

The Human Orphan Receptor PXR Messenger RNA Is Expressed in Both Normal and Neoplastic Breast Tissue¹

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ABSTRACT

The expression of PXR mRNA and a variant PXR mRNA, deleted in 111 nucleotides in the ligand-binding domain, was detected by reverse transcription-PCR amplification in both normal and neoplastic human breast tissues. The level of PXR mRNA did not differ between breast tumors and their adjacent matched normal breast tissues. However, the expression of PXR mRNA did vary among breast tumors. A statistically significant inverse relationship was found between the level of PXR mRNA expression and estrogen receptor (ER) status, as defined by ligand binding analysis. The level of PXR mRNA expression in ER+ tumors (median = 22.4, $n = 15$) was significantly lower ($P = 0.04$) than the level of PXR mRNA expression in ER– tumors (median = 46.7, $n = 15$). No relationship with progesterone receptor status was found. These data raise the possibility that PXR has a role in human breast tissues.

INTRODUCTION

A novel orphan receptor, PXR, activated by pregnanes, was originally cloned from a mouse liver cDNA library (1). Its human “homologue,” hPXR, has been recently cloned from a human liver cDNA library (2). This orphan receptor can be activated by natural and synthetic pregnenolone derivatives as well as the antiglucocorticoid/antiprogestin RU-486. A possible target gene of hPXR is *CYP3A*,³ which is thought to have a role in the biotransformation of many drugs (2). Also, the CYP3A enzymes have been implicated as major players in the metabo-

lism of the clinically useful antiestrogens tamoxifen and toremifene (3–5). Although the major site of expression of hPXR and CYP3A enzymes is the liver, CYP3A enzymes have also been found in human breast cancer tissue (6, 7). Because these enzymes are involved in the metabolism of several anti-hormonal agents used in the treatment of breast cancer and because their potential transcriptional regulator, hPXR, can be activated by another endocrine agent (*i.e.*, RU-486), we considered the possibility that the presence of such a pathway in breast cancers may have an influence on the efficacy of some endocrine therapies. To determine the potential existence of such a pathway in human breast cancer, we have investigated the expression of hPXR mRNA in both normal and neoplastic human breast tissue.

MATERIALS AND METHODS

Materials. All cell culture reagents were obtained from Life Technologies, Inc. (Burlington, Ontario, Canada). [α -³²P]dCTP was purchased from ICN (Montreal, Quebec, Canada).

Human Breast Tissues. Thirteen cases were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block was determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks, as described previously (8). The presence of normal ducts and lobules and the absence of any atypical lesion were confirmed in all normal tissue specimens. Histopathological analysis was performed on H&E-stained sections from the paraffin tissue block to estimate, for each case, the proportions of tumor and normal epithelial cells, fibroblasts, and fat as well as to determine the levels of inflammation and Nottingham grade scores. Six tumors were ER– (ER < 3 fmol/mg protein), with PR values ranging from 4.9 to 11.2 fmol/mg protein, as measured by ligand binding assay. Seven tumors were ER+ (ER values ranging from 3.5 to 134 fmol/mg protein), with PR values ranging from 5.8 to 134 fmol/mg protein. These tumors spanned a wide range of grade (grade 5–9), determined using the Nottingham grading system.

In a second experiment, 40 invasive ductal breast carcinomas were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected for ER and PR status, as determined by ligand binding assays. Ten tumors were classified as ER+/PR+ (ER range, 50–127 fmol/mg protein; PR range, 105–285 fmol/mg protein), 10 tumors were classified as ER+/PR– (ER range, 59–156 fmol/mg protein; PR range, 5–10 fmol/mg protein), 10 tumors were ER–/PR– (ER range, 0–2 fmol/mg protein; PR range, 0–10 fmol/mg protein), and 10 tumors were classified as ER–/PR+ (ER range, 5–9 fmol/mg protein; PR range, 51–271 fmol/mg protein).

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³ The abbreviations used are: CYP3A, cytochrome P450 monooxygenase 3A4; NCIC, National Cancer Institute of Canada; ER, estrogen receptor; PR, progesterone receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR.

Ligand Binding Assays for ER and PR. Available steroid receptors were assayed using a single saturating dose of [³H]ligand plus 500-fold excess of unlabeled ligand and incubated overnight at 4°C, and the bound and free steroids were separated using the charcoal-dextran method. ER and PR concentrations were expressed as fmol of steroid specifically bound per mg of cytosol protein, as described previously (9).

Cell Culture. Human breast cancer cells were grown in DMEM supplemented with 5% fetal bovine serum, 100 nM glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin, as described previously (10). The cells were grown until they were ~80% confluent, and they were harvested by scraping with a rubber policeman. After centrifugation, the cell pellet was frozen and stored at -70°C until RNA was isolated.

RNA Extraction and RT-PCR conditions. Total RNA was extracted from 20-μm frozen tissue sections (15 and 5 sections for normal and tumor breast tissue, respectively) or frozen cell pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY), according to the manufacturer's instructions. One μg of total RNA was reverse-transcribed in a final volume of 25 μl, as described previously (11).

The primers used consisted of hPXR-U primer (sense, 5'-CAAGCGGAAGAAAAGTGAACG-3'; nucleotides 678–698 of *hPXR*) and hPXR-L primer (antisense, 5'-CTGGTCCTCGATGGCAAGTC-3'; nucleotides 1119–1099 of *hPXR*). The nucleotide positions given correspond to published sequences of the human PXR cDNA (2). PCR amplifications were performed, and PCR products were analyzed as described previously, with modifications (11). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1.5 μCi of [α-³²P]dCTP (3000 Ci/mmol), 4 ng/μl hPXR-U/hPXR-L, and 0.4 unit of Hot Start Taq DNA polymerase (Qiagen, Mississauga, Ontario, Canada). Each PCR consisted of 20 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed *GAPDH* cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide, as described previously (11). PCR products were subcloned and sequenced as described previously (11).

Quantification and Statistical Analysis. Quantification of signals was carried out by excision of the bands corresponding to hPXR cDNAs (wild-type and variant cDNAs), addition of scintillant, and counting. Three independent PCRs were performed. To control for variations between experiments, a value of 100% was assigned to the sum of hPXR-related signals (wild-type plus variant, when present) measured in one of the tumor samples arbitrarily chosen (case 1) and used as the reference in all individual PCR assays. For each sample, the sum of hPXR-related signals (wild-type plus variant, when present) was expressed as the percentage of the case 1 value. In parallel, *GAPDH* cDNA was amplified, and following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using NIH Image 161/ppc software. Two independent *GAPDH* PCRs were performed. Each *GAPDH* signal was also expressed as a percentage of the *GAPDH* value measured in case 1. For each sample, the average of the hPXR values was

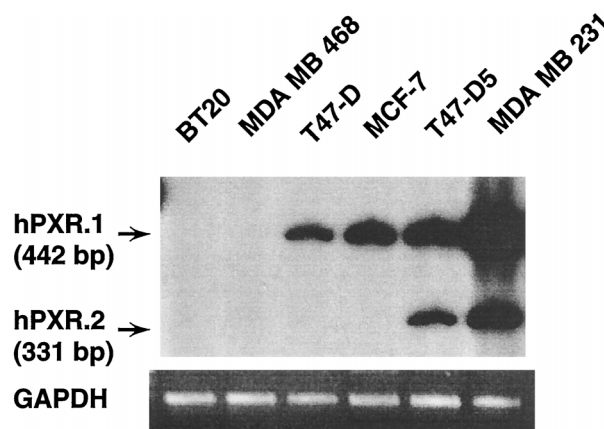


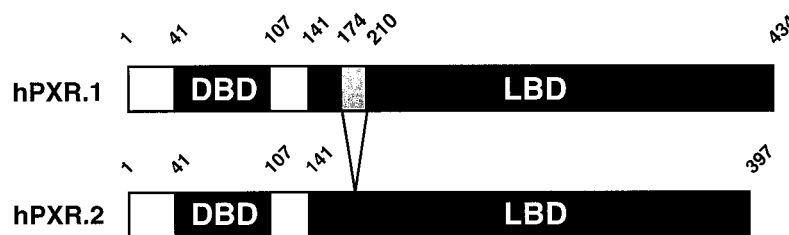
Fig. 1 Expression of hPXR mRNA and a variant hPXR mRNA in human breast cancer cell lines. *Top*, total RNA was extracted from six human breast cancer cell lines. Radiolabeled RT-PCR products using primers for human PXR and 35 cycles of PCR, separated by 6% PAGE containing 7 M urea, are shown. The hPXR.1 or wild-type product is 442 bp. The hPXR.2 or variant product is 331 bp. *Bottom*, amplification of *GAPDH* in the same six human breast cancer cell lines. The *GAPDH* PCR product was visualized by ethidium bromide staining of agarose gels.

then expressed as a percentage of the average of the *GAPDH* signals. Only samples ($n = 13$ for the tumor and matched normal experiment; $n = 30$ for the ER/PR tumor experiment), which demonstrated a reproducible wild-type hPXR signal (present or absent, see “Results”), were included in the statistical analysis. Differences in hPXR mRNA expression between groups were tested using the Mann-Whitney rank sum test (two-sided).

RESULTS

Expression of hPXR mRNA and a Variant hPXR mRNA in Human Breast Cancer Cell Lines. A RT-PCR approach was used to determine whether mRNA for hPXR was expressed in human breast cancer cell lines. In several human breast cancer cell lines, an expected 442-bp product was detected in T-47D, MCF-7, T-47D-5, and MDA-MB-231 but not in BT20 or MDA-MB-468 human breast cancer cells (Fig. 1). The ER- MDA-MB-231 cell line expressed the highest level of hPXR mRNA. However, the expression of hPXR mRNA was not related to the absolute ER status of the cell line in this small set of cell lines because both ER+ (T-47D and MCF-7) and ER- (MDA-MB-231) cells expressed hPXR mRNA. In some cell lines (T-47D-5 and MDA-MB-231), a smaller-sized PCR product of 331 bp was observed. Both of these PCR products were cloned and sequenced. The larger 442-bp product had a nucleotide sequence that was identical to that previously published for the human PXR cDNA (2). The 331-bp product was deleted in 111 nucleotides, which correspond to nucleotides 823–933 of the hPXR cDNA, as numbered in Lehmann *et al.* (2). This is an in-frame deletion in which 37 amino acids (residues 174–210) are deleted in the putative ligand-binding domain (Fig. 2). The protein predicted to be encoded by this variant hPXR mRNA, assuming there are no other alterations in

Fig. 2 Diagrammatic representation of the protein predicted to be encoded by the variant hPXR mRNA. hPXR.1 represents the wild-type human PXR (2). hPXR.2 represents the deleted PXR variant. Numbers represent the amino acid residues. DBD, DNA-binding domain; LBD, ligand-binding domain.



the mRNA, would be 397 amino acids, unlikely to bind ligand or have a markedly altered ligand profile compared to that of the “wild-type” hPXR (1). This variant human hPXR mRNA has not been reported previously and is similar but not identical to the mouse PXR.2, which contains an in-frame 123-nucleotide deletion predicting a 41-amino acid deletion in a similar region of the ligand-binding domain (1).

Identification of hPXR mRNA and a Variant hPXR mRNA in Human Breast Tissues. A RT-PCR approach was used to determine whether hPXR mRNA was expressed in human breast tissues *in vivo* (Fig. 3). The expected PCR products corresponding to the wild-type hPXR and the deleted hPXR cDNAs were detected in both normal and neoplastic human breast tissues. The normal tissues corresponded to the matched adjacent normal breast tissue of each breast tumor examined. Using parallel PCRs for the ubiquitous *GAPDH* mRNA for normalization, it appeared that the levels of hPXR and its deleted variant mRNA varied among the samples, but there were no quantitative differences seen between the normal tissues and their matched breast tumor samples (Fig. 3). Overall, there were no obvious differences in the pattern of hPXR mRNA expression between any one matched normal sample and its corresponding tumor, although the expression of the variant hPXR was not always seen (Fig. 3, Lanes 5, 6, and 8).

Relationship of hPXR mRNA Expression with ER and PR Status in Primary Human Breast Tumors. To address the question of whether the expression of hPXR in breast tumors could potentially influence the efficacy of endocrine therapy, we investigated the relationship of hPXR mRNA expression to the known prognostic and treatment response variables, ER and PR. Four groups of breast tumor samples were identified according to their ER/PR status, as defined by ligand binding analysis (see “Materials and Methods”). hPXR mRNA levels were measured by RT-PCR and normalized to the *GAPDH* mRNA level, as measured in parallel by RT-PCR. Results obtained are shown in Fig. 4. Only samples ($n = 13$ for the tumor and matched normal experiment; $n = 30$ for the ER/PR tumor experiment) that demonstrated a reproducible wild-type hPXR signal (present or absent, see “Materials and Methods”) were included in the statistical analysis. Furthermore, total hPXR-like PCR products (wild-type plus the variant) in any sample were used in the quantification of hPXR mRNA expression for statistical analysis. A statistically significant inverse relationship was found between the level of hPXR mRNA expression and ER status, as defined by ligand binding analysis. The level of hPXR mRNA expression in ER- tumors (median = 46.7, $n = 15$) was significantly higher ($P = 0.04$) than the level of hPXR mRNA

expression in ER+ tumors (median = 22.4, $n = 15$), as shown in Fig. 4B. No relationship with PR status was found.

Spearman correlation analysis showed no significant correlations of the level of hPXR mRNA with grade, age, nodal status, or the percentage of normal duct and lobular epithelium, stromal, or fat cell content within the tissue section analyzed. However, consistent with the above results (for which clinically relevant cutoff values for both ER and PR status were applied), a trend toward an inverse relationship was found between hPXR mRNA expression and the absolute level of ER, as measured by ligand binding analysis (Spearman $r = -0.50$, $P = 0.07$).

DISCUSSION

The data presented here provide evidence that the novel nuclear orphan receptor hPXR is expressed in both normal and neoplastic human breast tissues. As yet, there are no available antibodies to the hPXR protein to complement this investigation. Recombinantly expressed hPXR protein has been shown to activate transcription of reporter genes through a response element conserved in the promoter of the *CYP3A* genes (1, 2), suggesting the possibility that hPXR might be a transcriptional regulator of *CYP3A* enzyme expression (2). Because these *CYP3A* enzymes have also been found in human breast cancer tissues (6, 7), we hypothesized that hPXR/*CYP3A*-regulated pathways might play a role in human breast cancer.

The *CYP3A* enzymes, the expression of which is likely to be modulated, at least in part, by hPXR, are known to be involved in the metabolism of a wide range of xenobiotics, natural and synthetic steroids, and antisteroids, including tamoxifen and toremifene (3–5, 12, 13). Our observation that hPXR is expressed in both normal and neoplastic breast tissue opens the possibility that local metabolism and the factors regulating local metabolism may have a role in the responsiveness of human breast cancers to endocrine therapies. Indeed, altered uptake and retention of tamoxifen and possible altered local metabolism have been suggested as possible mechanisms of tamoxifen resistance in some human breast cancers (14, 15). Also, the antiglucocorticoid/antiprogesterin compound RU-486 was shown to be a relatively potent activator of hPXR (1). RU-486 has shown some efficacy as an endocrine therapy in human breast cancer (16); therefore, the interaction of RU-486 with PXR in human breast tissues, both normal and neoplastic, might be important to assess with respect to drug resistance and/or drug interactions.

Although the natural ligand for hPXR is unknown, the observations that high concentrations of several natural hormones, *e.g.*, pregnenolone and progesterone, and synthetic preg-

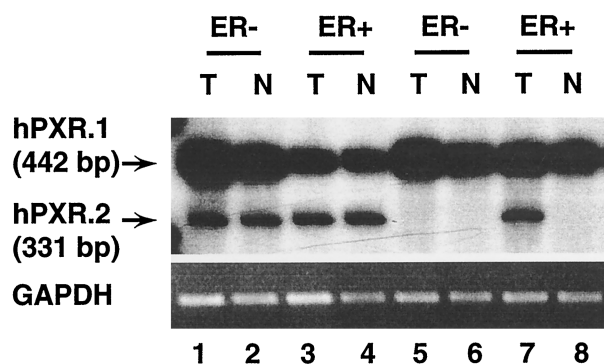


Fig. 3 Expression of hPXR mRNA and a variant hPXR mRNA in human breast tumor biopsies and their matched normal adjacent breast tissues. *Top*, total RNA was extracted from human breast cancer biopsy samples (*Lanes T*) and their matched adjacent normal breast tissues (*Lanes N*). Radiolabeled RT-PCR products using primers for human PXR and 35 cycles of PCR, separated on 6% PAGE containing 7 M urea, are shown. The hPXR.1 or wild-type product is 442 bp. The hPXR.2 or variant product is 331 bp. Both ER+ and ER- tumors were analyzed. *Bottom*, amplification of *GAPDH* in the same human breast tissue samples. The *GAPDH* PCR product was visualized by ethidium bromide staining of agarose gels.

nenolone derivatives, such as pregnenolone 16 α -carbonitrile, can activate hPXR have led to its being named the pregnane X receptor (1). It is unlikely that the concentrations required for any of these individual compounds that activate hPXR *in vitro* could be achieved in the circulation under physiological conditions; tissue accumulation levels and the possible additive effects of the various potential ligand activators of this receptor, which is activated by a wide spectrum of compounds, are unknown.

Our interest is in factors that could influence responsiveness to the so-called endocrine or hormonal therapies in human breast cancers, and we, therefore, investigated the relationship of hPXR expression to ER and PR status, which are known markers of treatment response to endocrine therapies and are also used as prognostic markers in human breast cancer (17). We observed that, in human breast tumors, the expression of hPXR was inversely correlated with the expression of ER, suggesting that hPXR-mediated pathways might be more active in breast tumors which are less likely to respond to endocrine therapies. This remains to be tested in samples of breast tumors from patients with known responses to endocrine therapies in clinical trials.

We have also investigated whether altered expression of hPXR occurs between matched normal and neoplastic breast tissues from histopathologically defined tissue sections. In this study, we found no significant difference in hPXR expression between matched normal and neoplastic breast tissues. The ER status of these normal tissues was not determined; however, given that normal tissue is often ER- and ER+ cells are a minor component (18) and that the epithelial cell content among the typical relatively sparse ducts and lobular units found in normal tissue is lower than in tumor sections ($13.5 \pm 6.6\%$ versus $40.4 \pm 13.6\%$, respectively, in this study), these results suggest that average hPXR expression is, in fact, high in normal epithelia, which is consistent with the inverse relationship be-

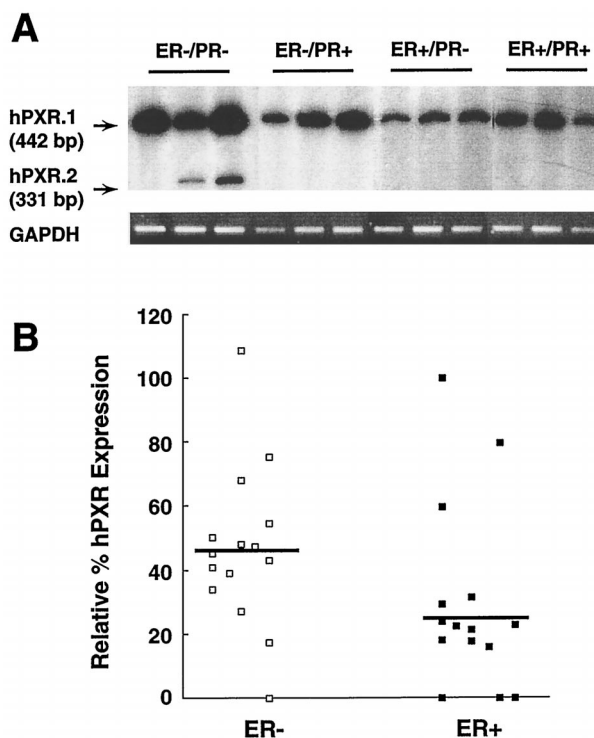


Fig. 4 Relationship of expression of hPXR-like mRNA to ER and PR status in human breast tumors. *A*, total RNA was extracted from human breast cancer biopsy samples of known ER (+ or -) and PR (+ or -) status, as defined by ligand-binding assays. Radiolabeled RT-PCR products using primers for human PXR and 35 cycles of PCR, separated on 6% PAGE containing 7 M urea, are shown. The hPXR.1 or wild-type product is 442 bp. The hPXR.2 or variant product is 331 bp. *Bottom*, amplification of *GAPDH* in the same human breast cancer biopsy samples. The *GAPDH* PCR product was visualized by ethidium bromide staining of agarose gels. *B*, relative PXR signals quantified as described in "Materials and Methods" are presented for ER- and ER+ breast tumors. The median of the values obtained in each group is indicated by a solid line.

tween ER status and hPXR seen in tumors. This interpretation suggests that hPXR is not significantly altered during tumorigenesis but may show changes in relation to altered differentiation and phenotype in tumors.

This is the first description of a human PXR variant mRNA that contains an in-frame deletion of 111 nucleotides corresponding to a deletion of nucleotides 823–933 of the wild-type hPXR mRNA and is most likely generated by an alternative splicing mechanism. This variant is similar but not identical to mouse PXR.2, which contains an in-frame 123-nucleotide deletion in a similar region of the ligand-binding domain (1). The mouse PXR.2, compared to the mouse PXR.1, showed a markedly reduced profile with respect to the agents that could activate the wild-type receptor in transient transfection analyses (1). It is possible that the human variant PXR may display a similar profile or may be unable to bind ligand. In this study, the presence or absence of this variant hPXR mRNA was not correlated with tumorigenesis or with ER/PR status.

In conclusion, we have identified the expression of human PXR mRNA and a hPXR variant mRNA in both normal and

neoplastic human breast tissues. The expression of human PXR mRNA in breast tumors was inversely correlated with expression of ER, as determined by ligand binding assay. These data raise the possibility that hPXR may play a role in human breast tissues.

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