staining with goat serum (20 min). Sections were further incubated with a primary anti-ALDH3A1 (Santa Cruz Biotechnology, clone B-8; sc-137168, dilution 1/200) antibody for 1 h at room temperature and washed with phosphate-buffered saline solution. After incubation with the Envision + System-labelled polymer–horseradish peroxidase (HRP) kit (Dako/Cytomation) and staining

**Table 1** Main clinical and pathological features

| | Microarray series ($n=60$) | Validation series ($n=81$) |
|---|---|---|
| Age>60 (%) | 66 | 46 |
| Sex ratio (M/F) | 7.6 | 5.2 |
| *Liver disease etiology* | | |
| Alcohol | 25 % (15/60) | 23 % (19/81) |
| HCV | 10 % (6/60) | 19 % (15/81) |
| HBV | 23 % (14/60) | 22 % (18/81) |
| HBV + alcohol | 0 % (0/60) | 4 % (3/81) |
| HCV + alcohol | 5 % (3/60) | 5 % (4/81) |
| HBV + HCV | 3 % (2/60) | 0 % (0/81) |
| Hemochromatosis | 7 % (4/60) | 9 % (7/81) |
| Hemochromatosis + alcohol | 0 % (0/60) | 2 % (2/81) |
| NASH | 3 % (2/60) | 5 % (4/81) |
| Auto-immune hepatitis | 0 % (0/60) | 1 % (1/81) |
| Unknown | 22 % (13/60) | 10 % (8/81) |
| Tumour size ≥ 50 mm | 55 % (33/60) | 55 % (45/81) |
| *Tumour differentiation* | | |
| Edmonson I–II | 60 % (36/60) | 43 % (35/81) |
| Edmonson III–IV | 40 % (24/60) | 57 % (46/81) |
| Vascular invasion | 37 % (22/60) | 55 % (45/81) |
| *Nontumourous liver fibrosis* (*METAVIR*[a]) | | |
| F0–F1 | 42 % (25/60) | 24 % (19/81) |
| F2–F3 | 28 % (17/60) | 44 % (36/81) |
| F4 | 30 % (18/60) | 32 % (26/81) |

[a] METAVIR equivalent in patients without HCV infection

**Table 2** Main clinical features of the HCA series

| | Patient | Gender | Age (years) | Tumour size (mm) |
|---|---|---|---|---|
| *CTNNB1*-mutated HCAs | 1 | F | 61 | 40 |
| | 2 | M | 15 | 40 |
| | 3 | F | 23 | 150 |
| | 4 | F | 42 | 90 |
| | 5 | F | 24 | 80 |
| | 6 | F | 67 | 60 |
| Inflammatory HCAs | 7 | F | 37 | 80 |
| | 8 | M | 27 | 135 |
| | 9 | M | 39 | 50 |
| | 10 | F | 26 | 45 |
| | 11 | F | 46 | 100 |
| | 12 | F | 30 | 35 |
| | 13 | M | 20 | 105 |
| | 14 | F | 45 | 50 |
| | 15 | F | 38 | 105 |
| HNF1A inactivated HCAs | 16 | F | 43 | 50 |
| | 17 | F | 43 | 50 |
| | 18 | F | 40 | 40 |
| | 19 | F | 44 | 36 |
| | 20 | F | 39 | 50 |
| Unclassified HCAs | 21 | F | 25 | 40 |
| | 22 | M | 32 | 75 |

*M* male, *F* female

with 3-diaminobenzidine (Dako) as chromogen, the slides were counterstained with Mayer's haematoxylin, dehydrated and coverslipped. Samples with at least 50 % of stained tumour cells were classified 'ALDH3A1 high'; samples with less than 50 % of stained tumour cells were classified 'ALDH3A1 low'.

Immunohistochemical staining for GS (BD Biosciences-Pharmigen, ref 610517, dilution 1/1,000) and ß-catenin (BD Biosciences-Pharmigen, ref 610153, dilution 1/4,000) was performed on an automated immunostainer (Leica Bond-Max), according to the manufacturer's instructions. GS expression was scored as follows: 0 (no staining), 1 (focal or diffuse and heterogeneous staining) and 2 (diffuse and strong staining). For ß-catenin, nuclear staining was considered as positive if at least one tumour cell had a stained nucleus.

### Mutation screening

Mutation status for CTNNB1 had been performed previously for the microarray series [16]. For the validation series, all HCC samples were sequenced for CTNNB1 (exons 2 to 4), using the same method [16]. All mutations were confirmed by sequencing a second independent amplification product on both strands.

### Statistical analysis

Differences in microarray expression of *ALDH1A1* and *ALDH3A1* between tumour subgroups were assessed using Student's *t* test. Correlations between immunohistochemical scores, clinical and molecular data was analysed using Fisher's exact test. Kaplan–Meier disease-specific survival and tumour recurrence analysis was performed and illustrated using GraphPad Prism 5 software and survival curve comparison analyses were performed using the log–rank (Mantel–Cox) test. $p$ Values<0.05 were considered statistically significant.

## Results

### ALDH1A1 and ALDH3A1 expression by microarray

Expression of *ALDH1A1* by gene expression array in HCC samples was not significantly different from that in tumour-free liver tissue samples ($n=5$; mean intensity 2,352 vs. 2,420, $p=0.9$). *ALDH1A1* expression was lower in G1 HCC than in

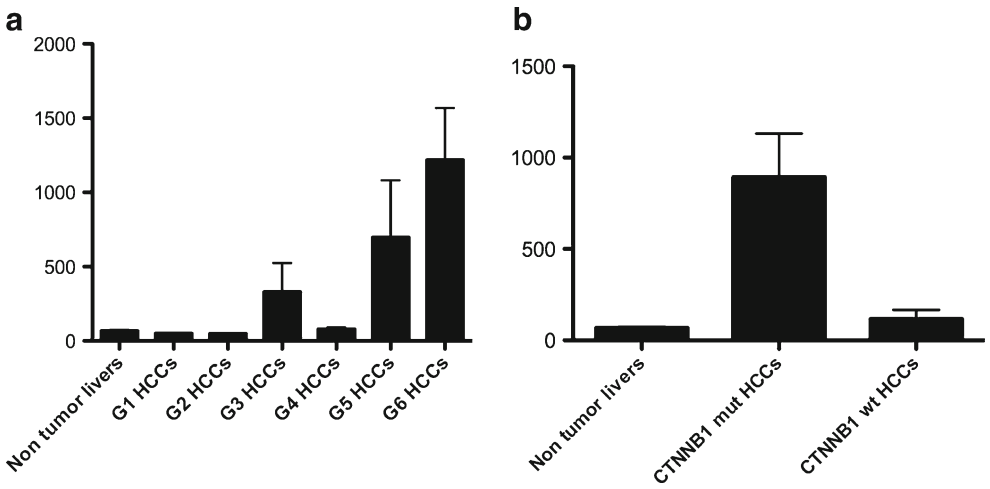


**Fig. 1** *ALDH3A1* expression in the microarray series according to HCC subgroup (**a**) and *CTNNB1* activating mutations (**b**)

tumour-free liver tissue (mean intensity 1,100 vs. 2,352, $p=0.02$); no other significant difference was observed between *ALDH1A1* expression in other subgroups and nontumour livers (data not shown).

Mean expression of *ALDH3A1* was higher in HCC samples than in tumour-free liver tissue, but this was not statistically significant (mean=363 vs. 68, $p=0.37$). *ALDH3A1* was strongly over-expressed in G5 and G6 subgroups of HCC ($p<0.001$; Fig. 1a). *ALDH3A1* expression was not correlated with other clinical or pathological parameters.

## ALDH3A1, GS and ß-catenin expression in the validation series

As most G5 and G6 HCC carry an activating CTNNB1 mutation, we investigated *ALDH3A1* expression in relation to *CTNNB1* mutational status in a separate set of tumours (validation set) including 81 HCC treated by resection in Créteil and Bordeaux hospitals. Immunohistochemical expression of *ALDH3A1* was negative in 31 cases (39 %), low in 17 cases (21 %) and high in 33 cases (40 %; Fig. 2a). Both cytoplasmic and nuclear staining of ALDH3A1 was observed (Fig. 2b). *ALDH3A1* expression was not significantly correlated with any clinical or pathological features.

In 28 cases (34 %), we identified a somatic activating *CTNNB1* mutation (Table 3). High *ALDH3A1* expression strongly correlated with *CTNNB1* mutations ($p<0.0001$). Sensitivity and specificity of high *ALDH3A1* expression for prediction of *CTNNB1* mutation were 71 % (20/28) and 75 % (40/53), respectively. Glutamine synthase over-expression (score 2), which is a reliable marker of ß-catenin activation in hepatocytes, was observed in 45 % of cases and significantly associated with *CTNNB1* mutation with a 78 % (22/28) sensitivity and 73 % (39/53) specificity (Fig. 3). Nuclear ß-catenin staining, a sign of ß-catenin activation, was observed in 20 out of 81 cases (24 %) with a sensitivity and specificity for predicting *CTNNB1* mutations of 53 % (15/28) and 89 % (47/53), respectively (Fig. 3).

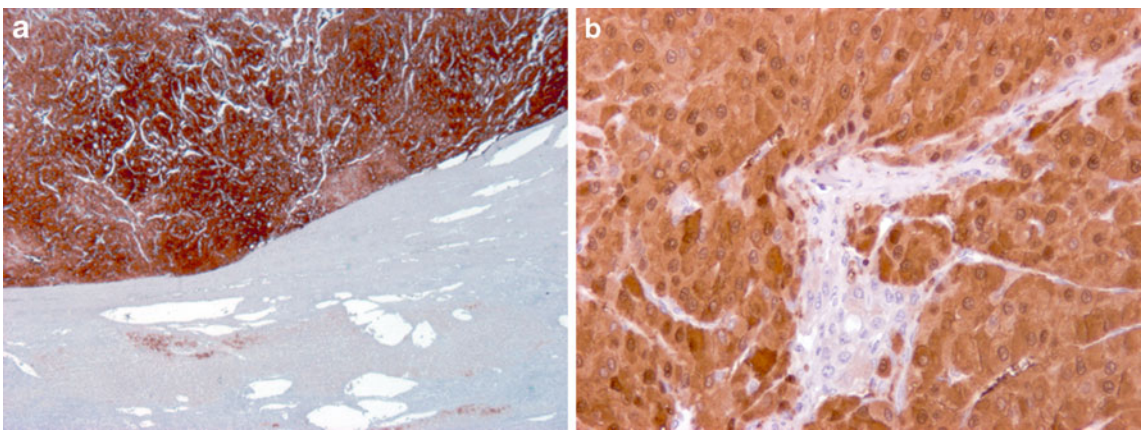


**Fig. 2** A case of HCC with high *ALDH3A1* expression, with scarce clusters of positive cells in the adjacent tumour-free liver tissue (**a**). Both cytoplasmic and nuclear ALDH3A1 immunoreactivity was observed (**b**)

**Table 3** *CTNNB1* mutations identified in the validation series

| Tumour ID | Nucleotide change | Aminoacid change |
|---|---|---|
| CHC1040T | c.133 T > C | p.Ser45Pro |
| CHC1041T | c.95A > T | p.Asp32Val |
| CHC1069T | c.95A > C | p.Asp32Ala |
| CHC1137T | c.94G > T | p.Asp32Tyr |
| CHC1138T | c.60_455del | p.Ala21_Ala152del |
| CHC1142T | c.47_400del | p.Pro16_Lys133del |
| CHC1146T | c.97 T > C | p.Ser33Pro |
| CHC1148T | c.121A > G | p.Thr41Ala |
| CHC1152T | c.139_140delinsCT | p.Ser47Leu |
| CHC1183T | c.95A > C | p.Asp32Ala |
| CHC1186T | c.121A > G | p.Thr41Ala |
| CHC1189T | c.98C > T | p.Ser33Phe |
| CHC1202T | c.122C > T | p.Thr41Ile |
| CHC1206T | c.107A > C | p.His36Pro |
| CHC1209T | c.101G > T | p.Gly34Val |
| CHC1715T | c.133 T > C | p.Ser45Pro |
| CHC1717T | c.95A > G | p.Asp32His |
| CHC1718T | c.134C > T | p.Asp32Gly |
| CHC1723T | c.121A > G | p.Ser45Phe |
| CHC1731T | c.95A > T | p.Thr41Ala |
| CHC1741T | c.98C > A | p.Ser33Tyr |
| CHC1744T | c.14–67_204del | indel. |
| CHC1745T | c.95A > T | p.Asp32Val |
| CHC1747T | c.95A > T | p.Asp32Val |
| CHC1751T | c.121A > G | p.Thr41Ala |
| CHC1756T | c.122C > T | p.Thr41Ile |
| CHC1760T | c.94G > C | p.Asp32His |
| CHC1763T | c.13+173_84del | indel. |

A strong positive correlation was observed between high *ALDH3A1* expression and score 2 GS expression ($p<0.0001$) and nuclear ß-catenin staining ($p<0.0001$). Of the 12 tumours with high *ALDH3A1* expression but *CTNNB1* wild type, eight cases had a GS score of 2, and five cases also featured nuclear ß-catenin staining. No correlation was observed between *ALDH3A1* expression and other clinical or pathological parameters.

Expression of *ALDH3A1* was further investigated in 23 HCA including six *CTNNB1* mutated, five *HNF1A* inactivated, ten inflammatory and two unclassified HCAs. We observed a higher percentage of positive cells in the *CTNNB1*-mutated HCA than in other subtypes

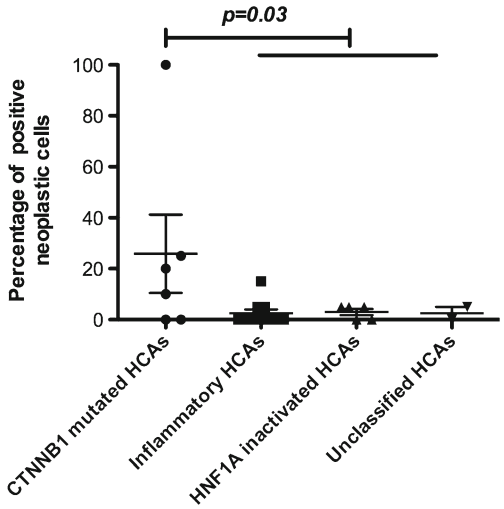


**Fig. 4** Percentage of neoplastic cells stained according to HCA molecular subgroup

($p=0.03$; Fig. 4). However, the total number of positive hepatocytes was lower in *CTNNB1*-mutated HCA than in mutated HCC, and two *CTNNB1*-mutated HCA were ALDH3A1-negative. Finally, no significant differences were observed between inflammatory, unclassified or HNF1A inactivated HCA.

## ALDH3A1 expression in nontumour liver

Next, we analysed *ALDH3A1* expression in tumour-free liver tissue samples remote from HCC in 71 cases (METAVIR F0 $n=3$; F1 $n=13$; F2 $n=13$; F3 $n=29$; F4 $n=23$). In liver tissue without extensive fibrosis (F0–F1; $n=16$), five cases featured striking centrilobular expression (Fig. 5a, b), ten cases did not show any *ALDH3A1* expression, and one case exhibited few clusters of positive cells without a particular pattern. In liver tissue with extensive fibrosis (F2 to F4), we observed a few cell clusters expressing *ALDH3A1*, most frequently located around fibrotic septa.

## ALDH3A1 expression, disease specific survival rate and tumour recurrence

There were no significant differences in disease-specific survival according to the level of *ALDH3A1* expression ($p=0.53$, Fig. 6a). A nonsignificant trend towards earlier tumour recurrence in ALDH3A1 high tumours was observed ($p=0.06$, Fig. 6b).


**Fig. 3** Schematic representation of immunohistochemical findings (*red boxes* yes, *green boxes* no)

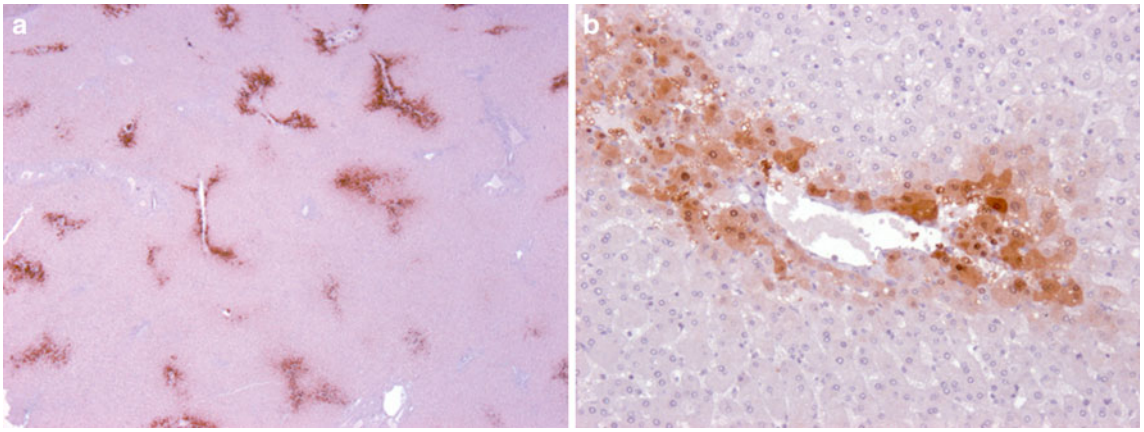


**Fig. 5** Striking centrilobular *ALDH3A1* expression in tumour-free liver tissue at low (**a**) and high magnification (**b**)

## Discussion

In several reports, *ALDH1A1* and *ALDH3A1* have been associated with resistance to chemotherapy and adverse clinical outcome in a wide variety of human malignancies. We present a large study on the expression of *ALDH1A1* and *ALDH3A1* in a series of human HCCs. We show that *ALDH3A1* expression is upregulated in a subset of HCCs with *CTNNB1* mutations activating the Wnt/ß-catenin pathway. In the microarray series of 60 samples analysed for mRNA expression and in the validation set of 81 HCCs studied by immunochemistry, our results show that *ALDH3A1* over-expression and *CTNNB1* mutations are strongly associated in HCC. Moreover, high *ALDH3A1* expression by immunohistochemistry had a sensitivity and specificity for predicting *CTNNB1* mutations comparable to that of other classical immunohistochemical markers (ß-catenin nuclear staining and GS overexpression). Interestingly, of the 12 cases with high *ALDH3A1* expression but no *CTNNB1* mutation, eight cases had a GS score of 2, and five cases also featured nuclear ß-catenin staining. These observations suggest that in those cases high *ALDH3A1* expression reflects activation of the Wnt/ß-catenin pathway caused by a different mechanism.

Consistent with our findings in HCC, percentage of neoplastic cells expressing *ALDH3A1* was higher in *CTNNB1*-mutated HCA, even if only one mutated case was classified as 'ALDH3A1 high', with two cases being completely negative. This finding casts doubt on the use of *ALDH3A1* expression as marker of *CTNNB1* mutation in HCA.

The Wnt/ß-catenin pathway plays a key role in liver zonation and differentiation [19, 24] and is strongly activated in centrilobular areas [19]. The striking centrilobular pattern of *ALDH3A1* expression observed in nonfibrotic tumour-free liver tissue is also consistent with a physiological link between ALDH3A1 and activation of the Wnt/ß-catenin pathway. Functional studies are needed to confirm that *ALDH3A1* transcription is directly or indirectly controlled by the Wnt/ß-catenin pathway.

Overall ALDH enzymatic activity, which partly relies on expression of *ALDH1A1* and *ALDH3A1*, is considered as a reliable marker of cancer stem cells [1, 5, 25–27]. In our series, *CTNNB1*-mutated HCC that also overexpressed *ALDH3A1* were well differentiated, with intra-tumour cholestasis. As reported before, these HCC often developed in a noncirrhotic liver [28]. In our series, 31 HCC did not express *ALDH3A1*, but in 33 cases, more than 50 % of the cells were positive. As cancer stem cells presumably represent a very

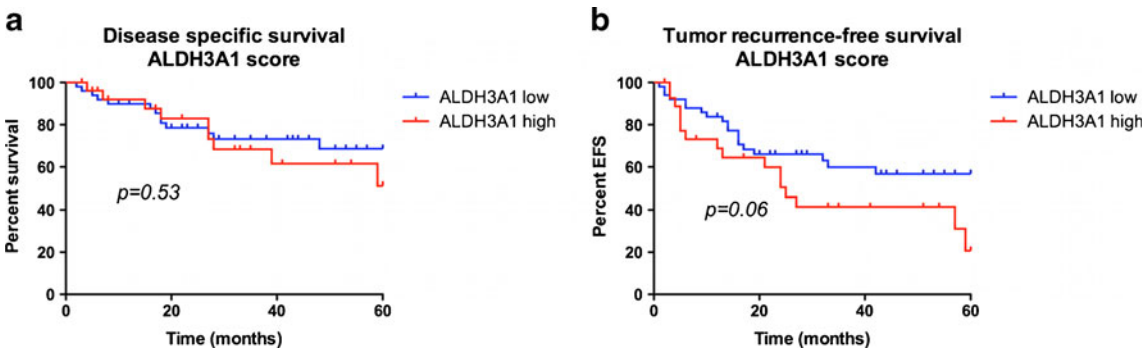


**Fig. 6** Disease specific survival (**a**) and recurrence-free survival (**b**) in relation to immunohistochemical expression of *ALDH3A1*

small proportion of tumour cells, this finding might argue against the validity of *ALDH3A1* expression as a marker of cancer stem cells in HCC.

In view of their detoxifying functions, ALDH isoforms are considered to be involved in tumour resistance to various anticancer drugs [11, 13, 14, 29, 30]. Hu et al. [31] reported that *ALDH3A1* knock down enhances sensitivity to paclitaxel, doxorubicin and 4-hydroxycyclophosphamide in a breast cancer cell line. Such experiments in HCC, modulating *ALDH3A1* expression, might clarify whether or not ALDH3A1 contributes to strong resistance to chemotherapy, in particular, in *CTNNB1*-mutated HCC.

We found a trend towards an association of high *ALDH3A1* expression with early recurrence, which suggests that *ALDH3A1* expression might be a reflection of tumour aggressiveness. In view of the on-going discussion on the relationship between WNT/ß-catenin pathway activation and survival [32–36], *ALDH3A1* expression might play a role in assessment of prognosis but this needs to be further validated in a larger series of patients.

In conclusion, we show that *ALDH3A1* expression is strongly upregulated in a subset of HCC with *CTNNB1* mutations activating the Wnt/ß-catenin pathway. Further studies will be needed to elucidate the potential role of ALDH3A1 in HCC development and resistance to chemotherapy.

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**Conflict of interest** None.

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