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Profiling of Aldehyde Dehydrogenase Isoforms in *In Vitro* Formed Tumorspheres

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Abstract. *Background/Aim: Aldehyde dehydrogenases (ALDHs) are considered as markers for normal and cancer stem cells (CSC) and are involved in cell metabolism, proliferation, differentiation, stemness, and retinoic acid (RA) biosynthesis. The aim of the present study was to identify the ALDH isoforms that are associated with the CSC phenotype in non-small cell lung and hepatocellular carcinomas. Materials and Methods: We utilized lung (A549) and hepatocellular (HepG2) cancer cells and generated tumor spheres to isolate the CSC sub-population. Results: The CSC enrichment was confirmed by the up-regulation of various CSC-related genes. Comparative qPCR analysis indicated the up-regulation of several ALDH isoforms in A549 and HepG2 spheres. Interestingly, cyclin D1 and Akt, down-stream targets of the RA signaling pathway, were also shown to be significantly up-regulated in both sphere populations. Conclusion: Specific ALDH isoforms appear to be important mediators for the acquisition of an CSC phenotype and thus, are potential promising targets for CSC-based therapeutic approaches in lung and hepatocellular carcinomas.*

Lung and liver cancer are common malignancies worldwide (1). Over the past years, the propagation of cancer and tumor heterogeneity have been explained by two different theories: the clonal/stochastic model and the hierarchical/cancer stem cell (CSC) model (2). CSCs, also known as tumor initiating cells, are characterized by a set of stem-like properties, such as self-renewal, differentiation, cell survival and asymmetric cell division (3, 4). They are crucial for tumor heterogeneity due to their ability to differentiate into multiple cell types, while they emerge as crucial therapeutic targets considering their enhanced chemo- and radio-resistance and their association with cancer initiation, progression and metastasis (4-6).

Aldehyde dehydrogenases (ALDHs) belong to a superfamily of multifunctional NAD(P)+-dependent enzymes, which catalyze the oxidation of endogenous (lipids, amino acids, vitamins) and exogenous (ethanol, drugs) aldehydes to their corresponding carboxylic acids (7). Nineteen [19] ALDH genes have been characterized and organized into 11 families and 4 sub-families; they encode enzymes that exhibit different cellular localization, substrate specificity, tissue distribution, and expression pattern (7). Although, ALDH activity has been referred as a marker for CSC populations (7), there is growing interest about the specific ALDH isoforms that are markers of CSCs (8, 9). More specifically, ALDHs up-regulation and/or high activity have been associated with tumorgenicity, clonogenicity, cell proliferation, invasion, chemo- and radio-resistance as well as hypoxia (10-14). In this study, we examined which specific ALDH isoforms correlate with sphere phenotype in lung and hepatocellular carcinoma.

Materials and Methods

Cell culture. A549 and HepG2 cells (ATCC) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM)

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Key Words: ALDH, ALDH3A1, cancer stem cells (CSCs), CSC markers, A549, HepG2.

Table I. *Primers used for the real-time PCR comparative quantification.*

Gene	Forward primer	Reverse primer
β-actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTTCC
EpCAM	TTATGATCCTGACTGCGATGAGA	GGTGCCGTTGCACTGCTT
ABCG2	ACCTGAAGGCATTTACTGAA	TCTTTCCTTGCAGCTAAGAC
SOX2	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTTCC
SOX4	CTGCGCCTCAAGCACATG	TTCTTCCTGGGCCGGTACT
JAG1	TGAAGTAGAAGAGGACGACATGGA	CGGCTGCTTGGCAAACC
NOTCH1	GCACCTCAGCCTGCACAGT	CTGTGTTGCTGGAGCATCTTCT
β-catenin	AAGGCTACTGTTGGATTGATTCT	CCCTGCTCACGCAAAGGT
OCT4	CGACCATCTGCCGCTTTG	GCCGCAGCTTACACATGTTCT
CXCR4	GGCCGACCTCCTCTTTGTC	TTGCCACGGCATCAACTG
CD49f	GATCCCGGCCTGTGATTAATATT	CTGGCGGAGGTCAATTCTGT
ALDH1A1	CAAGATCCAGGGCCGTACAA	CAGTGCAGGCCCTATCTTCC
ALDH1A3	CAGCCTCCAGGGTGTTCGT	CGCTCCGCCTGACAAACT
ALDH1B1	AGCCTCTGTTCAAGTTCAAG	CCTTAAAGCCTCCGAATGG
ALDH2	ATTTCCCGCTCCTGATGCAA	GGCCAAATCCAGGCACAATG
ALDH3A1	CAGCGGCATGGGATCCTA	GCGGCGGTGAGAGAAAGTC
ALDH3A2	TCAGCCACTGATAGGAGCCA	TCCGTGGTTTCCTCAACACC
ALDH3B1	TTCACCCCCACCCTAACTATCT	GCGTCAGGTTCAGCGGATA
ALDH4A1	CCTGCCTTGTGGGAGAAGAAA	TGCGTGAAGGCTAAGACGG
ALDH5A1	AGGGTCTCTATGGAGCTGGG	GCCTCGGCGAATGCTTTTAC
ALDH6A1	GGAACAAGGGAAGACCCTAGC	GTTCAGGCCAATGAAGGGGT
ALDH7A1	CCCTGTGGCAGTGTATGGTT	GCTCCACCACAAGTCAAGGA
ALDH9A1	GCAGCGTTGACTTGAGCAC	CAGCCAAGCCCGCATAATAC
ALDH16A1	GTGTCGCTCTTCGGACCTC	TCACATAGTGGCCCAAGCAC
ALDH18A1	GACCTGCAGGGGGTAAATGT	GTCCGTCAACAGATCAGCCA
c-MYC	TACAACACCCGAGCAAGGAC	AGCTAACGTTGAGGGGCATC
Cyclin D1	GAAGGAGACCATCCCCCTGA	GAAATCGTGCGGGGTCATTG
Akt	CAGGAGGTTTTTGGGCTTGC	TGTACTCCCCTCGTTTGTGC

(Biosera, Nuaille, France) supplemented with 10% fetal bovine serum (FBS) (Biosera), 100 units/ml penicillin (Biosera) and 100 μg/ml streptomycin (Biosera). Cells were cultivated at 37˚C with 5% CO_2 in a humidified incubator.

Sphere formation assay. Sphere formation assay was performed as described previously with minor modifications (15). In brief, A549 and HepG2 cells were enzymatically-dissociated in trypsin/EDTA (1X), washed with PBS (1X) and then seeded at clonal density (10 cells/μl) in a serum-free medium (DMEM/F-12; 3:1 mixture), containing 1X B-27 supplement (ThermoFisher Scientific, Waltham, MA, USA), 0.4% (w/v) BSA (Biosera), 10 ng/ml recombinant EGF (ThermoFisher Scientific) and 5 μg/ml insulin (Sigma-Aldrich, Burlington, MA, USA) in T75 flasks (SPL Life Sciences, Gyeonggi-do, Republic of Korea). The culture medium was changed every 2-3 days and spheres were formed in 8-12 days. First generation spheres were used in all subsequent experiments.

Real-time PCR. Total RNA was extracted from cultured cells using NucleoZOL reagent (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. For cDNA synthesis, 2 μg of total RNA and SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Waltham, MA, USA) were used according to the manufacturer's instructions. For Real-time PCR, KAPA SYBR Fast Master Mix (Kapa Biosystems, Basel, Switzerland) was conducted according to the manufacturer's instructions. Reactions were performed by using an Applied Biosystems Step One instrument. Primers were designed with the Primer Express 3.0 software (Applied Biosystems) and the sequences of these primers are presented in Table I. Reactions were run in triplicate in three independent experiments. Gene expression was normalized to *β-actin* using the $2^{-\Delta\Delta CT}$ method.

Table II. *ALDH activity in A549 and HepG2 cells.*

	nmol NAD(P)H produced/min/mg of protein	
Substrate	A549*	HepG2*
Benzaldehyde	278.18±12.32	Negligible
Acetaldehyde	26.19±4.98	Negligible
Propionaldehyde	94.34±1.91	Negligible

*Results are shown as mean±S.D. of three independent experiments.

Protein extraction, cell lysates preparation and western immunoblotting. Western immunoblotting was performed as described previously (15). Protein concentration was determined by using the Pierce™ BCA Protein Assay kit (ThermoFisher Scientific), according to the manufacturer's instructions. Polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA) were incubated overnight at 4˚C with primary antibodies at different dilutions: anti-ALDH3A1 (1:500)

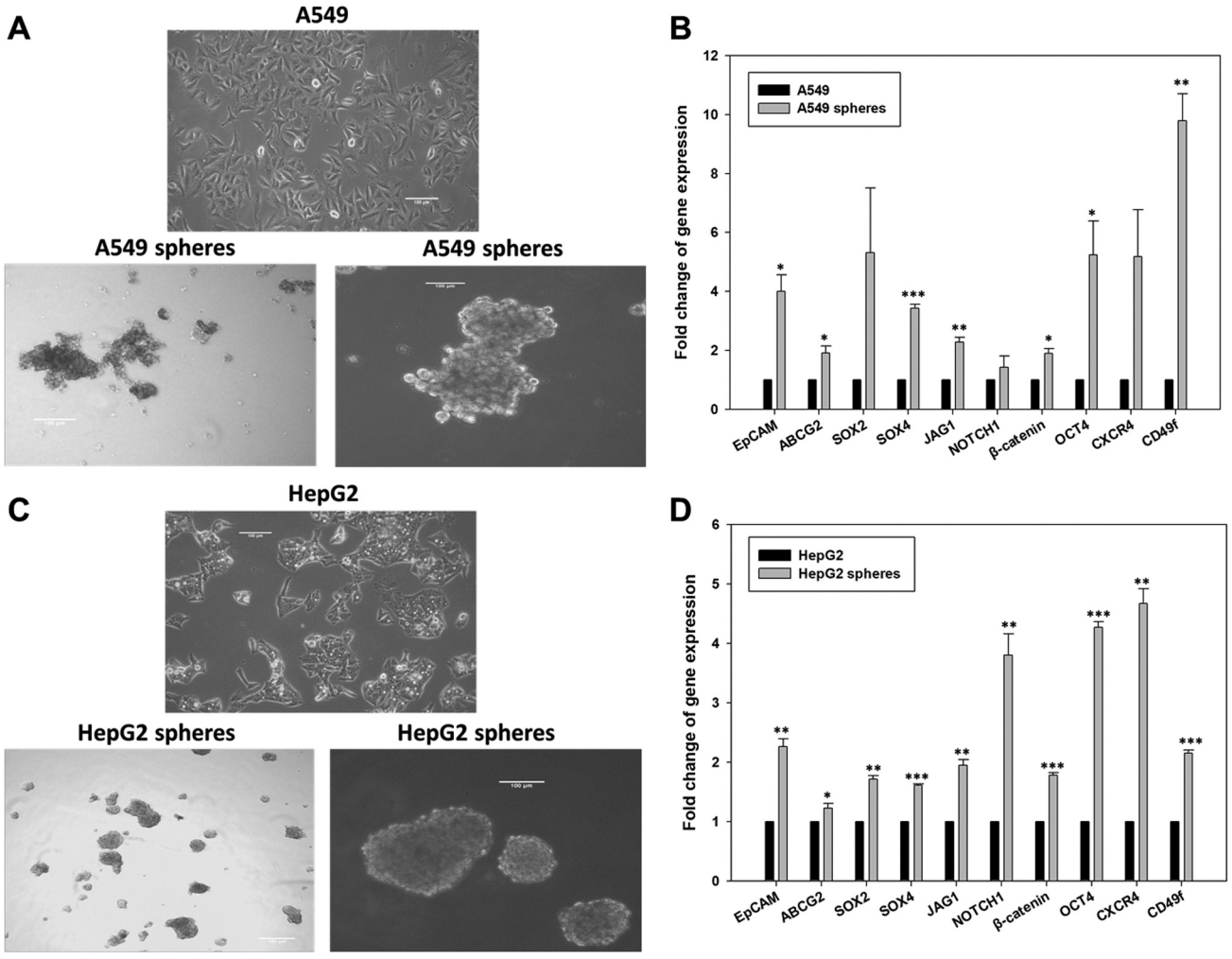

Figure 1. *Generation of A549 and HepG2 spheres and evaluation of their CSC phenotype. A549 (A) and HepG2 (C) cells were seeded at clonal density (10 cells/μl) in serum-free medium in T75 flasks. After 8-10 days, the size of the formed spheres was ≥50 μm. Total RNA from A549 (B), HepG2 (D) and their spheres was extracted and mRNA expression levels of various CSC markers were analyzed by qPCR (comparative quantification ΔΔCt method). Results are shown as mean±S.D. of three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001.*

(Abgent, San Diego, CA, USA) and anti-β actin (1:5,000) (Novus Biologicals, Building IVCentennial, CO, USA) and then with secondary horseradish peroxidase conjugated goat anti-rabbit and anti-mouse antibodies (Millipore) at room temperature. Immunoblot bands were detected by using Chemidoc MD Imaging System (Biorad, Hercules, CA, USA) and β-actin was used as loading control.

ALDH enzymatic activity. The enzymatic activity of ALDHs was estimated as described previously (11). In brief, a reaction mixture containing 75 mM sodium pyrophosphate pH 8.0, 1 mM pyrazole, 1 mM NAD^+ or 2.5 mM $NADP^+$ and 50 μl of cell lysates was prepared and used as a blank. The reaction was initiated by adding 100 μl of different substrates each time (1 mM acetaldehyde, 1 mM propionaldehyde or 0.5 mM benzaldehyde). ALDH enzymatic activity was determined by monitoring NAD(P)H production for 5 min at room temperature.

Statistical analysis. At least three independent experiments were performed. All values are expressed as the mean±S.D. GraphPad Prism software (version 8.3.0) was used for all statistical analyses. Comparison of results between two groups was performed by one sample Student's t test. A value of $p<0.05$ was considered to indicate statistically significant differences.

Results

Isolation of A549 and HepG2 spheres and CSC phenotype identification. A549 and HepG2 cells exhibited high and low ALDH activity, respectively (Table II). A549 and HepG2 spheres were generated to study the correlation of specific ALDH isoforms with the CSC phenotype. Spheres were typically formed in 8-12 days, when the size of each sphere was at least

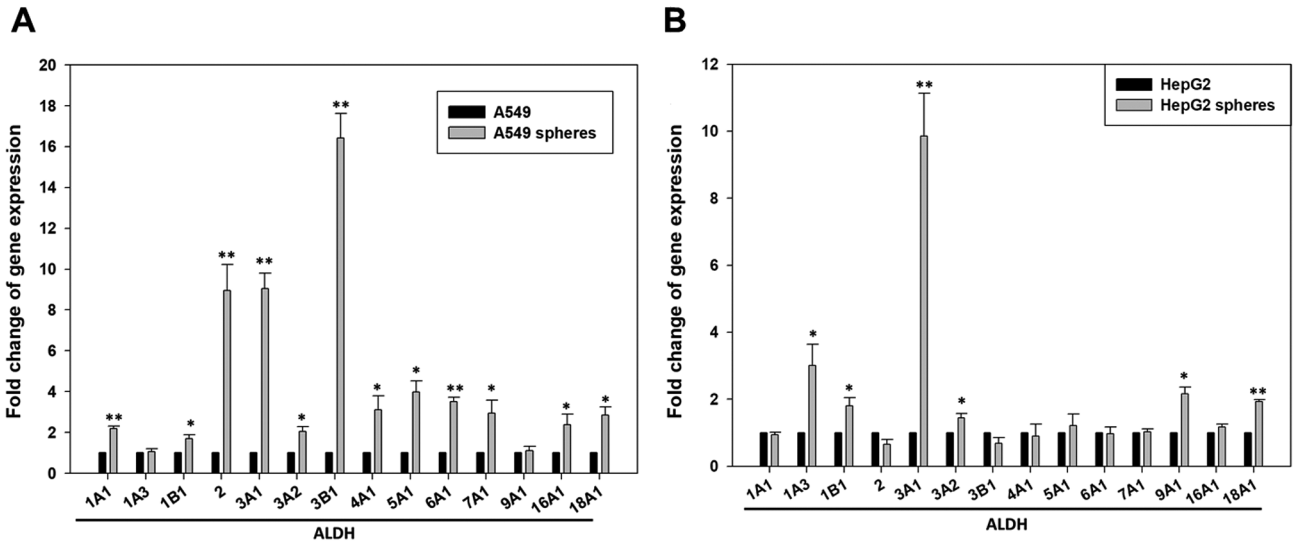

Figure 2. *Up-regulation of the mRNA levels of of specific ALDH isoforms in lung and hepatoma CSCs. Total RNA from A549 (A), HepG2 (B) and their spheres was extracted and the mRNA expression levels of 14 human ALDH isoforms were evaluated by qPCR (comparative quantification ΔΔCt method). The expression levels of the examined genes were normalized to those of β-actin, while the A549 and HepG2 cells were used as reference samples. Results are shown as mean±S.D. of three independent experiments. *p≤0.05, **p≤0.01.*

≥50 μm (Figures 1A and 1C). To confirm the CSC phenotype of the isolated-spheres, we evaluated the transcriptional expression levels of various CSC markers including *EpCAM*, *ABCG2*, *SOX2*, *SOX4*, *JAG1*, *NOTCH1*, *β-catenin*, *OCT4*, *CXCR4* and *CD49f*. All these markers were up-regulated in A549 and HepG2 spheres (when compared to the differentiated cells) in a statistically significant manner thereby demonstrating successful isolation of the CSC population (Figures 1B and 1D).

Differential up-regulation of ALDH isoforms in A549 HepG2 sphere populations. We assessed the mRNA expression levels of 14 different human ALDH genes in A549 and HepG2 spheres. The mRNA levels of 12 out of the 14 ALDH genes were up-regulated in A549 spheres, while 5 ALDHs were induced in HepG2 spheres. *ALDH3B1* demonstrated the highest up-regulation (>16-fold) in A549 spheres. The mRNA levels of *ALDH3A1* were >9- ($p \leq 0.01$) and >9.5-fold ($p \leq 0.01$) higher in spheres than in A549 and HepG2 cells, respectively (Figure 2). Other ALDH isoforms that were up-regulated in A549 CSC populations included *ALDH2* (8.9-fold), *ALDH5A1* (4.0-fold), *ALDH6A1* (3.5-fold), *ALDH4A1* (3.1-fold), *ALDH7A1* (2.9-fold), *ALDH18A1* (2.8-fold), *ALDH16A1* (2.3-fold), and *ALDH1A1* (2.1-fold). In the case of HepG2 CSC populations, the up-regulated ALDH isoforms were *ALDH1A3* (3-fold), *ALDH9A1* (2.1-fold), *ALDH18A1* (1.9-fold), and ALDH1B1 (1.8-fold).

ALDH3A1 protein is up-regulated in A549 and HepG2 sphere cultures. Next, we focused on the ALDH3A1 isoenzyme that was up-regulated in both A549 and HepG2 sphere populations and further assessed its protein levels. ALDH3A1 protein expression levels were significantly up-regulated in both A549 (>12.9-fold higher) and HepG2 (>1.7-fold higher) spheres compared to A549/HepG2 cells (Figures 3A and 3D). This expression pattern was consistent with the up-regulation of ALDH enzymatic activity levels in A549 cells (as measured by the utilization of different aldehyde substrates like benzaldehyde, propionaldehyde and acetaldehyde) (Figure 3E). Moreover, benzaldehyde is routinely used to determine ALDH3A1 activity and distinguish it from the activity of ALDH1/2 isozymes where propionaldehyde and/or acetaldehyde substrates are used (16).

ALDH1A isoforms are related to the up-regulation of downstream regulators of RA signaling. The observed up-regulation of ALDH1A in both A549 and HepG2 spheres, prompted us to further examine the gene expression profile of specific downstream targets of RA signaling. Our results indicated that *Cyclin D1* and *Akt* were significantly up-regulated (>1.5-fold) in both A549 and HepG2 spheres in comparison with the differentiated cells (Figures 4A and 4B).

Discussion

ALDHs are multifunctional enzymes, which play important roles in cellular and metabolic processes such as differentiation, embryogenesis, development, RA biosynthesis and tumor initiation and progression (17).

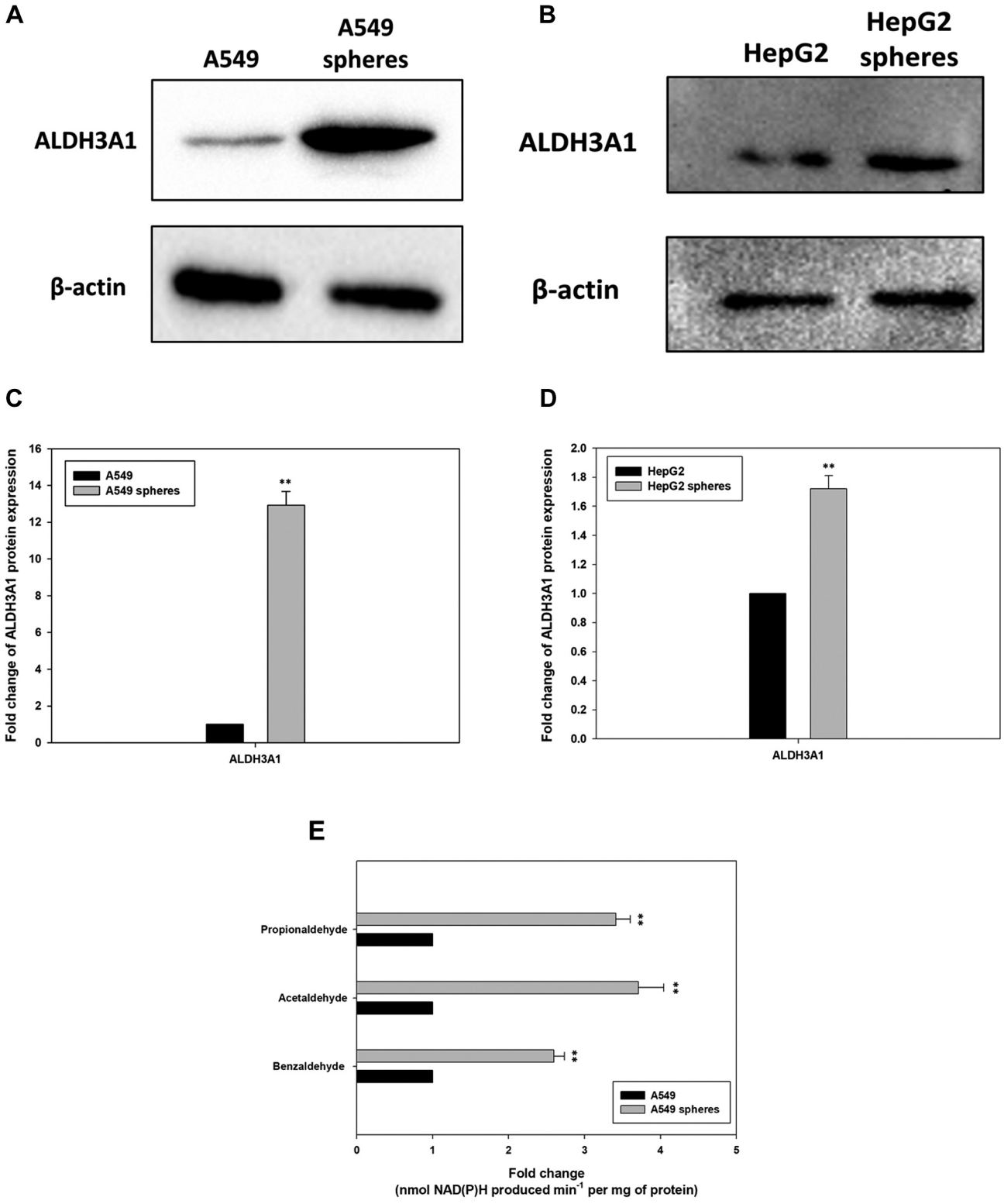

Figure 3. *ALDH3A1 is up-regulated in lung and hepatocellular CSC. A549 (A, C), HepG2 (B, D) and their spheres were lysed and ALDH3A1 protein levels were assessed by western blot. β-actin was used as loading control. (E) Evaluation of ALDH enzymatic activity in A549 cells and A549 spheres by using different aldehyde substrates (benzaldehyde, acetaldehyde and propionaldehyde). Results are shown as mean±S.D. of three independent experiments. **p≤0.01.*

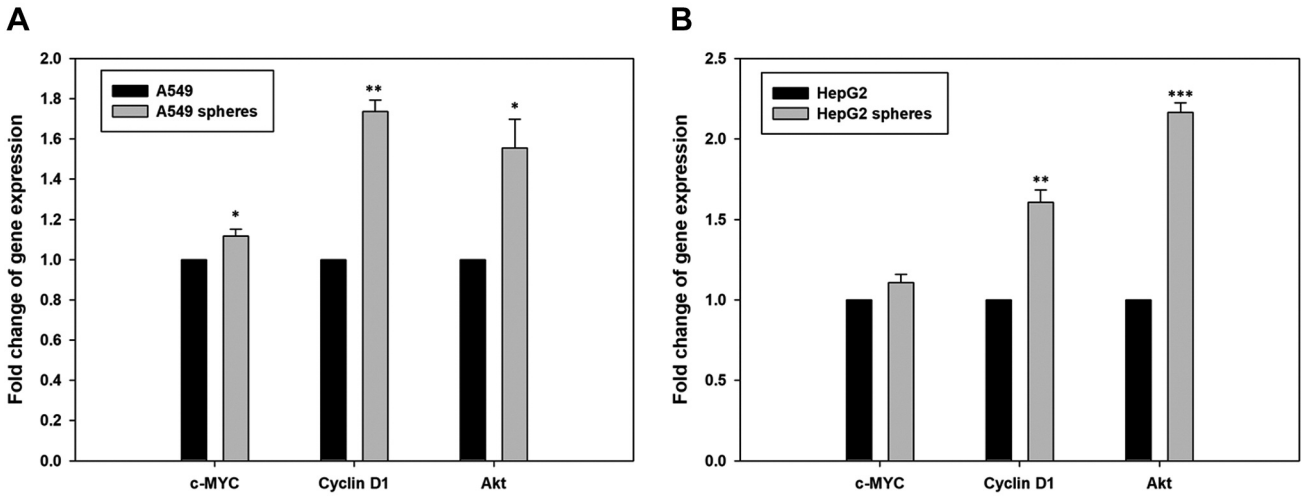

Figure 4. *Up-regulation of downstream RA signaling regulators in A549 and HepG2 spheres. Total RNA from A549 (A), HepG2 (B) and their spheres was extracted and the mRNA levels of downstream target genes of the RA signaling pathway were evaluated by qPCR (comparative quantification ΔΔCt method). The expression levels of the examined genes were normalized to those of β-actin, while the A549 and HepG2 cells were used as reference samples. Results are shown as mean±S.D. of three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001.*

Increased ALDH activity has been widely referred as a hallmark of the CSC phenotype (7); however, recent reports suggest that specific ALDH isoforms are responsible for the CSC-related ALDH activity (9). The main scope of our study was to investigate the specific ALDH isoforms that are related to lung and hepatocellular CSC populations.

Specific molecules of stem cell-related signaling pathways, such as Hedgehog, Wnt/β-catenin, and Notch can be used as biomarkers for the identification of the CSC populations (18, 19). Increased expression levels of specific CSC markers confer the CSC phenotype (20, 21). Recent studies revealed that A549 or HepG2 generated spheres express higher mRNA levels of *OCT4, CD133, NANOG* and *SOX2* transcription factors compared to differentiated cells (20, 21).

CSC identification is crucial in evaluating tumor aggressiveness and therefore could potentially be utilized as a prognostic index in cancer patients (22). Importantly, the identification of specific ALDH isoforms in various malignancies can improve their potential prognostic utilization (9). ALDH1 is associated with RA signaling pathway, which has a regulatory role in CSC differentiation (7).

ALDH3A1 is a homodimeric protein that detoxifies hexanal, octanal, benzaldehyde and 4-hydroxy-nonenal (4-HNE) and has been characterized as a corneal crystalline (16) with chaperone-like function (23). Recent studies have demonstrated that ALDH3A1 is associated with melanoma and lung tumor stemness and an aggressive mesenchyme and immunosuppressive profile (24). In this study, we showed that ALDH3A1 transcriptional and translational expression levels were up-regulated in A549 and HepG2 spheres (Figure 2 and Figure 3). Along these lines, Yan *et al*. demonstrated that ALDH3A1 is highly expressed on DU145 spheres and therefore its expression strong correlates with prostate CSCs and tumorigenesis (12). Wu *et al*. also showed the association of ALDH3A1 with gastric tumorigenesis and gastric CSCs by indicating its up-regulation on MKN-45 and SGC-7901 $ALDH^{bright}$ cells (13). Similarly, a recent study indicated that ALDH1A2, ALDH1L1 and ALDH3B2 were all up-regulated in NBTT2D-, NBTT1- and NBTT3-formed spheres, but only ALDH1A2 was considered as a neuroblastoma prognostic factor (25). Furthermore, ALDH2 enzymatic activity was correlated with aldefluor-positive cells in breast cancer samples (9) and hematopoietic stem cells (26). Finally, ALDH-mediated RA signaling pathway appeared to be critical for CSC self-renewal and differentiation (27).

The ALDH1A and ALDH8A1 subfamilies are well-known retinal dehydrogenases that metabolize retinal to RA, which then binds to RA response elements (RARE) and activates the transcription of genes critical for tumor growth, cell proliferation, stemness and apoptosis (7). Our data demonstrated elevated mRNA levels of *cyclin D1* and *Akt* in both A549 and HepG2 spheres. Cyclin D1 is a proto-oncogene, which regulates G1 to S cell cycle phase and it is important for tumor development and progression (28). Akt is a serine/threonine protein kinase that mediates various cellular processes such as cell survival, growth and metabolism, cell cycle, apoptosis and angiogenesis (29).

In conclusion, the characterization of specific CSC markers is important for the identification of CSC populations and the evaluation of tumor aggressiveness. Our results suggest that

specific ALDH isozymes play important roles in the acquisition of cancer stem-like traits, in lung and hepatocellular carcinomas, and may prove to be potentially useful targets for the development of CSC-based therapeutic approaches.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, I.T. and A.P.; methodology, I.T., G-P.V., and A.P.; validation, I.T.; formal analysis, I.T., and A.P.; investigation, I.T, G-P.V., A.G., M.I.K., M.I.P. and A.P.; resources, A.G., M.I.K., M.I.P and AP.; data curation, I.T.; writing – original draft preparation, I.T.; writing – review and editing, G-P.V., A.G., M.I.K., M.I.P. and A.P.; visualization, I.T; supervision, G-P.V., A.G., M.I.K and A.P.; project administration, A.P.; funding acquisition, A.P. All Authors have read and agreed to the published version of the manuscript.

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