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Clinical Implications of Cancer Stem Cell Markers and ABC Transporters as a Predictor of Prognosis in Colorectal Cancer Patients

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Abstract. *Background/Aim: Cancer stem cells (CSCs) and ABC transporters are associated with treatment resistance and outcomes of cancer patients. We aimed to investigate the prognostic implications of CSC markers and ABC transporters in colorectal cancer (CRC) patients. Materials and Methods: We collected 331 CRC samples and evaluated 3 CSC markers (SOX2, LGR5, and ALDH1) and 3 ABC transporters (ABCC2, ABCC3, and ABCG2) by immunohistochemistry. The association between the expression of these protein and patients' prognoses was statistically analyzed. Results: SOX2 was associated with longer overall survival (OS) (p<0.001). ABCG2 was associated with favorable overall survival (OS) p=0.001) and SOX2, and ABCC2 were associated with longer disease-free survival (DFS) (p=0.005 and 0.029, respectively). Multivariate analyses revealed that SOX2 was an independent prognostic factor for DFS [hazard ratio (HR)=2.701, p=0.044]. Conclusion: SOX2 and ABCC2 may be promising prognostic markers for CRC patients.*

Colorectal cancer (CRC) is one of the most common malignancies of the gastrointestinal tract and a leading cause of cancer-related death worldwide (1). Evaluation of prognostic and predictive markers enables stratification of patients with CRC into different risk categories and individualization of treatment to improve clinical outcomes.

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Cancer stem cells (CSCs) are a small population of cancer cells defined by their ability for self-renewal and multi-directional differentiation, which are common properties of normal stem cells. Because of these characteristics, CSCs are thought to be associated with aggressive tumor behavior such as metastasis, treatment resistance, and recurrence (2). Some biomarkers have been shown to have stem cell properties and are considered as prognostic or predictive biomarkers for cancer patients. G protein-coupled receptor 5 (LGR5) is a member of the G-protein-coupled receptor family of proteins and a regulated target of Wnt signaling. It is expressed in the crypt base of the intestines and has been suggested as a marker for normal colon stem cells and colon CSCs (2). Aldehyde dehydrogenases are a group of enzymes that catalyze the dehydrogenation of aldehydes to their corresponding carboxylic acids. Aldehyde dehydrogenase 1 (ALDH1) is widely used as a CSC marker in adult cancers (3). Sex-determining region Y-box 2 (SOX2), a transcription factor that acts as a critical regulator of stem cell maintenance and cell-fate decisions, is also used as a CSC marker in various malignant tumors. Several studies have revealed an association between the expression of LGR5, ALDH1, and SOX2 and patient prognoses in various malignant tumors (4-6).

ATP-binding cassette (ABC) transporter proteins act as active efflux pumps to extrude many substances including amino acids, polysaccharides, peptides, lipids, drugs, and toxins from cells. They are divided into seven subtypes from ABC-A-G based on their amino acid sequence in the ATP-binding domain (7). Differential expression of these transporters has been demonstrated in various tumor types (8) and suggested as one of the causes of multidrug resistance (9). Multiple ABC transporters such as breast cancer resistance protein (ABCG2) and multidrug resistance associated proteins, *i.e.* the C-family of ABC transporters

Table I. *Overview of antibodies for immunohistochemical staining.*

Antibody	Source	Catalog no.	Species	Clone	Dilution
ABCC2	Abcam	Ab3373	Mouse	Mono	1:50
ABCC3	Abcam	Ab3375	Mouse	Mono	1:20
ABCG2	Alexis Biochemicals	ALX 801-029-C125	Mouse	Mono	1:200
LGR5	Sigma-Aldrich	HPA012530	Rabbit	Poly	1:100
ALDH1	BD Bioscience	44/ALDH	Mouse	Mono	1:100
SOX2	Millipore	636675	Mouse	Mono	1:500

(10), have been identified in CSCs and found to be associated with CSC survival not only by increasing the clinical resistance to anticancer drugs but also by protecting CSCs from hypoxic-related cell damage (11-13). Thus, studies have been performed to examine whether ABC transporters are useful as prognostic or predictive biomarkers for patients with cancer (13-16).

Here, we evaluated the expression of multiple CSC markers and ABC transporters in cancer tissues from CRC patients by immunohistochemical analysis and statistically analyzed their prognostic significance. Our results provide fundamental data on the usefulness of CSC markers and ABC transporters as prognostic markers and target molecules.

Materials and Methods

Patients and tissue samples. Tissue specimens from 331 patients with CRC, for which paraffin blocks of the resected specimens and information on follow-up were available, were collected between May 2003 and December 2010 at Seoul National University Bundang Hospital. Clinical and follow-up data were also collected from medical records. The pathologic stage was determined according to the grading system of the 8th edition of the American Joint Committee on Cancer. The research protocol has been approved by the Seoul National University Bundang Hospital Ethic committee (IRB File No. 2018-12-003).

Construction of tissue microarrays (TMAs). Previously stained hematoxylin and eosin slides were reviewed, and a representative tumor section paraffin block (donor block) was collected from each case. Tumor cores (2 mm in diameter) were obtained from specific locations using a trephine apparatus. Trephined paraffin tissue cores were consecutively placed into recipient blocks. Each TMA block incorporated up to 60 samples.

Immunohistochemistry. Immunohistochemical staining was performed for 3 CSC markers, including LGR5, ALDH1, and SOX2, and 3 ABC transporters, including ABCC2, ABCC3, and ABCG2 (Table I), using a BenchMark XT automated immunostaining system (Ventana Medical System, Tucson, AZ, USA). Four-micrometer-thick sections from each TMA block were mounted on positively charged slides and dried at 62˚C for 30 min. After deparaffinization, heat pretreatment for epitope retrieval was performed for 60 min in ethylenediaminetetraacetic acid (pH 8.0) in the autostainer. The samples were incubated with individual primary antibodies and then treated with the UltraView Universal DAB kit (Ventana Medical Systems). The slides were counterstained with Harris hematoxylin.

Analysis of immunohistochemical staining. Immunostained slides were evaluated at 200× magnification in a blinded manner. Staining intensities were semi-quantitatively measured as negative (score=0), weak (score=1), moderate (score=2), or strong (score=3). The percentage of immune-reactive cells was also assessed. As there are no established absolute criteria for the positivity of proteins, by testing a series of different values, the staining results were considered as positive when >10% of tumor cells had intensity scores of ≥1. Representative immunostaining results are shown in Figure 1.

Statistical analysis. All analyses were performed using R (version 3.5.1) and SPSS (version 21.0; SPSS, Inc., Chicago, IL, USA) software. The chi-squared test and Fisher's exact test were used to assess the associations between the expression status of the CSC markers and ABC transporters and the clinicopathological features of the corresponding patients. To analyze the survival data, differences between survival rates were determined using the log-rank test. Disease-free survival (DFS) was defined as the time from the date of surgery until the date of first recurrence or death. Overall survival (OS) was defined as the interval from the date of surgery to the date of death. The plots of DFS and OS were drawn using the Kaplan–Meier method. Univariate analysis was conducted using the log-rank test, which nonparametrically compared the survival curves for the variables of CSC markers, ABC transporters, and clinicopathological features. Multivariate analysis was conducted by Cox proportional hazard modeling. The results were determined as significant when two-sided p-values were <0.05.

Results

Characteristics of patients. The demographic and clinicopathological characteristics of the patients are described in Table II. The mean (±SD) age of the patients at diagnosis was 63.0±12.4 years. A total of 95 (28.7%) patients had tumors localized to the right colon, whereas the other 236 (71.2%) patients had tumors localized to the left colon. For the TNM stage, 56 (16.9%), 98 (29.6%), 146 (44.1%), and 30 (9.1%) patients were classified as stage I, II, III, and IV, respectively. Invasion of lymphatic, vascular, and perineural tissues was observed in 107 (32.3%), 45 (13.6%), and 100 (30.2%) patients, respectively. The mean follow-up times for DFS and OS were 1,480 and 1,598 days,

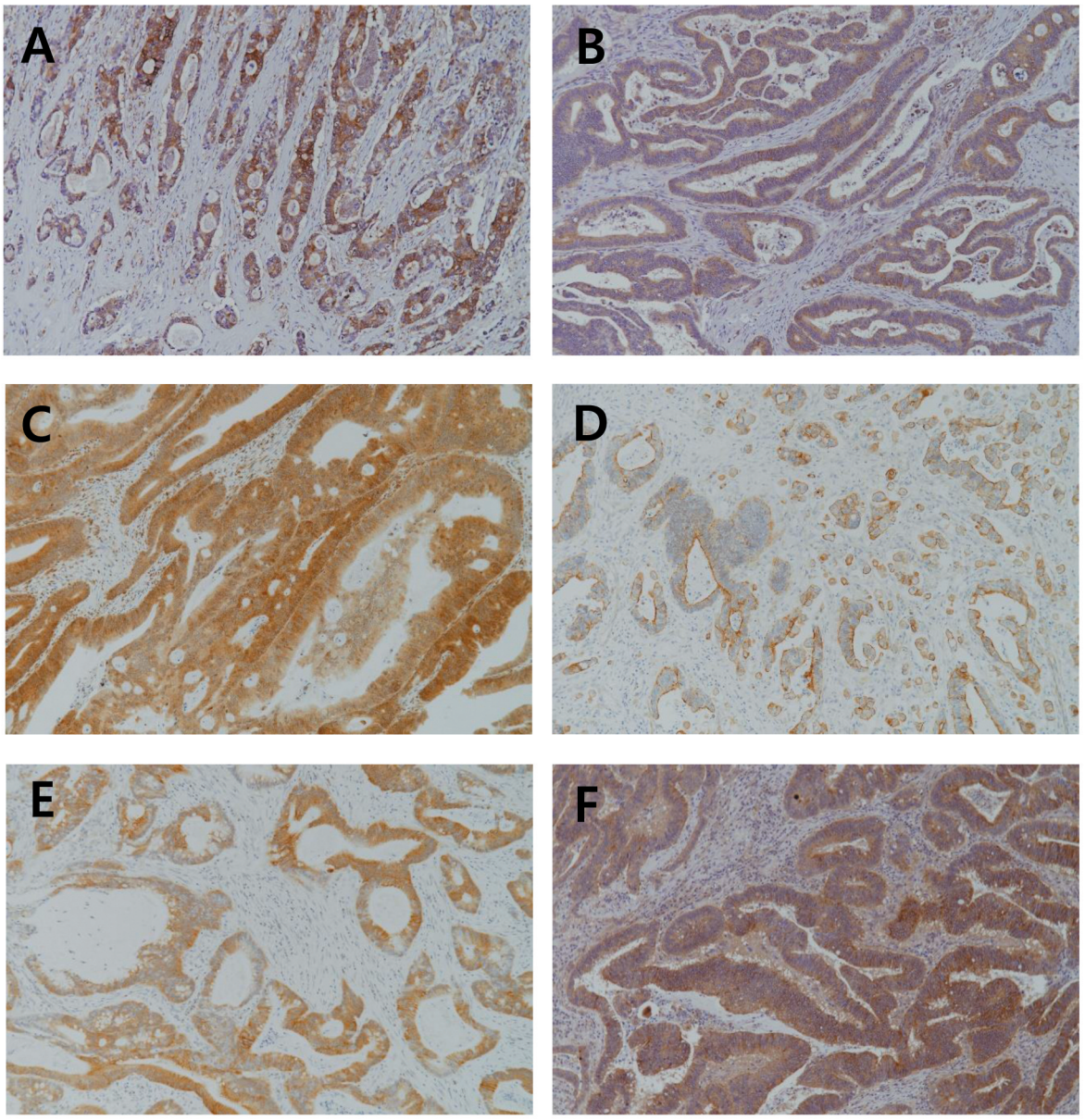

Figure 1. *Representative immunohistochemical staining images of cancer stem cell markers and BC transporters in colorectal cancer (A) ALDH1 (B) LGR5 (C) SOX2 (D) ABCC2 (E) ABCC3 (F) ABCG2 (original magnification ×200).*

respectively. During follow-up, 51 patients had local recurrences or distant metastases and 46 patients died.

Expression of CSC markers and ABC transporters. Immunohistochemical expression of each CSC marker was as follows: 15.2% (50/330) for LGR5, 20.1% (66/329) for ALDH1, and 31.7% (105/332) for SOX2. The values for ABC transporters were as follows: 64.2% (212/330) for ABCC2, 21.3% (69/324) for ABCC3, and 96.7% (320/331) for ABCG2. Association analysis of clinicopathological findings and the expression of CSC markers and ABC transporters showed that the following markers and transporters were significantly associated with clinicopathological characteristics: SOX2 *versus* stage ($p<0.001$), SOX2 *versus* perineural invasion ($p=0.003$), ABCC2 *versus* stage ($p=0.001$), ABCC2 *versus* vascular invasion ($p=0.014$), and ABCG2 *versus* perineural invasion ($p=0.014$) (Table III).

Association between OS and expression of ABC transporters and CSC markers. The median and mean OS at the last

Table II. *Demographic and clinicopathologic characteristics of colorectal cancer patients.*

Patients characteristics	Values* (n=331)
Age (years) at diagnosis	63.0±12.4 (32-89)
<50	51 (15.4%)
50-60	64 (19.3%)
60-70	110 (33.2%)
>70	106 (32.0%)
Gross	
Polypoid	47 (14.2%)
Ulcerofungating	185 (55.9%)
Ulceroinfiltrative	99 (29.9%)
Gender	
Female	133 (40.2%)
Male	198 (59.8%)
Location	
Right colon	95 (28.7%)
Left colon	236 (71.2%)
Tumor size (cm)	4.8±2.1 (1.0-13.0)
MSI status	
MSI-high	18 (5.4%)
MSI-low	30 (9.1%)
MSS	283 (85.5%)
Gross type	
Polypoid	47 (14.2%)
Ulcerofungating	185 (55.9%)
Ulceroinfiltrative	99 (29.9%)
Differentiation	
Well differentiated	14 (4.2%)
Moderately differentiated	300 (90.6%)
Poorly differentiated	17 (5.1%)
T status	
pTis	3 (0.9%)
pT1	18 (5.4%)
pT2	43 (13.0%)
pT3	223 (67.4%)
pT4a	30 (9.1%)
pT4b	14 (4.2%)
N status	
N0	159 (48.0%)
N1	94 (28.4%)
N2	78 (23.6%)
M status	
M0	301 (90.9%)
M1	30 (9.1%)
TNM stage	
I	56 (16.9%)
II	98 (29.6%)
III	146 (44.1%)
IV	30 (9.1%)
Lymphatic invasion	
Present	107 (32.3%)
Not identified	224 (67.7%)
Vascular invasion	
Present	45 (13.6%)
Not identified	286 (86.4%)
Perineural invasion	
Present	100 (30.2%)
Not identified	231 (69.8%)

*All the values, except for age and tumor size, are presented as a number of patients (%); both variables are presented as mean±standard deviation (minimum-maximum). MSI: Microsatellite instability.

follow-up appointment were 61.2 and 52.5 months, respectively. Kaplan–Meier curves for OS showed that patients with SOX2 expression had a better survival rate than patients without SOX2 expression ($p<0.001$, Figure 2). However, expression of the other proteins showed no association with OS (Figure 2). Additionally, univariate analysis suggested that clinicopathological parameters affected OS. A higher pathologic stage ($p<0.001$), presence of lymphatic invasion ($p<0.001$), vascular invasion ($p<0.001$), and perineural invasion ($p<0.001$) were associated with poorer OS. In multivariate Cox regression analyses, SOX2 expression and stage were prognostic indicators of OS: SOX2 [hazard ratio (HR)=2.701, 95% confidence interval (CI)=1.028-7.094, $p=0.044$) and Stage IV (HR=14.659, 95%CI=2.468-87.059, $p<0.001$) (Table IV).

Association between DFS and expression of ABC transporters and CSC markers. The median and mean DFS at the last follow-up were 59.8 and 48.7 months, respectively. Kaplan–Meier curves for DFS suggested that patients with ABCC2 and SOX2 expression had better survival rates than those without SOX2 and ABCC2 expression ($p=0.005$ for SOX2, $p=0.029$ for ABCC2) (Figure 3). Univariate analysis suggested that the clinicopathological parameters affecting DFS were gross type ($p=0.003$), lymphatic invasion ($p<0.001$), vascular invasion ($p<0.001$), perineural invasion ($p<0.001$), and stage ($p<0.001$). Multivariate Cox regression analyses of DFS showed that stage IV was associated with DFS (HR=42.525, 95%CI=3.787.009-328.621, $p=0.002$) (Table V).

Discussion

Biomarkers used as indicators of prognosis enable decisions on individual treatment plans for patients. CSCs and ABC transporters have been proposed as prognostic biomarkers, indicating the outcomes of patients with cancer. We evaluated the potential of using CSC markers and ABC transporters as prognostic biomarkers in patients with CRC and revealed that SOX2 and ABCC2 were associated with favorable prognosis. These results indicate that they may be useful prognostic markers in patients with CRC and also imply that markers for CSCs and ABC transporters may have variable biologic roles in cancer.

Previous studies have demonstrated an association between the expression of LGR5 and ALDH1 and the outcomes of patients with malignant tumors (4, 5, 17). In patients with CRC, a relationship between the expression of these proteins in the tumor and an unfavorable prognosis has been reported (18-21), although conflicting results have also been reported (22, 23). In the present study, the prognostic value of these proteins was not supported in patients with CRC. Previous studies have shown that SOX2 can serve as a poor prognostic

Table III. *Bivariate correlations between the clinicopathologic characteristics and biomarkers.*

	Stage					Lymphatic invasion			Vascular invasion			Perineural invasion		
	1	2	3	4	*p*-Value	Absent	Present	*p*-Value	Absent	Present	*p*-Value	Absent	Present	*p*-Value
ALDH1	19.3%	17.5%	22.1%	20.0%	0.856	19.7%	20.8%	0.828	20.1%	20.0%	0.991	21.0%	18.0%	0.537
LGR5	10.5%	11.2%	20.0%	13.3%	0.183	14.7%	16.0%	0.757	16.5%	6.7%	0.088	15.2%	15.0%	0.960
SOX2	54.4%	30.6%	26.0%	20.0%	<0.001	34.8%	25.2%	0.080	32.9%	24.4%	0.259	36.8%	20.0%	0.003
ABCC2	78.9%	70.4%	59.6%	37.9%	0.001	67.0%	58.5%	0.134	66.8%	47.7%	0.014	67.8%	56.0%	0.039
ABCC3	33.3%	15.3%	21.1%	20.0%	0.079	20.4%	23.3%	0.547	20.7%	25.0%	0.519	20.4%	23.5%	0.529
ABCG2	98.2%	98.0%	95.2%	96.7%	0.589	97.8%	94.4%	0.109	97.2%	93.3%	0.178	98.3%	93.0%	0.014

Table IV. *Multivariate Cox proportional hazard models for overall survival.*

	Hazard ratio	95%CI	*p*-Value
Stage			<0.001
Stage 1	1.000		
Stage 2	1.369	0.250-7.513	0.717
Stage 3	1.616	0.295-8.850	0.580
Stage 4	14.659	2.468-87.059	0.003
Perineural invasion			0.099
Absent	1.000		
Present	1.750	0.900-3.404	
Vascular invasion			0.414
Absent	1.000		
Present	1.375	0.641-2.950	
Lymphatic invasion			0.327
Absent	1.000		
Present	1.482	0.675-3.254	
Sox2			0.044
Positive	1.000		
Negative	2.701	1.028-7.094	

Table V. *Multivariate Cox proportional hazard models for disease-free survival.*

	Hazard ratio	95%CI	*p*-Value
Gross type			0.801
Polypoid	1.000		
Ulcerofungating	1.235	0.268-5.685	0.786
Ulceroinfiltrative	1.468	0.306-7.049	0.631
Stage			<0.001
Stage 1	1.000		
Stage 2	3.003	0.344-26.229	0.320
Stage 3	8.115	0.833-58.640	0.073
Stage 4	42.525	3.787-328.621	0.002
Perineural invasion			0.164
Absent	1.000		
Present	1.557	0.835-2.903	
Vascular invasion			0.942
Absent	1.000		
Present	1.028	0.489-2.162	
Lymphatic invasion			0.884
Absent	1.000		
Present	1.052	0.531-2.084	
Sox2			0.159
Positive	1.000		
Negative	1.756	0.803-3.840	
ABCC2			0.865
Positive	1.000		
Negative	1.053	0.583-1.901	

marker in patients with breast or oral squamous cell carcinoma (4, 6) and role of SOX2 in tumor initiation and progression has been also proved by *in vivo* experiment using cervical cancer cell line (24). However, the opposite results have also been reported in several types of malignancies such as cervical cancer, gastric cancer, and lung squamous cell carcinoma, where the expression of SOX2 was associated with favorable outcomes of patients (25-27). We also found that the expression of SOX2 indicated a favorable prognosis in patients with CRC. These results may be related to the role of SOX2 in the apoptotic pathway. SOX2 decreases the levels of cyclin D1 and phosphorylated Rb and increases the levels of p27, inducing cell-cycle arrest, and apoptosis (28). In addition, SOX2 directly trans-activates PTEN in gastric cancer (29). These findings suggest that the biologic role and prognostic value of CSC markers in cancers might depend on cancer type and stage.

ABC transporters have gained attention as the main factors provoking resistance to chemo- or chemoradiotherapy, and many researchers have reported a relationship between genetic pleomorphism or protein expression of ABCT transporters and treatment responses. Independent of their efflux potentials for chemotherapy agents, ABC transporters have been shown to be associated with clinical outcome parameters including patient survival and tumor progression, as they affect cancer cell survival by reducing the accumulation of cytotoxic metabolites under hypoxic conditions (12). However, in the present study, the expression of ABCC2 was associated with prolonged DFS, and ABCG2 showed a similar tendency,

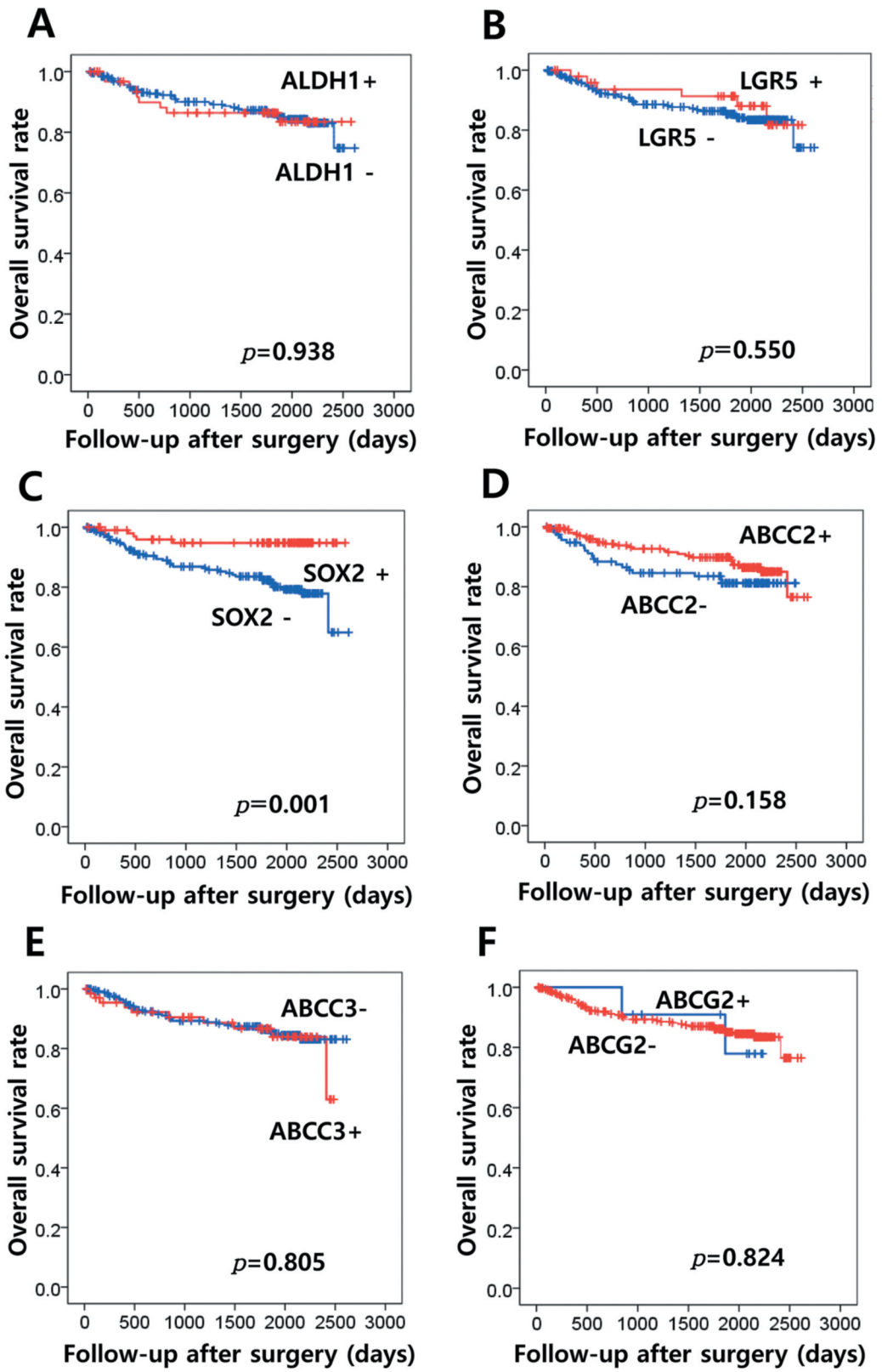

Figure 2. *Kaplan–Meier overall survival curve for patients with colorectal cancer according to the expression of cancer stem cell markers and ABC transporters; (A) ALDH1 (p=0.938), (B) LGR5 (p=0.550), (C) SOX2 (p=0.001), (D) ABCC2 (p=0.158), (E) ABCC3 (p=0.805), (F) ABCG2 (p=0.824).*

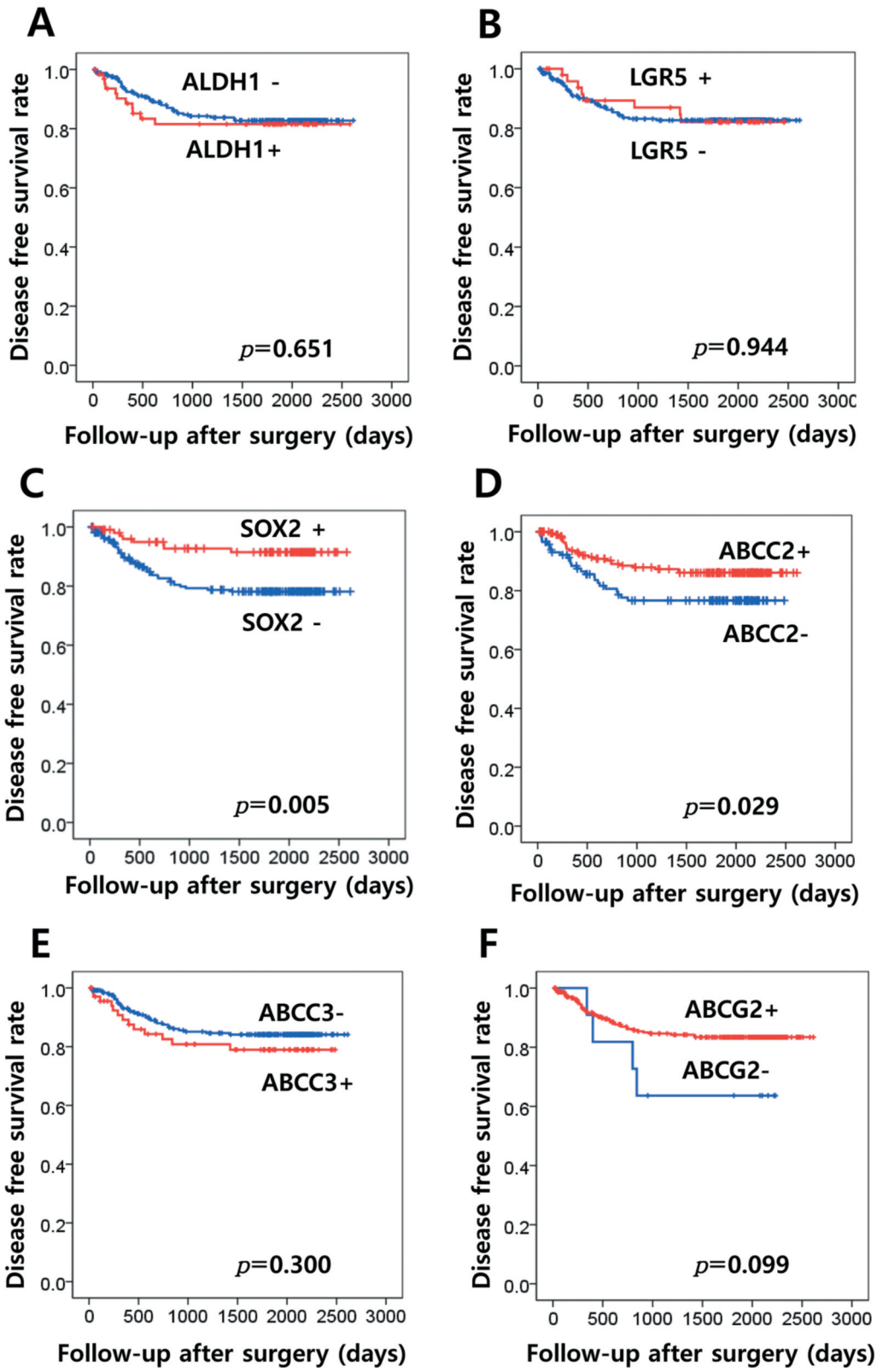

Figure 3. *Kaplan–Meier disease-free survival curve for patients with colorectal cancer according to the expression of ABC transporters and cancer stem cell markers; (A) ALDH1 (p=651), (B) LGR5 (p=0.944), (C) SOX2 (p=0.005), (D) ABCC2 (p=0.029), (E) ABCC3 (p=0.300), (F) ABCG2 (p=0.099).*

though the results were not significant. These findings militate against the supposition that ABC transporters may protect cancer cells from a harmful environment and contribute to cancer cell survival prolongation. Previous studies have reported similar results on the association between low expression of ABC transporters and poorer patient prognosis for various malignancies including CRC, intrahepatic cholangiocarcinoma, prostate cancer, and head and neck carcinoma (14, 30-32). These results may be related to the efflux of reactive oxygen species (ROS) by ABC transporters. Surgery-induced inflammation can create a ROS-rich environment. ROS signaling is a key regulator of tumor cell survival and cellular processes required for a successful metastatic cascade including invasion, adhesion, angiogenesis, and proliferation (33). In contrast, low expression of ABC transporters may indicate enhanced inflammatory processes in the microenvironment of the advanced tumor, as secreted inflammation-associated cytokines are very well-known to down-regulate drug transporters (34).

One limitation of this study is that it was retrospective in nature. Thus, further prospective studies of larger cohorts are needed to confirm the clinical use of these markers as prognostic marker for patients with CRC.

In conclusion, we found that expression of SOX2 and ABCC2 is associated with better outcomes in patients with CRC, and that these proteins may be useful prognostic markers in patients with CRC.

Conflicts of Interest

The Authors state that they have no conflicts of interest to declare in regard to this study.

Authors' Contributions

E.S. supervised the entire study, participated in study design and coordination as well as the writing of the manuscript; B.H.K. made substantial contributions to the acquisition, analysis, interpretation of data and writing of the manuscript; H.K.O., D.W.K. and S.B.K. made substantial contribution to case collection and data acquisition and revising this article; Y.C. was involved in revising the manuscript critically.

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