

Up-Regulation of the Peripheral Benzodiazepine Receptor during Human Colorectal Carcinogenesis and Tumor Spread

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ABSTRACT

The peripheral benzodiazepine receptor (PBR) is overexpressed in a variety of cancers. In Union Internationale Contra Cancrum (UICC) III colorectal cancers, a high level of PBR overexpression correlates with poor prognosis. However, little is known about the role of PBR in the development and progression of colorectal cancer. This study addresses the up-regulation of PBR during colorectal carcinogenesis and tumor spread. One hundred sixteen consecutive patients undergoing surgery for colorectal cancer with either regional (59 patients) or distant metastases (57 patients) were followed-up for 5 years or until death. Twenty-four of the 59 patients with initial UICC stage III cancers later developed distant metastases. PBR overexpression in tumor specimens was determined by immunohistochemistry. UICC stage III patients with colorectal primaries highly overexpressing PBR developed metastases significantly more often than patients with low PBR overexpression in their primary carcinoma. In 54 of the 116 patients adenomas and/or metastases and/or recurrences were available to be studied for PBR up-regulation during colorectal carcinogenesis and tumor spread. PBR was found to be overexpressed in 86% of early and late adenomas. Furthermore, 85% of primaries and of 86% of metastases displayed PBR overexpression. PBR overexpression was also detected at the mRNA level as revealed by real-time PCR. The extent of PBR protein overexpression was equivalent in colorectal adenomas and carcinomas but slightly increased in metastases. These data suggest a functional role of PBR during colorectal carcinogenesis and tumor spread. Thus, PBR qualifies as a target for innovative diagnostic and therapeutic approaches.

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INTRODUCTION

The peripheral benzodiazepine receptor (PBR) is a ubiquitously expressed 18 kDa protein which has been implicated in the regulation of various cellular processes including steroidogenesis, immune response, apoptosis, and proliferation (reviewed in refs. 1–3). Its expression levels range from very high in steroid-producing tissues to relatively low in breast or gut mucosa. However, PBR levels were shown to be increased in a variety of cancers indicating an important role of PBR in cancer development. The PBR was shown to be overexpressed in 80% to 90% of union internationale contre le cancer (UICC) stage III and IV colorectal cancers and advanced breast cancer (4–6). A high overexpression inversely correlated with overall survival of UICC stage III colorectal cancer patients (4) and with disease-free survival of patients suffering from lymph node-negative breast cancer (5). In human astrocytoma, PBR expression was associated with tumor grade (7). Moreover, *in vivo* and *in vitro* experiments showed a correlation of PBR levels with the ability of breast cancer cells to grow in severe combined immunodeficient mice (8) and with the protection against UV-induced apoptosis in lymphoma cells (9), indicating a functional role of PBR in the proliferation and apoptosis of cancer cells.

The development of colorectal carcinoma (CRC) is a multistep process evolving from normal colonic mucosa to adenoma, invasive cancer, and metastasis. Each step is characterized by well-defined alterations of oncogenes and tumor suppressor genes (10). Yet, only little is known about the occurrence and course of aberrant PBR expression in the distinct stages of colorectal carcinogenesis. Recently, it was suggested that colorectal adenomas displayed variable PBR levels with a trend to higher PBR expression levels in adenomas than in normal colonic mucosa (6). In the present study we focused on PBR overexpression during colorectal carcinogenesis and tumor spread. We studied PBR overexpression in the respective normal colorectal epithelia, adenomas, primary cancers, and metastases of the same patients suffering from UICC stage III and IV colorectal cancer.

PATIENTS AND METHODS

Patients and Tissue Samples. Normal gut mucosa, colorectal adenomas and cancers, as well as metastases were collected from 116 consecutive cancer patients who underwent surgery for UICC stage III and IV colorectal cancer at the Charité-Campus Benjamin Franklin (formerly University Hospital Benjamin Franklin) between 1989 and 1991. The human tumor material was used according to the standards set by the Ethics Committee of the Charité-Campus Benjamin Franklin. A complete follow-up of all patients was documented for at least 5 years or until death. There were 59 patients with UICC stage III and 57 with UICC stage IV cancers. In addition to the 57 patients with UICC IV cancers at time of diagnosis, 24 patients with UICC stage III disease at the time of initial

surgery developed distant metastases later on. The mean age of all 116 patients was 64.7 years (range 24-87 years). Clinicopathologic variables have already been described elsewhere (11). In 54 of the 116 patients, additional tissues from colorectal adenomas, recurrences, or metastases were available to be studied for PBR expression during carcinogenesis and tumor spread (Table 1): There were 28 patients with colorectal adenomas only, 12 patients with metastases only, 2 patients with local recurrences only, 9 patients with both adenomas and metastases, 2 patients with both metastases and local recurrences, and 1 patient with adenoma, metastases, and local recurrence.

Immunohistochemical Staining. Microsections (1-2 μm) of paraffin-embedded primary tumors were deparaffinized and rehydrated in a decreasing alcohol series (11, 12). Immunohistochemistry was done using a robotic system (Chem-mate, DAKO, Hamburg, Germany). Sections were incubated with the anti-PBR antibody 8D7 (0.5 $\mu\text{g}/\text{mL}$) for 30 minutes at room temperature. The antibody was kindly provided by P. Carayon (13). After washing, samples were incubated with anti-mouse immunoglobulin G (1:20 dilution) for 30 minutes at room temperature, and staining was detected by the "fast-red system" (DAKO). Samples were slightly counterstained in Mayer's hematoxylin.

Semiquantitative Evaluation of Peripheral Benzodiazepine Receptor Staining. Tissue staining was independently scored by three of the authors (Y.O., P.G., and K.M.) with an interobserver variation of less than 10%. The staining intensity

of tumor tissue was compared with that of the corresponding normal mucosa for each patient (0, no increase; 1, weak increase; 2, moderate increase; 2.5, moderate to strong increase; 3, strong increase). A score of 0 to 12 was calculated as the product of the increase in staining intensity and the frequency of stained cancer cells (0, 0%; 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, 76% to 100%). The overexpression was rated as low (score ≤ 6) or high (score > 6).

RNA Purification and cDNA Construction. Total RNA was isolated from biopsies using the RNeasy Kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. Briefly, the biopsies were lysed in 350 μL of lysis buffer for 3×10 seconds using an ultrasonic disintegrator (Sonoplus HD 70, Bandelin Electronic, Berlin, Germany) and homogenized using the QIAshredder Kit (Qiagen). After centrifugation, total RNA was purified from the supernatant by affinity chromatography using the RNeasy columns. Aliquots of 1.5 μg of total RNA were further purified by DNase I (Invitrogen, Paisley, United Kingdom) digestion and reverse transcribed into cDNA using oligo-dT primers and the SuperScript Preamplification-Kit (Invitrogen).

LightCycler PCR. Each 10 μL reaction volume contained $1 \times$ FastStart DNA Master Hybridization Probes Mix (Roche Diagnostics, Mannheim, Germany), 3 mmol/L of MgCl_2 , 0.5 $\mu\text{mol}/\text{L}$ of each primer, 0.2 $\mu\text{mol}/\text{L}$ of each hybridization probe (fluorescein and LC-Red 640), and 1 μL of cDNA. The amplified fragments measured 279 bp (*PBR*) and 235 bp (*β -actin*; Table 2). Real-time PCR was done in a LightCycler (Roche Diagnostics) under the following conditions: initial heating to 95°C for 10 minutes, 40 cycles at 95°C for 0 seconds, 62°C for 12 seconds, and 72°C for 12 seconds. The *PBR* and *β -actin* expressions were quantified using external standards (14). The ratio of *PBR* to *β -actin* copy numbers was calculated to compare PBR expression between specimens.

Statistical Analysis. For intervariable assessment, the Mann-Whitney *U* test for continuous variables and the χ^2 test for dichotomized variables were applied. Overall survivals were assessed by the Kaplan-Meier method, and the significance of differences was calculated by the log-rank test. The Wilcoxon rank sum test was applied to compare PBR overexpression in primary cancer with that in metastasis of the same patient. Differences of $P < 0.05$ were considered to be significant. All statistical analyses were done using SPSS software.

RESULTS

Peripheral Benzodiazepine Receptor Overexpression and Metastasis. PBR is known to be overexpressed in up to 90% of CRC and a high PBR overexpression significantly correlates with poor prognosis (4). We now investigated the risk of metastasis formation in relation to PBR overexpression in primaries of UICC stage III colorectal cancers. Patients who initially presented with UICC stage III cancers with high PBR overexpression developed distant metastases more frequently than patients with low PBR-overexpressing cancers (Fig. 1). As would be expected, UICC stage III patients who developed distant metastases later on had a reduced survival (log-rank

Table 1 Clinicopathologic variables of patients and removed tissues

	Patients (n)	Tissues (n)
Total	54	
Patients with adenomas	38	
Material		
1 adenoma	26	
2 adenomas	6	
3 adenomas	3	
4 adenomas	3	
Patients with metastases	24	
Material		
1 metastasis	15	
2 metastases	7	
3 metastases	1	
5 metastases	1	
Patients with recurrences	5	
Adenoma tissues		59
Histology		
Tubular		28
Villous		5
Tubulovillous		26
Size		
<1 cm		27
1-2 cm		17
>2 cm		9
Unknown		6
Dysplasia		
Low-grade		46
High-grade		13
Metastatic tissues		37
Localization		
Liver		15
Abdominal wall		8
Peritoneum		4
Distant lymph nodes		4
Ovary		2
Lung		1
Bladder		1
Colon		1
Small intestine		1

Table 2 Sequences of primers and hybridization probes used

Gene		Primer (5' → 3')	Location	GeneBank
PBR	Sense	CTTTggTgCCCgACAAATg	358-376	M36035
	Antisense	GgCCACCACATCACAAgC	636-619	
	Fluorescein	AgAgTgAgTgCCCggCCCACCA-FL	565-586	
	LC 640	LC-gACTgCAgCTgCACCAgCAggT-p	589-610	
β -Actin	Sense	GcgAgAAgATgACCCAgATCA	388-408	X00351
	Antisense	gTgAggATCTTCATgAggTAgTCAGTC	622-596	
	Fluorescein	ggTATgCCCTCCCCATgCC-FL	544-563	
	LC 640	LC-TCCTgCgTCTgACCTggCTg-p	565-585	

test, $P = 0.0002$). These data indicate that PBR expression in the primary might help to identify patients who develop distant metastases of colorectal cancer.

Distribution of Peripheral Benzodiazepine Receptor Overexpression during Carcinogenesis. Next we compared PBR expression in colorectal adenoma, CRC, and metastasis to further elucidate the role of PBR during carcinogenesis and tumor spread. PBR expression was detected in all tissues (Fig. 2). An equally high percentage of colorectal adenomas (86%), CRC (85%), and metastatic tissues (86%) showed PBR overexpression in comparison with the PBR expression in normal gut mucosa (Fig. 3A). A possible overexpression of PBR at mRNA level was studied using quantitative real-time PCR. PBR mRNA levels were increased in both adenomas and CRCs when compared with normal colorectal mucosa (Fig. 3B), reflecting the overexpression of PBR protein.

The subcellular distribution of PBR did not alter during carcinogenesis. As previously described for CRCs (4), PBR was detected in the cytoplasm of all neoplastic cells in a granular fashion (Fig. 2), indicating a mitochondrial localization.

Peripheral Benzodiazepine Receptor Overexpression in Adenomas. The extent of PBR overexpression was as high in adenomas as in CRC (Fig. 4A). The Mann-Whitney U test revealed no significant PBR staining difference between adenomas and CRCs. There was a high variability of PBR overexpression within adenomas. Moreover, PBR overexpression differed among multiple adenomas of an individual

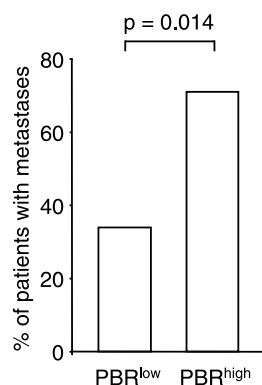


Fig. 1 Development of distant metastases in UICC stage III cancer patients. Patients with initial UICC stage III cancers at the time of diagnosis were followed up in respect to development of distant metastases. Patients with high PBR overexpression in the primary cancer developed distant metastases significantly more frequently than patients with low PBR overexpression did (χ^2 test).

patient (Fig. 4B). During carcinogenesis, adenomas grow and develop dysplasia with increasing grades leading to malignant transformation. Moreover, villous adenomas become malignant more often than tubular adenomas of similar size do. We investigated PBR overexpression in relation to histology, grade of dysplasia, and the size of adenomas to evaluate a possible correlation of PBR overexpression and adenoma characteristics. The frequency of PBR overexpression in small adenomas (<1 cm; 85%) was comparable to the one in large adenomas (≥ 1 cm; 89%). Similarly, the frequency of PBR overexpression in adenomas with low-grade dysplasia (87%) resembled the one in adenomas with high-grade dysplasia (83%). This indicates that PBR is already overexpressed during early stages (early adenomas) of colorectal carcinogenesis. Interestingly, 79% of the tubular adenomas displayed PBR overexpression, whereas adenomas with villous components showed more commonly PBR overexpression (94%), suggesting an association of the frequency of PBR overexpression with a more aggressive histology of adenomas. The extent of PBR overexpression did not significantly vary between adenomas of different grade of dysplasias, size, or histology.

Peripheral Benzodiazepine Receptor Overexpression in Metastases. All tissues investigated in this study (adenomas, CRCs, metastases, recurrences) originated from the 54 CRC patients described above. The metastases developed from the investigated primary CRC. Therefore, it is reasonable to directly compare the PBR expression in neoplastic tissues of the same patient by using the nonparametric paired Wilcoxon test. PBR expression significantly increased from primary cancer to metastasis (Fig. 4C): 21 of 37 (57%) metastases displayed a higher PBR overexpression in the metastases than in the respective primaries, in 11 cases (30%) the PBR expression was equal, and only 5 metastases (14%) had a lower PBR score than the primary cancer. PBR overexpression did not differ between tumor recurrences and the respective primary cancers. Different metastases of an individual patient displayed similar levels of PBR overexpression (data not shown).

DISCUSSION

PBR has been shown to be overexpressed in a variety of tumor entities including colorectal cancers. In patients with colorectal cancer a high PBR overexpression was associated with poor prognosis. This study aimed to clarify whether PBR overexpression represented an early or late event during carcinogenesis and to characterize PBR expression during tumor spread.

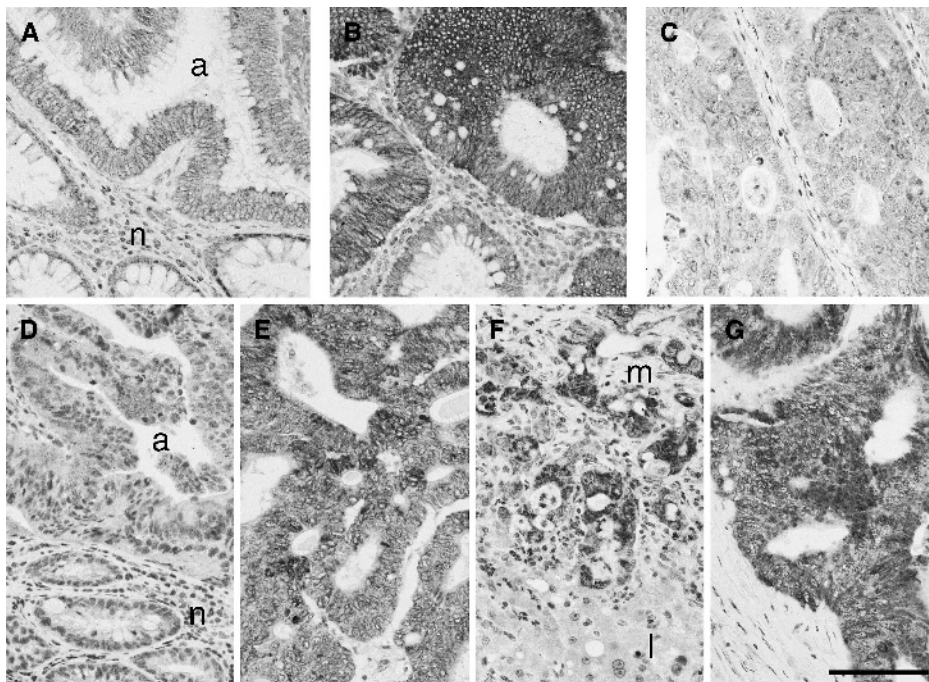


Fig. 2 Immunohistochemical detection of PBR. Immunohistochemical detection of PBR (red) in tissues of a single patient (A–C) and a second single patient (D–G). PBR staining in normal mucosa (A, n), adenoma with low-grade dysplasia (A, a), another adenoma with high-grade dysplasia (B), and primary cancer tissue (C) of a single patient are shown. PBR staining in the normal epithelia mucosa (D, n), adenoma (D, a), primary cancer (E), liver metastasis (F, m: metastatic compound, l: surrounding normal liver parenchyma), and lymph node metastasis of another patient is shown. The adenoma of the latter patient (D) is considered as the direct precursor lesion of the investigated primary cancer (E) due to its temporal and spatial proximity. Bar, 100 μ m.

Colorectal carcinogenesis is a multistep process developing from normal mucosa, aberrant crypt foci, early and late adenomas to carcinomas and metastasis (10). In this study, we show that PBR is generally overexpressed in both early and late adenomas, indicating that PBR overexpression is an early event in colorectal carcinogenesis. PBR overexpression persisted during later stages of carcinogenesis and even during tumor spread. A similarly high proportion of primary cancers and metastases displayed PBR overexpression. Moreover, we found a significant correlation between the extent of PBR overexpression in the primary CRC and the risk of metachronous metastases. The early up-regulation of PBR expression, its high incidence in all neoplastic tissues, and its association with metachronous metastases strengthen the concept of PBR playing an important role in colorectal carcinogenesis and tumor spread.

Early carcinogenesis is characterized by development of adenomas with increasing grades of dysplasia. However, the extent and frequency of PBR overexpression was comparably high in early and late adenomas as well as in CRC, indicating that PBR overexpression already occurs at the stage of early adenoma. The first morphologic alteration during carcinogenesis is the development of aberrant crypt foci. Whether PBR overexpression occurs as early as in aberrant crypt foci has yet to be elucidated.

We observed a high variability in the extent of PBR overexpression in adenomas of different patients confirming a previous study (6). Furthermore, we showed that even multiple adenomas of a single patient differed in the grade of PBR overexpression. In this study we exclusively investigated colorectal adenomas of patients with colorectal cancer. Because of removal of all detected adenomas as a good clinical practice, it is not possible to prospectively investigate the processes

involved in adenoma-carcinoma transition in the same patient. To explicitly answer the question of how and to which extent high PBR overexpression in adenomas contributes to malignant transformation, further studies are required including the use of animal models of colorectal carcinogenesis.

In this study, a significant correlation between PBR overexpression in CRC and metastases was observed. Most metastases (87%) displayed the same or even higher PBR overexpression than the respective primary cancer, indicating that PBR might play a role in metastatic disease as well. The maintenance or even increase of PBR overexpression in metastases may qualify PBR as a target for the diagnosis of micrometastases. PBR has already been used for diagnostic purposes. Radiolabeled PBR-specific ligands have been successfully employed to monitor neurodegenerative disorders or glioblastoma (15–17). Interestingly, the main target organs of colorectal metastases, such as liver, adipose and connective tissues of the abdomen, as well as normal gut mucosa, were shown to express PBR at a low level only (Fig. 2F; refs. 4, 6, 18–20). These marked difference of PBR expression in metastases versus the surrounding tissues might allow a PBR-based detection of metastases.

The abundant PBR overexpression in neoplastic colorectal tissues goes in line with the growth regulating functions attributed to PBR. The up-regulation of PBR during colorectal carcinogenesis has to be discussed in the light of the antiapoptotic and proliferative properties of PBR. It was shown that PBR protected cells from apoptosis (9) and promoted cellular proliferation (21). These effects could be reversed by PBR-specific ligands (22, 23). However, the main function ascribed to PBR is its involvement in cholesterol transport (24). Besides its function in steroid and bile acid formation, PBR has been proposed to be involved in cholesterol

compartmentalization and membrane biogenesis, events engaged in cell proliferation and death (25). The model for colorectal carcinogenesis of Fearon and Vogelstein (10) has recently been complemented. Whereas early carcinogenesis is determined by proliferation-regulating factors, the availability of substrate becomes more important for further carcinogenesis (26). Owing to PBR overexpression persisting during early and late carcinogenesis, elevated PBR levels might contribute to both cancer development and progression by the dysregulation of proliferation as well as by the increase of substrate (cholesterol) availability.

The mechanisms by which the increase in PBR expression was induced may involve two processes: gene amplification and transcriptional regulation. *PBR* gene was found to be amplified in a highly PBR-expressing, aggressive breast cancer cell line in contrast to a nonaggressive cell line that contains low levels of PBR (27). Moreover, differences in the expression of transcription factors and the usage of promoters have recently been shown for steroidogenic and non-steroidogenic cell lines expressing different levels of PBR (28). Whether these mechanisms also apply to PBR overexpression in colorectal cancers is not yet known.

To summarize, PBR overexpression already occurs in early carcinogenesis, persists during invasive cancer development, and slightly increases in metastasis. Thus, PBR may play a functional role in colorectal carcinogenesis and tumor spread. The understanding of the role of PBR in colorectal carcinogenesis and tumor spread will form the basis for future PBR-based diagnostic and/or therapeutic strategies.

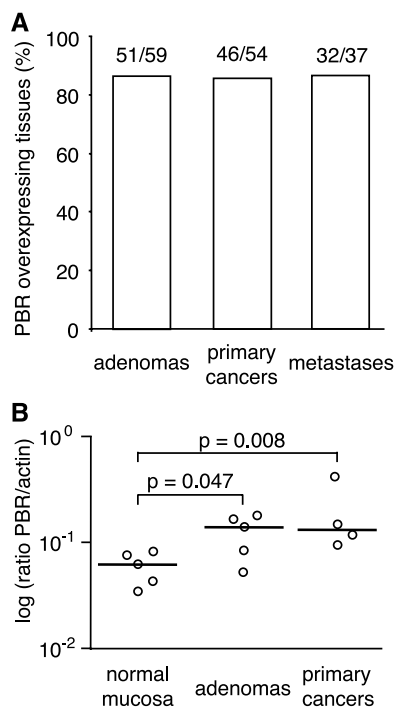


Fig. 3 Frequency of PBR overexpression in neoplastic tissues. *A*, colorectal adenomas, carcinomas, and metastases displayed a similar proportion of PBR-overexpressing tissues. *B*, PBR mRNA was increased in adenomas and carcinomas in comparison with normal epithelia cells (Mann-Whitney *U* test).

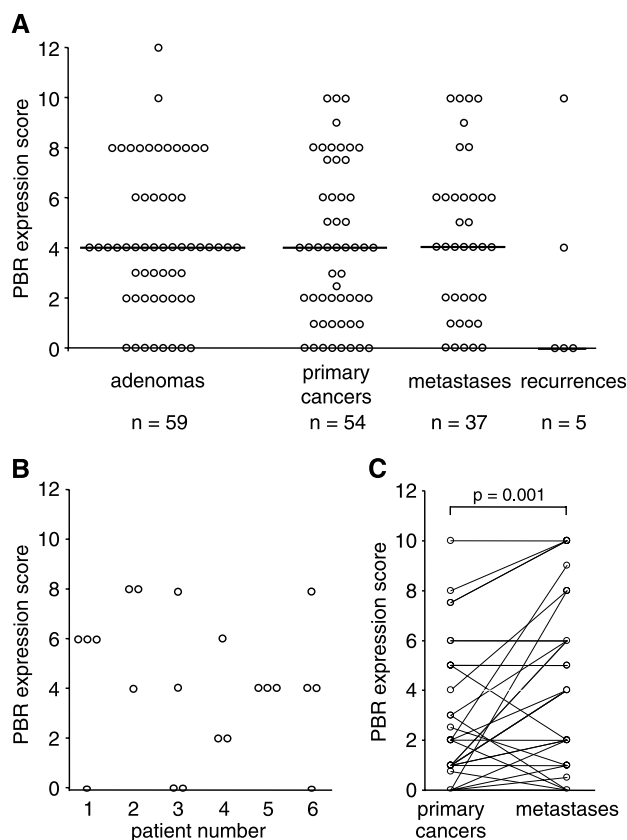


Fig. 4 The extent of PBR overexpression. *A*, PBR overexpression score ranged from 0 to 12 in adenomas and 0 to 10 in other neoplastic tissues. The median staining score was the same for adenomas, carcinomas, and metastases (median = 4). *B*, PBR overexpression staining scores in adenomas of six patients with three or more examined adenomas. *C*, direct comparison of PBR expression in CRC and metastases of individual patients revealed a significant increase of PBR expression during tumor spread (Wilcoxon test).

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