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Expression and Cellular Localization of Class IV Alcohol Dehydrogenase in Human Non-small Cell Lung Cancer

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Background: Class IV alcohol dehydrogenase (ADH7) acts as a multifunctional enzyme in the detoxification of endogenous aldehyde, biosynthesis of retinoids, and metabolism of bioactive eicosanoid. The aim of this study was to assess ADH7 expression and cellular localization in non-small cell lung cancer (NSCLC), including squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC). **Materials and Methods:** We used purified class-specific antibody for ADH7 to elucidate the cellular distribution in a human NSCLC tissue microarray. An overall score was obtained for each sample based upon multiplying the staining intensity (0-3) and the extensiveness (0-100%). Mean±S.E.M. for each experimental group was calculated and compared. **Results:** Our results indicated a significantly higher level of expression of ADH7 in SCC (116.7±13.7) than ADC (26.5±4.8), LCC (37.5±12.5) and normal lung tissues (11.4±3.7) (all $p < 0.005$). No statistically significant difference was observed between ADC, LCC, and normal lung tissues. ADH7 was mainly distributed in the epithelium of bronchiole, one of the origins of SCC. The tumor invasive front demonstrated a higher expression of ADH7, both in primary and metastatic SCC. **Conclusion:** Abundant expression of ADH7 in SCC but not ADC and LCC makes it a potential biomarker for diagnosis of lung SCC.

Key words: alcohol dehydrogenase, human non-small cell lung cancer

INTRODUCTION

Lung cancer accounts for 1.6 million new cancer cases annually in the world and is the most common and deadly form of cancer.[1] It is also the leading cause and responsible for 20.1% of cancer deaths in Taiwan in 2011.[2] Non-small cell lung cancer (NSCLC) comprises approximately 80% and small cell lung cancer 20% of all lung cancer.[3] NSCLC can be divided into three major histologic subtypes: squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell lung cancer (LCC). Although cigarette smoking is associated with more than 90% of cases of lung cancer[2], it is stronger for SCC than ADC.[4] In addition, SCC is usually found in the central part of the lung next to the bronchus where smoke or polluted air damage mainly occur. Cigarette smoke contains numerous aldehydes and is known to induce lipid peroxidation in the lung.[5,6]

Human class IV alcohol dehydrogenase (ADH), also known as ADH4 or ADH7, is a detoxifying enzyme involving in the metabolism of a wide variety of substrates, such as retinoids, steroids, biogenic amines, lipid peroxidation products, ω-hydroxyfatty acids as well as xenobiotic primary and secondary alcohols and aldehydes.[7-11] ADH7 express in various epithelia including the cornea, mouth, esophagus, stomach, vagina, nasopharynx and trachea.[12] Its localization is compatible with the importance of retinol in the integrity of these tissues. ADH7 also oxidizes 20-hydroxyeicosatetraenoic acid (20-HETE) to 20-carboxyeicosatetraenoic acid (20-COOH-AA), a bioactive eicosanoid that produces vasodilation in the microvasculature.[13]

Histopathological and immunohistochemistcal (IHC) analysis of biopsied tissue is still the current standard for diagnosing and classifying tumors. However, a scarcity of validated biomarkers for lung cancer is currently a problem requiring resolution.[14] It is worthy to note that the *ADH7* gene over-expressed in the squamous cell

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carcinoma of the lung using a combination of cDNA subtraction and microarray studies.[15] The authors proclaimed *ADH7* was a tumor specific gene and up-regulation of the enzyme might be associated with the hypoxic status of the tumor.[15] However, there is no published ADH7 protein expression and cellular localization in human lung cancer in the English literature. This may be partly due to no available class-specific, cross-reactivity free anti-ADH7 antibody for the study because the polyclonal anti-ADH7 antibody yielded obvious cross-reactivity with other classes of ADH.[16] We have previously purified the class-specific anti-ADH7 antibody by the cyanogen bromide affinity column and tested it in the human pancreas, small and large bowels.[17-19] In the present study, we repeated the purification and applied the specific antibody to the immunohistochemical analysis of NSCLC. Our results demonstrated the enzyme is differentially expressed in SCC, ADC, and LCC. The ADH7 protein appears to be more highly expressed in SCC.

MATERIALS AND METHODS

Human tissues

A human lung cancer tissue array (LC951) was purchased from US Biomax (Rockville, MD, USA) and tissue array analysis was conducted according to the manufacturer's suggested protocols. A total of 63 samples with normal (n = 15), squamous cell carcinoma (n = 24), adenocarcinoma (n = 18) and large cell carcinoma (n = 6) were included in this study. The data available for the samples included the age, gender, and pathological diagnosis of the patients. All samples were classified based on histological grading and TNM staging, and all tumors were staged according to the 1997 AJCC/TNM system (6th edition). An experienced pathologist (YCL) reassessed the slides to re-confirm the diagnosis.

Expression and purification of human ADH family

The expression of recombinant human class I-IV ADHs (i.e., ADH1C1, ADH4, ADH5, and ADH7), and the purification to apparent homogeneity were as described previously.[16,20-22] The purified ADH7 was used as immunogen for preparation of rabbit antiserum and the four class ADHs were used as the protein standard in western blot.

Purification of class-specific anti-human ADH7 antibody

Rabbit antiserum against human ADH7 was from those described earlier.[15] Class cross-reactivity of the polyclonal antisera was eliminated using affinity chromatography with sepharose covalently linked to the respective nonimmunogen isozymes, i.e., the ADH1C1, ADH4, ADH5 members, according to the manufacturer's procedure. The yielded class-specific antibodies were then further purified by affinity chromatography using the antigen isozymes, i.e., ADH7 immobilized on the sepharose resins. The immune-reactivities of anti-human ADH7 towards three ADH isozymes were assessed by Western blotting analysis. Concentration of the affinity-purified antibodies was assessed by enzyme-linked immunosorbent assay with commercially available rabbit IgG standard as described previously.[16]

Western Blot Analysis

The recombinant class I to IV ADHs were subjected to SDS-PAGE and electroblotted to the nitrocellulose membrane by a PhastSystem according to the manufacturer's protocol. The membrane was blocked with 1% BSA in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature and washed with PBST; then incubated with antiserum (1:1,000 dilution) or with the purified class-specific antibody for 1 h, washed; and finally incubated in a 1:2,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase and the western lightning chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA). The immunoreactive bands were evaluated by densitometric analysis using the Chemigenius 2 Chemiluminescent Image System (Synoptics LTD, Cambridge, UK). Local background was subtracted for each band using a separate nearby reference area.

Immunohistochemistry

The lung cancer tissue microarray was incubated with affinity-purified anti-human ADH7 antibody, followed by detection using the supersensitive nonbiotin horseradish peroxidase system (BioGenex Laboratories, San Ramon, CA, USA). Briefly, the slide was baked at 60 ^{o}C for 2 h, deparaffinized and rehydrated, followed by antigen retrieval in boiling sodium citrate buffer (10 mM, pH 6.0) for 30 min. Non-specific proteins were then blocked using Power Block^{TM} Reagent for 10 min. The sections were incubated at room temperature for 60 min with anti-human ADH7 antibody in Power Block^{TM}. The concentration of the antibody used in detecting ADH7 was 0.06 μg/ml. The slide was then incubated in the Super Enhancer^{TM} Reagent for 20 min after rinsing. The sections were rinsed again and incubated at room temperature for 30 min with Poly HRP Reagent. Chromogen development was performed by 3,3'-diaminobenzidine staining

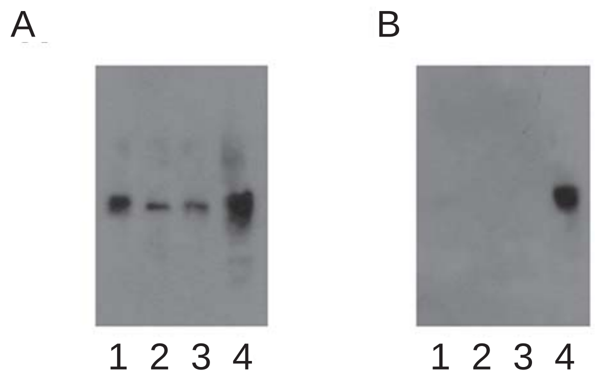

Fig. 1 Western blot analysis for class specificity of primary and affinity-purified antibodies. Immunodetection of cross-reactivities of the primary (A) and affinity-purified anti-ADH7 antibodies (B) to ADH1, ADH4, ADH5, and ADH7. Lanes 1–4 are the recombinant isozymes ADH1, ADH4, ADH5, and ADH7, respectively. All isozyme antigens were loaded at 25 ng. The concentrations of the antibodies used for detection were 0.03 μg/ml.

Table 1 Expression of ADH7 in human lung cancer by immunohistochemistry

Features of specimens	n/N[a]	I^b	E^c	S^d
Squamous cell carcinoma	24/24	1.56±0.12	70.4±5.4	116.7±13.7
Adenocarcinoma	15/18	0.97±0.08*	25.9±4.9*	26.5±4.8*
Large cell carcinoma	4/6	0.83±0.31†	31.7±10.7*	37.5±12.5*
Normal	7/15	0.63±0.08*	11.4±3.7*	11.4±3.7*

Results were expressed as mean±SEM.

[a] n denotes the number of cancer samples that showed immunopositivity of ADH7; N denotes the total number of sample sections that have been examined.

[b] The intensity (I) of ADH7 immunostaining in positively stained sections was scored as: 0 = no staining, 1 = faint staining, 2 = moderate staining, 3 = strong staining.

[c] The extensiveness (E) of ADH7 immunostaining in positively stained sections was scored as percent of total cancer cells.

[d] The overall immunopositivity score (S) of ADH7 was calculated as S = $I \times E$.

* $p < 0.005$ vs squamous cell carcinoma

† $p < 0.01$ vs squamous cell carcinoma

for 5 min. Counterstaining was carried out usinghaematoxylin slightly. The slides were rinsed with water and covered with mounting medium (Faramount aqueous S3025, Dako, Denmark) after dehydration.

Cancer sample analysis

The IHC images were viewed using a Leica DM-2500 microscope (Leica, Heiderberg, Germany) and captured using a SPOT CCD RT-slider integrating camera (Diagnostic Instruments Inc., Sterling Heights, MI). Tissue sections of lung cancer were present on the same tissue microarray slide and each was evaluated for the immunopositivity of ADH7 staining as described. Briefly, the IHC signal for each antibody in each tissue was rated semi-quantitatively in positively stained sections for intensity (I) on a scale of 0–3 (0 = no staining, 1 = faint staining, 2 = moderate staining, and 3 = strong staining) and for extensiveness (E, % of positive staining cells). Samples were examined and scored by two independent observers (YCL and CPC). Those samples lacking enough tumor tissue for evaluation were excluded from the final analysis. The IHC expression score (S) for each antibody in each sample was calculated by S = I×E. Data are reported as mean±SEM.

Statistics

The statistical differences between normal, squamous cell carcinoma and adenocarcinoma groups were evaluated by Bonferroni's test after one-way analysis of variance for multiple group comparisons. A confidence level of $p < 0.05$ was considered statistically significant. All analyses were conducted using the statistical package program of PASW statistics 18 Software (SPSS Inc., Chicago, IL, USA).

RESULTS

The polyclonal anti-human ADH7 antibody was again purified through a cyanogen bromide affinity column. We examined the immunoreactivity of the antibody towards four classes of human recombinant ADH proteins by Western immunoblotting analysis. Under the conditions we used, the purified anti-ADH7 antibody reacted exclusively with ADH7 (Fig. 1). Sixty-three human lung tissues were included from the human tissue microarray slide (LC951) for studies, including 24 squamous cell carcinoma, 18 adenocarcinoma, 6 large cell carcinoma and 15 normal tissues. The average age in years of the patients was 57±11, 60±9, and 56±11 for squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, respectively. Based on the scoring system, the expression profiles for ADH7 in these studied tissues are summarized in Table 1.

In apparently normal lesions, the staining intensity was very weak (I = 0.63±0.08) and the overall expression score was low (S = 11.4±3.7). The epithelial cells

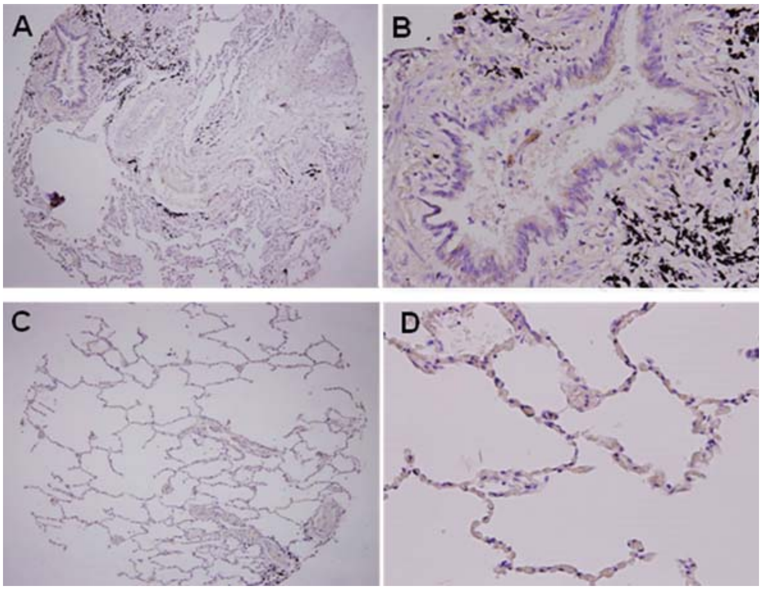

Fig. 2 Distribution of ADH7 in normal human lung tissues by immunohistochemistry. Positive staining (brown) displayed a diffuse cytoplasmic pattern for ADH7. Representative normal lung tissues with bronchiole (A and B) and alveola (C and D) are presented at two magnifications, with the low-power field in the left panel (200×) and enlarged in the right panel (800×).

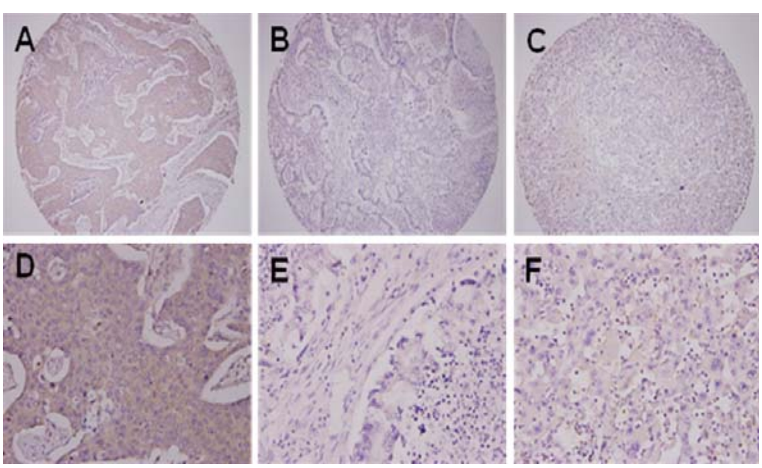

Fig. 3 Distribution of ADH7 in human squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC) by immunohistochemistry. Representation of different lung cancers for comparison presented at two magnifications, with the low-power field in the upper panel (A, B, and C; 200×) and enlarged in the lower panel (D, E, and F; 800×).

of bronchiole, however, showed relatively strong cytoplasmic immunopositivity; the pneumocytes of alveoli were weakly positive (Fig. 2). In squamous cell carcinoma of the lung, all samples were IHC-positive for ADH7. The overall staining intensity was moderate (I = 1.56±0.12) and found in 70.4±5.4 % of cancer cells. The overall expression score was 116.7±13.7 and much higher than normal lung tissues (p < 0.005). A strong cytoplasmic ADH7 immunostaining was noted in all the cancer cells of squamous cell carcinoma (Fig. 3). Only a few cells of the stromal compartment were positive, some of which appeared to be plasma cells. After analysis of the TNM staging in SCC, we noted the staining intensity and immunoreactive score of T4 were relatively higher than T2 (2.17±0.44 versus 1.48±0.11, p = 0.05 and 186.7±35.6 versus 106.7±13.7, p = 0.051, respectively). Besides, the staining intensity of M0 was significantly different from M1 (1.68±0.13 versus 1.10±0.10, p < 0.05). However, there was no statistically significant difference in node involvement, clinical staging, and the various histological grades of SCC. Interestingly, stronger ADH7 expressions were noted on the tumor invasion fronts of the primary and metastatic squamous cell carcinoma (Fig. 4).

In lung adenocarcinoma, 15 out of 18 (83%) samples were found to be weakly stained for ADH7 with intensity (I = 0.97±0.08), and present in about one fourth of the cancer cells (E = 25.9±4.9). The overall expression score (26.5±4.8) was 4.4-fold lower than that of squamous cell carcinoma (p < 0.005). The stromal tissue was almost negative (Fig. 3). In these 6 large cell carcinoma samples, 4 out of 6 (68%) samples were positive for ADH7 with low intensity (I = 0.83±0.31), and present in less than one third of the cancer cells (E = 31.7±10.7). The overall expression score (37.5±12.5) was also less than that of squamous cell carcinoma (p < 0.005) (Fig. 3). Besides, the small sample sizes restricted our ability to analyze TNM and perform a histological grading in ADC and LCC. There was no statistically significant difference between ADC, LCC, and normal lung tissues.

DISCUSSION

The current study applied a promising class-specific

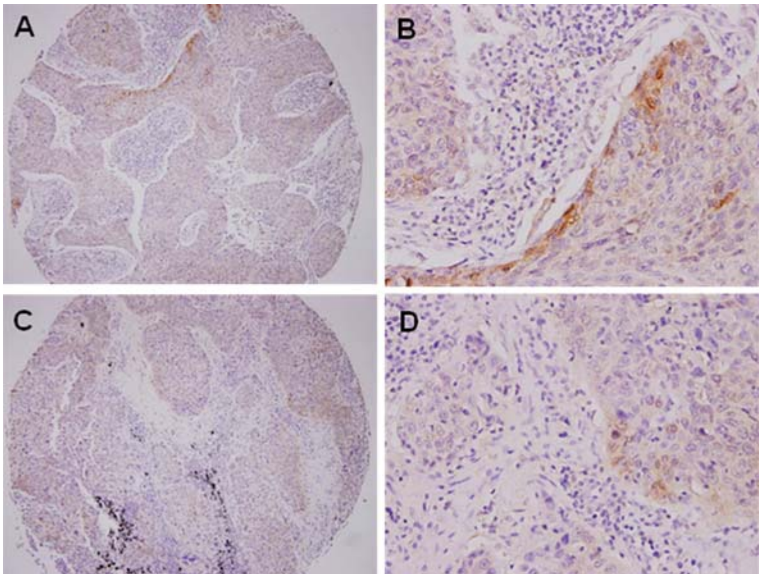

Fig. 4 Distribution of ADH7 in human lung squamous cell carcinoma (SCC) by immunohistochemistry. Lung SCC with primary (A and B) and metastatic (C and D) of the same patient presented at two magnifications, with the low-power field in the left panel (200×) and enlarged in the right panel (800×).

anti-ADH7antibody to identify the expression profile of ADH7 protein in NSCLC. As the pulmonary tract of the lung branches into the smaller bronchioles and alveoli of the distal airway, the columnar epithelial cells transition into a more cuboidal and relatively flat morphology. The alveoli are comprised of type 1 and type 2 pneumocytes. The lung epithelium consists of a large variety of functionally and morphologically different cell types, whose roles include detoxifying and clearing foreign agents, facilitating gas exchange, balancing fluids in the lung, and the activation of inflammation due to injury.[23,24] In our result, the ADH7 expressed at a moderate level in the bronchiole, but at low levels in the aveloli. The findings support the PubMed unigene expression profile of ADH7 in lung tissue and trachea.[25] ADH7 may play an important role in the detoxification of lipid peroxidation during proximal airway damage. In addition, ADH7 has been shown to be the most efficient form for oxidation of the retinol in the ADH family[20,26] and expression of ADH7 may contribute in part to maintaining epithelial differentiation through retinoic acid biosynthesis.

The expression of ADH7 was observed in all tumor samples of SCC. The results were compatible with the combination of cDNA subtraction and microarray analysis in lung squamous cell carcinoma[15]. In addition, the higher ADH7 expression in larger tumor volume may result from the necessity of more retinoic acid biosynthesis during tumor proliferation. A previous report also suggested the combination of beta carotene and vitamin A supplement (both precursors of retinoic acid) had no benefit and may have had an adverse effect on the incidence of lung cancer.[27] (Gilbert *et al*., 1996). It is notable that ADH7 expressed predominantly in the tumor invasion fronts of some primary and metastatic SCC. Tumor cell invasion, as a critical step for metastasis, depends on the interplay between the cancer cells and their microenvironment.[28,29] Thus, the enhanced expression of ADH7 on the tumor invasion front may involve the interaction between malignant cells and their surroundings and play an important role in SCC invasion and metastasis. However, the different finding between the staining intensity of M0 and M1 warrant further evaluation in a large sample sizes.

Interestingly, cigarette smoking decreased serum retinol levels in human body.[30] The finding of up-regulated ADH7, which encodes an enzyme most active as a retinol dehydrogenase, suggests a possible role for retinoic acid in lung SCC carcinogenesis, invasion and metastasis. In addition, the roles of ADH7 in eicosanoid, steroid and fat metabolism are also interesting because of the association of lung cancer with angiogenesis,[31,32] immunocompromised[33,34] and nutritional status.[35] In view of these possibilities, the molecular mechanism of ADH7 in squamous cell carcinoma should be assessed in the future.

In contrast, the expressions of ADH7 in adenocarcinoma and large cell carcinoma of lung were much lower than squamous cell carcinoma. In human, squamous cell carcinomas occur in the central region of the respiratory tract whereas adenocarcinomas and large cell carcinomas are located peripherally.[36] The histological and regional diversity found in different lung cancer may partly be due to the presence of diverse pools of self-renewing stem cells in the adult lung epithelium.[37] The higher expression of ADH7 in squamous cell carcinoma represents the regional distribution of the enzyme in the proximal respiratory tract as normal trachea, bronchus and bronchiole. The lower expression of ADH7 in adenocarcinoma and large cell carcinoma were also in consistent with low expression of ADH7 in the distal airway. The phenomenon of the reserved phenotype of ADH7 in squamous cell carcinoma of lung cancer may become a potential biomarker.

There are several limitations/unresolved issues that need to be acknowledged. First, we have a relatively small number of lung cancer cases to allow stratified analyses of age, gender, tumor staging and smoking history, etc. Second, we lack flesh tissues for ADH7 activity assay. Finally, we could not obtain tissue supernatants for immunoblotting study. For validation, further studies will be conducted on larger data sets by collaborating with thoracic surgeons and pathologists in our hospital.

In conclusion, ADH7 is expressed at high levels in the proximal airway and SCC in comparison to distal airway and ADC/LCC. Our results also show notable upregulation of the expression of the enzyme in the tumor invasion front. We believe the data presented herein may have implications for our understanding of the pathogenesis, diagnosis and management of lung cancer.

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