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The transcriptional activity profile of inhibitor apoptosis protein encoding genes in colon cancer patients

A STROBE-compliant study

Dariusz Waniczek, MD, PhD[a,*], Marcin Nowak, PhD[c], Justyna Lorenc-Góra, PhD[b], Małgorzata Muc-Wierzgoń, MD, PhD[d], Urszula Mazurek, MD, PhD[e], Magda Bichalska-Lach, MD[b], Zbigniew Lorenc, MD, PhD[c]

Abstract

The inhibitor of apoptosis family proteins (IAPs) plays a crucial role in the process of carcinogenesis by regulating apoptosis and maintaining the tissue balance.

In this study, a transcriptomic analysis of IAP-encoding genes in colon cancer was performed using oligonucleotide microarrays.

Adenocarcinoma and healthy colon tissue samples were collected from 32 patients (16 females and 16 males) who underwent surgery due to colon cancer. The mRNA was extracted from tissue samples and tested using oligonucleotide microarrays (Affymetrix). The results were validated using the qRT-PCR technique. Hierarchical grouping was used to allocate 37 samples of normalized mRNA concentrations into 4 groups, with statistically significant differences in gene expression between these groups. The group of genes associated with colon cancer, including IAP-encoding gene - BIRC5 (Survivin), was selected for further testing.

Our study confirmed an increased expression of BIRC5 in colon cancer tissue when compared to the control group. Increased levels of Neuronal Apoptosis Inhibitory Proteins were detected only in low-stage colon cancer, while the expression of Human X Chromosome-Encoded inhibitor of apoptosis family proteins decreased in colon cancer.

The transcriptional activity of IAP-encoding genes varied, depending on the severity of colon cancer. The concentration of mRNA, encoding BIRC5 was elevated in samples obtained from more advanced colon cancer. Hence BIRC5 could be used as a complementary parameter for the diagnosis and prognosis of colon cancer.

Abbreviations: BIRC5 = survivin, C1 = control group, C2 = second control group, CC = colon cancer, CS = clinical stage, HSC = high stage cancer, LSC = low stage cancer, NAIP = neuronal apoptosis inhibitory protein, XIAP = human X chromosome-encoded inhibitor of apoptosis family proteins.

Keywords: apoptosis, colon cancer, human X chromosome-encoded inhibitor of apoptosis family proteins, inhibitor of apoptosis family proteins, neuronal apoptosis inhibitory protein, survivin

1. Introduction

Colon cancer (CC) is the second most common cancer diagnosed in women and the third most in men.[1] The global incidence of this cancer is estimated at over 1 million people per year.[2] Experimental, genetic, epidemiological, and socioeconomic studies have suggested that CC results from complex interactions between inherited susceptibility, clinical conditions, and environmental or lifestyle-related risk factors.[3] New molecular

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[a] *Department of Oncological Surgery, Faculty of Medical Sciences in Zabrze, Medical University of Silesia, Katowice, Poland,* [b] *Department of Surgical Nursing and Propaedeutics of Surgery, Faculty of Health Sciences in Katowice, Medical University of Silesia, Katowice, Poland,* [c] *Department of General, Colorectal and Polytrauma Surgery, Faculty of Health Sciences in Katowice, Medical University of Silesia, Katowice, Poland,* [d] *Department of Internal Medicine, Faculty of Health Sciences in Bytom, Medical University of Silesia, Katowice, Poland,* [e] *Department of Molecular Biology, Faculty of Pharmaceutical Sciences in Sosnowiec Medical University of Silesia, Katowice, Poland.*

[*] *Correspondence: Dariusz Waniczek, Department of Oncological Surgery, Faculty of Medical Sciences in Zabrze, Medical University of Silesia, Katowice, Poland (e-mail: dwaniczek@sum.edu.pl).*

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markers are still being sought for earlier and more accurate diagnosis as well as a more effective treatment of CC.

The inhibitors of apoptosis family proteins (IAPs) participate in regulating apoptosis and maintaining the tissue balance. The impairment of apoptosis is a characteristic feature of neoplastic transformation. In many types of cancer, translocation or amplification of IAP-encoding genes has been detected, leading to reduced sensitivity of cancer cells to proapoptotic stimuli. Several studies reported the influence of IAPs on the growth of prostate, lung, breast, pancreatic, cervical, and head and neck cancers, and on hepatocellular carcinoma, chondrosarcoma, osteosarcoma.[4–11] In humans, 8 IAPs have been detected: neuronal apoptosis inhibitory protein (NAIP/BIRC1), cellular IAP1 (cIAP1/BIRC2), cellular IAP2 (cIAP2/BIRC3), X-chromosome-linked IAP (XIAP/BIRC4), Survivin (BIRC5), BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE/Apollon/BIRC6), LIVIN (BIRC7) and human IAP-like 2 (hILP2/BIRC8), and they have been grouped into 3 classes.[12] The characteristic molecular component of all IAPs is the Baculovirus IAP Repeat (BIR) domain. IAPs also have other molecular domains characteristic for a given protein. The BIR domains and the regions adjacent to these domains are responsible for binding IAPs to capsizes, thus leading to the inhibition of their activity. Other mechanisms of inhibiting apoptosis are also being investigated, such as ubiquitin ligase-like activity.[13] Due to their crucial role in the regulation of apoptosis, IAPs are being investigated as a prognostic factor, as well as a treatment target in cancer patients.[14–17]

BIRC5 is one of the best known IAPs and is a prime example of a multifunctional protein involved in a variety of regulatory circuits in tumor cells.[18] In addition, it is a radiation-inducible factor mediating the cellular radiation response in colon cancer.[19]

The XIAP is an IAPs that inhibits the active catalytic sites of caspases-3 and caspases-7 in a direct manner and interferes with the dimerization and activation of caspase-9.[20] Recent studies presented XIAP as a multifunctional protein involved in cellular and metabolic regulatory circuits such as invasion, migration, necroptosis, oxidative stress, inflammasome formation, and autophagy.[21]

NAIP is the least researched protein, however, comprehensive studies indicate the relevance of NAIP in various molecular mechanisms and diseases such as cytokinesis and inflammasome formation.[22]

Gene mutations and increased expression of IAPs are common in cancer cells. Thus, understanding the paths of apoptosis inhibition driven by IAPs may be the key to understanding the mechanism of cancer formation, its progression, and drug resistance. Moreover, research of IAPs as targets for cancer therapy encouraged scientists to better understand their role in carcinogenesis.[23] The expression of IAP-encoding genes varies depending on the stage of CC. However, there have not been many reports on the use of IAPs in colon cancer diagnostics.

This study aims to analyse the transcriptional activity of IAPs-encoding genes at different stages of CC, to propose them as potential complementary diagnostic and prognostic markers in this cancer.

2. Materials and methods

This study was performed at the Department of General, Colorectal and Polytrauma Surgery and the Department of Surgical Nursing and Propaedeutics of Surgery, Faculty of Health Sciences in Katowice, Medical University of Silesia, Poland. The samples were colected from 32 patients who were treated at our hospitals between May 2018 and December 2018 for CC.

2.1. Participants

Thirty two patients (16 females and 16 males), aged 39 to 86 years, were enrolled in the study. Based on the inclusion criteria of diagnosed CC in all stages of the disease, patients underwent elective surgery and provided written consent to participate in the study (Bioethics Committee, Medical University of Silesia, No. KNW/0022/KB1/21/I/10). Patient data has been encoded in accordance with the pseudonymisation procedure, which means that personal data is processed in such a way that it cannot be assigned to a specific data subject, without the use of an additional "key."

The exclusion criteria were a second surgery due to the underlying disease, no histopathological confirmation of CC, presence of co-existing genetic, systemic, or metabolic disorders (excluding obesity as an isolated disorder) and previous radio- or chemotherapy.

Tissue samples were obtained during surgical resection of the colon affected by cancer, which was performed according to surgical treatment standards. The tissue samples were collected using classical surgical techniques without the use of electric or ultrasound instruments. The material obtained consisted of tumor tissue and/or healthy colon tissue. Healthy control tissue specimens were collected from an area 5 cm outside of the histologically negative margin, during the operation because of CC. All materials were taken by the same operational team to minimize the mistakes. The cancer samples were obtained from the margin of the resected material to rule out the presence of necrotic tissue in the specimen.

In total, 37 samples were obtained (Table 1): 18 cancer tissue samples and 19 healthy tissue samples (used for control purposes). Some CC and healthy tissue samples were obtained from 2 separate specimens, collected from the same patients (13 CC samples, 14 control samples). In 5 cases (2 females and 3 males) the CC tissue and control tissue were obtained from the same resected material. Out of 18 cancer tissue samples, 3 were assessed as CC in clinical stage (CS) I, 5 in CS II, 6 in CS III, and 4 in CS IV. The method of hierarchical clustering was used to allocate samples to the groups of transcriptomes obtained from cancer samples and healthy tissue samples. Transcriptomes were divided into 4 groups. Two groups included samples from the histologically normal (healthy) colon and were labelled as the control group (C1). Samples from group C1, obtained from a wide margin, were assessed histologically and molecularly, confirming no neoplastic changes. In the C1 group, sample grouping was confirmed for 11 transcriptomes based on clinical, histopathological, and molecular analyses. One sample (33_CS III) in the C1 group did not pass the previously mentioned tests, and as a result, the abovementioned sample was excluded from further comparative analysis.

The second control group (C2) included histopathologically healthy tissue but with molecular characteristics typical of cancer cells. This group was considered intermediate, placed between the C1 and the groups of low stage cancer (LSC) and high stage cancer (HSC). The other 2 groups contained CC samples of LSC and HSC. The LSC group included mainly patients with histopathologically confirmed stage 1 (CS I) cancer, with

Table 1

Characteristics of patients enrolled in the study in whom transcriptomes were determined using expression microarray technique.

No.	Sample ID	Stage	Gender	Age	Tumor location	Extent of differ-entiation
1	1_CS I	CS_I	F	39	SIG	G1
2	11_CS I	CS_I	M	49	SIG	G2
3	2_CS I	CS_I	M	70	SIG	G2
4	24_CS II	CS_II	F	58	SIG	G2
5	29_CS II	CS_II	F	70	CAE	G1
6	3_CS II	CS_II	M	81	SIG	G1
7	31_CS II	CS_II	F	54	AC	G1
8	34_CS II	CS_II	M	57	DEC	G2
9	14_CS III	CS_III	F	86	SIG	G1
10	33_CS III	CS_III	M	61	AC	G3
11	38_CS III	CS_III	M	57	DEC	G2
12	4_CS III	CS_III	F	58	TRC	G2
13	5_CS III	CS_III	M	69	DEC	G2
14	6_CS III	CS_III	M	61	AC	G3
15	15_CS IV	CS_IV	F	71	SIG	G2
16	23_CS IV	CS_IV	M	69	SIG	G3
17	7_CS IV	CS_IV	F	73	CAE	G1
18	8_CS IV	CS_IV	F	70	CAE	G1
19	1_K	Control	F	39	SIG	–
20	12_K	Control	M	49	SIG	–
21	13_K	Control	F	69	SIG	–
22	16_K	Control	M	70	SIG	–
23	17_K	Control	F	58	SIG	–
24	18_K	Control	F	70	CAE	–
25	19_K	Control	F	54	AC	–
26	27_K	Control	M	81	SIG	–
27	28_K	Control	F	86	SIG	–
28	29_K	Control	F	70	CAE	–
29	30_K	Control	F	58	TRC	–
30	32_K	Control	M	69	DEC	–
31	35_K	Control	F	71	SIG	–
32	36_K	Control	M	69	SIG	–
33	37_K	Control	M	57	DEC	–
34	38_K	Control	M	57	DEC	–
35	39_K	Control	M	83	SIG	–
36	5_K	Control	M	69	DEC	–
37	6_K	Control	M	61	AC	–

AC = ascending colon, CAE = caecum, DEC = descending colon, F = female, M=male, SIG = sigmoid, TRC = transverse colon.

molecular and histopathological changes typical in stages T1 and T2 (Duke's A, according to Dukes' staging system).

Only 4 samples (out of 5 in total) in the LSC group were included in further testing, that is, 3_CS I samples and 1 control sample (5_K). One sample from this group was assessed as CS III (6_CS III) cancer and as a result, it was excluded from further comparative analysis. Thirteen samples were included in the HSC group with adenocarcinoma, that is, 5 samples in CS II, 4 samples in CS III, and 4 samples in CS IV.

Samples allocated to the control group were assessed as healthy colon samples on the gross examination and were dissected from the most distal part of the lesion (at least 5 cm from the healthy margin).

All of the collected cancer tissue and healthy tissue samples were split into 2 sections, one to be used in the standard histopathological evaluation and the second for the molecular analysis. The dissection of all samples was performed immediately after excision of the resected colon segment from the patient. The material prepared for analysis was submerged in the RNAlater (QIAGEN) and stored at −80°C until molecular analysis was performed.

2.2. Methods

The first step was to isolate the total RNA. The tissue material was homogenized (Kinematics, AG, Bern, Switzerland), then the total RNA was isolated according to the manufacturer's instructions using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Next, RNA was purified with the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) in combination with DNase I digestion. The Gene Quant II (Pharmacia Biotech, Uppsala, Sweden) spectrophotometer was used to quantify the RNA concentration on the basis of absorbance of 260 nm. The transcriptional activity of the genes was determined by the microarray technique (Affymetrix, Santa Clara, CA, USA) using the HG-U133A chip.

Following the isolation of total RNA, micro-RNA that is potentially involved in the regulation of apoptosis genes was selected. It was also confirmed that selected genes are involved in the development of adenocarcinoma. In the next step, the expression of genes involved in ubiquitin-mediated protein degradation was investigated. The quantitative mRNA amplification reaction was performed for the following genes: IAP - BIRC5, B-cell lymphoma 2 (BCL-2), β-actin and GAPDH. The

Table 2

The number of ID mRNA of inhibitors of apoptosis family proteins differentiating colon cancer transcriptomes, depending on the adopted criterion of p differentiation.

P value	*P* in total	$P < .05$	$P < .02$	$P < .01$	$P < .005$	$P < .001$
Number of ID mRNA	9	4	2	2	1	1

number of mRNA molecules of the investigated genes was defined based on the standard curve prepared for commercially available DNA templates of the β-actin gene using the TaqMan DNA Template Reagent (PE Applied Biosystems).

Microarray analysis was validated with qRT-PCR. Transcriptional activity of genes involved in apoptosis in CC tissue samples obtained from CC at 4 clinical stages (CSI, CSII, CSIII, CSIV) was investigated and compared to the control group. Obtained results are partially consistent with the microarray results.

Gene Chip Expression Analysis kit, Data Analysis Fundamentals (Affymetrix Inc., USA), Gene Spring GX 11.5 software, (Agilent Technologies), and SAM (Significance Analysis of Microarrays) statistical techniques were used for the comparative analysis of the transcriptomes. All microarrays were accepted for comparative analysis. The obtained results were normalized using the RMA software and the Gene Spring GX 11.5 software, which enabled the selection of genes differentiating transcriptomes, depending on the stage of progression of adenocarcinoma. The results obtained using the qRT-PCR technique were developed based on the statistical programs, that is, Microsoft Office Excel 2007 and STATISTICA 10. The analyses were started from the assessment of the normality of the distribution of numerical values of the results with the normal distribution using the Shapiro–Wilk test. Then ANOVA, Student *t* test or Mann–Whitney *U* and Wilcoxon tests were used ($P < .05$).

3. Results

The results included material from all collected CC and healthy control tissue samples. The patient group consisted of Caucasians, from different social groups, with a different family burden towards CC. During the collection of the database, none of the variables in both groups was omitted.

In order to confirm the genes involved in the regulation of apoptosis, based on the HGU133A microarray (Affymetrix) analysis, the Affymetrix database and the literature data, out of the 22283 ID mRNA obtained from the genes, 840 ID mRNA were selected for this study. Among the 840 selected transcripts, 9 ID mRNA of IAP-encoding genes were found.

The HGU133A microarray plate includes 3 sets of probes complementary to the mRNA of the IAP-encoding gene - BIRC5, complementary to the XIAP and the NAIP and a single ID mRNA complementary to the protein-coding genes - BIRC3 (Cellular IAP-2) and BIRC7 [ML-IAP/Livin (Melanoma IAP)]. The following IAPs were not analyzed in this study: BIRC8 [IAP-like Protein 2 (ILP-2)], BIRC2 [Cellular IAP-2 (cIAP1)], or BIRC6 (BRUCE). Table 3 shows the degree of differentiation of the transcriptome groups and the statistical variability of the differences between mRNA groups. Analysis was performed using the Gene Spring 11.0 software.

IAP-encoding mRNA detected in all analyzed transcriptome groups were compared using a one-way ANOVA, which revealed that out of 9 IAP-encoding ID mRNA, statistically significant differences between groups ($P < .05$) were observed in 4 ID mRNA that is, BIRC5-encoding ID mRNA in 2 isoforms and NAIP- and XIAP-encoding ID mRNA (Table 2). Further analysis included the comparison of transcriptomes from the C2, LSC, and HSC groups with the C1control group. Expression of BIRC5 was increased in both LSC and HSC groups. The expression of the NAIP-encoding gene was increased only in the LSC group and in the C2 group when compared to the control C1 group. The expression of the XIAP-encoding gene was decreased in all groups, that is, in the C2 control group, the LSC group and the HSC group, when compared to the C1 control group (Table 3).

4. Discussion

The inhibition of apoptosis is crucial in CC carcinogenesis, hence the following thought-provoking question – could IAPs be potential markers for CC?.[8,10,20,24,25,26,27,28]

In the colon, BIRC5 was detected in small amounts in mucosa obtained from healthy tissue margins. It has been reported that during tumorigenesis, the transition from adenoma (displaying a low to a high degree of dysplasia) to carcinoma, in accordance with the adenoma-carcinoma development stages, the expression of BIRC5 significantly and continuously increases.[28,29] Increased expression of BIRC5 has also been found in neoplastic cells, with simultaneously low presence in other terminal cells.[30]

Table 3

Genes differentiating transcriptomes of IAP-encoding genes in a one-way ANOVA (Fold Change).

		C2 vs C1			LSC vs C1			HSC vs C1		
ID	Gene symbol	FC (log2)	*P* value	Change	FC (log2)	*P* value	Change	FC (log2)	*P* value	Change
202094_at	BIRC5	1.288	.032	ß	1.371	.032	Ý	1.114	.032	Ý
202095_s_at	BIRC5	1.997	.00022	ß	1.918	.00022	Ý	1.216	.00022	Ý
204860_s_at	NAIP	1.278	.033	ß	1.176	.033	Ý	1.045	.033	ß
206536_s_at	XIAP	1.359	.00807	ß	1.154	.00807	ß	1.121	.00807	ß

BIRC5 = survivin, C1 = control group, C2 = second control group, HSC = high stage cancer, LSC = low stage cancer, NAIP = neuronal apoptosis inhibitory protein, XIAP = human X chromosome-encoded inhibitor of apoptosis family proteins.

The increased expression of BIRC5 is associated with a worse prognosis, decreased survival rate, and increased resistance to chemo- and radiotherapy in many cancers, which particularly indicates BIRC5 to be a valuable marker for diagnosis and prognosis.[9] In colorectal cancer, the high expression of BIRC5 correlated with shorter survival.[26,31] Our study gives further support to these findings and moreover, it has shown a statistically significant increase of BIRC5 in CC (both LSC and HSC), which was investigated using the oligonucleotide microarray method.[32] There are also studies on BIRC5 genetic polymorphisms, according to the research of Gazouli M. et al,[33] there are significant differences in the level of survival with the -31G/C genotype between CRC and healthy tissue, the authors also differentiate the level of this protein depending on the stage, tumor location, tumor size, growth and differentiation pattern.

The XIAP protein is usually overexpressed in a variety of cancers such as kidney, ovarian, lung, or thyroid cancers.[31,34,35] An increased level of XIAP and a correlation between high XIAP and CC progression was observed by Xian et al.[36] Our study did not confirm their findings. On the contrary, the number of XIAP transcripts decreased from the start of carcinogenesis. The XIAP activity was already decreased in cells that have characteristics of cancer cells (group C2); it was also low in LSC and HSC.

There is a clear interaction between BIRC5 and XIAP. It has been demonstrated that XIAP can directly reduce capsize activity in vivo, and it has been found that BIRC5 does not act alone. The combination of BIRC5 and XIAP inhibits the activation of caspase-9, while BIRC5 on its own does not show similar activity.[37,38] BIRC5 and XIAP can form a complex that protects XIAP from ubiquitination. Perhaps the reduced presence of XIAP in CC cancerogenesis acts as a defence mechanism. However, the increased level of BIRC5 is sufficient to decelerate the apoptosis despite a decrease in the number of XIAP transcripts. It is probable that the creation of a BIRC5-XIAP-like complex decreases XIAP degradation by ubiquitination, thereby stabilizing XIAP.[37]

Some research groups investigated the silencing of the expression of BIRC5, and other IAPs found in tumors. For this purpose, modern biotechnology and molecular biology techniques and approaches are used, such as antisense oligonucleotides (ASOs) acting against IAPs, small antisense molecules, antagonists and modulators of IAPs, siRNA and other molecules.[11,23,27,39,40,41] It seems that BIRC5 is the most promising target in CC. The probable synergistic effect of BIRC5 and XIAP is explained by the better induction of apoptosis when both proteins were blocked simultaneously, rather than each protein being blocked separately.[42]

In addition, the molecular pathway of IAP is not limited to the mechanism involving capsize. Not only BIR domains, but also the structures connecting individual BIR domains are important, as they can bind to capsize, executing capsize inhibition and thus the inhibition of apoptosis. The exception to the above is XIAP, in which the BIR3 domain directly affects the capsizes without affecting the binding structures.[13,43] XIAP can also inhibit apoptosis in a mechanism independent of its capsize-binding activity.[44] There are other known interactions between this and other families of proteins, so bearing in mind the complexity of the problem, we believe that the main emphasis should be placed on blocking BIRC5.

NAIP directly inhibits capsize-3, capsize-7, and indirectly capsize-9.[45,46] It is believed that NAIP participates in response to bacterial infection by binding bacterial lipopolysaccharides. It has been suggested that NAIP plays a role in the host response to intracellular bacterial infections by suppressing pro-inflammatory proteases by caspase-1.[47,48] Previous studies showed that the expression of NAIP mRNA is decreased in differentiated CC, when compared with healthy tissue, which is consistent with our observation.[49] Similar results were reported in studies on knockout mice, indicating that NAIP may play a crucial role at various stages of colon carcinogenesis, and the effect of NAIP on inflammatory-dependent colon carcinogenesis is of great importance.[49–51] Our study also indicates that a decrease in NAIP in advanced tumors is preceded by its increased activity and indicates an important role of NAIP in the early stage of cancer formation in the colon. The temporary increase in NAIP in LSC observed in our study may be indicative of initially increased anti-inflammatory mechanisms that are already impaired in advanced cancer.

This study has some limitations. Firstly, the availability of microarray analysis is expensive, which currently limits its widespread use in routine diagnostics due to colon cancer. Second, our research did not compare the protein expression methodology with the much more accessible method, Western Blot, which may be an exciting issue and allow faster implementation of IAPs determination in clinical practice. Third, this study included older age participants, who may have been less healthy and may not be representative of the general population. In future research, it is also worth analyzing gene expression within subgroups, taking into account confounding factors and calculating after obtaining additional material for research, and taking into account follow-up. Finally, more research is needed on the regulation of apoptosis using IAPs in other inflammatory diseases, such as inflammatory bowel disease. All these limitations are prompting us to conduct further research on this topic.

5. Conclusions

Inhibitors of apoptosis proteins have an important role in inhibiting apoptosis in both physiological and pathological conditions. Our study suggests that BIRC5 is a promising diagnostic and prognostic marker in CC. BIRC5 increases its transcriptional activity in CC cells in low and high cancer stages. Therefore, it may be used as the primary target of cancer treatment. It is worthwhile to mention the BIRC5- XIAP complex. Increased transcriptional activity of NAIP was confirmed only in LSC, so possible cancer prevention using anti-NAIP molecules in CC is unlikely. NAIP, however, can be used for the diagnosis of early CCs. The roles of both XIAP and NAIP, although difficult to explain, seem to be undisputed in CC. The remaining proteins from the IAP group, that is, BIRC3 and BIRC7, were not considered to significantly alter the transcriptional activity in CC cells.

Our results could be an inspiration for further studies, focusing on the development of new cancer treatment strategies, combining pharmacological treatment and molecular biology methods. Such strategies could be based on selective blocking of BIRC5 expression and possibly other IAPs. The molecular assessment of cellular metabolism disturbances, especially apoptotic disorders, deserves particular attention for better prevention and treatment of CC. Comprehensive studies on the role of IAPs and their inhibitors in cancer diseases seem to be warranted.

Author contributions

Conceptualization: Dariusz Waniczek, Justyna Lorenc-Góra, Małgorzata Muc-Wierzgoń, Zbigniew Lorenc.
Data curation: Małgorzata Muc-Wierzgoń.
Formal analysis: Justyna Lorenc-Góra, Małgorzata Muc-Wierzgoń, Urszula Mazurek, Magda Bichalska-Lach, Zbigniew Lorenc.
Investigation: Justyna Lorenc-Góra, Magda Bichalska-Lach.
Methodology: Urszula Mazurek.
Project administration: Dariusz Waniczek, Magda Bichalska-Lach, Zbigniew Lorenc.
Software: Marcin Nowak.
Supervision: Małgorzata Muc-Wierzgoń, Urszula Mazurek, Zbigniew Lorenc.
Validation: Małgorzata Muc-Wierzgoń, Urszula Mazurek, Magda Bichalska-Lach.
Visualization: Dariusz Waniczek, Magda Bichalska-Lach.
Writing – original draft: Dariusz Waniczek, Marcin Nowak, Justyna Lorenc-Góra, Małgorzata Muc-Wierzgoń, Magda Bichalska-Lach.
Writing – review & editing: Dariusz Waniczek, Marcin Nowak, Justyna Lorenc-Góra, Małgorzata Muc-Wierzgoń, Magda Bichalska-Lach.

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