

# Expression of ALDH1A1 and CD44 in primary head and neck squamous cell carcinoma and their value for carcinogenesis, tumor progression and cancer stem cell identification

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**Abstract.** In head and neck squamous cell carcinoma (HNSCC), aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) and hyaluronan receptor cluster of differentiation 44 (CD44) are often used as cancer stem cell (CSC) markers. The aim of the present study was to examine the relevance of these proteins for HNSCC in general and for the identification of CSCs. Tumors from 48 patients with primary HNSCC were analyzed for the expression of ALDH1A1 and CD44. Additionally, the association of the proteins with the proliferation rate and epidermal growth factor receptor (EGFR) expression was analyzed. ALDH1A1 was expressed in 54.2% of the carcinoma samples while CD44 was expressed in 89.6% of the carcinoma samples. Most notably, these proteins were often not expressed exclusively in a subpopulation, but also in the majority of tumor cells (ALDH1A1: 30.8% of ALDH1A1<sup>+</sup> tumors; CD44: 65.1% of CD44<sup>+</sup> tumors). Furthermore, patients with ALDH1A1<sup>+</sup> tumors exhibited worse survival rates. CD44 and EGFR expression patterns were overlapping within the tumors and the expression rates were significantly connected. Ki-67<sup>+</sup> tumor cells often expressed CD44. ALDH1A1 and CD44 expression patterns only partly overlapped. Consequently, ALDH1A1 and CD44 play significant roles in carcinogenesis and tumor progression. Within the present study, CD44 appeared to interact with EGFR and was more often expressed in primary HNSCC than the marker ALDH1A1. However, ALDH1A1 was a better marker to define a subpopulation of tumor cells. Finally, neither ALDH1A1 nor CD44, alone or combined, were sufficient to determine the CSC population in HNSCC.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common entity of the upper aerodigestive tract (1). Carcinoma of this area is the fourth most common region of cancer incidence and the second most common region for cancer-related mortality worldwide (2). The intramural heterogeneity may be an explanation for the worse survival rates recorded in HNSCC. Cancer stem cells (CSCs) are the most important subpopulation within carcinoma. CSCs are associated with a worse response to therapy, metastasis and cancer relapse (3). Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) and hyaluronan receptor cluster of differentiation 44 (CD44) are common markers for CSCs in HNSCC (4-6).

ALDH1A1 belongs to the ALDH enzyme family and catalyzes the oxidation of aldehydes to carboxylic acids (7). This enzyme mediates protection from chemotherapy and reactive oxygen species (ROS) (7,8).

CD44 is an important hyaluronan receptor. CD44 acts in cell aggregation, proliferation and migration (9). In HNSCC, CD44 is associated with tumor invasion (10) and the poor survival of tumor patients (11).

The present study analyzed the expression of ALDH1A1 and CD44 for their relevance in human primary HNSCC. The prognostic value of ALDH1A1 expression was estimated. Moreover, the utility of ALDH1A1 and CD44 was evaluated in order to identify CSCs. To define the CSC population and its activity more precisely, features such as proliferation (Ki-67) and growth receptor expression [epidermal growth factor receptor (EGFR)] were included into the analysis.

## Materials and methods

**Patients.** In this study, 48 patients with primary HNSCC (35 male and 13 female) were investigated. Tumor samples were collected between 1997 and 2008. Experiments were approved by the Ethics Committee of the Department of Medicine of the Johann Wolfgang Goethe-University, Frankfurt am Main (276/12).

**Sample preparation and analysis.** All available samples of each primary tumor were examined. For immunohistochemistry,

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Table I. ALDH1A1 and/or CD44 expression in human primary tumors.<sup>a</sup>

Expression	ALDH1A1 and/or CD44 expression, n (%)			
	ALDH1A1 <sup>+</sup>	CD44 <sup>+</sup>	ALDH1A1 <sup>+</sup> /CD44 <sup>+</sup>	ALDH1A1 <sup>-</sup> /CD44 <sup>-</sup>
General expression	26 (54.2)	43 (89.6)	24 (50.0)	3 (6.3)
Majority ALDH1A1 <sup>+</sup>	8 (30.8)	8 (18.6)	8 (33.3)	-
Minority ALDH1A1 <sup>+</sup>	18 (69.2)	16 (37.2)	16 (66.7)	-
ALDH1A1 <sup>-</sup>	-	19 (44.2)	-	3 (100.0)
Majority CD44 <sup>+</sup>	13 (50.0)	28 (65.1)	11 (45.8)	-
Minority CD44 <sup>+</sup>	11 (42.3)	15 (34.9)	13 (54.2)	-
CD44 <sup>-</sup>	2 (7.7)	-	-	3 (100.0)

<sup>a</sup>CD44 and/or ALDH1A1 were expressed in 93.8% of carcinoma samples. CD44 was more often expressed, but ALDH1A1 more frequently represented a minority of tumor cells. ALDH1A1, aldehyde dehydrogenase 1 family, member A1; CD44, hyaluronan receptor cluster of differentiation 44.

Table II. Staining intensity of CD44 and EGFR in human primary tumors. Linear regression of expression (P≤0.01).

Protein	Staining intensity of CD44 and EGFR, n (%)			
	-	+	++	+++
EGFR	1 (2.1)	12 (25.0)	9 (18.8)	26 (54.2)
CD44	5 (10.4)	5 (10.4)	14 (29.2)	24 (50.0)

ALDH1A1, aldehyde dehydrogenase 1 family, member A1; CD44, hyaluronan receptor cluster of differentiation 44.

formaldehyde-fixed paraffin-embedded tumor samples, cut to a size of 5 μm, were used. Antigen retrieval was performed in boiling citrate buffer (pH 6.0; S1699; Dako, Glostrup, Denmark). ALDH1A1 and CD44 are common markers for the identification of CSC (4-6), therefore, these markers were used to identify tumor cell subpopulations comprised of CSCs. The expression of anti-human rabbit monoclonal ALDH1A1 (1/100, ab52492; Abcam, Cambridge, UK), anti-human mouse monoclonal CD44 (1/100, MU310-UC; BioGenex, Fremont, CA, USA), anti-human mouse monoclonal EGFR (1/50, ab49716; Abcam) and anti-human rabbit monoclonal Ki-67 (1/200, KI68IC01: DCS, Hamburg, Germany) were detected. The samples were incubated with primary antibody for 1 h at room temperature. In the next step of immunohistochemistry staining procedure, the DCS Detection Line system staining kits (AD050POL-K, PD000RP and DD006RAP; DCS, Hamburg, Germany) were used. Staining was developed with DAB reagent (DC137C100; DCS) and a Fuchsin-Substrate-Chromogen System (K0625; Dako). Images were captured with a Zeiss Axioplan2 (AxioCam ICc1 camera; Zeiss, Oberkochen, Germany). The staining intensity of CD44 and EGFR in the tumor cells was scaled as follows: Strong (+++), moderate (++), weak (+) and none (-). Weak staining was described as less intensive and/or discontinuous membrane staining of the tumor cells.

So far as documented, the clinical history of the patients and the tumor-node-metastasis (TNM) status (12) of the carcinoma were analyzed.

**Statistical analysis.** The statistical analysis of data was performed with BiAS software for Windows (version 10.12; Epsilon-Verlag, Frankfurt, Germany). The significance between CD44 and EGFR was analyzed by Yates-Cochran test of trends. The significance of cancer relapse for the ALDH1A1 expression groups (+/-) was analyzed by Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**General ALDH1A1 and CD44 expression.** The majority of primary HNSCC tumors (45/48; 93.8%) expressed a minimum of one protein (Table I). Only three samples (6.3%) expressed neither ALDH1A1 nor CD44. CD44 expression was detected in 89.6% (43/48) of studied tumors. CD44 expression intensity was scaled within the tumor cells (Table II). In 55.8% (24/43) of the CD44<sup>+</sup> tumors expression for the receptor was strong (+++), in 32.6% (14/43) the CD44 expression was moderate (++) and in 11.6% (5/43) of samples the CD44 level was weak (+). In 65.1% (28/43) of the CD44<sup>+</sup> tumors this receptor was expressed in almost all tumor cells. The majority of CD44<sup>+</sup> tumors (38/43, 88.4%) expressed this receptor at a strong to moderate level (++/+++). Tumor cell nests were solely CD44<sup>+</sup> or CD44-expressing tumor cells were located in the periphery of the tumor cell nests and close to the stroma (Fig. 1D).

ALDH1A1 expression was detected in 54.2% (26/48) of the studied tumor samples (Table I). In 30.8% (8/26) of the

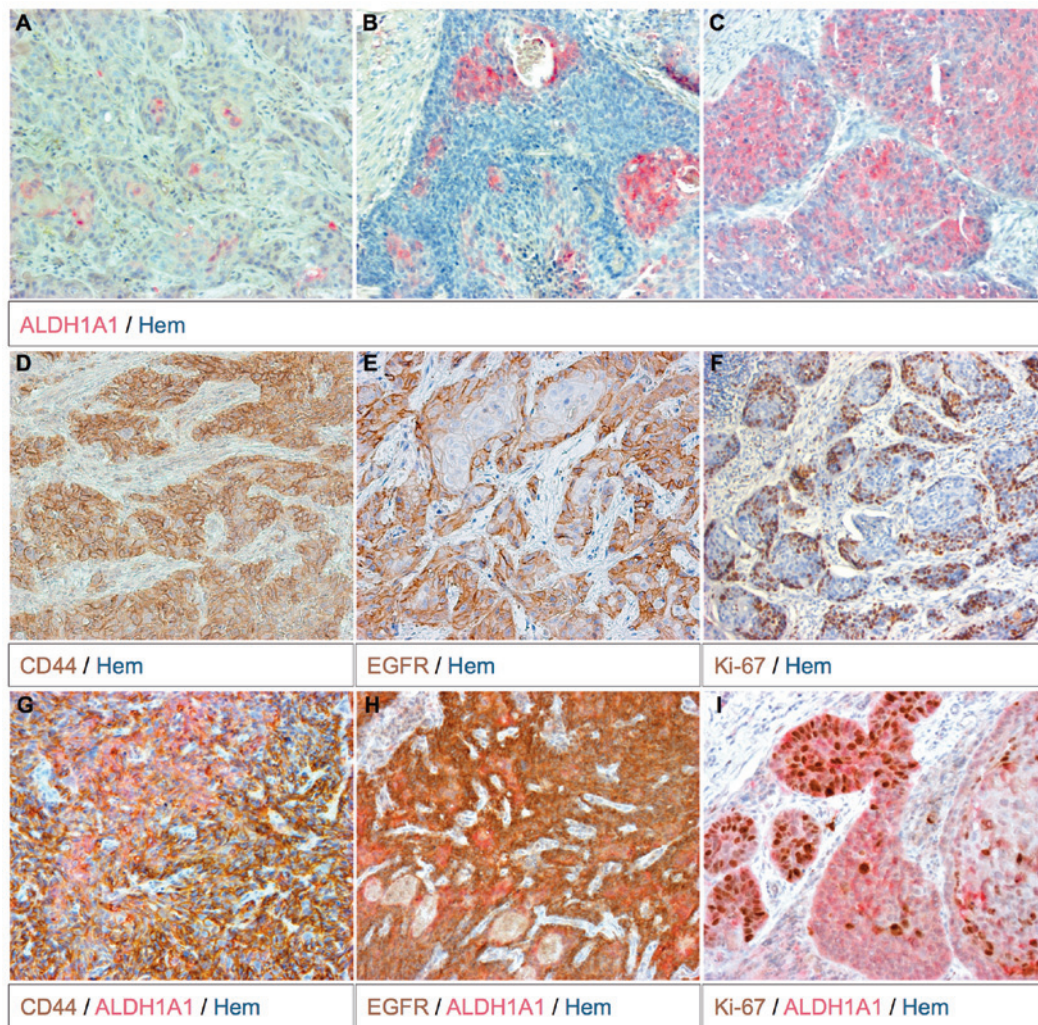


Figure 1. Immunohistochemistry of human primary tumors. (A and B) ALDH1A1<sup>+</sup> cells were located singularly, in groups and more centrally within cell nests. (C) Tumor expressing ALDH1A1 completely. (D-F) CD44, EGFR and Ki-67 were expressed marginally to generally within the cell nests. (G and H) ALDH1A1 expression pattern only partly overlapped with CD44 or EGFR. (I) The rate of Ki-67<sup>+</sup> cells was independent from ALDH1A1 expression. Magnification, x200; hem counterstaining. ALDH1A1, aldehyde dehydrogenase 1 family, member A1; CD44, hyaluronan receptor cluster of differentiation 44; EGFR, epidermal growth factor receptor; hem, hematoxylin.

ALDH1A1<sup>+</sup> tumors this enzyme was expressed in the majority of tumor cells (Fig. 1C). In the remaining ALDH1A1<sup>+</sup> tumors (18/26, 69.2%) ALDH1A1-expressing cells were found to be singular, in groups or throughout the entirely cell nest (Fig. 1A and B). More often ALDH1A1<sup>+</sup> tumor cell groups were located more centrally within the cell nests (Fig. 1A and B).

Half (24/48) of the analyzed tumors expressed ALDH1A1 and CD44 (Table I). In these tumors the expression pattern of ALDH1A1 and CD44 intersected, but did not completely overlap (Fig. 1G). With the exception of one tumor (7/8, 87.5%), the human papilloma virus (HPV)<sup>+</sup> tumors expressed ALDH1A1 and CD44. The remaining HPV<sup>+</sup> tumor showed CD44 staining (Table III).

**EGFR and Ki67 status of ALDH1A1<sup>+</sup> or CD44<sup>+</sup> tumor cells.** With the exception of one tumor, the examined HNSCC tumors expressed EGFR (47/48, 97.9%) (Table II). In most tumors the majority of tumor cells were EGFR<sup>+</sup>. The staining intensity of EGFR was scaled as strong to moderate (++) in 74.5% (35/47) of EGFR<sup>+</sup> tumors. The staining patterns of CD44 and

EGFR were mostly overlapping. The position of the EGFR<sup>+</sup> and CD44<sup>+</sup> tumor cells was generally identical. However, EGFR<sup>+</sup> tumor cells were located more marginally within the tumor cell nests (Fig. 1E). The association between EGFR and CD44 staining intensity could be described as an linear regression ( $P \leq 0.01$ ). In the majority of tumors, ALDH1A1<sup>+</sup> tumor cells were not necessarily CD44<sup>+</sup> (Fig. 1G). EGFR staining gave similar results (Fig. 1H). The amount of Ki-67<sup>+</sup> tumor cells also appeared to be independent of ALDH1A1 expression (Fig. 1I). Closer to the stroma the proliferation rate was higher (Fig. 1F).

**Analysis of ALDH1A1<sup>+</sup> and ALDH1A1<sup>-</sup> patients.** The patient collective was divided into ALDH1A1<sup>+</sup> and ALDH1A1<sup>-</sup> primary tumor samples. Notably, the majority of confirmed tonsillar tumors (11/16; 68.8%) were ALDH1A1<sup>+</sup>. Due to the low number of CD44<sup>-</sup> tumors (5/48; 10.4%), an analysis of CD44<sup>+</sup> and CD44<sup>-</sup> samples was not meaningful.

The appearance of metastasis and relapse was analyzed (Table III). In the majority of cases, cancer relapse was

Table III. Evaluation of available patient data for ALDH1A1 expression and HPV status.<sup>a</sup>

Factor	Evaluation of patient data, n (%)			
	ALDH1A1 <sup>+</sup>	ALDH1A1 <sup>+</sup> /HPV <sup>-</sup>	ALDH1A1 <sup>-</sup>	ALDH1A1 <sup>-</sup> /HPV <sup>-</sup>
Total patients	26 (100.0)	19 (100.0)	22 (100.0)	21 (100.0)
Gender				
Male	20 (76.9)	14 (73.7)	15 (68.2)	15 (71.4)
Female	6 (23.1)	5 (26.3)	7 (31.8)	7 (33.3)
Cancer relapse				
Positive	6 (23.1)	6 (31.6)	12 (54.5)	12 (57.1)
Negative	20 (76.9)	13 (68.4)	10 (45.5)	9 (42.9)
Age, years				
<60	10 (38.5)	7 (36.8)	13 (59.1)	12 (57.1)
60-70	9 (34.6)	9 (47.4)	7 (31.8)	7 (33.3)
>70	7 (26.9)	3 (15.8)	2 (9.1)	2 (9.5)
T stage	25 (100.0)	18 (100.0)	18 (100.0)	17 (100.0)
T1	0 (0.0)	0 (0.0)	4 (22.2)	4 (23.5)
T2	12 (48.0)	9 (50.0)	7 (38.9)	7 (41.2)
T3	9 (36.0)	5 (27.8)	5 (27.8)	4 (23.5)
T4	4 (16.0)	4 (22.2)	2 (11.1)	2 (11.8)
N stage	24 (100.0)	17 (100.0)	18 (100.0)	17 (100.0)
N0	8 (33.3)	4 (23.5)	6 (33.3)	6 (35.3)
N1-N3	16 (66.7)	13 (76.5)	12 (66.7)	11 (64.7)

Patients with ALDH1A1<sup>+</sup> tumors exhibited a reduced risk for relapse ( $P \leq 0.05$ ). Metastasis into lymph nodes (N status) between ALDH1A1<sup>+</sup> and ALDH1A1<sup>-</sup> tumors was equal. ALDH1A1, aldehyde dehydrogenase 1 family, member A1; CD44, hyaluronan receptor cluster of differentiation 44; HPV, human papilloma virus.

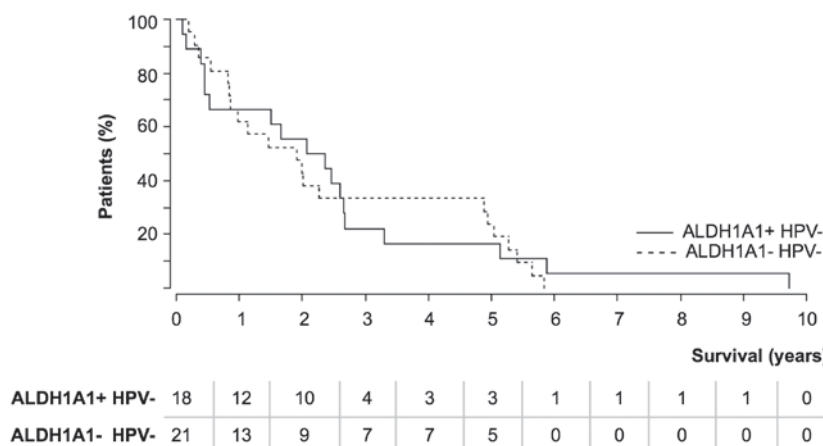


Figure 2. Survival rates of patients with ALDH1A1<sup>+</sup> or ALDH1A1<sup>-</sup> tumors with known day of mortality. Patients with ALDH1A1<sup>+</sup> tumors appeared to exhibit reduced survival. (HPV was an independent prognostic factor for better survival). ALDH1A1, aldehyde dehydrogenase 1 family, member A1; CD44, hyaluronan receptor cluster of differentiation 44; HPV, human papilloma virus.

observed up to 2 years after primary tumor detection. Cancer relapse occurred in 23.1% (6/26) of patients with ALDH1A1<sup>+</sup> primary tumors. The number of relapses was significantly higher ( $P \leq 0.05$ ) for patients with ALDH1A1<sup>-</sup> primary tumors (12/22; 54.5%). No notable differences were found between the TNM stage of ALDH1A1<sup>+</sup> and ALDH1A1<sup>-</sup> tumors. However, one explanation for the increased number of relapses resulted

in the comparison of overall survival (Fig. 2). As HPV is a prognostic factor for longer survival (13), HPV<sup>+</sup> cases were excluded.

The majority of patients in the two groups succumbed within the first 3 years of tumor appearance. Within the ALDH1A1<sup>+</sup> (HPV<sup>-</sup>) tumor group, fewer patients (4/18; 22.2%) survived longer than 3 years compared with the ALDH1A1<sup>-</sup> (HPV<sup>-</sup>)

tumor group (7/21; 33.3%). This issue could not be explained by a younger age of the ALDH1A1<sup>-</sup> tumor patients, as the analysis of different age groups (<60, 60-70 and >70 years) gave comparable results (data not shown). Survival was not linked to the percentage of ALDH1A1<sup>+</sup> cells within the tumor.

## Discussion

ALDH1A1 and CD44 function in the carcinogenesis and tumor progression of HNSCC. ALDH1A1 is not expressed in the normal oral mucosa (14,15). However, other ALDH isoenzymes, such as ALDH1A3 and ALDH3A1, are found (14). The expression of the retinoic acid receptors in the oral mucosa underlines the function of ALDH isoenzymes in the normal mucosa (16). However, ALDH1A1 expression is increased in dysplasia and HNSCC (6). In the present study, it was found that 54.2% of primary HNSCC tumors were ALDH1A1<sup>+</sup>. In 30.8% of the ALDH1A1<sup>+</sup> tumors, ALDH1A1 was expressed in the majority of tumor cells. This observations confirm a more general function of ALDH1A1 in the carcinogenesis of HNSCC. One explanation is that ALDH1A1 is stress-induced. The ALDH1A1 promoter has binding sites for the AP-1 (17) and Oct-1 (18) transcription factors. Oct-1 is stabilized and translocated into the nucleus due to radiation, ROS (19) and HPV16 infection (20). The low expression of ALDH1A1 has also been recognized in the normal tonsillar squamous epithelium (21). Additionally, ALDH1A1 protects from oxidative stress-induced reactive aldehydes (8). In the present study, ALDH1A1<sup>+</sup> tumor cells were mostly located in the middle of tumor cell nests and assumed hypoxic tumor sites. Furthermore, ALDH1A1 appeared to be a prognostic factor for worse survival. This was in agreement with the results of a study by Qian *et al* (22). The reduced relapse rates for patients with ALDH1A1<sup>+</sup> tumors may arise from worse survival rates. Additionally, the majority of HPV-associated carcinomas are ALDH1A1<sup>+</sup>, and HPV-induced HNSCC exhibits a better prognosis (13). These observations possibly verify the role of ALDH1A1 in tumor progression.

CD44 is expressed in the basal layer of the normal oral mucosa (21,23-25). As a consequence, CD44 was overexpressed in many of the examined tumors in the present study. CD44 was located entirely throughout the cell nest or peripheral to the stroma. CD44 can be linked to the invasion of HNSCC (10). However, the present results indicate that CD44 has a more general function in carcinogenesis. CD44 was often coexpressed with EGFR. Additionally the expression intensities of CD44 and EGFR were correlated significantly throughout the tumor. It is known that CD44 can interact with tyrosine kinase receptors, such as EGFR (26). Interaction of these surface receptors can cause proliferation, chemotherapy resistance and invasion in HNSCC cell lines (27,28). EGFR also increases CD44 mRNA expression (29). Additionally, proliferating tumor cells (Ki-67) are often CD44<sup>+</sup>. Consequently, CD44 also has a significant role in HNSCC tumor progression.

ALDH1A1 and CD44 are common CSC markers in HNSCC (4-6). The present study examined the ability of the two proteins to identify CSCs. Corresponding to the hierarchical tumor model, CSCs exist in every tumor (30,31). In the present study, the ALDH1A1 and CD44 proteins

were expressed in the majority of tumors and also in HPV<sup>+</sup> carcinoma. Consequently, ALDH1A1 and CD44 could also be expressed in the CSCs of these tumors. As a CSC marker, CD44 was more often expressed in the tumors than ALDH1A1. However, a lot of tumors expressed CD44 in almost all tumor cells. To identify CSCs, the markers must isolate CSCs from the tumor bulk. ALDH1A1 was a better marker to define a subpopulation of tumor cells. Finally, the two markers were not sufficient to isolate the CSCs from the bulk of tumor cells. Further CSC markers should be used to define and isolate the CSC population.

In addition, ALDH1A1 und CD44 expression did not completely overlap. In the majority of tumors ALDH1A1<sup>+</sup>/CD44<sup>+</sup>, ALDH1A1<sup>+</sup>/CD44<sup>-</sup> and ALDH1A1<sup>-</sup>/CD44<sup>+</sup> populations were observed. This observation may indicate that different CSC populations could exist within one tumor. For HNSCC, this theory of different CSC phenotypes was first suggested by Biddle *et al* (32). This hypothesis has also been discussed for breast (33,34) and colorectal (30) cancer. Different phenotypes would make it more difficult to determine the CSC population.

In conclusion, CD44 and ALDH1A1 appear to be important factors in carcinogenesis and tumor progression. ALDH1A1 was shown to be a possible prognostic marker for worse survival, while ALDH1A1 and CD44 may be expressed in the CSCs of most examined tumors. However, these markers are not sufficient to precisely isolate the CSC subpopulation from the tumor bulk.

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