Translational Oncology
www.transonc.com

Volume 9 Number 3 June 2016 pp. 251–255 251

Detection of Biomarkers with Solid-Phase Proximity Ligation Assay in Patients with Colorectal Cancer

Lana Ghanipour*, Spyros Darmanis†, Ulf Landegren†, Bengt Glimelius‡, Lars Påhlman* and Helgi Birgisson*

*Department of Surgical Science, University of Uppsala, Uppsala, Sweden; †Department of Immunology, Genetics and Pathology, Science for Life Laboratory, University of Uppsala, Uppsala, Sweden; ‡Department of Radiology, Oncology and Radiation Science, University of Uppsala, Uppsala, Sweden

Abstract

BACKGROUND: In the search for prognostic biomarkers, a significant amount of precious biobanked blood samples is needed for conventional analyses. Solid-phase proximity ligation assay (SP-PLA) is an analytic method with the ability to analyze many proteins at the same time in small amounts of plasma. The aim of this study was to explore the potential use of SP-PLA for biomarker validation in patients with colorectal cancer (CRC). *MATERIAL AND METHODS:* Plasma samples from patients with stage I to IV CRC, with ($n = 31$) and without ($n = 29$) disease dissemination at diagnosis or later, were analyzed with SP-PLA using 35 antibodies targeting an equal number of proteins in 5-μl plasma samples. Carcinoembryonic antigen (CEA), analyzed earlier in this cohort using a different technology, was used as a reference. *RESULTS:* A total of 21 of the 35 investigated proteins were detectable with SP-PLA. Patients in stage II to III with disseminated disease had lower plasma concentrations of HCC-4 ($P = .025$). Low plasma levels of tissue inhibitor of metalloproteinases–1 were seen in patients with disseminated disease stage II ($P = .003$). The level of CEA was higher in patients with disease dissemination compared with those without ($P = .007$). *CONCLUSION:* SP-PLA has the ability to analyze many protein markers simultaneously in a small amount of blood. However, none of the markers selected for the present SP-PLA analyses gave better prognostic information than CEA.

Translational Oncology (2016) 9, 251–255

Introduction

Tumor markers in colorectal cancer (CRC) have the potential to play a crucial role in screening, prognostication, and therapeutic monitoring. Carcinoembryonic antigen (CEA) is the most known and widely used marker. Because of its low sensitivity for identifying individuals with CRC, CEA is not recommended for screening [1]. Even though CEA has low sensitivity, it is the only marker certificated for detection of early recurrence, although many patients with tumor relapse have normal CEA levels [2].

Earlier survival analyses in the present CRC cohort revealed that patients with stage I disease have no risk of developing tumor recurrence for up to 5 years, although the 5-year overall survival (OS) was only 75% due to death from other diseases. In CRC stage II, only 14% developed cancer recurrence, and the 5-year OS was similar to stage I or 74%. In CRC stage III, 40% developed disease recurrence, and the 5-year OS was 54% [3]. For stage III CRC patients, adjuvant treatment improves OS by 15% to 20%, whereas 60% are already cured by the primary surgery alone [4]. The use of adjuvant therapy is not routinely recommended for patients with CRC stage II, but for groups at high risk for recurrence like those with T4, emergency surgery and analysis of a few lymph nodes may be offered treatment, although the benefits are not well proven [5].

It would certainly be of great benefit to find biomarkers that could identify those 14% of stage II and 40% with stage III CRC who will have cancer recurrence. This could radically change the strategy of

Address all correspondence to: L Ghanipour, Department of Surgical Science, University of Uppsala, Uppsala, Sweden.
E-mail: arezo_g@hotmail.com
Received 25 November 2015; Accepted 10 April 2016

1936-5233/16
http://dx.doi.org/10.1016/j.tranon.2016.04.001

adjuvant chemotherapy use, sparing those not in need of the treatment with all its side effects and improving the surveillance of those at higher risk of tumor recurrence.

The proximity ligation assay (PLA) is a recently described protein detection assay. Pairs of oligonucleotide-labeled antibodies (PLA-probes) are used to detect the target antigen. When two such PLA-probes bind the same antigen, the probes are brought in proximity, leading to formation of a template DNA strand by ligation. The DNA strand is then amplified by quantitative real-time polymerase chain reaction to detectable signals [6]. Solid- phase PLA (SP-PLA) is a form of PLA where antibodies immobilized on a solid support act as capture agents for the target proteins before the PLA [7]. There are several advantages in detecting plasma biomarkers using PLA, such as increased specificity, minimal sample consumption, and the capacity to simultaneously analyze numerous targets in a multiplex format [8].

The primary aim of this study was to explore the use of SP-PLA to evaluate the concentrations of a set of potential biomarkers in clinical plasma samples from patients with CRC according to disease stage and recurrence in relation to CEA. For this purpose, a strategic sample of a limited number of patients was selected.

Material and Methods

Sixty patients from a previously described cohort of 320 patients [9], operated for CRC at the Central District Hospital, Västerås, County of Västmanland, were strategically selected. Nine patients had disease stage I, one of whom had a recurrence after 7 years. Twenty-two patients had stage II disease, 10 of whom had a recurrence; 19 had stage III disease, with 9 having a recurrence; and 10 patients were at stage IV at diagnosis. Patients were divided into two groups: those with disease dissemination (stage I-III with recurrence and stage IV) and those without dissemination (stage I-III without recurrence). The purpose was to select approximately 10 patients each with stage I, stage II with/without dissemination, stage III with/without dissemination, and stage IV. Among patients with nondisseminated disease, six received postoperative adjuvant chemotherapy of which all were in stage III of the disease. The patients were treated between August 2000 and December 2003. Information about stage, localization, differentiation, and vascular and neural invasion was received from pathology records. Information on death and cancer recurrence was received from surgical and oncology records and from the Clinical Database for Colorectal Cancer held at the Regional Oncologic Centre in Uppsala/Örebro.

Preoperative collection of blood samples was drawn into EDTA tubes and processed for plasma by centrifugation. The samples have been stored in −70°C for at least 10 years before being analyzed.

Methods

SP-PLA. From each patient, 5 μl of plasma was used for protein detection with multiplex SP-PLA, as described by Darmanis [6,7]. The multiplex protein detection panel was preselected by the science group of U Landegren for explorative studies and comprised 35 proteins previously reported as biomarkers for cancer, inflammation, or cardiovascular disease, and 1 internal control (mouse IgG) [6]. For each protein in the panel, individual dilutions of recombinant proteins were prepared at high, medium, or low concentrations (1 nM, 10 pM, and 0.1 pM, total volume of 45 μl). Quantification of each protein was made by real-time polymerase chain reaction of the DNA reporter strands that formed in the detection reactions. Molar protein concentrations were converted to pg/μl before analysis.

Statistical Analyses

The χ^2 and Fisher's exact tests were used for comparisons of categorical variables. The Mann-Whitney *U* test was used to compare the plasma levels of selected biomarkers and dissemination status and between each disease stage. Kruskal-Wallis test was used to compare values of plasma levels biomarkers and stage of the disease.

Statistical significance was set at $P < .05$. All observations were censored at the end of the study period (15th April 2010).

SPSS statistics version 21 (SPSS Inc., Chicago, IL) was used for statistical analysis.

Ethical approval (number 2000:001) was obtained from the Ethics Committee at Uppsala University, Uppsala, Sweden.

Results

Patient Characteristics

Of 60 patients, 65% had colon cancer; and 35%, rectal cancer. Thirty-one patients had disseminated disease, and 29 were without dissemination. The median age of patients with dissemination was 69 years (range, 34-85), and for patients with nondisseminated disease, it was 76 years (range, 49-91) (Table 1). There were no statistically significant differences in age, gender, tumor localization, tumor differentiation, and presence of vascular and neural invasion between the two groups. The cases with vascular or neural invasion were, however, confined to the disseminated group. Patients with disease dissemination had higher CEA levels than those without ($P = .007$) (Table 1), which could also be seen when comparing the disease stages ($P = .040$) (Table 2).

Plasma Analyses of Detectable Biomarkers

The plasma level of 35 biomarkers (Table 3), of which 21 were detectable, and also including one internal control were analyzed with

Table 1. Comparison of Clinicopathological Characteristics in Patients with and without Disease Dissemination

Characteristics	No Dissemination (*n* = 29)	Dissemination (*n* = 31)	*P* Value
Age (years)			
Median (range)	76 (49-91)	69 (34-85)	NS
Gender			
Female	13	18	NS
Male	16	13	
Localization			
Right/left colon	14/15	9/22	NS
Colon/rectum	20/9	19/12	
Disease stage			
I	8	1	*
II	12	10	
III	9	10	
IV	-	10	
Differentiation			
Well-moderate	26	25	NS
Poor	3	9	
Mucinous			
Yes	3	4	NS
No	26	26	
Vascular invasion			
Yes	0	5	NS
No	29	25	
Neural invasion			
Yes	0	2	NS
No	29	28	
CEA			
<6 ng/ml	24	15	.007
≥6 ng/ml	5	16	

* Not relevant testing due to strategic selection of patients.

Table 2. Comparison of Preoperatively Taken CEA between Disease Stages I and IV CRC

Disease Stage	CEA <6 ng/ml	CEA ≥6 ng/ml	*P* Value
I	8	1	.040
II	14	8	
III	14	5	
IV	3	7	

SP-PLA, and the results are based on these cases. Table 4 shows descriptive results for the detectable biomarkers.

Plasma concentrations of detectable biomarkers in patients with disease stage I to 1V CRC revealed no statistically significant differences between the different stages. Neither were any significant differences seen when analyzing the relations to disease dissemination (data not shown). Patients with disseminated disease stage II to III did have lower median plasma concentration of 1 out of the 21 detectable markers, the "cancer marker" HCC-4, compared with those with no dissemination (P = .025) (Table 5, Figure 1). Low plasma levels of tissue inhibitor of metalloproteinases (TIMP)–1 were also seen in patients with disseminated disease stage II (P = .003, Figure 2). The patients with disseminated disease tended to be younger than those with nondisseminated disease (median age, 68 and 79 years, respectively) (data not shown). We could not detect any significant relation between age and TIMP-1 plasma levels (data not shown).

Discussion

SP-PLA has the potential to become an important tool in the search for new prognostic biomarkers. With only a few microliters necessary for the analysis of, as in our case, 35 different proteins, it enables efficient use of limited, often precious biobanked serum or plasma samples in search for new biomarkers in CRC. This work was a pilot study and the first to test the multiplex SP-PLA method on a clinical series of cancer patients. A strategic selection of cases was done to maximize the chances to obtain potentially clinically valuable information from a limited number of samples. Nonetheless, the small sample size provides insufficient power for statistical analysis, which makes it difficult to draw definitive conclusions regarding relations between plasma level of biomarkers and disease dissemination. It is still reasonable to conclude that none of the analyzed markers will have a future clinical value in separating stages and disseminated cases of CRC. The two findings that achieve statistical significance may well be a reflection of multiple testing, presenting many opportunities for isolated observation below the probability threshold we have applied because great number of comparisons were done. We found low plasma levels of HCC-4 in patients with disseminated disease stage II-III. It has been described that HCC4, also known as CCL16, LEC, LCC1, NCC4, LMC, exerts

Table 4. Descriptive Value of Detectable Biomarkers Plasma Levels (pM/µl) Analyzed with SP-PLA in CRC Patients Stages I to IV

Biomarkers	1st Quartile	2nd Quartile	3ed Quartile	Min Value	Max Value
TIMP1	2951.58	3281. 14	3767.52	2083.76	6254.69
VEGF	3.12	4.39	5.81	1.66	21.71
E-Selectin	242.36	395.93	547.36	81.08	1437.95
Cystatin C	14,138.27	15,761.99	18,358.41	9828.02	27,761.97
Cathepsin B	8161.66	10,054.53	11,968.79	5220.28	20,118.34
IL-8	1.69	2.26	2.98	0.99	155.63
CF3	14.58	18.44	26.23	5.82	856.83
ICAM-1	2408.89	2888.55	3470.66	1671.87	6862.89
Cathepsin S	385.41	430.31	549.40	191.09	1126.73
CCL2	24.53	32.11	41.51	13.77	123.99
CCL4	15.74	21.46	31.87	5.20	145.89
Fas	53.13	70.23	102.14	24.75	637.96
CCL5	1839.26	2095.30	2316.75	1019.98	4372.21
Follistatin	18.08	24.51	30.60	1.21	73.95
CXCL5	37.18	54.84	72.92	11.41	422.44
HCC-4	1381.14	1489.09	1670.85	407.89	2379.58
IL17a	1.34	1.89	2.64	0.81	38.67
P-selectin	897.43	1273.11	1706.64	499.77	10,369.68
TIMP4	184.90	249.85	408.40	77.99	1485.46
Kallikrein 6	131.06	149.93	177.50	79.34	1002.89
GDF15	71.54	124.95	258.40	22.69	1008.22

Table 3. Detectable and Nondetectable Proteins Analyzed with SP-PLA in Patients with CRC

Biomarkers	Detectable (n = 21)	Nondetectable (n = 14)
Markers for inflammation	Interleukin 8 (IL8)	Interleukin 7 (IL7)
	Interleukin 17A (IL17A)	Interleukin 6 (IL6
	Vascular endothelial growth factor (VEGF)	Interleukin 4 (IL4)
		Interleukin 10 (IL10)
		Interleukin 1alpha (IL1α)
		Tumor necrosis factor alpha (TNF-α)
Markers for cardiovascular disease	Growth differentiation factor 15 (GDF-15)	CD40 ligand
	Intercellular adhesion molecule 1 (ICAM-1)	Cystatin B
	P-selectin	
	E-selectin	
	Chemokine (C-C) ligand 2 (CCL2)	
	Cystatin C	
	Coagulation factor III (CF3)	
Markers for cancer	Chemokine (C-XC) ligand 5 (CXCL5)	Epidermal growth factor (EGF)
	Kallikrein 6	Growth hormone
	Chemokine (C-C) ligand 16 (HCC-4)	Artemin
	Tissue inhibitor of metalloproteinase 1 (TIMP-1)	p53
	Tissue inhibitor of metalloproteinase 4 (TIMP-4)	PSA
	Follistatin	Nerve growth factor beta (NGF-β)
	Chemokine (C-C) ligand 4 (CCL4)	
	Chemokine (C-C) ligand 5 (CCL5)	
	Cathepsin B	
	Cathepsin S	
	Fas	
Internal control	Mouse Ig G	

Table 5. Comparison in Median Value of Detectable Biomarker's Plasma Levels in Patients with Disseminated or Nondisseminated CRC, Disease Stage II to III

Biomarkers	Dissemination, Stage II to III (n = 20)	Nondissemination, Stage II to III (n = 21)	P Value
TIMP-1	3136.75	3324.83	NS
VEGF	4.33	5.45	NS
E-Selectin	271.15	343.12	NS
Cystatin C	15,923.75	15,248.01	NS
Cathepsin B	10,048.99	9863.31	NS
IL-8	2.23	2.14	NS
CF3	16.60	17.21	NS
ICAM-1	2575.66	2713.22	NS
Cathepsin S	421.78	452.24	NS
CCL2	29.51	21.01	NS
CCL4	19.57	22.54	NS
Fas	73.44	82.72	NS
CCL5	2101.09	2051.12	NS
Follistatin	24.39	26.02	NS
CXCL5	54.25	53.84	NS
HCC-4	1428.29	1527.38	**.025**
IL17a	1.96	1.60	NS
P-selectin	1183.69	1149.97	NS
TIMP-4	278.40	261.90	NS
Kallikrein 6	159.76	158.57	NS
GDF15	133.71	115.84	NS

chemotactic activity on monocytes and lymphocytes [10]. HCC-4 impaired tumour cell growth in a mouse adenocarcinoma cell line [11].

Tissue inhibitor of metalloproteinases is involved in cancer progression and could be used as a selective marker for metastatic disease, especially TIMP-1 [12]. Our study failed to corroborate a significant relationship between high plasma levels of TIMP-1 and disease dissemination. Contrary to what would be expected, patients in stage II disseminated disease had lower average TIMP-1 plasma levels. Patients in stage II with dissemination tended to be younger than those without disease dissemination. It has been shown that TIMP-1 plasma levels increase with age [12]. Birgisson et al. showed that patients in stage III with high TIMP-1 levels not receiving adjuvant chemotherapy had the highest risk of recurrence [12]. Our inability to confirm this herein is likely a problem of power for our study design.

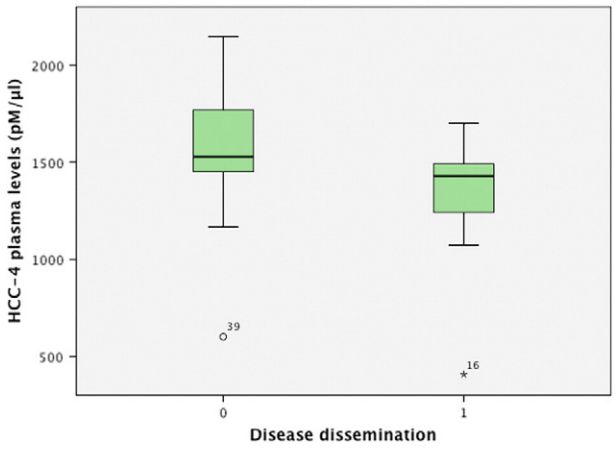

Figure 1. Plasma levels of HCC-4 in patients with and without dissemination, stages II to III CRC. The boxes represent quartiles and medians, and bars minimum and maximum. Asterisks are extremes and circles are outliers.

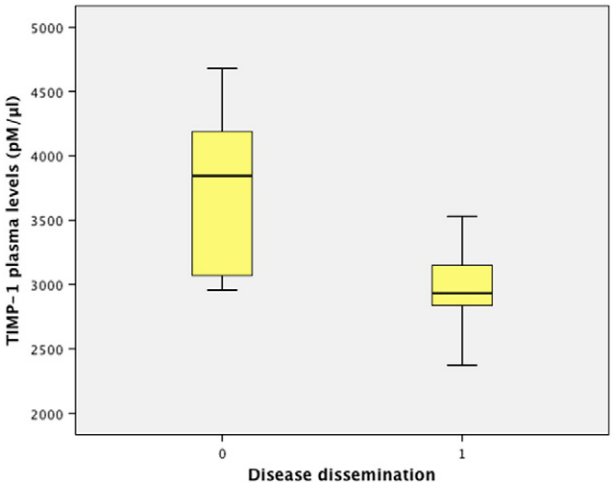

Figure 2. Plasma levels of TIMP-1 in patients with CRC stage II, with and without dissemination.

The outcome of the study necessarily also depends on the selection of biomarkers to be tested. The majority of the selected biomarkers with some roles in cancer, inflammation, and cardiovascular pathways were detectable with SP-PLA in the samples. Several inflammatory proteins are described to have important roles in tumor biology. Overexpression of IL-8 has been linked to upregulation of matrix metalloproteinases, a group of proteins with roles in cancer progression and metastasis [13]. Our multiplex panel includes several proteins that have previously been described to have prognostic value in CRC [14,15]. Angiogenesis plays an important role in tumor growth and metastasis. The marker protein VEGF included in our panel is involved in regulation of both normal and tumor angiogenesis, and by binding to its receptor, cell proliferation and vascularization are induced [16]. Bevacizumab is an anti-VEGF monoclonal antibody used as additional treatment in patients with metastatic CRC [17].

Preoperatively taken CEA was the marker exhibiting highest significance among the investigated individuals. Increased plasma levels correlated with increasing stage and with disseminated disease as opposed to localized disease. CEA is routinely taken as a gold standard marker, although its clinical value in patients with primary CRC is limited. It was reassuring that the levels correlated with both stage and the presence of tumor dissemination, whether known (stage IV) or occult (stage II+III with recurrence), despite the limited number of patients. Herein CEA was not measured using multiplex SP-PLA because the biomarkers were already preselected. In the clinical setting, tumor–lymph node–metastasis staging system is the most precise prognostic factor in therapeutic decision making. Few, if any, biomarkers have been convincingly shown to offer more accurate prediction of prognosis in combination with tumor–lymph node–metastasis staging. CEA is the most widely used protein biomarker in CRC and considered as an independent prognostic marker, especially in patients with stage II CRC and for monitoring therapy in advanced disease [18,19]. Regular measurement of CEA in patients with curatively resected CRC is recommended, and Figueredo et al. also showed that CEA testing and/or liver imaging improves the survival rate [20].

Enzyme-linked immunosorbent assay is today the gold standard method for plasma protein detection; however, SP-PLA uses only a

small sample volume to analyze numerous biomarkers with minimal cross-reactivity and high sensitivity [8]. Other than plasma and serum, SP-PLA can be applied in whole blood, cerebrospinal fluid, brain homogenates, and tissue and cell lysates [7]. Darmanis et al. used SP-PLA to investigate the same preselected proteins as used herein in plasma samples from patients with cardiovascular disease compared with matched controls. The study revealed three potentially relevant diagnostic markers for cardiovascular disease [6]. The authors concluded that the technique could provide a platform for validation of diagnostic biomarkers both in biobanked samples and for clinical use [6]. Wallin et al. used multiplex SP-PLA to show that patients with CRC and high levels of the protein GDF-15 in tumor tissues had shorter time to recurrence and reduced overall survival [21]. In that study, plasma levels of GDF-15 were reported for the same 60 patients as used herein. The plasma levels correlated weakly with CEA levels, but as also reported here, no correlations were seen with stage and dissemination.

The present work represents one of the first to test the SP-PLA method on plasma from patients with CRC, and the interpretation of the results must be taken with some caution. The method is rapidly developing, and analyses of up to 92 proteins and 4 internal controls at the same time are now possible using SP-PLA or a variant of the technique: proximity extension assays [22]. Simultaneous analyses of large sets of proteins require appropriate study design to avoid risks of chance findings due to the multiple variables being tested.

In conclusion, SP-PLA is a suitable method for detecting and validating prognostic and predictive biomarkers. Because CRC is a heterogeneous disease, understanding its molecular pathogenesis can offer enormous advantages in clinical decision making with important treatment implications; herein, multiplex SP-PLA might give valuable guidance in pursuing new biomarkers.

Acknowledgements

The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement no. 294409 (ProteinSeq), the European Community's 7th Framework Program (FP7/2007-2013) under grant agreement no. 259796 (DiaTools), and the IngaBritt and Arne Lundbergs Foundation.

References

[1] Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, Somerfield MR, Hayes DF, and Bast Jr RC, ASCO (2006). ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* **24**(33), 5313–5327 [Epub 2006/10/25].

[2] NIH consensus conference (1990). Adjuvant therapy for patients with colon and rectal cancer. *JAMA* **264**(11), 1444–1450 [Epub 1990/09/19].

[3] Birgisson H, Wallin U, Holmberg L, and Glimelius B (2011). Survival endpoints in colorectal cancer and the effect of second primary other cancer on disease free survival. *BMC Cancer* **11**, 438 [Epub 2011/10/13].

[4] Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, Nordlinger B, van del Velde CJ, Balmana J, and Regula J, et al (2012). ESMO consensus guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. *Ann Oncol* **23**(10), 2479–2516 [Epub 2012/09/27].

[5] O'Connell MJ, Mailliard JA, Kahn MJ, Macdonald JS, Haller DG, Mayer RJ, and Wieand HS (1997). Controlled trial of fluorouracil and low-dose leucovorin given for 6 months as postoperative adjuvant therapy for colon cancer. *J Clin Oncol* **15**(1), 246–250 [Epub 1997/01/01].

[6] Darmanis S, Nong RY, Vanelid J, Siegbahn A, Ericsson O, Fredriksson S, Bäcklin C, Gut M, Heath S, and Gut IG, et al (2011). ProteinSeq: high-performance proteomic analyses by proximity ligation and next generation sequencing. *PLoS One* **6**(9)e25583 [Epub 2011/10/08].

[7] Nong RY, Wu D, Yan J, Hammond M, Gu GJ, Kamali-Moghaddam M, Landegren U, and Darmanis S (2013). Solid-phase proximity ligation assays for individual or parallel protein analyses with readout via real-time PCR or sequencing. *Nat Protoc* **8**(6), 1234–1248 [Epub 2013/06/01].

[8] Blokzijl A, Nong R, Darmanis S, Hertz E, Landegren U, and Kamali-Moghaddam M (2013). Protein biomarker validation via proximity ligation assays. *Biochim Biophys Acta* **1844**(5), 933–939.

[9] Ghanipour A, Jirstrom K, Ponten F, Glimelius B, Pahlman L, and Birgisson H (2009). The prognostic significance of tryptophanyl-tRNA synthetase in colorectal cancer. *Cancer Epidemiol Biomarkers Prev* **18**(11), 2949–2956 [Epub 2009/11/11].

[10] Youn BS, Zhang S, Broxmeyer HE, Antol K, Fraser Jr MJ, Hangoc G, and Kwon BS (1998). Isolation and characterization of LMC, a novel lymphocyte and monocyte chemoattractant human CC chemokine, with myelosuppressive activity. *Biochem Biophys Res Commun* **247**(2), 217–222 [Epub 1998/06/27].

[11] Guiducci C, Di Carlo E, Parenza M, Hitt M, Giovarelli M, Musiani P, and Colombo MP (2004). Intralesional injection of adenovirus encoding CC chemokine ligand 16 inhibits mammary tumor growth and prevents metastatic-induced death after surgical removal of the treated primary tumor. *J Immunol* **172**(7), 4026–4036 [Epub 2004/03/23].

[12] Birgisson H, Nielsen HJ, Christensen IJ, Glimelius B, and Brunner N (2010). Preoperative plasma TIMP-1 is an independent prognostic indicator in patients with primary colorectal cancer: a prospective validation study. *Eur J Cancer* **46**(18), 3323–3331 [Epub 2010/07/14].

[13] Mian BM, Dinney CP, Bermejo CE, Sweeney P, Tellez C, Yang XD, Gudas JM, McConkey DJ, and Bar-Eli M (2003). Fully human anti-interleukin 8 antibody inhibits tumor growth in orthotopic bladder cancer xenografts via down-regulation of matrix metalloproteases and nuclear factor-kappaB. *Clin Cancer Res* **9**(8), 3167–3175 [Epub 2003/08/13].

[14] Dymicka-Piekarska V and Kemona H (2009). Does colorectal cancer clinical advancement affect adhesion molecules (sP-selectin, sE-selectin and ICAM-1) concentration? *Thromb Res* **124**(1), 80–83 [Epub 2009/01/13].

[15] Christodoulou S, Alexopoulou DK, Kontos CK, Scorilas A, and Papadopoulos IN (2014). Kallikrein-related peptidase-6 (KLK6) mRNA expression is an independent prognostic tissue biomarker of poor disease-free and overall survival in colorectal adenocarcinoma. *Tumour Biol* **35**(5), 4673–4685 [Epub 2014/01/17].

[16] Weng W, Feng J, Qin H, and Ma Y (2014). Molecular therapy of colorectal cancer: progress and future directions. *Int J Cancer* **136**(3), 493–502.

[17] Degirmenci M, Karaca B, Gorumlu G, Durusoy R, Demir Piskin G, Bozkurt MT, Cirac Y, Tunali D, Karabulut B, and Sanli UA, et al (2010). Efficacy and safety of bevacizumab plus capecitabine and irinotecan regimen for metastatic colorectal cancer. *Med Oncol* **27**(3), 585–591 [Epub 2009/06/16].

[18] Duffy MJ, Lamerz R, Haglund C, Nicolini A, Kalousová C, Holubec L, and Sturgeon C (2013). Tumor markers in colorectal cancer, gastric cancer and gastrointestinal stromal cancers: European group on tumor markers 2014 guidelines update. *Int J Cancer* **134**(11), 2513–2522.

[19] Huh JW, Oh BR, Kim HR, and Kim YJ (2010). Preoperative carcinoembryonic antigen level as an independent prognostic factor in potentially curative colon cancer. *J Surg Oncol* **101**(5), 396–400 [Epub 2010/02/02].

[20] Figueredo A, Rumble RB, Maroun J, Earle CC, Cummings B, McLeod R, Zuraw L, and Zwaal C, Gastrointestinal Cancer Disease Site Group of Cancer Care Ontario's Program in Evidence-based Care (2003). Follow-up of patients with curatively resected colorectal cancer: a practice guideline. *BMC Cancer* **3**, 26 [Epub 2003/10/08].

[21] Wallin U, Glimelius B, Jirström K, Darmanis S, Nong RY, Pontén F, Johansson C, Påhlman L, and Birgisson H (2011). Growth differentiation factor 15: a prognostic marker for recurrence in colorectal cancer. *Br J Cancer* **104**(10), 1619–1627 [Epub 2011/04/07].

[22] Lundberg M, Eriksson A, Tran B, Assarsson E, and Fredriksson S (2011). Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. *Nucleic Acids Res* **39**(15)e102 [Epub 2011/06/08].