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Comparison of Quantum Dot Technology with Conventional Immunohistochemistry in Examining Aldehyde Dehydrogenase 1A1 as a Potential Biomarker for Lymph Node Metastasis of Head and Neck Cancer

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Abstract

This study explored whether expression of aldehyde dehydrogenase 1 (ALDH1A1) in the primary tumor correlated with lymph node metastasis (LNM) of squamous cell carcinoma of the head and neck (HNSCC). We used both quantum dot (QD)-based immunohistofluorescence (IHF) and conventional immunohistochemistry (IHC) to quantify ALDH1A1 expression in primary tumor samples taken from 96 HNSCC patients, 50 with disease in the lymph nodes and 46 without. The correlation between the quantified level of ALDH1A1 expression and LNM in HNSCC patients was evaluated with univariate and multivariate analysis. The prognostic value of ALDH1A1 was examined by Kaplan-Meier analysis and Wald test. ALDH1A1 was highly correlated with LNM in HNSCC patients ($p < 0.0001$ by QD-based IHF and 0.039 by IHC). The two methods (QD-based IHF and conventional IHC) for quantification of ALDH1A1 were found to be comparable ($R = 0.75$, $p < 0.0001$), but QD-IHF was more sensitive and objective than IHC. The HNSCC patients with low ALDH1A1 expression had a higher 5-year survival rate than those with high ALDH1A1 level ($p = 0.025$). Our study suggests that ALDH1A1 is a potential biomarker for predicting LNM in HNSCC patients, though it is not an independent prognostic factor for survival.

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of HNSCC patients. Furthermore, QD-IHF has advantages over IHC in quantification of ALDH1A1 expression in HNSCC tissues.

Keywords

Aldehyde dehydrogenase 1; biomarker; metastasis; immunohistochemistry; head and neck cancer; quantum dot; nanotechnology

1. Introduction

Aldehyde dehydrogenase (ALDH), a cytosolic enzyme, is responsible for catalyzing the pyridine nucleotide-dependent oxidation of aldehydes to carboxylic acids. To date, 17 different isoforms of ALDH have been identified in a variety of human tissues, including liver, kidney, erythrocytes, skeletal muscle, lung, breast, brain, prostate, pancreas, and others¹. In the liver, ALDH1 functions mainly as a retinoic acid biosynthetic enzyme and catalyzes the conversion of retinol (vitamin A) to retinoic acid. It is known that in mammals, retinoids are essential for normal development and homeostasis as they play a crucial role in the physiology of many organs and act as powerful morphogens during embryogenesis²³. Therefore, the lack of functional ALDH1 might result in early embryogenesis disruption. Furthermore, ALDH1 was found to be highly expressed in stem cells in which its major function might be linked to retinoid metabolism⁴. ALDH1 has been reported largely as a marker for cancer stem cells or tumor-initiating cells in many types of solid tumors, including hematopoietic, breast, colon, pancreatic, prostate, liver, and head and neck cancers⁵⁻¹⁶.

Recently, high expression of ALDH1 has been proposed to be responsible for chemoresistance to cyclophosphamide (CPA) in a mouse xenograft model^{17,18}. In a study of human samples, high ALDH1 expression in breast cancer was correlated with poor clinical outcome, and increased ALDH1 expression was seen in breast tissue from patients with metastases compared to those without metastases¹⁹. Higher levels of ALDH1A1 were also detected in non-small cell lung cancer (NSCLC) as compared with small cell lung cancer²⁰. The elevated expression of this enzyme in normal pneumocytes was found to be correlated with history of cigarette smoking. Expression of one of the ALDH1 family members, ALDH1A1, in squamous cell carcinoma of the head and neck (HNSCC) and dysplastic oral mucosa tissue samples was examined by Visus *et al*²¹. They found that 12 of 17 HNSCC and 30 of 40 dysplastic mucosa tissues expressed this protein. However, this study did not correlate ALDH1A1 expression status with adverse clinical features, such as metastasis, chemoresistance, or short survival. Recently, Yu *et al*. reported that co-expression of Bmi-1/ Snail/ALDH1 correlated with poor prognosis of HNSCC, but the study did not examine the direct correlation of ALDH1 with HNSCC metastasis¹³.

Head and neck cancers comprise the sixth most common cancer and are responsible for almost 200,000 deaths worldwide each year²²⁻²⁴. Ninety percent of all head and neck cancers are squamous cell carcinomas. Lymph node metastasis (LNM) of HNSCC is a strong indicator of poor prognosis. The five-year survival rate of HNC is less than 50% for patients with a single ipsilateral LNM and less than 25% for patients with bilateral LNM^{25,26}. An accurate biomarker might obviate the need for diagnostic neck dissections.

In this study, we examined expression of ALDH1A1 in clinical specimens from both non-metastatic and metastatic HNSCC by using a nanoparticle quantum dot (QD)-based technique and conventional immunohistochemistry (IHC). We looked for correlations between the expression of this enzyme with LNM and survival of the HNSCC patients.

QDs are nanoscale particles made from inorganic semiconductors and have novel optical properties that can produce fluorescence emission at different wavelengths depending on their size and composition²⁷. QDs have large molar extinction coefficients, meaning that they are very efficient at absorbing light at specific wavelengths. Not only do QDs have molar extinction levels 10-50 times that of organic dyes, QDs have longer excited state lifetimes compared with organic dyes, providing an optimized separation of QD fluorescence from the background fluorescence²⁸. This study provides not only a basic understanding of ALDH1A1 expression and its potential biological significance in HNSCC, but also a new strategy for quantification of biomarkers in cancer tissue specimens, using a QD-based technology, which our experience demonstrates to be superior to conventional IHC.

2. Materials and Methods

2.1. Patients, tissue samples and cell lines

Utilizing an institutional review board-approved consent for tissue acquisition, specimens for this study were obtained from the primary surgical specimens of patients who were diagnosed with HNSCC at the Emory University Hospital and whose initial treatment was surgery with no prior treatment with radiation and/or chemotherapy. The selection criteria for the available formalin-fixed and paraffin-embedded tissue blocks included two groups: primary SCC with positive lymph nodes (Tu⁺LN, n = 50) and primary SCC with negative lymph nodes (Tu⁻LN, n = 46). In the Tu⁻LN group, if any patient developed lymph node metastases within 2 years of the initial procedure they were excluded from this group. The clinical information associated with the samples was obtained from the Surgical Pathology files in the Department of Pathology at Emory University following the regulations of the Health Insurance Portability and Accountability Act (HIPAA).

Lung cancer cell line A549, purchased from American Type Culture Collection (Manassas, VA), was used to check the specificity of the anti-ALDH1A1 antibody²⁰²⁹. The cells were grown in DMEM:F12 (1:1) supplemented with 5% fetal bovine serum.

2.2. Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections were used for IHC, as described previously³⁰. In brief, the slides were incubated with primary antibody against ALDH1A1 (N-19, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). The staining of the antibody was observed by diaminobenzidine tetrahydrochloride peroxidase substrate solution (Vector Laboratories, Burlingame, CA). Cell nuclei were counterstained by hematoxylin QS (Vector Laboratories). Immunoglobulin G (IgG) was used as a negative control, and adjacent normal epithelial tissue with known positive immunoreactivity to anti-ALDH1A1 antibody was used as positive control. The intensity of IHC staining was scored according to the following scheme: 0: no staining, 1+: <5%; 2+: 5-25%; 3+: 25-50%; 4+: >50% cells staining. In order to compare IHC with QD-based immunohistochemistry (IHF), the intensity of IHC staining of 30 samples was re-quantified as a weighted index [WI= % positive staining x intensity score (0, no expression; 2+, moderate expression; and 3+, strong expression)], as described in our previous publication³⁰. The score was determined by 2 individuals, and the final values were the average of the 2 readings.

2.3 Immunoblotting analysis

Whole cell lysates were extracted from drug-treated cells using lysis buffer. Fifty micrograms of protein was separated by SDS-PAGE, transferred onto a PVDF membrane (Millipore Co., Bedford MA), and immunostained with the same antibody used in the IHC.

Immunostained protein bands were detected with an enhanced chemiluminescence kit (ECL, Amersham, Buckinghamshire, UK).

2.4. QD-based immunohistofluorescence (IHF)

Secondary antibodies labeled with QDs (QD655-2nd rabbit anti-goat Abs and QD605-2nd goat anti-mouse Abs) were purchased from Invitrogen Life Technologies Inc (Carlsbad, CA). QD-based IHF staining was performed as described previously by our laboratory³¹. In brief, after the preparation steps, a primary antibody against pan-cytokeratin (clone AE1/AE3, Dako North America Inc., Carpinteria, CA) and the ALDH1A1-specific antibody used in IHC (1:200) were simultaneously applied to the surface of the slide and incubated overnight at 4°C. After washing with phosphate-buffered saline (PBS) 3 times, the QD655-2nd and QD605-2nd Abs were added to the slides with further incubation for 1-hour at 37°C. The slides were washed 3 times with PBS, counterstained with 4', 6-diamidino-2-phenylindole (DAPI), mounted, and stored at 4°C under dark conditions.

2.5. Quantification of QD-based IHF

The Nuance™ fluorescence microscope system (Cambridge Research & Instrumentation [Cri] Biomedical Systems Inc. Woburn, MA) was used for quantification of the QD-IHF signals. As illustrated in Figure 1A-1D, a spectral library composed of DAPI, autofluorescence, QD605, and QD655 was set up initially. The acquired QD-IHF signals as a cube were unmixed according to their wavelengths in the library. For each slide, 10 cubes were taken randomly, DAPI and pan-cytokeratin (QD605) images were used as tumor masks to determine the area of interest (AOI). Based on both cytokeratins and DAPI, 50 cells were randomly selected in each cube. The total signals of the 10 cubes, as arbitrary unit (au), were averaged and used for correlation with clinical characteristics, including LNM. In order to compare IHC with QD-based IHF, the intensity of IHF staining of 30 samples was re-quantified as a WI (% positive staining x au).

2.6. Data and Statistical Analyses

The correlation between ALDH1A1 expression levels measured by IHC and that by QD-IHF was calculated using Spearman's rank correlation. The Wilcoxon-Mann-Whitney two sample rank-sum test was applied to assess differences in the distribution of ALDH1A1 expression in Tu^{-LN} and in Tu^{+LN}. Correlations between ALDH1A1 expression level and clinical characteristics were performed in all patients and within Tu^{-LN} and Tu^{+LN} separately. The Wilcoxon-Mann-Whitney two sample tests were also applied to assess the association between the level of ALDH1A1 and binary clinical characteristics (gender, tobacco history, whether received chemotherapy, and whether received radiation therapy). Kruskal-Wallis tests were applied for categorical variables with more than two categories (tumor site, stage, differentiation, T, and node status). Receiver operator characteristic curve (ROC) analysis was performed to define the cut off for expression value with best predictive power on metastasis, by maximizing the sum of sensitivity and specificity in the curve. Patients were classified into low or high groups based on this cut-off value. Kaplan-Meier survival estimates were generated for these two groups of patients. Log-rank test was employed to compare the difference in overall survival or disease free survival between the patients with low and high ALDH1A1 expression level. The five-year survival estimates of the two groups of patients were compared by using Wald test. Multivariable logistic regression analysis was performed to examine the adjusted association between ALDH1A1 expression value and the outcome (metastatic status or 5-year survival rate) after adjusting for age, sex, T-stage, differentiation, tumor site, and smoking. Tests were considered significant at $P < 0.05$. The SAS statistical package V9.2 (SAS Institute, Inc., Cary, North Carolina) was used for all data management and analyses.

3. Results

3.1. Clinical characterization of patient samples

The clinicopathologic parameters for the study groups, including age, gender, tobacco history, tumor site, and stage and their correlations with LNM are listed in Table 1. Patients' age ranged from 22 to 88 with a median 61.5 years. The median follow up was 5.2 years with a range of 1 to 12.5 years. Gender distribution was 69% male and 31% female. The majority of patients were smokers (85%). The lesion sites involved were oropharynx (OP: 30%), larynx (L: 26%), and oral cavity (OC: 44%), with 69% moderately differentiated, 15% poorly differentiated, and 16% well differentiated tumors. 28%, 36%, 17%, and 19% of the patients had T1, T2, T3, and T4 tumors, respectively, while 14%, 18%, 21%, and 47% were staged to I, II, III, and IV, accordingly. Most of the patients had treatment with radiation (70%), but not chemotherapy (11%) after the surgery.

3.2. IHC and QD-IHF are comparable in quantification of ALDH1A1 in HNSCC tissues

To develop the QD-IHF technique, we initially checked the specificity of the anti-ALDH1A1 antibody using A549 cells, which overexpress ALDH1A1 and ALDH3A1²⁰. Western blot analyses showed a single band at the 55kDa position corresponding to ALDH1A1, but did not reveal a band at 50kDa corresponding to ALDH3A1 (Figure 1E). No non-specific bands were observed under the exposure conditions used. We then validated the QD-IHF method by comparing it with conventional IHC since this was the first reported use of QD-IHF to quantify ALDH1A1 expression in formalin fixed and paraffin embedded (FFPE) tissues. Figure 2A and 2B show that ALDH1A1 expression was predominantly found in the cytoplasm. Quantification of ALDH1A1 staining using the IHC and QD-IHF methods was comparable ($R = 0.75$, $p < 0.0001$) (Figure 2C), but the QD-IHF signals are expected to be more sensitive and its quantification is more objective than that of conventional IHC.

3.3. Correlation of ALDH1A1 expression with clinical characteristics including LNM of HNSCC

ALDH1A1 expression was significantly higher in the metastatic Tu^{+LN} tumors than in the non-metastatic Tu^{-LN} group, when compared by both IHC (1.52 ± 1.64 vs. 2.34 ± 1.99 , $p = 0.039$) and QD-IHF (1.79 ± 0.44 vs. 4.99 ± 0.84 , $p < 0.0001$, Figure 2D) analyses. However, it was observed that the standard deviation of the mean values determined by IHC analysis was much higher than that determined by QD-IHF (% of SD over mean value: 108% vs. 25% for the Tu^{-LN} group and 85% vs. 17% for the Tu^{+LN} group). Therefore, the expression level of ALDH1A1 determined by QD-IHF was used to explore correlations with clinical characteristics. Univariate analysis showed that ALDH1A1 was not correlated with most clinicopathologic characteristics in patients with and without metastasis (Table 2). However, both univariate (Figure 2D) and multivariable (Table 3) analyses, which considered T stage, smoking, age, tumor site, differentiation and sex as covariates, showed that ALDH1A1 expression level was highly associated with positive lymph node status in HNSCC patients (OR: 1.667, 95%, CI: [1.162, 2.393], $p = 0.0055$).

3.4. Prognostic value of ALDH1A1 expression determined by QD-IHF

Patients were classified into low (< 1.3) or high (> 1.3) groups based on their ALDH1A1 values compared to the cut-off value of ALDH1A1 expression (1.3) from ROC analysis, which had the best predictive power of spread to lymph nodes. Kaplan-Meier survival estimates indicated that patients with higher ALDH1A1 level had worse overall survival and disease free survival than those with lower ALDH1A1, although this was not statistically significant (Figure 3). Five-year survival and disease-free survival rates were significantly

and near significantly higher in patients with low ALDH1A1 level than those with high ALDH1A1 level ($p = 0.025$ and 0.065 , respectively) (supplementary data Table S1). This significant difference was not observed when the IHC method was applied ($p > 0.05$). Since patients with oropharyngeal (OP) HNSCC are known to have better outcomes than other HNSCC patients due to the high incidence of human papillomavirus (HPV) infection in OP HNSCC^{32,33}, it is of interest to determine whether ALDH1A1 status may be a stronger prognostic marker when excluding the OP population. Five-year survival was more significantly correlated with low ALDH1A1 level when the OP population was excluded from the analysis ($p = 0.012$), but the five-year disease-free survival rate was not significantly correlated with ALDH1A1 level ($p = 0.181$, supplementary data Table S2). Multivariable logistic regression analysis showed that the dichotomized ALDH1A1 level had no significant adjusted effect on the 5-year survival rate after considering other covariates, including the metastasis status which was a significant factor in the 5-year survival rate ($p = 0.037$, supplementary data Table S3). These data suggest that ALDH1A1 expression is not an independent prognosis factor and its effect on survival results from its correlation with metastasis.

4. Discussion

Our study clearly showed that ALDH1A1 expression was highly associated with lymph node metastasis of HNSCC and was associated with poor prognosis in patients with HNSCC. The significant correlation between disease in the lymph nodes and the level of ALDH1A1 expression in the HNSCC samples as determined by QD-IHF was not observed when ALDH1A1 expression was examined with conventional IHC, though the two methods were significantly comparable. Due to the semi-quantitative nature, large variations were obtained when quantifying ALDH1A1 expression by the IHC method, suggesting that QD-IHF can more accurately and objectively determine protein levels in FFPE tissues than conventional IHC. This notion is supported by comparison of the standard deviation when using the two methods, where it is common to observe a high level of standard deviation in IHC analysis.

ALDH1, not its specific isotype ALDH1A1, has been described as a biomarker for cancer stem cells or tumor initiating cells in many types of cancers, including head and neck cancer^{57,12-14,34,35}. Which ALDH1 isoforms, including ALDH1A1, are associated with cancer stem-like cells has not been addressed in the current literature including our study, which focuses on lymph node metastasis of HNSCC and did not explore other ALDH1 isoforms.

Increasing evidence shows a correlation between ALDH1 with metastasis and poor prognosis in breast, prostate, and adenoid cystic cancers. As a biomarker of normal and malignant human mammary stem cells, ALDH1 was first reported as a predictor of poor outcome for breast cancer by Ginestier *et al*⁷. Recently, Charafe-Jauffret *et al*. found that ALDH1 mediated metastasis in a breast cancer animal model and was associated with poor prognosis in inflammatory breast cancer³⁶. Similar observations were also reported in prostate cancer¹², adenoid cystic carcinoma³⁷, and pancreatic adenocarcinoma⁶. In head and neck cancer, Yu *et al* reported that in the ALDH1-positive population, Bim regulated the expression of Snail, an E-cadherin suppressor, and promoted lung metastasis in an animal model¹³. In another study, Chen *et al*. reported that Snail was co-expressed with the CD44⁺/CD24⁻/ALDH1⁺ population isolated from HNSCC patients, suggesting that the ALDH1-positive population may hold the property of epithelial-mesenchymal transition which is essential for cancer metastasis³⁴. It is currently unknown whether ALDH1 is a biomarker of metastasis or actually promotes metastasis.

Based on our observations, total ALDH1A1 expression may not be a biomarker for HNSCC stem cells since its percentage expression was high (larger than 25%) in the majority of HNSCC tissues, particularly in those with metastases. Due to the commercial availability of the antibody, current studies of ALDH1A1 expression in FFPE tissue cannot distinguish the total level of the protein from its active form, which might affect the accuracy of using ALDH1A1 as a biomarker for HNSCC stem cells.

Our current study suggests that ALDH1A1 is a potential biomarker for prediction of HNSCC lymph node metastasis. The possibility for quantification of multiple biomarkers using QD-IHF technology may enhance the specificity and sensitivity of metastasis prediction when compared with single biomarkers. A new QD-IHF-based multiplex biomarker technology is currently under development in our laboratory for the prediction of HNSCC metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Sladek NE. Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. *Journal of biochemical and molecular toxicology*. 2003; 17(1):7–23. [PubMed: 12616643]
- Duester G. Alcohol dehydrogenase as a critical mediator of retinoic acid synthesis from vitamin A in the mouse embryo. *The Journal of nutrition*. 1998; 128(2 Suppl):459S–62S. [PubMed: 9478048]
- Ross SA, McCaffery PJ, Drager UC, De Luca LM. Retinoids in embryonal development. *Physiological reviews*. 2000; 80(3):1021–54. [PubMed: 10893430]
- Dockham PA, Lee MO, Sladek NE. Identification of human liver aldehyde dehydrogenases that catalyze the oxidation of aldophosphamide and retinaldehyde. *Biochemical pharmacology*. 1992; 43(11):2453–69. [PubMed: 1610409]
- Clay MR, Tabor M, Owen JH, Carey TE, Bradford CR, Wolf GT, et al. Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. *Head Neck*. 2010; 32(9):1195–201. [PubMed: 20073073]
- Rasheed ZA, Yang J, Wang Q, Kowalski J, Freed I, Murter C, et al. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. *J Natl Cancer Inst*. 2010; 102(5):340–51. [PubMed: 20164446]
- Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell stem cell*. 2007; 1(5):555–67. [PubMed: 18371393]
- Chu P, Clanton DJ, Snipas TS, Lee J, Mitchell E, Nguyen ML, et al. Characterization of a subpopulation of colon cancer cells with stem cell-like properties. *International journal of cancer*. 2009; 124(6):1312–21.
- Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, et al. High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. *Journal of cellular and molecular medicine*. 2008
- Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene*. 2008; 27(47):6120–30. [PubMed: 18591932]

11. Ma S, Chan KW, Lee TK, Tang KH, Wo JY, Zheng BJ, et al. Aldehyde dehydrogenase discriminates the CD133 liver cancer stem cell populations. *Mol Cancer Res*. 2008; 6(7):1146–53. [PubMed: 18644979]
12. van den Hoogen C, van der Horst G, Cheung H, Buijs JT, Lippitt JM, Guzman-Ramirez N, et al. High aldehyde dehydrogenase activity identifies tumor-initiating and metastasis-initiating cells in human prostate cancer. *Cancer research*. 2010; 70(12):5163–73. [PubMed: 20516116]
13. Yu CC, Lo WL, Chen YW, Huang PI, Hsu HS, Tseng LM, et al. Bmi-1 Regulates Snail Expression and Promotes Metastasis Ability in Head and Neck Squamous Cancer-Derived ALDH1 Positive Cells. *J Oncol*. 2011; 2011
14. Douville J, Beaulieu R, Balicki D. ALDH1 as a functional marker of cancer stem and progenitor cells. *Stem cells and development*. 2009; 18(1):17–25. [PubMed: 18573038]
15. Joshua B, Kaplan MJ, Doweck I, Pai R, Weissman IL, Prince ME, et al. Frequency of cells expressing CD44, a Head and Neck cancer stem cell marker: Correlation with tumor aggressiveness. *Head Neck*. 2011
16. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104(3):973–8. [PubMed: 17210912]
17. Magni M, Shammah S, Schiro R, Mellado W, Dalla-Favera R, Gianni AM. Induction of cyclophosphamide-resistance by aldehyde-dehydrogenase gene transfer. *Blood*. 1996; 87(3):1097–103. [PubMed: 8562935]
18. Dylla SJ, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, et al. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS ONE*. 2008; 3(6):e2428. [PubMed: 18560594]
19. Sreerama L, Sladek NE. Primary breast tumor levels of suspected molecular determinants of cellular sensitivity to cyclophosphamide, ifosfamide, and certain other anticancer agents as predictors of paired metastatic tumor levels of these determinants. *Rational individualization of cancer chemotherapeutic regimens. Cancer chemotherapy and pharmacology*. 2001; 47(3):255–62. [PubMed: 11320670]
20. Patel M, Lu L, Zander DS, Sreerama L, Coco D, Moreb JS. ALDH1A1 and ALDH3A1 expression in lung cancers: correlation with histologic type and potential precursors. *Lung cancer (Amsterdam, Netherlands)*. 2008; 59(3):340–9.
21. Visus C, Ito D, Amoscato A, Maciejewska-Franczak M, Abdelsalem A, Dhir R, et al. Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck. *Cancer research*. 2007; 67(21):10538–45. [PubMed: 17974998]
22. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin*. 2010; 60(5):277–300. [PubMed: 20610543]
23. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005; 55(2):74–108. [PubMed: 15761078]
24. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA: a cancer journal for clinicians*. 2007; 57(1):43–66. [PubMed: 17237035]
25. Lentsch EJ. Management of cervical metastasis. *Curr Oncol Rep*. 2004; 6(2):141–51. [PubMed: 14751092]
26. Ferlito A, Silver CE, Rinaldo A. Neck dissection: present and future? *Eur Arch Otorhinolaryngol*. 2008; 265(6):621–6. [PubMed: 18286295]
27. Bruchez M Jr, Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. *Science*. 1998; 281(5385):2013–6. [PubMed: 9748157]
28. Smith AM, Gao X, Nie S. Quantum dot nanocrystals for in vivo molecular and cellular imaging. *Photochem Photobiol*. 2004; 80(3):377–85. [PubMed: 15623319]
29. Sreerama L, Sladek NE. Class 1 and class 3 aldehyde dehydrogenase levels in the human tumor cell lines currently used by the National Cancer Institute to screen for potentially useful antitumor agents. *Adv Exp Med Biol*. 1997; 414:81–94. [PubMed: 9059610]

30. Muller S, Su L, Tighiouart M, Saba N, Zhang H, Shin DM, et al. Distinctive E-cadherin and epidermal growth factor receptor expression in metastatic and nonmetastatic head and neck squamous cell carcinoma: predictive and prognostic correlation. *Cancer*. 2008; 113(1):97–107. [PubMed: 18473353]
31. Huang DH, Peng X, Su L, Wang D, Khuri FK, Shin DM, Chen ZG. Comparison and optimization of multiplexed quantum dot-based immunohistofluorescence. *Nano Res*. 2010; 3:61–68.
32. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010; 363(1): 24–35. [PubMed: 20530316]
33. Lowy DR, Munger K. Prognostic implications of HPV in oropharyngeal cancer. *N Engl J Med*. 2010; 363(1):82–4. [PubMed: 20530315]
34. Chen YC, Chen YW, Hsu HS, Tseng LM, Huang PI, Lu KH, et al. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. *Biochem Biophys Res Commun*. 2009; 385(3):307–13. [PubMed: 19450560]
35. Krishnamurthy S, Dong Z, Vodopyanov D, Imai A, Helman JJ, Prince ME, et al. Endothelial cell-initiated signaling promotes the survival and self-renewal of cancer stem cells. *Cancer research*. 2010; 70(23):9969–78. [PubMed: 21098716]
36. Charafe-Jauffret E, Ginestier C, Iovino F, Tarpin C, Diebel M, Esterni B, et al. Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor clinical outcome in inflammatory breast cancer. *Clin Cancer Res*. 2010; 16(1):45–55. [PubMed: 20028757]
37. Sun S, Wang Z. ALDH high adenoid cystic carcinoma cells display cancer stem cell properties and are responsible for mediating metastasis. *Biochem Biophys Res Commun*. 2010; 396(4):843–8. [PubMed: 20450887]

Figure 1. Quantification of QD-IHF signals using Nuance™ (CRi) imaging system

A: A spectral library composed of DAPI (blue), autofluorescence (black), QD605 (green) and QD655 (red) were pre-set before the analysis; B: cytokeratin (QD605) and DAPI; C: ALDH1A1(QD655) and DAPI; D: Mixed image of cytokeratin (QD605), ALDH1A1(QD655), and DAPI. DAPI and cytokeratin were used as tumor masks to define the area of interest (tumor cells). Based on the tumor mask, fifty cells were randomly selected from each of images acquired. Ten images obtained from each sample were used for the quantification. E: Immunoblotting analysis of ALDH1A1 in A549 cells which are known to express ALDH1A1 and ALDH3A1. A549 cells serve as a specificity control for anti-ALDH1A1 antibody.

Figure 2. Correlation between QD-based IHF and conventional IHC

A: ALDH1A1-positive tumor; B: ALDH1A1-negative tumor; C: Correlation between ALDH1A1 expression levels measured by IHC and by QD-IHF was calculated using Spearman's rank correlation. The result shows that these two methods are comparable ($R=0.75$, $p<0.0001$). D: Box-Plot for ALDH1A1 in non-metastatic and metastatic groups. The expression of ALDH1A1 in the metastatic group is significantly higher than that in the non-metastatic group ($p < 0.0001$).

Figure 3. Kaplan-Meier overall and disease free survival estimates for HNSCC patients stratified by ALDH1A1 expression level

All 96 patients were classified into low and high groups based on the cut-off value determined by the ROC model. HNSCC patients with low ALDH1A1 expression have higher 5-year survival rate ($p = 0.025$) (A) and 5-year disease free survival rate ($p = 0.065$) (B) than those with high ALDH1A1 expression.

Table 1

Summary of the clinicopathologic characteristics of patients with (Met) and without (Non-Met) lymph node metastasis

		No. of patients (%)		P
		Non-Met (n = 46)	Met (n = 50)	
Average age (SD), years		64.4 (11.3)	59.3 (12.3)	0.038 ⁺
Sex				0.188 [*]
	Male	29 (63.0)	38 (76.0)	
	Female	17 (37.0)	12 (24.0)	
T Stage				0.873 [*]
	T1	14 (30.4)	13 (26.0)	
	T2	17 (37.0)	18 (36.0)	
	T3	8 (17.4)	8 (16.0)	
	T4	7 (15.2)	11 (22.0)	
Tumor Stage				<0.0001 [*]
	I	14 (30.4)	0	
	II	17 (37.0)	0	
	III	8 (17.4)	12 (24)	
	IV	7 (15.2)	38 (76)	
Lymph node status				-
	N1	-	10 (20.0)	
	N2	-	36 (72.0)	
	N3	-	4 (8.0)	
Differentiation				0.004 [*]
	PD	6 (13.0) ^{**}	10 (20.0)	
	MD	27 (58.7)	38 (76.0)	
	WD	13 (28.3)	2 (4.0)	
Site				<0.0001 [*]
	Oropharynx (OP)	5 (10.9)	24 (48.0)	
	Larynx (L)	16 (34.8)	9 (18.0)	
	Oral cavity (OC)	25 (54.3)	17 (34.0)	
Smoking				0.366 [*]
	No	8 (20.0)	5 (10.9)	
	Yes	32 (80.0)	41 (89.1)	
Data missing		6	4	

^{*} Fisher exact test;

⁺ Two sample t test;

^{**} Including two UD (undifferentiated), WD, Well-differentiated; MD, Moderately-differentiated; PD, Poorly-differentiated.

Table 2

Univariate analysis of ALDH1A1 correlation with clinicopathologic characteristics in patients with (Met) and without (Non-Met) lymph node metastasis

Variable	Non-Met (n=46)				Met (n=50)			
	Estimate (SE)	P	Estimate (SE)	P	Estimate (SE)	P	Estimate (SE)	P
Age, β coef (SE)	0.039 (0.043)	0.367	0.014 (0.069)	0.841				
Variable	N	Mean(SD)	P	N	Mean(SD)	P		
Sex			0.619 ⁺			0.266 ⁺		
Male	29	1.343 (1.453)		38	5.034 (4.731)			
Female	17	2.804 (4.923)		12	4.853 (8.954)			
Smoking			1.000 ⁺			0.451 ⁺		
No	8	2.116 (2.818)		5	2.454 (1.157)			
Yes	32	1.358 (1.450)		41	5.477 (6.419)			
Site			0.712 [*]			0.045 [*]		
OP	5	1.692 (2.638)		24	3.902 (3.442)			
L	16	1.396 (1.340)		9	11.81 (10.18)			
OC	25	2.234 (4.123)		17	2.918 (2.365)			
T Stage			0.773 [*]			0.732 [*]		
T1	14	2.006 (2.114)		13	4.908 (5.038)			
T2	17	2.534 (4.905)		18	3.344 (2.570)			
T3	8	1.069 (0.997)		8	3.924 (2.836)			
T4	7	0.987 (0.493)		11	8.559 (10.19)			
Tumor stage			0.773 [*]			0.550 ⁺		
I	14	2.006 (2.114)		0	-			
II	17	2.534 (4.905)		0	-			
III	8	1.069 (0.997)		12	4.436 (5.091)			
IV	7	0.987 (0.493)		38	5.166 (6.196)			
Chemotherapy			0.313 ⁺			0.167 ⁺		
No	43	1.968 (3.327)		42	5.343 (6.232)			
Yes	2	0.530 (0.057)		8	3.140 (3.490)			

Variable	N	Mean(SD)	P	N	Mean(SD)	P
Radiation						
No	24	1.733 (1.922)	0.491 ⁺	4	3.550 (1.620)	0.609 ⁺
Yes	21	2.100 (4.373)		43	4.961 (6.214)	
Differentiation						
PD	6	1.715 (2.074)	0.578 [*]	10	3.017 (2.703)	0.308 [*]
MD	27	1.203 (1.228)		38	5.634 (6.525)	
WD	13	3.373 (5.535)		2	2.640 (1.810)	

^{*} Kruskal-Wallis test;

⁺ Wilcoxon Two-sample test

Table 3

Multivariable logistic regression model to assess independent association of ALDH1A1 expression level with lymph node metastasis

Parameter	Estimate	Standard Error	P	Odds Ratio	95% Confidence Interval
<i>Intercept</i>	0.1067	2.0521	0.9585	-	-
<i>ALDH1A1</i>	0.5112	0.1843	0.0055	1.667	1.162 2.393
<i>Age</i>	-0.0360	0.0279	0.1974	0.965	0.913 1.019
<i>Sex</i>	-0.3245	0.4459	0.4668	0.523	0.091 3.001
	<i>Male</i>				
	<i>Female</i>				
<i>T Stage</i>					
	<i>T4</i>	0.7598	0.6857	0.2678	0.604 39.005
	<i>T3</i>	0.7479	0.7608	0.3256	0.499 46.090
	<i>T2</i>	-0.6877	0.5514	0.2123	1.141 0.263 4.958
	<i>T1</i>	Baseline			
<i>Differentiation</i>					
	<i>WD</i>	-1.9462	0.8489	0.0219	0.057 0.003 0.926
	<i>MD</i>	1.0230	0.5128	0.0460	1.105 0.226 5.412
	<i>PD</i>	Baseline			
<i>Smoking</i>					
	<i>Yes</i>	0.6957	0.9394	0.4590	2.005 0.318 12.641
	<i>No</i>	Baseline			
<i>Site</i>					
	<i>OC</i>	0.1120	0.4989	0.8224	0.209 0.040 1.102
	<i>L</i>	-1.7870	0.6271	0.0044	0.031 0.004 0.257
	<i>OP</i>	Baseline			

Notes: OP, Oropharynx; L, Larynx; OC, Oral cavity; Logistic regression model is modeling the probability of metastasis.