RESEARCH ARTICLE

ALDH1 in Combination with CD44 as Putative Cancer Stem Cell Markers are Correlated with Poor Prognosis in Urothelial Carcinoma of the Urinary Bladder

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Abstract

    <u>Background</u>: The aldehyde dehydrogenase 1 family member A1 (ALDH1A1) is one of the promising markers for identifying cancer stem cells in many cancer types, along with other markers including CD44. The aim of the present study was to evaluate the expression and clinical significance of putative cancer stem cell markers, CD44 and ALDH1A1, in a series of urothelial carcinomas of urinary bladder (UCUB) by tissue microarray (TMA). <u>Materials and Methods</u>: A total of 159 Urothelial Carcinomas (UC) including 96 (60%) low grade and 63 (40%) high grade carcinomas were immunohistochemically examined for the expression of CD44 and ALDH1A1. Correlations of the relative expression of these markers with clinicopathological parameters were also assessed. <u>Results</u>: High level expression of ALDH1A1 was found in 16% (25/159) of bladder UC which was significantly correlated with increased tumor size (p value=0.002), high grade (p value<0.001), pathologic stage (T1, p value=0.007 and T2, p value<0.001) and increased rate of recurrence (p value=0.013). A high level of CD44 expression was found in 43% (68/159) of cases, being positively correlated with histologic grade (p value=0.032) and recurrence (p value=0.039). <u>Conclusions</u>: Taken together, our results showed that ALDH1 was concurrently expressed in a fraction of CD44+ tumors and its expression correlated with poor prognosis in UCs. ALDH1A1 could be an ideal marker for targeted therapy of UCs in combination with conventional therapies, particularly in patients with high grade carcinomas. These findings indicate that cells expressing ALDH1A1 along with CD44 can be a potential therapeutic target in bladder carcinomas.

    Keywords: ALDH1A1 - CD44 - TMA - bladder carcinoma - targeted therapy

Introduction

    Urothelial carcinoma (UC) is the fifth most common cancer throughout the world (Hatina and Schulz, 2012). Based on the report of the International Agency for Research on Cancer (GLOBOCAN 2008), there are about 386,000 new cases of urothelial bladder cancer and 150,000 deaths annually worldwide (Ferlay et al., 2010; Hatina and Schulz, 2012). In Iran, bladder cancer is the third most common cancer among males and the ninth most common among females with an incidence of 13.03 and 3.32 per 100000 population among men and women, respectively (Salehi et al., 2011). Several risk factors can cause bladder cancer including tobacco, non-occupational and industrial carcinogens, population aging, gender, residential area, consumption of contaminated water, family history of cancer, drinking tea and coffee, pelvic irradiation, cyclophosphamide, schistosomia infection or chronic cystitis (Salehi et al., 2011; Ahmad et al., 2012; Ferris et al., 2013).

    Two different carcinogenic pathways also called dual track carcinogenesis are involved in the pathogenesis of UC including low-grade noninvasive papillary and high-grade flat-invasive sub-types (Brandt et al., 2009; Hatina and Schulz, 2012). Low-grade (G1, G2) noninvasive papillary (pTa and pT1/pTis) UC account for approximately 70-80% of all bladder cancers which are caused by mutations in the fibroblast growth receptor (FGFR3) gene which causes activation of receptor tyrosine kinase-Ras pathway (Harvey Rasor PI3-kinase) (Billerey et al., 2001; van Rhijn et al., 2002; Wu, 2005; Su et al., 2010). Compared to low-grade tumors, the FGFR3 mutation was not seen in high-grade (G3) flat- muscle invasive (greater than pT2) cancers which have the oncogenic mutations with loss of p53 and retinoblastoma (RB) tumor-suppressor gene activity (Cordon-Cardo et al.,

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1997; Cote et al., 1998; Wu, 2005; Brandt et al., 2009; Su et al., 2010). Half of patients diagnosed with high grade muscle-invasive UC progress to have metastases in the lungs and liver (Cordon-Cardo et al., 1997; Cote et al., 1998; Brandt et al., 2009; Overdevest et al., 2011). High recurrence rate is another feature of urothelial carcinoma that can occur at every clinical stage (Brandt et al., 2009). It has been reported that the highest recurrence rate among all cancers is related to Urothelial carcinoma of urinary bladder (UCUB) which is attributed to the heterogeneous feature of cancer stem cells residing in the tumor (Yang and Chang, 2008).

Cancer stem cells (CSCs) or tumor-initiating cells are subpopulation of undifferentiated tumorigenic cells within the tumors that represent unique characteristics such as tumor-initiation, self-renewal, and the ability of proliferation which are responsible for tumor progression, relapse, metastasis and also tumor heterogeneity resulting from differentiation (Heppner, 1984; Reya et al., 2001; Clarke et al., 2006; Jordan et al., 2006; Dwyer et al., 2007; Chang et al., 2008; Bentivegna et al., 2010; Su et al., 2010; Bohl et al., 2011; Ho et al., 2012; Madjd et al., 2013). CSCs also mediate tumor resistance to common treatments such as chemotherapy and radiation (Bao et al., 2006; Li et al., 2008; Bentivegna et al., 2010; Ho et al., 2012; Madjd et al., 2013). In a large number of studies, CSCs have been isolated from patient specimens, cancer cell lines and xenografts using fluorescence-activated cell sorting (FACS) and with a combination of suitable cell surface markers, cytokeratin markers, drug transporters and side population (SP) in Hoechst 33342 staining (Yang and Chang, 2008; Su et al., 2010; Ho et al., 2012; Luo et al., 2012; Zhang et al., 2012). There are functionally distinct subpopulations of bladder CSCs which require further investigation to identify their cells of origin and self-renewal and differentiation features (Chan et al., 2010; Ho et al., 2012).

Cancer stem cell markers including basal urothelial cell cytokeratins (CK5, CK14, CK17) (Ho et al., 2012) or adhesion molecules (CD44) (Immervoll et al., 2011; Ho et al., 2012), aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) (Ho et al., 2012), and tumor protein 63 (p63) (Ho et al., 2012) have been previously used as prognostic factors and applied to identify urothelial carcinoma stem cells (Hatina and Schulz, 2012; Ho et al., 2012). Further studies suggest that CSCs markers are composed of CD133 (Immervoll et al., 2008; Jaggupilli and Elkord, 2012), CD24 (Jaggupilli and Elkord, 2012), CD90, CD34, CD117 and CD20 as other potential CSC markers in bladder carcinomas (Komuro et al., 2007; Mehrazma et al., 2013). CD44v6+/EMA- cells were used for isolating bladder CSCs in some studies (Yang and Chang, 2008; Chan et al., 2010; Ho et al., 2012).

In addition, several Wnt signaling components are variously expressed in urothelial CSCs and non-tumorigenic cancer cells, including Wnt10a ligand and the MYC oncogene (He et al., 2009). These components have been applied to distinguish urothelial CSCs from non-tumorigenic cancer cells (Urakami et al., 2006; Brandt et al., 2009).

ALDH1A1, a putative CSC marker, belongs to the ALDH1 family (Pearce et al., 2005; Balicki, 2007) and is expressed in normal and malignant human mammary stem cells (Ginestier et al., 2007); over expression of this marker is associated with poor prognosis in breast (Ginestier et al., 2007), bladder (Su et al., 2010) and lung cancers (Huang et al., 2009).

CD44, a specific receptor for hyaluronic acid and adhesion/homing molecule (Naor et al., 2008; Jaggupilli and Elkord, 2012), is a multifunctional class I trans-membrane glycoprotein expressed in almost all normal and cancer cells (Naor et al., 2008; Jaggupilli and Elkord, 2012). In several previous studies, CD44 individually or in combination with other putative CSC markers has been applied to isolate CSCs in various solid tumors including breast (Al-Hajj et al., 2003), prostate, pancreas (Immervoll et al., 2011), ovarian, colorectal (Wielenga et al., 1993; Woodman et al., 1996; Choi et al., 2009), head and neck (Satpute et al., 2013) and bladder cancers (Yang and Chang, 2008; Chan et al., 2009; Slomiany et al., 2009; Lee et al., 2010; Su et al., 2010; Jaggupilli and Elkord, 2012). Both in vitro and in vivo studies have shown that CD44+ bladder cancer cells have higher tumorigenic potential compared to CD44- bladder cancer cells; whereas they have lower tumorigenic potential compared to ALDH1A1+ cells (Su et al., 2010).

Considering the absence of detailed reports focusing on the association between concurrent expression of two most frequent putative CSC markers ALDH1 and CD44 with prognostic factors in UC of urinary bladder, in the present study we aimed to investigate the prognostic significance of these markers; also the prevalence of combined ALDH1/CD44 phenotype in a large scale series of Iranian UC of urinary bladder tumors using tissue microarray (TMA). This would help to determine the potential utility of the combination of these two molecular markers in the targeted therapy of bladder cancers.

Materials and Methods

Patients and specimens

Tumor samples from 159 paraffin embedded UC of urinary bladder of patients underwent either transurethral resection of bladder tumor (TURB-BT, 81%) or bladder biopsy (19%) were assembled in tissue microarray format. All patients were diagnosed between 2008 and 2009 (mean follow up of 46 months) in Hasheminejad Kidney Center, a major referral university-based Urology hospital in Tehran, Iran. Medical records and clinicopathologic features including tumor size, tumor grade, lamina propria invasion, muscular invasion, and pathologic tumor stage of cases were collected and recorded in the database (Table 1).

Follow-up data regarding the date and cause of death for part of (120 cases) this cohort of patients has been provided prospectively, since the specimen collected form national referral hospital , some patients of other cities could not be followed. Follow-up was calculated from the date of operation, and all surviving cases were censored for survival analysis at 31 Sep 2013. None of these patients received adjuvant chemotherapy or radiation therapy before surgery. Tissue sections (4-μm thick) were obtained

Table 1. Association between ALDH1A1 and CD44 Expression (Intensity, Percentage of Positive Cells and H-Score) and Clinicopathological Parameters of Bladder Cancer (p value; Pearson χ^2)

Patients and tumor characteristics		No. of cases (%)	Expression of ALDH1A1 (p value; Pearson χ^2)			Expression of CD44 (p value; Pearson χ^2)		
			Intensity of staining	Percentage of positive cells	H-score (cut-off=8.5)	Intensity of staining	Percentage of positive cells	H-score (cut-off=90)
All cases		159						
Age (years)	<64	75 (47)	0.006	0.153	0.074	0.38	0.597	0.446
	>64	84 (53)						
Gender	Male	138 (87)	0.45	0.675	0.118	0.609	0.85	0.4
	Female	21 (13)						
Tumor Grade	High	63 (40)	<0.001	0.046	<0.001	0.899	0.032	0.305
	Low	96 (60)						
Tumor Size(cm)	<3	124 (78)	0.002	0.604	0.066	0.367	0.36	0.432
	>3	35 (22)						
Tumor Recurrence	Yes	21 (13)	0.013	0.269	0.135	0.039	0.211	0.159
	No	138 (87)						
Lamina propriainvasion	Present	41 (26)	0.007	0.4	<0.001	0.408	0.252	0.503
	Absent	118 (74)						
Muscularinvasion	Present	13 (8)	<0.001	0.069	0.019	0.33	0.487	0.289
	Absent	146 (92)						

from each block, stained with Hematoxylin and Eosin (H&E), and reviewed independently by two pathologists (H. K. & M. A.) to confirm the diagnosis and the presence of tumor. The best area for preparing TMA of each samples were determined after reviewing all H&E stained slides. Furthermore, normal bladder specimens were obtained from non-cancerous bladder biopsy specimens, which were used as normal controls and included in TMAs to compare the staining patterns of ALDH1 and CD44 in a range of different tissue samples.

Tumor grade was determined according to 2004 WHO grading system (Grignon, 2009). The staging of UC was assessed according to AJCC (American joint committee on cancer) (Cheng et al., 2009). Patients' data were fully anonymous. This research study was approved by Iran University of Medical Sciences Research Ethics Committee.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on TMA slides (Superfrost plus, Thermo Scientific, Germany) prepared from formalin-fixed paraffin-embedded tissues (FFPE) using a protocol as previously described (Mohsenzadegan et al., 2013; Taeb et al., 2014). Briefly, after deparaffinizationin xylene, tissues were rehydrated by immersion in decreasing grades of ethanol and endogenous peroxidase activity was blocked by immersing the tissues for 20 min in methanol containing 0.3% hydrogen peroxide and retrieved by boiling in a microwave for 20 minutes with sodium citrate buffer (pH 6.0). To detect ALDH1A1 and CD44 staining in tissue sections, after antigen-retrieval, tissues were incubated overnight with primary antibodies including rabbit monoclonal antibody (mAb) to ALDH1A1 (Abcam 52492, UK) and CD44 (NE 12 8 EW, Novocastra, UK) with optimal dilution of 1:200 and 1:40, respectively. After washing with Tris-Buffered Saline (TBS), tissues were incubated in the secondary antibody which was EnVision™+/HRP, Dual Link Rabbit/Mouse (Dako, Denmark) for 15 minutes at room temperature. The slides were then washed with TBS, and the antigens were visualized by 10 minutes incubation with the addition of 3, 3'-diaminobenzidine (DAB, Dako). In

the final step, tissue sections were lightly counterstained with haematoxylin (Dako), dehydrated in alcohol, cleared in xylene (Dako) and mounted for visualization. The entire tissues of tonsil and liver specimen were used as positive controls for CD44 and ALDH1A1 antibodies, respectively. Negative control, consisting of TBS instead of primary antibody, confirmed the specificity of the staining.

Preparing tissue microarray (TMA)

Tissue microarray blocks were prepared as described previously (Kononen et al., 1998; Mehrazma et al., 2013). In each case, 5-μm H and E slides were used to mark out representative areas of tumor tissue. From each corresponding paraffin-embedded block, five representative tumor regions were marked to cover heterogenicity of expression of ALDH1A1. Cores with a diameter of 0.6 mm were punched from selected regions of each "donor" block and precisely arrayed into a new recipient paraffin block using Tissue Arrayer Minicore (ALPHELYS, Plaisir, France). Tissue microarray (TMA) blocks were constructed in five copies for each specimen; the mean scoring of five cores was then calculated as the final score (Camp et al., 2000).

Evaluation of immunostaining

The staining intensity of antibodies were evaluated applying a semi-quatitaive system, ranging from negative to strong; 0 (negative), 1 (weak), 2 (moderate), and 3 (strong staining), by one pathologist (HK) after a series was observed on a multi-headed microscope by other observer (ZM) in a coded manner. In controversy cases, the scoring was confirmed by two observers and an agreement achieved. The percentage of positive cells for each antibody was graded as: 1 (<25% positive cells), 2 (25-50% positive cells), 3 (50-75% positive cells), 4 (>75% positive cells). The overall score was calculated by H-score (Histochemical score) for each case by multiplying the intensity of staining by the percentage of positive cells and a final score of 0 to 300 was given (McCarty et al., 1985). The mean of H-scores was chosen as cut-off value to classify the samples as high or low expression. The cut off value found to be 8.5 for

ALDH1A1 and 90 for CD44.

Statistical analysis

The data were analyzed using the SPSS statistical software package version 20 (SPSS Inc, ChicagoIL, USA). Pearson's χ^2 and Pearson's R tests were used to analyze the significance of correlation between expression of two putative CSC markers, ALDH1A1 and CD44 and clinicopathologic variables. Survival rates were examined by the Kaplan-Meier method for analysis of censored data. The statistical significance of differences between the survivals rates of groups with different expression of these markers were analyzed using the log-rank test. Patients whose deaths related to bladder cancer were considered in the disease-specific survival calculations. Deaths resulting from non bladder cancer related causes were censored at the time of death. A p value of <0.05 was considered to be statistically significant.

Results

Study population and demographic features of patient

A total of 173 patients which diagnosed with Urothelial carcinoam of urinary bladder (UCUB) were collected for this study. Due to technical problems in tissue processing or absence of tumor cells within the cores, 14 samples were excluded from the study, leaving 159 tumors for final scoring. Of this collection, 138 (87%) cases were male and 21 (13%) cases were female (M:F ratio was 6.57). The mean age at the time of diagnosis was 64±12 years (range 23-87 years).

Follow up data were available for 120 patients, from whom 94 cases were still alive and 22 were deceased from non-bladder cancer related causes, only 4 cases had died from cancer related disease. The median length of follow-up available for surviving patients was 49 months (range 1-77 months).

Seventy five (47%) patients were less than 64 year age, whereas 84 (53%) cases were older than mean age. In terms of histologic grade, 96 (60%) of tumors were low grade, whereas 63 (40%) of tumors were high grade. Tumor size ranged 0.3-6 cm (mean= 2.3 cm) in largest diameter. In 124 (78%) cases, tumor size was less than 3cm (cut off= 3 cm) in the largest diameter and 35 (22%) tumors were more than 3cm (Cheng et al., 2009). Of this series of patients, in 41(26%) of cases the lamina propria were involved and 13 (8%) tumors showed muscular invasion. The recurrence of disease was observed in 21 (13%) of patients, while other 138 (87%) cases had no recurrence. The clinicopathologic and demographic features of patients, tumor characteristics and tissue involvement data are summarized in Table 1.

Expression of ALDH1A1 and CD44 in UCUB

Immunohistochemical analysis of two putative stem cell markers, ALDH1 and CD44, was performed on a series of 159 paraffin embedded UCUB samples which were included in TMA. Level of expression was assessed by three scoring methods; intensity of staining, percentage of positive cells, and H-score. Normal liver and tonsil tissues, which were used as positive controls for ALDH1 and CD44 antibodies, showed strong and uniform staining (Figure 1 A, B). Staining of ALDH1 was mainly cytoplasmic, whereas CD44 was mainly expressed on the cell membrane of tumor cells (Figure 2 A, B). Only 33% (52/159) of TMA cores stained with ALDH1, whereas the majority of tumors (67%) were negative for ALDH1 staining. Among the tumors stained with ALDH1, weak, moderate and strong intensity was observed in 6 % (10), 11% (17) and 16 % (25) of samples, respectively (Table 2). In contrast, CD44 was expressed in 87% (139/159) of tumors and only 13% (20) of cases did not show any staining. Weak, moderate and strong staining of CD44 were observed in 24% (39), 39% (62) and 24% (38) of tumors, respectively (Figure 3) (Table 2). The average intensity of CD44 expression (mean= 1.7) was higher than ALDH1 expression (mean= 0.7). Similarly, the overall staining (H-score) of CD44 (mean= 90) was also higher than H-score of ALDH1 (mean H-score= 8.5).

Association of ALDH1 expression with clinicopathological parameters

Higher level of expression of ALDH1A1 was observed in 16% (25 of 159) of UCUB. Univariate analysis showed a significant correlation between expression of ALDH1 in terms of intensity of staining (p value<0.001), percentage of positive cells (p value=0.046) and H-score (cut-off= 8.5, p value<0.001) with tumor grade, indicating that higher level of ALDH1expression was more often found in high grade tumors. Moreover, the stronger intensity of ALDH1 was significantly associated with age (p value=0.006), increased tumor size (p value=0.002), lamina propria involvement (p value=0.007), muscular invasion (p value<0.001), and increased rate of recurrence
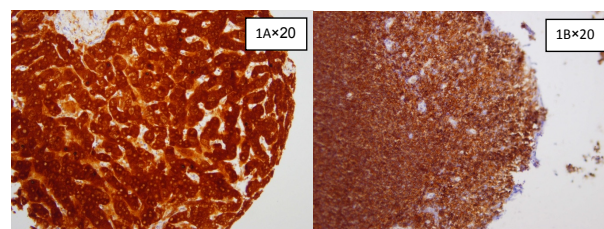

Figure 1. Expression of ALDH1A1 and CD44 Proteins Observed in Normal Controls. (A) Normal liver for ALDH1A1: ×20, (B) Normal tonsil for CD44: ×20

Table 2. Expression of ALDH1A1 and CD44 (intensity, percentage of positive cells and H-score) in UCUB

Pattern of expression		Intensity of staining				Percentage of positive cells				H-score cut-off=8.5(ALDH1A1) cut-off=90(CD44)	
		Negative	Weak	Moderate	Strong	<25	25-50	50-75	>75	Low	High
Expression of ALDH1	No (%)	10 9(67)	10 (6)	17 (11)	25 (16)	154(97)	3 (2)	0 (0)	2 (1)	134 (84)	25 (16)
Expression of CD44	No (%)	20 (13)	39 (24)	62 (39)	38 (24)	57 (36)	49 (31)	31 (19)	22 (14)	91 (57)	68 (43)

Figure 2. Cytoplasmic Expression of ALDH1A1 Proteinobserved in Bladder Carcinomas. (A) Strong with original magnification: ×20 (B) moderate with original magnification: ×20

Figure 3. Cell Membrane Staining of CD44 Observed in Bladder Carcinomas. (A) Strong with original Magnification: ×20, (B) Moderate with original Magnification: ×20

(p value=0.013). Similarly, the overall staining of ALDH1 (H-score) was positively correlated with lamina propria involvement (p value<0.001), muscular invasion (p value=0.019), and a relative association with tumor size (p value=0.066).

Low expression of ALDH1A1 (in terms of H-score) was significantly correlated with T1 stage (p value<0.001), whereas moderate and strong expression of ALDH1A1 showed highly significant correlation with T2 stage (p value<0.001).

There was no significant correlation with age (p value=0.074), gender (p value=0.118) and tumor recurrence (p value=0.135).

In contrast, on Kaplan-Meier analysis, no association was found between ALDH1 expression and patient survival (log rank test, p value=0.159).

Association of CD44 expression with clinicopathological parameters

High level of CD44 expression (intensity) was found in 43% (68 of 159) of UCUB samples, which was significantly correlated with higher histologic grade (p value=0.032), and recurrence (p value=0.039). To assess the clinical significance of CD44 expression in bladder cancer patients, we used H-score (cut-off= 90) to determine whether a high or low expression of CD44 expression was correlated with clinicopathologic features. However, there was no significant association between overall staining of CD44 (H-score) and clinicopathologic features such as patient's age (p value=0.44), gender (p value=0.40), tumor grade (p value=0.30), tumor size (p value=0.43), lamina propria involvement (p value=0.50), muscular invasion (p value=0.28), and tumor recurrence (p value=0.15). Similarly, no significant association was evident between CD44 expression and survival on Kaplan-

Meier analysis (log rank test, p value=0.444).

Combined analysis of ALDH1/CD44

Comparing the results for both CSC markers ALDH1 and CD44, a significant reciprocal pattern of expression was seen (p value<0.001). Combined analysis of the expression of these two markers indicated that a total of 47/159 (30%) cases displayed the ALDH1+/CD44+ phenotype, occurring more frequently in poor prognosis tumors. Strong correlation was found between ALDH1+/CD44+ phenotype and high grade tumors (p value<0.001), larger tumor size (p value=0.013), lamina propria involvement (p value=0.002), muscular invasion (p value<0.001), and tumor recurrence (p value<0.001) on univariete analysis. Whereas 9% (15/159) of tumors showed ALDH1-/CD44- phenotype, 58% (92/159) of cases were ALDH1-/CD44+ phenotype, and just 3% (5/159) of tumors possessed ALDH1+/CD44- phenotype.

Kaplan-Meier analysis did not reveal a significant survival advantage in ALDH1 high/CD44 high phenotype tumors compared with the remaining cases (log rank test, p value=0.115).

Discussion

Studies on phenotypic and functional properties of urothelial CSCs revealed that these cells can be identified by a panel of markers. Among various and proposed cancer stem cell surface markers used to isolate different subtypes of urothelial carcinomas, ALDH1A1 is one of the promising prognostic markers for CSCs of UCUB (Hatina and Schulz, 2012; Ho et al., 2012). In the present study, we examined the immunohistochemical expression of two putative cancer stem cell markers, CD44 and ALDH1A1, using tissue microarray method for the first time among 159 Iranian patients suffering from UCUB and then we evaluated the correlation between expressions of each marker with clinicopathological parameters. Our finding showed that high expression of ALDH1A1 was found in 16% of bladder cancers which was significantly correlated with high grade carcinomas, older patients, increased rate of tumor stage and recurrence.

Therefore, overexpression of ALDH1A1 was positively associated with clinicopathological and prognostic factors of bladder cancers indicating that ALDH1A1 could be applied as a prognostic marker in urothelial carcinomas.

Only two studies have been previously performed on bladder cancer regarding ALDH1A1 which revealed the prognostic value of ALDH1A1 for urothelial carcinomas. In Su et al study ALDH1A1+ cells isolated from bladder cancer cells with Aldefluorassay and the expression of ALDH1A1 in bladder tissues examined using immunohistochemistry method. Significant association was found between high expression of ALDH1A1 in patients with bladder urothelial carcinomas and tumor progression compared to those with low expression of ALDH1A1 (Su et al., 2010). In another study on the prognostic value of the ALDH1A3 and HOXA9, ISL1 as novel methylation markers was evaluated using microarray
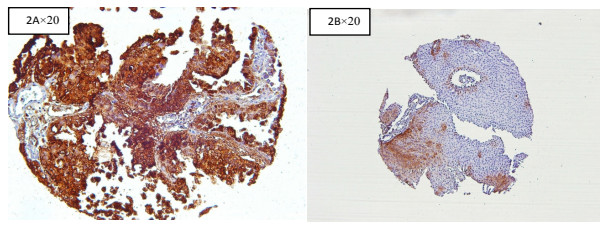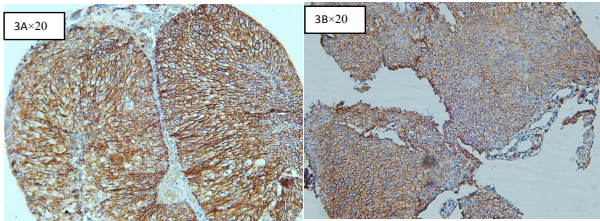
analysis of DNA methylation and RNA expression patterns in non-muscle invasive bladder cancer (NMIBC) (Kim et al., 2013). Our study in a series of Iranian UCUB patients reveals the same characteristics of ALDH1A1 expression in urothelial carcinomas as previous studies in western populations. In a review performed by Januchowski et al, the association between high level expression of ALDH1A1 and poor prognosis was shown in breast (Black et al., 2009; Tanei et al., 2009), bladder (Su et al., 2010) and prostate cancer patients (Li et al., 2010). It has also been shown to be a marker of CSCs in many types of solid tumors, including liver (Lingala et al., 2010), head and neck (Clay et al., 2010), pancreas (Rasheed et al., 2010), lung (Rasheed et al., 2010) ovary (Deng et al., 2010), and colon carcinomas (Lugli et al., 2010). Similar results were reported by Tanei et al in another study on breast cancer indicating the association between high expression of ALDH1A1 with lower overall survival and increase the number of ALDH1A1+ cells after chemptherapy (Tanei et al., 2009), since chemotherapy cannot eradicate cancer stem cells (CSCs) expressing ALDH1A1. Moreover another study performed by Deng et al. using tissue array of serous ovarian cancers showed that high expression of ALDH1A1 was correlated with shorter disease-free and overall survival times compared to those with low ALDH1A1(Deng et al., 2010). Recently, ALDH1A1 has been reported to be an independent prognostic factor for patient free survival and overall survival in astrocytomas (Liu et al., 2012). Our findings showed that only 33% of TMA cores stained with ALDH1A1 and high level expression of ALDH1A1 was found in 16% of samples, whereas the majority of tumors (67%) were negative for ALDH1A1. These results support that the ALDH1A1+ cancer cells constitute only a small fraction of cancer stem cells in bladder cancer. Our study was also consistent with other IHC studies illustrating that ALDH1A1 was expressed mainly in cytoplasm of 26% of bladder tumors (Su et al., 2010), 30% of breast tumors (Ginestier et al., 2007), and 29% of lung cancers (Jiang et al., 2009).

Since the assessment of markers in combination may perform better than those considered individually, therefore, we emphasize the prognostic value of combined ALDH1/CD44 status in bladder UCUB. Our data from combined analysis of both ALDH1A1/CD44 showed that 30% of urothelial carcinomas displayed the ALDH1A1+/CD44+ phenotype; whereas 58% of cases were ALDH1A1-/CD44+ phenotype and only 3% tumors expressed ALDH1A1+/ CD44- phenotype. This finding suggests that the ALDH1A1+ population is a subset of the CD44+ bladder cancer cells.

In conclusion, ALDH1A1, a bladder CSC associated marker is a potential prognostic factor for identifying and treatment of high grade urothelial carcinomas. Therefore, it can be used as a new marker for localized targeting of CSCs (with monoclonal antibodies against ALDH1A1) and thus control of the tumor in advanced cases. Further stratification of patients by combined ALDH1+/CD44+ phenotype identifies a subset of bladder cancer patients for whom more aggressive treatment is appropriate.

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