Aldehyde Dehydrogenase-1 Predicts Favorable Prognosis in Patients with Vulvar Squamous Cell Carcinoma

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Abstract. *Backgrounds: Aldehyde dehydrogenase-1 (ALDH1) has been considered as a potential cancer stem cell marker in different types of cancer. In the present study, we investigated the expression of ALDH1 in vulvar squamous cell carcinoma, and evaluated its correlation with clinicopathological factors in patients suffering from this disease. Materials and Methods: One hundred and fifty-four patients with vulvar squamous cell carcinoma, together with their verified histopathological and complete clinical data in Norway were included in the study. All paraffin-embedded samples of the primary vulvar carcinoma were recruited. The presence of ALDH1 was detected by immunohistochemistry and compared against commonly recognized prognostic factors. Results: By immunohistochemical staining, the expression of ALDH1 was observed in 10/154 (6.5%) vulvar squamous cell carcinomas, while being extensively expressed in the suprabasal cells in normal vulvar epithelia from patients with benign gynecological disease and non-malignant epithelia adjacent to the tumor cells. In addition, ALDH1 was highly expressed in stromal fibroblasts, blood vessels and keratinized pearl of the carcinoma in all the samples. Patients with ALDH1-positive tumors had a significantly longer disease-specific survival (p=0.042). Conclusion: Contrary to the characteristics of cancer stem cells shown in other types of cancer with positive expression of ALDH1, the positive expression of ALDH1 in patients with vulvar squamous cell carcinoma indicates a significantly better prognosis. Furthermore, there is a trend that the expression of ALDH1 is associated with better histological differentiation.*

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Vulvar carcinoma is an uncommon type of tumor accounting for approximately 3% to 5% of all female genital tract cancers and 6% of all cancers in women (1). In Norway, vulvar carcinoma accounts for 5% of all female gynecological cancers (http://www.oncolex.no/en/Gynecological-cancer/Diagnoses/Vulvar-cancer/Background/References). The etiology of this type of cancer is still not clear, since it is associated with both chronic vulvar inflammatory lesions and vulvar intraepithelial neoplasia. Squamous cell carcinoma accounts for 85-95% of invasive carcinomas of the vulva, which includes the following common types: keratinized squamous cell, non-keratinized squamous cell, basaloid and warty (condylomatous) carcinoma (2). Verrucous carcinoma is a distinct variant of squamous cell carcinoma and associated with human papillomavirus type 6. The important prognostic factors for survival of patients with vulvar carcinoma are age, lymph node metastasis, tumor grade and International Federation of Gynecology and Obstetrics (FIGO) stage (3), which predict for metastatic progression and outcome of different treatments for individual patients. The molecular mechanism and pathogenic processes of this disease have not been yet elucidated. More biomarkers are required to define the profiles of vulvar carcinoma.

In recent years, the cancer stem cell (CSC) model has been proposed to explain the development of different kinds of cancer, including acute myeloid leukemia, and cancer of the breast, brain, prostate, colon and pancreas (4-9). Cancer is driven by a small sub-population of stem cells with the properties of self-renewal, chemoresistance and tumor-initiating ability. However, little is known about CSC-associated properties in vulvar carcinoma.

Aldehyde dehydrogenase-1 (ALDH1), a detoxifying enzyme that oxidizes intracellular aldehydes (10), is considered a putative and universal marker for the identification and isolation of CSCs in various types of human epithelial cancer (11-14).

In the present study, we investigated the expression of ALDH1 in vulvar squamous cell carcinoma and clarified its relation with clinicopathological characteristics in a cohort of Norwegian patients with vulvar squamous cell carcinoma. Expression of ALDH1 in stromal tissues and normal vulvar epithelia was also evaluated.

Materials and Methods

Patient and samples. A retrospective study was performed on 154 patients diagnosed with vulvar squamous cell carcinoma who underwent operation at The Norwegian Radium Hospital of Oslo University between 1977 and 2004. The detailed clinical data of the patients including age, FIGO stage, grade of differentiation, tumor size, depth of invasion, and infiltration of vessels are summarized in Table I. Tumors were all staged in terms of the FIGO classification from the 2009 Revised FIGO staging (15) for carcinoma of the vulva, cervix, and endometrium. Histological type was classified according to the World Health Organization recommendation for vulvar tumors (16). The type and grade of histology in these specimens were reviewed by two pathologists in the Department of Pathology, the Norwegian Radium Hospital, who were blinded to clinical information. This study was approved by the regional Committee for Medical Research Ethics South of Norway (S-06012), the Social and Health Directorate (04/2639 and 06/1478) and the Data Inspectorate (04/01043), Norway.

Immunohistochemistry. Immunohistochemical staining was applied on the formalin-fixed, paraffin-embedded sections using the Dako EnVision™ Flex+ System (K8012; Dako, Glostrup, Denmark) and the Dako Autostainer. Briefly, 4 μm-thick serial sections were prepared. De-paraffinization and unmasking of epitopes were performed using PT-Link and EnVision™ Flex target low PH retrieval solution (Dako, Denmark). To block endogenous peroxidase, the sections were treated with 0.03% hydrogen peroxide for 5 min. The sections were incubated with the following reagents: rabbit polyclonal antibody against human ALDH1A1 (1:3000; ab63026, Cambridge, UK) for 30 min, EnVision™ Flex+ rabbit linker for 15 min and EnVision™ Flex/HRP (horse radish peroxydase) enzymes for 30 min. The staining was visualized using 3'3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. All sections were finally dehydrated and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Scientific, Waltham, MA, USA). Appropriate negative controls (replacement of the mouse monoclonal antibody with non-immune mouse IgG at the same concentration as the primary antibody) and positive controls (human liver tissue) were included. As normal controls, the normal vulvar epithelia collected from 10 patients who had undergone surgery for benign gynecological disease were used in the study.

Evaluation of immunostaining was executed independently by two observers with no clinical patient information. All discordant cases were reviewed by a third observer to reach a final agreement. *Statistical analysis*. The Chi-square test and Spearman correlation coefficient were performed for statistical analysis. The correlation of the expression of ALDH1 with clinicopathological variables was analyzed. Disease-specific survival curve was processed through Kaplan and Meier method and compared with the use of the two-sided log-rank test. *p*-Values less than 0.05 were regarded as statistically significant in all of the analyses. Statistical analyses of data were processed by using the SPSS17.0 statistical software package (SPSS, Chicago, IL USA).

Results

Characteristics of the studied population. The clinicopathological characteristics of the 154 patients with vulvar carcinoma are listed in Table I. Briefly, the median age of the patients at-diagnosis was 71 (range=35-96). All patients were followed-up from the confirmed diagnosis until death or September 1st, 2009. The median follow-up time was 90 (range=22 days to 378). Three types of vulvar carcinoma were involved, 145 (94.2%) were keratinizing/non-keratinizing, seven (4.6%) were basaloid and two (1.2%) were veruccoid. The overall survival of the patients with vulvar carcinoma was significantly associated with age, FIGO stage, differentiation, tumor diameter and infiltration of vessels ($p<0.05$).

Expression of ALDH1 protein in vulvar carcinomas is lower than that in non-malignant vulvar epithelia. The results of immunohistochemical staining of vulvar carcinomas and normal vulvar epithelia are shown in Figure 1. In normal vulvar epithelia, ALDH1 protein was expressed in suprabasal cells (Figure 1a and b). In the 154 cases of vulvar carcinomas, expression of ALDH1 was detected as having two different patterns: expression of ALDH1 was discovered in the well-keratinized tumor cells surrounding the keratin pearl and inside the keratin pearl (Figure 1c and d); ALDH1 protein was homogeneously expressed throughout the tumoros areas (Figure 1e). Only positive staining in non-keratinized tumor cells was considered as positive tumor expression, while positivity in keratinized tumor cells was regarded as negative.

Among the 154 samples, only 10 (6.5%) expressed ALDH1, and the rest was negative for ALDH1 expression (Figure 1f). All samples exhibited scattered keratinizing cells both in the tumor and in the relative normal vulvar epithelial adjacent to the tumor cells stained positively by an antibody against ALDH1. In addition, ALDH1 protein was also expressed in stromal fibroblasts, blood vessels or lymphocytes infiltrating tumor cells in all specimens, including vulvar carcinomas and non-malignant vulvar epithelia. ALDH1 expression was present in all histological subtypes. As a positive control, liver tissue showed strong and uniform staining of ALDH1 (Figure 1g).

Table I. *Clinicopathological features of patients with vulvar carcinoma and their relationship with expression of Aldehyde Dehydrogenase-1 (ALDH1).*

Variable	Total	Expression		*p*-Value
		Negative	Positive	
Age, years				0.529
25-69	69	66	3	
70-84	72	66	6	
85+	13	12	1	
Differentiation grade				0.622
High	34	32	2	
Middle	85	78	7	
Low	35	34	1	
FIGO stage				1.0
Ia-Ib	13	12	1	
II	66	61	5	
IIIa, IIIb, IIIc	67	63	4	
IVb	5	5	0	
Tumor diameter (mm)				0.524
0.3-2.5	30	29	1	
2.6-4.0	58	55	3	
4.1-20	64	58	6	
Depth of invasion (mm)				0.471
0-4	29	26	3	
4.1-8	54	52	2	
8.1-40	71	66	5	
Infiltration of vessels				0.233
No	121	115	6	
Yes	33	29	4	

Correlations between ALDH1 expression and clinicopathological factors. To evaluate the prognostic value of ALDH1 in vulvar carcinoma, survival curve was calculated by the Kaplan–Meier method and compared using the log-rank test. The overall survival of the patients with positive ALDH1 expression was better-compared to those without expression or with only scarcely-scattered expression in keratinized squamous cells (Figure 2).

Nine out of the 10 ALDH1-positive vulvar carcinomas were well-to moderately-differentiated; the tenth was poorly-differentiated, however, fewer than 50% of tumor cells were ALDH1-positive (Figure 1h) in this case whereas more than 90% of tumor cells were positive in the other nine samples.

In univariate analysis, no significant correlations between intra-tumoral epithelial expression of ALDH1 and age, FIGO stage, vessel infiltration, tumor diameter and histological type were found.

Discussion

ALDH1 is a cytosolic detoxifying enzyme responsible for converting aldehydes to carboxylic acids (10) and is distributed ubiquitously in a wide range of normal tissues (11, 17-19). In recent years, it has been identified with stem cells characteristics. Firstly, it was suggested as a surrogate biomarker for hematopoietic stem cells (20, 21). Further studies showed that it is related to the cancer stem cell phenotype. Ginestier *et al*. first showed that a very low number of ALDH1-positive cells was able to form a breast tumor when implanted in the fat pad of nude mice, and the presence of cancer cells with such a stem cell marker was associated with a worse outcome (11). These studies demonstrated that ALDH1 may be a specific marker for breast CSCs. ALDH1 was later identified as a CSC-specific marker in other types of human solid cancer, such as colorectal cancer (22) and lung cancer (23). It should be noted that ALDH1 may be involved in regulating cellular differentiation (24, 25) and stem cell proliferation (22) particularly through the retinoid signaling pathway (11, 24). Since it is involved in detoxification (24, 25), ALDH1 has also been proposed as a therapeutic target because of its function in resistance to chemotherapy in some types of cancer (26-28).

However, the theory of CSCs has not been applied very well to vulvar carcinoma. No publication has shown any reliable CSC markers involved in this type of cancer. Therefore, in the present study, the promising cancer stem cell marker ALDH1 was evaluated in vulvar carcinoma. Nine out of the ten samples of vulvar carcinoma with ALDH1 expression in more than 90% of the tumor cells were found in the group of moderately- and well-differentiated tumors, whereas one sample of low histological grade had fewer than 50% ALDH1-positive tumor cells. It seems that positive expression of ALDH1 is associated with better differentiation, although without statistical significance, which is in accordance with previous studies (29-31). The more significant finding is that positive expression of ALDH1 is associated with favorable prognosis in patients with vulvar carcinoma, which differs from the previous impression that ALDH1 was inversely associated with most malignant tumors (13, 32-35). However, it has recently been suggested that ALDH1 may not have the same implication as a CSC marker in all cases. ALDH1 expression was shown to predict favorable prognosis in pancreatic (35), ovarian (36), and non-small cell lung cancer (37), and hepatocellular carcinoma (38). This discrepancy might be due to the heterogeneous expression in tumor samples, or the different role of ALDH1 in individual tumor types. Another important reason is that there was no unique scoring standard used to evaluate the immunostaining results. In the articles by Kitamura *et al*. (39), Charafe-Jauffret *et al*. (40) and Zhang *et al*. (41), tumors presenting at least one ALDH1-positive cancer cell were considered to be ALDH1-positive. In other studies (11, 42, 43), tumors with more than 1% of cells with ALDH1 expression were evaluated as positively-stained. Moreover, both the extensity and intensity were considered in evaluation of the ALDH1 expression (44).

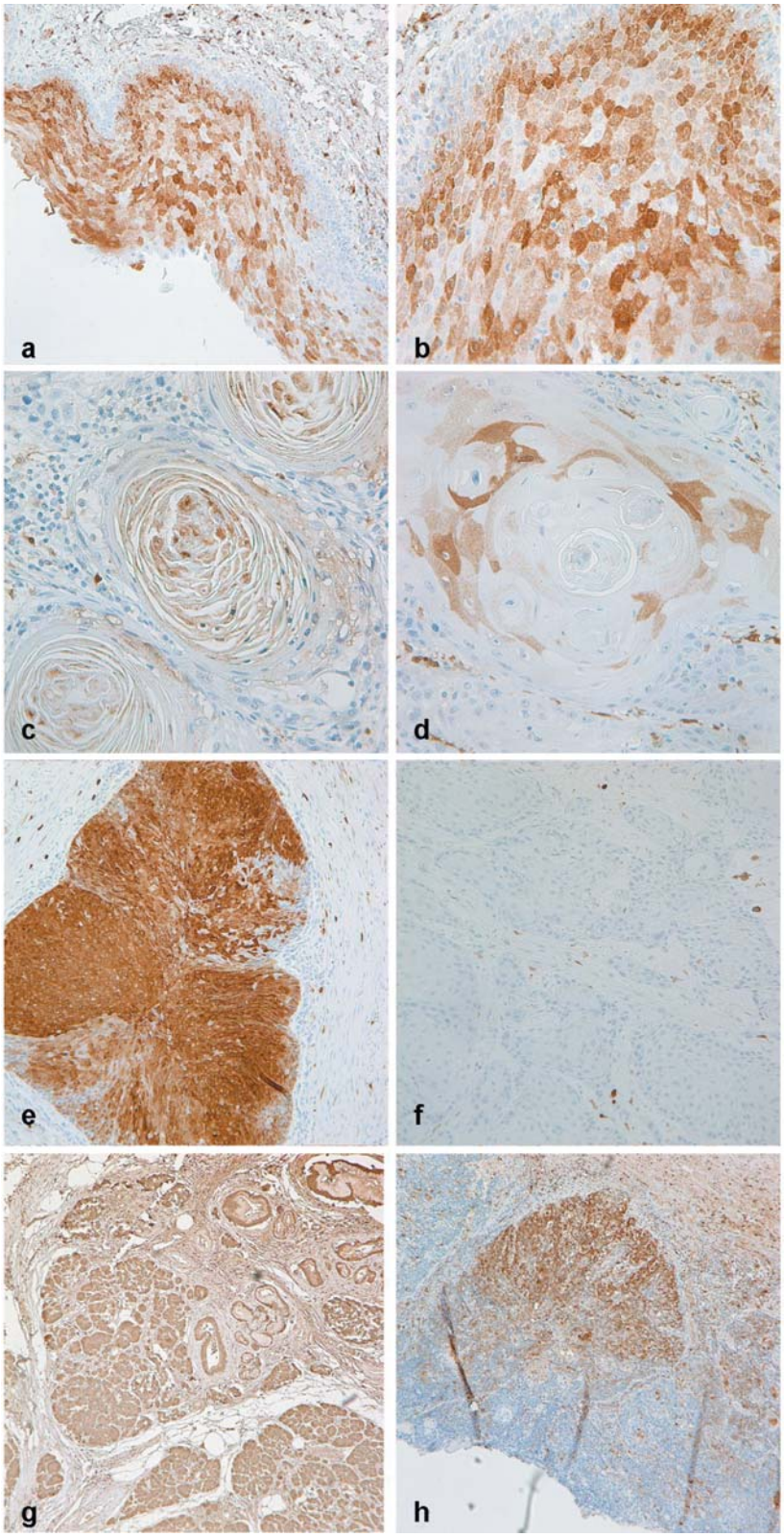

Figure 1. *Expression of Aldehyde dehydrogenase-1 (ALDH1) in normal vulva and vulvar carcinomas. Expression of ALDH1 in suprabasal cells in normal vulvar epithelia (a, b); in keratinized pearl in vulvar carcinoma (c); in keratinized cells (d); in vulvar carcinioma (e); negative expression of vulvar carcinoma (f); positive control (g); in poorly differentiated vulvar carcinoma (h).*

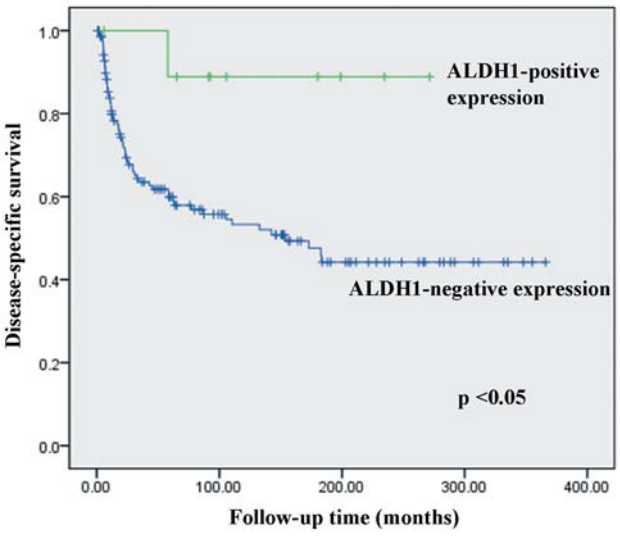

Figure 2. *Positive expression of ALDH1 is associated with better survival in patients with vulvar carcinoma.*

Distinctive ALDH1 expression patterns in normal tissues and correlations with their corresponding tumors have been reported. As documented by Deng *et al*. (17), there were three types of ALDH1 expression pattern in normal tissues: absent or limited expression in breast and lung, relatively weak expression in colon and gastric epithelia, and extensive and high expression in liver and pancreas. According to Yoshitaka *et al*., no expression of ALDH1 was identified in normal oral squamous cell epithelia (45). ALDH1 expression was significantly reduced in malignant ovarian tumors, while relatively unchanged in benign tumors compared to normal ovary (46). In the present study, scarcely scattered ALDH1-positive cells were found in all samples which had well-keratinized cells. The expression of ALDH1 in normal epithelia of vulva was high but not that extensive, and the same pattern was found in non-malignant epithelia adjacent to tumor cells. ALDH1 may serve as a marker of progenitor cells in normal tissue, or loss of ALDH1 may be an additional step of carcinogenesis in specific tumor types.

In the present study, expression of ALDH1 was detected all over the stroma both in non-malignant vulvar epithelia and vulvar carcinomas, as seen in other study (11). Tumors with high stromal expression had the best outcome (47). The tumor microenviroment may be as important as tumor cells in determining prognosis. However, there was no difference in the expression of ALDH1 in stroma between normal epithelia and vulvar carcinomas in the present study.

Generally, tumor stage is the most informative baseline information that defines prognosis and aids treatment decision. Other histological factors are also involved in the prognosis. In this cohort of vulvar carcinomas, the unfavorable prognosis of these patients were also found to be related to increased age, FIGO stage, grade of differentiation, tumor diameter and infiltration of vessels. However, the expression of ALDH1 in the tumor cells was not correlated with these factors. This is in line with most of the previous studies (18, 48, 49), although Yoshioka *et al*. (50) showed that ALDH1 expression was correlated with larger tumor size in node-positive breast cancer, and correlated with lymph node metastasis in oral squamous cell carcinoma (45). Usually in solid tumors, cancer stem cells are only a small population of cells, express a stem cell phenotype and have higher tumor initiating ability (51). In the present study, in nine vulvar carcinomas, the extensity of ALDH1 expression was more than 50%. Therefore, we postulate that ALDH1 might not be a cancer stem marker in vulvar carcinoma.

In summary, we found that ALDH1 predicts favorable prognosis in patients with vulvar carcinoma, indicating that ALDH1 may be a marker of well-differentiated keratinized cells. However, further studies on larger cohorts are required to confirm the value of ALDH1 as a biomarker for prognosis and uncover the function of ALDH1 in vulvar carcinogenesis.

Conflicts of Interest

None declared.

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