

Immunohistochemical expression of Snail1 protein in colorectal adenocarcinoma samples and its prognostic activity in Caucasian patients

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Abstract

Introduction: The current study investigated the expression of Snail1 protein in colon adenocarcinoma samples to assess its prognostic significance by correlating its expression with the clinicopathological variables and survival of Caucasian patients.

Aim: To investigate the clinicopathological and prognostic roles of Snail1 expression, the immunohistochemical analysis was performed in colon tumour tissues and adjacent non-pathological mucosa.

Material and methods: It should be noted that only trace expression of this protein was revealed in adjacent non-tumour colorectal mucosa, whereas the high expression was demonstrated in well differentiated, moderately differentiated, and poorly differentiated tumours.

Results: The Kaplan-Meier survival analysis showed that the overall survival rate in the group of patients with a low expression level of Snail1 was significantly longer than that for patients with a moderate or strong level of Snail1 immunoreactivity ($p < 0.001$). The 5-year overall survival for patients with a low, moderate, or strong level of Snail1 immunoreactivity was 100%, 25.7%, and 2%, respectively. The Snail1-moderate patients had an average survival time of 44.886 months (95% CI: 40.855–48.916), whereas the Snail1-strong expression groups had an average survival time of 21.706 (95% CI: 17.863–25.549). The statistical analysis revealed that the level of the Snail1 immunohistochemical reactivity was correlated with the grade of the histological differentiation. A multivariate analysis demonstrated that the grade of tumour differentiation (HR = 2.150; 95% CI: 1.380–3.349, $p = 0.001$) and Snail1 expression (HR = 3.901; 95% CI: 2.436–6.247, $p < 0.001$) were the independent risk factors for worse survival.

Conclusions: In colon adenocarcinoma patients, the expression of Snail1 may act as the independent risk factors for worse survival.

Introduction

It is generally accepted that colon cancer is the most commonly diagnosed gastrointestinal cancer and a major public health problem. As revealed by studies, the clinical outcomes of the patients, especially those with lymph node-positive status, are still unsatisfactory [1, 2]. It is worth noting that about 20% of the diagnoses are made in the metastatic stage of the disease, where the percentage of 5-year overall survival is less than 15%. However, the prognoses are not satisfactory even in the case of patients with primary tumours [3–5].

Therefore, the identification of novel approaches to describe patients from the high-risk group, particular-

ly in the context of the clinical outcomes, has become a major challenge.

Snail1 is a zinc-finger transcription factor, which plays a role in colorectal cancer development by silencing E-cadherin expression and inducing epithelial-to-mesenchymal transition (EMT). Therefore, in colon cancer patients, enhanced levels of Snail1 are usually associated with poor clinical outcome, probably due to down-regulation of E-cadherin expression [6–8].

Aim

The current study investigated the expression of Snail1 protein in colon adenocarcinoma samples to assess its prognostic significance by correlating its expres-

sion with the clinicopathological variables and survival of Caucasian patients.

Material and methods

Tissue samples

The study was conducted on formalin-fixed and paraffin-embedded colon adenocarcinoma samples archived in the files of the Department of Pathomorphology in Zabrze (Poland). Tissue specimens were received from 97 colon adenocarcinoma patients who underwent surgical resection on surgical clinics in 2012.

Table I. Demographic and clinical characteristics of colon adenocarcinoma patients included in the study ($n = 97$)

Parameter	N	%
Gender:		
Females	49	51
Males	48	49
Age [years]:		
≤ 60	26	27
61–75	37	38
≥ 75	34	35
M ± SD	67.81 ± 12.18	
Me [Q1–Q3]	68 [59–79]	
Min.–max.	33–89	
Grade of histological differentiation:		
G1	14	14
G2	52	54
G3	31	32
Depth of invasion:		
T1	3	3
T2	10	10
T3	67	69
T4	17	18
Regional lymph node involvement:		
N0	48	49
N1	37	38
N2	12	12
Location of tumour:		
Proximal	50	52
Distal	47	48

The exclusion criteria were as follows: (1) history of previous malignant disease, (2) familial adenomatous polyposis, (3) inflammatory bowel disease, (4) preoperative anti-cancer treatment, and (5) evidence of distant metastasis. The clinicopathological characteristics obtained from the medical records were as follows: age, gender, location of tumour, grade of tumour differentiation, depth of invasion, lymph node metastasis, operation record, treatment record, recurrence, and vital status at the last follow-up date.

The colon adenocarcinoma specimens belonged to 48 men and 49 women (mean age: 68 years; range: 33–89 years). Tumours were located in the proximal part of the colon in 50 (51.5%) cases and in the distal part of the colon in 47 (48.45%) cases. Three levels of differentiation were used to classify the grading as follows: well differentiated (G1), 14 (14.43%) cases; moderately differentiated (G2), 52 (53.61%) cases; and poorly differentiated (G3), 31 (31.96%) cases (Table I).

Immunohistochemical staining

For the immunohistochemical studies the paraffin-embedded specimens were cut into 4- μ m-thick sections, fixed on Polysine slides, deparaffinised in xylene, and rehydrated through a graded series of alcohol. To retrieve the antigenicity, the tissue sections were treated twice with microwaves in a 10-mM citrate buffer (pH 6.0) for 8 min each. Subsequently, sections were incubated with rabbit polyclonal antibody to Snail1 (final dilution 1 : 500) (GeneTex; cat. number GTX100754). For visualisation of protein expression, the sections were treated with a BrightVision detection system and Permanent AP Red Kit (Zytomed). Mayer's haematoxylin was used to counterstain the nuclei.

Semi-quantitative analysis of Snail1 expression in colorectal adenocarcinoma samples

The scores were assigned separately for the stained area and for the intensity of the immunohistochemical reaction. Quantification connected to the stained area of the tissue section was performed as follows: (1) < 33% of cells showed immunoreaction, (2) 33–66% of the cells had positive reaction to Snail1, and (3) > 66% of the cells were positive. The intensity of the immunohistochemical reaction was quantified as follows: (1) absent or weak, (2) moderate, and (3) strong. Each tissue section was characterised by a final grade derived from the multiplication of the stained area and the intensity of the staining. The Snail1 expression was considered to be: absent/low for grade 1; moderate for grades 2, 3, and 4; and strong for grades 6 and 9.

Survival analysis

Survival analysis was conducted in 97 patients. The survival curves were generated using the Kaplan-Meier method. The overall survival (OS) was defined as the length of time between surgery and death. The follow-up period was 60 months. Patients alive were censored at 5 years.

Statistical analysis

Statistical analyses were conducted using Statistica 9.1 (StatSoft, Poland). The clinical characteristics of the patients in relation to vimentin immunoreactivity were assessed by performing the Kruskal-Wallis test and *U* Mann-Whitney test. Additionally, using Spearman's rank correlation coefficient, the relationship between the immunoreactivity of GRP94 and age, grade of tumour differentiation, and size of primary tumour was assessed. The Kaplan-Meier method was used to study

survival curves, and the long-rank test was used to compute differences between the curves.

Results

To investigate the clinicopathological and prognostic roles of Snail1 expression, the immunohistochemical analysis was performed in colon tumour tissues and adjacent non-pathological mucosa. It should be noted that only trace expression of this protein was revealed in adjacent non-tumour colorectal mucosa, whereas high expression was demonstrated in well, moderately, and poorly differentiated tumours (Figure 1). Among the 97 samples, 11 (11.34%) showed a weak immunohistochemical reaction, 35 (36.08%) demonstrated moderate immunoreactivity, and 51 (52.58%) revealed strong expression.

The relationships between the Snail1 levels and each clinicopathological parameter are summarised in Table II. As demonstrated, the level of the Snail1 immunohistochemical reactivity was correlated with the

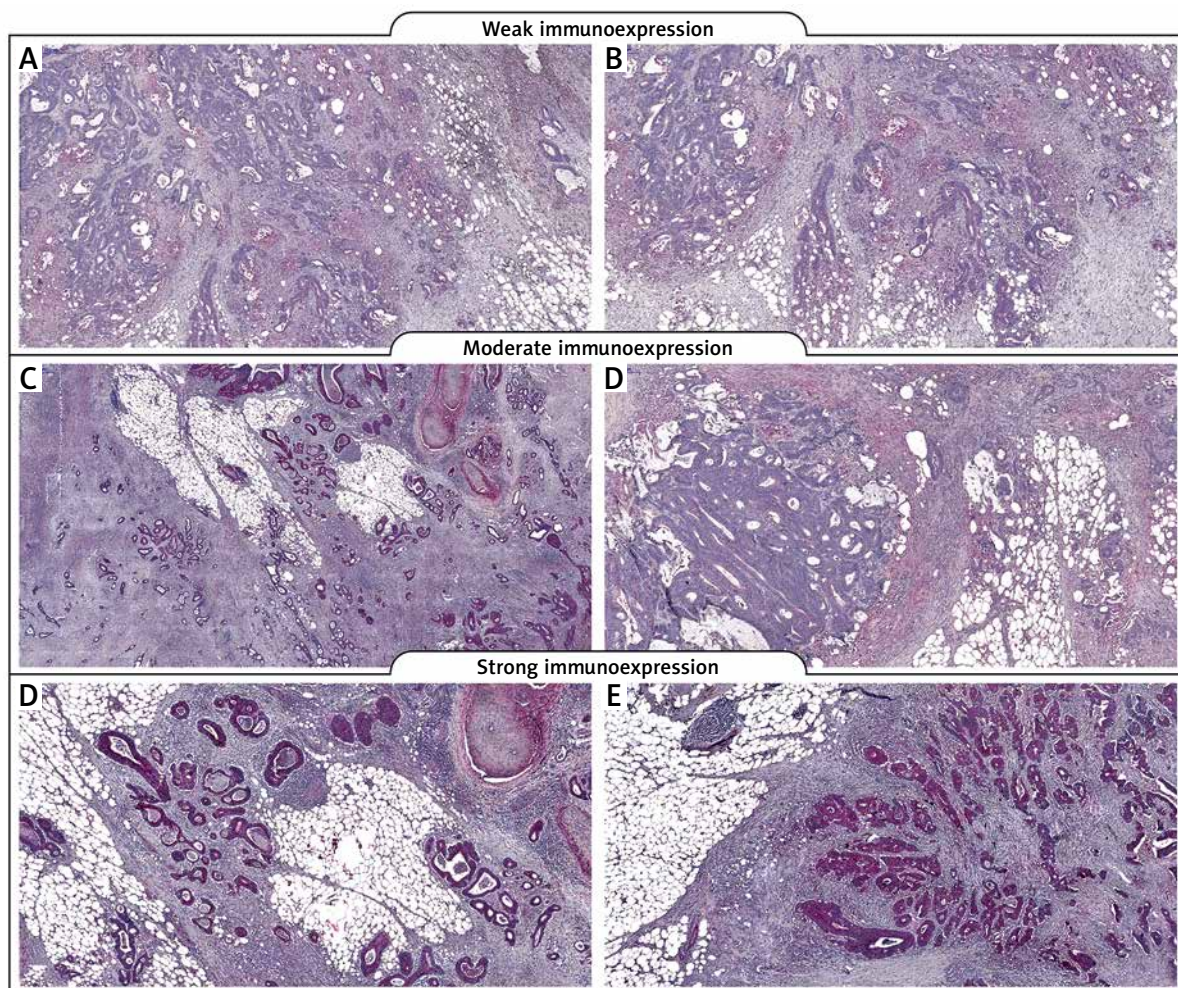


Figure 1. Immunohistochemical expression of Snail1 in colon adenocarcinoma patients according to intensity of immunohistochemical reaction

Table II. Correlations between Snail1 immunoeexpression and clinicopathological characteristics in colon adenocarcinoma patients

Parameter	The immunoeexpression level of Snail1			The mean range	Statistical analysis
	1	2	3		
Age [years]:					
< 60	1 3.85%	13 50.00%	12 46.15%	47.96	H (2.97) = 1.865. p = 0.394 R = -0.067, p = 0.516
61–75	4 10.81%	10 27.03%	23 62.16%	53.24	
> 75	6 17.65%	12 35.29%	16 47.06%	45.18	
Gender:					
Females	6 12.24%	20 40.82%	23 46.94%	46.37	Z = -1.033, p = 0.302
Males	5 10.42%	15 31.25%	28 58.33%	51.69	
Grade of histological differentiation:					
G1	10 71.43%	4 28.57%	0 0.00%	12.57	H (2.97) = 37.949 p < 0.001 G1 < G2, G1 < G3
G2	1 1.92%	24 46.15%	27 51.92%	50.89	
G3	0 0.00%	7 22.58%	24 77.42%	62.29	
Depth of invasion:					
T1/T2	4 30.77%	5 38.46%	4 30.77%	35.15	H (2.97) = 4.651 p = 0.098
T3	5 7.46%	24 35.82%	38 56.72%	51.67	
T4	2 11.76%	6 35.29%	9 52.94%	49.06	
Regional lymph node involvement:					
N0	7 14.58%	19 39.58%	22 45.83%	45.35	H (2.97) = 3.851 p = 0.146
N1	4 10.81%	13 35.14%	20 54.05%	49.76	
N2	0 0.00%	3 25.00%	9 75.00%	61.25	
Location of tumour:					
Proximal	6 12.00%	19 38.00%	25 50.00%	47.74	Z = -0.502, p = 0.615
Distal	5 10.64%	16 34.04%	26 55.32%	50.34	

Z – result of U Mann-Whitney test, H – result of Kruskal-Wallis test, R – Spearman's rank correlation coefficient.

grade of the histological differentiation ($H [2.97] = 37.949; p < 0.001$). A strong expression of Snail1 protein was detected more frequently in patients with G3 tumours than in patients with G1 tumours. Moreover, a significant difference was also detected between the patients with G1 tumour and G2. In the G2 patients a strong reactivity was described more frequently. The statistical evaluation of Snail1 immunoreactivity according to age, sex, tumour location, and depth of invasion revealed no significant difference among these variables (all $p > 0.05$; Table II).

The Kaplan-Meier survival analysis showed that the overall survival rate in the group of patients with a low expression level of Snail1 was significantly longer than that for patients with a moderate or strong level of Snail1 immunoreactivity ($p < 0.001$; Figure 2). The 5-year overall survival for patients with a low, moderate, or strong level of Snail1 immunoreactivity was 100%, 25.7%, and 2%, respectively. The Snail1-moderate patients had an average survival time of 44.886 months (95% CI: 40.855–48.916), whereas the Snail1-strong expression groups had an average survival time of 21.706 months (95% CI: 17.863–25.549) (Table III). In the univariate analysis the grade of tumour differentiation and Snail1 immunoreactivity were found to be significantly associated with reduced 5-year survival. Moreover, a multivariate analysis demonstrated that grade of tumour differentiation (HR = 2.150; 95% CI: 1.380–3.349, $p = 0.001$) and Snail1 expression (HR = 3.901; 95% CI: 2.436–6.247, $p < 0.001$) were the independent risk factors for worse survival (Table IV).

Discussion

The results of our study demonstrate that in comparison to samples of non-tumour colon mucosa, the samples of colon cancer were characterized by high levels of Snail1 immunoreactivity. Moreover, the high levels of vimentin immunoreactivity were demonstrated to be associated with malignancy-related clinicopathological factors and 5-year overall survival among Caucasian patients. It should be pointed out that the high

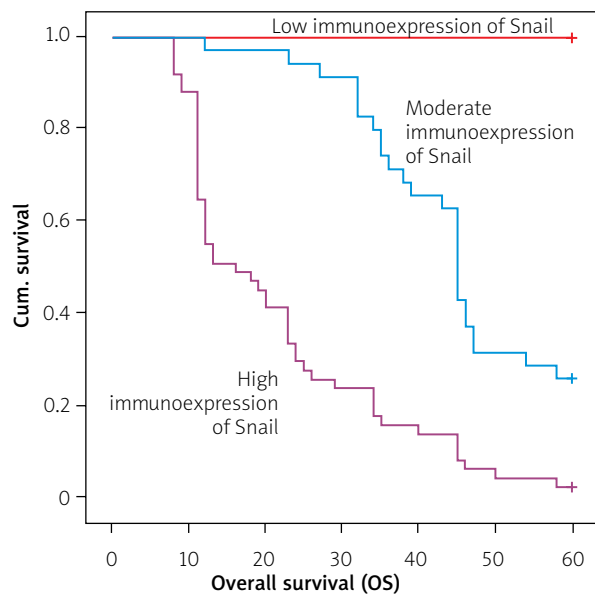


Figure 2. Kaplan-Meier survival curves of colon adenocarcinoma patients with weak, moderate, and strong expression of Snail1 protein; follow-up period = 60 months

immunoreexpression of Snail1 was significantly related to advanced histological tumour grade. The Kruskal-Wallis test showed a statistical difference between the patients with G1 tumours and those with G2 tumours, and between the patients with G1 tumours and those with G3 tumours. In both cases, patients with G1 tumours were described by a low level of vimentin immunoreexpression ($p < 0.001$). As revealed by the studies during colorectal cancerogenesis, the adenoma is the first stage exhibiting Snail1 expression, which is inversely correlated with expression of E-cadherin [7]. This finding is in agreement with the statement that Snail1 acts as a transcriptional repressor of *CDH1*. It is well demonstrated that repression of *CDH1* is a consequence of molecular reaction cascade, during which a major role

Table III. Means and medians for survival time of colon adenocarcinoma patients according to Snail1 immunoreexpression

IHC immunoscores	Mean				Median			
	Estimate	Std. error	95% confidence interval		Estimate	Std. error	95% confidence interval	
			Lower bound	Upper bound			Lower bound	Upper bound
1	–	–	–	–	–	–	–	–
2	44.886	2.056	40.855	48.916	45.000	0.836	43.360	46.640
3	21.706	1.961	17.863	25.549	16.000	2.678	10.752	21.248
Overall	31.140	1.887	27.441	34.838	32.000	4.211	23.747	40.253

Table IV. Multivariate analyses of various prognostic parameters in colon adenocarcinoma patients using Cox-regression analyses

Prognostic parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender	0.9	0.583–1.436	NS	–	–	–
Age	0.9	0.970–1.005	NS	–	–	–
Grade	3	2.063–4.388	< 0.001	2.150	1.380–3.349	0.001
	1.3	0.913–2.012	NS	–	–	–
Regional lymph node involvement	1.1	0.802–1.528	NS	–	–	–
Location of tumour	1.2	0.777–1.912	NS	–	–	–
Snail1	5	3.004–7.252	< 0.001	3.901	2.436–6.247	< 0.001

is played by interactions with regions containing the consensus core sequence (CAGGTC), a motif that is a subset of E-box (CANNTG) [9, 10]. The expression of Snail1 in adenoma tissue suggests that the EMT process promoting cancer metastasis is already active in precancerous lesions. Studies with the use of transgenic mouse models such as HER-2 and PyMT have revealed that tumour cells may disseminate in morphologically benign hyperplastic lesions [11]. It should be stressed here that in the case of colon tissue, Snail1 expression has also been revealed in normal mucosa in close vicinity to the tumour lesions. Interestingly, some differences between non-pathological tissue and cancer tissue have been demonstrated at the subcellular level, where the shift of the protein to the cytoplasm with mixed cytoplasmic and nuclear or pure cytoplasmic expression has been detected [12]. Regarding Snail1 expression in colorectal mucosa with no specific pathological changes, some published results suggest that Snail1 is not associated with malignant transformation only. In non-pathological mucosa, this factor may be involved in the maintenance of homeostasis by regulation of processes at the cellular level such as proliferation, differentiation, and apoptosis [12].

Moreover, up-regulation of Snail1 expression has been reported to promote regional and distant lymph node metastasis [13, 14]. Snail1 expression as a marker for lymph node metastasis has also been reported in the case of breast cancer and oesophageal squamous carcinoma [14–16]. Some authors revealed that about 77% of colon cancer samples display Snail1 immunostaining in the stroma, especially in the stromal cells with fibroblast-like phenotype [17–19]. Expression of this factor in the stroma will indicate how many cells possess the ability to escape from the primary tumour mass and how many cells may invade the basal lamina to colonize distant target organs [20]. Therefore, the presence of Snail1 immunoreactive cells in the stroma may serve as prognostic marker in patients with colon cancer. It is worth

noting that intratumoural injection with Snail1-specific monoclonal antibody inhibits tumour growth and metastasis followed by an increased number of stromal tumour-infiltrating lymphocytes (TILs). Snail1 immunoreactivity in tumour stroma is related to distant metastasis and lower specific survival of patients [18]. Importantly, this correlation was also detected in stage I and II, which are known to display a variable prognosis. The capability to predict recurrence at these stages would be important from a clinical point of view because markers for them have not yet been identified. Thus, the expression of Snail1 would help to distinguish patients with an unfavourable clinical outcome and would be applicable for chemotherapy, which is not a treatment commonly used in the early stages of colorectal tumours [18]. It is worth noting that no association between Snail1 expression and clinical parameters has been revealed in adenocarcinomas of the upper gastrointestinal tract [21]. Probably, there are other more powerful mechanisms, which are implicated in EMT triggering during colon cancer development, e.g. factors associated with the Wnt signalling pathway. Larriba *et al.* revealed that Wnt signalling and Snail1 may be interconnected by multiple positive loops, possibly adding to the robustness of both [22].

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Conflict of interest

The author declares no conflict of interest.

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