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Prognostic value of cancer stem cell markers CD133, ALDH1 and nuclear β-catenin in colon cancer

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Abstract ***Objective:*** Colon cancer is one of the most common human malignancies. Cancer stem cells (CSCs), despite being only a small subset of cancer cells, have the capability to self-renew and sustain the tumor. They also have the ability to proliferate. Multiple CSCs-associated markers have been identified in colon cancer including CD133, ALDH1 and β-catenin. The aim of the work was to study the prognostic value of CSCs markers (CD133, ALDH1 and β-catenin), as well as their relationship to clinicopathological features of colon cancer. ***Methods:*** CD133, ALDH1 and β-catenin proteins expression was assessed immunohistochemically in a series of colon cancers and their prognostic significance was evaluated. ***Results:*** CD133 expression showed significant relationship to tumor stage and lymph node metastasis (*P*-value 0.004 & < 0.001 respectively), and near significant relationship to liver metastasis (*P*-value 0.092). ALDH1 was significantly associated with tumor grade, stage and nodal metastasis (*P*-value 0.021, 0.001 and 0.026 respectively), but its relationship to liver metastasis was near significant (*P*-value 0.068). Nuclear β-catenin was significantly related to tumor grade, stage, nodal and liver metastasis (*P*-value 0.001, < 0.001, < 0.001 and 0.008 respectively). Overall survival (OS) was associated inversely with CD133, ALDH1 positivity, and directly with nuclear β-catenin positivity (*P*-value < 0.001, 0.0001 and < 0.001 respectively). Also recurrence free survival (RFS) was associated inversely with CD133, ALDH1 and directly with nuclear β-catenin positivity (*P*-value 0.0001, 0.001 and < 0.001 respectively). ***Conclusion:*** CD133, ALDH1 and β-catenin expressions of tumor cells have significant impact upon malignant progression of colon cancer and thus patient survival and tumor recurrence. Hence they can be used to predict outcome of colon cancer patients.

Key words colon cancer; cancer stem cells (CSCs); CD133; ALDH1; β-catenin; overall survival (OS); recurrence free survival (RFS)

Colon cancer is one of the most common human malignancies and the third most common gastrointestinal tumor. It shows a high incidence in 40 to 60 years old people with male to female ratio of 2–3:1 [1].

Tumor cells show heterogeneity regarding their morphology, inheritance and functions. However, some cells present not only heterogeneity but also hierarchy. Cancer stem cells (CSCs), despite being only a small subset of cancer cells, have the capability to self-renew and sustain the tumor. They also have the ability to proliferate, resulting in expansion of the CSCs pool, and to differentiate into the heterogeneous cancer cells that may not themselves be tumorigenic but usually constitute the majority of the tumor [2]. These cells are thought to initiate tumor metastasis and relapse after therapy. A better characterization of tumor initiating cells could lead to improvement of cancer therapies [3].

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In spite of the marked progress in the treatment strategies in the last few decades, a large percent of advanced tumors has poor outcome, which makes it critical to find specific biomarkers to identify CSCs as well as to predict patient prognosis [3].

Multiple CSCs-associated markers have been identified, among which CD133 has received considerable attention. CD133 (prominin-1) gene is located on chromosome 4p15.32 and encodes a cell surface glycoprotein compromising five transmembrane domains and two large glycosylated extracellular loops. The transcription of CD133 is initiated at five tissue specific promoters, yielding eight alternatively spliced transcripts [4].

β-Catenin is a key component of the adherens junctions, that is necessary for hemophilic cell-cell adhesions. In addition, β-catenin plays a role in cell-signaling and gene transcription [5]. In the presence of a Wnt signal, β-catenin translocates to the nucleus, where it promotes transcription of several target genes involved in cell proliferation [6].

Aldehyde dehydrogenase 1 isoform A1 (ALDH1, ALDH1A1) is a member of the ALDH family of enzymes. Its expression was originally found in a small subpopulation of neuronal tumor cells which were linked to CSCs. ALDH1 is involved in tumor growth as well as detoxification which confers therapeutic resistance against cyclophosphamide. Thus, ALDH1 is likely to be a driver of carcinogenesis and metastases [7].

This study was designed to study the expression of CSC markers (CD133, nuclear β-catenin, and ALDH1) in colon cancer by immunohistochemical methods, to evaluate their relationship to clinicopathological characteristics and to determine their prognostic significance.

Materials and methods

Tissues and patient history

For this retrospective cohort study, formalin-fixed paraffin-embedded tissue samples from 36 patients with colon cancer, who underwent intentionally curative surgical resection at the Zagazig University Hospitals (Egypt) between 2008 and 2013, were selected retrospectively. None of the patients received any preoperative treatment, but most of the stages II–IV patients received adjuvant chemotherapy. The tissue blocks were collected from the archives of Pathology Department, Faculty of Medicine, Zagazig University, Egypt. Patients were followed up till death or their most recent medical examination in Clinical Oncology and Nuclear Medicine Department of the same institute.

Histopaothologic characteristics were confirmed by blinded review of the original pathology slides. The TNM classification was used for pathologic staging, and the World Health Organization classification was used for pathologic grading [8].

Overall survival was defined as the time from first surgery to death or censored at the last known alive data. Recurrence was defined as initial tumor recurrence [9].

Proximal colon was defined as the large bowel proximal to the splenic flexure, and distal colon was defined as the large bowel distal to the splenic flexure excluding rectum. The study complied with the guidelines of the local ethics committee.

Immunohistochemistry

Formalin-fixed, paraffin-embedded archived tissues were cut into 4-μm thick sections. Then, sections were subjected to dewaxing, rehydration, blocking with hydrogen peroxide, and antigen retrieval with microwave in a 10 mM citrate buffer (pH 6.0) for 10 min and cooled to room temperature. After being blocked with 1% goat serum albumin, sections were incubated with the mouse monoclonal antibodies against human CD133 at a dilution of 1:150 (Abcam, Cambridge, UK), β-catenin monoclonal antibody at a dilution of 1:500 (Abcam, Cambridge, UK) and ALDH1 rabbit monoclonal antibody at a dilution of 1:100 (Abcam, Cambridge, UK) overnight at 4 ℃, followed with horseradish peroxidase labeled secondary antibodies for 30 minutes at room temperature.

The sections were incubated with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. To prevent antigen degradation sections were stored at 4 ℃ before immunohistochemical analysis. Human kidney, liver tissues and tissue from breast cancer was used as positive control to confirm the specificity of staining with CD133, ALDH1 and β-catenin respectively. Negative controls were made with primary antibody replaced by PBS. Positive and negative control slides were included within each batch of slides.

Assessment of immunohistochemistry

CD133 positivity was defined as membranous staining of the glandular-luminal surface of tumor epithelial cells and the intraglandular debris of shed tumor cells [10].

ALDH1 was located in the cytoplasm. Intensity of staining was scored as 0 (no expression), 1 (weak expression), 2 (moderate expression), and 3 (strong expression). The overall score was obtained by H-score for each case by multiplying the intensity of staining by the percentage of positive cells. The cut-off value (median) was calculated to define groups showing ALDH1 negative (H-score < 80) and ALDH1 positive (H-score > 80) [11].

When evaluating the β-catenin protein immunoreaction, we regarded β-catenin overexpression in the nucleus as a positive result [12].

Statistical analysis

Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. A P-value < 0.05 was considered significant.

Results

The demographic data of our patients were summarized in Table 1. The mean age ± SD was (51.3 ± 12.9) years, and the mean follow up period ± SD was (25.1 ± 11.9) months.

Table 1 Demographic data of colon cancer patients

Characteristics	No. of patients	Percent (%)
Age (years)		
≤ 55	21	58.3
> 55	15	41.7
Gender		
Male	24	66.7
Female	12	33.3
Site		
Proximal	19	52.8
Distal	17	47.2
Type		
Adenocarcinoma	34	94.4
Mucinous	2	5.6
Grade		
I	5	13.9
II	21	58.3
III	10	27.8
TNM stage		
I	5	13.9
II	13	36.1
III	10	27.8
IV	8	22.2
Nodal status		
N0	18	50.0
N+	18	50.0
Liver metastasis		
Present	7	19.4
Absent	29	80.6
CD133 immunoreactivity		
–ve	17	47.2
+ve	19	52.8
ALDH1 immunoreactivity		
–ve	10	27.8
+ve	26	72.2
Nuclear β-catenin immunoreactivity		
–ve	18	50.0
+ve	18	50.0
OS		
Alive	16	44.4
Dead	20	55.6
RFS		
Not recurrent	16	44.4
Recurrent	20	55.6

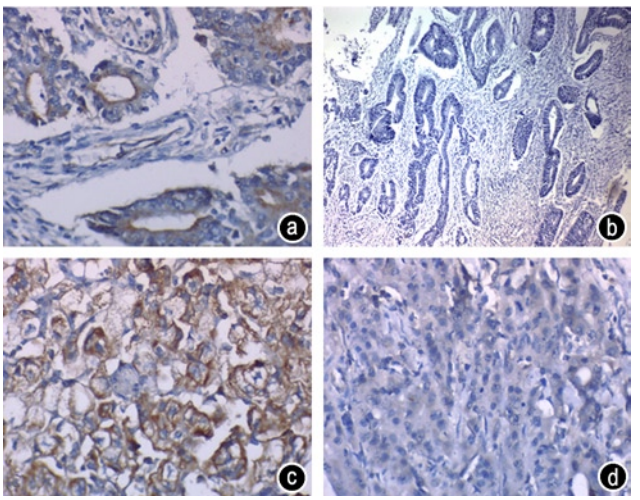

Fig. 1 CD133 expression in colon cancer. (a) positive staining of glandular-luminal surface of epithelial cells in grade I tumor (× 200); (b) negative staining of grade I tumor (× 100); (c) positive membranous staining in grade III tumor (× 400); (d) negative staining in grade III tumor (× 400)

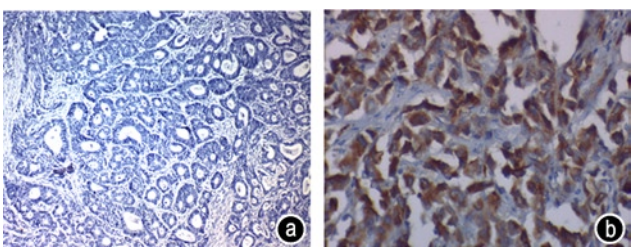

Fig. 2 ALDH1 expression in colon cancer. (a) negative staining of grade I tumor (× 100); (b) positive cytoplasmic staining of grade III tumor (× 400)

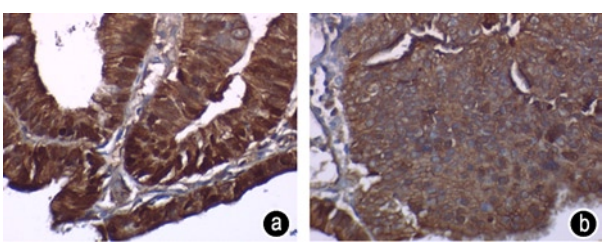

Fig. 3 Nuclear β-catenin expression in colon cancer. (a) positive nuclear staining of grade I tumor (× 400); (b) negative nuclear staining of grade III tumor (× 400)

Relationship between stem cell markers expression and clinicopathological characteristics of patients

Regarding CD133 immunoexpression, it was directly related to tumor stage and lymph node metastasis (*P*-value 0.004 & < 0.001 respectively), and near significant relationship was noted with liver metastasis (*P*-value 0.092). While non significant relationship was noted with tumor grade (*P*-value 0.188; Table 2 and Fig. 1).

As for ALDH1 positivity, it was significantly associated with higher tumor grade, advanced stage and nodal metastasis (*P*-value 0.021, 0.001 and 0.026 respectively), but its relationship to liver metastasis was near significant (*P*-value 0.068; Table 2 and Fig. 2).

Nuclear β-catenin was inversely related to tumor grade, stage, nodal and liver metastasis (*P*-value 0.001, < 0.001, < 0.001 and 0.008 respectively; Table 2 and Fig. 3).

Patient age, sex and tumor site were not significantly related to any of the studied stem cell markers (data

Table 2 Relation of stem cell markers to clinicopathological characteristics of patients [*n* (%)]

Characteristics	No.	CD133 expression			ALDH1 expression			Nuclear beta-catenin expression		
		–ve	+ve	*P*-value	–ve	+ve	*P*-value	–ve	+ve	*P*-value
Age (years)				0.158			0.379			0.310
≤ 55	21	12 (57.1)	9 (42.9)		7 (33.3)	14 (66.7)		9 (42.9)	12 (57.1)	
< 55	15	5 (33.3)	10 (66.7)		3 (20.0)	12 (80.0)		9 (60.0)	6 (40.0)	
Gender				0.813			0.599			1.000
Male	24	11 (45.8)	13 (54.2)		6 (25.0)	18 (75.0)		12 (50.0)	12 (50.0)	
Female	12	6 (50.0)	6 (50.0)		4 (33.3)	8 (66.7)		6 (50.0)	6 (50.0)	
Site				0.516			0.836			0.317
Proximal	19	8 (42.1)	11 (57.9)		5 (26.3)	14 (73.7)		11 (57.9)	8 (42.1)	
Distal	17	9 (52.9)	8 (47.1)		5 (29.4)	12 (70.6)		7 (41.2)	10 (58.8)	
Grade				0.188			0.021*			0.001*
I	5	4 (80.0)	1 (20.0)		3 (60.0)	2 (40.0)		0 (0.0)	5 (100.0)	
II	21	10 (47.6)	11 (52.4)		7 (33.3)	14 (66.7)		9 (42.9)	12 (57.1)	
III	10	3 (30.0)	7 (70.0)		0 (0.00)	10 (100.0)		9 (90.0)	1 (10.0)	
TNM stage				0.004*			0.001*			< 0.001*
I	5	4 (80.0)	1 (20.0)		5 (100.0)	0 (0.00)		0 (0.0)	5 (100.0)	
II	13	10 (76.9)	3 (23.1)		3 (23.1)	10 (76.9)		3 (23.1)	10 (76.9)	
III	10	2 (20.0)	8 (80.0)		2 (20.0)	8 (80.0)		7 (70.0)	3 (30.0)	
IV	8	1 (12.5)	7 (87.5)		0 (0.00)	8 (100.0)		8 (100.0)	0 (0.0)	
Nodal status				< 0.001*			0.026*			< 0.001*
N0	18	14 (77.8)	4 (22.2)		8 (44.4)	10 (55.6)		3 (16.7)	15 (83.3)	
N+	18	3 (16.7)	15 (83.3)		2 (11.1)	16 (88.9)		15 (83.3)	3 (16.7)	
Liver metastasis				0.092			0.068			0.008
Absent	29	16 (55.2)	13 (44.8)		10 (34.5)	19 (65.5)		11 (37.9)	18 (62.1)	
Present	7	1 (14.3)	6 (85.7)		0 (0.00)	7 (100.0)		7 (100.0)	0 (0.0)	

* significant *P* value

shown in Table 2).

Relationship between stem cell markers expression and overall survival

The median overall patient survival (OS) was 32 (17.5–46.5) months. Worse OS was directly associated with CD133, ALDH1 positivity and inversely associated with nuclear β-catenin positivity (*P*-value < 0.001, 0.0001 and < 0.001 respectively; Fig. 4).

Relationship between stem cell markers expression and recurrence free survival

The median recurrence free survival (RFS) in our study was 23.0 (12.4–33.6) months. Worse RFS of patients was directly associated with CD133, ALDH1 positivity and inversely associated with nuclear β-catenin positivity (*P*-value 0.0001, 0.001 and < 0.001 respectively; Fig. 5).

Discussion

A basic problem in cancer research is identifying the cells responsible for tumor formation. CSCs are long-lived cells that accumulate cancer-inducing mutations. Furthermore, they have the unique ability to self-renew and, to generate mature non-tumorigenic cancer cells of all lineages through differentiation. These differentiated cells appear to constitute the bulk of the tumor. Therefore, we have to reconsider treatment regimens that eradicate the bulk of cancer cells, but may not target the cell of origin. These cells are thought to be refractory to classic chemotherapy and responsible for metastasis and relapse. Further characterization of the stem cell population is required to identify potential targets for prospective therapies [13].

Preliminary evidence proposed that expression of CD133 is associated with the activation of stemness-related signal pathway, resistance to apoptosis and bioenergetic stress. Its immunopositivity is localized to apical/endoluminal surfaces and to luminal contents. Its concentration in plasma membrane protrusions suggests that CD133 may play a role in cell-cell and cell-matrix contact formation [10].

We observed a significant positive relationship between CD133 immunoreactivity and tumor stage (*P* value 0.004), which was consistent with the findings of Ong *et al* [14] and Galizia *et al* [15].

CD133 protein over-expression was significantly associated with lymph node metastasis (*P* value < 0.001) in our cases. Similarly, Galizia *et al* [15] reported that CD133 positivity has a linear relationship with nodal metastasis, therefore CD133 expression is associated with tumor pro-

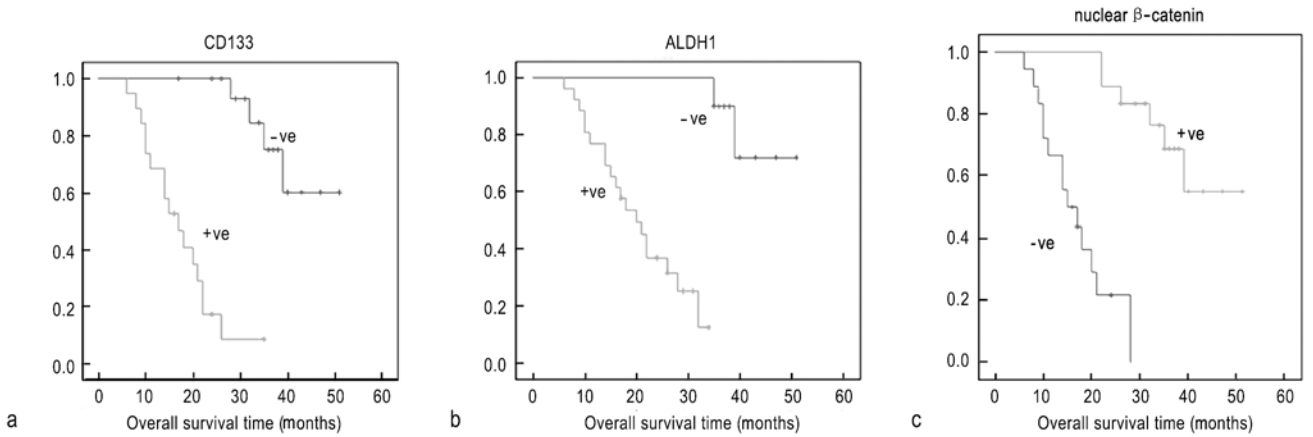

Fig. 4 (a) Kaplan-Meier survival plot of CD133 expression in cases of colon cancer; (b) Kaplan-Meier survival plot of ALDH1 expression in cases of colon cancer; (c) Kaplan-Meier survival plot of nuclear β-catenin expression in cases of colon cancer

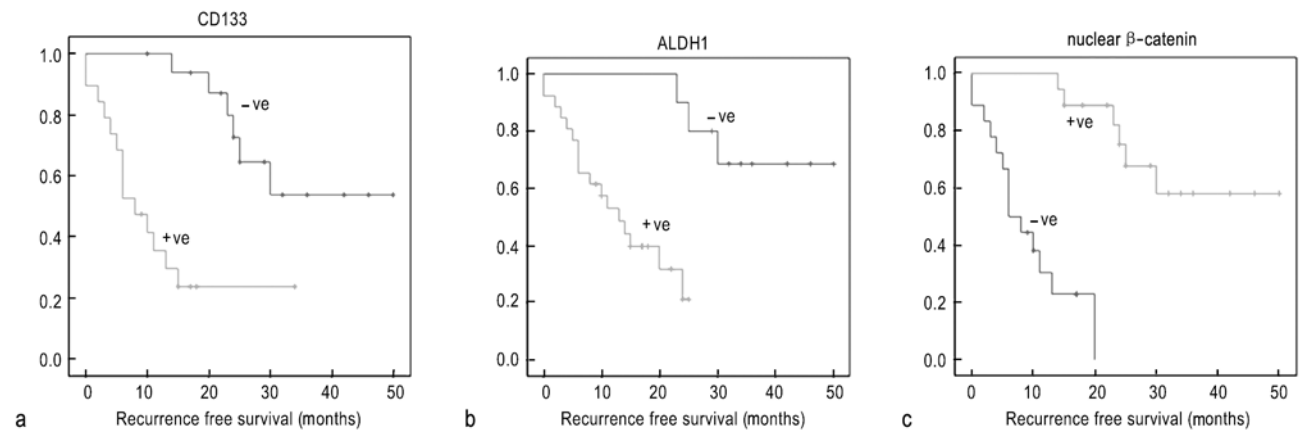

Fig. 5 (a) Kaplan-Meier plot of recurrence free survival of CD133 expression in cases of colon cancer; (b) Kaplan-Meier plot of recurrence free survival of ALDH1 expression in cases of colon cancer; (c) Kaplan-Meier plot of recurrence free survival of nuclear β-catenin expression in cases of colon cancer

gression. Horst *et al* [16] speculated that CD133-expressing tumor cell population may have additional cellular characteristics that contribute to the aggressiveness (tumor progression) of colorectal cancer, such as increased cell motility, invasion, or migration capabilities; colonization of distant organs; and growth of metastatic tumors, besides pure tumor initiation.

A near significant relationship was found between CD133 immunoreactivity and liver metastasis (*P* value 0.092), which confirms the results of Huang *et al* [17]. CD133 can be found in primary tumors, as well as metastatic tumors such as liver metastases. It was reported that the CD133 (+) subpopulation of cells was responsible for this metastasis [18].

Knockdown of CD133 in hepatocarcinoma cells results in decreased expressions of matrix metalloproteinase (MMP)-2 and ADAM9. These lead to decreased invasion [19]. In addition, blocking of chemokines and their receptors, that are upregulated in CD133 (+) CSCs, effectively inhibits the invasive capacity of these cells [20]. Therefore, CD133 (+) CSCs may confer metastatic potential to their progenies.

Kaplan-Meier analysis of patient survival proved that CD133 immunoreacitivity was significantly associated with both patient death due to the disease and tumor recurrence (*P* value < 0.001 and 0.0001 respectively). These findings are in agreement with previous evidence suggesting a potential prognostic role of the protein in colon cancer patients [9–10, 14–16, 21–22].

One possible explanation for the poor outcome in patients with high CD133 levels may be due to the fact that cytotoxic drugs do not target the CSCs, which then are able to recapitulate the bulk tumor population [23].

A promising marker for CSCs is aldehyde dehydrogenase 1 (ALDH1). ALDH is a detoxifying enzyme that oxidizes intracellular aldehydes, thereby confers resistance

to alkylating agents. In fact, the detoxification capacity of ALDH, by protecting CSCs against oxidative insult, might underlie their well-recognized longevity. ALDH also converts retinol to retinoic acid, a modulator of cell proliferation, which may also modulate CSCs proliferation [24].

Regarding the expression of ALDH1 in colon cancer, we detected a significant direct relationship between ALDH1 over-expression and tumor grade, stage and nodal metastasis (*P* value 0.021, 0.001 and 0.026 respectively), but we found near significant relationship between ALDH1 over-expression and liver metastasis (*P* value 0.068). Similar results were reported by Hou *et al* [1]. They showed increasing level of ALDH1 protein expression from normal colon tissue to invasive cancer. So it can be a valuable marker in early detection of colon cancer and the development process of the disease.

Previous experiments showed that ALDH1 protein expression was related to long-term survival of many tumors such as breast and prostate cancer [25–26]. In this study, ALDH1 protein over-expression was significantly associated with worse OS and RFS (*P* value 0.0001 and 0.001 respectively). Volger *et al* [27] and Hou *et al* [1] reported similar findings. They suggested that inhibiting the expression of ALDH1 protein could reduce colon cancer growth, invasion and metastasis ability to improve the survival rate of patients. However, no significant correlation could be found by Lugli *et al* [28]. This might be due to a considerably different study designs where multiple types of colorectal cancer were used, whereas we focused on colon adenocarcinoma.

Evidence of somatic mutations and nuclear accumulation of β-catenin in various pediatric cancers signifies a role of the Wnt-signaling pathway in their tumorigenesis. Accumulation of β-catenin is a result of defects in its degradation process, which usually takes place in the cytoplasm by an interaction between β-catenin and a complex of APC, AXIN and GSK-3-beta. Stabilized β-catenin translocates into the nucleus where the protein activates target genes with important functions and promotes tumor growth [29].

In this work, a significant inverse relationship was observed between nuclear β-catenin and tumor grade (*P* value 0.001). Cheah *et al* [30] reported that nuclear β-catenin is associated with tumor differentiation. This finding suggests that β-catenin plays a role in maintaining good differentiation status in cancer cells. However, these findings were not consistent with those of Kobayashi *et al* [31] and Pancione *et al* [32].

Nuclear β-catenin was inversely related to tumor stage of our patients (*P* value < 0.001). Also, we and others have shown that reduced or absent β-catenin expression are associated with more frequent nodal and liver metastasis (*P* value < 0.001& 0.008 respectively), supporting the notion that alternative pathways play a role in colon carcinogenesis [32–34]. β-Catenin is a key component of the adherens junctions, and thus, a significant reduction of the protein level, even in the absence of its own gene mutations, may promote cell spreading [35].

In the present work, nuclear β-catenin was significantly associated with better patient OS and RFS (*P* value < 0.001). Similar results were reported by previous studies [16, 33–34]. Outcome prognosticating ability of non-membranous β-catenin was also supported by two separate studies from Europe. Lines of epidemiological evidence suggested that nuclear accumulation of β-catenin could be employed as a factor identifying patients outcome [36].

Coexpression of colorectal CSC markers associated with patient survival may be more meaningful for clinical application in CRC. Studies have shown that CSC-related factors are associated with cancer progression [27]. In addition, CSCs have major phenotypic and functional heterogeneity which may help distinguish them from cancer cells, and may be of potential benefit in the development of anti-cancer therapies to improve clinical outcome [37].

Conclusion

Our findings demonstrate that the CD133, ALDH1 and β-catenin expression of tumor cells has significant impact upon malignant progression of colon cancer and thus patient survival and tumor recurrence. Hence they can predict patient outcome. In order to discover the functional role of their activity for colon carcinogenesis and to clarify the connection between them, further investigation of signaling and function are required. Moreover, it might also be of interest to investigate the connection between CD133, ALDH1 and β-catenin and CSCs, for which the proteins may be more than just markers.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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