Original Research

The Prognostic Significance of Apoptotic Protease Activating Factor (Apaf-1) Protein Expression in Colon Adenocarcinoma Tissue-Preliminary Report

Marlena Brzozowa-Zasada[1],*👤, Natalia Matysiak[1], Adam Piecuch[1], Ewa Gawełek[1], Marek Michalski[1], Marek Kucharzewski[2], Marek J. Łos[3,4,5]👤

[1]Department of Histology and Cell Pathology in Zabrze, Faculty of Medical Sciences in Zabrze, Medical University of Silesia in Katowice, 40-055 Katowice, Poland
[2]Departmentof Descriptive and Topographic Anatomy, Faculty of Medical Sciences in Zabrze, Medical University od Silesia, 40-055 Katowice, Poland
[3]Department of Pathology, Pomeranian Medical University, 71-344 Szczecin, Poland
[4]Autophagy Research Center, Shiraz University of Medical Sciences, 7134845794 Shiraz, Iran
[5]Linkocare Life Sciences AM, 583-30 Linkoping, Sweden
*Correspondence: marlena.brzozowa@op.pl (Marlena Brzozowa-Zasada)
Academic Editors: Francesca Bruzzese and Antonio Barbieri
Submitted: 17 November 2022 Revised: 5 January 2023 Accepted: 12 January 2023 Published: 16 February 2023

Abstract

Background: The Apoptotic protease activating factor 1 (Apaf-1) protein, as one of the factors involved in the activation of the mitochondrial apoptotic pathway, plays an important role in cancer biology. Apaf-1 expression in tumour cells has been shown to be downregulated, with significant implications for tumour progression. Hence, we investigated the expression of Apaf-1 protein in the Polish population of patients with colon adenocarcinoma without any therapy prior to radical surgery. Moreover, we assessed the relation between Apaf-1 protein expression and the clinicopathological factors. The prognostic activity of this protein was analyzed in relation to 5-year survival of patients. In order to show the localization of Apaf-1 protein at the cellular level, the immunogold labelling method was used. **Methods**: The study was conducted using the colon tissue material from patients with histopathologically confirmed colon adenocarcinoma. Immunohistochemical expression of Apaf-1 protein was performed using Apaf-1 antibody at dilution 1:600. The associations between the immunohistochemistry (IHC) expression of Apaf-1 and clinical parameters were analyzed using the Chi^2 test and Chi^2_{Yatesa} test. Kaplan-Meier analysis and the log-rank test were used to verify the relationship between the intensity of Apaf-1 expression and 5-year survival rate of patients. The results were considered statistically significant when $p < 0.05$. **Results**: Apaf-1 expression was evaluated by immunohistochemical staining in whole tissue sections. Thirty-nine (33.23%) samples had strong Apaf-1 protein expression and 82 (67.77%) samples were characterized by low expression. The high expression of Apaf-1 was clearly correlated with the histological grade of the tumour ($p = 0.001$), proliferating cell nuclear antigen (PCNA) immunohistochemical expression ($p = 0.005$), age ($p = 0.015$), depth of invasion ($p < 0.001$) and angioinvasion ($p < 0.001$). The 5-year survival rate was significantly higher in the group of patients with high expression of this protein (log-rank, $p < 0.001$). **Conclusions**: We can conclude that Apaf-1 expression is positively correlated with reduced survival of colon adenocarcinoma patients.

Keywords: immunohistochemistry; prognostic marker; 5-year survival rate; colon adenocarcinoma; apoptosis; immunogold labelling

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers of the gastrointestinal tract with approximately 90% of histopathological diagnoses being colorectal adenocarcinoma (COAD) [1]. Factors that significantly influence the development of this type of malignancy include lifestyle, e.g., obesity, prolonged and frequent smoking, alcohol consumption and a diet low in fibre but rich in red meat. Other risk factors include the presence of chronic inflammatory bowel disease (IBD), inadequate exposure to sunlight, including vitamin D3 deficiency, and genetically determined changes leading to colonic mucosal cell dysplasia [2–4]. It should also be noted that CRC is a highly invasive type of cancer and shows a high level of heterogeneity. This in turn raises many questions and concerns regarding the timely di-

agnosis and treatment of patients. The 5-year survival rate applies to approximately 60–95% of patients with early-stage disease but drops significantly (approximately 35%) with the presence of lymph node metastases [5–7]. Hence there is a need for early diagnosis, which would certainly improve patient survival.

Apoptotic protease activating factor 1 (Apaf-1) is a protein with multiple domains in its structure, including a caspase recruitment domain (CARD), a nucleotide-binding domain and an oligomerization domain (NOD). A WD-40 repeat region (WDR) is also present in the Apaf-1 protein framework [8]. The released cytochrome C associates with a single Apaf-1 molecule within the WD40 domain to generate a heptameric apoptosome, ultimately leading to caspase activation [9,10]. As Apaf-1 is known to be a key

molecule during the activation of mitochondrial apoptosis pathway apoptosis, and changes in its expression may contribute to many diseases including cancer [11,12]. Therefore, a detailed understanding of the function and regulation of Apaf-1 may influence the development of new better anti-cancer therapies.

Zlobec *et al*. [13,14] revealed that in Canadian patients Apaf-1 is a marker of tumour progression in MMR- proficient colorectal cancer and an independent adverse prognostic factor in MLH1-negative colorectal cancer. Nevertheless, we have little data on Apaf-1 protein expression in patients suffering from colon adenocarcinoma, especially in the European population. With this in mind, we decided to investigate the expression of Apaf-1 protein in the Polish population of patients with colon adenocarcinoma without any therapy prior to radical surgery. Moreover, we also investigated the association between Apaf-1 protein expression and the clinicopathological factors of colon adenocarcinoma patients. The prognostic activity of this protein was analyzed in relation to 5-year survival of patients. It should be pointed out that our study is the first that assessed the correlation between immunohistochemical expression of Apaf-1 and Proliferative Cell Nuclear Antigen (PCNA) in the population of patients with colon adenocarcinoma. Yang *et al*. [15] revealed that PCNA expression has been found to correlate with the degree of malignancy, vascular infiltration, distant metastasis and survival. This antigen has been described as a biomarker of colorectal adenocarcinoma [16]. Nevertheless, the relationship between Apaf-1 and PCNA in colon adenocarcinoma has not yet been assessed.

2. Materials and Methods

2.1 Patients and Tumour samples

Tissue colon material collected from the patients undergoing colon resection at the Municipal Hospital in Jaworzno between January 2014 and December 2015 with histopathologically confirmed colon adenocarcinoma was used for the study. Patients who received preoperative radiotherapy or chemotherapy, patients with distant metastasis, patients undergoing resection from tumour recurrence, patients with adenocarcinoma in the setting of inflammatory bowel disease and patients with histopathologically confirmed subtype other than adenocarcinoma were excluded from the study. Based on an established protocol, histopathological sections containing tumour fragments and adjacent tissue sections without tumour lesions were taken from each surgical specimen. The collected samples were fixed in formalin and embedded in paraffin blocks. In the next step, the paraffin blocks were cut and sections were routinely stained with hematoxylin-eosin staining (H&E) to confirm the histopathological diagnosis. Sections containing tissue margins were also assessed. If tumour cells were found, the material was excluded from the study. To determine whether Apaf-1 protein had prognostic significance, patients were followed up for 5 years to estimate the 5-year survival rate.

2.2 Immunohistochemical Staining

Paraffin-embedded tissue blocks with formalin-fixed colon adenocarcinoma specimens and resected margins were cut into 4-m-thick sections, fixed on Polysine slides and deparaffinized in xylene and rehydrated through a graded series of alcohol. To retrieve the antigenicity, the tissue sections were treated with microwaves in a 10 mM citrate buffer (pH 6.0) for 8 min each. Subsequently, sections were incubated with antibody to Apaf-1 (Zytomed Systems. polyclonal antibody. order no. 501395. final dilution 1:600, Berlin, Germany) and Proliferating Cell Nuclear Antigen (PCNA) (GeneTex. polyclonal antibody. Cat. No. GTX100539, final dilution 1:600, Irvine, CA, USA). For visualization of protein expression, the sections were treated with Bright Vision detected system and Permanent AP Red Kit (Zytomed, Cat. No. ZUCC001-125, Berlin, Germany). Mayer's haematoxylin was used to counterstain the nuclei). In addition, the Expression of Apaf-1 and PCNA was studied in sections of healthy mucosa from patients undergoing screening colonoscopy with no inflammatory or cancerous lesions. The scoring of Apaf-1 and PCNA expression was based on both the intensity and frequency of immunohistochemical reaction determining the presence of Apaf-1 and PCNA. The intensity was graded as follows: 0, no signals; 1, weak; 2, moderate; and 3, strong staining. The frequency of positive tumour cells was determined semiquantitatively by assessing the whole section, and each sample was scored on a scale of 0 to 4: 0, negative; 1, positive staining in 10–25% cells, 2, 26–50% cells; 3, 51–75% cells; and 4, 76–100% cells. A total score of 0–12 was finally calculated and graded as; I, score 0–1; II, 2–4; III, 5–8; IV, 9–12. Grade I was considered negative; and grades II, III and IV were positive. Grades I and II represented no or weak staining (low expression), and grades III and IV represented strong staining (strong expression). The evaluation was carried out by two independent pathologists. Differences were again assessed until consensus was obtained.

2.3 Statistical Analysis

The associations between the IHC expression of Apaf-1 and clinical parameters were analyzed statistically with Statistica 9.1 (Software, StatSoft, Cracow, Poland). All the quantitative variables were described as medians and ranges. The Chi^2 test and Chi^2_{Yatesa} test were used to compare the analyzed groups. Kaplan-Meier analysis and the log-rank test were used to verify the relationship between the intensity of Apaf-1 expression and 5-year survival rate of patients. The results were considered statistically significant when $p < 0.05$.

All figures in this manuscript were generated by the use of Xara Designer Pro X 18 (ProX 18, MAGIX Software GmbH, Berlin, Germany).

2.4 Immunogold Electron Microscopy

For the study with the use of immunogold labelling methods, the tissue samples were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 2 hours at room temperature and then washed several times in PBS. After washing, the specimens were dehydrated in a graded ethanol series and infiltrated in a 2:1 (v:v) ethanol/LR White mixture and 1:2 (v:v) for 30 min each on ice. Afterwards, the samples were infiltrated in pure LR White. Ultra-thin sections (70 nm) were cut with a RMC Boeckeler Power Tomo PC ultramicrotome with a diamond knife (45°; Diatom AG, Biel, Switzerland). Ultrasections were mounted on 200-mesh nickel grids coated with Formvar and immunolabelled. Sections on the grids were preincubated first for 30 minutes by floating on drops of 50 mM NH_4Cl in PBS and subsequently blocked for 30 minutes on drops of 1% BSA- Bovine Serum Antigen (BSA) in PBS. The grids were then incubated overnight (16–18 h) at 4 °C with primary anti-Apaf-1 antibody (Zytomed Systems, polyclonal antibody; order no. 501395, Berlin, Germany) diluted 1:20 in BSA. The bound antibodies were localized by incubating the sections for 1 h on Immunogold-conjugated goat anti-mouse IgG 15 nm (BB International, BBI Solutions, Sittingbourne, UK) diluted 1: 100. Lastly, the grids were washed on PBS drops (five changes, 5 min each) and water (three changes, 3 min each) before staining with 0.5% aqueous uranyl acetate. In controls, the primary antibody was not used. The grids were then air-dried and analyzed in a company transmission electron microscope at 120 kV (Tecnai 12 G2 Spirit BIOTWIN, FEI, Hillsboro, OR, USA). Images were captured using a Morada CCD camera (Catan RIO 9, Pleasanton, CA, USA).

3. Results

3.1 Patients' Characteristics

The patient characteristics are summarized in Table 1.

The colon adenocarcinoma specimens belonged to 63 men and 58 women (mean age: 64; range: 55–78 years). Tumours were located in the proximal part of the colonin64 (52.89%) cases and in the distal part of the colon in 57 (47.11%). Three levels of histological differentiation were used to classify the grading as follows: G1, 20 cases (16.53%), G2, 64 cases (52.89%) and G3, 37 (30.58%).

A positive immunohistochemical reaction determining the expression of Apaf-1 protein was found in cancerous tissues and surgical margins of colon adenocarcinoma patients (Fig. 1).

It should be noted that in the vast majority of patients, the expression in cancer tissue was determined to be low. The cytoplasmic or membranous expression was detected in cancer cells and stromal cells. In contrast, expression in surgical margins and healthy colon mucosa was described as strong. In healthy colon mucosa, the expression was detected in glandular cells and in infiltrating cells of lamina propria.

Table 1. Characteristics of Patients Included in the Study (n = 121).

		N (number of cases)	%
Gender	Females	58	47.93
	Males	63	52.07
Age [years]	≤60 years	47	38.84
	61–75 years	36	29.75
	>75 years	38	31.45
	M ± SD	65.89 ± 13.46	
	Me [Q1–Q3]	64 [55–78]	
	Min–Max	33–89	
Grade of histological differentiation	G1	20	16.53
	G2	64	52.89
	G3	37	30.58
Depth of invasion	T1	11	9.09
	T2	14	11.57
	T3	75	61.98
	T4	21	17.36
Regional LymphNode involvement	N0	49	40.49
	N1	43	35.54
	N2	29	23.97
Location of tumour	Right sided tumours	64	52.89
	Left sided tumours	57	47.11
Angioinwasion	No	26	21.49
	Yes	95	78.51
PCNA expression	Low	33	27.27
	High	88	72.73
Staging	I	18	14.88
	II	34	28.10
	III	69	57.02

3.2. Correlations between Apaf-1 Immunohistochemical Expression and Clinicopathological Parameters

39 (33.23%) samples of colon adenocarcinoma specimens had strong Apaf-1 protein expression whereas a low level of immunoreactivity was observed in 82 samples (67.77%). The high expression of Apaf-1 was inversely related to histological grade of the tumour ($p = 0.001$, Chi^2 test). A high level of Apaf-1 expression was noted in 17 (85%), 18 (28.13%) and 4 (10.81%) tumours at G1, G2 and G3 respectively. Moreover, the expression of Apaf-1 was related to PCNA immunohistochemical expression ($p = 0.005$, Chi^2 test; Sperman coefficient R = –0.253, $p = 0.005$) (Fig. 2, Table 2). The high level of Apaf-1 protein expression was revealed in 17 (51.52%) and 22 (25%) samples with low and high expression of PCNA respectively (Fig. 3).

It should be pointed out that Apaf-1 protein expression was markedly correlated with age ($p = 0.015$, Chi^2 test), depth of invasion ($p < 0.001$, Chi^2 test) and angioinvasion ($p < 0.001$, Chi^2_{Yatesa} test). However, there were no significant correlations between Apaf-1 protein expression and gender, lymph node metastasis and localization (Table 3).
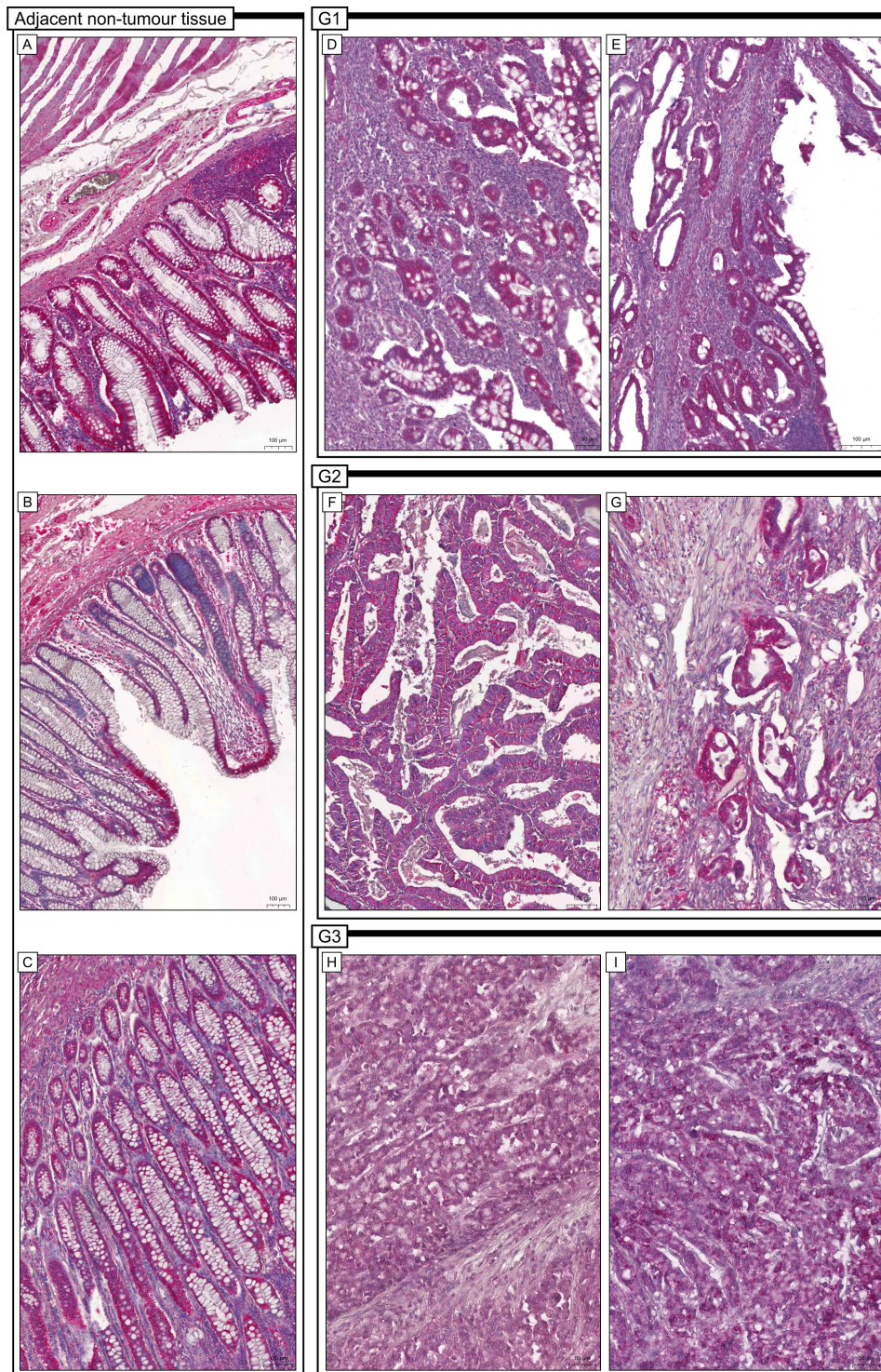
Fig. 1. Representative microphotographs of immunohistochemical expression of Apaf-1 (Apaf-1 antibody at dilution: 1:600) in colon adenocarcinoma tissue (G1, G2, G3) and tissue margins with no cancerous lesions (adjacent non-tumour tissue). (A,B,C-magnification ×200) The strong immunohistochemical expression of Apaf-1 in control samples of the colon mucosa. (D,E,G) Strong Apaf-1 immunostaining in G1, G2 and G3 tumours, respectively. (F,I) Low level of immunohistochemical reaction in G2 and G3 tumours respectively (H). The scale bar is 100 μm (A,B-magnification ×100, C-magnification ×200, E-magnification ×100, F-magnification ×150, G-magnification×100, H, I-magnification ×300) and 50 μm (D). Red colour in adenocarcinoma tissues (cancer tissue and stromal tissue) shows positive immunohistochemical reaction indicating the presence of Apaf-1.Mayer's haematoxylin was used to counterstain the nuclei.

Table 2. Correlations between the Expression of Apaf-1 Protein and PCNA protein.

		The immunoexpression level of Apaf-1				Statistical analysis	
		Low		High			
PCNA expression	Low	16	(48.48%)	17	(51.52%)	Chi^2 = 7.725	p = 0.005
	High	66	(75.00%)	22	(25.00%)	R = −0.253	p = 0.005

Table 3. Correlations between the Expression of Apaf-1 Protein and Clinicopathological Characteristics in Colon Adenocarcinoma Patients.

		The immune expression level of Apaf-1				Statistical analysis
		Low		High		
Age [Years]	≤60 years	37	(78.72%)	10	(21.28%)	Chi^2 = 8.402
	61–75 years	26	(72.22%)	10	(27.78%)	p = 0.015
	>75 years	19	(50.00%)	19	(50.00%)	df = 2
Gender	Females	41	(70.69%)	17	(29.31%)	Chi^2 = 0.435
	Males	41	(65.08%)	22	(34.92%)	p = 0.509
						df = 1
Grade of histological differentiation	G1	3	(15.00%)	17	(85.00%)	Chi^2 = 33.763
	G2	46	(71.88%)	18	(28.13%)	p < 0.001
	G3	33	(89.19%)	4	(10.81%)	df = 2
Depth of invasion	T1	1	(9.09%)	10	(90.91%)	Chi^2 = 19.733
	T2	9	(64.29%)	5	(35.71%)	p < 0.001
	T3	56	(74.67%)	19	(25.33%)	df = 3
	T4	16	(76.19%)	5	(23.81%)	
Regional Lymph Node involvement	N0	30	(61.22%)	19	(38.78%)	Chi^2 = 2.731
	N1	29	(67.44%)	14	(32.56%)	p = 0.255
	N2	23	(79.31%)	6	(20.69%)	df = 2
Angioinwasion	No	8	(30.77%)	18	(69.23%)	Chi^2 $_{Yatesa}$ test = 0.18653
	Yes	74	(77.89%)	21	(22.11%)	p < 0.001
						df = 1
Localisation	Right sided tumours	42	(65.63%)	22	(34.38%)	Chi^2 = 0.286
	Left sided tumours	40	(70.18%)	17	(29.82%)	p = 0.593
						df = 1
Staging	I	6	(33.33%)	12	(66.67%)	Chi^2 = 11.481
	II	25	(73.53%)	9	(26.47%)	p = 0.003
	III	51	(73.91%)	18	(26.09%)	df = 3

3.3. Prognostic Role of Apaf-1 Expression in Colon Adenocarcinoma

The prognostic significance of Apaf-1 expression in colon adenocarcinoma patients was analyzed in relation to 5-year survival rate. All samples were assessed by Kaplan-Meier survival curves. The 5-year survival rate was significantly higher in the group of patients where high Apaf-1 expression was found (log-rank, $p < 0.001$) (Fig. 4).

Additionally, the value of Apaf-1 expression in the context of the 5-year survival rate was evaluated in patients' subgroups stratified by PCNA expression, grade of histological differentiation, depth of invasion and lymph node involvement. The expression of Apaf-1 was strongly associated with the 5-year survival rate of patients with a high level of PCNA expression. The patients with a high level of this antigen and low level of Apaf-1 expression has significantly lower 5-year survival rate (log rank test, $p = 0.012$).

Interestingly, in the group of patients without lymph node involvement and with low level of Apaf-1 expression, the 5-year survival rate was also reduced (log rank test, $p = 0.002$). Similar results have been obtained in patients with T3/T4 depth of invasion (log rank test, $p = 0.003$) and low levels of Apaf-1 immunohistochemical reaction (Fig. 5).

Univariate Cox regression analyses revealed that Apaf-1 protein level, the grade of histological differentiation, and depth of invasion are significant prognostic factors. However, multivariate analysis showed that Apaf-1 cannot be considered an independent indicator associated with the 5-year survival rate in patients with colon adenocarcinoma. Additionally, the multivariate analysis showed that only one clinicopathological factor should be considered an independent prognostic marker. In our group of patients, this factor was associated with the grade of histological differentiation (Table 4).

Table 4. Univariate and Multivariate Analyses of Various Prognostic Parameters in Colon Adenocarcinoma Patients using Cox Regression Analyses.

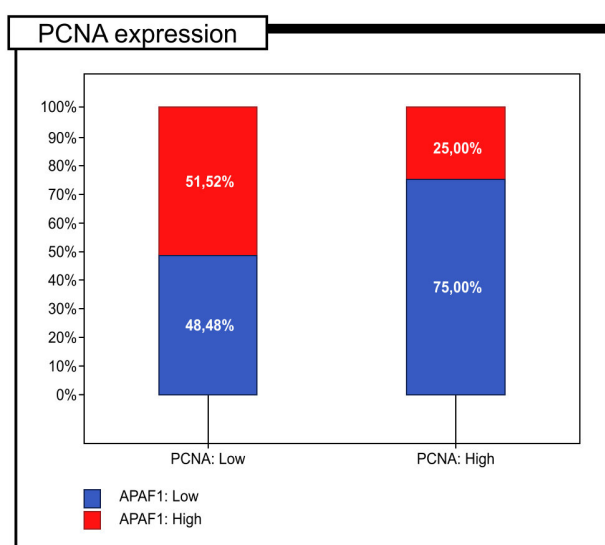| Prognostic parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
Gender	1.088	0.718–1.651	0.690	–	–	–
Age	1.006	0.991–1.021	0.408	–	–	–
Grade	2.907	2.062–4.097	<0.001	2.402	1.596–3.614	<0.001
Depth of invasion	1.824	1.367–2.434	<0.001	1.265	0.899–1.780	0.177
Regional Lymph Node involvement	1.202	0.924–1.563	0.171	–	–	–
Angioinwasion	3.598	1.855–6.981	<0.001	1.097	0.500–2.406	0.818
Localisation	1.166	0.769–1.768	0.469	–	–	–
PCNA expression	2.061	1.226–3.465	0.006	1.461	0.794–2.690	0.223
Staging	1.357	1.018–1.808	0.037	0.866	0.592–1.269	0.461

HR, hazard ratio.

Fig. 2. Percentage of immunohistochemical expression of PCNA defined as high and low expression in colon adenocarcinoma patients (n = 121).

3.4. Intracellular Localization of Apaf-1 by the Method of Immunogold Labelling with the Use of Transmission Electron Microscopy (TEM)

In order to show the localization of Apaf-1 protein at the cellular level of colon adenocarcinoma tissue, the immunogold labelling method was used (Fig. 6). The black electron-dense granules formed by immunohistochemical reaction indicating the presence of Apaf-1 protein were found both in cancer cells and in cells of colon epithelium in control samples. The small gold particles conjugated to the anti-Apaf-1 antibody were dispersed in the cytoplasm in vicinity of the endoplasmic reticulum and Golgi Apparatus. Moreover, black electron-dense granules were found in mitochondria. Interestingly, in the control samples, the gold granules associated with the Apaf-1 antibody were detected within the endoplasmic reticulum and small vesicles, especially in the apical part of cells (Fig. 6).

4. Discussion

Apoptosis is a process that is critical to the systemic homeostasis of multicellular organisms. Considering that Apaf-1 is a focal molecule during activation of the intrinsic apoptotic pathway, deregulation of its expression is unavoidably implicated in human diseases, including cancer [17–19]. Inactivation of the *Apaf-1* gene is implicated in disease progression and chemoresistance of some malignancies [20]. Unfortunately, little is known about the prognostic significance of the Apaf-1 protein in patients with colon adenocarcinoma, especially in the European population. Paik *et al*. [21] have shown that specimens of primary colorectal adenocarcinomas and around 88% of metastatic lesions exhibited impairment in Apaf-1 expression. This could suggest that impairment of Apaf-1 expression is implicated in the malignant transformation from adenoma to adenocarcinoma, particularly during the later stage of colorectal carcinogenesis. In addition, the lower expression of Apaf-1 in samples with metastatic lesions compared to primary colorectal adenocarcinoma would suggest that loss of Apaf-1 expression may promote metastasis. Moreover, a study of allelic imbalance of the Apaf-1 locus in colorectal cancer revealed that allelic imbalance of the Apaf-1 locus on 12q23 was absent in adenoma tissue, but was commonly noted in adenocarcinoma and metastatic tumour tissue. What is more, mRNA levels were markedly reduced, which was consistent with their allelic imbalance [22]. Results of our study demonstrated that expression of Apaf-1 in colon cancerous tissue was lower than that observed in normal tissue margin or healthy colon tissue. Moreover, by the use of the immunogold labelling method, we have confirmed the Apaf-1 presence in the cytoplasm of tumour cells, mostly in the vicinity of membranous organelles including the endoplasmic reticulum, Golgi Apparatus and mitochondria. The presence of Apaf-1 protein was also observed in normal cells of non-tumour colonic mucosa. Our study revealed that approximately 32% of colon adenocarcinoma specimens demonstrated strong Apaf-1 protein expression, while low levels of immunoreactivity were found
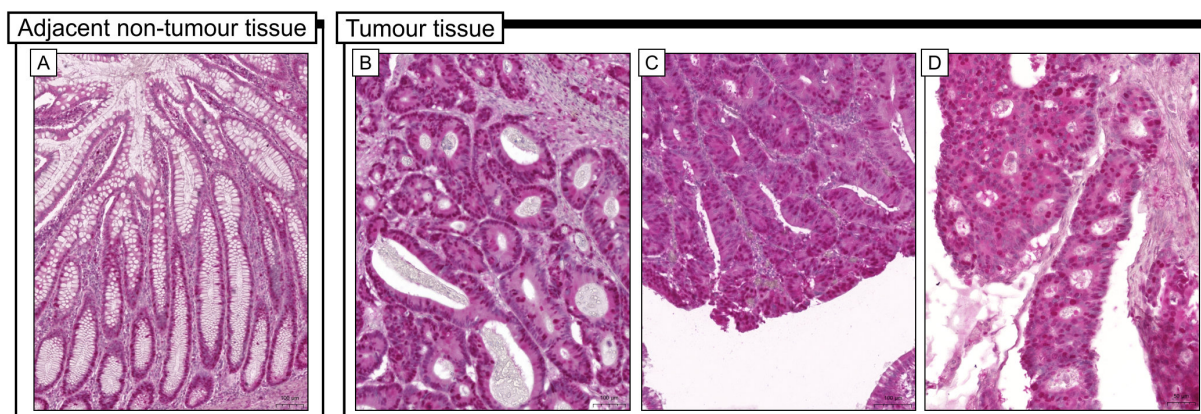
Fig. 3. Representative microphotographs of immunohistochemical expression of PCNA (dilution 1:600) in colon adenocarcinoma tissue (B-magnification ×200, C-magnification ×1500, D-magnification ×200) and tissue margins with no cancerous lesions (A-magnification ×100). (B,C,D) The strong immunohistochemical expression of PCNA in the nuclei of cancer cells (red colour). (A) Low level of immunohistochemical reaction in adjacent non-tumour tissue. The scale bar is 100 μm (A, B, C) and 50 μm (D). Red colour in adenocarcinoma tissues (cancer cells and stromal cells) shows positive immunohistochemical reaction indicating the presence of PCNA. Mayer's haematoxylin was used to counterstain the nuclei.
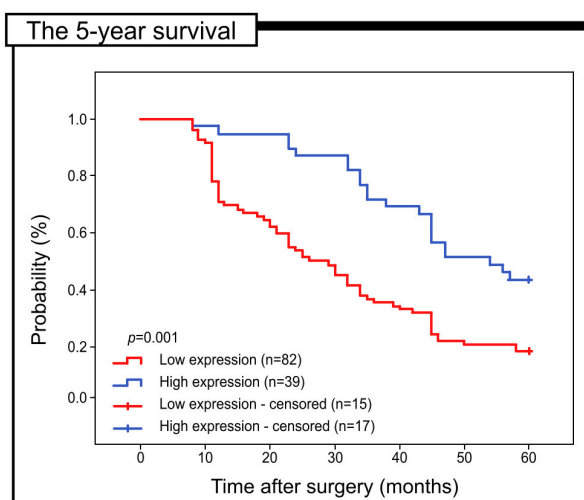

Fig. 4. Kaplan-Meier curves of univariate analysis date (log-rank test) showing the 5-year survival rate for patients with high versus low Apaf-1 expression.

in 68% of cases. High Apaf-1 expression was inversely related to the histological grade of the tumour ($p = 0.001$, Chi^2 test). The strong expression of Apaf-1 protein was noted in 85% of G1 tumours, 30% of G2 tumours and 10% of G3 tumours. Moreover, the expression of Apaf-1 was associated with PCNA immunohistochemical expression ($p = 0.005$, Chi^2 test). The high level of Apaf-1 reactivity was revealed in 52% and 25% of samples with low and high PCNA expression, respectively. PCNA, a non-histamine nuclear protein, has a molecular mass of 36 kDa and is a specific marker of cell division. Its action is associated with DNA polymerase, synthesized shortly before the S-phase of the cell cycle [23]. It should be noted that the expression of PCNA was the most intensive in those patients who were characterized by a low level of Apaf-1 expression. Expression of Apaf-1 was also correlated with the age of patients ($p = 0.015$, Chi^2 test), depth of invasion ($p < 0.001$, Chi^2 test) and angioinvasion ($p < 0.001$, Chi$^2_{\text{Yatesa}}$ test).

The multivariate analysis revealed that expression of Apaf-1 in colorectal cancer tissue showed no prognostic significance in terms of 5-year survival time. In the studied group of patients, only the parameter of histological differentiation shows prognostic value. However, the obtained findings indicate that patients with a high level of Apaf-1 protein expression confirmed by immunohistochemistry have significantly longer survival times and the large majority of them achieve a 5-year survival time, which, in the context of clinical oncology, may be important. In addition, when patients are stratified according to clinical parameters, e.g., PCNA expression, the expression of Apaf-1 can have a prognostic value in the context of 5-year survival. Patients with high Apaf-1 expression and high PCNA expression had a statistically better survival time (median overall survival 44 vs. 29.50 in patients with low Apaf-1 and high PCNA). Our study is the first which demonstrated that patients with tumours at the G1 stage and characterized by a strong level of Apaf-1 immunoreactivity had a relatively higher 5-year survival rate in comparison to patients with low expression of this protein. Moreover, in patients with tumours characterized by T3/T4 depth of invasion, the expression of Apaf-1 was also enhanced. These results may indicate that Apaf-1 plays an important role in colon adenocarcinoma progression and may be an identification biomarker for patients with a more aggressive form of this malignancy.
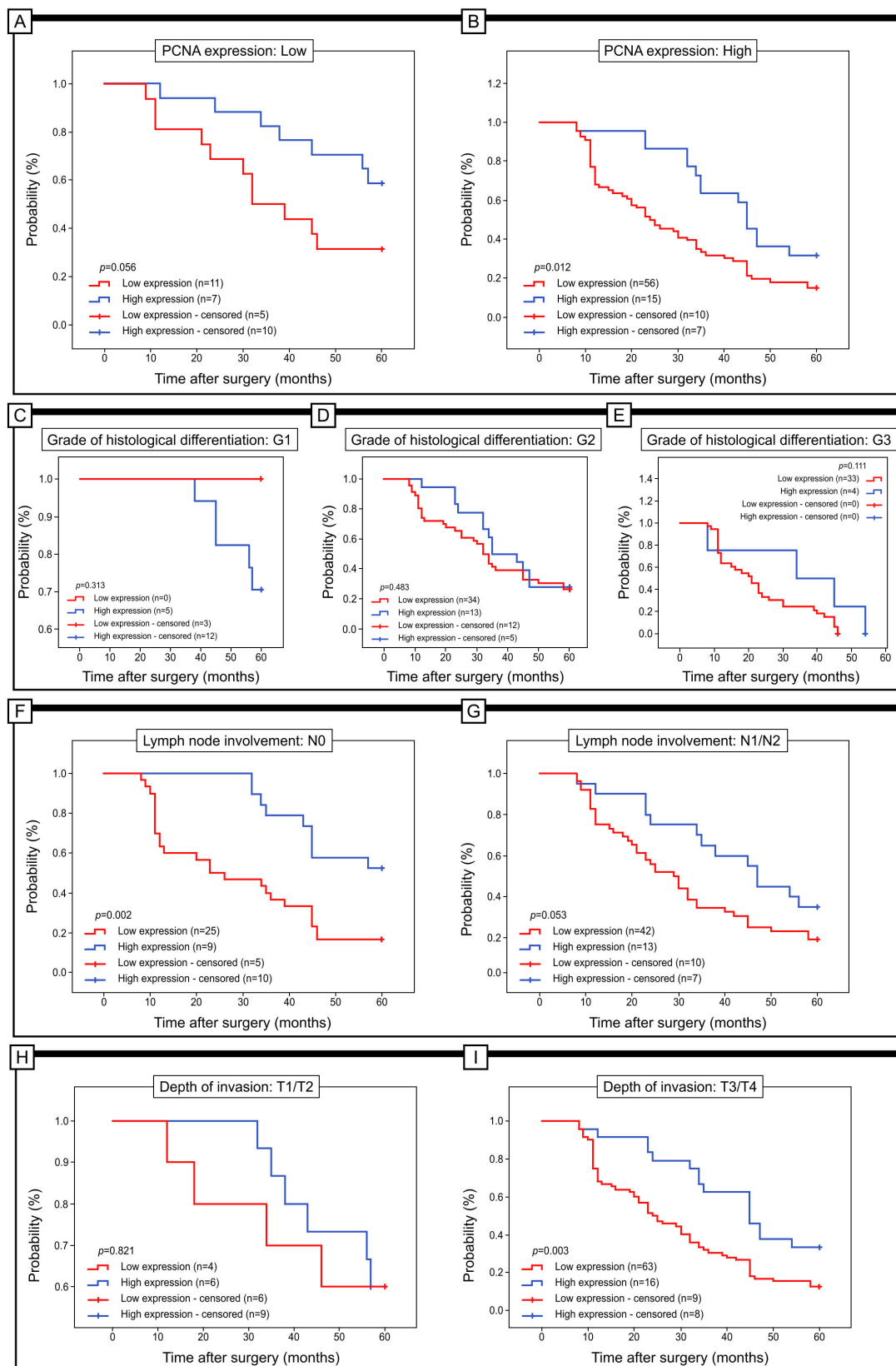
Fig. 5. Kaplan-Meier curves of univariate analysis date (log-rank test) of patients with high versus low level of Apaf-1 immunore-activity. (A,B) 5-year survivaval of patients with low (A) and high (B) PCNA expression. (C,D,E) 5-year survival rate of patients with tumour at G1 (A), G2 (B) and G3 (C). (F,G) 5-year survivaval of patients without lymph node involvement (F) and with lymph node involvement (G). (H,I) 5-year survivaval of patients with T1/T2 (H) and with T3/T4 depth of invasion (I).
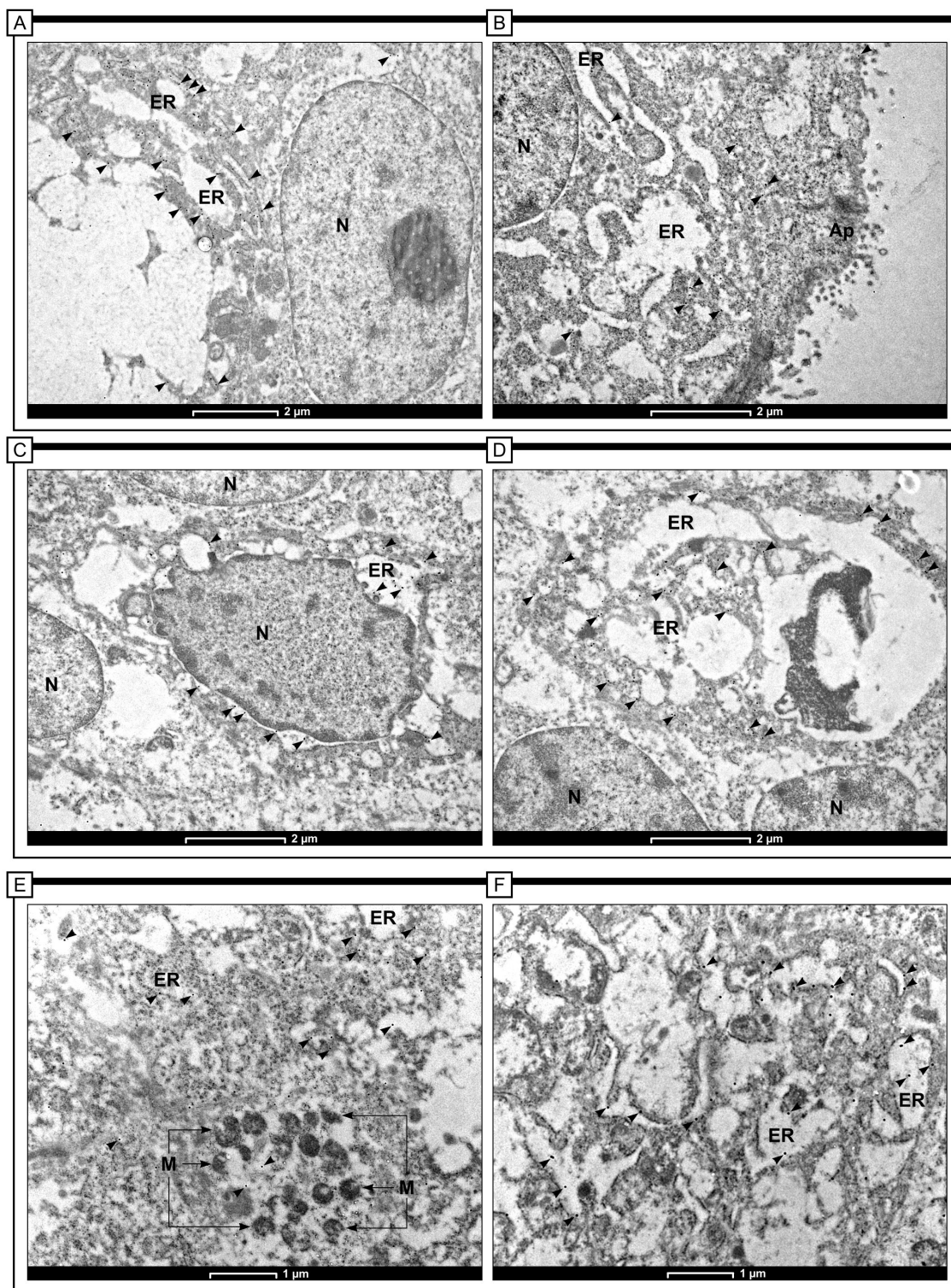
Fig. 6. Immunogold labeling of Apaf-1 protein in colon adenocarcinoma tissue in control cells (A-magnification ×9900, B-magnification ×11500) and cancer cells (C-magnification ×11500, D-magnification ×11500, E-magnification ×16500, F-magnification ×16500). (A,B) The small electron-dense particles (arrowheads) were found to be associated with membrane of the endoplasmic reticulum (ER). (B) The positive reaction (arrowheads) were also found in small vesicles especially in the apical part (Ap) of the cells. In cancer cells gold granules conjugated to the anti-Apaf-1 antibody (arrowheads) were connected with the membranes of endoplasmic reticulum (ER) and mitochondria (M). The scale bar is 2 μm (A–D) and 1 μm (E,F).

As numerous studies have shown, colon adenocarcinoma is not the only cancer type to have reduced expression of Apaf-1 protein. Lukosiute-Urbonieniehas shown that in pancreatic ductal adenocarcinoma (PDAC) downregulation of Apaf-1 expression was detected both at mRNA and protein levels [24]. Decreased level of Apaf-1 was also found in serum samples of patients [25]. In breast adenocarcinoma, Apaf-1 expression was downregulated at both the transcriptional and posttranslational levels [26]. Wang *et al*. [27] showed that the low expression level of Apaf-1 in breast cancer patients was probably related to the hypermethylation process. In contrast, Lauber *et al*. [28] identified cleavage of Apaf-1 in cytosolic extracts of Jurkat or MCF-7 cells after incubation with cytochrome c/dATP or caspase, suggesting another potential mechanism for reduced Apaf-1 levels in breast cancer. In addition, Fang *et al*. [29] showed that increased miR-937 expression has the effect of reducing Apaf-1 protein levels through binding to the Apaf-1-3′UTR. Reduced Apaf-1 expression levels were also found in serous ovarian cancer treated with first-line chemotherapy including paclitaxel and carboplatin [30]. Similar results have been obtained in lung cancer [31,32] and head and neck cancer [33,34]. Interestingly, Soengas *et al*. [35] revealed that in metastatic melanomas Apaf-1 is inactivated which leads to defects in the execution of apoptotic cell death. Apaf-1 loss may contribute to the low frequency of p-53 mutations observed in this highly chemoresistant tumour type. In the context of Apaf-1 prognostic significance, it should be mentioned that expression of Apaf-1 protein is under epigenetic control, with Apaf-1 gene hypermethylation being associated with poor prognosis in the case of melanoma [34], renal cell carcinoma [36], neuroblastoma [37] and bladder cancer [38].

5. Conclusions

Based on the results obtained in the Cox regression model, Apaf-1 has been identified as a protein connected with the reduced 5-year survival rate in the univariate analysis. The multivariate analysis revealed that only the grade of histological differentiation might be considered a prognostic factor. However, we can conclude that Apaf-1 expression is positively correlated with reduced survival of colon adenocarcinoma patients.

Nevertheless, our study has some limitations that need to be mentioned. The size of the studied group of patients was limited and the patients came from a single hospital, which may introduce selection bias into the study. Future studies should be conducted to increase the sample size in multicentre studies. A large number of patients will also allow the parameter of *RAS* gene mutations to be addressed. Moreover, the prognostic activity of Apaf-1 should be investigated in the context of Disease Free Survival, especially in relation to chemotherapy that depends on apoptosis and *in vivo* tests to understand the mechanism of action of the Apaf-1 factor during the development of colon adeno-

carcinomas. It would be interesting also to know the mRNA levels of PCNA and Apaf-1 in colon adenocarcinoma tissue versus normal adjacent tissue.

Availability of Data and Materials

The data used to support the findings of this research are available upon request.

Author Contributions

MB-Z designed the research study. MB-Z, AP and NM performed the research. MB-Z and MK provided help and advice on raising funds on research. MB-Z and EG analyzed the data. MM drafted the figures. MB-Z and MŁ wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Academy of Physical Education in Katowice (Resolution no. 1/2013 aproved on 21 November 2013).

Conflict of Interest

The authors declare no conflict of interest. Prof. Łos'es role in the compay is dedicated to the development of specific cellular elements within artificial corneas; it has nothing to do with the research area presented in our paper.

References

[1] Pericleous S, Bhogal RH, Mavroeidis VK. The Role of Circulating Biomarkers in the Early Detection of Recurrent Colorectal Cancer Following Resection of Liver Metastases. Frontiers in Bioscience-Landmark. 2022; 27: 189.

[2] Daniel CL. Colorectal cancer disparities beyond biology Screening treatment access. Frontiers in Bioscience-Landmark. 2017; 22: 465–478.

[3] Harbiyeli IFC, Burtea DE, Ivan ET, Streață I, Nicoli ER, Uscatu D, *et al*. Assessing Putative Markers of Colorectal Cancer Stem Cells: From Colonoscopy to Gene Expression Profiling. Diagnostics. 2022; 12: 2280.

[4] Latos W, Aebisher D, Latos M, Krupka-Olek M, Dynarowicz K, Chodurek E, *et al*. Colonoscopy: Preparation and Potential Complications. Diagnostics. 2022; 12: 747.

[5] Sawicki T, Ruszkowska M, Danielewicz A, Niedźwiedzka E, Arłukowicz T, Przybyłowicz KE. A Review of Colorectal Can-

cer in Terms of Epidemiology, Risk Factors, Development, Symptoms and Diagnosis. Cancers. 2021; 13: 2025.

[6] Xi Y, Xu P. Global colorectal cancer burden in 2020 and projections to 2040. Translational Oncology. 2021; 14: 101174.

[7] Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. Nature Reviews Gastroenterology Hepatology. 2019; 16: 713–732.

[8] Benedict MA, Hu Y, Inohara N, Núñez G. Expression and Functional Analysis of Apaf-1 Isoforms. Journal of Biological Chemistry. 2000; 275: 8461–8468.

[9] Dorstyn L, Akey CW, Kumar S. New insights into apoptosome structure and function. Cell Death Differentiation. 2018; 25: 1194–1208.

[10] Yuan S, Akey C. Apoptosome Structure, Assembly, and Procaspase Activation. Structure. 2013; 21: 501–515.

[11] Cecconi F, Gruss P. Apaf1 in developmental apoptosis and cancer: how many ways to die? Cellular and Molecular Life Sciences. 2001; 58: 1688–1697.

[12] Cavalcante GC, Schaan AP, Cabral GF, Santana-da-Silva MN, Pinto P, Vidal AF, et al. A Cell's Fate: An Overview of the Molecular Biology and Genetics of Apoptosis. International Journal of Molecular Sciences. 2019; 20: 4133.

[13] Zlobec I, Minoo P, Baker K, Haegert D, Khetani K, Tornillo L, et al. Loss of APAF-1 expression is associated with tumour progression and adverse prognosis in colorectal cancer. European Journal of Cancer. 2007; 43: 1101–1107.

[14] Zlobec I, Lugli A, Baker K, Roth S, Minoo P, Hayashi S, et al. Role of APAF-1, E-cadherin and peritumoral lymphocytic infiltration in tumour budding in colorectal cancer. The Journal of Pathology. 2007; 212: 260–268.

[15] Yang H, Hsu P, Chan S, Lee J, Shin J, Chow N. Growth kinetics of colorectal adenomacarcinoma sequence: an immunohistochemical study of proliferating cell nuclear antigen expression. Human Pathology. 1996; 27: 1071–1076.

[16] Guzińska-Ustymowicz K, Pryczynicz A, Kemona A, Czyzewska J. Correlation between proliferation markers: PCNA, Ki-67, MCM-2 and antiapoptotic protein Bcl-2 in colorectal cancer. Anticancer Research. 2009; 29: 3049–3052.

[17] Morana O, Wood W, Gregory CD. The Apoptosis Paradox in Cancer. International Journal of Molecular Sciences. 2022; 23: 1328.

[18] Kashyap D, Garg VK, Goel N. Intrinsic and extrinsic pathways of apoptosis: Role in cancer development and prognosis. Advances in Protein Chemistry and Structural Biology. 2021; 20: 73–120.

[19] Vaghari-Tabari M, Ferns GA, Qujeq D, Andevari AN, Sabahi Z, Moein S. Signaling, metabolism, and cancer: an important relationship for therapeutic intervention. Journal of Cellular Physiology. 2021; 236: 5512–5532.

[20] Furukawa Y, Sutheesophon K, Wada T, Nishimura M, Saito Y, Ishii H, et al. Methylation silencing of the Apaf-1 gene in acute leukemia. Molecular Cancer Research. 2005; 3: 325–334.

[21] Paik SS, Jang K, Song YS, Jang S, Min K, Han HX, et al. Reduced Expression of Apaf-1 in Colorectal Adenocarcinoma Correlates with Tumor Progression and Aggressive Phenotype. Annals of Surgical Oncology. 2007; 14: 3453–3459.

[22] Umetani N, Fujimoto A, Takeuchi H, Shinozaki M, Bilchik AJ, Hoon DSB. Allelic imbalance of APAF-1 locus at 12q23 is related to progression of colorectal carcinoma. Oncogene. 2004; 23: 8292–8300.

[23] Kelman Z. PCNA: structure, functions and interactions. Oncogene. 1997; 14: 629–640.

[24] Lukosiute-Urboniene A, Mazeike A, Kazokaite M, Silkuniene G, Silkunas M, Barauskas V, et al. Epigenetic Regulation of APAF-1 through DNA Methylation in Pancreatic Cancer. Anticancer Research. 2020; 40: 3765–3779.

[25] Jia K, Zhao X, Dang X. Mass spectrometry-based iTRAQ analysis of serum markers in patients with pancreatic cancer. Oncology Letters. 2020; 19: 4106–4114.

[26] Vinothini G, Murugan RS, Nagini S. Mitochondria-mediated apoptosis in patients with adenocarcinoma of the breast: Correlation with histological grade and menopausal status. Breast. 2011; 20: 86–92.

[27] Loginov VI, Pronina IV, Burdennyi AM, Pereyaslova EA, Braga EA, Kazubskaya TP, et al. Role of Methylation in the Regulation of Apoptosis Genes APAF1, DAPK1, and BCL2 in Breast Cancer. Bulletin of Experimental Biology and Medicine. 2017; 162: 797–800.

[28] Lauber K, Appel HAE, Schlosser SF, Gregor M, Schulze-Osthoff K, Wesselborg S. The Adapter Protein Apoptotic Protease-activating Factor-1 (Apaf-1) is Proteolytically Processed during Apoptosis. Journal of Biological Chemistry. 2001; 276: 29772–29781.

[29] Fang H, Jiang W, Jing Z, Mu X, Xiong Z. miR-937 regulates the proliferation and apoptosis via targeting APAF1 in breast cancer. Onco Targets and Therapy. 2019; 12:5687–5699.

[30] Abdelrahman AE, Fathy A, Elsebai EA, Nawar N, Etman WM. Prognostic impact of Apaf-1, Cyclin D1, and AQP-5 in serous ovarian carcinoma treated with the first-line chemotherapy. Annals of Diagnostic Pathology. 2018; 35: 27–37.

[31] Zang Y, Zhong Y, Fang Z, Li B, An J. MiR-155 inhibits the sensitivity of lung cancer cells to cisplatin via negative regulation of Apaf-1 expression. Cancer Gene Therapy. 2012; 19: 773–778.

[32] Li T, Ding Z, Zheng Y, Wang W. MiR-484 promotes non-small-cell lung cancer (NSCLC) progression through inhibiting Apaf-1 associated with the suppression of apoptosis. Biomedicine Pharmacotherapy. 2017; 96: 153–164.

[33] Kuwahara D, Tsutsumi K, Oyake D, Ohta T, Nishikawa H, Koizuka I. Inhibition of caspase-9 activity and Apaf-1 expression in cisplatin-resistant head and neck squamous cell carcinoma cells. Auris Nasus Larynx. 2003; 30: 85–88.

[34] SUN X, LIU B, ZHAO X, WANG L, JI W. MicroRNA-221 accelerates the proliferation of laryngeal cancer cell line Hep-2 by suppressing Apaf-1. Oncology Reports. 2015; 33: 1221–1226.

[35] Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature. 2001; 409: 207–211.

[36] Christoph F, Weikert S, Kempkensteffen C, Krause H, Schostak M, Köllermann J, et al. Promoter Hypermethylation Profile of Kidney Cancer with New Proapoptotic p53 Target Genes and Clinical Implications. Clinical Cancer Research. 2006; 12: 5040–5046.

[37] Grau E, Martinez F, Orellana C, Canete A, Yañez Y, Oltra S, et al. Hypermethylation of apoptotic genes as independent prognostic factor in neuroblastoma disease. Molecular Carcinogenesis. 2011; 50: 153–162.

[38] Christoph F, Weikert S, Kempkensteffen C, Krause H, Schostak M, Miller K, et al. Regularly methylated novel pro-apoptotic genes associated with recurrence in transitional cell carcinoma of the bladder. International Journal of Cancer. 2006; 119: 1396–1402.