

Thesis

Analysis of Prolactin Receptor Expression in Primary Colorectal Cancers, Corresponding Metastases and Colorectal Cancer Cell Lines

Therapeutic Option for Affected Patients?

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Graz, 4th September 2010

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Declaration of Authorship

I declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. Where I have quoted from the work of others, the source is always given at their point of use. This work has been published in the journal *Modern Pathology* (Harbaum et al. Mod Pathol. 2010 Jul;23(7):961-71.).

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Abbreviations

AJCC	American joint committee on cancer
CRC	Colorectal Cancer
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
Grb	Granzyme B
hGH	Human growth hormone
JAK	Janus-kinase
MAPK	Mitogen-activated protein kinases
MRT	Magnet resonance tomography
PE	Pseudomonas exotoxin A
PMSF	Phenylmethanesulfonyl fluoride
PRL-R	Prolactin Receptor
Ras	Retrovirus-associated DNA sequences
RIPA	Radioimmunoprecipitation
SDS-Page	Sodium dodecyl sulfate polyacrylamide gel
SHC	Signaling adaptor proteins
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TMA	Tissue microarray
UICC	Union internationale contre le cancer
VEGFR	Vascular endothelial growth factor

Abstract

Background: The role of human prolactin and its receptor, the prolactin receptor, in colorectal cancer has been controversially discussed over the past two decades. Recent data indicates that prolactin receptor signaling contributes to tumor growth through an auto-/paracrine loop. Our study aimed to assess the prevalence of prolactin receptor expression, its association with clinicopathological variables, as well as its prognostic value, comparing results of primary tissues with those of corresponding metastases. **Methods:** 373 primary colorectal cancer and 171 corresponding metastases were evaluated for prolactin receptor expression by immunohistochemistry using a tissue microarray technique. Immunoreactivity was semiquantitatively scored as either focal (<10% of tumor cells positive), moderate (10-50%), or extensive (>50%). Prolactin receptor expression was related to clinicopathological parameters as well as patient's outcome. To substantiate our findings, prolactin receptor expression was additionally assessed in HT-29 and SW-480 colorectal cancer cell lines using western blot. **Results:** Prolactin receptor expression was observed in 360 out of 373 (97%) primary tumors, with 21 (6%) cases showing focal, 55 (15%) moderate and 284 (76%) extensive expression, respectively. Extensive prolactin receptor expression was significantly associated with tumor size ($P=0.002$) and grade ($P<0.001$) as well as histological subtype ($P<0.001$). The expression of prolactin receptor in metastatic tissues matched well with that of corresponding primary tumors: Somer's D coefficients for concordance of primary tumors with corresponding lymph node and distant metastases were $D=0.719$ ($P<0.001$) and $D=0.535$ ($P=0.001$), respectively. Extensive prolactin receptor expression was significantly associated with disease progression ($P=0.03$) and cancer-specific survival ($P=0.04$) in patients with high grade cancers. **Conclusion:** Prolactin receptor expression is common in colorectal cancer, with high concordance between primary tumors and corresponding metastases. In view of evolving targeted therapy concepts in colorectal cancer, widespread prolactin receptor expression may offer a therapeutic perspective in affected patients.

Zusammenfassung

Hintergrund: Die Rolle des humanen Prolaktin und des dazugehörigen Prolaktin Rezeptors beim kolorektalen Karzinom ist weitgehend unbekannt. Ziel dieser Studie war es, die Häufigkeit der Expression des Prolaktin Rezeptors bei primären kolorektalen Karzinomen und korrespondierenden Lymph- und Fernmetastasen unter Einbeziehung unterschiedlicher klinisch-pathologischer Parameter, sowie Daten zum Follow-up der Patienten zu untersuchen. **Methoden:** 373 primäre kolorektale Karzinome und 171 korrespondierende Metastasen wurden mit einem monoklonalen Antikörper gegen den Prolaktin Rezeptor immunhistochemisch mittels „Tissue Micro Array (TMA)“ Technik untersucht. Immunoreaktivität wurde semiquantitativ als entweder fokal (<10% der Tumorzellen sind positiv), moderate (<50%) oder extensiv (>50%) beurteilt. Die Ergebnisse sind mit unterschiedlichen klinisch-pathologischen Parametern sowie mit dem Follow-up der Patienten korreliert worden. Zur Validierung dieser immunhistochemischen Ergebnisse wurden zusätzlich die Kolonkarzinom-Zelllinien HT-29 und SW-480, sowie die Mammakarzinom-Zelllinie T47D mittels Western Blot Technik hinsichtlich der Prolaktin Rezeptor Expression untersucht. **Ergebnisse:** Insgesamt exprimierten 360 von 373 (97%) Tumore den Prolaktin Rezeptor, darunter zeigte sich die Expression fokal bei 21(6%), moderate bei 55 (15%) und extensiv bei 284 (76%) Tumoren. Extensive Expression des Prolaktin Rezeptors war signifikant assoziiert mit der TumorgroÙe ($p=0,002$), der Tumordifferenzierung ($p<0,001$) sowie dem histologischen Subtyp ($p<0,001$). Es bestand eine hohe Konkordanz zwischen der Expression beim Primärtumor und korrespondierender Metastase. Der Somer's D Koeffizient für die Konkordanz zwischen Primärtumoren und Lymphknoten-metastasen betrug $D=0.719$ ($P<0.001$) und $D=0.535$ ($P=0.001$) für Fernmetastasen. Extensive Prolaktin Rezeptor Expression war bei Tumoren mit schlechter Differenzierung (G3 und G4) signifikant assoziiert mit Progression-Freiem ($p=0,03$) und Tumor-Spezifischem Überleben ($p=0,04$). **Schlussfolgerung:** Unsere Ergebnisse zeigen, dass der Prolaktin Rezeptor sehr häufig in kolorektalen Karzinomen exprimiert wird und eine hohe Konkordanz der Expression zwischen primärem und metastatischem Tumorgewebe besteht. Mit Hinblick auf neue zielgerichtete Therapieansätze beim kolorektalen Karzinom, vor allem hinsichtlich metastasierter kolorektaler Karzinome, kann die ausgeprägte Expression des Prolaktin Rezeptors eine therapeutische Option bieten.

Introduction

Incidence of colorectal cancer ranks fourth in men (after lung, prostate and stomach) and third in women (after breast and cervix uteri) accounting for about 1 million new cancer cases occurring every year worldwide with a similar number of cases in men and women for colon cancer and a male prevalence for rectal cancer.¹ In the United States, colorectal cancer accounts for approximately 10% of annual new cancer cases. About 150,000 newly diagnosed cases and nearly 50,000 deaths of the disease have been estimated for 2008.² The overall lifetime risk in the United States for developing colorectal cancer is as high as 5.3%.³

Owing to advanced surgical techniques, new drugs and multimodal therapy regimens, outcome and prognosis of colorectal cancer patients have markedly improved. Thus, chemotherapy based on 5-fluoruracil has decreased tumor recurrence in patients with nodal disease, while neoadjuvant chemoradiotherapy and total mesorectal excision have improved local control of rectal cancer.⁴⁻⁶ The introduction of new drugs, the so-called biologicals, which selectively target pathways implicated in tumor growth and development, such as monoclonal antibodies directed against the ligand-binding extracellular domain of the epidermal growth factor receptor (EGFR) or the vascular endothelial growth factor (VEGFR), has further improved outcome of affected patients.⁷⁻⁹ The role of human prolactin and its receptor, the prolactin receptor, in cancer has been investigated for almost two decades. Prolactin receptor is expressed in various extrapituitary cells, including breast, liver, pancreas, and gastrointestinal tissues. In the latter, prolactin has been reported to act on water and electrolyte transport through the mucosa.^{10,11} Numerous studies have provided evidence implicating a pathogenetic role of prolactin in breast cancer. Upon prolactin binding, the receptor exerts mitogenic effects involving various intracellular signaling cascades, such as JAK-STAT, ras-MAPK, and SHC-Grb pathways (Figure 1).¹²⁻¹⁴ Thus, prolactin and its receptor are promising therapeutic targets, wherein prolactin receptor antagonism appears to be the most promising interventional approach.¹⁵⁻¹⁷

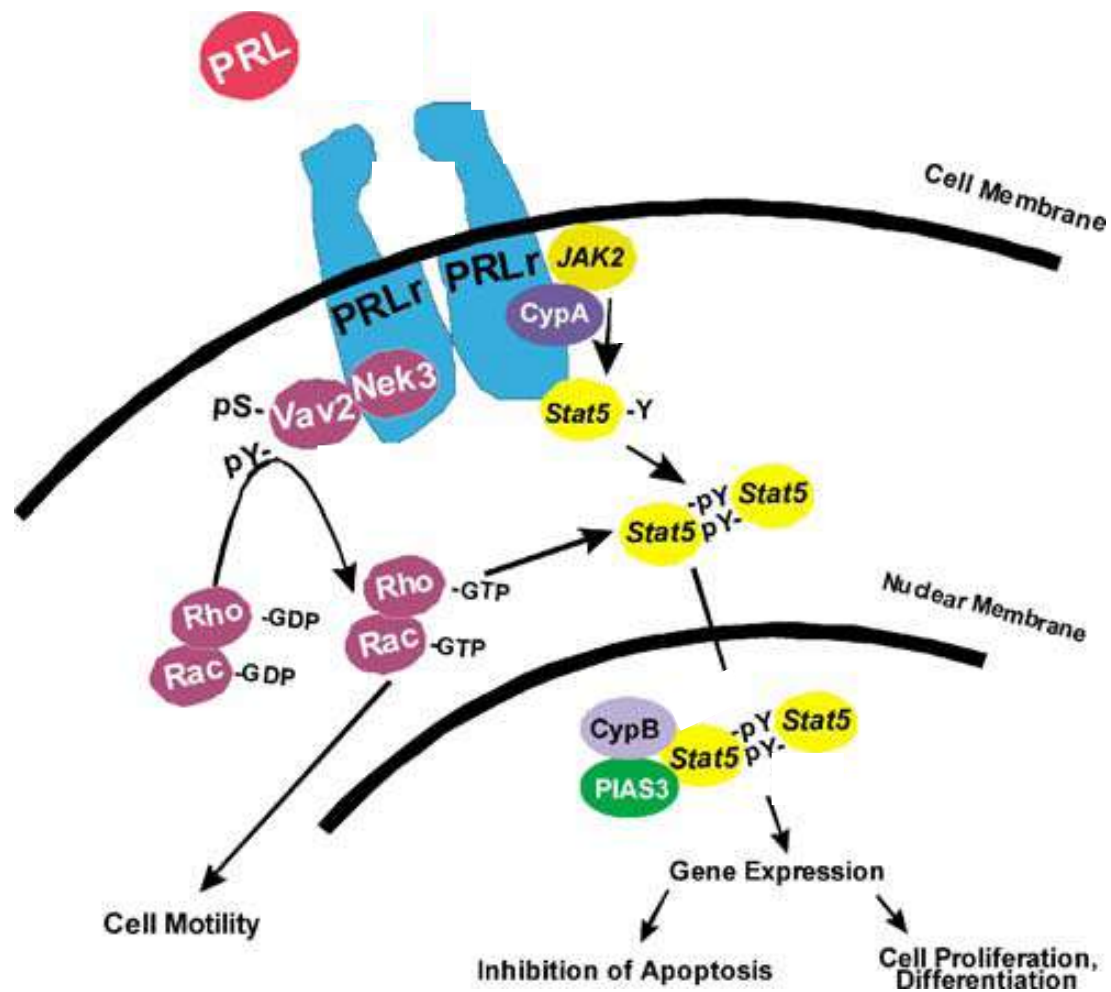


Figure 1 The intracellular signaling pathway upon prolactin binding eventually leads to increased cell motility, inhibition of apoptosis, enhanced cell proliferation and differentiation: The arrows demonstrate the signaling relationships. Adapted by Clevenger et al.¹²

The significance of prolactin and its receptor in colorectal cancer, however, is largely unknown. The expression of prolactin receptor in primary colorectal cancer has so far been investigated by three studies, reporting significant higher level of prolactin receptor protein and mRNA expression in cancer tissue as compared with normal colorectal tissue.¹⁸⁻²⁰ A systematic correlation of prolactin receptor expression with other tumor variables including analysis of prognostic impact is currently lacking.

Our study aimed to assess the frequency of prolactin receptor expression, its association with other clinicopathological variables, as well as its prognostic value in a large cohort of colorectal cancer patients. Herein, we evaluated the expression profile of primary tumor tissues and corresponding metastases, which appears to be of particular interest regarding the possible therapeutic role of prolactin receptor

antagonists in patients with metastatic disease. To substantiate our immunohistochemical findings in cancer tissues, we assessed prolactin receptor expression in HT-29 and SW-480 colorectal cancer cell lines with different methodological approaches.

Material and Methods

Patient Selection

During the period from January 1, 1984 to December 31, 2005, a total of 7909 colorectal cancers from 7564 patients (4095 males, 3469 females; ratio 1.2:1) were identified in the colorectal cancer database of the Institute of Pathology, Medical University of Graz, Austria. Of these, 400 (5%) patients were randomly sampled from January 1992 through December 2000 with the aim of obtaining identical adjuvant treatment modalities (see below) as well as at least 5 years' follow-up. The following patients were excluded:

- (i) those who underwent endoscopic polypectomy for low-risk T1 cancer due to missing data regarding nodal status;
- (ii) patients who underwent neoadjuvant chemotherapy due to presumptive treatment-related changes in T classification;
- (iii) patients with synchronous or metachronous secondary colorectal cancer; and
- (iv) patients with competitive invasive cancers originating from other sites if metastatic deposits were not assessed by histology.

In total, 381 specimens from 400 patients (95%) were available for review pathology. There were 215 males (56%) and 166 females (44%) with a median age of 68.5 (range 27.6-93.1) years. Of these, 191 (50%) were older than 70 years.

Tumors were located in the caecum in 49 patients (13%) in the ascending colon in 27 (7%), at the hepatic flexure in 18 (5%), in the transverse colon in 13 (3%), at the splenic flexure in 13 (3%), in the descending colon in 15 (4%), in the sigmoid colon in 82 (22%), at the rectosigmoid junction in 15 (4%) and in the rectum in 149 patients (39%). Thus, 107 tumors (28%) were found on the right side, 110 (29%) on the left side and 164 (43%) in the rectum (including rectosigmoid junction) (Figure 2).

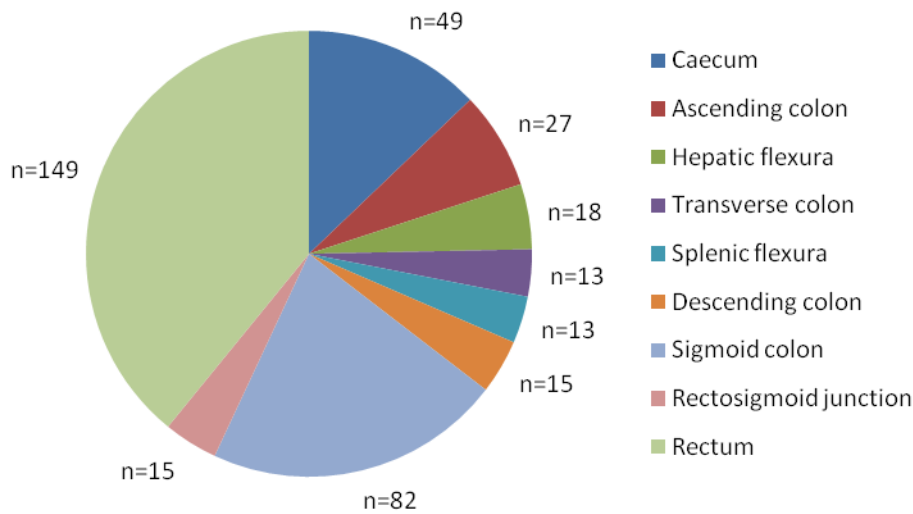


Figure 2 Distribution of the location of primary colorectal cancer.

Adjuvant chemotherapy was guided by AJCC/UICC stage: stage I (T1 N0 M0 or T2 N0 M0) and stage II (T3 N0 M0 or T4 N0 M0) patients did not receive adjuvant therapy, whereas, stage III (any T N1 M0 or any T N2 M0) patients were given 5-fluorouracil/folinic acid according to the Mayo Clinic protocol.⁴

Follow-up included laboratory testing (including blood count, liver enzymes and tumor markers (CEA and CA19-9) at 3-month intervals; after 3 years the interval was extended to 6 months. Chest X-ray and abdominal ultrasound were performed at 6-month intervals; after 3 years the interval was extended to 12 months. Patients with rectal cancer underwent computerized tomography every 12 month.

Institutional review board approval (Ethic commission vote 21-414 ex 09/10) was received from the Ethic's Committee of the Medical University of Graz, Austria.

Histopathology

Slide review was independently performed by two investigators (Dr. med univ. Marion J Pollheimer and Univ. Doz. Dr. med. Cord Langner). Discrepancies were resolved by simultaneous re-examination of the slides by both investigators using a double-headed microscope. The following parameters were reviewed: TNM classification, tumor size and tumor localisation (both taken from patients records), tumor grading (by evaluating the grad of tubular differentiation), histological subtype, as well as presence of lymphatic and venous invasion. T and N classification were adjusted to

the 2002 edition of the AJCC/UICC TNM system.²¹ Histological tumor type and tumor grades were assessed according to the WHO classification.²²

Immunohistochemistry

Using a tissue microarray technique provided the immunohistochemical evaluation. The details of this technique have been described previously.²³ Briefly, tissue microarrays were constructed using a manual tissue-arraying instrument (Beecher, Silver Spring, MD, USA). To account for tumor heterogeneity, between 3 and 14 (mean 5.03, median 5) cylindrical core biopsies, 0.6mm in diameter, were taken from different sites of each tumor and arrayed in recipient paraffin tissue microarrays block (Figure 3). Corresponding lymph node and distant metastases were included in 143 and 42 cases, respectively. Four micrometer tissue microarrays sections were stained using an automated staining system (BenchMark™, Ventana Medical Systems, S.A, Illkirch, CEDEX, France) (Figure 4). Enzymatic digestion was performed using Protease Type I (concentration 0.5 enzyme unit / ml, Catalog No. 760-2018, Ventana) for 32 minutes. The primary prolactin receptor antibody (Ab-1, Clone B6.2; Thermo Fisher Scientific, Fremont, CA, USA) was applied at 1:400 dilution, and the reaction was visualized using the ultraVIEW Universal DAB Detection Kit™ (Catalog No. 760-500 Ventana).

Immunoreactivity was independently assessed by two investigators (cand. med. Lars Harbaum and Univ. Doz. Dr. med. Cord Langner), who were blinded to clinicopathologic data, using a semiquantitative scoring system. Discrepancies were resolved by simultaneous re-examination of the slides by both investigators using a double-headed microscope. A distinct membranous and/or granular cytoplasmic staining was considered positive, and immunoreactivity was semiquantitatively categorized as “focal” (<10% of tumor cells positive), “moderate” (10-50%), or “extensive” (>50%). Each tumor was scored assessing the average positivity of the core biopsies. Slides of breast cancer, known to express prolactin receptor, served as positive control. Negative controls included omission of the primary antibody and incubation with Ventana Antibody Diluent (Catalog No. 251-018).



Figure 3 Paraffin tissue microarray block: Cylindrical core biopsies were taken from different sites of tumor tissue using a manual tissue arraying instrument (Beecher, Silver Spring, MD, USA) and arrayed in recipient paraffin tissue microarray block.

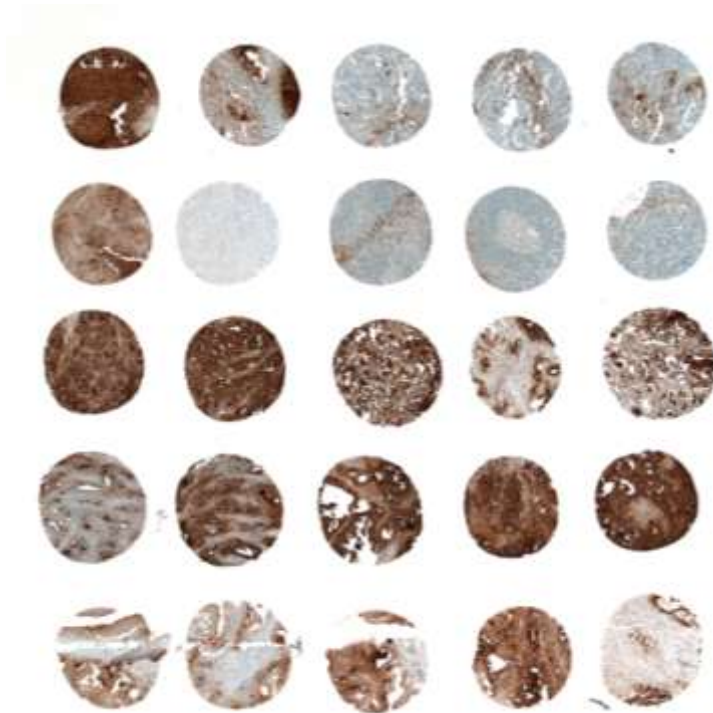


Figure 4 Four micrometer tissue microarrays sections were taken from the paraffin tissue microarray block and were stained using an automated staining system (BenchMark™, Ventana Medical Systems, S.A, Illkirch, CEDEX, France).. Each core represents a different area of either primary or metastatic tumor tissue. (Original x2)

Cell Lines

Commercially available colon cancer cell lines HT-29 and SW-480 and the breast cancer cell line T47D were used for experiments. HT-29 and T47D were purchased from American Type Culture Collection (Manassas, VA, USA, www.atcc.org; HT-29 Catalog No. HTB-38, T47D Catalog No. HTB-133) and SW-480 from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Braunschweig, Germany, www.dsmz.de; Catalog No. ACC 313). Cells were cultivated according to the manufacturer's instructions. The media, the fetal bovine serum "Gold" EU approved (FBS "Gold") as well as the Penstrep were obtained from PAA Laboratories (Pasching, Austria). Twenty four hours before harvesting, cells were cultivated in appropriate media supplemented with charcoal stripped 10% fetal bovine serum (Bio Products, Grossmugl, Austria) to avoid blocking of prolactin receptor on cell surface by prolactin contained in unmanipulated fetal bovine serum. For prolactin receptor immunostaining, cells were harvested and cell pellets were embedded in bacto agar (Becton Dickinson and Company, Franklin Lakes, NJ, USA), fixed in formalin and embedded in paraffin following standard procedures as published previously.²⁴

Western Blot

Cell lines were harvested and lysed using RIPA Buffer (Radioimmunoprecipitation Buffer, formulation according to Abcam Inc. Cambridge, USA) supplemented with 1mM PMSF (phenylmethanesulfonyl fluoride), 10mM DTT (both purchased from Sigma-Aldrich, St. Louis, MO, USA) and 1x Protease Inhibitor Cocktail (Invitrogen Leek, The Netherlands). The amount of extracted protein was detected with 660nm Protein Assay Kit (Thermo Fisher Scientific). SDS-Page electrophoresis was performed using 10% polyacrylamide dissolving gels with 2.5 to 20µg protein per lane. Proteins were transferred onto a nitrocellulose membrane (Applichem, Darmstadt, Germany). After the membrane was blocked with 5% skim milk powder in TBS-T (Abcam), the primary prolactin receptor antibody (Ab-1, Clone B6.2; Thermo Fisher Scientific) was incubated at a dilution of 1:1500 in 0.5% skim milk powder TBS-T (at 4°C, overnight). Anti-β-actin (Sigma-Aldrich) was incubated at 1:20000 under the same conditions as described above and served as a positive loading control. As an isotype control universal mouse negative control IgG (Dako, Glostrup, Denmark) was diluted at 1:100. Next, the membrane was washed and incubated with

the secondary antibody (polyclonal rabbit anti-mouse immunoglobulins/HRP, Dako) at a dilution of 1:1000 (at room temperature, for two hours). Visualization of x-ray films was performed using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and Photo developer Curix 60 (Wiroma, Niederscherli, Switzerland) according to the manufacturer's instructions. T47D cell line was used as positive control and supernatant of the cell cultures as well as the cell culture media served as negative controls. The molecular weight of the protein bands was analyzed using SeeBlue Plus2 Pre-Stained Standard (Invitrogen).

Statistical Analysis

Progressive disease was defined as either local recurrence (any detectable local disease at follow-up, occurring either alone or in conjunction with generalized recurrence) or systemic recurrence (as any detectable disease at follow-up, except local disease).

Associations between prolactin receptor expression and other tumor parameters, such as T classification, N classification, tumor differentiation, angioinvasion, and AJCC/UICC stage, were analyzed using Chi-square or Fisher's exact test, respectively. Disease-free (progression-free) and cancer-specific survival was assessed with the Kaplan-Meier method and compared by the log-rank test. For multivariate testing, Cox's proportional hazards regression model was used. All reported p-values were 2-sided with significance at $p < 0.05$.

To assess concordance of immunostaining results between primary and corresponding lymph node and/or distant metastases the Somer's D rank-order correlation coefficient was used.

All statistical calculations were performed using NCSS (Hintze, J. (2007); NCSS, LLC. Kaysville, Utah, U.S.A.) and StatXact (Cytel Software Corporation, Cambridge, MA, U.S.A.).

Results

Histopathology

The tumor distribution according to T and N classification, AJCC/UICC stage and tumor grade is shown in Table 1. The majority of tumors extended beyond the bowel wall. Positive lymph nodes were detected in more than 40% of cases. Tumor grades were G1 in 121 (32%), G2 in 138 (36%), G3 in 99 (26%), and G4 in 23 (6%) cases, respectively.

Overall, 316 (83%) tumors were adenocarcinomas, 45 (12%) mucinous adenocarcinomas, and 13 (3%) undifferentiated carcinomas. Seven cases presented rare histological subtypes, including three signet-ring cell, two medullary, one adenosquamous, and one amphicrine carcinoma, respectively (Figure 5). Lymph vessel and blood vessel invasion were detected in 126 (33%) and 87 (23%) cases. Tumor size, measured by the largest horizontal diameter, varied between 0.6cm and 16cm (Median 4.5cm).

Table 1

Patient cohort: Distribution of T / N classification, AJCC/UICC stage and tumor grade

T	n (%)	N	n (%)	Stage	n (%)	G	n (%)
T1	28 (7%)	N0	213 (56%)	I	81 (21%)	1	121 (32%)
T2	70 (18%)	N1	83 (22%)	IIA	110 (29%)	2	138 (36%)
T3	218 (57%)	N2	85 (22%)	IIB	10 (3%)	3	99 (26%)
T4	65 (17%)			IIIA	5 (1%)	4	23 (6%)
				IIIB	61 (16%)		
				IIIC	60 (16%)		
				IV	54 (14%)		

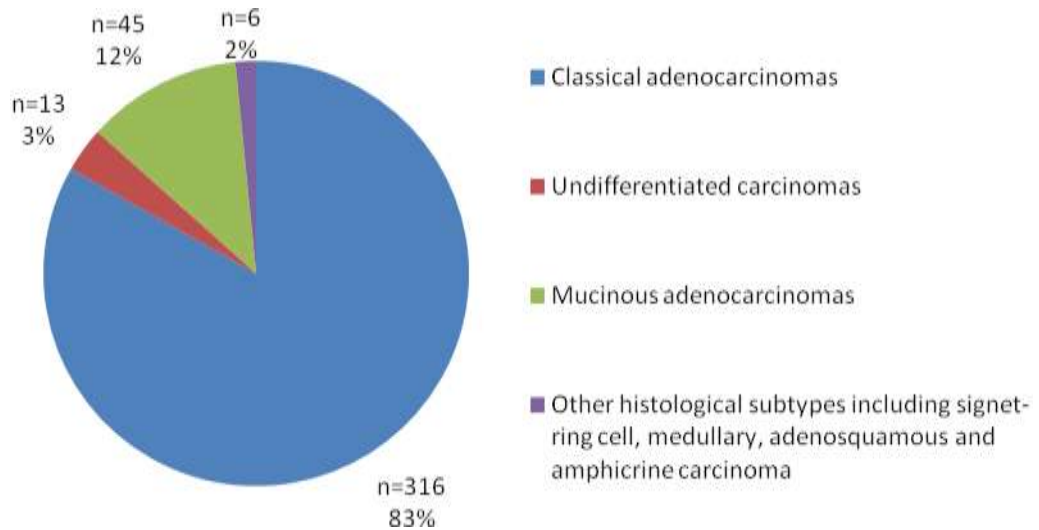


Figure 5 Distribution of histological subtypes of primary colorectal cancer.

Immunohistochemistry

Prolactin receptor expression was observed in 360/373 (97%) evaluable primary tumors, with 21 (6%) cases showing focal, 55 (15%) moderate and 284 (76%) extensive expression, respectively (Figure 6). Staining was homogenous, mainly cytoplasmic, but with membranous accentuation (Figure 7-9). Adjacent non-neoplastic epithelial cells showed comparatively weak immunoreactivity with staining accentuation at the mucosal surface (Figure 10).

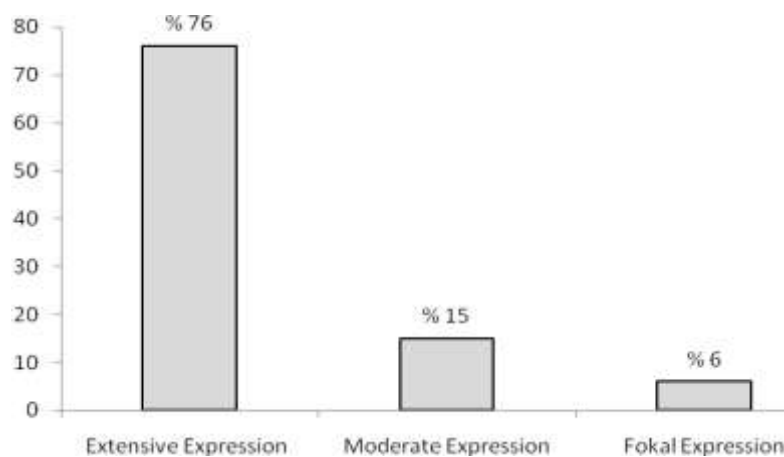


Figure 6 Distribution of prolactin receptor expression in colorectal cancer positive for prolactin receptor expression.

Extensive prolactin receptor expression in primary tumors was significantly associated with histological subtype, tumor size and grade (Table 2). Thus, extensive prolactin receptor expression was observed in 205/254 (81%) low grade (G1/G2), yet in only 79/119 (66%) high grade (G3/G4) cancers ($P=0.004$). This finding, however, is most probably related to the fact, that extensive prolactin receptor expression was comparably low in mucinous adenocarcinomas, which, according to the current WHO classification,²² are by convention considered poorly differentiated (G3). Thus, restricting analysis to classical (non-mucinous) adenocarcinomas, extensive prolactin receptor expression was observed in similar amounts in low grade (G1/G2: 205/254 or 81%) and high grade (G3/G4: 45/58 or 78%) cancers ($P=0.59$). Anyhow, it should be noted that only about 50% of undifferentiated tumors showed extensive prolactin receptor expression. Sample size, however, was small (Table 2).

Immunoreactivity of metastatic tissues matched well with that of corresponding primary tumors. If primary tumors were negative for prolactin receptor, metastatic sites similarly lacked immunolabelling. If primary tumors, however, showed extensive prolactin receptor expression, 96/104 (92%) corresponding nodal and 28/32 (86%) corresponding distant metastases also showed high expression (Table 3). Somer's D coefficients for concordance of primary tumors with corresponding lymph node and distant metastases were $D=0.719$ ($P<0.001$) and $D=0.535$ ($P=0.001$), respectively.

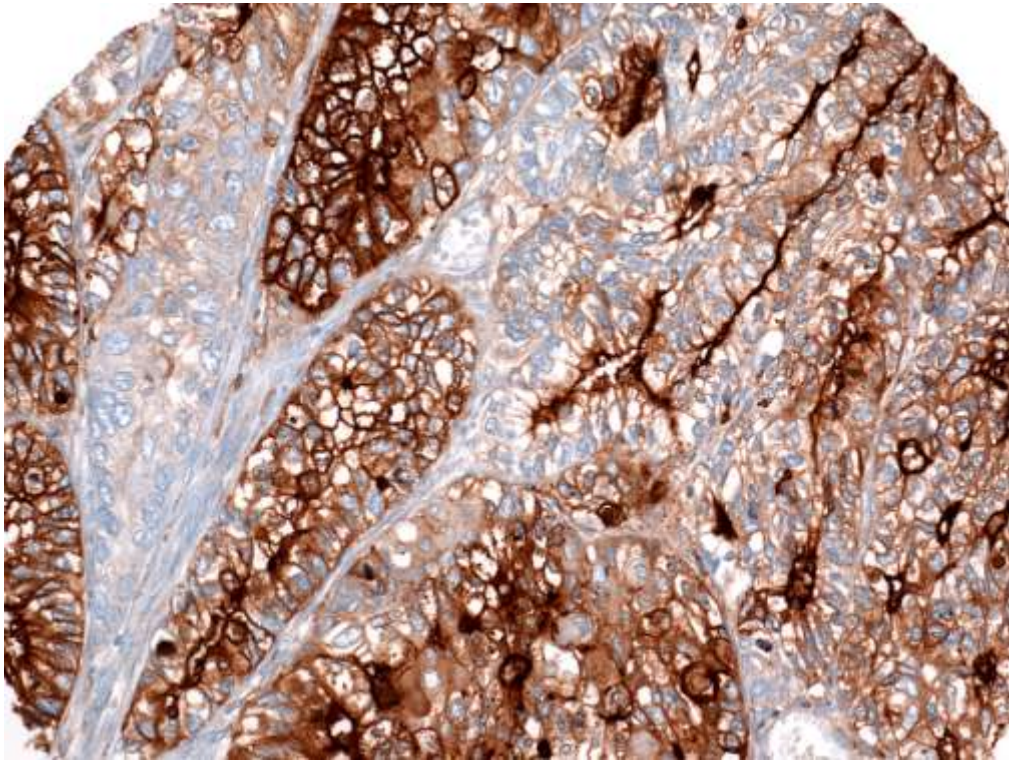


Figure 7 Expression of prolactin receptor in classical (non-mucinous) colorectal adenocarcinoma (original x100),

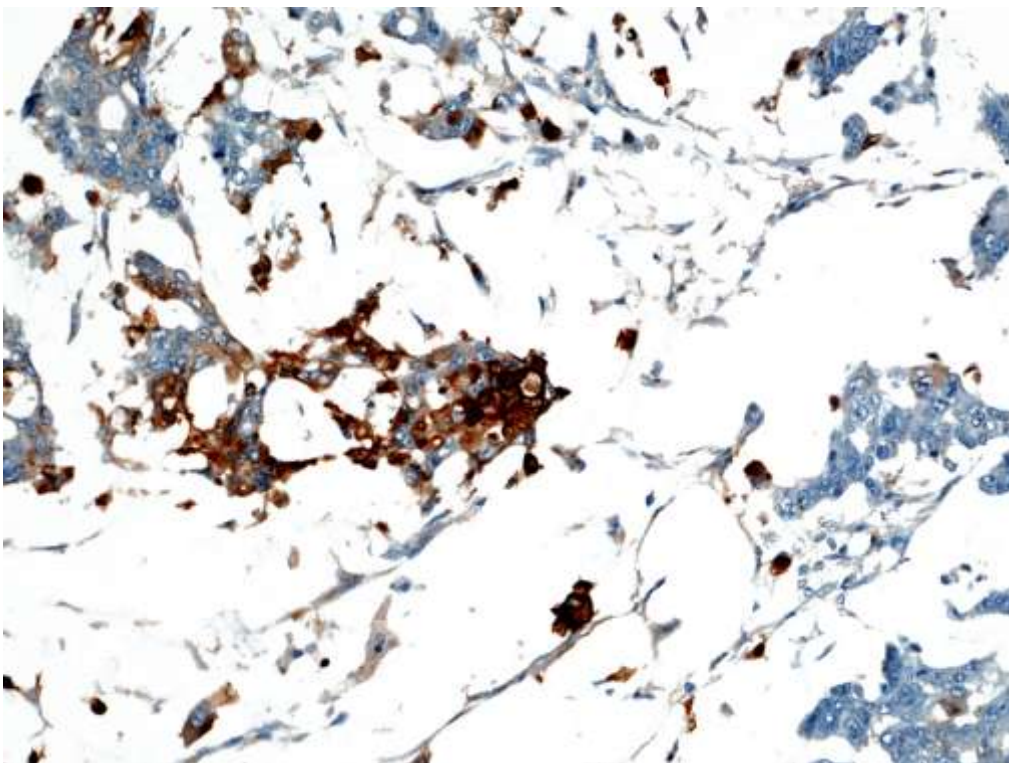


Figure 8 Expression of prolactin receptor in mucinous colorectal adenocarcinoma (original x100).

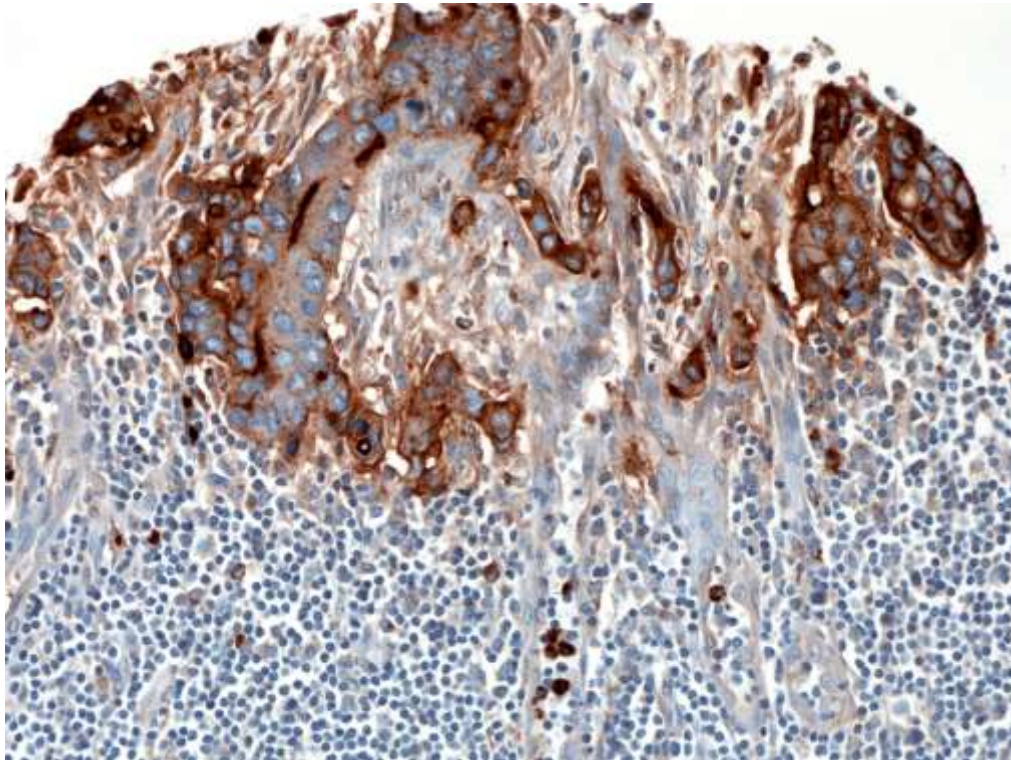


Figure 9 Expression of prolactin receptor in lymph node metastasis of colorectal cancer (originalx100).

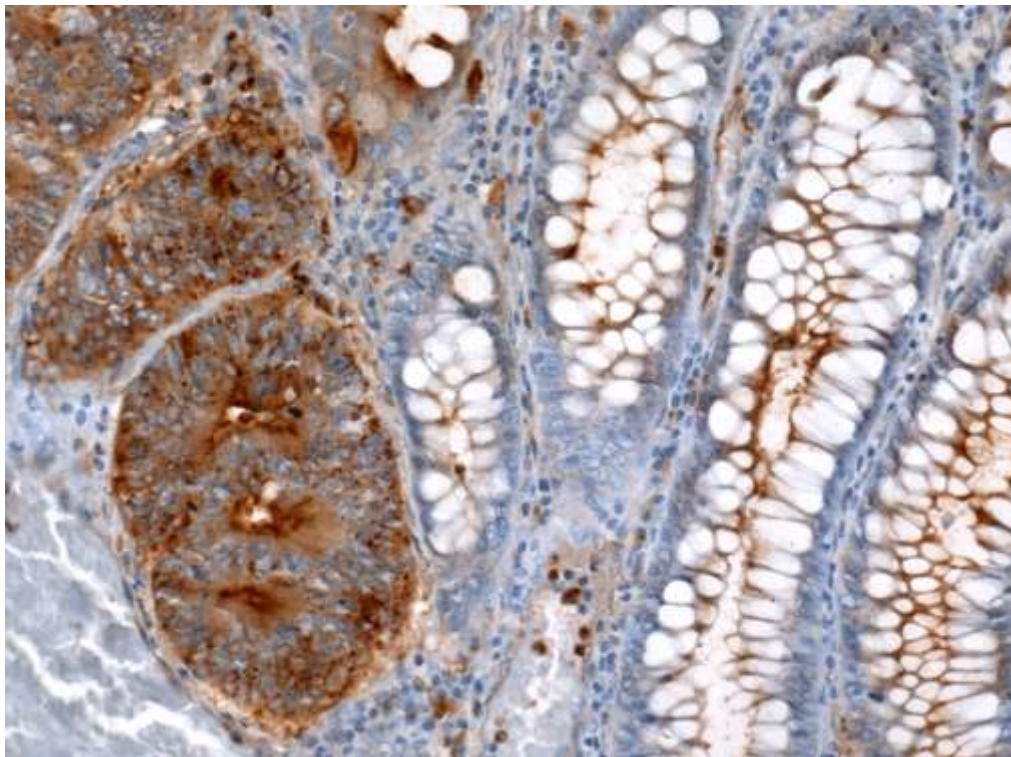


Figure 10 Expression of prolactin receptor in classical (non-mucinous) colorectal; note comparably weak prolactin receptor expression in adjacent non-neoplastic mucosa (original x100).

Table 2

Association of prolactin receptor expression with different tumor characteristics

		Prolactin Receptor Immunoreactivity		P-Value
		Positive	Extensive Expression	
T classification	T1	24/26 (92%)	20/26 (77%)	<i>P</i> =0.15
	T2	66/69 (96%)	48/69 (70%)	
	T3	206/213 (97%)	160/213 (75%)	
	T4	64/65 (98%)	56/65 (86%)	
N classification	N0	199/208 (96%)	152/208 (73%)	<i>P</i> =0.29
	N1	80/82 (98%)	66/82 (80%)	
	N2	81/83 (98%)	66/83 (75%)	
AJCC/ UICC Stage	I	75/80 (88%)	55/80 (69%)	<i>P</i> =0.30
	II	112/116 (97%)	88/116 (76%)	
	III	121/124 (98%)	99/124 (80%)	
	IV	52/53 (98%)	42/53 (79%)	
Grade	G1	114/118 (97%)	87/118 (74%)	<i>P</i> <0.001
	G2	135/136 (99%)	118/136 (87%)	
	G3	90/97 (93%)	7/97 (69%)	
	G4	21/22 (95%)	12/22 (55%)	
Histology	Adeno-Ca.	304/312 (97%)	250/312 (80%)	<i>P</i> <0.001
	Mucinous Ca.	37/42 (88%)	23/42 (55%)	
	Undiff. Ca.	12/12 (100%)	7/12 (58%)	
	Other Ca.	7/7 (100%)	4/7 (57%)	
Tumor size	≤4.5 cm	191/194 (98%)	161/194 (83%)	<i>P</i> =0.002
	>4.5 cm	148/157 (94%)	108/157 (69%)	
Lymphatic invasion	L0	241/250 (96%)	190/250 (76%)	<i>P</i> =1
	L1	119/123 (97%)	94/123 (76%)	
Venous invasion	V0	277/286 (97%)	216/286 (76%)	<i>P</i> =0.67
	V1	83/87 (95%)	68/87 (78%)	
Age	≤70	179/184 (97%)	139/184 (76%)	<i>P</i> =0.81
	>70	181/189 (96%)	145/189 (77%)	
Gender	Female	161/164 (98%)	123/164 (75%)	<i>P</i> =0.71
	Male	199/209 (95%)	161/209 (77%)	

Table 3

Concordance of prolactin receptor expression in primary and corresponding metastatic tumor tissues (n=151 primary tumors with lymph node (n=132) and/or distant (n=39) metastases).

Primary Tumor	Lymph Node Metastasis		Distant Metastasis	
T negative 4/151 (3%)	Negative	4/4 (100%)	Negative	-
	Focal	-	Focal	-
	Moderate	-	Moderate	-
	Extensive	-	Extensive	-
T focal 9/151 (6%)	Negative	-	Negative	-
	Focal	3/6 (50%)	Focal	1/3 (33%)
	Moderate	1/6 (17%)	Moderate	2/3 (66%)
	Extensive	2/6 (33%)	Extensive	-
T moderate 19/151 (13%)	Negative	-	Negative	-
	Focal	-	Focal	-
	Moderate	15/18 (83%)	Moderate	2/4 (50%)
	Extensive	3/18 (17%)	Extensive	2/4 (50%)
T extensive 119/151 (79%)	Negative	-	Negative	-
	Focal	1/104 (1%)	Focal	1/32 (3%)
	Moderate	7/104 (7%)	Moderate	3/32 (9%)
	Extensive	96/104 (92%)	Extensive	28/32 (86%)

Survival Analysis

Follow-up data were available for 350 (92%) patients. Median follow-up was 45 months (mean 56, range 0-180). At the time of last follow-up, 173 (49%) patients showed no evidence of disease. Progressive disease was observed in 141 (40%) patients including 117 (33%) patients who died from cancer and 11 (3%) patients who currently are alive with metastatic disease. Median time to progression was 7 months (mean 15, range 0-88). 49 (14%) patients died from causes not related to colorectal cancer.

Disease progression occurred in 24/78 (31%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression and 116/264 (44%) patients with tumors showing extensive prolactin receptor expression ($P=0.08$, log-rank test; Figure 11A). Actuarial 5-year progression-free (disease-free) survival rates for patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression and patients with tumors with extensive prolactin receptor expression were 68% and 56%, respectively. In addition, 19/78 (24%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression and 98/264 (37%) patients with tumors showing extensive prolactin receptor died of disease ($P=0.08$, log-rank test; Figure 11B). Actuarial 5-year cancer-specific survival rates for patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression and patients with tumors with extensive prolactin receptor expression were 73% and 62%, respectively. In Cox's proportional hazards regression models lymph node metastasis, tumor size >4.5cm in largest diameter and T classification proved to be independent predictor of both progression-free and cancer-specific survival. Tumor grade was an independent prognostic factor only in respect of cancer-specific survival. Patients with tumors showing extensive prolactin receptor expression were more likely to experience disease progression (hazard ratio 1.37, 95% confidence interval 0.86-2.17) or to die of disease (hazard ratio 1.67, 95% confidence interval 0.99-2.81), but differences were not statistically significant (Table 4).

Regarding only patients with high grade (G3/G4) tumors, 43/73 (59%) patients with tumors showing extensive prolactin receptor expression experienced disease progression compared with 11/33 (33%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression ($P=0.03$, log-rank

test; Figure 12A). Similarly, 39/73 (53%) patients with tumors with extensive prolactin receptor expression died of disease in this group compared with 9/33 (27%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression ($P=0.04$, log-rank test; Figure 12B). In Cox's proportional hazards regression models restricted to patients with high grade (G3/G4) tumors presence of lymph node metastasis proved to be the only independent predictor of both progression-free and cancer-specific survival. Patients with tumors showing extensive prolactin receptor expression were more likely to experience disease progression (hazard ratio 1.62, 95% confidence interval 0.77-3.40) or to die of disease (hazard ratio 2.01, 95% confidence interval 0.89-4.53), but differences were not statistically significant (Table 5).

To appraise the possible impact of histological subtype on analysis, we recalculated data in classical (non-mucinous) adenocarcinomas and mucinous adenocarcinomas separately.

In the group of classical adenocarcinomas for which follow-up data were available ($n=289$) 99/233 (42%) patients with tumors showing extensive prolactin receptor expression experienced disease progression compared with 17/56 (30%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression ($P=0.19$, log-rank test). In addition, 84/233 (36%) patients with tumors with extensive prolactin receptor expression died of disease compared with 14/56 (25%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression ($P=0.23$, log-rank test).

In the much smaller group of mucinous adenocarcinomas ($n=39$) data were nearly significant, thus indicating stronger prognostic value of prolactin receptor immunostaining in this distinct type of cancer. 12/23 (52%) patients with tumors showing extensive prolactin receptor expression experienced disease progression compared with 4/16 (25%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression ($P=0.06$, log-rank test; Figure 13A). Similarly, 10/23 (43%) patients with tumors with extensive prolactin receptor expression died of disease compared with 3/16 (19%) patients with tumors either lacking prolactin receptor expression or showing only focal/moderate expression ($P=0.08$, log-rank test; Figure 13B). In Cox's proportional hazards regression models restricted to patients with mucinous adenocarcinoma presence of lymph node metastasis proved to be an independent predictor of progression-free survival. No

other predictor emerged to be statistical significant. Patients with tumors showing extensive prolactin receptor expression were more likely to experience disease progression (hazard ratio 3.19, 95% confidence interval 0.84-12.12) or to die of disease (hazard ratio 3.96 95% confidence interval 0.80-19.52), but differences were not statistically significant (Table 6).

Cell Lines

To substantiate the specificity of prolactin receptor staining results we thought to examine prolactin receptor protein expression in the colon cancer cell lines HT-29 and SW-480 employing immunohistochemistry as well as Western blotting. Intensive prolactin receptor staining was observed in HT-29 cells (and in T47D breast cancer cells, serving as positive control), whereas SW-480 cells showed weak to moderate immunolabeling of single cancer cells (Figure 14-16). In accordance, Western blotting revealed a prominent band at 78kDa corresponding to the prolactin receptor protein in the lysate of HT-29 cells, whereas no band was detected in the lysate of SW-480 cells (Figure 17). Although we increased the amount of protein of SW-480 cell lysate by 1^{10^9} compared with HT-29 cell lysate we were not able to detect a signal suggesting a very low expression of prolactin receptor corresponding to findings in immunohistochemistry.

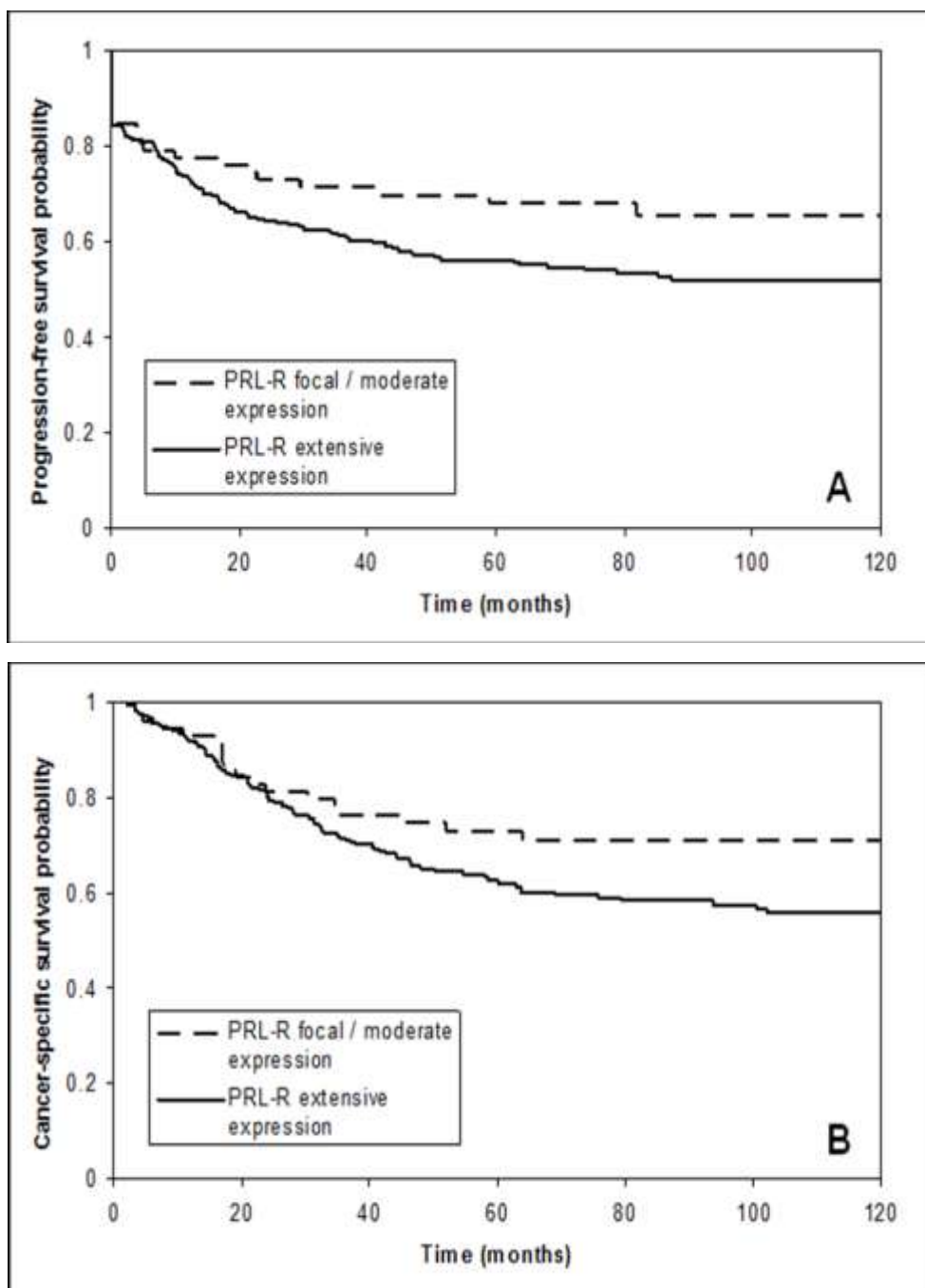


Figure 11 Progression-free (A; $P=0.08$, log-rank test) and cancer-specific (B; $P=0.08$, log-rank test) survival in patients with colorectal carcinoma related to prolactin receptor (PRLR) expression (extensive prolactin receptor expression vs. focal/moderate prolactin receptor expression).

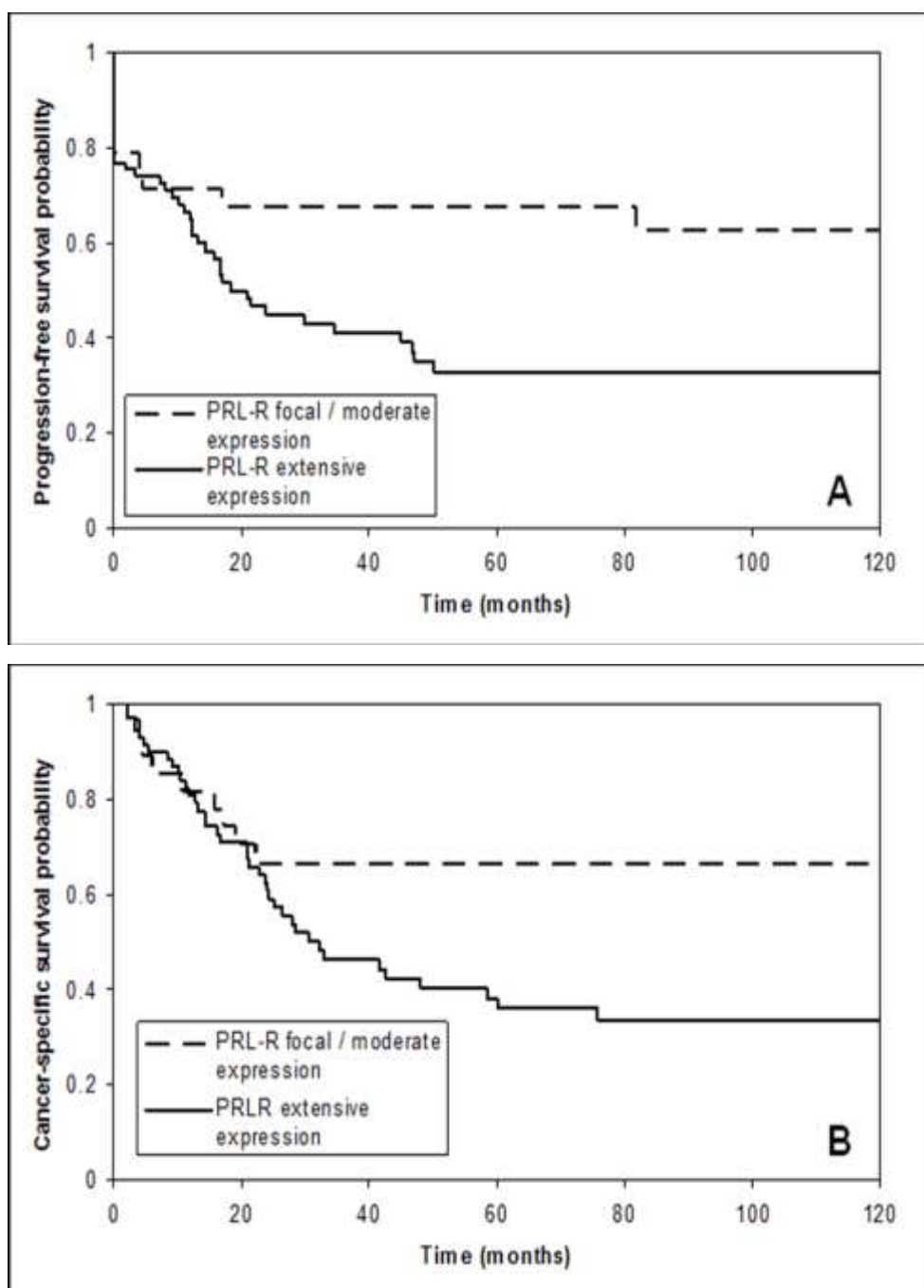


Figure 12 Progression-free (A; $P=0.03$, log-rank test) and cancer-specific (B; $P=0.04$, log-rank test) survival in patients with high grade (G3/G4) colorectal carcinoma related to prolactin receptor (PRLR) expression (extensive prolactin receptor expression vs. focal/moderate prolactin receptor expression).

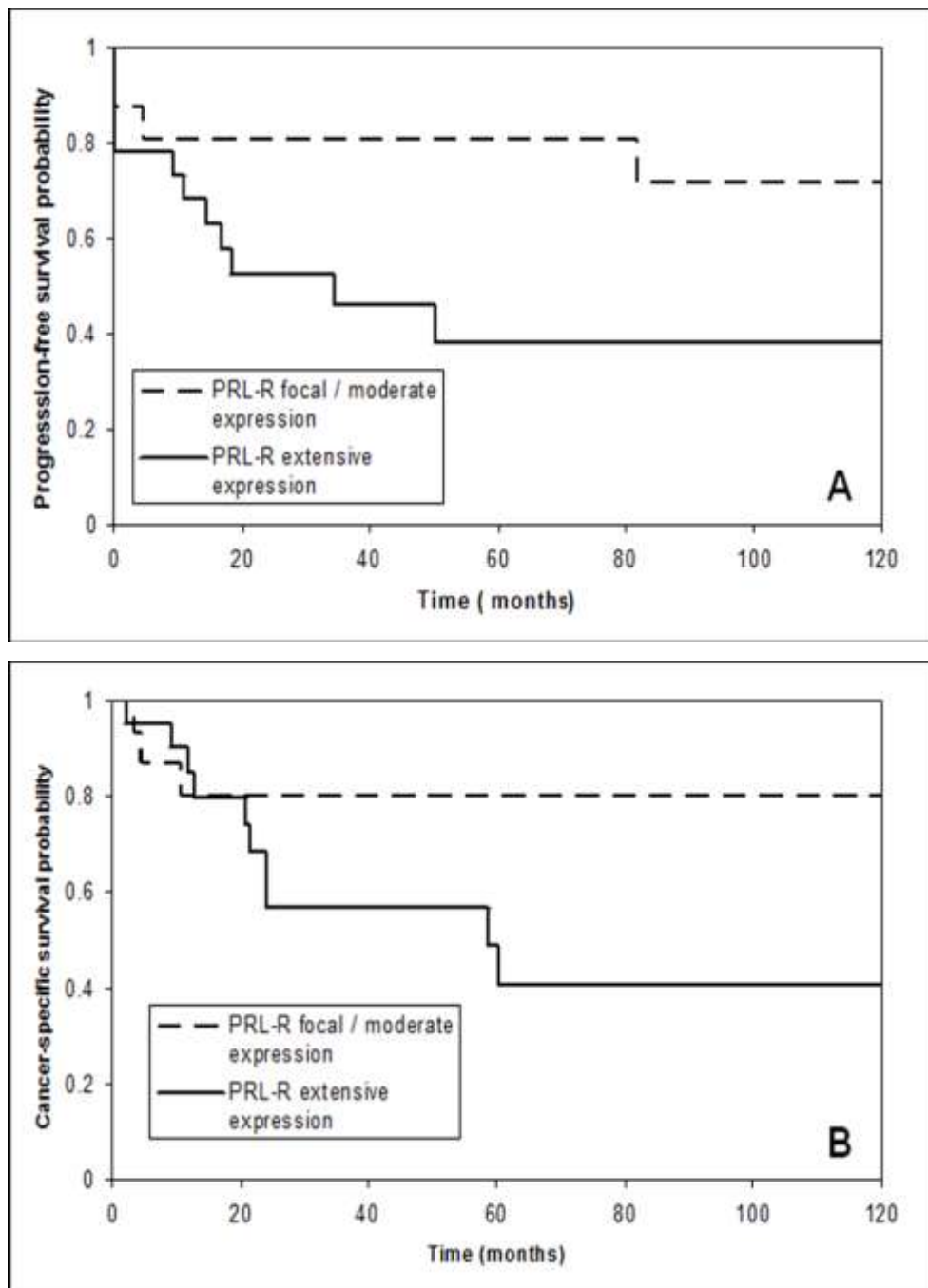


Figure 13 Progression-free (**A**; $P=0.06$, log-rank test) and cancer-specific (**B**; $P=0.08$, log-rank test) survival in patients with mucinous adenocarcinoma related to prolactin receptor (PRLR) expression (extensive prolactin receptor expression vs. focal/moderate prolactin receptor expression).

Table 4 Cox's proportional hazards regression model of all patients with colorectal carcinoma related to prolactin receptor expression (n=360) in respect to progression-free and cancer-specific survival.

	Progression-Free Survival			Cancer-Specific Survival		
	Hazard Ratio	95% CI	<i>P</i> -Value	Hazard Ratio	95% CI	<i>P</i> -Value
Age (>70)	1.21	0.85-1.71	<i>P</i> =0.29	1.59	1.09-2.32	<i>P</i> =0.02
Male gender	0.92	0.65-1.31	<i>P</i> =0.66	0.96	0.65-1.40	<i>P</i> =0.82
T classification > 2	1.98	1.10-3.57	<i>P</i> =0.02	2.46	1.26-4.82	<i>P</i> =0.01
Tumor size > 4.5cm	1.53	0.86-2.19	<i>P</i> =0.02	1.62	1.10-2.38	<i>P</i> =0.02
Nodal Disease	4.16	2.80-6.20	<i>P</i> <0.0001	4.12	2.68-6.34	<i>P</i> <0.0001
High grade (G3/4)	1.14	0.79-1.65	<i>P</i> =0.47	1.60	1.08-2.37	<i>P</i> =0.02
Extensive prolactin receptor expression	1.37	0.86-2.17	<i>P</i> =0.18	1.67	0.99-2.81	<i>P</i> =0.06

Table 5 Cox's proportional hazards regression model of patients with high grade (G3/G4) colorectal cancer (n=119) in respect to progression-free and cancer-specific survival.

	Progression-Free Survival			Cancer-Specific Survival		
	Hazard Ratio	95% CI	<i>P</i> -Value	Hazard Ratio	95% CI	<i>P</i> -Value
Age (>70)	1.14	0.65-2.01	<i>P</i> =0.64	1.29	0.7-2.37	<i>P</i> =0.41
Male gender	0.98	0.56-1.73	<i>P</i> =0.95	1.30	0.70-2.40	<i>P</i> =0.41
T classification > 2	2.35	0.56-9.96	<i>P</i> =0.25	2.57	0.61-10.79	<i>P</i> =0.20
Tumor size > 4.5cm	1.56	0.86-2.86	<i>P</i> =0.15	1.74	0.92-3.29	<i>P</i> =0.09
Nodal Disease	4.28	2.06-8.86	<i>P</i> =0.0001	4.10	1.92-8.74	<i>P</i> =0.0003
Extensive prolactin receptor expression	1.62	0.77-3.40	<i>P</i> =0.21	2.01	0.89-4.53	<i>P</i> =0.09

Table 6 Cox's proportional hazards regression model of patients with mucinous colorectal adenocarcinoma (n=42) related to prolactin receptor expression in respect to progression-free and cancer-specific survival.

	Progression-Free Survival			Cancer-Specific Survival		
	Hazard Ratio	95% CI	<i>P</i> -Value	Hazard Ratio	95% CI	<i>P</i> -Value
Age (>70)	1.37	0.41-4.58	<i>P</i> =0.61	0.81	0.20-3.33	<i>P</i> =0.78
Male gender	0.52	0.10-2.64	<i>P</i> =0.43	0.99	0.18-5.55	<i>P</i> =0.99
Tumor size > 4.5cm	2.00	0.43-9.37	<i>P</i> =0.38	1.63	0.32-8.28	<i>P</i> =0.56
Nodal Disease	7.01	1.35-36.52	<i>P</i> =0.02	4.17	0.84-20.77	<i>P</i> =0.08
Extensive prolactin receptor expression	3.19	0.84-12.12	<i>P</i> =0.09	3.96	0.80-19.52	<i>P</i> =0.09



Figure 14 Intensive prolactin receptor staining in HT-29 colon cancer cells.

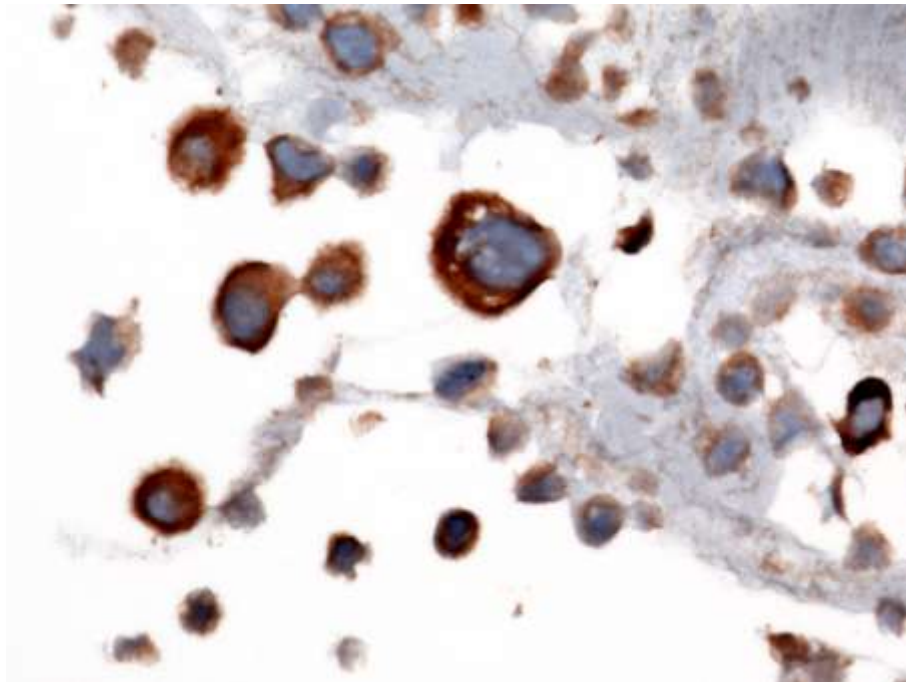


Figure 15 Intensive prolactin receptor staining in T47D breast cancer cells, which served as positive control.

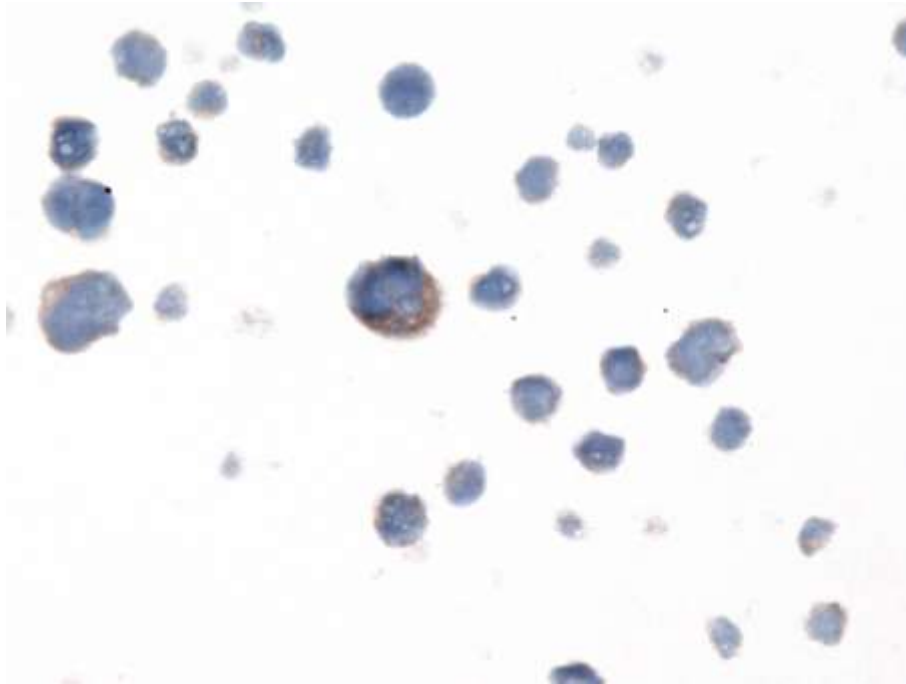


Figure 16 Weak prolactin receptor staining of single cells in SW-480 colon cancer cells.

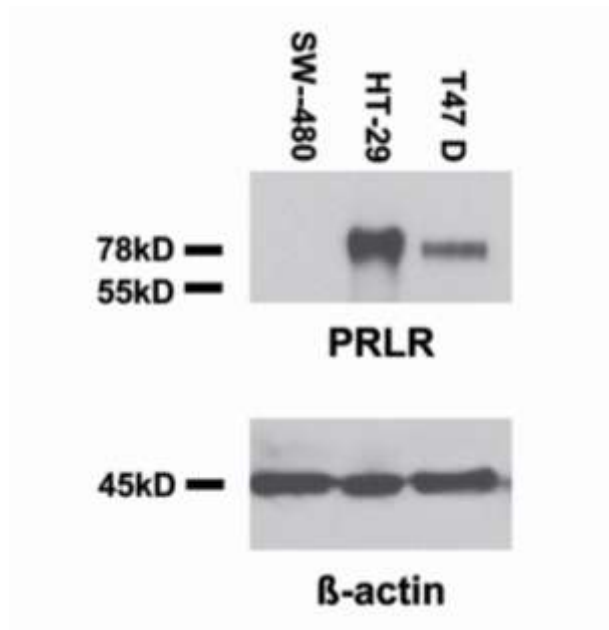


Figure 17 Western blotting reveals a prominent band at 80kDa corresponding to the prolactin receptor protein in the lysate of HT-29 cells, whereas no band was detected in the lysate of SW-480 cells. The expression of prolactin receptor in HT-29 cells was approximately 10-fold compared to T47D cells (D). Anti- β -actin served as a positive loading control. Please note that the protein concentration of SW-480 and T47D cell lysates was 20 μ g, and that of HT-29 was 2.5 μ g, respectively, due to the 1log difference in prolactin receptor expression. The western blot presented is representative for five experiments.

Discussion

The prolactin hormone, synthesized by lactotrophic cells of the anterior pituitary gland, has been attributed with numerous biological effects, including functions linked to reproduction, metabolism, water and electrolyte balance, growth and development, as well as immunoregulation.¹¹ However, prolactin exerts its effects not only through an endocrine but also through autocrine and/or paracrine activity.²⁵

The human prolactin receptor is a single-pass transmembrane, non-tyrosine kinase receptor belonging to the class 1 cytokine receptor superfamily. The gene encoding the receptor is localized on chromosome 5p13-14 and contains at least 10 exons. Alternative splicing of those yields multiple isoforms, which are differing in length and composition of their cytoplasmatic component and are referred to as short, intermediate or long prolactin receptor depending on their size.¹¹

Prolactin binding leads to homodimerization of two receptor molecules, thereby activating different signal transduction cascades, which ultimately lead to cell proliferation. Data obtained from breast cancer cell lines and *in vivo* models show that intracellular signaling involves phosphorylation of cytoplasmatic transcription factors, e.g. signal transducer and activator of transcription (STAT) 1, STAT3 or STAT5, via recruited kinases, e.g. janus-kinase JAK2, or activation of the ras-MAPK pathway.^{12,14}

According to our data, prolactin receptor is widely expressed in colorectal cancer. Immunoreactivity was significantly associated with tumor differentiation and histological subtype which is reported here for the first time. Patients with extensive prolactin receptor expression were more likely to experience disease progression and cancer-related death. This effect was pronounced in high grade tumors and in mucinous adenocarcinomas. Specificity of immunohistochemical staining was endorsed by cell line experiments. Interestingly, the colon cancer cell lines HT-29 and SW-480 used in our study differentially expressed prolactin receptor. SW-480 cell line showed weak to no expression of prolactin receptor whereas HT-29 cell line showed a high expression of prolactin receptor. The difference in prolactin receptor expression goes parallel to different *in vivo* behavior of HT-29 and SW-480 cells, with high expression levels in cells exhibiting an aggressive phenotype. Thus, HT-29 cells have been reported to show local invasion and marked metastatic capacity after subcutaneous and intracaecal xenografting in nude mice, whereas SW-480 showed

solid growth without invasion in the bowel wall or the development of metastases.²⁶ This pair of cell lines may consequently be used as a model system for further research of the role of prolactin and its receptor in the biology of colon cancer.

To the best of our knowledge, only three studies have so far addressed the topic of prolactin receptor expression in colorectal cancer.¹⁸⁻²⁰ The first study assessed prolactin receptor protein by radioligand assay using ¹²⁵I-hGH. Tumor specimens of 71 male patients were investigated and prolactin receptor was found to be positive in 36 (51%) patients.¹⁸ No associations with clinicopathological variables were noted and prolactin receptor protein content was independent from outcome. In a second study, the same group of authors noted prolactin receptor immunoreactivity in 26 out of 56 (46%) tumors.¹⁹ The authors used a monoclonal mouse anti-human prolactin receptor antibody different from the one used in our study which may have accounted for the low prevalence of prolactin receptor expression. Data were not correlated with follow-up data in this investigation.

In the third study by Otte et al.²⁰ the long transcript of prolactin receptor mRNA was detected in 45 out of 48 (94%) of cancer specimens as well as in 22 out of 23 (96%) normal colonic tissues. Expression in cancer tissue did not differ significantly between tumors of different grades. mRNA encoding for the short form of prolactin receptor was not found in any samples investigated. In addition, prolactin receptor protein was detected by Western blotting in 39 out of 45 (87%) cancer specimens and in 17 out of 22 (77%) normal tissues. Similar to our data, the prolactin receptor was detected on the membrane and in the cytoplasm of epithelial cells in normal and cancer tissue. Data regarding the percentage of tumors stained, however, were not provided and follow-up information was not given.

Remarkably, in all three studies the patient cohorts consisted only of male patients and/or postmenopausal women. Thus our study is the first to investigate an unselected cohort of patients, including a random sample of female and male patients of all ages. It is known that prolactin secretion is greatly affected by the menstrual cycle.¹¹ It is, however, largely unknown whether prolactin receptor expression, particularly in the gastrointestinal mucosa, varies accordingly in female patients. Of note, we did not find an association between prolactin receptor expression and gender. Moreover, there was no difference in immunoreactivity among pre- and postmenopausal women (data not shown).

Data in the literature regarding the ectopic expression of prolactin in colorectal cancer are controversial, with prevalence rates ranging from 0 to 77% .^{20,27-32} One study described positive immunoreactivity in 51% (50/98) of the specimen, whereas others have found fewer immunoreactivity for prolactin ranging from 20% (3/15) and 3% (1/32) to none reactivity found in three studies including altogether 119 patients.²⁷⁻³² The expression of the prolactin mRNA has been investigated by two groups and was 88% (44/50) and 27% (13/48), respectively.^{20,30} Western blot method showed a detectable level of prolactin protein in 77% (10/13) and 0% (0/4) of colorectal cancer tissue sample.^{20,29}

Of note, compared with normal colorectal tissue, cancer tissues demonstrate a significantly higher rate of co-expression of prolactin and its receptor. Thus, in addition to systemic effects, prolactin presumably acts through an auto-/paracrine loop in promoting cell proliferation, as demonstrated in colorectal cancer cell lines (CaCO-2, HT-29 and LoVo cells).^{20,25}

Furthermore, the question whether circulating serum prolactin is reliable as a diagnostic and/or prognostic marker in colorectal cancer patients is still ongoing. Significant elevated serum levels in colorectal cancer patients with hyperprolactinemia (>20ng/ml serum) predicting a deteriorated prognosis have been reported and were disputed by other authors.²⁷⁻³³ Most recently a study of 82 colorectal cancer patients showed that within normal range a high prolactin (>11.6ng/ml serum) predicts a lower overall survival in colon adenocarcinoma and a higher overall survival in rectal adenocarcinoma.³⁴

Interestingly, there was no significant association of hyperprolactinemia (>20ng/ml serum) and prolactin expression in colorectal cancer tissue in any of these studies, suggesting that prolactin expression in colorectal cancer not affects the systemic level and in cases of hyperprolactinemia the tumor is unlikely to be the source.

As pointed out above, prolactin and its receptor are promising anti-cancer targets, wherein prolactin receptor antagonism appears to be the most promising interventional approach.^{15-17,35} First results demonstrating a beneficial effect of prolactin/prolactin receptor -targeted therapy in breast cancer have been reported from both *in vitro* and *in vivo* models using either pure prolactin receptor antagonists, such as Δ 1-9-G129R-hPRL or Δ 1-14-G129R-hPRL, or fusion peptides between a prolactin receptor antagonist and potential anti-tumor peptides, such as G129R-hPRL-IL2, G129R-hPRL-endostatin or G129R-hPRL-PE40-KDEL.^{12,36-38} However,

these drugs are in early stages of development and application, and difficulties regarding stability and binding affinity of prolactin receptor antagonist must be overcome before entering the clinic.³⁵ Nevertheless, a single clinical therapy trial using the anti-prolactinemic drug cabergoline combined with docetaxel in woman with metastatic breast cancer and concomitant hyperprolactinemia demonstrated that tumor regression rate was significantly higher in patients treated with cabergoline than in those who received docetaxel alone.³⁹ To the best of our knowledge, other cancer trials targeting the prolactin/prolactin receptor axis, in breast cancer or even other types of cancer, have not been published.

According to Carver et al.³⁵ the relatively restricted distribution of prolactin receptor as well as the limited disturbance in phenotype of the prolactin receptor $-/-$ transgenic mice apart from morphological changes occurring within the mammary gland suggest that therapeutics directed toward prolactin/prolactin receptor should be well tolerated, increasing its appeal. According to our data, the widespread expression of prolactin receptor in primary tissues as well as in regional and distant metastatic sites of colorectal cancer makes this type of cancer an ideal candidate for prolactin/prolactin receptor -targeted therapy. Moreover, the concordant expression profiles of primary tumors and corresponding metastases, facilitates the promise of individualized treatment fro metastatic disease, since tailored medicine can be accomplished without taking additional biopsies from metastatic deposits.

In conclusion, prolactin receptor is widely expressed in colorectal cancer. High concordance rates between primary tumor and metastatic tissues make this protein a promising target in patients with advanced disease. Moreover, prolactin receptor expression may be an additional prognostic variable, particularly in high grade and mucinous cancers.

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