INTRODUCTION

Prolactin, a polypeptide hormone of the growth hormone/cytokine family, is produced by lactotrophs in the anterior pituitary gland and exerts diverse functions in its several target tissues through specific membrane receptors [prolactin receptors (PRLRs; Ref. 1)]. Multiple forms of the single transmembrane domain PRLR are present in several species. The primary actions of prolactin are to promote growth and differentiation of the mammary gland during pregnancy and to initiate and maintain lactation. It is also a mammary tumor promoter (2). PRLRs are present in most human breast cancer cells (3, 4), and prolactin stimulates the proliferation of these cells (5). There is local prolactin production in mammary epithelial cells, increased expression of PRLR in human mammary tumors, and a correlation between serum prolactin with the incidence and progression of breast tumors (6–9). This evidence suggests that prolactin has a role in human breast cancer. Acting through its cognate receptor, prolactin activates the tyrosine kinase, Jak2, and transcription factor Stat5 in target cells (1). Also, prolactin can activate other tyrosine kinases, including Src family kinases, focal adhesion proteins, Tec kinase, and ErbB tyrosine kinase (10).

Previous studies in our laboratory have mapped and resolved the genomic structure of the human PRLR (hPRLR) gene (>200 kb) and have demonstrated that its complex structure is subject to alternative splicing with generation of several forms of the receptor (11–13). PRLRs are composed of an extracellular ligand domain, a single transmembrane region, and an intracellular domain of variable length. The gene contains 11 exons, including 6 noncoding exons (1 generic and 5 human specific) that are alternative spliced to a common noncoding exon 2 (11–13). Exons 3–10 encode the full length of long receptor form (LF) and intermediate form with partial deletion of exon 10 of the receptor (14). Exon 8 encodes the transmembrane domain, and exon 10 encodes most of the intracellular domain. In the human, exon 11 of the PRLR was found to be distinct from its rodent counterpart (13). Two novel short forms (SFs) of the hPRLR first identified in our laboratory, termed S1a and S1b, are derived from alternative splicing of exons 10 and 11 and possess a truncated intracellular domain. These forms are inhibitory of the activation induced by prolactin through LF form of the receptor (13). In addition, exon 11 is shared by three other species of unknown function, which are S1a and S1b variants. These include soluble forms lacking the transmembrane domain Δ7/11-S1a and Δ4-Δ7/11 S1a with deletion of exons 8–10 and 8–10, respectively, and Δ4 S1b with deletion of exon 4 (15).

The role of PRLR variants in the actions of prolactin in breast cancer is presently unknown. However, there is increasing evidence that PRLR expression is altered in some neoplasms. An increased level of PRLR expression in tumors, compared with that of normal tissue, has been observed in a high proportion of breast tumors by immunohistochemistry and quantitated PCR (16–18). However, rigorous and specific quantification of PRLR variants and their expression in breast tumors remained to be determined. In the present study, we initially screened breast cancer profile arrays with specific LF or SF probes to compare the relative expression of the PRLR variants. In subsequent studies, we applied real time PCR methodology to quantify the mRNA expression levels of PRLR forms (LF, S1a, and S1b) in normal and breast cancer tissue and cell lines. Our studies have revealed a consistent pattern of low SFs to LF ratio associated with breast tumors. This suggests that a reduction of the inhibitory role of SF on LF action may be operative in mammary tumors.

MATERIALS AND METHODS

Cell Lines and Tissues. Human breast cancer and normal mammary gland cell lines used in Fig. 5 of this study were obtained from American Type Culture Collection (Manassas, VA). Cells grown according to American Type Culture Collection protocols in T75 culture flasks to 90% confluency were collected for total RNA preparation. Frozen tissues of matched normal and tumor breast from 10 patients were obtained from Cooperative Human Tissue Network. Human breast-matched cDNA pairs and human cancer profiling arrays were from Clontech (Palo Alto, CA). The arrays used to determine the differential expression of hPRLR forms (LF and SFs) contained normalized SMART-amplified cDNA from breast tumors and corresponding normal tissues from 47 patients.

Southern Blot Analysis of Human Cancer Profiling Array and PCR Products. Southern blot analyses following our previously described procedure (11) were performed using specific oligonucleotides or DNA fragment to analyze the RNA expression of hPRLR forms in cancer profiling arrays as well.
as to confirm the expected size of specific PCR products in real time PCR analysis (see below). Oligonucleotides probes were γ-32P-ATP end-labeled using T4 polynucleotide kinase as described previously (11). Membrane filter was autoradiographed on Kodak X-Omat film. Radioactive signals obtained in the cancer profiling array were quantified by means of a Bio-Rad GS-800 calibrated densitometer (Bio-Rad). The relative intensity in each pair of normal and tumor-immobilized cDNA samples from individual patients was normalized by ubiquitin before data analysis.

Real-Time PCR Analysis. Total RNA levels of hPRLR forms were assessed using two-step real-time PCR in a LightCycler (Roche Molecular Biochemicals). Total RNA was extracted from cells or tissues (Invitrogen) and treated with DNase I for 30 min at 37°C followed by phenol chloroform extraction to avoid genomic DNA contamination. Samples (200–1000 ng) were reverse transcribed to synthesize first-strand cDNA using oligo dT and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Specific primer pairs used in the PCR amplification were selected based on the LightCycler probe design software program (Roche) to avoid dimer or nonspecific product formation. Forward (F) and reverse (R) primer pairs located at nucleotide position as follows: LF, 1175/1196 bp (F) and 1386/1407 bp (R); S1a, 866/887 bp (F) and 1189/1210 bp (R); and S1b, 839/860 bp (F) and 1035/1056 bp (R). Primers designed from a common region present in all variants were located at nucleotide −34/−14 bp at exon 3 (F) and +330/+350 at exon 5 (R).

All reactions were performed in glass capillaries (Roche) using LightCycler-FastStart DNA Master SYBR Green I kit. The reaction mixture contained FastStart TaqDNA polymerase, reaction buffer, deoxynucleoside triphosphate mixture, 3 nM MgCl₂, SYBR Green I dye, 0.5 μM of each primer, and 5 μl of a standard dilution or first-strand cDNA specimen from cells or tissues. On the basis of the expression level of hPRLR in the individual samples, cDNA concentration varying from 10⁻² to 10⁻³ attomoles corresponding to the original RNA concentration (indicated in the figure legends) was used for PCR analysis. The final reaction volume was 20 μl. Water was used as a negative control. All samples were kept on ice during preparation. Thermocycling conditions were designed to program in four consecutive steps. It was programmed in step number 1 to denature the specimen for 10 min at 95°C prior amplification of all different variants for one cycle. This was followed in the second step (step 2) by denaturation for 1 s at 95°C, annealing 7 s at 60°C (LF), 65°C (S1a), 67°C (S1b), or 60°C (β-actin) and extension at 72°C for 11 s for 45 cycles (LF), 15 s (S1a), 10 s (S1b) for 55 cycles, and 15 s for 40 cycles (β-actin). The melting program (step 3) consisted of 30 s at 65°C (LF), 70°C (S1a) and 72°C (S1b) for one cycle. The cooling program (step 4) was for 30 s at 40°C for one cycle. All standard or samples were analyzed in quadruplicate and repeated at least three times.

All fluorescence data were analyzed by LightCycler 3.5 software. Quantification was derived from the concept that the logarithm of template concentration is proportional to the cycle number where the amplicons become detectable. Concentrations of hPRLR variants were determined by comparing the samples to the standard curve generated from external standards.
RESULTS

hPRLRs Expression in Breast Cancer Profile Array. To determine the presence of the hPRLR forms in breast cancer patients, we first examined the relative expression of the LF and SFs of the receptor in matched tumor and normal breast tissues from 47 patients. The relative levels of expression derived from the array hybridization signals (Fig. 1A) normalized by housekeeping ubiquitin gene (Fig. 1B) were classified into three groups according to the level of LF in the tumor in relation to the adjacent normal tissue for each sample (LF increase, decrease, or no change). Compared with the normal tissues, 62% of breast cancer tissues expressed increased level of LF (group I) and 21 and 17% of the patients revealed either decrease (group II) or no change (group III), respectively. Twenty-two of 29 patients in group I showed increased level of SF, whereas the remainder of patients in this group and all patients in groups II and III displayed lower levels of SF. However, the ratio of SF to LF was consistently lower and statistically significant in breast tumor regardless of the level of hPRLR variants when compared with the normal tissue adjacent to the tumor (Fig. 1C). There is no specific correlation in the change of hPRLR variants level with the type of breast tumor (Table 1A).

Table 1 Breast tumor backgrounds

<table>
<thead>
<tr>
<th>Morphology of neoplasm</th>
<th>LF increase</th>
<th>LF decrease</th>
<th>LF no change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninfiltrating intraductal carcinoma</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobular carcinoma</td>
<td>B, F</td>
<td>K, M</td>
<td>F§</td>
</tr>
<tr>
<td>Infiltrating intraductal carcinoma</td>
<td>C</td>
<td></td>
<td></td>
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<tr>
<td>Infiltrating ductal carcinoma</td>
<td>D, E</td>
<td></td>
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</tr>
<tr>
<td>Fibrosarcoma</td>
<td>X</td>
<td></td>
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<tr>
<td>Mucinous adenocarcinoma</td>
<td>T</td>
<td></td>
<td></td>
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<tr>
<td>Infiltrating lobular carcinoma</td>
<td>Y</td>
<td></td>
<td></td>
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<tr>
<td>Medullary carcinoma</td>
<td>Q†</td>
<td></td>
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<tr>
<td>Mixed lobular-ductal carcinoma</td>
<td>BB</td>
<td></td>
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<tr>
<td>Tubular adenocarcinoma</td>
<td>C†</td>
<td></td>
<td></td>
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<tr>
<td>Tubular carcinoma</td>
<td>W‡</td>
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Matched cDNA pairs of individual patients

<table>
<thead>
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<th>LF increase</th>
<th>LF decrease</th>
<th>LF no change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrating ductal carcinoma</td>
<td>D1 (62 yrs)</td>
<td>A† (40 yrs)</td>
<td>E‡ (71 yrs)</td>
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<tr>
<td>Tubular adenocarcinoma</td>
<td>C1 (47 yrs)</td>
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<tr>
<td>Lobular carcinoma</td>
<td>B2 (58 yrs)</td>
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</table>

Individual patients from Cooperative Human Tissue Network

<table>
<thead>
<tr>
<th>Morphology of neoplasm</th>
<th>LF increase</th>
<th>LF decrease</th>
<th>LF no change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrating ductal carcinoma</td>
<td>SC157†† (34 yrs)</td>
<td>14,001†† (30 yrs)</td>
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<tr>
<td></td>
<td>14,012 (45 yrs)</td>
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<td></td>
<td>14,042 (65 yrs)</td>
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<td></td>
<td>14,009† (44 yrs)</td>
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<td>14,091†† (N/A)</td>
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<td>14,099 (69 yrs)</td>
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<td>14,103†† (N/A)</td>
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<td></td>
<td>685†† (69 yrs)</td>
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<tr>
<td>Medullary carcinoma</td>
<td>14,008 (47 yrs)</td>
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</tbody>
</table>

Abbreviations: N/A, information not available.
* Columns 1 and 2, capital; columns 3 and 4, italic.
† Stage I.
‡ Stage II.
§ Stage IIA.
¶ Stage IIB.
†† Stage IIIA.
‡‡ Stage information not available.

were calculated by the number of molecules amplified in attomole/ng RNA or normalized by the internal amplified β-actin and human ribosomal protein S9. Both normalization gave comparable results. SDs were obtained based on three time experiments and in quadruple for each time. Within assay variation was 4% and between assays was 6%.

hPRLR Isoform Constructs for Used as RNA Standards. Three hPRLR variants were separately inserted into PcDNA3.1+ at HindIII and EcoRV for LF, KpnI and ApaI for S1a and S1b. All constructs were linearized by XhoI at their COOH-terminal and purified by extracting DNA from the gel for in vitro transcription (Qiagen number 28704) of RNA standard using T7 promoter. RNAs were additionally purified by gel extraction to remove DNA template contamination (Qiagen number 28704). The concentrations of LF-2.7 Kb, S1a-1.8 Kb and S1b-1.6 Kb were measured by Biophotometer AG (made in Germany). First-strand cDNA was synthesized using specific reverse primer for the variant indicated above followed by real-time PCR analysis. Ten-fold series dilution of (10^2 to 10^−7) attomole) of the purified RNA of the individual form in water was used as the external standards.

Statistical Analysis. The significance of the differences in the SF/LF ratios between tumor and matched normal tissue specimens, and cell lines were determined by the Mann-Whitney test and Kruskal-Wallis test (nonparametric test ANOVA analysis), respectively.

RESULTS

hPRLRs Expression in Breast Cancer Profile Array. To determine the presence of the hPRLR forms in breast cancer patients, we first examined the relative expression of the LF and SFs of the receptor in matched tumor and normal breast tissues from 47 patients. The relative levels of expression derived from the array hybridization signals (Fig. 1A) were calculated by the number of molecules amplified in attomole/ng RNA or normalized by the internal amplified β-actin and human ribosomal protein S9. Both normalization gave comparable results. SDs were obtained based on three time experiments and in quadruple for each time. Within assay variation was 4% and between assays was 6%.
Linear standard curves of each variant plot in log (attomole) versus cycle was established from $10^{-2}$ to $10^{1.1}$ attomole (Fig. 2A–C). At a given molar concentration, standards for all forms displayed comparable amplification and identical melting points when we used common primers for all these forms (see “Materials and Methods;” Fig. 2E). Standard curve of β-actin used for normalization of individual test samples was also established (Fig. 2F).

**Expression of Three hPRLR Variants in Breast Cancer Tissues.** We first analyzed five commercial cDNA pairs of normal versus breast tumor samples using the established real-time PCR conditions (Fig. 3A). LF was significantly increased in patients B–D, no change in patient E, and decrease in patient A. Except for patients C and D, S1a level was decreased in all other patients. Decreased S1b level was observed in all patients. Four of five patients with breast tumor revealed significant decrease in both S1a and S1b to LF ratios when compared with the adjacent normal tissue (Fig. 3, B and C). Southern analysis of individual forms showed comparable changes to that assessed by real time PCR (Fig. 3D). We additionally analyzed the expression of hPRLR forms in the breast tumor and adjacent normal tissue of 10 patients obtained from Cooperative Human Tissue Network. The tumor in 8 of 10 patients showed elevated LF expression with no consistent pattern change of either S1a or S1b (Fig. 4A). However, all patients displayed ratios of S1a or S1b to LF that were significantly lower in the tumor than in normal tissues (Fig. 4, B and C). This finding is consistent with studies shown in Figs. 1 and 3, which demonstrated that low ratio of SF to LF is associated with breast tumor tissues.

**Quantitative Analysis of Normal and Breast Cancer Cell Lines of hPRLR Expression.** Ten mammary cancer cell lines and two normal cell lines (Hs578Bst and MCF10A) were evaluated for the concentration of hPRLR forms (Fig. 5). The highest level of hPRLR expression was observed in T47D cells, MDA-MB-134-V1 and MCF7. S1b was low in both MDA-MB-157 and MDA-MB-330. The relative distribution of LF among different cell lines was comparable.
were normalized by real-time PCR. Patients (A–E) from Clontech human breast matched cDNA pairs. Results
logarithmic scale. Results represent mean C ratios in normal and tumor tissues for each patient. Results were plotted in
S1b/LF (1 for background of individual patients. See Table 1 for background of the patients.

Southern blot analysis of the samples using specific probes indicated in Fig. 2. We have demonstrated that the generalized concept of LF increased expression level of any of the hPRLR forms (LF; S1a and S1b). On the other hand, we have demonstrated a general pattern of low ratio of SFs to LF associated with both breast tumor tissues and cancerous cell lines when compared with normal samples.

We have demonstrated that the generalized concept of LF increased in breast tumors does not apply to all of the population of breast tumor patients. The Southern blot analysis of cancer profile array have clearly indicated that LF and the SF variants could also decrease or no change in the tumor versus normal breast samples, and this occurs regardless of the pathological background of the tumor. This is additionally supported by RNA quantification of the individual forms using a sensitive real time reverse transcription-PCR approach. The difference between our present study and a previous study of an additional patients. A, results represent the expression of each hPRLR variant normalized by β-actin in the normal (N) and tumor (T) breast tissue of individual patients. B and C, comparison of SF/LF ratios in normal and breast cancer patients. Results were calculated in number of molecules (attomoles) encoding individual hPRLR variants amplified by PCR/ng of RNA used in the study. See Table 1 for background of the patients.
used in the study. The fold-change of LF expression in the several cell lines examined (Fig. 5C) relative to MCF7A was comparable with earlier studies using either real-time PCR (4) or Northern blot analysis (19) where only LF was examined. It is noted that there is a marked difference in hPRLR variants expression between normal cell lines Hs578Bst and MCF10A. The expression of hPRLR variants in atto-mole/ng RNA ranged from 6.94 to 0.24 × 10^3 in LF, 1.8 to 0.03 × 10^3 in S1a, and 8 to 1.7 × 10^5 in S1b. This type of variation was also observed in the other report for the LF in the normal and tumor breast tissue (16). Our analysis indicates at least 10^3-fold difference in the expression of each form among normal and tumor samples. Among the cancer cell lines, T47D, MDA-MB-134VI, and MCF7A expressed higher levels of LF, S1a, and S1b, where other cell lines expressed comparable LF to Hs 578Bst with variation of SF expression. Although there is no general rule for the expression of hPRLR forms associated with the tumor background of the cell lines, the ratio of SF to LF was consistently lower in most cancer cell lines when compared with either Hs 578Bst or MCF10A (normal epithelial cells). This finding is of significance because it not only further supports the SF/LF findings in breast tumor tissues but also provides useful information to select specific cell lines for PRLR variants related projects.

The present observation provides an additional index for evaluation of human breast cancer. The regulation of alternative splicing for the expression of PRLR forms may provide a mean for the regulation of mammary epithelial cells growth, differentiation and tumorigenesis.

REFERENCES


Human Prolactin Receptor Variants in Breast Cancer: Low Ratio of Short Forms to the Long-Form Human Prolactin Receptor Associated with Mammary Carcinoma

Jianping Meng, Chon-Hwa Tsai-Morris and Maria L. Dufau


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