Altered expression of 17-β-hydroxysteroid dehydrogenase type 2 and its prognostic significance in non-small cell lung cancer

HANNA DRZEWIECKA[1], DONATA JARMOŁOWSKA-JURCZYSZYN[2], ANDRZEJ KLUK[2], BARTŁOMIEJ GAŁĘCKI[3], WOJCIECH DYSZKIEWICZ[3] and PAWEŁ P. JAGODZIŃSKI[1]

[1]Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, 60-781 Poznan; [2]Department of Clinical Pathomorphology, Poznan University of Medical Sciences, 60-355 Poznan; [3]Department of Thoracic Surgery, Poznan University of Medical Sciences, 60-569 Poznan, Poland

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Abstract. Numerous studies have reported that oestrogens may contribute to the development of non-small cell lung cancer (NSCLC). Although different steroidogenic enzymes have been detected in the lung, the precise mechanism leading to an exaggerated accumulation of active oestrogens in NSCLC remains unexplained. 17-β-Hydroxysteroid dehydrogenase type 2 (HSD17B2) is an enzyme involved in oestrogen and androgen inactivation by converting 17-β-oestradiol into oestrone, and testosterone into 4-androstenedione. Therefore, the enzyme serves an important role in regulation of the intracellular availability of active sex steroids. This study aimed to determine the expression levels of *HSD17B2* in lung cancer (LC) and adjacent histopathologically unchanged tissues obtained from 161 patients with NSCLC, and to analyse the association of *HSD17B2* with clinicopathological features. For that purpose, reverse transcription-quantitative PCR, western blotting and immunohistochemistry were conducted. The results revealed that the mRNA and protein expression levels of HSD17B2 were significantly decreased in LC tissues compared with matched controls ($P<10^{-6}$). Conversely, strong cytoplasmic staining of HSD17B2 was detected in the unchanged respiratory epithelium and in glandular cells. Notably, a strong association was detected between reduced *HSD17B2* expression and advanced tumour stage, grade and size. Furthermore, it was revealed that HSD17B2 may have potential prognostic significance in NSCLC. A log-rank test revealed the benefit of high HSD17B2 protein expression for the overall survival (OS) of patients (P=0.0017), and multivariate analysis confirmed this finding (hazard ratio=0.21; 95% confidence interval=0.07-0.63; P=0.0043). Stratified analysis in the Kaplan-Meier Plotter database indicated that patients with higher *HSD17B2* expression presented better OS and post-progression survival. This beneficial effect was particularly evident in patients with adenocarcinoma and during the early stages of NSCLC. Decreased expression of *HSD17B2* appears to be a frequent feature in NSCLC. Retrospective analysis suggests that the HSD17B2 mRNA and protein status might be independent prognostic factors in NSCLC and should be further investigated.

Correspondence to: Ms. Hanna Drzewiecka, Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, 6 Święcickiego Street, 60-781 Poznan, Poland
E-mail: hdrzewiecka@gmail.com; hdrzewiecka@ump.edu.pl

Abbreviations: Ab, antibody; ADC, adenocarcinoma; CI, confidence interval; E1, oestrone; E2, 17-β-oestradiol; ERs, oestrogen receptors; FP, first progression; GPER, G protein-coupled oestrogen receptor; hMRPL19, human mitochondrial ribosomal protein L19; HR, hazard ratio; HRP, horseradish peroxidase; HSD17B1, 17-β-hydroxysteroid dehydrogenase type 1; HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; LC, lung cancer; LCC, large cell carcinoma; NSCLC, non-small cell lung cancer; OS, overall survival; PBGD, porphobilinogen deaminase; POLR2A, RNA polymerase II subunit A; PPS, post-progression survival; RA, retinoic acid; RT-qPCR, reverse transcription-quantitative PCR; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SCC, squamous cell carcinoma; T, testosterone; TNM, tumour-node-metastasis

Key words: NSCLC, HSD17B2, E2, clinicopathological variables, OS

Introduction

Lung cancer (LC) is the leading cause of cancer-associated mortality and represents the most common malignancy worldwide (1). The outcome of LC remains unsatisfactory, with low survival rates, presumably due to a late diagnosis made in the advanced stages (2). Therefore, it is important to identify novel biomarkers that can contribute to a better understanding of LC biology and serve as new therapeutic targets.

Non-small cell LC (NSCLC) is the major type of LC, which accounts for ~85% of all LC cases. NSCLC comprises adenocarcinoma (ADC), squamous cell carcinoma (SCC), large cell carcinoma (LCC) and other rare subtypes (3).

Although smoking remains the main risk factor for LC and the lung is not classically thought to be a target tissue for sex steroid hormones, numerous studies have revealed sex differences in LC pathogenesis (4,5). These observations have shed light on the potential role of oestrogens in the development

of LC. Among never-smokers, women are more likely to develop LC, with ADC being the predominant subtype (6). Considering the same amount of smoked cigarettes, women are more frequently diagnosed with NSCLC than men are (7). These data raise the question of whether there is a link between carcinogens from cigarette smoke and oestrogens. Notably, several studies have revealed that women are more vulnerable to the hazardous effects of smoking (7-9). Smoking has been reported to enhance the expression and activity of cytochrome P450 family 1 subfamily B member 1 (CYP1B1) in lung tissue (10), which may consequently lead to the formation of 2- and 4-catechol oestrogens. Subsequently, the conversion of these oestrogens to toxic metabolites mediates DNA damage (11,12). These reports support the role played by oestrogens during LC development.

In addition, LC in young women demonstrates aggressive biology with rapid growth, whereas postmenopausal women with advanced NSCLC have survival advantages over men and younger counterparts (13,14). Furthermore, high levels of serum 17-β-oestradiol (E2) have been associated with shorter survival of men with advanced NSCLC (15), and high plasma concentrations of dihydrotestosterone and testosterone (T) have been correlated with an increased LC incidence in older men (16).

Numerous studies have confirmed the presence of classical oestrogen receptors (ERs), ER-α and ER-β, as well as a membrane G protein-coupled ER (GPER) in NSCLC tumours, regardless of sex (17-23). A growing body of evidence has indicated that oestrogen signalling mediated by these receptors may promote the growth, survival and aggressiveness of human NSCLC cells, contributing to cancer development and progression (18,24-27).

Although the ovaries are the main source of oestrogens in women before menopause and the testicles are mostly responsible for the synthesis of T in men, it is well known that active sex steroids can be synthesized locally, in peripheral tissues, from inactive precursors of adrenal origin (28,29). Studies by Luu-The (28) and Labrie (29) revealed that the majority of peripheral tissues possess different sets of steroidogenic enzymes involved in the local interconversion of inactive precursors into their active forms. Synthesized sex steroids act in an intracrine manner without being released into the circulation (28,29). It was previously indicated that oestrogens and androgens are metabolized within the lung and that NSCLC cells are able to produce their own sex steroid hormones (22,30). Niikawa *et al* (31) detected higher concentrations of E2 in LC tissues than in corresponding non-neoplastic tissues from patients with NSCLC, regardless of sex, suggesting that oestrogens may be synthesized locally during LC development. Therefore, an altered expression of enzymes responsible for the generation, activation or inactivation of sex steroids may lead to a local imbalance in androgen/oestrogen concentrations and trigger carcinogenesis.

Earlier studies associated an elevated level of E2 in LC tissues with an increased expression of aromatase, which converts 4-androstenedione into oestrone (E1) and T into E2 (31,32). However, the research carried out by Verma *et al* (33), and the results of our previous studies (34,35), pinpointed another important pathway for E2 synthesis in NSCLC. The presence of 17-β-hydroxysteroid dehydrogenase type 1 (HSD17B1), which catalyses the reduction of weak E1 into highly potent E2, was detected in the investigated NSCLC cells. Our previous study identified the ability of HSD17B1 to convert E1 into E2 *in vitro*, and demonstrated an elevated expression of *HSD17B1* in NSCLC tissues compared with matched, histopathologically unchanged specimens (34,35). Encouraged by these results, we decided to continue our research related to enzymes belonging to the HSD17B family.

The present study focused on 17-β-hydroxysteroid dehydrogenase type 2 (HSD17B2), an enzyme that catalyses the oxidation of active steroids into their corresponding 17-keto forms, efficiently inactivating E2, T and dihydrotestosterone in various tissues (36,37). Therefore, HSD17B2 may regulate the amount of active sex steroids within the lung, thus protecting cells from their excess.

The aim of this study was to evaluate the expression levels of HSD17B2 in LC and corresponding histopathologically unchanged tissues from patients with NSCLC at the mRNA and protein levels, and to determine the association between HSD17B2 and clinicopathological features. Three different methods, which complement each other, were conducted: Reverse transcription-quantitative PCR (RT-qPCR), western blotting and immunohistochemistry. In addition, a retrospective analysis was performed to investigate whether HSD17B2 mRNA or protein expression may have prognostic significance in the survival outcome of patients with NSCLC. To date, to the best of our knowledge, only one study investigated the amount of HSD17B2 protein in NSCLC clinical specimens. However, it was performed exclusively by immunohistochemistry, and only in cancer tissues, without comparison with adjacent normal tissues (33). To the best of our knowledge, this study is the first to compare HSD17B2 mRNA and protein expression between LC and adjacent histopathologically unchanged tissues, and to evaluate their prognostic significance in NSCLC.

Materials and methods

Antibodies and reagents. The following antibodies (Abs) were used for western blotting: Rabbit polyclonal anti-HSD17B2 Ab (cat. no. ab103161; Abcam), rabbit polyclonal anti-GAPDH Ab (FL-335; cat. no. sc-25778; Santa Cruz Biotechnology, Inc.) and goat anti-rabbit horseradish peroxidase (HRP)-conjugated Ab (cat. no. 7074S; Cell Signaling Technology, Inc.). For immunohistochemistry, rabbit polyclonal anti-HSD17B2 Ab (cat. no. 10978-1-AP) was purchased from ProteinTech Group, Inc. TRI Reagent® for RNA isolation and RIPA lysis buffer for protein isolation were provided by Sigma-Aldrich (Merck KGaA).

Patient samples. Primary LC tissues and histopathologically unchanged lung tissues, the latter located at a distance of 10-20 cm from the cancerous lesions were obtained from 161 patients diagnosed with NSCLC between March 2012 and May 2016. All patients underwent surgical resection at the Department of Thoracic Surgery, Poznan University of Medical Sciences (Poznan, Poland). None of the patients received any preoperative chemotherapy or radiation therapy. All patients provided written informed consent for the use of clinical specimens. The procedures of the study were approved by the Local Ethical Committee of Poznan University of

Medical Sciences, and all procedures were in accordance with the 1964 Declaration of Helsinki and its later amendments. After surgical intervention, tissue samples were divided into two sets. One set was immediately snap-frozen in liquid nitrogen for further processing (homogenization, RNA and protein isolation). The other set was used for histopathological examination, which was performed by an experienced pathologist according to the 7th edition of the tumour-node-metastasis (TNM) staging system (38). The residual tumour status after surgical resection was also examined and classified in accordance with the TNM staging system.

Measurement of overall survival (OS). For the measurement of OS, patients were observed from the moment of surgery (the first patient underwent surgery on March 6, 2012) until death or December 31, 2017. In the OS analysis, patients who died of non-cancer-related causes were excluded. Follow-up data concerning OS were available for 147 patients. None of the patients received any preoperative chemotherapy or radiation therapy.

Kaplan-Meier Plotter database and survival analysis. To analyse the prognostic value of HSD17B2 expression in a wider group of patients, the online tool Kaplan-Meier Plotter (http://kmplot.com/analysis/) was used. This database contains gene expression data from multiple microarrays [from the Gene Expression Omnibus (Affymetrix microarrays only), European Genome-Phenome Archive and The Cancer Genome Atlas databases] and survival information for 1,926 patients with NSCLC (39). This study focused on OS, post-progression survival (PPS) and first progression (FP) survival.

The Affymetrix probe set ID for *HSD17B2* was 204818_at. This study used 'JetSet best probe set', 'Auto select best cutoff' options, and an array quality control set at 'exclude biased arrays' during analysis. Patients were divided into high and low *HSD17B2* expression groups, and the Kaplan-Meier survival plots with P-values from the log-rank test, hazard ratio (HR) with 95% confidence intervals (CI) and numbers-at-risk were obtained. In addition, plot data were exported as text and P-values were calculated using the Gehan-Breslow-Wilcoxon test when the log-rank test was not applicable (when survival curves were not parallel at late stages of analysis). $P<0.05$ was considered to indicate a statistically significant difference.

RT-qPCR analysis. All investigated tissues (cancerous and histopathologically unchanged tissues obtained from 161 patients) were homogenized into a powder in liquid nitrogen, and total RNA was isolated using TRI Reagent® (Sigma-Aldrich; Merck KGaA). Subsequently, RNA samples were treated with recombinant DNase I using a DNA-free™ DNA Removal kit (Ambion; Thermo Fisher Scientific, Inc.) to eliminate any residual DNA. RNA quality and concentration were determined by denaturing agarose gel (1.2%) electrophoresis and by absorbance measurements using a NanoDrop One spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). Quantified samples were then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. For cDNA synthesis, a mixture of oligo$(dT)_{23}$ and random hexaprimers was used, with 1 μg isolated total RNA as a template. RT-qPCR was conducted using a Light Cycler® 480 Real-Time PCR system (Roche Diagnostics GmbH) with SYBR Green I used as the fluorophore. Target cDNA was quantified according to the relative quantification method using a calibrator. For the calibrator, 1 μl cDNA from each tissue sample was mixed together. Consecutive dilutions of the calibrator served for the generation of standard curves for all investigated genes, as provided in the Relative Quantification Manual (Roche Diagnostics GmbH) (40). The quantity of *HSD17B2* transcript in each sample was standardized by the geometric mean of porphobilinogen deaminase, human mitochondrial ribosomal protein L19 and RNA polymerase II subunit A cDNA levels. The PCR amplification efficiency for target and reference genes was 92, 100, 92 and 98%, respectively. For amplification, 1 μl total (20 μl) cDNA solution was added to 9 μl 1X concentrated Light Cycler® 480 SYBR Green I Master mix (Roche Diagnostics GmbH), containing 2.5 mM $MgCl_2$, and 0.5 μM or 1 μM of primers, and subjected to 40 PCR cycles preceded by 10 min of activation at 95˚C. Primer sequences and specific amplification conditions are presented in Table I. A sample of non-reverse-transcribed RNA and a no-template control were included in each batch of samples as negative controls. Melting curve analysis and agarose gel (1.5%) electrophoresis were applied to confirm the specificity of the amplified products. All cDNA samples were applied in triplicate for each gene of interest. *HSD17B2* transcript levels in the investigated tissues were expressed as the decimal logarithm of multiplicity of cDNA concentrations in the calibrator.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Proteins for western blotting were isolated as previously described (41). Briefly, all tissue specimens obtained from 161 patients were homogenized in liquid nitrogen and treated with RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH). Samples were incubated on ice for 30 min and were then centrifuged at 10,000 x g for 10 min at 4˚C. Supernatants were collected for western blotting. Subsequently, 2 μl isolated proteins from each sample were denatured in sample loading buffer and separated by standard SDS-PAGE on 10% Tris-glycine gels. Gel proteins were electrotransferred to a nitrocellulose membrane, which was then blocked in 5% non-fat dry milk in 1X concentrated Tris-buffered saline/Tween-20 (0.1%) for 1 h at room temperature. After blocking, each membrane was incubated overnight at 4˚C with rabbit anti-HSD17B2 Ab (1:800), followed by 2-h incubation with goat anti-rabbit HRP-conjugated Ab (1:2,500) at room temperature. Bands were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). To ensure equal protein loading, membranes were stripped and incubated with rabbit polyclonal anti-GAPDH Ab (1:3,300) for 2 h, followed by incubation with goat anti-rabbit HRP-conjugated Ab (1:2,500) for 1 h at room temperature. Bands were revealed using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.). For signal visualization, the Biospectrum Imaging system 500 (UVP, LLC) was used. The time of exposure and camera settings were identical for all membranes. The amount of HSD17B2 protein is presented as the decimal logarithm of

Table I. Primer sequences used for reverse transcription-quantitative PCR analysis.

Gene	Sequence (5'-3')	ENST/ENSG number (www.ensembl.org/)	Product size (bp)	Final primer concentration	RT-qPCR cycling conditions (denaturation; annealing; extension)
HSD17B2	F: CAATGCTGCAGGACAGAGGA R: GTTCACGGCCATGCATTGTT	ENST00000199936	116	0.5 μM	95˚C/6 sec; 60˚C/6 sec; 72˚C/6 sec
PBGD	F: GCCAAGGACCAGGACATC R: TCAGGTACAGTTGCCCATC	ENST00000278715	160	0.5 μM	95˚C/6 sec; 61˚C/6 sec; 72˚C/6 sec
hMRPL19	F: ACTTTATAATCCTCGGGTC R: ACTTTCAGCTCATTAACAG	ENST00000393909	171	1 μM	95˚C/10 sec; 56˚C/10 sec; 72˚C/10 sec
POLR2A	F: AAGTGGTGGAGGGAGTCAAG R: CGCAGGTGGATGTTGAAGAG	ENST00000617998	115	0.5 μM	95˚C/6 sec; 58˚C/6 sec; 72˚C/6 sec

F, forward; R, reverse; HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; PBGD, porphobilinogen deaminase; hMRPL19, human mitochondrial ribosomal protein L19; POLR2A, RNA polymerase II subunit A.

the HSD17B2-to-GAPDH band optical density ratio, which was measured using ImageJ2x program (https://imagej.net/ImageJ2). Samples were run in technical duplicates to confirm the reproducibility of the results.

Immunohistochemistry. Cancer tissues (n=161) were fixed in 10% formalin for 24 h at room temperature, embedded in paraffin and cut into 4-μm sections. Samples were then mounted on SuperFrost® Plus adhesion microscope slides (Menzel; Thermo Fisher Scientific, Inc.) and stained according to the presented optimized procedure. Briefly, slides were deparaffinized (overnight at 60˚C and immersed in xylene for 30 min) and rehydrated (two washes in 100, 96, 75 and 50% ethanol and distilled water; 5 min/wash). Subsequently, heat-induced epitope retrieval was carried out by heating slides in low pH EnVision FLEX Target Retrieval Solution (Dako; Agilent Technologies, Inc.) for 50 min at 97˚C in a water bath. The sections were then incubated for 10 min at room temperature in Novocastra Peroxidase Block reagent (Leica Microsystems, Ltd.) to neutralize endogenous peroxidase activity and were treated with Novocastra Protein Block (Leica Microsystems, Ltd.) for 10 min at room temperature to reduce non-specific binding of Ab and polymer. Sections were then incubated overnight with rabbit polyclonal anti-HSD17B2 Ab at a dilution of 1:120 in a humidified chamber at 4˚C. The primary Ab was diluted in EnVision FLEX Antibody Diluent (Dako; Agilent Technologies, Inc.). Immunodetection was achieved using the Novolink Polymer Detection system (Leica Microsystems, Ltd.), which is a two-step streptavidin-biotin-HRP method. Each slide was incubated for 30 min at room temperature, and between each step, slides were washed in EnVision FLEX Wash Buffer (Dako; Agilent Technologies, Inc.). Finally, the reaction product was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) prepared from Novolink Polymer DAB Chromogen and Novolink DAB Substrate Buffer (two incubations for 5 min at room temperature). Sections were counterstained with Mayer's haematoxylin for 5 min at room temperature, dehydrated, cleared and mounted in DPX. Sections from formalin-fixed and paraffin-embedded normal human liver were used as a positive control for anti-HSD17B2 Ab. Normal liver tissue was obtained from a patient with squamous lung cancer with liver metastasis, and written informed consent was provided for the use of tissue samples. As a negative control, sections were incubated with an omission of the primary Ab.

Evaluation of immunohistochemical staining was scored independently by two experienced pathologists and was repeated twice for each sample. Both pathologists were blinded to the clinical characteristics of the patients and scores were accepted if investigators agreed with each other. Otherwise, specimens were re-evaluated until a consensus was reached. Immunohistochemical reactivity was assessed using a semi-quantitative method based on staining intensity, with a score of 0, negative staining; 1, weak staining; and 2, strong staining, in >60% of tumour cells. Moderate staining was omitted, as it is highly subjective. Furthermore, in each group, subgroups were extracted based on the percentage of positively stained tumour cells (2/1, strong staining in >60% and weak staining in 30-40% of tumour cells; 1/2, weak staining in >60% and strong staining in 30-40% of tumour cells; 1/0,

Table II. Differences in HSD17B2 transcript and protein levels between lung cancer and corresponding histopathologically unchanged tissues from patients with non-small cell lung cancer, including clinicopathological characteristics.

		HSD17B2 transcript level			HSD17B2 protein level		
		Cancerous tissues	Histopathologically unchanged tissues		Cancerous tissues	Histopathologically unchanged tissues	
Variables	Number of cases	Median (range)	Median (range)	P-value	Median (range)	Median (range)	P-value
No. of patients	161	2.65 (0.13-4.39)	3.43 (0.07-5.61)	$<10^{-6}$	2.54 (0.07-4.39)	3.60 (1.03-6.81)	$<10^{-6}$
Sex							
Male	98	2.60 (0.18-4.06)	3.31 (0.07-5.61)	$<10^{-6}$	2.50 (0.38-4.05)	3.40 (1.03-6.81)	$<10^{-6}$
Female	63	2.70 (0.13-4.39)	3.63 (2.46-5.28)	$<10^{-6}$	2.58 (0.07-4.39)	4.06 (2.36-6.30)	$<10^{-6}$
Patient age (years)							
≤60 (males:females)	45 (29;16)	2.61 (0.13-3.81)	3.41 (1.53-5.45)	$<10^{-6}$	2.50 (0.07-3.97)	3.56 (2.06-6.53)	$<10^{-6}$
>60 (males:females)	116 (69:47)	2.66 (0.13-4.39)	3.45 (0.07-5.61)	$<10^{-6}$	2.55 (0.43-4.39)	3.63 (1.03-6.81)	$<10^{-6}$
Histological type							
Adenocarcinoma (males:females)	70 (37:33)	2.49 (0.13-.39)	3.53 (0.07-5.49)	$<10^{-6}$	2.34 (0.07-4.39)	3.65 (1.03-6.30)	$<10^{-6}$
Squamous cell carcinoma (males:females)	71 (50:21)	2.76 (0.58-3.89)	3.35 (1.53-5.61)	$<10^{-6}$	2.66 (0.38-4.04)	3.48 (1.80-6.81)	$<10^{-6}$
Large cell carcinoma (males:females)	12 (9:3)	2.55 (0.43-3.21)	3.50 (2.83-4.54)	0.0029	2.66 (0.43-3.26)	3.54 (2.81-5.25)	0.0022
Carcinoid (males:females)	8 (2:6)	2.09 (0.13-3.67)	3.35 (2.70-4.64)	0.012	2.47 (1.12-3.77)	3.36 (2.67-5.19)	0.012
Lung cancer stage							
0 (males:females)	6 (3:3)	2.02 (0.13-3.67)	3.07 (2.70-4.01)	0.028	1.88 (1.12-3.77)	3.18 (2.67-4.95)	0.028
IA-IB (males:females)	63 (29:34)	2.84 (0.13-4.15)	3.50 (2.46-5.61)	$<10^{-6}$	2.77 (0.07-4.21)	3.84 (2.36-6.81)	$<10^{-6}$
IIA-IIB (males:females)	53 (35:18)	2.41 (0.15-4.39)	3.60 (0.07-5.49)	$<10^{-5}$	2.30 (0.10-4.39)	3.64 (1.03-6.53)	$<10^{-6}$
IIIA-IV (males:females)	39 (31:8)	2.60 (1.20-3.81)	3.24 (2.42-5.11)	$<10^{-5}$	2.41 (1.04-3.97)	3.34 (2.38-6.11)	$<10^{-6}$
Lung cancer grade[a]							
G1 (males:females)	12 (7:5)	2.94 (1.64-4.39)	3.31 (2.76-5.21)	0.21	2.89 (1.44-4.39)	3.36 (2.72-6.30)	0.019
G2 (males:females)	68 (44:24)	2.71 (0.62-4.15)	3.29 (2.34-5.49)	$<10^{-6}$	2.59 (0.42-4.21)	3.39 (1.80-6.53)	$<10^{-6}$
G3 (males:females)	73 (47:26)	2.58 (0.13-3.89)	3.59 (0.07-5.61)	$<10^{-6}$	2.46 (0.07-4.05)	3.84 (1.03-6.81)	$<10^{-6}$
Tumour size							
Tis (males:females)	6 (3:3)	2.02 (0.13-3.67)	3.07 (2.70-4.01)	0.028	1.88 (1.12-3.77)	3.18 (2.67-4.95)	0.028
T1 (males:females)	23 (13:10)	3.11 (2.04-3.89)	3.71 (2.42-5.25)	0.00092	3.04 (1.92-4.04)	4.19 (2.85-6.29)	0.00013
T2 (males:females)	99 (55:44)	2.59 (0.13-4.39)	3.48 (1.53-5.61)	$<10^{-6}$	2.53 (0.07-4.35)	3.64 (1.803-6.81)	$<10^{-6}$
T3-T4 (males:females)	33 (27:6)	2.51 (0.58-4.35)	3.23 (0.07-4.91)	0.0003	2.39 (0.38-4.39)	3.31 (1.03-5.63)	$<10^{-4}$

Table II. Continued.

		HSD17B2 transcript level			HSD17B2 protein level		
		Cancerous tissues	Histopathologically unchanged tissues		Cancerous tissues	Histopathologically unchanged tissues	
Variables	Number of cases	Median (range)	Median (range)	P-value	Median (range)	Median (range)	P-value
Lymph node metastasis							
N0 (males:females)	95 (49:46)	2.73 (0.13-4.35)	3.46 (0.07-5.61)	$<10^{-6}$	2.61 (0.07-4.39)	3.63 (1.03-6.81)	$<10^{-6}$
N1 (males:females)	44 (34:10)	2.37 (0.15-4.39)	3.34 (1.53-5.50)	$<10^{-6}$	2.27 (0.10-4.35)	3.45 (1.80-6.53)	$<10^{-6}$
N2 (males:females)	22 (15:7)	2.66 (1.73-3.81)	3.42 (2.42-5.11)	0.00098	2.47 (1.63-3.97)	3.50 (2.53-6.11)	$<10^{-4}$
Distant metastasis							
M0 (males:females)	158 (95:63)	2.60 (0.13-4.38)	3.45 (0.07-5.61)	$<10^{-6}$	2.53 (0.07-4.39)	3.62 (1.03-6.81)	$<10^{-6}$
M1 (males:females)	3 (3:0)	2.78 (2.65-3.05)	3.33 (2.82-3.34)	-	2.73 (2.55-2.81)	3.34 (2.72-3.37)	-
Smoking							
Yes (males:females)	146 (92:54)	2.66 (0.13-4.39)	3.34 (0.07-5.61)	$<10^{-6}$	2.55 (0.07-4.35)	3.58 (1.03-6.81)	$<10^{-6}$
No (males:females)	15 (6:9)	2.41 (0.18-4.35)	3.41 (2.70-5.21)	0.0045	2.29 (1.13-4.39)	3.63 (2.67-6.30)	0.0015
Residual tumour status							
R0 (males:females)	144 (84:60)	2.60 (0.13-4.39)	3.42 (0.07-5.61)	$<10^{-6}$	2.53 (0.07-4.39)	3.56 (1.03-6.81)	$<10^{-6}$
R1 (males:females)	16 (13:3)	2.94 (1.87-3.81)	3.43 (2.84-5.44)	0.0072	2.85 (1.75-3.77)	3.78 (2.85-6.53)	0.0013
R2 (males:females)	1 (1:0)	--	-	-	-	-	

HSD17B2 transcript levels were standardized by the geometric mean of porphobilinogen deaminase, human mitochondrial ribosomal protein L19 and RNA polymerase II subunit A cDNA levels, and expressed as the decimal logarithm of multiples of these cDNA copies in the calibrator. HSD17B2 protein expression detected by western blotting was presented as the decimal logarithm of the HSD17B2-to-GAPDH band optical density ratio. The normality of the observed patient data distribution was assessed by the Shapiro-Wilk test. The Wilcoxon matched-pairs signed rank test was used to compare the mean values. $P<0.05$ was considered to indicate a statistically significant difference. [a]Lung cancer grading was evaluated for 153 patients, excluding eight patients with carcinoid tumours. HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; Tis, carcinoma in situ.

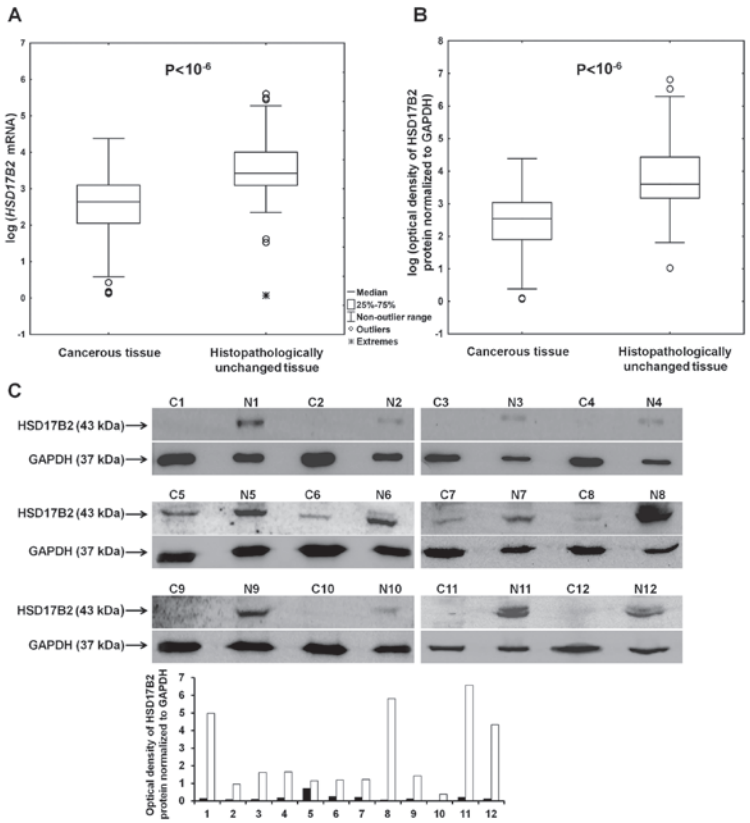

Figure 1. HSD17B2 mRNA and protein expression levels in lung cancer and histopathologically unchanged tissues obtained from patients with NSCLC. (A) *HSD17B2* transcript levels in the investigated tissues obtained from 161 patients with NSCLC (63 women and 98 men). Total RNA was reverse-transcribed and mRNA expression was studied by reverse transcription-quantitative PCR relative quantification analysis. The mRNA expression levels of *HSD17B2* in each sample were standardized by the geometric mean of porphobilinogen deaminase, human mitochondrial ribosomal protein L19 and RNA polymerase II subunit A cDNA levels, and were quantified using a calibrator. The amount of *HSD17B2* mRNA was expressed as the decimal logarithm of multiples of cDNA copies in the calibrator. (B) HSD17B2 protein expression levels in investigated tissues were detected by western blotting. The amount of HSD17B2 proteins was presented as the decimal logarithm of the HSD17B2-to-GAPDH band optical density ratio. (C) Representative image of from western blot analysis of HSD17B2 protein expression in lung cancer tissues (C) and their matched histopathologically unchanged tissues (N) obtained from the same patient. Optical density is presented as a bar graph; black bars, lung cancer tissues; white bars, matched histopathologically unchanged tissues. Data were evaluated using the Wilcoxon matched-pairs signed rank test. HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; NSCLC, non-small cell lung cancer.

weak staining in >60% and negative staining in 30-40% of tumour cells; and 0/1, negative staining in >60% and weak staining in 30-40% of tumour cells). Based on this evaluation, cases were considered to have high (2 and 2/1) and low (1/2; 1/0; 0/1; and 0) expression of HSD17B2 protein. In order to perform univariate survival analysis based on the immunohistochemical results, LC specimens were subdivided into three groups: High (score 2 and 2/1), intermediate (score 1/2) and low (score 1/0, 0/1 and 1) HSD17B2 protein levels.

Statistical analysis. Statistical analyses were performed using STATISTICA 12 software (StatSoft, Inc.) and GraphPad Prism 8.3.0 version (GraphPad Software, Inc.). The Shapiro-Wilk test was used to assess the normality of the observed patient data distribution. Because HSD17B2 mRNA and protein expression levels were not normally distributed, the Wilcoxon matched-pairs signed rank test was used to consider statistically significant differences in HSD17B2 mRNA and protein levels between LC and histopathologically unchanged tissues ($P<0.05$). χ^2 and Fisher's exact tests were used to determine whether there was a significant association between HSD17B2 expression and various clinicopathological parameters in LC tissues. U-Mann-Whitney (for comparisons between two groups) and Kruskal-Wallis tests (for comparisons between three or more groups) were performed to evaluate the relationship between HSD17B2 expression and clinicopathological parameters in histopathologically unchanged lung tissues. Survival curves were plotted using the Kaplan-Meier method, and differences were estimated by log-rank test or Gehan-Breslow-Wilcoxon test. Univariate and multivariate Cox proportional hazard models were used to estimate HR. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. In total, 161 patients diagnosed with NSCLC were included in the present study. The median age of patients at the moment of resection was 65 years (range, 29-80). Among the 63 women enrolled, all patients were postmenopausal. Only four women received hormone replacement therapy (E2 transdermal patches). The majority of patients were smokers (n=146). LC grading was evaluated for 153 patients, excluding eight patients with carcinoid tumours. Detailed clinicopathological characteristics of the patients are presented in Table II.

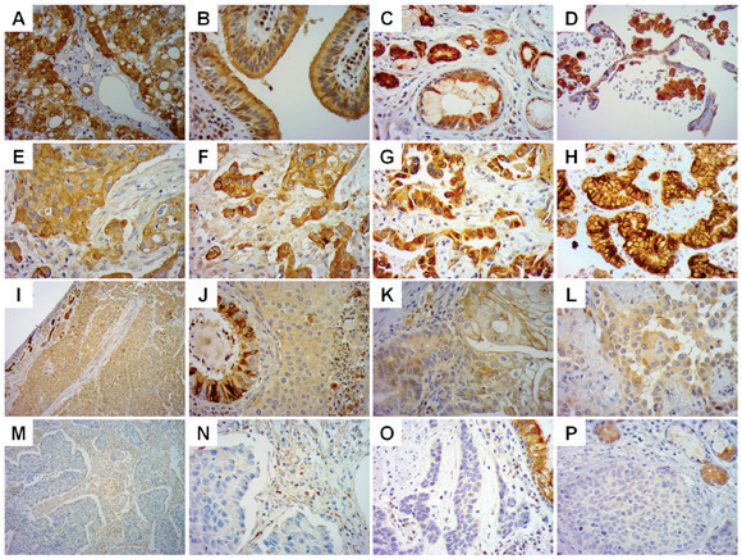

Figure 2. Representative immunohistochemical staining of HSD17B2 in formalin-fixed, paraffin-embedded clinical tissue specimens. Positive immunoreactivity of HSD17B2 protein was indicated by brown staining. (A) Normal human liver sections with strong HSD17B2 staining in hepatocytes were used as a positive control. (B-D) Representative images of lung specimens with strong HSD17B2 staining; (B) normal respiratory epithelium, (C) normal submucosal seromucous glands and (D) macrophages. (E-H) Representative images of strong HSD17B2 staining in (E and F) SCC cells and (G and H) ADC cells. (I-L) Representative images of weak HSD17B2 staining in (I and J) SCC cells and (K and L) ADC cells. (M-P) Representative images of negative HSD17B2 staining in (M and N) ADC cells and (O and P) SCC cells. The presence of strong HSD17B2 staining in (J and O) normal respiratory epithelium and in (I and P) normal glands, located within lung cancer tissue specimens served as an internal positive control. A weak positive reaction was detected in the (M and N) tumour stroma. Original magnifications, x100 (I and M) and x400 for all other images. ADC, adenocarcinoma; HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; SCC, squamous cell carcinoma.

Differences in HSD17B2 transcript and protein levels between LC tissues and adjacent histopathologically unchanged tissues from patients with NSCLC. RT-qPCR and western blotting were performed to compare the expression status of HSD17B2 in LC and histopathologically unchanged tissues. HSD17B2 mRNA ($P<10^{-6}$) and protein ($P<10^{-6}$) expression levels were significantly lower in cancer tissues compared with in adjacent normal tissues (Fig. 1A and B; Table II). A representative image of western blotting results with a bar graph including optical density measurements of bands is presented in Fig. 1C. The clinicopathological characteristics of patients whose samples were used to generate the image presented in Fig. 1C are summarized in Table SI.

In addition, this study aimed to determine whether the differences in HSD17B2 mRNA and protein levels among the investigated tissues were associated with various clinicopathological features (Table II). HSD17B2 mRNA and protein expression levels were significantly lower in cancer tissues regardless of sex or age ($P<10^{-6}$). With regards to NSCLC histological subtypes, a substantial decrease in HSD17B2 mRNA and protein expression was detected in ADC and SCC specimens ($P<10^{-6}$). Although only 12 patients included in this study presented with LCC, HSD17B2 mRNA and protein expression was also significantly diminished in cancer tissues compared with histopathologically unchanged specimens (P=0.0029 and P=0.0022, respectively). Furthermore, the expression of HSD17B2 was detected in tissues from eight patients with carcinoid tumours; notably, reduced mRNA and protein expression levels of HSD17B2 were detected in tumour tissues compared with matched normal specimens (P=0.012 and P=0.012, respectively). Statistically significant differences in HSD17B2 mRNA and protein levels between investigated tissues were also detected in all LC stages and all tumour sizes (Table II). Notably, only six patients were diagnosed with stage 0 cancer and carcinoma *in situ*; therefore, this is not a representative group to consider the statistical significance of HSD17B2 expression. HSD17B2 mRNA and protein expression levels were decreased in LC tissues compared with their matched normal counterparts in all grades of lymph node metastasis (Table II), and were associated with G2 and G3 LC histological grades ($P<10^{-6}$; Table II). In the group of patients with low-grade tumours (G1), the difference in HSD17B2 protein levels was statistically significant, whereas a tendency towards lower *HSD17B2* mRNA levels in cancer tissues was observed; however, this was not significant. Furthermore, HSD17B2 mRNA and protein levels were significantly decreased in LC tissues regardless of smoking status and residual tumour status (Table II). The majority of patients included in this study (n=158) presented no distant metastasis (M0); therefore, no conclusions can be made concerning this parameter. A strong positive association between HSD17B2 mRNA expression and protein expression was detected in all investigated tissues (data not shown).

HSD17B2 immunoreactivity in clinical tissue specimens. Although LC tissues used for RT-qPCR and western blotting were obtained from the centre of the tumour, it is possible that these specimens contained some non-tumoural cells and stromal cells. Therefore, to verify the present results and to reveal the location of HSD17B2 protein, immunohistochemical staining was performed with anti-HSD17B2 Ab (Fig. 2).

Sections of normal human liver were used as a positive control for anti-HSD17B2 Ab (Fig. 2A). A positive reaction, with strong cytoplasmic staining, was detected in normal

Table III. Association between HSD17B2 mRNA and protein levels and clinicopathological parameters in lung cancer tissues from patients with non-small cell lung cancer.

		HSD17B2 mRNA expression			HSD17B2 protein expression		
Variable	Number of cases	High	Low	P-value	High	Low	P-value
Sex				0.13			0.07
Male	98	44 (45%)	54 (55%)		30 (31%)	68 (69%)	
Female	63	36 (57%)	27 (43%)		28 (44%)	35 (56%)	
Patient age (years)				0.63			0.51
≤60	45	21 (47%)	24 (53%)		18 (40%)	27 (60%)	
>60	116	59 (51%)	57 (49%)		40 (34%)	76 (66%)	
Histological type				0.11			0.089
Adenocarcinoma	70	29 (41%)	41 (59%)		30 (43%)	40 (57%)	
Squamous cell carcinoma	71	43 (61%)	28 (39%)		23 (32%)	48 (68%)	
Large cell carcinoma	12	5 (42%)	7 (58%)		1 (8%)	11 (92%)	
Carcinoid	8	3 (37%)	5 (63%)		4 (50%)	4 (50%)	
Lung cancer stage				0.017			0.0006
0	6	2 (33%)	4 (67%)		1 (17%)	5 (83%)	
IA-IB	63	41 (65%)	22 (35%)		35 (56%)	28 (44%)	
IIA-IIB	53	20 (38%)	33 (62%)		12 (23%)	41 (77%)	
IIIA-IV	39	17 (44%)	22 (56%)		10 (26%)	29 (74%)	
Lung cancer grade[a]				0.14			$<10^{-4}$
G1	12	9 (75%)	3 (25%)		10 (83%)	2 (17%)	
G2	68	37 (54%)	31 (46%)		31 (46%)	37 (54%)	
G3	73	33 (45%)	40 (55%)		14 (19%)	59 (81%)	
Tumour size				0.0061			0.046
Tis	6	2 (33%)	4 (67%)		1 (17%)	5 (83%)	
T1	23	19 (83%)	4 (17%)		14 (61%)	9 (39%)	
T2	99	46 (46%)	53 (54%)		33 (33%)	66 (67%)	
T3-T4	33	13 (39%)	20 (61%)		10 (30%)	23 (70%)	
Lymph node metastasis				0.0055			0.028
N0	95	56 (59%)	39 (41%)		38 (40%)	57 (60%)	
N1	44	13 (30%)	31 (70%)		9 (20%)	35 (80%)	
N2	22	11 (50%)	11 (50%)		11 (50%)	11 (50%)	
Distant metastasis				0.55			0.19
M0	158	78 (49%)	80 (51%)		58 (37%)	100 (63%)	
M1	3	2 (67%)	1 (33%)		0	3 (100%)	
Smoking				0.81			0.82
Yes	146	73 (50%)	73 (50%)		53 (36%)	93 (64%)	
No	15	7 (47%)	8 (53%)		5 (33%)	10 (67%)	
Residual tumour status				0.29			0.51
R0	144	70 (49%)	74 (51%)		51 (35%)	93 (65%)	
R1	16	10 (62%)	6 (38%)		7 (44%)	9 (56%)	

χ^2 and Fisher's exact tests were used to analyze data. $P<0.05$ was considered to indicate a statistically significant difference. [a]Lung cancer grading was evaluated for 153 patients, excluding eight patients with carcinoid tumours. HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; Tis, carcinoma *in situ*.

respiratory epithelium, histopathologically unchanged glands, and macrophages (Fig. 2B-D). In addition, weak staining was detected in lung endothelial cells (Fig. 2D). Among all NSCLC specimens, only 58 (36%) demonstrated high cytoplasmic staining of HSD17B2 in cancer cells (Fig. 2E-H). A total of 72 specimens (45%) exhibited weak HSD17B2 immunoreactivity

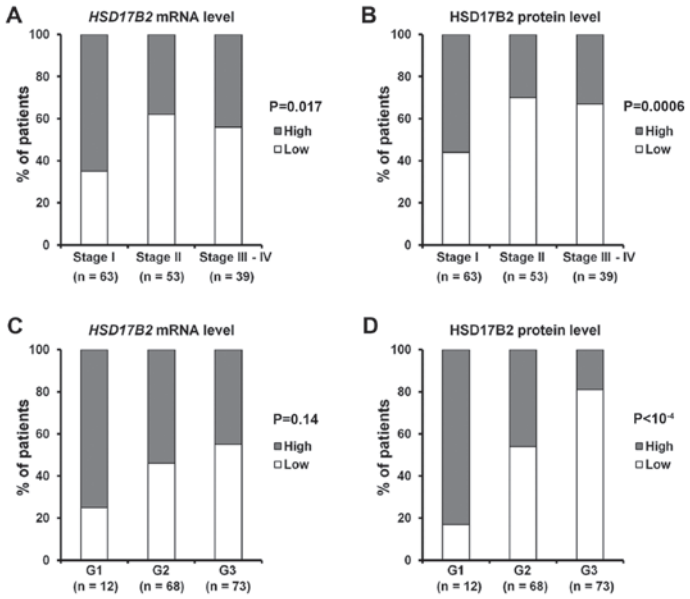

Figure 3. Association between HSD17B2 mRNA and protein levels in cancer tissues, and lung cancer stage and grade. Association between HSD17B2 (A) mRNA and (B) protein levels in NSCLC tissues and LC stages. Association between HSD17B2 (C) mRNA and (D) protein levels in NSCLC tissues and LC grades. (A and C) *HSD17B2* mRNA levels were divided into high and low according to the median value from reverse transcription-quantitative PCR analysis. Relative cDNA concentrations higher than the median were classified as high, and concentrations equal to or lower than the median were assigned as low. (B and D) HSD17B2 protein levels in lung cancer cells were estimated by immunohistochemical staining and separated into two groups; those with high and low protein content, according to immunohistochemistry staining classification. P<0.05 was considered to indicate a statistically significant difference. HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; LC, lung cancer; NSCLC, non-small cell lung cancer.

in cancer cells (Fig. 2I-L), whereas 31 (19%) were negative (Fig. 2M-P). A weak positive reaction was revealed in tumour stromal cells. The presence of HSD17B2 protein was also confirmed in normal submucosal glands (Fig. 2I and P) and in normal bronchial epithelial cells (Fig. 2J and O), located in the tumour field. The clinicopathological characteristics of the patients whose tissues were used to generate the staining images presented in Fig. 2 are detailed in Table SII.

Association between HSD17B2 mRNA and protein levels in LC tissues and various clinicopathological parameters. To evaluate the clinical significance of HSD17B2 expression in NSCLC, this study investigated whether there was an association between HSD17B2 mRNA and/or protein expression and clinicopathological features in tumour specimens. The results from RT-qPCR analysis were divided into two groups according to the median value of the detected *HSD17B2* expression in cancer tissues. Tissues that displayed values higher than the median were classified as having high expression, and those that displayed values equal to or lower than the median were classified as having low expression. With regards to HSD17B2 protein levels, immunohistochemistry results were used. LC specimens were classified into two groups: Those with high and low levels of HSD17B2 protein in cancer cells, according to immunohistochemistry staining classification.

In cancer tissues, HSD17B2 mRNA and protein expression levels were significantly associated with LC stages (P=0.017; P=0.0006), tumour size (P=0.0061; P=0.046), and lymph node metastasis (P=0.0055; P=0.028; Table III). Notably, 65% of patients with stage I disease presented a high level of *HSD17B2* transcripts, and this feature was diminished to 38% of patients with stage II disease and 44% of patients with stage III disease (Fig. 3A; Table III). The difference was even more substantial for HSD17B2 protein expression. Only 44% of patients with stage I disease had low HSD17B2 levels in cancer cells, whereas as much as 77 and 74% of patients with stage II and III-IV disease showed decreased amounts of HSD17B2 protein in cancer specimens, respectively (Fig. 3B; Table III). Similarly, the same tendency was maintained regarding tumour size. Furthermore, *HSD17B2* expression was strongly associated with LC grading. Although the difference in transcript levels among patients with various LC grades appeared to be not statistically significant, the tendency towards lower mRNA expression in higher grade LC (moderately or poorly differentiated tumours, G2 and G3) was revealed (Fig. 3C; Table III). Notably, a great majority of patients with G3 LC (81%) exhibited weak or negative staining for HSD17B2 protein, whereas in the G1 group (well differentiated, low grade tumours), 83% of patients had high amounts of HSD17B2 protein ($P<10^{-4}$; Fig. 3D; Table III). Furthermore, lower levels of HSD17B2 mRNA and protein were observed in LC tissues from patients with N1 lymph node metastasis.

Association between HSD17B2 mRNA and protein levels in histopathologically unchanged lung tissues and various clinicopathological parameters. In addition, the relationship between the clinicopathological features of patients with NSCLC and HSD17B2 expression status in histopathologically unchanged lung tissues was assessed (Table IV). RT-qPCR and western blotting results were used, because tumour-adjacent, histopathologically unchanged lung specimens were not available for immunohistochemical staining. Notably, women had significantly higher levels of HSD17B2 mRNA and protein in tumour-matched, macroscopically unchanged tissue specimens than men (P=0.0012; P=0.0022). The expression levels of HSD17B2 mRNA and protein were also associated

Table IV. Association between HSD17B2 mRNA and protein levels and clinicopathological parameters in lung histopathologically unchanged tissues from patients with non-small cell lung cancer.

		HSD17B2 mRNA		HSD17B2 proteinb	
Variable	Number of cases	Median (range)	P-value	Median (range)	P-value
Sex			0.0012[c]		0.0022[c]
Male	98	3.31 (0.07-5.61)		3.40 (1.03-6.81)	
Female	63	3.63 (2.46-5.28)		4.06 (2.36-6.30)	
Patient age (years)			0.69[c]		0.75[c]
≤60	45	3.41 (1.53-5.45)		3.56 (2.06-6.53)	
>60	116	3.45 (0.07-5.61)		3.63 (1.03-6.81)	
Histological type			0.67[d]		0.68[d]
Adenocarcinoma	70	3.53 (0.07-5.49)		3.65 (1.03-6.30)	
Squamous cell carcinoma	71	3.35 (1.53-5.61)		3.48 (1.80-6.81)	
Large cell carcinoma	12	3.50 (2.83-4.54)		3.54 (2.81-5.25)	
Carcinoid	8	3.35 (2.70-4.64)		3.36 (2.67-5.19)	
Lung cancer stages			0.10[d]		0.14[d]
0	6	3.07 (2.70-4.01)		3.18 (2.67-4.95)	
IA-IB	63	3.50 (2.46-5.61)		3.84 (2.36-6.81)	
IIA-IIB	53	3.60 (0.07-5.49)		3.64 (1.03-6.53)	
IIIA-IV	39	3.24 (2.42-5.11)		3.34 (2.38-6.11)	
Lung cancer grades[a]			0.17[d]		0.051[d]
G1	12	3.31 (2.76-5.21)		3.36 (2.72-6.30)	
G2	68	3.29 (2.34-5.49)		3.39 (1.80-6.53)	
G3	73	3.59 (0.07-5.61)		3.84 (1.03-6.81)	
Tumour size			0.030[d]		0.021[d]
Tis	6	3.07 (2.70-4.01)		3.18 (2.67-4.95)	
T1	23	3.71 (2.42-5.25)		4.19 (2.85-6.29)	
T2	99	3.48 (1.53-5.61)		3.64 (1.80-6.81)	
T3-T4	33	3.23 (0.07-4.91)		3.31 (1.03-5.63)	
Lymph node metastasis			0.8[d]		0.87[d]
N0	95	3.46 (0.07-5.61)		3.63 (1.03-6.81)	
N1	44	3.34 (1.53-5.50)		3.45 (1.80-6.53)	
N2	22	3.42 (2.42-5.11)		3.50 (2.53-6.11)	
Distant metastasis			0.31[c]		0.22[c]
M0	158	3.45 (0.07-5.61)		3.62 (1.03-6.81)	
M1	3	3.33 (2.82-3.34)		3.34 (2.72-3.37)	
Smoking			0.66[c]		0.52[c]
Yes	146	3.34 (0.07-5.61)		3.58 (1.03-6.81)	
No	15	3.41 (2.70-5.21)		3.63 (2.67-6.30)	
Residual tumour status			0.85[c]		0.62[c]
R0	144	3.42 (0.07-5.61)		3.56 (1.03-6.81)	
R1	16	3.43 (2.84-5.44)		3.78 (2.85-6.53)	

[a]Lung cancer grading was evaluated for 153 patients, excluding eight patients with carcinoid tumours; [b]HSD17B2 protein levels were evaluated by western blotting; [c]U-Mann-Whitney test; [d]Kruskal-Wallis test. P<0.05 was considered to indicate a statistically significant difference. HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; Tis, carcinoma in situ.

with tumour size (P=0.030; P=0.021). The expression levels of HSD17B2 tended to decrease in histopathologically unchanged tissues obtained from patients diagnosed with larger tumour dimensions. This tendency was also maintained for advanced LC stages. No significant associations were detected between HSD17B2 expression and the other clinicopathological variables in the investigated macroscopically unchanged tissues.

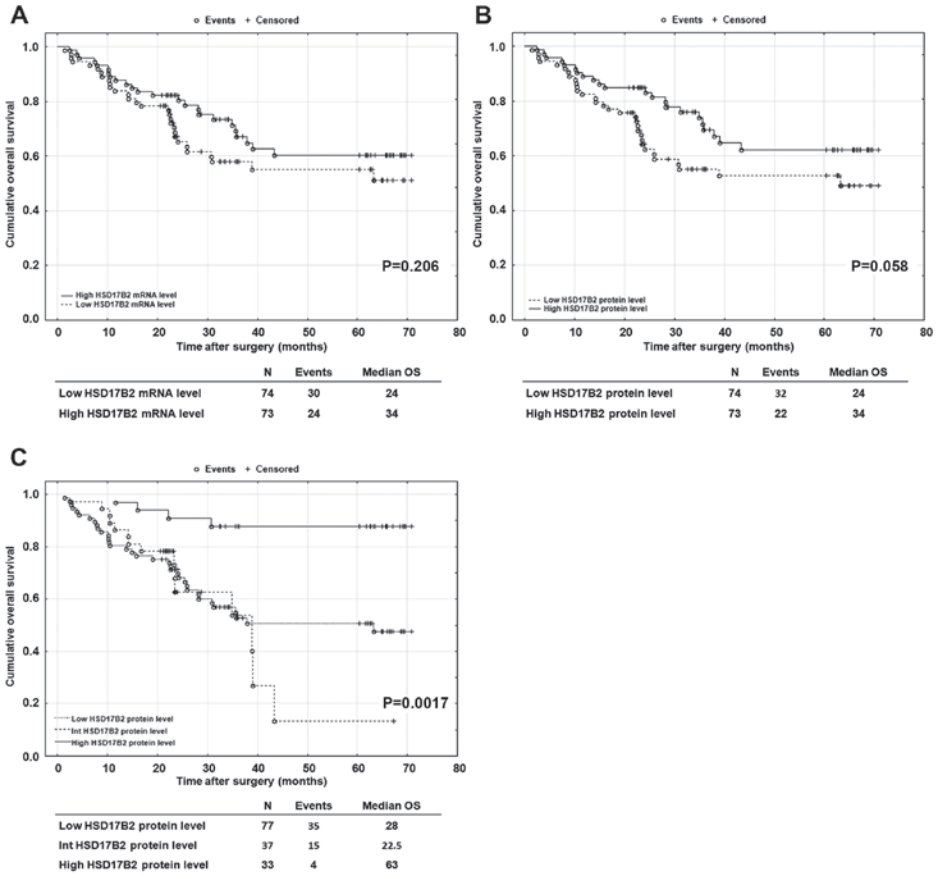

Figure 4. Kaplan-Meier survival analysis among 147 patients with non-small cell lung cancer according to HSD17B2 mRNA and protein levels in cancer tissues. (A) OS curves according to the *HSD17B2* transcript level. (B) OS curves according to the HSD17B2 protein level, as determined by western blotting. (A and B) Patients were divided into two groups: High and low HSD17B2 mRNA and protein levels according to the median value of measurements. (C) OS curves according to the HSD17B2 protein level, as determined by immunohistochemical analysis. Patients were subdivided into three groups: High, intermediate and low HSD17B2 protein levels. The number of patients classified into each group, number of events and median OS are presented at the bottom of each graph. P-values for OS were determined by log-rank test. P<0.05 was considered to indicate a statistically significant difference. HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; OS, overall survival.

Association of HSD17B2 mRNA and protein levels in cancer tissues with clinical outcome in patients with NSCLC. A retrospective analysis was performed to investigate whether HSD17B2 mRNA or protein expression may have prognostic significance in outcomes for patients with NSCLC. The median OS among 147 patients enrolled in this analysis was 30.7 months (range, 1.3-80 months). The results from RT-qPCR and western blotting in cancer tissues were divided into two groups, high and low mRNA and protein levels, according to the median value of measurements. Although univariate analysis revealed results that did not reach statistical significance, median survival was longer for patients with higher HSD17B2 mRNA and protein expression (Fig. 4A and B). In particular, a certain trend towards significance was observed for HSD17B2 protein content (P=0.058; Fig. 4B). As aforementioned, specimens used for western blotting may be burdened with the presence of non-tumoural cells. Therefore, univariate analysis was performed based on the immunohistochemical results. LC specimens were subdivided into three groups: High (score 2 and 2/1), intermediate (score 1/2) and low (1/0; 0/1 and 1) HSD17B2 protein levels. Notably, using the log-rank test, patients with high levels of HSD17B2 protein presented a significant increase in OS (P=0.0017; Fig. 4C). The median value of OS for those patients was 63 months vs. 28 and 22.5 months for patients with low and intermediate HSD17B2 protein levels, respectively. Univariate Cox regression analysis revealed that high HSD17B2 protein content was associated with a better prognosis in patients with NSCLC (HR=0.18; 95% CI=0.06-0.51; P=0.0012; Table V). Furthermore, a large tumour size and LCC subtype were poor predictors in this study (Table V). Subsequently, variables with a P-value <0.15 in the univariate analysis (HSD17B2 protein level, LC histological type, tumour size and residual tumour status) were included in the multivariate analysis during stepwise selection to determine independent predictors of outcome in patients with NSCLC.

Results from multivariate analysis revealed that HSD17B2 protein levels in cancer tissues could serve as an independent prognostic factor for OS (HR=0.21; 95% CI=0.07-0.63; P=0.0055). The rate of mortality for patients with low HSD17B2 protein expression within cancer tissues was 4.75 times higher than that for patients with high HSD17B2 expression. As well as HSD17B2 protein content, a large tumour size (T3-T4), LCC subtype and R1 residual tumour status were also found to predict a poorer OS in patients with NSCLC in an independent manner (Table V).

Prognostic significance of HSD17B2 expression in the NSCLC patient cohort from the Kaplan-Meier Plotter database. To

Table V. Univariate and multivariate Cox regression analyses for overall survival in patients with non-small cell lung cancer.

	Univariate analysis		Multivariate analysis	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value
HSD17B2 protein level				
Low	1 (Reference)		1 (Reference)	
Intermediate	1.17 (0.63-2.17)	0.62	1.25 (0.67-2.34)	0.48
High	0.18 (0.06-0.51)	0.0012	0.21 (0.07-0.63)	0.0055
Sex				
Male	1 (Reference)			
Female	0.75 (0.43-1.31)	0.31		
Patient age (years)				
≤60	1 (Reference)			
>60	1.08 (0.59-1.96)	0.81		
Histological type				
Adenocarcinoma	1 (Reference)		1 (Reference)	
Squamous cell carcinoma	1.23 (0.69-2.21)	0.48	0.76 (0.41-1.4)	0.38
Large cell carcinoma	3.64 (1.58-8.39)	0.0025	2.49 (1.06-5.84)	0.036
Tumour size				
Tis-T1	1 (Reference)		1 (Reference)	
T2	2.56 (0.99-6.59)	0.051	1.31 (0.49-3.52)	0.59
T3-T4	5.42 (1.98-14.83)	0.001	2.95 (1.03-8.49)	0.044
Lung cancer stage				
0-I	1 (Reference)			
II	1.15 (0.62-2.15)	0.65		
III-IV	1.31 (0.66-2.58)	0.44		
Lymph node metastasis				
N0	1 (Reference)			
N1	1.07 (0.60-1.91)	0.82		
N2	0.68 (0.26-1.74)	0.42		
Lung cancer grade				
G1	1 (Reference)			
G2	1.09 (0.38-3.18)	0.87		
G3	1.07 (0.38-3.05)	0.9		
Smoking				
No	1 (Reference)			
Yes	1.15 (0.38-3.05)	0.75		
Residual tumour status				
R0	1 (Reference)		1 (Reference)	
R1	1.94 (0.91-4.11)	0.085	2.47 (1.12-5.46)	0.025

P-values were calculated using the Cox proportional hazard model. P<0.05 was considered to indicate a statistically significant difference. Variables with a P-value <0.15 in the univariate analysis were included in the multivariate analysis. CI, confidence interval; HR, hazard ratio; HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2.

investigate whether downregulation of *HSD17B2* expression was associated with unfavourable survival in a larger group of patients with NSCLC, Kaplan-Meier Plotter was used to generate survival curves for all available patient cohorts. Since some clinical characteristics of patients are available in the Kaplan-Meier Plotter database, a stratified analysis of *HSD17B2* expression in different subgroups of patients with NSCLC was also performed. This approach allows us to distinguish groups of patients in which *HSD17B2* expression may have prognostic significance. The Kaplan-Meier Plotter database also offers an opportunity to perform multivariate analysis. However, it is important to note that some patients do not have complete clinical information and therefore are not included in such analyses. The total number of cases enrolled

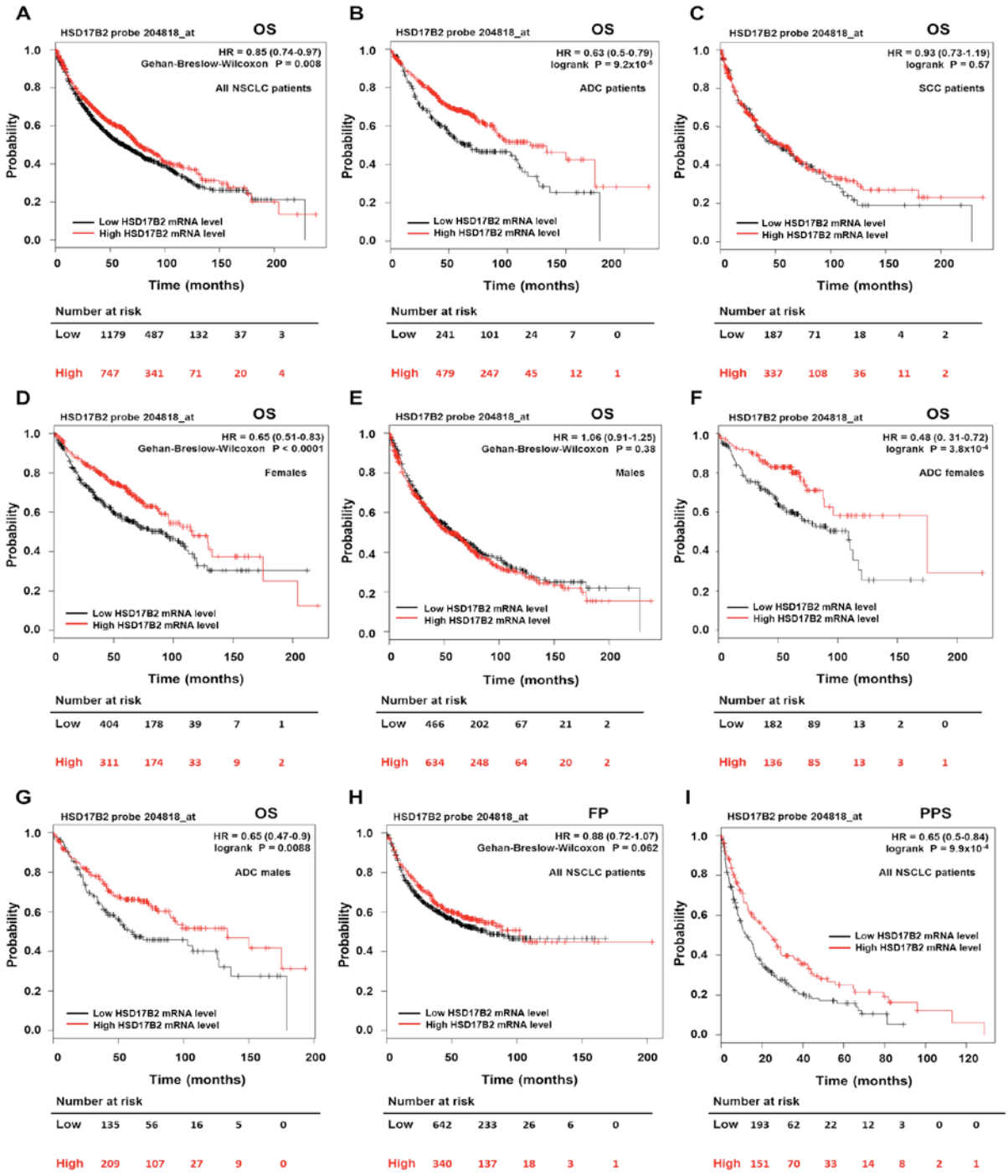

Figure 5. Prognostic value of *HSD17B2* expression in the NSCLC patient cohort from the Kaplan-Meier Plotter online database. OS, FP survival and PPS survival curves demonstrating survival rates of patients with NSCLC and high (red line) or low (black line) *HSD17B2* expression levels. *HSD17B2* expression was categorized into high and low according to the 'Auto select best cutoff' value. The number of patients at risk at specific time (in months) is presented in tables below each graph. (A-G) Kaplan-Meier survival curves showing the OS of patients with NSCLC. (A) Survival curves were plotted for all patients with NSCLC (n=1,926). (B) Survival curves were plotted only for patients with ADC (n=720). (C) Survival curves were plotted only for patients with SCC (n=524). (D) Survival curves were plotted only for female patients (n=715). (E) Survival curves were plotted only for male patients (n=1,100). (F) Survival curves were plotted only for female patients with ADC (n=318). (G) Survival curves were plotted only for male patients with ADC (n=344). (H) Kaplan-Meier survival curves showing FP survival for all patients with NSCLC (n=982). (I) Kaplan-Meier survival curves showing PPS for all patients with NSCLC (n=344). P<0.05 was considered to indicate a statistically significant difference. ADC, adenocarcinoma; FP, first progression; HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; n NSCLC, non-small cell lung cancer; OS, overall survival; PPS, post-progression survival; SCC, squamous cell carcinoma.

in each univariate or multivariate analysis is presented in Table VI. When the group had <50 patients, analysis was not performed. For all survival analyses, patients were divided into two groups, with low and high *HSD17B2* mRNA expression classified according to the 'auto select best cutoff' value.

The results of univariate analysis revealed that high *HSD17B2* expression was significantly associated with a favourable prognosis (HR=0.85; 95% CI=0.74-0.97; P=0.008) in an independent verification cohort of 1,926 patients with NSCLC (Fig. 5A; Table VI). The median OS in the group of

Table VI. Stratified univariate analysis of prognostic significance of *HSD17B2* expression for OS, FP survival and PPS in non-small cell lung cancer patient cohorts from the Kaplan-Meier Plotter database.

	OS			FP			PPS		
Variable	No. of cases	HR (95% CI)	P-value	No. of cases	HR (95% CI)	P-value	No. of cases	HR (95% CI)	P-value
Total no. of patients	1,926	0.85 (0.74-0.97)	0.008[a]	982	0.88 (0.72-1.07)	0.062[a]	344	0.65 (0.5-0.84)	$9.9x10^{-4}$[b]
Histological type									
Adenocarcinoma	720	0.63 (0.5-0.79)	$9.2x10^{-5}$[b]	461	0.76 (0.55-.04)	0.088[b]	125	0.5 (0.3-0.82)	0.0056[b]
Squamous cell carcinoma	524	0.93 (0.73-1.19)	0.57[b]	141	0.76 (0.44-1.29)	0.3[b]	20	-	-
Sex									
Male	1,100	1.06 (0.91-1.25)	0.38[a]	514	1.28 (0.96-1.7)	0.098[b]	179	0.74 (0.5-1.08)	0.078[a]
Female	715	0.65 (0.51-0.83)	<0.0001[a]	468	0.81 (0.6-1.1)	0.17[b]	165	0.46 (0.31-0.69)	$9.1x10^{-5}$[b]
Tumour size									
T1	437	0.87 (0.65-1.17)	0.17[a]	177	0.63 (0.34-1.17)	0.14[b]	61	0.32 (0.16-0.61)	$3.1x10^{-4}$[b]
T2	589	1.09 (0.86-1.39)	0.48[b]	351	1.57 (1.16-2.13)	0.0032[b]	169	0.63 (0.44-0.9)	0.0095[b]
T3	81	0.63 (0.37-1.08)	0.09[b]	21	-	-	17	-	-
T4	46	0.52 (0.25- 1.05)	0.065[b]	7	-	-	5	-	-
Lung cancer stage									
I	577	0.65 (0.49-0.87)	0.0033[b]	325	0.55 (0.35-0.87)	0.0097[b]	78	0.41 (0.21-0.79)	0.0059[b]
II	244	0.74 (0.51-1.09)	0.17[a]	130	0.71 (0.42-1.19)	0.19[b]	58	0.47 (0.24-0.95)	0.03[b]
III	70	0.74 (0.43-1.28)	0.32[a]	19	-	- 10	-	-	
Lymph node metastasis									
N0	781	1.14 (0.92-1.41)	0.23[a]	374	1.72 (1.22-2.43)	0.0019[b]	146	0.5 (0.34-0.75)	$5.4x10^{-4}$[b]
N1	252	0.87 (0.63-1.2)	0.17[a]	130	0.78 (0.49-1.24)	0.26[a]	71	0.57 (0.33-0.99)	0.042[b]
N2	111	0.66 (0.44 - 0.99)	0.05[b]	51	0.81 (0.38-1.72)	0.85[a]	35	-	-
Lung cancer grade									
G1	201	0.83 (0.56-.21)	0.33[b]	140	1.41 (0.91-2.18)	0.12[b]	79	0.65 (0.37-1.15)	0.13[b]
G2	310	1.15 (0.81-1.63)	0.44[b]	165	1.29 (0.81-2.04)	0.29[a]	89	0.67 (0.41-1.1)	0.11[b]
G3	77	0.52 (0.24-1.08)	0.22[a]	51	0.58 (0.24-1.4)	0.22[b]	24	-	-

[a]Gehan-Breslow-Wilcoxon test; [b]log-rank test. P<0.05 was considered to indicate a statistically significant difference. CI, confidence interval; FP, first progression; HR, hazard ratio; HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; OS, overall survival; PPS, post-progression survival.

Table VII. Stratified multivariate analysis of prognostic significance of *HSD17B2* expression for OS, FP survival and PPS in non-small cell lung cancer patient cohorts from the Kaplan-Meier Plotter database.

	OS[a]		FP[b]		PPS[c]	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
HSD17B2 mRNA level	0.73 (0.59-0.9)	0.0031	0.72 (0.49-1.04)	0.082	0.6 (0.39-0.92)	0.019
Histological type	1.45 (1.23-1.7)	<0.0001	0.9 (0.61-1.33)	0.6	1.94 (1.17-3.23)	0.011
Sex	1.34 (1.09-1.66)	0.0059	1.26 (0.91-1.75)	0.17	1.24 (0.79-1.94)	0.35
Lung cancer stage	1.52 (1.33-1.74)	<0.0001	2.25 (1.74-2.91)	<0.0001	1.32 (0.95-1.82)	0.098

[a]890 cases assessed; [b]469 cases assessed; [c]145 cases assessed. P<0.05 was considered to indicate a statistically significant difference. CI, confidence interval; FP, first progression; HR, hazard ratio; HSD17B2, 17-β-hydroxysteroid dehydrogenase type 2; OS, overall survival; PPS, post-progression survival.

patients with high HSD17B2 expression was 76 months, in comparison with 62.3 months in the low expression group. Next, the relationship between *HSD17B2* mRNA levels and clinical outcomes in various subgroups of patients with NSCLC was investigated. The results indicated that high *HSD17B2* expression was significantly associated with a better OS in patients with ADC (HR=0.63; 95% CI=0.5-0.79; $P=9.2x10^{-5}$), whereas it was not associated with OS in patients with SCC (HR=0.93; 95% CI=0.73-1.19; P=0.57) (Fig. 5B and C; Table VI). Furthermore, during the preliminary analysis, it was revealed that high *HSD17B2* expression significantly improved the OS rates in female patients, but not in male patients (Fig. 5D and E; Table VI). Subsequently, sex-stratified analyses were performed in the ADC and SCC patient subgroups; it was observed that high mRNA expression levels of *HSD17B2* predicted better OS in women and men with ADC (Fig. 5F and G), but it had no impact on OS in either women or men with SCC (Fig. S1A and B). Furthermore, high *HSD17B2* expression significantly improved OS rates in patients with stage I LC (Fig. S1C; Table VI). In addition, multivariate Cox regression analysis was performed, which revealed that *HSD17B2* mRNA expression, LC histological type, stage and sex were associated with OS. Notably, high *HSD17B2* expression exerted a protective effect, as it was associated with an improved OS in an independent manner (HR=0.73; 95% CI=0.59-0.9; P=0.0031; Table VII).

No association between *HSD17B2* mRNA expression and FP survival was detected in the entire cohort (Fig. 5H; Table VI). However, when patients were divided according to clinicopathological variables, *HSD17B2* expression influenced FP survival in particular subgroups. Patients diagnosed with stage I LC, presenting high *HSD17B2* transcript levels, had better FP survival times than patients with lower expression (Fig. S1D; Table VI). Unexpectedly, higher *HSD17B2* expression was negatively associated with FP survival in patients with T2 tumour size and no regional lymph node metastases (Fig. S1E and F; Table VI). The multivariate analysis revealed that *HSD17B2* expression had no prognostic impact on FP survival (HR=0.72; 95% CI=0.49-1.04; P=0.082; Table VII).

This study revealed that *HSD17B2* mRNA levels were strongly associated with improved PPS in the whole cohort of patients (HR=0.65; 95% CI=0.5-0.84; $P=9.9x10^{-4}$) as well as in various cohort subsets (Fig. 5I; Table VI). The median PPS in the group of patients with high *HSD17B2* expression was 24.5 months, in comparison with 12.3 months in the low expression group. According to the univariate analysis, low expression of *HSD17B2* was a significant unfavourable prognostic factor in terms of PPS in patients with ADC, in women, in patients diagnosed with early stages of LC (I and II), with T1 and T2 tumour size, and with N0 and N1 lymph node metastasis (Fig. S2A-H; Table VI). Because of the lack of data, we were unable to perform analysis regarding PPS for patients with SCC, advanced LC stages and larger tumour sizes. The prognostic effect of *HSD17B2* expression on PPS remained significant and independent from other risk factors, as determined by multivariate analysis (HR=0.6; 95% CI=0.39-0.92; P=0.019; Table VII).

Discussion

At present, numerous studies have demonstrated that oestrogens may exert an impact on LC development (22,42). It has been shown that NSCLC cells express classical ERs as well as the membrane GPER, and the administration of oestrogens has been reported to activate genomic and non-genomic signalling pathways in these cells (17-21,25,43). Many studies have confirmed that the activation of ERs and GPER by their agonists and oestrogens was associated with enhanced proliferation and migration of human NSCLC cells *in vitro* and *in vivo*, whereas their inhibition or knockdown significantly reduced these events (18,26,27,44). For example, an administration of E2 initiated rapid activation of the p42/p44 MAPK signalling cascade and promoted the phosphorylation of nuclear (steroid) receptor coactivator SRC-3, which may enhance ER transcriptional activity in NSCLC cells, whereas downregulation of ERs by small interfering RNA diminished cancer cell proliferation (18). Furthermore, E2 treatment in lung adenocarcinoma mouse models with expression of oncogenic *K-ras* and concurrent deletion of *Tp53* significantly increased the number of tumours and their volume in male and ovariectomized female mice (44). Fan *et al* (26) indicated that E2 and ERβ agonists increased the protein levels of ERβ and matrix metalloproteinase-2, leading to increased proliferation, migration and invasion of NSCLC cells.

Using an experimental lung metastatic mouse model, it was confirmed that oestrogens enhanced NSCLC aggressiveness, which resulted in an increased number of lung metastatic lesions in the group of mice treated with E2 (26). Previous studies have indicated that both oestrogens and androgens are metabolized within the lung, and can be synthesized locally in lung tumours by various steroidogenic enzymes (30-35). However, all of the mechanisms and pathways that may lead to an exaggerated accumulation of active sex steroids in LC tissue are currently not known.

The present study demonstrated, using three different techniques, that the expression of HSD17B2 was significantly reduced in NSCLC tissues compared with adjacent histopathologically unchanged tissue specimens. Because HSD17B2 inactivates biologically potent steroid hormones and regulates their balance in various tissues, these results may indicate the protective role of this enzyme within the lung. This study complemented and expanded the results of a previous study performed by Verma *et al* (33). This previous study evaluated HSD17B2 protein levels in NSCLC specimens for the first time (33); however, the evaluation was made exclusively using immunohistochemical staining, and the authors did not compare the differences in HSD17B2 mRNA and protein levels between NSCLC tissues and histopathologically unchanged tissue specimens. The present study revealed that downregulation of HSD17B2 expression may be a frequent feature in LC tissues. Similar to Verma *et al* (33), this study detected that the immunoreactivity of HSD17B2 was mostly located in the cytoplasm of cells. In their previous study, higher HSD17B2 immunoreactivity was associated with SCC and adenosquamous cell carcinoma subtypes (33). The present study also observed that among all histological subtypes of NSCLC, the highest amount of *HSD17B2* mRNA was detected in SCC. However, the expression of *HSD17B2* was substantially decreased in all LC subtypes in comparison with adjacent histopathologically unchanged counterparts, and no significant association among subtypes alone was identified.

The present study demonstrated that normal respiratory epithelium and normal glands within the lung were positively stained for HSD17B2 protein. Low HSD17B2 mRNA and protein expression levels in cancer tissues were associated with LC stage, tumour size, lymph node metastasis and LC grading. Notably, in higher grade, poorly differentiated tumours, most often characterized by a poor prognosis, and in advanced NSCLC, the amount of HSD17B2 expression was significantly diminished. Lower expression was also detected in LC tissues from patients with N1 lymph node metastasis; however, in patients with N2, HSD17B2 mRNA and protein expression was higher than that in N1 and comparable with that in N0. Notably, during the evaluation of immunohistochemical staining, it was revealed that the amount of HSD17B2 protein was elevated in apoptotic regions of tumour specimens (data not shown). A recent study by Hilborn *et al* (45) revealed that E2 may regulate *HSD17B2* in breast cancer cells; the long-term exposure to E2 (7 days) resulted in increased *HSD17B2* mRNA levels in the MCF7 cell line. Therefore, it may be possible that decreased expression of *HSD17B2* is crucial during the first steps of NSCLC development, as it could provide a high level of sex steroids, which may support cancer progression. Furthermore, it has been reported that oestrogens stimulated proliferation of preneoplastic parenchymal cells in the lung, suggesting that those hormones may be a driver of LC at early stages of the disease (44). On the other hand, the reactivation of *HSD17B2* expression in NSCLC apoptotic cells after prolonged exposure to elevated levels of active sex steroids may exert a protective role. This process may eliminate an excessive amount of potent E2 and therefore diminish its pro-apoptotic properties, as reported previously (46).

This study also carried out a retrospective analysis to estimate the prognostic significance of *HSD17B2* expression in cancer tissues in patients with NSCLC. To date, to the best of our knowledge, only one study has raised this issue. Verma *et al* (33) reported that patients with a negative HSD17B2 protein status in NSCLC cells had poorer OS than HSD17B2-positive patients. The results based on immunohistochemical analysis revealed that only negative cases possessed prognostic significance because patients with high HSD17B2 protein levels presented a steeper Kaplan-Meier curve than patients with a low amount of HSD17B2 protein (33). In the aforementioned study, multivariate analysis demonstrated that HSD17B2 protein content could not be considered an independent prognostic factor in the investigated group of patients the NSCLC. The present study used a log-rank test to assess the impact of HSD17B2 mRNA and protein levels on the OS of patients. Even though univariate analysis revealed no statistically significant benefits of high HSD17B2 mRNA and protein levels (from western blotting), a clear trend towards longer survival rates in these groups of patients was detected. Because specimens used for western blotting usually represent a mixture of various cells, it was decided that this study would focus on immunohistochemical results considering only cancer cells. The specimens were subdivided into three groups (high, intermediate and low HSD17B2 protein levels); high expression levels of HSD17B2 protein in cancer cells were significantly associated with better OS of patients with NSCLC. The median OS was clearly longer for those patients than for patients with low or intermediate HSD17B2 immunoreactivity. This result indicated the potential value of HSD17B2 as a predictor of survival in patients with NSCLC. Subsequently, multivariate Cox regression analysis was performed, which revealed that a high level of HSD17B2 protein in NSCLC cells was associated with prolonged patient survival. The current study indicated that the HSD17B2 protein expression in cancer cells could serve as a prognostic factor in NSCLC. In addition, stratified survival analysis was performed in an independent cohort of patients with NSCLC from the Kaplan-Meier Plotter database. The analysis clearly demonstrated that patients with higher expression levels of *HSD17B2* presented better OS and PPS. This favourable prognosis was particularly observed in women, patients with ADC of both sexes, and patients with early stages of LC. This online analysis confirmed that HSD17B2 expression may possess a prognostic value concerning OS and PPS, at least in some groups of patients with NSCLC. Unfortunately, no online database that contained data concerning HSD17B2 protein status in patients with NSCLC was found, which could verify the preliminary results. The present study revealed that high protein expression of HSD17B2 in LC tissues of patients was an independent factor associated with favourable OS. However, because of limited follow-up data, we were not able to perform

stratified survival analysis concerning HSD17B2 protein significance in various subgroups of patients, or to assess its association with FP survival or PPS. Thus, the present study is still preliminary, and it postulates that HSD17B2 could be a promising prognostic factor for NSCLC, but further studies are required to confirm its value in this disease.

The disturbed expression of *HSD17B2* and its prognostic significance have also been demonstrated for other types of cancer. This fact emphasizes the role of this enzyme during carcinogenesis. HSD17B2 appears to be an important player during breast cancer development; while an oxidative pathway is preferred in normal breast epithelium, where HSD17B2 activity may protect cells from an excess of E2 (47), in malignant breast tumours, the reductive pathway becomes dominant, with considerably elevated expression of *HSD17B1*, resulting in an elevated E2/E1 ratio (48-50). Gunnarsson *et al* (51) reported that *HSD17B2* expression was lost in breast cancer tissues, particularly in ER-positive tumours, whereas it was detectable in normal mammary gland specimens. Diminished *HSD17B2* transcript levels were correlated with a higher risk of later recurrence in the group of investigated patients. Furthermore, in a later study, the same team revealed that the expression of *HSD17B2* can be a valuable predictor for the prognosis of patients with breast cancer, as its low or absent transcript levels were associated with decreased survival (52). Immunohistochemical staining of HSD17B2 in breast cancer revealed a positive reaction in only 20% of cancer specimens, while 83% of adjacent non-malignant tissues exhibited immunoreactivity (53). In the present work, only 36% of NSCLC specimens demonstrated high HSD17B2 protein content.

Additionally, reduced expression of *HSD17B2* has been associated with advanced stages of urothelial carcinoma and was postulated to be an unfavourable prognostic factor (54). Lower levels of HSD17B2 mRNA and protein were also detected in gastric tumour tissues (55). Conversely, *HSD17B2* expression was significantly upregulated in non-responding patients with colorectal cancer treated with preoperative chemoradiotherapy (56). Its overexpression was associated with a poor prognosis and with an aggressive phenotype of cancer. It is thought that in this type of cancer, E2 may serve a protective role (57). Therefore, in different types of cancer, *HSD17B2* expression is regulated in a different manner, presumably to sustain the best environmental conditions for cancer development.

Notably, the disturbed expression of *HSD17B2*, with the concomitant induction of the expression of reductive *HSD17B* genes, led to an elevated E2/E1 ratio in breast cancer cells and was associated with a poorer outcome in patients (49,50,52). Recently, a relationship between *HSD17B1* and *HSD17B2* expression levels, and an outcome in patients with endometrial cancer has also been established. Concerning *HSD17B1* and *HSD17B2* mRNA levels analysed in combination, patients with tumours exhibiting high HSD17B1 and low HSD17B2 transcript levels had the worst prognosis (58). Previous studies have also revealed that the expression of *HSD17B1* was significantly increased in NSCLC tissues (33,35). In the present work, a substantial decrease in HSD17B2 mRNA and protein levels was detected in NSCLC tissue specimens. Therefore, another important pathway that may contribute to an elevated E2 concentration in the lung tumour milieu has emerged. Therefore, it is important to clarify the exact role played by the HSD17B2 enzyme in the process of sex steroid inactivation in normal lung and LC tissues.

As HSD17B2 catalyses the conversion of T into 4-androstenedione, androstenediol into dehydroepiandrosterone, and dihydrotestosterone into androstanedione (29,36,37), it is inarguably involved in androgen inactivation within the lung. An androgen receptor and the formation of active androgens were detected in NSCLC cells and led to a significant growth response (30,59,60). High plasma levels of T and dihydrotestosterone have also been associated with an increased incidence of LC in men (16). Androgen deprivation therapy, applied after, or before and after, the diagnosis of LC in men, contributed to a greater survival rate among patients (61). Collectively, these reports suggested that androgens are also implicated in LC pathogenesis. The present results indicated an alteration in NSCLC tissues that may maintain a relatively high concentration of not only oestrogens but also androgens, enhancing cancer development.

Smoking remains the most important risk factor for LC, and an increasing number of studies have aimed to investigate the interaction between tobacco exposure and oestrogen signalling in lung tissue. It has been reported that polycyclic aromatic hydrocarbons found in cigarette smoke stimulated the expression of *CYP1B1* in lung tissue (10). As this enzyme possesses an affinity not only for tobacco carcinogens but also for the most potent oestrogen, E2, the reaction of hydroxylation may result in the generation of 2- and 4-catechol oestrogens with their subsequent conversion to quinones. These compounds are able to damage DNA by inducing single-strand breaks, 8-hydroxylation of guanine bases, and may enhance formation of free radicals and DNA adducts (11,12). Huuskonen *et al* (62) revealed that among the HSD17B enzyme family, the expression of HSD17B2 was repressed in the human placenta of smokers (62). However, this study did not find any important relationship between the smoking status of patients and the differences in *HSD17B2* expression in LC specimens. In smokers and non-smokers, the expression of HSD17B2 mRNA and protein was significantly diminished in LC tissues compared with adjacent histopathologically unchanged tissues. However, in the present study, a great majority of patients were smokers, so further investigations are required to determine whether smoking may have an influence on *HSD17B2* expression in NSCLC.

Very little is known about the mechanisms responsible for the regulation of *HSD17B2* gene expression. It has been reported that retinoic acid (RA) increased the transcriptional activity of the *HSD17B2* gene in breast cancer, endometrial cancer cells and in human placental endothelial cells (63-65). RA exerts its effect through RA receptors, which are thought to be tumour suppressors in several types of cancer. RA receptor β was reported to be severely hypermethylated in NSCLC tissues, particularly in smokers (66). In addition, recent evidence suggests that the lack of nuclear RA receptors may serve a critical role during lung carcinogenesis (67). Therefore, alterations in the RA signalling pathway in LC tissues may contribute to the disturbed expression of *HSD17B2*. Furthermore, the *HSD17B2* gene is located at chromosome 16q24.1, and an allelic loss at this region was reported as a frequent event in prostate and breast cancer (68,69). This

allelic deletion was also detected as a common feature in LC (70). Collectively, these mechanisms may be responsible for inactivation of *HSD17B2* expression.

Additionally, previous studies showed that hormone replacement therapy or oral contraceptives may influence the expression level of steroidogenic enzymes (48). Hilborn *et al* (45) investigated the impact of active sex steroids on *HSD17B2* expression in breast cancer cell lines. It was reported that E2 stimulation significantly altered the expression status of *HSD17B2*, but the final effect was dependent on the cell type and time of exposure (45). In the present study, there was a small group of patients who were treated by hormone replacement therapy (n=4), and all women were postmenopausal. Therefore, this study cannot verify how and whether postmenopausal oestrogen therapy may affect *HSD17B2* expression in LC or histopathologically unchanged tissues. Because of the lack of premenopausal women in the patient group, this study was not able to compare *HSD17B2* expression levels in LC as well as in histopathologically unchanged tissues between pre- and postmenopausal women. Further investigations are required to clarify this issue.

In conclusion, this study demonstrated that HSD17B2 transcript and protein levels were significantly reduced in LC tissues compared with in matched, normal specimens. However, this study investigated tissues obtained only from patients suffering from NSCLC and a lack of healthy control patients may be a limitation. It would be helpful and interesting to compare the expression levels of *HSD17B2* in lung tissues from healthy individuals with expression levels in histopathologically unchanged tissues and LC tissues from patients with NSCLC. Further investigations are required to obtain a comprehensive view on this point. The present study also revealed that normal respiratory epithelium and histopathologically unchanged glandular cells exhibited strong cytoplasmic staining for HSD17B2. A strong association between reduced HSD17B2 mRNA and protein expression, and NSCLC stages, tumour size, lymph node metastasis and LC grading was detected in the group of cancer tissues. The majority of the investigated cancer tissue specimens exhibited weak or negative staining with increasing tumour grade. The results from the log-rank test and a multivariate Cox regression model revealed a beneficial effect of high HSD17B2 protein levels in cancer tissues on OS, and indicated that HSD17B2 protein status may be a prognostic factor of OS in patients with NSCLC. Furthermore, online survival analysis using an independent verification cohort revealed statistically significant differences in OS and PPS according to *HSD17B2* mRNA level. The survival time of patients with high *HSD17B2* expression was significantly longer than that of patients with low expression levels. This beneficial effect was particularly evident in patients with ADC of both sexes, and in patients with early stages of LC. The results from multivariate analysis pinpointed HSD17B2 as an independent prognostic factor.

Collectively, decreased expression of HSD17B2 is frequently observed in NSCLC; however, the mechanisms underlying the disturbed expression of HSD17B2 in NSCLC remains unexplained. The restoration of HSD17B2 expression in NSCLC tissues may lead to patient benefits and may be a target for future therapy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HD developed the concept of the project, designed and performed the experiments, analysed the results and wrote the manuscript. DJJ was responsible for the histopathological classification and evaluation of HSD17B2 staining in the investigated specimens. AK performed immunohistochemical staining. BG informed patients about the aim of the research study, collected and analysed clinical specimens, and collected all clinical data. WD and PPJ participated in design and coordination of the study, were involved in statistical analysis of the obtained results and were responsible for revision of the manuscript for substantive and methodological correctness. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Local Ethical Committee of Poznan University of Medical Sciences. All procedures performed in this study were in accordance with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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