Regional Expression of the BCRP/ABCG2 Transporter in Term Human Placentas

Naureen Memon#, Kristin M Bircsak#, Faith Archera, Christopher J Gibsob, Pamela Ohman-Stricklandc, Barry I Weinbergera, Mana M Parasid, Anna M Vetranoe, and Lauren M Aleksunesb,e

aDepartment of Pediatrics, Rutgers University Robert Wood Johnson Medical School, 1 Robert Wood Johnson Place, New Brunswick, NJ, 08901 USA
bDepartment of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, 170 Frelinghuysen Rd., Piscataway, NJ, 08854 USA
cDepartment of Biostatistics, Rutgers University School of Public Health, 683 Hoes Lane W, Piscataway, NJ, 08854 USA
dDepartment of Pathology, University of California, San Diego, 200 West Arbor Drive, San Diego, California 92035 USA
eEnvironmental and Occupational Health Sciences Institute, 170 Frelinghuysen Rd., Piscataway, NJ, 08854 USA

#These authors contributed equally to this work.

Abstract

The breast cancer resistance protein (BCRP, ABCG2) is an efflux transporter that removes xenobiotics that cross the placenta back to the maternal circulation, thereby limiting exposure of the fetus to drugs and chemicals. Currently, variability of BCRP expression within the placenta is not known. Ten placentas were collected from healthy women undergoing elective Cesarean sections at term. Villous samples were dissected in defined regions (medial, intermediate, and peripheral) and BCRP mRNA and protein were quantified. There were no regional differences in mRNA expression of housekeeping genes (GAPDH, RPL13a, PRL, 18S). GAPDH had the lowest correlation with BCRP Ct values and was used for BCRP mRNA normalization. No differences in placental BCRP mRNA and protein were observed among the sample sites (<20% variability). Sampling site does not affect the expression of BCRP, supporting the utility of single site sampling protocols to assess the interindividual regulation of this transporter in human placentas.

Keywords

BCRP; ABCG2; Transporter; Placenta; Housekeeping Gene
1. Introduction

The breast cancer resistance protein (BCRP) is an ATP-binding cassette (ABC) transporter that is expressed on the apical surface of placental syncytiotrophoblasts [1]. This transporter extrudes a wide range of xenobiotics that cross the placenta back into the maternal circulation, thereby protecting the fetus from drug and chemical accumulation and toxicity. The BCRP gene (ABCG2) is expressed in the human placenta approximately 100 times greater than that of any other organ [2, 3], suggesting that BCRP plays a critical fetoprotective role. Studies of placental cell lines and placental primary cultures [4, 5], mutant/knockout mice [6–9] and a more recent study of an ex vivo human placental perfusion model [10] have all documented the importance of the BCRP transporter in determining fetal exposure to a number of drugs and environmental chemicals, including antineoplastic agents (mitoxantrone, topotecan), antidiabetic medications (glyburide), antibiotics (nitrofurantoin), and phytoestrogens (genistein).

When studying the expression of specific transcripts and proteins in the placenta, it is important to consider sampling methodologies and to control for intraplacental variability. Studies have shown that placental structure and blood flow patterns across the human placental disk are not uniform (reviewed in [11]). Histological studies have also shown that placental tissue near the chorionic surface and in the placentals margins may have more syncytial knots and villous fibrin, which is typically characteristic of underperfused villi [12–14]. In fact, studies have suggested that the mRNA expression of hypoxia-related transcripts, such as vascular endothelial growth factor and connective tissue growth factor, is dependent upon sampling site within the placental disc, with up-regulation noted in the presumed hypoxic lateral-chorionic sites as compared to medio-basal sites [14]. It is unknown whether BCRP exhibits such regional differences in mRNA and protein expression in term human placentas. Therefore, the purpose of this study was to examine the mRNA and protein expression of BCRP in defined regions of healthy term human placentas. Knowledge of the intraindividual expression of BCRP in the placenta may provide insight into its regulation and is important for future studies assessing the interindividual expression of this transporter.

2. Materials and Methods

2.1 Patient Selection

Ten placentas were obtained after written informed consent from healthy women with uncomplicated pregnancies following term delivery (as defined by standard clinical criteria) by scheduled Cesarean section. Inclusion criteria included healthy women between the ages of 18–40 years, term gestation (≥ 36 weeks), and scheduled Cesarean section without labor. Table 1 includes patient demographic data (Table format modified from [15]). Exclusion criteria included chronic medical conditions (e.g. hypertension, diabetes, autoimmune disorders), pregnancy-induced medical conditions (e.g. pregnancy-induced hypertension, preeclampsia, gestational diabetes), maternal infection, clinical chorioamnionitis, medication use (with the exception of prenatal vitamins), maternal smoking, alcohol or drug abuse, multiple pregnancies, and known fetal chromosomal abnormalities. The study was approved by the Institutional Review Boards of Robert Wood Johnson Medical School (Protocol #0220100258) and Rutgers University (Protocol #E12-024).

2.2 Sample Collection

All placentas were obtained within ten minutes of delivery and processing was completed within one hour. Each placenta was rinsed thoroughly with phosphate-buffered saline (PBS) three times. Placentas were carefully inspected for any visible abnormalities and location of
the umbilical cord. Only placentas with central or eccentric cord insertion were used. The maternal decidua and the chorionic plate along with overlying membranes were removed. Using a scalpel, six tissue samples were collected along the long axis per placenta according to the scheme in Figure 1. ‘M1’ and ‘M2’ samples were categorized as “medial” or from the area closest to the umbilical cord insertion. These samples were obtained approximately 1 cm distal to the cord insertion site. ‘I1’ and ‘I2’ samples were categorized as “intermediate”, and were mid-distance from cord insertion and periphery. ‘P1’ and ‘P2’ samples were classified as “peripheral” and were obtained approximately 2 cm away from the lateral placental margin (to avoid fibrin-rich areas). Each sample was approximately 2 cm × 1 cm × 0.5 cm in dimension.

Individual samples were dissected into three sub-segments of equal size. The first subsegment was placed in a PAXgene Tissue Container (Qiagen, Germantown, MD) in the PAXgene Tissue Fix for 4 h, moved to PAXgene Tissue Stabilizer, and stored at −20°C for histologic and RNA analysis. The second and third sub-segments were frozen and stored at −80°C for protein analysis.

2.3 Histology

The first sub-segment of each placenta sample was embedded in paraffin and sectioned into 5 μm thick sections. Placenta sections were stained with hematoxylin and eosin, examined, and imaged by light microscopy (VS120-S5, Olympus, Center Valley, PA).

2.4 RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated using a Tissue miRNA Kit (Qiagen, Germantown, MD) according to the manufacturer's instruction. The concentration of total RNA in each sample was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and purity was confirmed with a Bioanalyzer Instrument (Agilent, Santa Clara, CA). Complementary DNA (cDNA) was generated using the Ovation Pico WTA System (NuGEN, San Carlos, CA) in combination with the Biomek FXP Laboratory Automation Workstation (Beckman Coulter, Inc., Brea, CA). Quantitative analysis of mRNA was performed with 500 ng cDNA, commercially-available primers for each gene, and Taqman probes (Applied Biosystems, Foster City, CA) using a Viia7 RT-PCR system (Applied Biosystems) in the Bionomics Research and Technology Center at Rutgers University. For the comparison of threshold cycle (Ct) values across the 3 placenta regions, the mean of two sample sites (run in quadruplicate) per placenta were used (for example, M1 and M2). Ct values for BCRP mRNA were first converted to delta Ct values by comparing to the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then to delta delta Ct values by designating the average delta Ct values for medial samples from each placenta as the control group. Supplemental Table 1 includes the gene name and assay ID information.

2.5 Western Blot Analysis

Placentas were homogenized in sucrose (250 mM)-Tris base (10 mM) buffer (pH 7.5) containing protease inhibitors (Sigma Aldrich, St. Louis, MO). Homogenates were centrifuged (100,000 × g) for 1 hr at 4°C and the pellets, or crude membrane fractions, were resuspended in sucrose-Tris base buffer with protease inhibitors. Protein concentrations were determined by a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed by loading 10 μg placenta membrane protein and 5 μg human embryonic kidney-293 cells overexpressing an empty vector or wild-type BCRP on polyacrylamide 4–12% Bis-Tris gels (Life Technologies, Carlsbad, CA) that were resolved by electrophoresis. Gels were transblotted onto polyvinylidene fluoride membranes in a 7-minute transfer apparatus (Life Technologies, Carlsbad, CA). Membranes were then blocked
in 5% non-fat dairy milk in PBS with 0.5% Tween-20 for 1 h. BCRP (BXP-53, Abcam, Cambridge, MA) and β-actin (ab8227, Abcam, Cambridge, MA) primary antibodies were diluted in 2% non-fat dairy milk in PBS with 0.5% Tween-20 and incubated with the membranes at dilutions of 1:5000 and 1:2000, respectively. Primary antibodies were probed using species-specific HRP-conjugated secondary antibodies (Sigma Aldrich, St. Louis, MO) and the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL). Detection and semiquantitation of protein bands was performed with a FluorChem imager (ProteinSimple, Santa Clara, CA).

2.6 Statistical Analysis

Intraclass correlation coefficients were calculated using SAS (SAS Institute, Cary, NC). Linear regression analysis and one-way analysis of variance followed by a Newman-Keuls multiple comparison post hoc test were performed using GraphPad Prism v5 (GraphPad Software, La Jolla, CA). Significance was set at p < 0.05.

3. Results

3.1 Histologic Evaluation of Human Placentas

The health and sampling quality of term human placentas were assessed by histologic examination (n=10). Eight of the placentas consisted of mostly normal tissue (Figure 2), while two placentas displayed minor areas of patchy villous edema or avascular villi (not shown). Small areas of maternal decidua were noted in three of the placentas (not shown). All placenta samples were included in subsequent data analysis.

3.2 Assessment of Placental Housekeeping Genes

Total RNA was isolated from term human placentas (n=10) and four housekeeping genes were evaluated to determine the appropriate placental housekeeping gene for the expression of BCRP mRNA levels. There were no differences in the regional (medial, intermediate, peripheral) Ct values of the four housekeeping genes: GAPDH, ribosomal protein L13a (RPL13a), prolactin (PRL), and 18S ribosomal RNA (18S) (Figure 3A). Between placentas, GAPDH, RPL13a, and 18S Ct values were not significantly different. However, interplacental PRL Ct values varied greatly. Using linear regression analysis, BCRP Ct values correlated least with GAPDH Ct values ($R^2=0.003$) (Figure 3B). In addition, intraclass correlation of BCRP and GAPDH was 0.06 compared to 0.46, −0.45, and 0.16 for 18S, PRL, and RPL13a, respectively. In line with the recommendation of Adibi et al. 2009 [16], the low intraclass correlation of BCRP with GAPDH was used as a criterion for selecting GAPDH as standard for normalizing BCRP mRNA levels. Furthermore, intraclass correlation analysis of BCRP mRNA normalized to each of the tested housekeeping genes were 0.70, 0.32, 0.65, and 0.35 yielding $p$ values of 0.61, 0.54, 0.33, and 0.056 for GAPDH, 18S, RPL13a, and PRL, respectively. These data further support the conclusion that expression of BCRP mRNA is not related to GAPDH mRNA and therefore, GAPDH is a suitable gene for normalization.

3.3 BCRP mRNA and Protein Expression in Term Human Placentas

BCRP mRNA and protein expression levels were compared between three regions (six sub-regions) of the placenta. BCRP mRNA expression was normalized to GAPDH and compared to the medial regions of each placenta. As displayed in Figure 4, there was up to a 20% difference in mean mRNA expression level between the medial, intermediate, and peripheral sub-regions of the placenta that was not statistically significant. Likewise, the protein expression of BCRP, normalized to β-actin loading control, was similar in the three different regions of the placenta, with a statistically insignificant difference (up to 20%
variability between sub-region means) (Figure 5). It should be noted that the raw blot is only shown for one placenta (Figure 5A), however all ten placentas were used for graphical representation (Figure 5B).

4. Discussion

In this study, we investigated the intraplacental variability in mRNA and protein expression of the BCRP efflux transporter in term human placentas. Regional variation in the expression of several genes and proteins within healthy, term human placentas has previously been reported in a number of studies [14, 17, 18]. Though many different factors contribute to this site-to-site variability, it is speculated that the physical location of cells and their relative exposure to oxygen is responsible for the variation seen in several hypoxia-related transcripts.

Hypoxia has been shown to alter BCRP expression in different experimental models, and depending on the tissue sample, hypoxic conditions can either up-regulate or down-regulate BCRP expression. For instance, BCRP mRNA expression is increased under hypoxic conditions in hematopoietic stem cells, and this increase is associated with up-regulation of hypoxia-inducible factor 1 [19]. In contrast, BCRP mRNA expression is decreased in placentas from growth-restricted infants exposed to hypoxia in utero, when compared to placentas of healthy infants [5]. In the present study, we found that expression of BCRP was not dependent upon location within the placental disc. Our findings suggest that differences in maternal blood flow and oxygen tension across the placenta do not affect BCRP mRNA and protein expression. However, it should be noted that local oxygen levels were not quantified in these 10 placentas, and our findings were not correlated with histopathological changes consistent with hypoperfusion.

The lack of intraplacental variability in levels of BCRP mRNA and protein suggests that a small number of samples from each placenta may be sufficient when comparing BCRP expression between placentas. However, our results must be generalized with caution. Firstly, three of the ten placentas studied were contaminated with small amounts of maternal decidua, thereby potentially confounding the above results as the samples may have contained maternal mRNA and protein. Although, it should be noted that BCRP mRNA expression in human uterus is lower than levels observed in the placenta (reviewed in [20]). Secondly, only healthy women who were not in labor and who had reached term gestation were included in this study. Based on previous studies showing that BCRP expression does not change with labor or mode of delivery (Cesarean or vaginal) [21, 22], we speculate that our findings of negligible sampling site variability can be generalized regardless of the absence or presence of labor and the type of delivery. However, our findings cannot be generalized to the study of placentas with underlying pathologic conditions, such as preeclampsia, diabetes, or fetal growth restriction. Studies have shown that there may be intraplacental variation in overall gene expression in diseased states, such as fetal growth restriction [23]. Therefore, sampling from a single site in the placenta may be sufficient to detect differences in BCRP expression between healthy placentas, whereas further studies are needed to assess site-to-site variability for BCRP expression in the setting of pathological conditions of pregnancy.

In addition to analyzing intraplacental BCRP expression, the expression of the four potential internal controls, GAPDH, RPL13a, PRL, and 18S, was also evaluated. Several studies have previously demonstrated that GAPDH and 18S are stably expressed in healthy placentas and that they can reliably be used as housekeeping genes [24, 25]. The present study confirms minimal intraplacental variability in expression of these two genes. RPL13a was recently proposed as a superior reference gene in bone-marrow and placental-derived human
mesenchymal stromal cells [26]. Although there is no literature on the use RPL13a as an endogenous control in placental tissue, our results demonstrate minimal site-to-site variability in its expression. On the basis of this observation, it can be suggested that RPL13a can also be used as a reference gene for placental studies. PRL expression across the placental disc was also analyzed. A previous study has shown that there is insignificant intraplacental variability in its expression [27]. Although our results confirmed the lack of intraplacental variability, an extensive amount of interplacental variability in the expression of PRL was noted, thereby limiting its utility as a reference gene. Therefore, it can be concluded that GAPDH, RPL13a, and 18S are all suitable as internal controls for normalization in placental tissues, with GAPDH being superior to both RPL13a and 18S in this sample set.

5. Conclusion

To our knowledge, this is the first study that has evaluated the intraplacental variability in BCRP mRNA and protein expression in healthy, term human placentas. Our results demonstrate that BCRP mRNA and protein levels are similar across the placental disc and sampling site does not contribute to variation in its expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>Ct</td>
<td>threshold cycle</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PRL</td>
<td>prolactin</td>
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<tr>
<td>RPL13a</td>
<td>ribosomal protein L13a</td>
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<tr>
<td>18S</td>
<td>18S ribosomal RNA</td>
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References


Highlights

- BCRP mRNA and protein are uniform across the term human placental disc.
- GAPDH mRNA was the most appropriate housekeeping gene for expression of BCRP mRNA.
- Single site sampling can be used to assess the interindividual regulation of BCRP.
Figure 1. Regional sampling of term human placentas
Placentas were collected and processed within one hour of birth. Six regionally-defined samples (medial: M₁ and M₂, intermediate: I₁ and I₂, peripheral: P₁ and P₂) measuring 2 cm × 1 cm × 0.5 cm in dimension were collected along the long axis of the placenta. Each sample was split into three sub-segments and processed for histologic, mRNA, and protein analysis.
Figure 2. Histology of the medial region of term human placentas
Placenta subsegments were fixed in PAXgene Tissue Fix for (4 h) and then placed in PAXgene Tissue Stabilizer and stored at −20°C prior to routine tissue processing and paraffin embedding. Sections (5 μM) of placentas were stained with hematoxylin and eosin and imaged by light microscopy for histopathologic examination. All placentas displayed mostly normal histologic features of the placenta. Three representative images of the M2 medial region from three placentas are shown above (A–C) (× 4 magnification). Insets demonstrate the villous structure at a higher magnification (× 40 magnification).
Figure 3. Messenger RNA expression of housekeeping genes in term human placentas
Regional Ct values of four housekeeping genes (GAPDH, RPL13A, Prolactin, 18S) and BCRP in human term placenta was determined by qPCR. (A) Data are presented as box and whisker plots with median, mean and range of the housekeeping gene Ct values for each sampling location (medial, intermediate, peripheral) (n=10). The average Ct value for two sites was used for each region of the placenta (for example, average of M1 and M2 sites for ‘Medial’). The overall median is marked by a line and the mean is marked by a plus sign (+). The box represents 50% of the data while the whiskers represent those data that fall in the 25th and 75th quartiles. (B) Correlation between housekeeping gene and BCRP Ct values was determined by linear regression.
Figure 4. Regional BCRP mRNA expression in term human placentas
Placental mRNA expression of BCRP was quantified by qPCR. Ct values for BCRP mRNA were first converted to delta Ct values by comparing to the reference gene, GAPDH, and then to delta delta Ct values by designating the average delta Ct values for medial samples from each placenta as the control group. Data are presented as a box and whisker plot with median, mean and range of values (n=10), relative to the medial mRNA expression (set to 1.0). The median is marked by a line and the mean is marked by a plus sign (+). The box represents 50% of the data while the whiskers represent those data that fall in the 25th and 75th quartiles.
Figure 5. Regional BCRP protein expression in term human placentas
Protein expression of BCRP was quantified by western blot (10 μg membrane fraction protein/lane) from placentas. β-actin was used as a loading control. The western blot data are presented as (A) a representative western blot from one placenta including human embryonic kidney-293 cells overexpressing an empty vector and wild-type BCRP, or the negative and positive control, respectively, and (B) semi-quantification of protein band density for each region normalized to the medial BCRP protein expression. Data are presented as mean ± standard error (n=10).
### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clinical Characteristics of Pregnancies for Placentas Studied</th>
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<tr>
<td>Gestational age (weeks)</td>
<td>Mean = 39</td>
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<tr>
<td>Maternal age (years)</td>
<td>Mean = 29.3, SD = 4.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Maternal Ethnicity</td>
<td>Caucasian = 4, African American = 1, Asian = 1, Hispanic = 4, Other = 0</td>
</tr>
<tr>
<td>Paternal Ethnicity</td>
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<tr>
<td>Birth weight (grams)</td>
<td>Mean = 3400, SD = 425.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Placental weight (grams)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean = 663, SD = 217.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Baby’s sex</td>
<td>Female = 4, Male = 6</td>
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<tr>
<td>Delivery to processing (mins)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mean = 40</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are presented as means ± standard deviation (SD)

<sup>b</sup> Wet, untrimmed weight

<sup>c</sup> Time from delivery to placement of specimen in PAXgene FIX in a PAXgene container