Studies on Expression of Aldehyde Dehydrogenase in Normal and Cancerous Tissues of Thyroids

Running Head: ALDH in Thyroids

Tomomi Kurashige[1], Mika Shimamura[1], Kazuaki Yasui[1], Norisato Mitsutake[2], Michiko Matsuse[2], Masahiro Nakashima[3], Shigeki Minami[4], Susumu Eguchi[4], Yuji Nagayama[1]

Departments of [1]Molecular Medicine, [2]Radiation Medical Science and [3]Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, 852-8523 Japan; [4]Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501 Japan

Corresponding author: Yuji Nagayama, M.D., Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523 Japan (TEL) 81+95-819-7173 (FAX) 81+95-819-7175 (E-mail) nagayama@nagasaki-u.ac.jp

Abstract

Recently published articles have reported the controversial data regarding expression of aldehyde dehydrogenase isozyme 1A1 (ALDH1A1), a potential candidate marker for normal and cancer stem cells (CSCs), in thyroid tissues. These data prompted us to re-evaluate expression of ALDH1A1 in normal and cancerous thyroid tissues by two different means. The first method was immunohistochemistry with two different anti-ALDH1A1 antibodies from distinct companies. Following validating the integrity of these two antibodies by western blotting with ALDH-expressing and -non-expressing cancer cell lines and immunohistochemistry with breast and colon tissues, we here demonstrated significant and comparable expression of ALDH1A1 in both normal and cancerous thyroid tissues with both antibodies. Next, relative expression levels of ALDH isozymes were evaluated by reverse transcription-polymerase chain reaction (RT-PCR), revealing that ALDH1A1 was the most highly expressed isozyme followed by ALDH9A1 and relative expression patterns of isozymes were very similar in normal and cancerous tissues. All these data demonstrate that thyroid cells of normal and cancer origins *do* express ALDH1A1 and to a lesser extent 9A1. Further study will be necessary to study functional significance of ALDH1A1 in the function and behaviors of thyroid normal and cancer stem cells.

Key words - stem cells; immunohistochemistry; thyroid cancer

Introduction

It has recently become apparent that a small subpopulation of cancer cells, called cancer stem cells (CSCs) or cancer initiating cells, are crucial not only for the initiation, but also for the propagation, recurrence and metastasis of cancers [1] . Enormous efforts have so far been made to identify CSC's biological markers, of which aldehyde dehydrogenase (ALDH) appears to be highly promising [2]. Indeed Todaro *et al*. [3] have recently reported the usefulness of ALDH1A1 as a CSC marker for thyroid cancers. In their work, ALDH1A1-positive thyroid cancer cells directly isolated from cancer tissues were shown to be capable of forming spheres *in vitro*, a biological marker for CSCs, and tumors *in vivo*. Importantly, it has also been demonstrated by immunohistochemistry that normal thyroid cells rarely express ALDH1A1, but thyroid cancers express higher levels of ALDH1A1 as they dedifferentiate (3 ± 1.2 % in follicular thyroid cancers, 7 ± 1.8 % in papillary thyroid cancers (PTC) and 16 ± 4 % in undifferentiated thyroid cancers).   In contrast, however, another report by Deng *et al*. [4] has shown the high levels of ALDH1A1 expression in virtually all the normal thyroid cells using the same antibody (BD Biosciences, clone #44/ALDH) and the same method. They thus proposed that ALDH1A1 should not be used as a CSC marker in tissues that normally express a high level of ALDH1A1 including thyroids.

In the present study, we therefore re-evaluated expression of ALDH1A1 by immunohistochemistry, and, because there are 19 ALDH isozymes in humans [2,5], also examined relative expression levels of each ALDH isozymes by reverse transcription-polymerase chain reaction (RT-PCR), in normal and cancerous thyroid tissues. We here show that normal and cancerous thyroid tissues clearly express the significant and comparable levels of ALDH with ALDH1A1 isozyme being dominant (and to a lesser degree ALDH9A1).

Methods

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues from 18 pairs of normal thyroid and PTC

tissues and three pairs of normal and cancerous tissues of colon and breast were obtained from

the surgical pathology archives of Department of Tumor and Diagnostic Pathology, Nagasaki

University, Japan. All experiments were performed after obtaining approval by the Ethical

Committee of Nagasaki University Hospital. The tissues were cut into 4-μm-thick sections,

deparaffinized and subjected to antigen retrieval by microwave treatment in citrate buffer.

Following blockade of endogenous peroxidase with 0.3 % hydrogen peroxide in methanol for

20 min, the tissue sections were incubated with the first antibodies of mouse anti-ALDH1A1

antibody (#44/ALDH, BD Biosciences, San Jose, CA, USA; diluted at 1:1,500 to 6,000 or

sc-374149, Santa Cruz (SC) Biotechnology Inc., Santa Cruz, CA, USA; diluted at 1:1,500 to

10,000). The reaction products were visualized by the avidin-biotin-peroxidase technique using

a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and liquid DAB+

Substrate (K3468, Dako Corporation, Carpinteria, CA, USA). Finally, the sections were

counterstained with hematoxylin and mounted. The staining was quantified by scoring the

percentage of ALDH1A1-positive cells; score 4 indicates that >75% cells express ALDH1A1,

score 3 51-75%, score 2 25-50% and score 1 <25%.

Real-Time Quantitative RT-PCR

Ten pairs of frozen PTC and adjacent normal tissue samples were used. Again, all

experiments were performed after obtaining approval by the Ethical Committee of Nagasaki

4

University Hospital. Written informed consent was obtained from each individual. Total RNA was extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan). Complementary DNA was synthesized from 500 ng of total RNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in the presence of random hexamers according to the protocol recommended by the manufacturer. Quantitative PCR was then carried out in a Thermal Cycler Dice Real-time system (TaKaRa Bio, Tokyo, Japan) using SYBR Premix Ex Taq II (Takara). The sequences of primer pairs used were described in the Supporting information. The cycle threshold values, which were determined using second derivative, were used to calculate the normalized expression of the indicated mRNAs using Q-Gene software using β-actin for normalization.   The PCR product sizes were also confirmed with 2% agarose gel electrophoresis.

Western Blotting

Total cell lysates were isolated from a human hepatoma cell line HepG2 and a human PTC cell line TPC-1. Fifty μg proteins were used for immunoblotting. The signal was developed with two anti-ALDH1A1 antibodies mentioned above and horse radish peroxidase ABC method (Vectastain ABC kit). To confirm equal loading, membranes prepared in parallel were probed with mouse anti-β-actin antibody (sc-47778, SC) diluted at 1:1,000.

ALDEFLUOR assay

ALDH activity was measured with an ALDEFLUOR assay kit (Aldagen/Cytomedix Inc. Gaithersburg, MD, USA) according to the manufacturer's protocol; labeling was performed for 30 min. Diethylaminobenzaldehyde (DEAB), an inhibitor of ALDH activity, was used as a negative control. The labeled cells were analyzed using a FACS Vantage SE with a ClonCyt

option (BD Biosciences).   Data analysis was done with FlowJo software (Tree Star, Ashland, OR, USA).

Results

Two anti-ALDH1A1 monoclonal antibodies were used in this study. One is an anti-ALDH1A1 monoclonal antibody from BD (#44/ALDH), used by Todaro *et al*. [3] and Deng *et al*. [4]. The antigen used to isolate this antibody was amino acids 7-128 of human ALDH1A1. The other is a monoclonal antibody from SC (sc-374149) whose epitope is mapped to amino acids 105-137.

To validate the integrity of these two antibodies, we first used two cancer cell lines HepG2 and TPC. The former was positive, but the latter was negative, for ALDH expression as demonstrated by ALDOFLUOR assay and RT-PCR (Fig. 1A and B). As shown in Fig. 1C, the appropriate sizes of ALDH1A1 protein (~55 kDa) were visualized in HepG2, but not in TPC, cells with both antibodies. The next experiments, that is, immunohistochemical analysis of ALDH1A1 expression with these two antibodies in formalin-fixed, paraffin-embedded colon and breast tissues, were performed to determine the specificities and appropriate dilutions of each antibody. In normal parts of these tissues, putative localization sites of ALDH-positive stem/progenitor cells are known [4]. Among the different dilutions of antibodies (see Methods), BD antibody at a 1:1,500 dilution and SC antibody at a 1:10,000 dilution gave the best staining of normal stem cells in these tissues. The representative pictures are shown in Fig.1 D. Cancerous lesions of these tissues also expressed variable levels of ALDH1A1 as reported in the previous report [4] (Fig.1 D). No staining was confirmed by omitting addition of the first antibodies (data not shown). Altogether, these data clearly show that both antibodies react well with ALDH1A1 protein.

Eighteen pairs of formalin-fixed, paraffin-embedded normal and cancerous thyroid tissues

were then subjected to immunohistochemical analysis. The aforementioned dilutions of each antibody were used in these experiments. As shown in Fig. 2A and B, very similar results were obtained with two antibodies. Thus, over 75 % cells were positive for ALDH1A1 staining in approximately two thirds of normal tissues, and 50-75 % positivity in the rest of tissues. The similar staining pattern was also observed in cancerous tissues despite totally negative staining in a few samples. The staining scores were not significantly different between cancerous and normal tissues [3.8 + 0.1 (mean $\pm$ SE) in cancers and 3.2 $\pm$ 0.3 in normal tissues with BD antibody and 3.2 $\pm$ 0.3 in cancers and 3.7 $\pm$ 0.1 in normal tissues with SC antibody]. The pictures of representative staining were shown in Fig. 2A.

As mentioned earlier, 19 different isozymes have so far been identified in human ALDH [2,5]. Therefore, expression of all these isozymes was quantified by RT-PCR in 10 pairs of normal and cancerous thyroid tissues. Fig. 3 shows the average expression levels in 10 tissues (Fig. 3B) and also those in the representative three cases (Fig. 3A). Our data clearly demonstrated that ALDH1A1 was the most highly expressed isozyme in most of normal and cancerous tissues, followed by 9A1, although expression levels of each isozyme were variable in individual tissues. However, it should be noted that this kind of comparison may not be accurate because of different primer efficiencies in distinct primer sets. Also importantly, relative expression levels of each isozyme between normal and cancer tissues (Fig. 3B) were not significantly different.

It may be worth noting that our preliminary microarray data obtained with a normal thyroid tissue (Mitsutake *et al*. unpublished) and the data from Geo DataSets with seven thyroid normal and cancer sets (GDS1732; http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1732) both demonstrated that the transcripts of eight isozymes are clearly expressed (ALDH1A1, 1B1, 1A3, 2, 3A2, 7A1, 9A1 and 18A1).

Discussions

Recent contradictory data on ALDH1A1 expression in thyroid tissues [3,4] prompted us to re-evaluate these data by immunohistochemistry and also by RT-PCR. Two different anti-ALDH1A1 antibodies we used in this study were from distinct companies (BD and SC) and both recognized approximately 55 kD protein in immunoblotting, a size compatible to that of ALDH1A1, in the cells dominantly expressing 1A1 isozyme of ALDH and also detected normal and cancer stem cells in colon and breast tissues, validating the integrity of these antibodies, although the possibility of cross-reactivity of these antibodies with other ALDH isozymes cannot completely be excluded due to a high degree of amino acid sequence similarity [6]. Indeed one of these antibodies was used in the previously published controversial studies [3,4]. Our data obtained with these antibodies demonstrate significant and comparable levels of ALDH1A1 expression in normal and cancerous thyroid tissues. Furthermore, we also show clear expression of ALDH mRNA by RT-PCR in thyroid tissues with predominantly expressed ALDH isozyme being ALDH1A1 followed by ALDH9A1. We thus interpret all these data as indicating that thyroid cells of normal and cancer origins *do* express ALDH1A1. These data support those by Deng *et al*. [4], but sharply contradict those by Todaro *et al* [3].

The similar discrepant data can also be reported in pancreas. Thus almost all normal and cancerous pancreatic cells express ALDH1A1 in one study [4] while the positivity is much lower in other studies with primary pancreatic tumors and xenografts [7].

As mentioned in the Introduction, ALDH is increasingly recognized as an important biological marker for CSCs. As with the data by Todaro *et al*. [3], we have also recently found that CSCs can be enriched for ALDH activity, determined by an ALDEFLUOR assay, in some, but not all, thyroid cancer cell lines [8]. Thus, we do not here intend to negate the importance of ALDH for thyroid CSC study and instead we think that ALDH may still be a useful marker to enrich thyroid CSCs.

Expression levels of ALDH isozymes vary in different cancerous tissues. Prostate CSCs express mainly ALDH7A1 and to a lesser degree 4A1 and 9A1 [9]; breast cancer CSCs

ALDH1A3 (and to a lesser degree 2, 4A1, 5A1, 6A1 and 7A1) [10]; and primary melanoma cells 1A1 and 1A3 and melanoma cell lines 1A3 [11].

It is reported that ALDH is not just a marker for isolating or enriching CSCs but can modulate CSC properties [9,12-15], although the data are controversial. On the one hand, some articles reported the crucial role for ALDH in maintaining CSC characteristics. For example, ALDH1A1 silencing by siRNA sensitizes ovarian cancer cells to chemotherapy and reduces their tumorigenesis [14]; siRNA-mediated silencing of ALDH1A1 and 3A1 suppresses cell proliferation and motility in cancer cell lines [15]; and knockdown of ALDH7A1 by shRNA inhibits the clonogenic, migratory and metastatic activities in prostate cancer cells [9]. On the other hand, however, other reports demonstrated inhibition of CSC function by ALDH. Thus, inhibition of ALDH activity by DEAB and addition of all-trans retinoic acid (ATRA), a product by ALDH, augments and suppresses, respectively, sphere formation in cancer cell lines [13]. In thyroid cancers, anti-tumor effect of ATRA is also reported [16]. It thus may be of interest to clarify how ALDH activity modulates the characteristics of thyroid cancer cells and particularly thyroid CSCs.

In conclusion, we here show the significant expression of ALDH1A1 (and to a lesser extent 9A1) in normal and cancerous thyroid tissues. Further study will definitely be necessary to study functional significance of the predominantly expressed ALDH1A1 in the behaviors of thyroid cells and CSCs.

Figure legends

Figure 1. Expression of ALDH in ALDOFLUOR assay and RT-PCR, and of ALDH1A1 in western blotting and immunohistochemistry. (A) ALDEFLUOR assay with/without DEAB treatment, (B) RT-PCR analysis of mRNA for 19 ALDH isozyme expression, in two cancer cell lines and (C) immunoblotting of the total cell lysates extracted from two cancer cell lines with two anti-ALDH1A1 antibodies and anti-β-actin antibody were performed as described in the Materials and Methods. (D) Immunohistochemistry of ALDH1A1 expression with normal and cancerous tissues of colon and breast with two antibodies. The representative staining with 1:1,500 diluted BD antibody and 1:10,000 diluted SC antibody was shown.   In (B), the arrows indicate β-actin or ALDH1A1 proteins, and * non-specific reaction.

Figure 2. Expression of ALDH1A1 proteins in normal and cancerous thyroid tissues in immunohistochemical analysis. (A) The representative pictures of ALDH1A1 staining in normal and cancerous thyroid tissues with two different anti-ALDH1A1 antibodies are shown. Antibody dilutions were 1:1,500 for BD antibody and 1:10,000 for SC antibody. (B) ALDH1A1 expression in 18 pairs of normal and cancerous thyroid tissues estimated by immunostaining with two antibodies were summarized. The data are categorized into four groups according to percentage of ALDH1A1-positive cells: >75, 75 to 51, 50 to 25 and <25 %.

Figure 3. Expression of mRNAs for 19 ALDH isozymes analyzed by RT-PCR. Total RNAs extracted from pairs of normal and cancerous thyroid tissues were subjected to RT-PCR as described in the Materials and Methods. (A) The representative data in three pairs are shown as means $\pm$ ranges (n=2). (B) The averages of the data obtained with 10 pairs are summarized and shown as means $\pm$ S.D.

References

1. *Valent P, Bonnet D, De Maria R, Lapidot T, Copland M, Melo JV, Chomienne C, Ishikawa F, Schuringa JJ, Stassi G, Huntly B, Herrmann H, Soulier J, Roesch A, Schuurhuis GJ, Wohrer S, Arock M, Zuber J, Cerny-Reiterer S, Johnsen HE, Andreeff M, Eaves C.* Cancer stem cell definitions and terminology: the devil is in the details. Nat Rev Cancer 2012; 12: 767-775

2. *Marcato P, Dean CA, Giacomantonio CA, Lee PW.* Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform. Cell Cycle 2011; 10: 1378-1384

3. *Todaro M, Iovino F, Eterno V, Cammareri P, Gambara G, Espina V, Gulotta G, Dieli F, Giordano S, De Maria R, Stassi G.* Tumorigenic and metastatic activity of human thyroid cancer stem cells. Cancer Res 2010; 70: 8874-8885

4. *Deng S, Yang X, Lassus H, Liang S, Kaur S, Ye Q, Li C, Wang LP, Roby KF, Orsulic S, Connolly DC, Zhang Y, Montone K, Butzow R, Coukos G, Zhang L.* Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. PLoS One 2010; 5: e10277

5. *Marchitti SA, Brocker C, Stagos D, Vasiliou V.* Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. Expert Opin Drug Metab Toxicol 2008; 4: 697-720

6. *Ma I, Allan AL.* The role of human aldehyde dehydrogenase in normal and cancer stem cells. Stem cell reviews 2011; 7: 292-306

7. *Jimeno A, Feldmann G, Suarez-Gauthier A, Rasheed Z, Solomon A, Zou GM, Rubio-Viqueira B, Garcia-Garcia E, Lopez-Rios F, Matsui W, Maitra A, Hidalgo M.* A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Mol Cancer Ther 2009; 8: 310-314

8.   *Shimamura M, Nagayama Y, Matsuse M, Yamashita S, Mitsutake N.* Analysis of multiple markers for cancer stem-like cells in human thyroid carcinoma cell lines. Endocr J 2014; 6: 481-490

9.   *van den Hoogen C, van der Horst G, Cheung H, Buijs JT, Pelger RC, van der Pluijm G.* The aldehyde dehydrogenase enzyme 7A1 is functionally involved in prostate cancer bone metastasis. Clin Exp Metastasis 2011; 28: 615-625

10.   *Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, Helyer L, Pan L, Leidal A, Gujar S, Giacomantonio CA, Lee PW.* Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells 2011; 29: 32-45

11.   *Luo Y, Dallaglio K, Chen Y, Robinson WA, Robinson SE, McCarter MD, Wang J, Gonzalez R, Thompson DC, Norris DA, Roop DR, Vasiliou V, Fujita M.* ALDH1A Isozymes are Markers of Human Melanoma Stem Cells and Potential Therapeutic Targets. Stem Cells 2012; 30: 2100-2113

12.   *Chute JP, Muramoto GG, Whitesides J, Colvin M, Safi R, Chao NJ, McDonnell DP.* Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. Proc Natl Acad Sci U S A 2006; 103: 11707-11712

13.   *Ginestier C, Wicinski J, Cervera N, Monville F, Finetti P, Bertucci F, Wicha MS, Birnbaum D, Charafe-Jauffret E.* Retinoid signaling regulates breast cancer stem cell differentiation. Cell Cycle 2009; 8: 3297-3302

14.   *Landen CN, Jr., Goodman B, Katre AA, Steg AD, Nick AM, Stone RL, Miller LD, Mejia PV, Jennings NB, Gershenson DM, Bast RC, Jr., Coleman RL, Lopez-Berestein G, Sood AK.* Targeting aldehyde dehydrogenase cancer stem cells in ovarian cancer. Mol Cancer Ther 2010; 9: 3186-3199

15.   *Moreb JS, Ucar D, Han S, Amory JK, Goldstein AS, Ostmark B, Chang LJ.* The enzymatic activity of human aldehyde dehydrogenases 1A2 and 2 (ALDH1A2 and ALDH2) is detected by Aldefluor, inhibited by diethylaminobenzaldehyde and has

significant effects on cell proliferation and drug resistance. Chem Biol Interact 2012; 195: 52-60

16. *Malehmir M, Haghpanah V, Larijani B, Ahmadian S, Alimoghaddam K, Heshmat R, Ghavamzadeh A, Adabi K, Ghaffari SH.* Multifaceted suppression of aggressive behavior of thyroid carcinoma by all-trans retinoic acid induced re-differentiation. Mol Cell Endocrinol 2012; 348: 260-269

Table 1. The sequences of primers used to quantify mRNAs for ALDH isozymes.

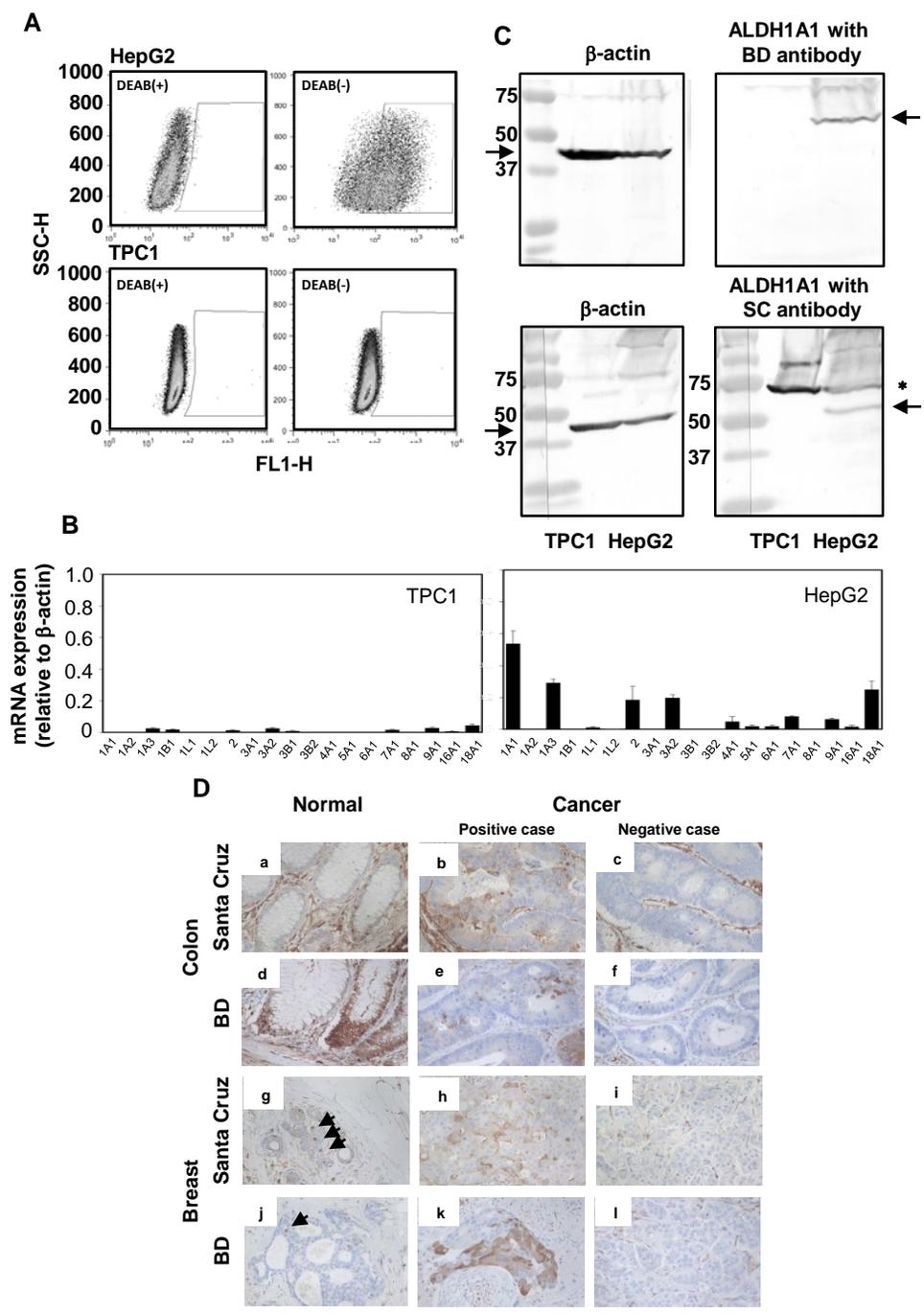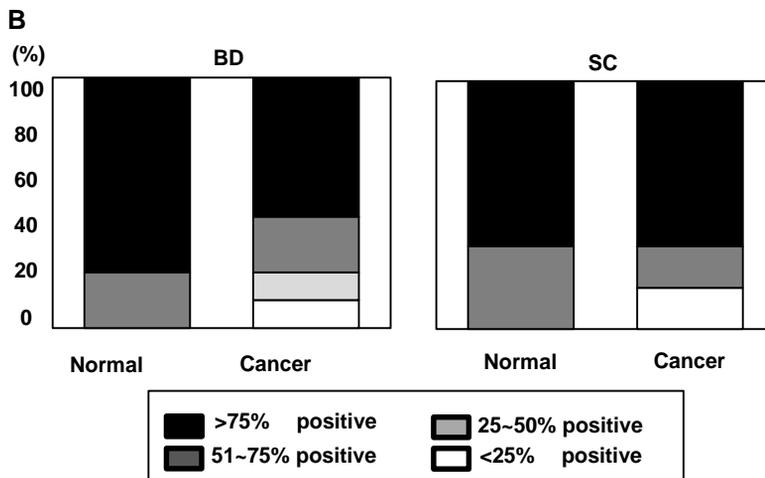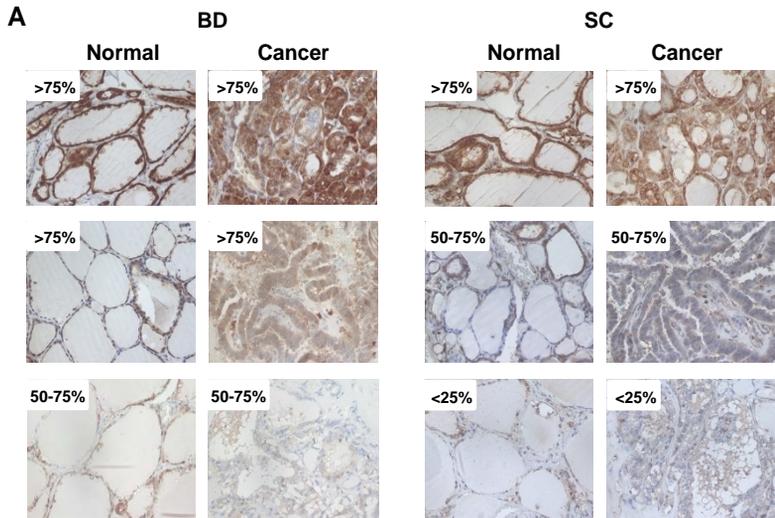| Primers used	Forward	Reverse
1A1	TGTTAGCTGATGCCGACTTG	TTCTTAGCCCGCTCAACACT
1A2	CACAGTGTTTTCCAACGTCACT	GGGCCTTGTTGATGTCATTAGT
1A3	TCTCGACAAAGCCCTGAAGT	TATTCGGCCAAAGCGTATTC
1B1	AACCAGAACCCAAGCGTGAT	TTGTTGATGAACAGCTGGTTGTA
1L1	ATCTTTGCTGACTGTGACCT	GCACCTCTTCTACCACTCTC
1L2	GCCTGGTCTCGTTACCAAAA	GCCACTTTCACCTCTTCAGC
2	GGGTCAACTGCTATGATGTGTTT	TTTCCATCAATGGCTGAGGGAGG
3A1	GCAGACCTGCACAAGAATGA	TGTAGAGCTCGTCCTGCTGA
3A2	CCCCTTCGTTCTCACCATTC	CTGAGGGAGAAGCTTTGCCA
3B1	GGGCTTCTGTGGGAACGAC	AGAACTTGCCATGGTACCGG
3B2	ACAGAGGAAGGTCCTGGCTGA	CATGACAATCTTGCCCACAC
4A1	GTACGGTGGCCAGAAGTGTT	TCTTGATACGGGCAAAGGAC
5A1	ACCAATTCTTGGTGCAAAGG	GTTGGTGTCGTTTTCCACCT
6A1	GGCTCTTTCAACAGCAGTCC	ATGGAAGCTCCCTCCTTTGT
7A1	CAACGAGCCAATAGCAAGAG	GCATCGCCAATCTGTCTTAC
8A1	GCAGAGTGCCAAATAGTGGA	AACTCCTCCAGGGACTGCT
9A1	CACTCATCAACCGACCACAC	GGACATAACAGGCCCAAAGA
16A1	AGGATCCCATCACAGGAGAG	ACTCCAGCCCTTAAATGCC
18A1	CTGAGTATGGGGACCTGGAA	GCGGTAACCATCAGAAAAGC

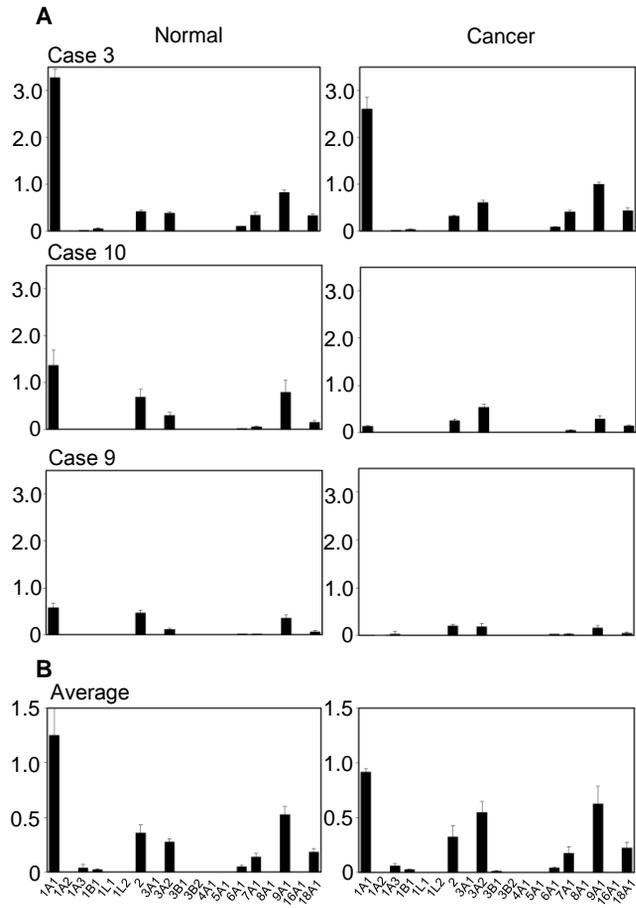**A**

HepG2

DEAB(+)   DEAB(-)

TPC1

DEAB(+)   DEAB(-)

SSC-H

FL1-H

B

mRNA expression (relative to β-actin)

TPC1

HepG2

C

β-actin   ALDH1A1 with BD antibody

75
50
37

β-actin   ALDH1A1 with SC antibody

75
50
37

75
50
37

*

TPC1 HepG2   TPC1 HepG2

D

Normal   Cancer

Positive case   Negative case

Colon

Santa Cruz

a   b   c

BD

d   e   f

Breast

Santa Cruz

g   h   i

BD

j   k   l

Fig.1

Fig.2

A

Normal | Cancer

Case 3

Case 10

Case 9

B Average

Fig.3