Prognostic implication of SOX2 expression in small intestinal adenocarcinoma

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Research article

Keywords: Small intestine; Adenocarcinoma; Prognosis; Cancer stem cell; KRAS

Posted Date: March 31st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-18397/v1

Version of Record: A version of this preprint was published at Virchows Archiv on October 25th, 2020. See the published version at https://doi.org/10.1007/s00428-020-02946-x.

Abstract

Background: The presence of KRAS mutation enhance the stem cell features of colorectal carcinoma cells containing mutant adenomatous polyposis coli (APC). However, their potential role in small intestinal adenocarcinoma remains elusive. Here, we aimed to investigate the clinical significance of cancer stem cell markers in the context of small intestinal adenocarcinoma with the KRAS genotype.

Methods: SOX2, NANOG, and OCT4 expression were assessed by immunohistochemistry and digital image analysis, and their potential association with KRAS was further examined in 185 small intestinal adenocarcinoma patients.

Results: Positive expression of SOX2, NANOG, and OCT4 was detected in 65 (35.1%), 94 (50.8%), and 82 (44.3%) of patients, respectively. SOX2-/wild-type KRAS (KRASWT) was often observed in low-grade carcinoma (P = 0.048). SOX2+ and SOX2+/mutant KRAS (KRASMT) were significantly associated with shorter overall survival relative to SOX2- and others (P < 0.001, both). Multivariate analysis revealed SOX2+ (HR=1.929 [95% CI, 1.320-2.819], P = 0.001) as an independent prognostic factor of worse overall survival among small intestinal adenocarcinoma patients.

Conclusions: These results suggest that SOX2 expression, in conjunction with KRAS, is a potential prognostic marker for small intestinal adenocarcinoma.

Background

Small intestinal adenocarcinoma is a relatively rare malignancy, accounting for 1–3% of all gastrointestinal tumors [1, 2]. Small intestinal adenocarcinomas are most commonly found in the duodenum, followed by the jejunum and ileum [3]. Moreover, duodenal adenocarcinomas are often observed in patients over the age of 60 years [3]. The early diagnosis of small intestinal adenocarcinoma is uncommon because of its rarity and non-specific symptoms. Clinical symptoms may differ depending on tumor location, size, or polypoid growth pattern [4]. Unfortunately, approximately 90% of patients are only diagnosed once the cancer has reached stage III and IV [5, 6]. The prognosis for patients with disseminated disease remains significantly poor, with a median survival of 2 to 14.4 months after diagnosis, and less than 5% of patients surviving for 5 years [3, 4, 6]. The mortality rate from small intestinal adenocarcinoma has not improved over recent decades. There is no standard adjuvant chemotherapy due to the lack of conclusive data. Thus, there is a strong need for a more effective and systematic individualized treatment option for advanced-stage, metastatic small intestinal adenocarcinoma.

Cancer stem cells (CSCs) are a small subpopulation of tumor cells that have the capacity for self-renewal, differentiation, and tumorigenicity [7, 8]. Furthermore, CSCs are proposed to be responsible for metastasis, relapse of cancer cells, and drug resistance [8, 9]. Thus, targeting CSCs is a promising therapeutic strategy because it allows complete eradication of CSCs and prevents recurrence. Identification and eradication of CSCs are not easy because they are highly plastic and hidden within the tumor cell population, usually in specialized hypoxic microenvironments [7, 10]. CSC identification generally relies on CSC markers, such as sex-determining region Y-box 2 (SOX2), NANOG, octamer-binding transcription factor 4 (OCT4), and DNA methyltransferase 1 (DNMT1). The SOX2, NANOG, and OCT4 genes play important roles in regulating pluripotency [11] and have been spotlighted in association with the treatment responses to, and prognoses of, various cancers [12]. High SOX2 expression has also been correlated with colorectal cancer metastasis and lymph node infiltration [13]. Interestingly, NANOG expression is regulated by the OCT4/SOX2 complex, and its high expression is positively correlated with tumor progression and poor prognosis among patients with colorectal cancer [14]. OCT4 expression has been reported in colorectal cancer cells undergoing epithelial-mesenchymal transition (EMT) [15], and its expression is associated with poor clinical outcomes [16]. Although there is increasing evidence of the importance of CSCs in cancer progression, the clinical significance of NANOG, OCT4, and SOX2 expression in small intestinal adenocarcinoma remains unknown.

KRAS encodes a protein that is linked with the extracellular-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathways. The activation of these pathways leads to cellular growth and proliferation. Indeed, KRAS mutations are one of the most prevalent oncogenic mutations found in human cancers, with approximately 40% of colorectal cancer patients exhibiting the mutated genotype [17]. In genetic models of colorectal cancer, KRAS mutations with mutated adenomatous

polyposis coli (APC)-induced colorectal cancers led to tumor growth and liver metastasis [18, 19]. Notably, Moon et al. also demonstrated that KRAS mutations induce the stemness of colorectal cells harboring APC mutations [20]. In addition, Jun et al. reported that KRAS mutations were found in one-thirds of patients with small intestinal adenocarcinoma and that these mutations were associated with a poor prognosis in the early stages [21]. However, a recent study demonstrated that small intestinal adenocarcinoma presents lower APC mutation frequency (26.8%) in comparison to that of colorectal cancer (75.9%) [22]. In this context, it is unclear whether KRAS mutation, in conjunction with low APC mutation rates play a role in the regulation of CSCs associated with small intestinal adenocarcinoma tumorigenesis

We investigated the clinical values of SOX2, NANOG, and OCT4 and expression in surgically resected small intestinal adenocarcinoma specimens by immunohistochemistry (IHC) and quantitative image analysis. Furthermore, we examined the potential association between the expression of CSC markers and KRAS genotypes in patients with small intestinal adenocarcinoma.

Methods

Patients specimens

A total of 197 surgically resected primary small intestinal adenocarcinomas were collected from the surgical pathology archives of 22 South Korean institutions by the Korean Small Intestinal Cancer Study Group, as previously reported [4]. Only carcinomas originating from the mucosa of the small intestine, including the duodenum, jejunum, and ileum, were included in the present study. This retrospective study was approved by the Institutional Review Board of Incheon St. Mary's Hospital (OC14OIMI0133). All procedures were conducted in accordance with the Declaration of Helsinki.

Clinical and pathologic data that were collected as part of our previous study were used again in this study [4]. Clinical data included patient sex, age, tumor location, operation date, T- and N-categories and stage grouping according to the eighth edition of the American Joint Committee on Cancer (AJCC) cancer staging system, most recent follow-up examination, survival status, presence or absence of synchronous or metachronous malignancies, and presence or absence of conditions predisposing patients to small intestinal adenocarcinomas (including Crohn's disease, familial adenomatous polyposis, Lynch syndrome, Peutz-Jeghers syndrome, Gardner's syndrome, gluten-sensitive enteropathy, intestinal duplication, Meckel's diverticulum, or heterotopic pancreas). Pathological data obtained from the gross examination included tumor size and growth pattern. The macroscopic growth patterns of the small intestinal adenocarcinomas were divided into 3 groups, including polypoid pattern, exophytic with predominantly intraluminal growth; nodular pattern, endophytic/ulcerative with intramural growth; and infiltrative pattern, annular with circumferential involvement or diffusely infiltrative [23]. The microscopic characteristics included histologic subtype, tumor grade, depth of invasion, peritoneal seeding, pancreatic and other intestinal loop invasions, nodal metastasis, and perineural and lymphovascular invasion. Histologic types and differentiations were classified according to the 2019 World Health Organization (WHO) classification [23]. Tumors were graded as low-grade (well and moderately differentiated, > 50% gland formation) and high-grade (poorly differentiate and undifferentiated, < 50% gland formation) carcinomas following the criteria for the histological grading of colorectal adenocarcinoma, which was described in the 2019 WHO classification [23].

Tissue microarray (TMA) and IHC

TMAs were constructed from the archived formalin-fixed and paraffin-embedded tissue blocks, as previously described [24]. Briefly, representative areas with invasive adenocarcinomas and normal small intestinal mucosa were identified on the corresponding hematoxylin and eosin-stained slides. Three cores from each tumor and 1 matched core from normal mucosa were sampled using a 1.0-mm punch, and 7 TMA blocks were constructed using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI).

TMA slides sectioned at 5-μm thickness were deparaffinized in xylene and rehydrated with a series of graded ethanols. Heat-mediated antigen retrieval was performed using a pressure chamber (Pascal; Dako, Carpinteria, CA, USA) with pH 6.0 citrate buffer (Dako) for SOX2 and OCT4, and pH 9.0 citrate/EDTA (Dako) for NANOG. To block endogenous activity, slides were incubated with 3% H_2O_2 for 10 minutes with additional protein serum block (Dako) applied to NANOG to reduce background

staining for 20 minutes at room temperature. Primary antibodies were incubated with the following conditions: rabbit polyclonal anti-OCT4 (Abcam, Cambridge, MA; cat# ab19857) at 1:1200 for 1 hour; rabbit monoclonal anti-SOX2 antibody (Cell Signaling Technology, Danvers, MA; clone D6D9; cat# 3579) at 1:500 for 1 hour, and rabbit monoclonal anti-NANOG (Cell Signaling Technology; clone D73G4; cat#4903) at 1:200 for 1 hour at room temperature, respectively. Antigen-antibody reactions were detected with Envision^{+}Ms/Rb HRP dual link secondary (Dako) and visualized with 3,3'-diaminobenzidine (Dako) followed by hematoxylin counterstain, dehydration, and clearing to coverslip prior to examination by light microscopy. The negative control was performed by omitting the primary antibody and rabbit immunoglobulin, and human testis tissue with seminoma was used as a positive control in each staining run.

Quantitative evaluation of immunostaining

All stained slides were digitalized using an Aperio AT2 digital scanner (Leica Biosystems, Vista, CA) in × 40 objective magnification. Subsequently, the images were automatically analyzed using Visiopharm software v6.9.1 (Visiopharm, Hørsholm, Denmark). In brief, screenshots of single relevant areas of regions of interest were generated by a pathologist (JWK) who was blind to clinicopathological information. Blue-colored (hematoxylin) tumor nuclei were initially defined, and then brown-colored (DAB) nuclei and cytoplasm were separated spectrally. For SOX2 IHC, a brown nuclear staining intensity (0 = negative, 1 = weak, 2 = moderate, and 3 = strong) and the percentage were obtained using a predefined algorithm and optimized settings. Histoscores, calculated by the percentage of positive cells multiplied by their staining intensity, were assigned to evaluate SOX2 IHC results. For NANOG and OCT4 IHC, a brown cytoplasmic intensity (weak and strong) with or without nuclear staining was obtained, and each proportion was analyzed. Expression values for histoscores (SOX2) and cytoplasmic staining (NANOG and OCT4) were dichotomized (negative vs. positive), with cut-off values showing the most discriminative power. Cut-off values of NANOG and OCT4 were 5.7% and 40.0% with strong cytoplasmic staining with and without nuclear expression, respectively, and the SOX2 cut-off histoscore was 2.5.

KRAS mutation

KRAS mutations were previously evaluated in the same cohort. KRAS mutations in codons 12 and 13 of KRAS exon 1 were identified by cycle sequencing, as previously described. In brief, 10 sections (each 10 μm in thick) from formalin-fixed paraffin-embedded tissue blocks were used to extract genomic DNA with a QIAmp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The KRAS genes were polymerase chain reaction (PCR)-amplified with primers for KRAS (F: 5'-TGACATGTTCTAATATAGTCAC-3', R: 5'-ACAAGATTTACCTCTATTGTT-3'). PCR reactions were run in a total volume of 25 μl with 0.3 μM of each primer using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Samples were subjected to initial denaturation at 95 °C for 15 minutes, 40–45 cycles at 95 °C for 50 seconds, annealing for 50 seconds, and elongation at 72 °C for 1 minute, followed by final elongation at 72 °C for 7 minutes. PCR products were column-purified using a QIAquick PCR Purification Kit (Qiagen) or enzymatically treated with ExoSAP-IT (USB, Cleveland, OH). The sequencing primers were identical to the PCR primers, and all samples were sequenced in both directions using a BigDye Terminator Cycle Sequencing Kit, version 1.1 (Applied Biosystems). The sequencing reactions were analyzed on an ABI Prism 3100 Genetic Analyzer with Sequencing Analysis software, version 3.7 (Applied Biosystems).

Statistical analysis

Data analysis was performed using the SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, NY, USA). Unpaired Student's t-test was applied to compare continuous variables. The χ^2 or Fisher's exact tests were used to characterize relationships between categorical variables. All survival analyses used an overall survival (OS) model, which captured all patient deaths as events and censored other patients at their last visit dates. Survival curves were constructed using the Kaplan–Meier method, and differences between groups were assessed using the log-rank test. Multivariate analysis was performed using Cox proportional hazards modeling to investigate the significance of any prognostic factors. P values less than 0.05 were considered statistically significant.

Results

Patient characteristics

Out of 197 total patients, 185 (93.9%) with interpretable immunohistochemical and molecular results were included in our study cohort. Eleven cases were not successfully immunostained due to tissue loss, folding during sectioning, or the staining process.

The patients' clinicopathological characteristics are summarized in Supplementary Table 1. Their ages ranged between 23 and 86 years (mean, 58.9 years; standard deviation [SD], 14.1 years). The sex ratio (men-to-women) was 1.4. Tumors were located in the duodenum in 103 cases (55.7%), the jejunum in 54 cases (29.2%), and the ileum in 28 cases (15.1%) (Fig. 1A). The follow-up period after surgical resection ranged between 1 and 168 months (median, 28.8 months). Several predisposing conditions were observed in 20 cases (10.8%), including 14 cases of sporadic adenomas, 3 cases of Peutz-Jeghers syndrome, 2 cases of Meckel's diverticulum, and 1 case of Crohn's disease. There were no patients with familial adenomatous polyposis, Gardner syndrome, gluten-sensitive enteropathy, intestinal duplication, and heterotopic pancreas.

KRAS mutational analysis

Overall, KRAS mutations were detected in 60 patients (32.4%), including codon 12 mutations in 49 patients (81.7%) and codon 13 in 11 patients (18.3%). Among KRAS variants, p.G12D (30/60 cases, 50.0%) was the most frequent, followed by p.G13D (11/60, 18.3%), p.G12C (7/60, 11.7%), p.G12V (6/60, 10.0%), p.G12A (4/60, 6.7%), p.G12R (1/60, 1.7%) and p.G12S (1/60, 1.7%) (Fig. 1B).

SOX2, NANOG, and OCT4 protein expression

Representative immunohistochemical images of SOX2, NANOG, and OCT4 are presented in Fig. 2. Of the cancer specimens, 65 (35.1%), 94 (50.8%), and 82 (44.3%) of 185 cases exhibited positive expression of SOX2, NANOG, and OCT4, respectively. Although the SOX2 (mean ± SD; 8.5 ± 26.1) and NANOG (mean ± SD; 25.5 ± 32.3%) expression levels in small intestinal adenocarcinoma tissues were higher than that in corresponding nonadjacent normal intestinal tissues (mean ± SD; 4.0 ± 7.4 and mean ± SD; 11.2 ± 20.8%, respectively), the differences were not statistically significant (P = 0.050 and P = 0.116, respectively). There was also no significant difference in OCT4 expression between small intestinal adenocarcinoma (mean ± SD; 6.0 ± 14.7%) and normal intestinal tissues (mean ± SD; 12.4 ± 18.2%) (P = 0.134). As summarized in Supplementary Table 2, positive expression of OCT4 was significantly associated with low pT category (P = 0.022) and stage grouping (P = 0.020). In terms of SOX2 and NANOG, there were no meaningful differences between negative and positive expression with respect to the investigated clinicopathological variables. Furthermore, there was no association between CSC marker expression and KRAS genotype.

Thirty-two (11.9%), 29 (15.7%), and 45 (24.3%) small intestinal adenocarcinoma cases exhibited dual positive expression of SOX2/NANOG, SOX2/OCT4, and NANOG/OCT4, respectively (Supplementary Table 3). Dual positive expression of NANOG/OCT4 was more frequently observed in association with low pT category (P = 0.031) and early-stage carcinoma (P = 0.032), while there were no significant associations of clinicopathological factors with SOX2/NANOG and SOX2/OCT4 expression. Triple-positive expression for SOX2/NANOG/OCT4 was observed in 14 cases (7.6%).

Associations between CSC marker expression and KRAS mutations

The associations between CSC marker expression and clinicopathologic factors according to KRAS genotype status are summarized in Tables 1 and 2. In the subgroup with wild-type KRAS ($KRAS^{WT}$), the rates of positive expression of SOX2, NANOG, and OCT4 were 32.8% (41/125), 50.4% (63/125), and 44.8% (56/125), respectively (Table 1 and Fig. 3). Low-grade carcinomas were more common among those with $SOX2^{-}/KRAS^{WT}$ (P = 0.048) whereas $NANOG^{-}/KRAS^{WT}$ was associated with mucinous and signet ring cell subtypes (P = 0.035). In the subgroup with mutant KRAS ($KRAS^{MT}$), the rates of positive expression of SOX2, NANOG, and OCT4 were 40.0% (24/60), 51.7% (31/60), and 43.3% (26/60), respectively (Table 2 and Fig. 3). Patients with $KRAS^{MT}$ and positive NANOG expression was associated with the absence of lymphovascular invasion (P = 0.010). Patients with $KRAS^{MT}$ plus OCT4 expression tended to develop tumors with early stage group (P = 0.024). There was no association between KRAS mutation subtype, CSC marker expression, and other clinicopathologic factors, including age and gender, growth pattern, histologic type, nodal metastasis, and perineural invasion.

Table 1

Correlation between clinicopathologic factors and CSC marker expression among small intestinal adenocarcinoma patients with $KRAS^{WT}$

Category (n = 125)	SOX2, No. (%)			NANOG, No. (%)			OCT4, No. (%)		
	Positive	Negative	P	Positive	Negative	P	Positive	Negative	P
Age			0.657			0.128			0.124
<60 years	19 (46.3)	44 (52.4)		27 (42.9)	36 (58.1)		33 (58.9)	30 (43.5)	
≥60 years	22 (53.7)	40 (47.6)		36 (57.1)	26 (41.9)		23 (41.1)	39 (56.5)	
Sex			0.530			1.000			0.968
Male	28 (68.3)	51 (60.7)		40 (63.5)	39 (62.9)		36 (64.3)	43 (62.3)	
Female	13 (31.7)	33 (39.3)		23 (36.5)	23 (37.1)		20 (35.7)	26 (37.7)	
Tumor location			0.661			0.658			1.000
Proximal	20 (48.8)	46 (54.8)		35 (55.6)	31 (50.0)		30 (53.6)	36 (52.2)	
Distal	21 (51.2)	38 (45.2)		28 (44.4)	31 (50.0)		26 (46.4)	33 (47.8)	
Type of growth			0.348			0.276			0.447
Polypoid	5 (12.8)	18 (22.8)		11 (18.3)	12 (20.7)		11 (20.0)	12 (19.0)	
Nodular	2 (5.1)	6 (7.6)		2 (3.3)	6 (10.3)		2 (3.6)	6 (9.5)	
Infiltrative	32 (82.1)	55 (69.6)		47 (78.3)	40 (69.0)		42 (76.4)	45 (71.4)	
Histological subtype			0.557			0.035*			0.387
Adenocarcinoma	35 (85.4)	76 (90.5)		60 (95.2)	51 (82.3)		52 (92.9)	59 (85.5)	
Mucinous carcinoma	4 (9.8)	3 (3.6)		1 (1.6)	6 (9.7)		1 (1.8)	6 (8.7)	
Signet ring cell carcinoma	1 (2.4)	3 (3.6)		0 (0.0)	4 (6.5)		2 (3.6)	2 (2.9)	
Undifferentiated carcinoma	1 (2.4)	2 (2.4)		2 (3.2)	1 (1.6)		1 (1.8)	2 (2.9)	
Grade			0.048*			0.799			0.803
Low	24 (58.5)	65 (77.4)		46 (73.0)	43 (69.4)		41 (73.2)	48 (69.6)	
High	17 (41.5)	19 (22.6)		17 (27.0)	19 (30.6)		15 (26.8)	21 (30.4)	

SOX2 positive, Histoscore of > 2.5; NANOG positive, IHC score of > 5.7%; OCT4 positive, IHC score of > 40.0%

*Statistically significant (P < 0.05)

Category (n = 125)	SOX2, No. (%)			NANOG, No. (%)			OCT4, No. (%)		
	Positive	Negative	P	Positive	Negative	P	Positive	Negative	P
Lymphovascular invasion			0.745			0.246			0.157
Absent	18 (43.9)	41 (48.8)		26 (41.3)	33 (53.2)		22 (39.3)	37 (53.6)	
Present	23 (56.1)	43 (51.2)		37 (58.7)	29 (46.8)		34 (60.7)	32 (46.4)	
pT category			0.356			0.346			0.167
pT_{is}-pT_2	2 (4.9)	11 (13.1)		9 (14.3)	4 (6.5)		9 (16.1)	4 (5.8)	
pT_3	17 (41.5)	30 (35.7)		22 (34.9)	25 (40.3)		19 (33.9)	28 (40.6)	
pT_4	22 (53.7)	43 (51.2)		32 (50.8)	33 (53.2)		28 (50.0)	37 (53.6)	
pN category			1.000			0.156			0.809
pN_0	17 (45.9)	36 (47.4)		31 (54.4)	22 (39.3)		26 (49.1)	27 (45.0)	
$pN_1 + pN_2$	20 (54.1)	40 (52.6)		26 (45.6)	34 (60.7)		27 (50.9)	33 (55.0)	
Stage group			0.253			0.198			0.308
0-I	1 (2.7)	9 (11.8)		7 (12.3)	3 (5.4)		7 (13.2)	3 (5.0)	
II	16 (43.2)	27 (35.5)		24 (42.1)	19 (33.9)		19 (35.8)	24 (40.0)	
III	20 (54.1)	40 (52.6)		26 (45.6)	34 (60.7)		27 (50.9)	33 (55.0)	
SOX2 positive, Histoscore of > 2.5; NANOG positive, IHC score of > 5.7%; OCT4 positive, IHC score of > 40.0%									
*Statistically significant (P < 0.05)									

Table 2

Correlation between clinicopathologic factors and CSC marker expression among small intestinal adenocarcinoma patients with $KRAS^{MT}$

Category (n = 60)	SOX2, No. (%)			NANOG, No. (%)			OCT4, No. (%)		
	Positive	Negative	P	Positive	Negative	P	Positive	Negative	P
Age			1.000			0.986			0.394
< 60 years	13 (54.2)	19 (52.8)		16 (51.6)	16 (55.2)		16 (61.5)	16 (47.1)	
≥ 60 years	11 (45.8)	17 (47.2)		15 (48.4)	13 (44.8)		10 (38.5)	18 (52.9)	
Sex			0.357			0.462			0.432
Male	17 (70.8)	20 (55.6)		21 (67.7)	16 (55.2)		18 (69.2)	19 (55.9)	
Female	7 (29.2)	16 (44.4)		10 (32.3)	13 (44.8)		8 (30.8)	15 (44.1)	
Location			0.213			1.000			1.000
Proximal	12 (50.0)	25 (69.4)		19 (61.3)	18 (62.1)		16 (61.5)	21 (61.8)	
Distal	12 (50.0)	11 (30.6)		12 (38.7)	11 (37.9)		10 (38.5)	13 (38.2)	
Type of growth			0.520			0.669			0.134
Polypoid	3 (13.0)	7 (19.4)		4 (13.3)	6 (20.7)		4 (15.4)	6 (18.2)	
Nodular	2 (8.7)	1 (2.8)		2 (6.7)	1 (3.4)		3 (11.5)	0 (0.0)	
Infiltrative	18 (78.3)	28 (77.8)		24 (80.0)	22 (75.9)		19 (73.1)	27 (81.8)	
Histological subtype			NA			NA			NA
Adenocarcinoma	22 (91.7)	35 (97.2)		29 (93.5)	28 (96.6)		25 (96.2)	32 (94.1)	
Mucinous carcinoma	1 (4.2)	1 (2.8)		2 (6.5)	0 (0.0)		1 (3.8)	1 (2.9)	
Signet ring cell carcinoma	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Undifferentiated carcinoma	1 (4.2)	0 (0.0)		0 (0.0)	1 (3.4)		0 (0.0)	1 (2.9)	
Grade			0.941			0.914			1.000
Low	21 (87.5)	30 (83.3)		27 (87.1)	24 (82.8)		22 (84.6)	29 (85.3)	
High	3 (12.5)	6 (16.7)		4 (12.9)	5 (17.2)		4 (15.4)	5 (14.7)	
Lymphovascular invasion			0.429			0.010*			0.434

NA, not applicable; SOX2 positive, histoscore > 2.5; NANOG positive, IHC score > 5.7%; OCT4 positive, IHC score > 40.0%

*Statistically significant (P < 0.05)

Category (n = 60)	SOX2, No. (%)			NANOG, No. (%)			OCT4, No. (%)		
	Positive	Negative	P	Positive	Negative	P	Positive	Negative	P
Absent	10 (41.7)	20 (55.6)		21 (67.7)	9 (31.0)		15 (57.7)	15 (44.1)	
Present	14 (58.3)	16 (44.4)		10 (32.3)	20 (69.0)		11 (42.3)	19 (55.9)	
pT category			0.509			0.354			0.052
pT_{is}-pT_2	2 (8.3)	4 (11.1)		3 (9.7)	3 (10.3)		5 (19.2)	1 (2.9)	
pT_3	7 (29.2)	6 (16.7)		9 (29.0)	4 (13.8)		7 (26.9)	6 (17.6)	
pT_4	15 (62.5)	26 (72.2)		19 (61.3)	22 (75.9)		14 (53.8)	27 (79.4)	
pN category			0.234			0.669			1.000
pN_0	9 (39.1)	20 (58.8)		17 (54.8)	12 (46.2)		13 (50.0)	16 (51.6)	
$pN_1 + pN_2$	14 (60.9)	14 (41.2)		14 (45.2)	14 (53.8)		13 (50.0)	115 (48.4)	
Stage group			0.312			0.806			0.024*
0-I	2 (8.7)	3 (8.8)		3 (9.7)	2 (7.7)		5 (19.2)	0 (0.0)	
II	7 (30.4)	17 (50.0)		14 (45.2)	10 (38.5)		8 (30.8)	16 (51.6)	
III	14 (60.9)	14 (41.2)		14 (45.2)	14 (53.8)		13 (50.0)	15 (48.4)	
NA, not applicable; SOX2 positive, histoscore > 2.5; NANOG positive, IHC score > 5.7%; OCT4 positive, IHC score > 40.0%									
*Statistically significant (P < 0.05)									

Prognostic significance of CSC marker expression

Kaplan–Meier plots demonstrated that patients with positive immunoreactions for SOX2 (median, 20.2 months) had significantly shorter OS times that those with negative reactivity (median, 50.1 months; P < 0.001) (Fig. 4A). However, there were no significant differences according to NANOG and OCT4 expression status (Fig. 4B and C). The survival of patients with $SOX2^+/OCT4^+$ (median, 17.8 months) revealed worse OS relative to that of patients with $SOX2^-/OCT4^-$ (median, 39.7 months) (P = 0.021, Fig. 4D), while patients with $SOX2^+/NANOG^+$ and $NANOG^+/OCT4^+$ expression did not significantly differ in OS relative to patients with $SOX2^-/NANOG^-$ and $NANOG^-/OCT4^-$ expression (Fig. 4E and F). Patients with $SOX2^+/NANOG^+/OCT4^+$ (median, 13.3 months) had shorter survival times than patients exhibiting other expression patterns (median, 36.5 months, Supplementary Fig. S1).

Notably, patients with positive SOX2 expression harboring $KRAS^{MT}$ (median, 11.1 months; OS rate, 4.2%) had significantly worse outcomes than those with $SOX2^-/KRAS^{WT}$ (median, 53.6 months; OS rate, 40.5%), $SOX2^-/KRAS^{MT}$ (median, 50.1 months; OS

rate, 36.1%), or $SOX2^+/KRAS^{WT}$ (median, 28.4 months; OS rate, 26.8%) (P < 0.001) (Fig. 5A). In subgroup analysis of $SOX2^+$ expression, patients harboring $KRAS^{MT}$ had worse OS than those with $KRAS^{WT}$ (P = 0.038) (Fig. 5B). Patients with $SOX2^+/KRAS^{MT}$ had poorer OS than those with $SOX2^-/KRAS^{WT}$ and other expression ($SOX2^-/KRAS^{WT}$ and $SOX2^+/KRAS^{WT}$) patterns (median, 30.0 months; OS rate, 31.2%; P < 0.001) (Fig. 5C).

Cox multivariate proportional hazard analysis indicated that distal location (hazard ratio [HR] 1.245 [95% CI, 1.022–1.517], P = 0.029), high pT category (≥ pT_3) (HR 1.439 [95% CI, 1.084–1.910], P = 0.012), lymph node metastasis (HR 1.799 [95% CI, 1.212–2.671], P = 0.004), and positive SOX2 expression (HR 1.929 [95% CI, 1.320–2.819], P = 0.001) were independent negative prognostic factors (Table 3).

Table 3
Univariate and multivariate analysis of overall survival among small intestinal adenocarcinoma patients

Variables	Univariate analysis		Multivariate analysis	
	HR [95% CI]	P	HR [95% CI]	P
Age (≥ 60 years)	1.220 [0.860–1.730]	0.274	NA	
Sex (female)	1.110 [0.780–1.600]	0.557	NA	
Location (distal)	1.280 [1.070–1.530]	0.007*	1.245 [1.022–1.517]	0.029*
Grade (high)	1.240 [0.840–1.850]	0.280	NA	
pT category (≥ pT_3)	1.460 [1.160–1.840]	0.001*	1.439 [1.084–1.910]	0.012*
LN metastasis	2.160 [1.470–3.170]	< 0.001*	1.799 [1.212–2.671]	0.004*
$KRAS^{MT}$	1.410 [0.980–2.030]	0.064	NA	
$SOX2^+$	1.890 [1.330–2.710]	< 0.001*	1.929 [1.320–2.819]	0.001*
$NANOG^+$	0.760 [0.530–1.080]	0.121	NA	
$OCT4^+$	0.920 [0.640–1.310]	0.641	NA	
CI, confidence interval; LN, lymph node; MT, mutant type; NA, not applicable				
*Statistically significant (P < 0.05)				

Discussion

As the CSC model has evolved, many biomarkers have been identified in various tumors to better understand CSCs and elucidate their roles in conferring stemness. SOX2, NANOG, and OCT4, contributing to the “core pluripotency network,” are transcription factors that regulate the development of embryonic stem cells (ESCs). These transcription factors are thought to regulate pluripotency and lead to self-renewal in embryonic and induced pluripotent stem cells [25, 26]. Moreover, it has been reported that SOX2 and OCT4 play roles in inducing the stemness of various cancer cells, as well as embryonic cells [26–28]. However, due to its rarity and a lack of understanding of its pathogenesis, only a few studies have been conducted on the clinical value of stem cell markers in small intestinal adenocarcinoma. Here, we investigated the clinical significance of SOX2, NANOG, and OCT4 expression in small intestinal adenocarcinomas.

We first determined SOX2 (35.1%), NANOG (50.8%), and OCT4 (44.3%) expression in small intestinal adenocarcinoma samples and demonstrated that SOX2 expression is an independent negative prognostic factor. Due to the lack of a standardized method for assessing SOX2 expression [13, 25], and the paucity of SOX2 nuclear expression, we used a histoscore method and digital image analysis to objectively quantify the results. In colorectal cancer samples, SOX2 expression has been analyzed with an

absolute quantitative or semiquantitative scoring system via microscopy. It has been reported that SOX2 expression ranges from 11–45.6% and that it correlates with lymph node metastasis, tumor grade, TNM categories, and BRAF mutations [13, 25].

In this study, we observed that OCT4 is mostly expressed in the cytoplasm and is associated with low pT category and stage grouping. When considering different isoforms of OCT4, OCT4A is a nuclear transcription factor responsible for the pluripotency properties of ESCs, while OCT4B resides in the cytoplasm, where it may respond to cellular stress [29]. Therefore, it has been suggested that OCT4B expression is predominantly found in small intestinal adenocarcinoma cells, which correlates with low tumor stage. Additionally, alternatively spliced OCT4 transcripts may exhibit diverse functions in different tissues, considering that OCT4 expression (mean, 12.4%) is also found in normal small intestinal mucosa.

There is ongoing debate about the contribution of the Wnt signaling pathway to self-renewal and differentiation in human ESCs; however, many members of the Wnt signaling pathway are implicated in stem-cell proliferation [26, 30]. Recently, Moon et al. demonstrated that the initial activation of β-catenin, by APC loss, and further enhancement through mutated KRAS induces CD44, CD133, and CD166 expression in colorectal cancer [20]. Moreover, many studies suggest that SOX2, NANOG, and OCT4 expression may contribute to both the EMT and stemness of cancer cells in the digestive system [14, 15, 31]. Although small intestinal adenocarcinomas express notably different levels of APC (26.8% vs 75.9%), CDKN2A (14.5% vs 2.6%) and TP53 (58.4% vs 75.0%), they are characterized by alterations in both KRAS (53.6%) and PIK3CA (16.1%), which are involved in dedifferentiation and disease progression [22]. Therefore, we analyzed the expression of SOX2, NANOG, and OCT4 in conjunction with the KRAS genotype in small intestinal adenocarcinoma patients. SOX2 expression was higher in the $KRAS^{MT}$ subgroup (40.0%) than in the $KRAS^{WT}$ (32.8%) subgroup, but this difference was not statistically significant. We found that patients with $KRAS^{MT}$ and SOX2 expression had significantly worse OS outcomes than those with $KRAS^{WT}$ without SOX2 expression. This implies that both SOX2 and KRAS genotypes are important prognostic factors for small intestinal adenocarcinomas. The $KRAS^{WT}/SOX2^{+}$ expression pattern was more frequently found in association with high-grade carcinomas than with low-grade carcinomas, which suggests that SOX2 may play a certain role in the dedifferentiation of small intestinal adenocarcinoma cells, independently of KRAS genotype. Although there was no statistical significance with $SOX2^{+}$ expression and tumor differentiation, we observed $SOX2^{+}$ expression to be more common in high-grade tumors (44.4%, 20/45) than low-grade tumors (32.1%, 45/140) (Supplementary Table 2).

We found small intestinal adenocarcinomas with $KRAS^{MT}$ and $NANOG^{+}$ expression to be associated with a lack of lymphovascular tumor invasion and small intestinal adenocarcinomas with $KRAS^{MT}$ and $OCT4^{+}$expression to be associated with eary stage group. Regarding the combined expression of SOX2, NANOG, and OCT4, we observed that $NANOG^{+}/OCT4^{+}$ expression was more common in early-stage carcinoma, whereas $SOX2^{+}/OCT4^{+}$ and $SOX2^{+}/NANOG^{+}/OCT4^{+}$ expression were associated with short OS. It is well known that the transcription factor NANOG is localized to the nucleus [32]. We detected that NANOG is expressed in the cytoplasm of small intestinal cancer cells with and without nuclear accumulation, which corroborates the findings of other colorectal cancer studies [14, 33]. This aberrant expression pattern is frequently found in a variety of cancers, with testicular germ cell tumors being a notable exception [34]. The regulation mechanism for localization is currently unknown and should be elucidated in the future. Recent studies also indicate that NANOG is a negative prognostic factor among colorectal cancer patients. Meng et al. revealed that NANOG expression significantly correlates with poor prognosis, lymph node metastasis, and TNM categories [14], and Xu et al. reported that NANOG may be a potential biomarker for the postoperative hepatic metastasis of colorectal cancer [33]. These results suggest that SOX2, NANOG, and OCT4 play complex roles in small intestinal adenocarcinoma. Further studies are needed to clarify the interaction between SOX2, NANOG, OCT4, and KRAS mutations in small intestinal adenocarcinoma.

The main challenge of this study was that it relied on a relatively imbalanced cohort, despite collecting patient samples from multiple institutions. In this study cohort, 57% of the patients had pT4 tumors, and 52% had cancers with AJCC stage group III, even though inoperable stage IV cases with distant metastases were not included. However, this deviation seems to be characteristic of small intestinal adenocarcinoma. Indeed, the findings of a previous large single-center study agreed with our findings when comparing just the percentages of stage I, II, and III cases, which were 12%, 45%, and 43%, respectively. Additionally, other epidemiologic characteristics, such as age, sex, and location, also paralleled those of our cohort [3].

Conclusions

We demonstrated that SOX2 overexpression to be an independent negative prognostic factor for small intestinal adenocarcinoma. $SOX2^{+}/OCT4^{+}$ and $SOX2^{+}/NANOG^{+}/OCT4^{+}$ expression were also associated with short OS. $SOX2^{+}/KRAS^{MT}$ was significantly associated with poor survival, and the $SOX2^{+}/KRAS^{WT}$ expression pattern may be associated with high-grade carcinomas. These findings imply that SOX2 expression, in conjunction with mutated KRAS, is a potential prognostic marker for small intestinal adenocarcinoma. The results also shed light on the potential mechanistic relationship between stemness and molecular alterations.

Abbreviations

APC:adenomatous polyposis coli; CSC:cancer stem cells; SOX2:sex-determining region Y-box 2; OCT4:octamer-binding transcription factor 4; EMT:epithelial-mesenchymal transition; ERK:extracellular-regulated kinase; PI3K:phosphatidylinositol 3-kinase; IHC:immunohistochemistry; TMA:tissue microarray; OS:overall survival; SD:standard deviation; HR:hazard ratio; CI:confidence interval; ESC:embryonic stem cell; LN:lymph node; MT:mutant type; NA:not applicable.

Declarations

Ethics approval and consent to participate

This retrospective study was approved by the Institutional Review Board of Incheon St. Mary's Hospital (OC14OIMI0133), and written informed consent was obtained from all patients. All procedures were conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

Data is available in the supporting files.

Competing interests

The authors declare that there is no conflict of interest.

Funding

This study was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, and the Basic Science Research Program, through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2017R1D1A1B03031817, awarded to S.-Y. J.).

Authors' contributions

JWK, J-YC, S-MH and SMH designed the study. JWK, J-YC, KY, and YP collected the data and analysed the data. JWK and J-YC drafted and edited the manuscript. S-YJ, S-MH, and SMH reviewed and edited the manuscript.

Acknowledgements

Not applicable.

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Supplementary Files Legend

Additional file1: Supplementary Figure 1

Supplementary Fig. S1 Kaplan–Meier plots analyzing the overall survival (OS) of small intestinal adenocarcinoma patients. Patients with combined $SOX2^+$/$NANOG^+$/$OCT4^+$ (median 13.3 months) expression had significantly shorter OS (log-rank test, $P = 0.031$, respectively) than other expression patterns (median, 36.5 months).

Additional file 2: Supplementary Table 1-3

Supplementary Table 1 Clinicopathologic characteristics of patients with small intestinal adenocarcinomas.

Supplementary Table 2 Correlation between clinicopathologic factors and CSC marker expression among patients with small intestinal adenocarcinoma.

Supplementary Table 3 Correlation between clinicopathologic factors and combinational CSC marker expression among patients with small intestinal adenocarcinoma.

Figures

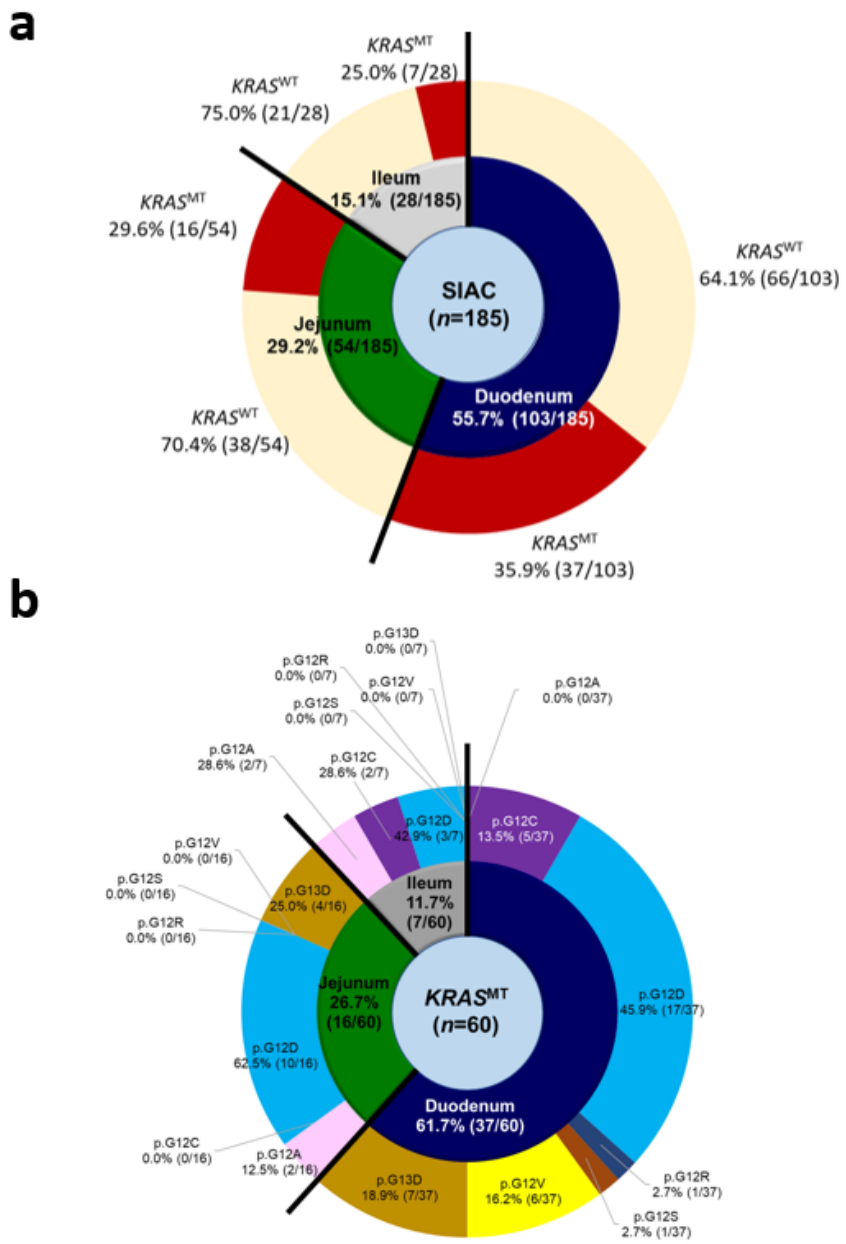

Figure 1

The KRAS genotypes analysis according to the location in patients with small intestinal adenocarcinoma. (a) Small intestinal adenocarcinomas were detected in 55.7% (103/185) duodenum, 29.2% (54/185) jejunum, and 15.1% (28/185) ileum. (b) In subgroup analysis of KRAS mutation group, mutant KRAS (KRASMT) was detected in 61.7% (37/60) duodenum, 26.7% (16/60) jejunum, and 11.7% (7/60) ileum.

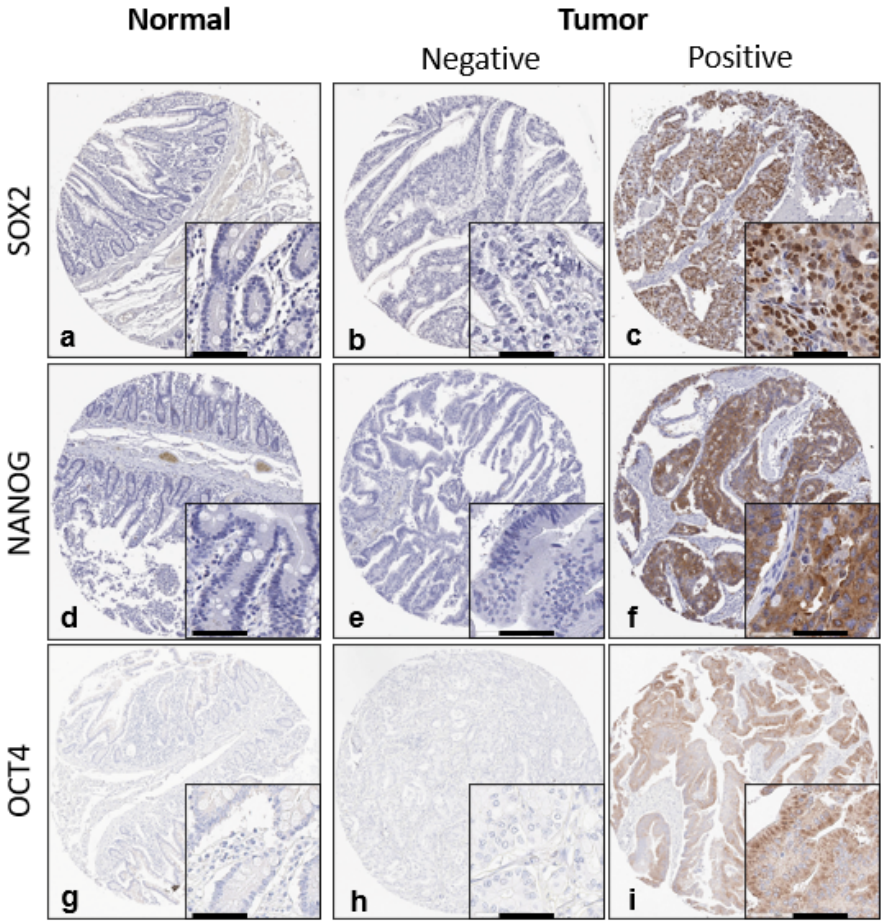

Figure 2

Representative immunohistochemical staining of NANOG, SOX2, and OCT4 expression in small intestinal adenocarcinoma and normal mucosal tissue. In the normal small intestinal epithelial cells (control), NANOG, SOX2, and OCT4 were not stained (a, d, & g). We considered cytoplasmic staining for NANOG and OCT4 (c & i) and nuclear staining for SOX2 (f) as a successful staining. The middle columns show representative negative staining (b, e, & h) (Original magnification, ×8; inset, ×40; scale bar, 60 μm).

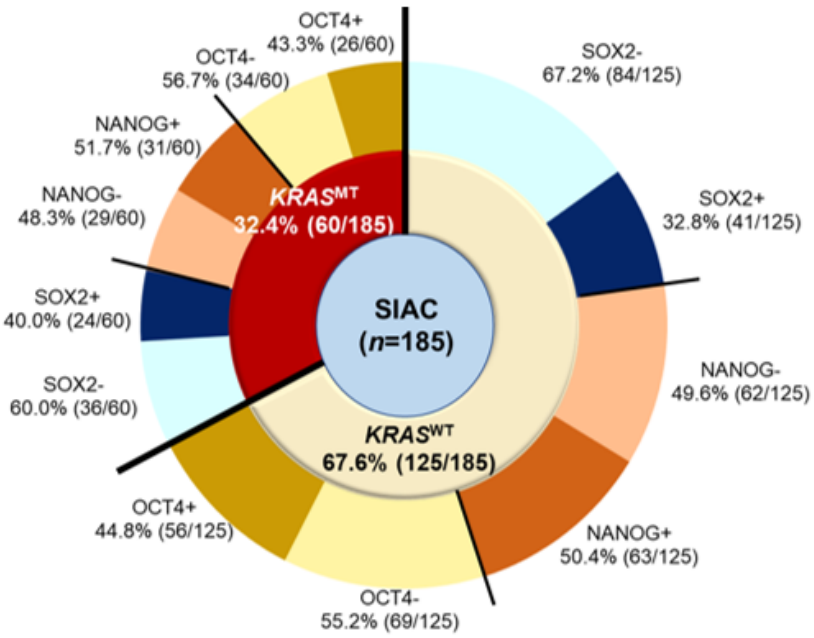

Figure 3

Cancer stem cell markers (SOX2, NANOG, and OCT4) expressional profiling according to KRAS genotypes.

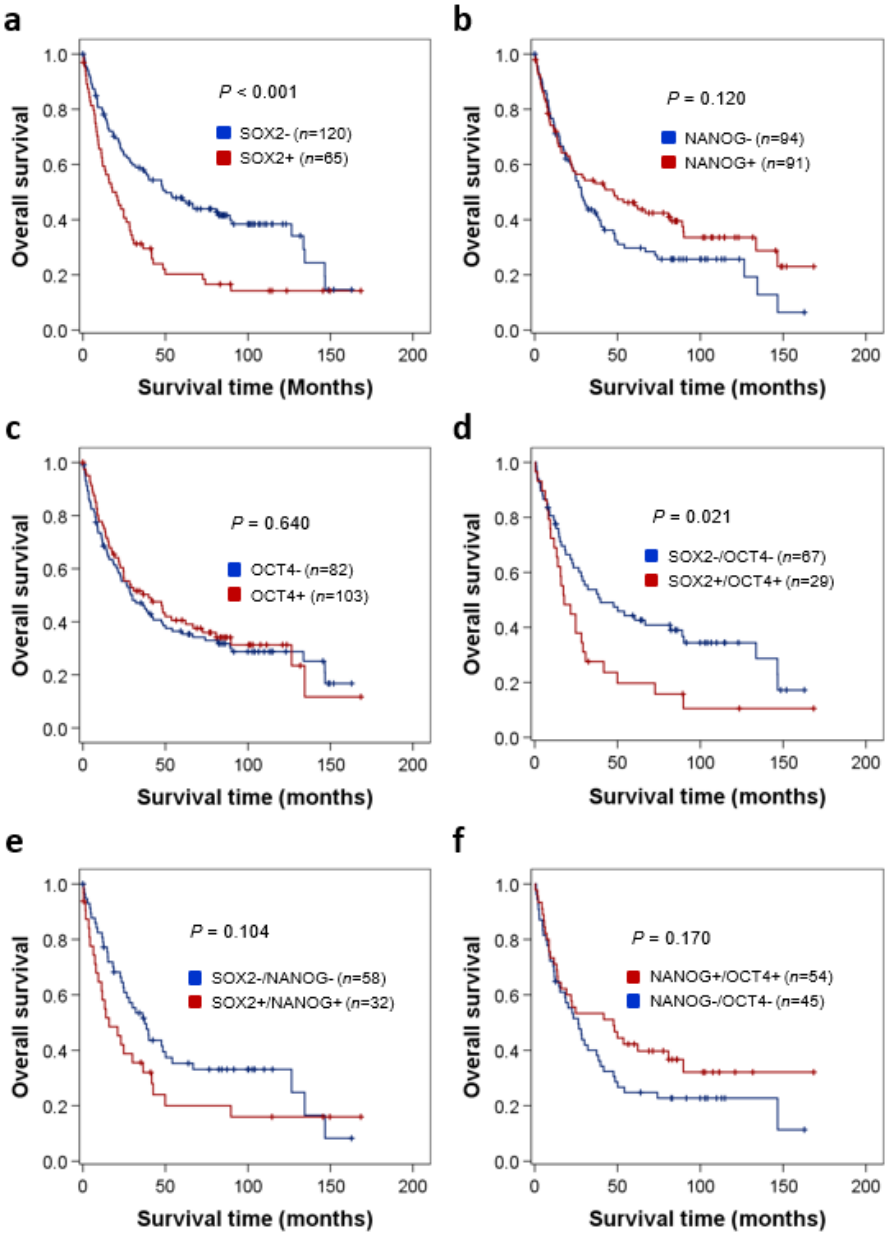

Figure 4

The Kaplan–Meier survival analysis of small intestinal adenocarcinoma patients. (a) Patients with SOX2-positive adenocarcinoma demonstrated lower survival than those with SOX2-negative tumors (log-rank test, P < 0.001). The expression of NANOG (b) and OCT4 (c) were not associated with patient survival. Patients with combined (d) SOX2+/OCT4+ (median 17.8 months) had significantly shorter OS (log-rank test, P = 0.021) than patients with SOX2-/OCT4- (median 39.7 months). There were no meaningful OS differences for the combinations of SOX2/NANOG (e) and NANOG/OCT4 (f) expression.

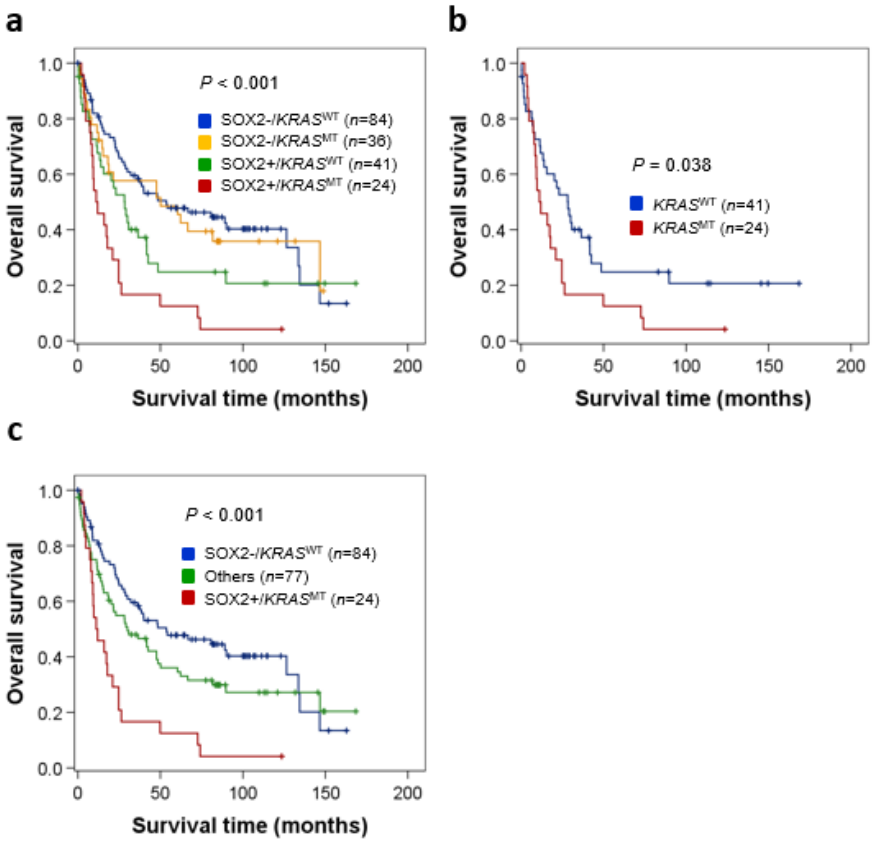

Figure 5

Survival analysis of small intestinal adenocarcinoma patients with SOX2 expression according to KRAS genotypes. (a) Survival differences were observed among 4 small intestinal adenocarcinoma patient groups classified according to their SOX2 expression and KRAS genotype (P < 0.001). (b) Patients with mutant KRAS (KRASMT) (median, 11.1 months) had a short OS rates than those with wild type of KRAS (KRASWT) (median, 28.4 months) in subgroup of SOX2 positive. (c) The survival times for patients with both SOX2-positive and KRASMT was significantly shorter than those with other expression (SOX2-/KRASMT and SOX2+/KRASWT) (median, 30.0 months), and SOX2-/KRASWT (P < 0.001).

Supplementary Files

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- SOX2inSIACSupplTable13BMCGatsroenterology2020v1.docx
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