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Down-regulation of BCRP/ABCG2 in colorectal and cervical cancer

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Abstract

Expression of Breast Cancer Resistance Protein (BCRP/ABCG2) in tumor cells is associated with resistance to multiple chemotherapeutic agents. BCRP also protects against phototoxicity by mediating the efflux of protoporphyrins from cells. However, chemotherapy and photodynamic therapy are effective treatment options for cancer. Furthermore, protoporphyrins are essential, in the form of heme, for the synthesis of nitric oxide, over-production of which is associated with cancer. This raises the question as to whether the expression of this transporter is altered in cancer. To address this question, we investigated the expression of BCRP in colorectal cancer and cervical cancer. Paired normal and cancer tissues from colectomy specimens were used for the analysis of BCRP mRNA by RT-PCR and Northern blot. BCRP was analyzed by immunohistochemistry/immunofluorescence. Similar studies were also done with specimens of normal cervix and cancer cervix. A commercial dot blot was probed to quantify the expression of BCRP in paired normal and cancer cDNA samples from 154 patients with tumors in 19 different tissues. BCRP mRNA was present in normal colorectal tissue and showed a 6-fold decrease in cancer. BCRP was abundant in the normal colon and showed a decrease in colon cancer. The down-regulation of BCRP mRNA and protein was also evident in cervical cancer. There was also a decrease in BCRP mRNA in cancer in 12 of the 19 different tissues collected from 154 patients. These data show that cancer-associated down-regulation of BCRP is likely to be a common phenomenon in several tissues. Decreased expression of BCRP may have a role in tumorigenesis by allowing accumulation of genotoxins and over-production of nitric oxide.

Keywords: Colorectal cancer; Cervical cancer; ABCG2 protein; Breast Cancer Resistance Protein; Drug resistance and cancer; ATP-binding cassette transporters; Protoporphyrins; iNOS

Breast Cancer Resistance Protein (BCRP, ABCG2, MXR, and ABCP) represents the newest member to be identified in the ATP-binding cassette (ABC) transporter superfamily [1,2]. Its over-expression in vitro in drug-selected cell lines is associated with resistance to commonly used chemotherapeutic agents [3,4]. Though the role of this transporter in multidrug resistance has been the subject of numerous studies, the pattern of differential expression of BCRP in normal and cancer tissue in vivo and a possible relevance of BCRP to the pathophysiology of tumorigenesis and tumor progression has not been elucidated.

BCRP is expressed abundantly in the apical membrane of normal intestinal and colonic epithelium in vivo [5]. The expression of BCRP in the normal intestinal tract lends credence to its proposed physiologic role in protecting normal cells from malignant change by effluxing a variety of diet-derived carcinogens [6]. Ironically however, the camptothecin irinotecan, which is a BCRP substrate, is an effective agent used in several clinical protocols (IFL, FOLFIRI) for the treatment of advanced colorectal cancer [7–9], suggesting that colon cancer may not express the

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camptothecin-resistance conferring BCRP to the same extent as the normal colon does.

Studies with a BCRP knockout mouse have provided evidence for the ability of BCRP to efflux protoporphyrins and protect cells from phototoxicity [10]. One particular mode of therapy for some forms of cancer is based on the selective accumulation of photosensitive protoporphyrins in cancer cells by a hitherto unexplained mechanism. The selective accumulation of these phototoxins in cancer cells renders them susceptible to apoptosis on exposure to red light. This therapy is called photodynamic therapy (PDT) and colorectal cancer is one of the gastrointestinal malignancies that is uniquely susceptible to this treatment [11]. The apparent contradiction of the efficacy of protoporphyrin-based PDT in colorectal cancer when normal colon abundantly expresses the protoporphyrin-effluxing BCRP led to the hypothesis that this transporter may be down-regulated with malignant change in the colon.

The possible down-regulation of BCRP in colorectal cancer was of particular interest since protoporphyrins are precursors of heme, an essential cofactor for nitric oxide synthases [12]. These enzymes produce nitric oxide from arginine, and over-expression of iNOS, one of the isoforms of NOS, is associated with cancer [13]. We have recently shown that the amino acid transporter $ATB^{0,+}$, which transports arginine in a Na^+- and Cl^--coupled manner, is up-regulated in colorectal cancer, thus providing a mechanism for the delivery of increased amounts of substrate to iNOS for nitric oxide production [14]. A similar co-ordinated up-regulation of $ATB^{0,+}$ and iNOS is also seen in cervical cancer [15]. Down-regulation of BCRP and the resultant accumulation of protoporphyrins may lead to increased generation of heme, a cofactor for iNOS. Thus, the increased availability of arginine and heme in cancer tissues may support the catalytic activity of the over-expressed iNOS and sustain nitric oxide production during malignancy.

In this study, we show that malignant transformation of the colonic epithelium in vivo is accompanied by a significant down-regulation of BCRP mRNA and protein expression. We demonstrate a similar phenomenon in cervical cancer. We also used a commercial array of paired normal and cancer cDNA to demonstrate that down-regulation of BCRP with malignant change occurs in 12 different tissues, suggesting that decreased expression of BCRP may be a widespread phenomenon in human cancers.

Methods

Patients. This study received the Medical College of Georgia Institutional Review Board's approval. Thirteen adult patients with colorectal adenocarcinoma and one patient with a hepatic metastasis from a previously resected colon cancer were included in this study after obtaining their informed consent. Details of some of these patients have been published earlier [14]. A similar procedure was used to collect specimens from patients with cervical cancer [15].

Tissue collection and processing. A pathologist harvested normal and tumor tissue from freshly resected surgical specimens [14,15]. Portions of the tissues (0.3–0.5 g from each site) from each patient were processed for total RNA extraction using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The integrity of the isolated RNA was confirmed by size fractionation on a denaturing agarose gel. Tissues from each site were fixed by immersion in neutral-buffered formalin for immunohistochemical studies and also snap-frozen in liquid nitrogen for immunofluorescent studies. A commercial array of standardized cDNA blots isolated from paired human normal and cancer tissues from 19 different sites in 154 patients (Cancer Profiling Array II, BD Biosciences Clontech, Palo Alto, CA, USA) was obtained for analysis of BCRP mRNA by Southern blot.

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR was performed as previously described [14,15]. The PCR primers for human BCRP were designed based on its published sequence (Gene Accession No.: AF098951) using OLIGO Primer Analysis Software (Molecular Biology Insights Inc, Cascade, Colorado, USA). The primers were: sense 5′-CACAGGTGGAGGCAAATCTT-3′ and antisense 5′-CAGCTCTGT TCTGGATTCCA-3′. The predicted size of the PCR product with these primers is 1039 bp. The PCR cycle number was determined such that the PCR amplification occurred within the linear range. As an internal control, 18S rRNA was amplified with a primer-competimer combination from Quantum<u>mRNA</u>™ Universal 18S rRNA Internal Standards Kit (Ambion Inc, Austin, TX, USA). The levels of BCRP mRNA were normalized to the corresponding 18S rRNA, and the relative expression in cancer compared to that in normal tissue from the same patient was determined in terms of BCRP mRNA/18S rRNA. The RT-PCR product was sequenced to confirm its molecular identity and then used as a probe for Northern blot analysis and hybridization with the commercial cancer array.

Northern blot analysis. Total RNA was used to prepare poly(A)$^+$ RNA using oligo(dT)-microbeads (Miltenyi Biotec Inc, Auburn, CA, USA). Northern blot analysis was carried out as described previously [14] using high stringency conditions. The BCRP-specific cDNA probe was labeled with ^{32}P by random priming using the Ready-to-go Oligo labeling beads (Amersham Biosciences) and $\alpha[^{32}P]$-dCTP (3000 Ci/mmol, Amersham Biosciences). After quantifying the hybridization signal by densitometry, the membrane was stripped and re-probed with a $[^{32}P]$-labeled cDNA probe specific for β-actin. The hybridization signals were expressed as a ratio of the target mRNA signal to the β-actin mRNA signal.

Immunohistochemistry. Tissue samples obtained for immunohistochemistry were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (5 μm) cut from the paraffin block were deparaffinized in xylene and rehydrated through graded alcohols. Normal and cancer tissue from the same patient was mounted on the same slide to ensure identical conditions. Immunohistochemistry was performed using the Labeled Streptavidin-Biotin 2 (LSAB2) detection system (DAKO Corp., Carpenteria, CA, USA). 3,3-Diaminobenzidine tetrahydrochloride was used as the chromogen. A monoclonal anti-BCRP antibody (Kamiya Biomedical) was used as the primary antibody at a dilution of 1:20. The secondary antibody was goat anti-mouse IgG. Negative controls unexposed to primary antibodies were processed in the same manner.

Immunofluorescence studies. Tissue samples were fixed in 7% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h and then washed in PBS three times at 4 °C. They were kept in 30% sucrose overnight at 4 °C and mounted in Tissue-Tek® OCT (Sakura Finetek USA Inc, California, USA). Sections were cut at 10 μm thickness and treated in 0.2% Triton X-100 in PBS for 10 min. Blocking was done with 4% normal goat serum for 1 h at room temperature. A monoclonal anti-BCRP antibody (Kamiya Biomedical) was used as the primary antibody. Sections were then immersed in PBS-Tween and incubated with the secondary antibody, goat anti-mouse IgG coupled to Alexafluor 568 (Molecular Probes), at a dilution of 1:1000. Sections were washed again in PBS-Tween and coverslips mounted with Vectashield Antifade Mounting Medium + DAPI (Vector Labs) and viewed using a Zeiss Axioplan 2 fluorescent microscope equipped with a Spot Camera and Spot Software. Appropriate negative controls were obtained.

Cancer profiling array. The differential expression of BCRP mRNA in normal and cancer tissues from 19 different tissues was determined using a