Re-evaluation of the prolactin receptor expression in human breast cancer

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Abstract

The pituitary hormone PRL is involved in tumorigenesis in rodents and humans. PRL promotes proliferation, survival and migration of cancer cells acting via the PRL receptor (PRLR). Aiming to perform a large-scale immunohistochemical (IHC) screening of human mammary carcinomas for PRLR expression, we evaluated the specificity of commercially available anti-human PRLR antibodies (B6.2, U5, PRLRI pAb, 1A2B1, 250448 and H-300). The latter three antibodies were found to specifically recognise PRLR. The relative PRLR expression level detected with these antibodies closely reflected the level of 125I-PRL binding to the cell surface. The monoclonal antibody (mAb) 250448 was specific for the N-glycosylated form of PRLR and blocked PRL binding and signalling. The PRLRI polyclonal antibody recognised cytokeratin-18. The mAb B6.2, previously used in a number of studies, was found to lack specificity for PRLR and to rather recognise a PRLR-associated protein. The mAb U5 raised against the rat PRLR did not cross-react with the human receptor. Only one mAb, 1A2B1, was found useful for detection of PRLR in IHC applications. This antibody recognised PRLR expressed in human breast cancer cell lines and decidual cells in tissue sections of human placenta. Screening of 160 mammary adenocarcinomas demonstrated significant immunoreactivity in only four tumours, indicating that PRLR is generally not strongly upregulated in human breast cancer. However, even a very low level of PRLR expression was found to be sufficient to mediate PRL responsiveness in breast cancer cell lines.

Introduction

PRL is a polypeptide hormone secreted by the anterior pituitary. It functions systemically as a classical endocrine factor. PRL has been known for many decades to be a potent differentiation factor for the mammary epithelium (reviewed in Ben-Jonathan et al. 2008). Extrapituitary synthesis of PRL was discovered in T-lymphocytes, placenta, breast cancer cells and surrounding normal breast epithelium (Montgomery et al. 1990, Kenner et al. 1991, Gellersten et al. 1994, Clevenger et al. 1995, Ginsburg & Vonderhaar 1995). Since the same tissues express PRL receptor (PRLR), it was suggested that PRL may act in an autocrine/paracrine fashion. The role of PRL in promoting carcinogenesis in rodents is well established. PRL was also suggested to contribute to tumour development and progression in humans. A number of in vitro studies proved the role of PRL in a variety of biological processes, which can contribute to cancer progression including regulation of cell proliferation, motility, survival and possibly angiogenesis (reviewed in Clevenger et al. 2003). PRL exerts its actions via PRLR, a member of the cytokine receptor superfamily. These receptors are non-tyrosine kinases, single-pass transmembrane proteins organised into three domains: an extracellular ligand-binding domain (ECD); a hydrophobic transmembrane domain (TM); and an intracellular signalling domain (ICD) containing a proline-rich motif. Multiple PRLR isoforms resulting from alternative splicing events have been identified. Most of them are similar in their ECD, but differ in the intracellular part (reviewed in Clevenger et al. 2003). Thus, multiple isoforms potentially can activate distinct intracellular signalling events. The long receptor isoform was studied in detail. It is a 598 amino acid residue (aa) glycoprotein with an electrophoretic mobility of 80–85 kDa. Upon ligand binding and sequential dimerisation, it activates multiple signalling systems including JAK2/STAT5, STAT3, MAPK p44/42 and PI3K pathways. It has been suggested that the long isoform is responsible for the pro-proliferative and anti-apoptotic effects of PRL on cancer cells. Specific functions of the other PRLR isoforms are much less investigated (reviewed in Ben-Jonathan et al. 2008). The intermediate
PRLR isoform, PRLRi was originally cloned from the human breast cancer cell line T47D (Kline et al. 1999). This isoform is a 349 aa protein that was reported to be identical to the long PRLR in the ECD and TM domains, but to differ in the cytoplasmic domain. As a result of out-of-frame splicing, it is shorter, lacks STAT docking sites and harbours a unique 13 aa C-terminal sequence.

Reports on the expression of PRLR in human breast cancer and normal tissues are contradictory. This is in part due to the use of different methodologies and hence sensitivity and/or specificity of the probes. In earlier days, binding of radiolabelled PRL to tissues was used to detect the presence of PRLR and quantify its relative expression level (Holdaway & Friesen 1977, Di Carlo & Muccioli 1979, Partridge & Hahn 1979, Rae-Venter et al. 1981, Turcot-Lemay & Kelly 1982). These studies revealed that 20–60% of human breast cancers express PRLR. Later, PRLR expression was re-examined on the mRNA level using PCR or in situ hybridisation approaches. The PRLR transcripts were found in 95–100% of breast carcinomas and 93–100% of normal surrounding mammary epithelium (Ormandy et al. 1997, Reynolds et al. 1997, Touraine et al. 1998). Expression of the PRLR mRNA was found to correlate with expression of oestrogen receptor (ER) and progesterone receptor (PR; Ormandy et al. 1997). However, these data were not confirmed when immunological methods were applied for detection of the PRLR protein (Reynolds et al. 1997). Touraine et al. (1998) established a quantitative PCR technique to detect PRLR mRNA in human tumours and normal breast tissues. In parallel, the same samples were analysed by immunohistochemical (IHC) staining. The authors did not find a correlation between relative PRLR expression levels assessed by the two techniques.

Only a few mid-scale studies on the PRLR protein expression in breast carcinomas were conducted so far. Several monoclonal antibodies (mAb) were generated. The three of them being most frequently used in the IHC studies on human tissues and cell lines, namely B6.2 (Mertani et al. 1998, Bhatavdekar et al. 2000, Gill et al. 2001, Glasow et al. 2001), U6 (Clevenger et al. 1995, Reynolds et al. 1997) and U5 (Touraine et al. 1998). Use of the different antibodies raised another level of complexity in the data interpretation. Though all these studies revealed that PRLR is expressed in more than two-thirds of human breast cancers, data on the expression level, expression pattern in tumours, subcellular localisation and correlation between the PRLR and ER expressions were not consistent for the three antibodies used. None of these IHC studies found a correlation between the PRLR expression and clinico-pathological parameters of tumours. However, earlier studies suggested prognostic significance of PRLR in some subgroups of breast cancer patients (Bonneterre et al. 1987). Taking into consideration the contradictory data generated with the use of different techniques, the clinical relevance of PRLR expression deserves re-evaluation.

In this study, we have evaluated six commercially available anti-PRLR antibodies and identified those that can specifically recognise a functional PRLR for in vitro studies and IHC screening of human mammary carcinomas.

Materials and Methods

Cell lines

Human breast cancer cell lines, T47D, MCF7-S8 and AU565, colon cancer cell lines LS174T and HT-29, hamster ovary epithelial cells CHO–K1 and the B6.2 hybridoma were purchased from American Type Culture Collection (ATCC). The cells were propagated in a 5% CO2 humidified atmosphere in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 4.5 g/l glucose, 100 U penicillin and streptomycin (Invitrogen). In case of T47D, human insulin (Novo Nordisk A/S, Bagsvaerd, Denmark) was added to 0.2 U/ml.

Establishment of the CHO–K1 clones expressing human PRLR

The human PRLR long isoform cDNA was subcloned from the IMAGE clone 30343995 into the pIRESneo2 mammalian expression vector (Clontech). The cDNA was amplified by PCR using the following primers: 5’-aacagccgacgcaaaagaagcccagaagctgaga-3’ and 5’-aacagcagctggaggggtaaaagca-3’ harbouring NotI and KpnI restriction sites respectively, to facilitate cloning into the corresponding sites of pIRESneo2. After sequence verification, the construct was used for transfection of CHO–K1 cells. Stably transfected clones were selected in the presence of 500 µg/ml genetin (Invitrogen). Expression of PRLR was confirmed by RT-PCR using the following set of primers: forward 5’-ccctgtctacagtcatgg-3’ and reverse 5’-ccctgtctacagtcatgg-3’.

Antibodies and recombinant proteins

The six anti-PRLR antibodies used in this work are listed in Table 1. The B6.2 mAb was either purchased from the commercial source or purified from the hybridoma medium using a protein G column affinity chromatography (HiTrap Protein G FF, GE Healthcare, Fairfield, CT, USA) according to the manufacturer’s protocol. Experiments conducted with the B6.2 mAb were reproducible independently of the source used. For IHC applications, the 1A2B1 ascites-derived mAb was purified on a column loaded with the recombinant extracellular domain of PRLR (rECD–PRLR) according to the protocol described in (la Cour et al. 2003). Mouse IgG1 and IgG2b isotype control antibodies were purchased from DAKO (Glostrup, Denmark). Rabbit non-immune IgG was purchased from Zymed (Invitrogen).

Mouse IgG1 and IgG2b isotype control antibodies were purchased from DAKO (Glostrup, Denmark). Rabbit non-immune IgG was purchased from Zymed (Invitrogen). The anti-phospho-STAT5 Tyr694 rabbit polyclonal antibody (pAb) was purchased from Cell Signaling Technologies (Danvers, MA, USA), anti-phospho-MAPK p44/p42 mouse

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mAb (anti-active MAPK) was from Promega, anti-β-actin mAb—from AbCam (Cambridge, UK) and anti-human keratin-8/18 mouse mAb—from Cell Signaling Technologies.

For Western blotting, secondary HRP-conjugated anti-mouse and anti-rabbit immunoglobulins were purchased from DAKO. For IHC, secondary biotinylated donkey anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Human recombinant PRL and rECD-PRLR were produced in E. coli and purified as described by Svensson et al. (2008).

Human recombinant cytokeratin-18 and cytokeratin-19 were purchased from US Biological (Swampscott, MA, USA). Human recombinant cytokeratin-8 was from Cell Sciences (Canton, MA, USA).

**Western blotting and immunoprecipitation**

Cells grown to 80% confluence were lysed in a buffer containing 1×PBS (137 mM sodium chloride, 10 mM phosphate and 2·7 mM potassium chloride; pH 7·4), 20 mM EDTA, 0·1% w/v SDS and 1·0% v/v NP-40, supplemented with a cocktail of protease inhibitors (Complete, Roche) and a phosphatase inhibitors cocktail (Calbiochem, Merck) on ice for 30 min. The protein extracts were clarified by centrifugation at 15 000 g for 10 min, 4°C. The concentration of the protein extracts was measured using a BCA protein assay kit (Pierce, Thermo Scientific). Image acquisition was performed using an LAS-3000 image reader (Fujifilm, Tokyo, Japan) and analysed using an Image Gauge V4 software.

For immunoprecipitation experiments, 2–3 µg of an antibody were incubated with 500 µg of protein extracts overnight at +4°C under gentle rotation. The immune complexes were captured using either protein G- or protein A sepharose 4B Fast Flow beads (Sigma-Aldrich) upon incubation for 3 h at room temperature under gentle rotation. The sepharose beads were spun down at 400 g, resuspended in a gel-loading buffer, boiled for 10 min and spun at 10 000 g, 5 min prior to loading on a gel.
Deglycosylation of cellular proteins

N-linked glycans were removed from cellular proteins using N-glycosidase F (PNGase F; Roche) as follows. The cells were lysed as described above. The protein extracts were divided into two aliquots, of which one was treated with PNGase F (1 U per 30 μg protein) for 3 h at 37 °C, while the other aliquot was left untreated.

PRL binding to the cell surface

The recombinant PRL protein was iodinated with Na\textsuperscript{125}I using H\textsubscript{2}O\textsubscript{2}/lactoperoxidase at neutral pH (Jørgensen & Larsen 1990) followed by anion exchange chromatography (NAP-5, GE-Healthcare). Cells were plated at 2 \times 10^6 per well of a six-well plate and grown for 2 days to a confluent monolayer. Cell monolayers were washed with the binding buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 4-7 mM KCl, 2-5 mM CaCl\textsubscript{2}, 1-24 mM MgSO\textsubscript{4} and 0-1% w/v BSA). Na\textsuperscript{125}I-PRL (373 Ci/mmol) was added to the cells either alone at 1 nM in a binding buffer (for estimation of B\textsubscript{max}), or in the presence of 1 μM of unlabelled PRL (for measurements of non-specific binding). The plates were incubated at room temperature for 90 min, set on ice and washed twice in an ice-cold-binding buffer. The cells were lysed in 1% w/v SDS for 1 h at 37 °C. Radioactivity was counted using a TopCounter (PerkinElmer, Waltham, MA, USA). Data treatment was done using the GraphPad Prism 4.0 software.

Intacellular signalling

T47D cells were grown until ~80% confluence in a complete growth medium and then starved for 20 h in a growth medium omitting FCS. Cells were pre-treated for 30 min with 40 μg/ml of 1A2B1, 250448, U5 or B6.2 antibodies (endotoxin free, sodium azide free) and then stimulated with 10 nM of human recombinant PRL (endoxin free) for 20 min. Cells were washed in ice-cold PBS and snap frozen in liquid nitrogen. After partial thawing at 4 °C, cells were lysed in a buffer containing 50 mM HEPES, pH 7-4, 0-1% w/v SDS, 1% w/v NP-40 and 150 mM NaCl supplemented with protease inhibitors (Complete, Roche) and a phosphatase inhibitor's cocktail (Calbiochem, Merck). The protein extracts were analysed by western blotting as described above.

Identification of the protein recognised by the anti-PRLR\textsubscript{i} pAb

The antigen was purified from T47D cells by affinity chromatography using the anti-PRLR\textsubscript{i} pAb cross-linked to the protein–A sepharose as described by Schneider et al. (1982). The eluted proteins were subjected to gel filtration chromatography to exchange the buffer to 8 M Urea, 4% w/v CHAPS (2D gel sample kit, Pierce, Thermo Scientific) and separated using a two dimensional gel electrophoresis. Isoelectricfocusing was performed using a pH 3 to pH 10 ampholyte carrier (GE Healthcare) for 30 min at 500 V, followed by 30 min at 1000 V and 1 h 40 min at 5000 V in an Ettan IPGphor–II apparatus (GE Healthcare). The strips were equilibrated in a buffer containing 2% w/v SDS, 50 mM Tris–HCl pH 8-8, 6 M urea, 30% v/v glycerol and 0:002% w/v bromophenol blue for 15 min. Dithiothreitol was added to the final concentration of 10 mg/ml and proteins were separated by the second dimension 12% SDS–PAGE. The gels were stained using a Silver Stain Plus kit (Bio–Rad). Protein spots were excised from a gel and digested with trypsin. The peptides were analysed using a Matrix-Assisted Laser Desorption Ionisation Time–of–Flight Tandem Mass–Spectrometry (MALDI–TOF MS/MS) technique (the service was provided by Alphalyse A/S, Odense, Denmark).

Immunohistochemistry

Cultured cells were detached using Versene’s solution (Invitrogen), fixed in 4% v/v paraformaldehyde overnight at 4 °C, gently pelleted and embedded in 3% w/v agar cylinders, which were processed and paraffin embedded.

Formalin-fixed and paraffin-embedded human placenta tissue samples were provided by Dr Tornehave, Odense Hospital, Denmark. Formalin-fixed, paraffin-embedded human breast carcinoma samples from 160 patients were drawn from the tissue bank at Department of Pathology, Nordjaellands Hospital, Hilleroed, Denmark. The material has been treated in a uniform manner with a fixation time of 24 h, according to the European guidelines for quality assurance in breast cancer screening and diagnosis (Fourth ed. European commission, Brussels, 2006). The study was approved by the local ethical committee.

Tissue micro arrays (TMA) were generated as follows: each sample was represented by two cores (diameter: 2 mm), which were placed in different TMA blocks. In addition, cores representing normal human kidney and normal human liver were included in all TMA blocks for orientation. In total, ten TMA blocks were generated with eight blocks containing 35 cores and two blocks containing 30 cores.

Sections (3 μm) of cells, placenta and breast tumours (full size as well as TMA) were deparaffinised and brought to water. Heat-induced antigen retrieval was performed in a buffer containing 10 mM Tris–HCl, 0.5 mM EDTA, pH 9, for 15 min in a microwave oven. Sections were rinsed in TBS after this and each of the following steps. Endogenous peroxidase activity and biotin were blocked by H\textsubscript{2}O\textsubscript{2} (0.5% v/v) and avidin–biotin blocking kit (DAKO) respectively. Non-specific antibody binding was blocked with 10% v/v normal donkey serum and 3% w/v non-fat milk in TBS for 2 h at room temperature. The anti-PRLR antibodies were titrated by serial dilutions in TBS containing 7% v/v donkey serum, 3% w/v BSA and 0-5% w/v non-fat milk. The optimal concentration was determined for each antibody: 5 μg/ml B6.2, 3 μg/ml 250448, 3 μg/ml 1A2B1, 3 μg/ml U5, 0-013 μg/ml H-300 and 0-035 μg/ml PRLR\textsubscript{i} pAb. Staining of sections was performed overnight at 4 °C. The secondary anti–mouse and anti–rabbit antibodies were diluted
1:3000 and 1:4000 respectively, in the same buffer and applied to sections for 1 h at room temperature. Binding of the B6.2 mAb was detected by peroxidase-conjugated avidin–biotin complex (VectorStain, Vector Laboratories, Burlingame, CA). Binding of the mAb 250448, mAb U5, pAb H-300 and pAb PRLRi was detected with peroxidase-conjugated streptavidin (Strep<sup>PO</sup>, DAKO), indirect biotin-conjugated tyramide signal amplification (TSA) system (NEL700 from NEN, Boston, MA, USA) and Strep<sup>PO</sup>. For 1A2B1, antibody binding was detected with avidin–biotin complex, TSA and avidin biotin complex. Visualisation of antibody binding was performed with diaminobenzidine (DAB). Cell nuclei were counter stained with haematoxylin. Finally, sections were dehydrated in alcohol and xylene and mounted with Pertex. Immunostained slides were analysed by light microscopy using an Olympus AX-70 microscope. Pictures were captured with an Olympus DP50 digital camera and figures were generated with Adobe Photoshop. Staining was not detectable when isotype controls (mouse IgG1 for B6.2, U5 and 250448; mouse IgG2b for 1A2B1; rabbit IgG for H-300 and PRLRi) were applied in corresponding concentrations.

The breast cancer TMA blocks were also immunostained for the clinical markers: ER (NCL-L-ER-6F11/2, NovoCastra), PR (clone PGR 636, DAKO), HER2 (kit from DAKO) and the proliferation marker Ki67 (clone MIB1, DAKO).

Results

Correlation between PRLR expression analysed by Western blotting and PRL binding to the cells

CHO cells stably transfected with the long isoform of PRLR and a panel of breast and colon cancer cell lines were analysed for PRL binding to the surface of live cells (Fig. 1A). PRL bound strongly to T47D– and PRLR-transfected CHO cells,
while binding to AU565, MCF-7 and LS174T cells was much weaker. PRL binding to HT-29 cells was comparable with that of the untransfected CHO indicating a very low expression of PRLR in these cell lines. Thus, most of the analysed cell lines expressed a functional PRLR receptor.

Expression of the functional PRLR receptors in breast cancer cell lines was confirmed by analysing the PRL-induced intracellular signalling (Fig. 1B). PRL stimulated a robust phosphorylation of STAT5 in T47D, the cells with a high level of PRLR expression, as well as in AU565 and MCF7 cells expressing low levels of the receptor. Thus, even a low expression level of PRLR was sufficient to mediate a potent response to PRL in breast cancer cell lines.

The selected panel of cell lines was used to characterise specificity of six commercially available antibodies by Western blotting (Fig. 1C). Three of the antibodies, 1A2B1, 250448 and H-300, revealed similar staining pattern of immuno-blotted proteins with the major band having relative electrophoretic mobility of approximately 80 kDa. This band most likely represented the long receptor isoform as a protein with the same electrophoretic mobility that was detected in the CHO–PRLR transfected cells. The weaker bands with higher electrophoretic mobility might represent other isoforms of PRLR expressed in cancer cells or degradation products similar to those present in the CHO–PRLR lysates. The relative PRLR expression level detected with these antibodies correlated with PRL binding (Fig. 1A).

Specificity of the PRLR antibodies assessed by immunoprecipitation and western blotting

The B6.2 mAb was generated by Colcher et al. (1981) against membranes of metastatic human breast cancer cells. The antigen of B6.2 was described as being an N-glycosylated form of PRLR (Banerjee et al. 1993). The authors reported that blocking N-linked glycosylation by tunicamycin treatment fully abrogated binding of B6.2 to the T47D breast cancer cells. Thus, the differences in staining pattern shown in Fig. 1C may be attributed to a cancer-specific glycosylation pattern in the selected cell lines. To check this possibility, the protein extracts from two cell lines, T47D and AU565, were treated with PNGase F to remove N-linked carbohydrates and immunoblotted with the six antibodies (Fig. 2). The protein recognised by 1A2B1 and H-300 had a higher electrophoretic mobility when treated with PNGase F, indicating that N-linked glycans were successfully removed from the detected protein. These antibodies seem to recognise the glycosylated and non-glycosylated antigen equally well. The mAb 250448, however, preferentially stained the glycosylated form of the antigen. Unexpectedly, the electrophoretic mobility of the protein recognised by B6.2 was unaffected by PNGase F treatment, indicating that the mAb B6.2 may not recognise the same protein as 1A2B1 and H-300. Immunoreactivity of PRLRi was not affected by PNGase F treatment either, suggesting that the PRLRi pAb antigen is not N-glycosylated.

The specificity of the antibodies supposedly recognising the same protein, namely the long PRLR isoform, was further analysed by cross-immunoprecipitation of their antigens from the T47D cell extracts, untreated or treated with PNGase F (Fig. 3). All analysed antibodies efficiently pulled down their antigens. 1A2B1, 250448 and H-300 recognised
proteins of approximately 80 and 75 kDa for the glycosylated and deglycosylated forms respectively. In addition, the ICD-specific H-300 pAb pulled down a protein of \( \sim 70 \) kDa, which may represent another isoform of PRLR, which is not recognised by the ECD-specific 1A2B1 and 250448. The B6.2 mAb failed to detect the antigen immunoprecipitated by 1A2B1, 250448 and H-300. However, H-300 and 1A2B1 detected PRLR among the proteins pulled down by B6.2. Taken together, these data indicate that the B6.2 epitope is not present on PRLR itself, but rather on a protein associated with PRLR.

Antibody-mediated blocking of PRL signalling

The B6.2 mAb was reported to block PRL binding to the cell membranes and to inhibit proliferation of T47D cells (Banerjee et al. 1993). Here, we analysed B6.2 along with the three other anti-ECD PRLR antibodies, 1A2B1, 250448 and U5, for the ability to block PRL-induced signalling in T47D (Fig. 4). Only mAb 250448 was able to inhibit ERK1/2 and STAT5 phosphorylation, the two major PRLR-mediated signalling events.

Identification of the protein detected by the PRLRi antibody

The fact that the PRLRi antigen was not N-glycosylated was surprising as the intermediate PRLR isoform was described to be identical to the long PRLR isoform in its extracellular part, and thus the N-glycosylation pattern was expected to be the same. Therefore, we attempted to identify the protein recognized by the PRLRi pAb. The PRLRi antigen was purified by affinity chromatography on a PRLRi pAb–protein

![Diagram](image-url)
A sepharose column. The proteins eluted from the column were separated on a two-dimensional gel and subjected to a sequence analysis. Surprisingly, three eluted proteins with the electrophoretic mobility of \( \approx 48 \) kDa and approximate pI 5.0–6.0 were identified as cytokeratin-8. Indeed, the protein(s) eluted from the column, was cross-reactive with an anti-cytokeratin 8/18 mAb as assessed by western blotting (Fig. 5A). The anti-PRLR ECD mAb 1A2B1 did not recognise the eluted protein(s). Since the technique used here allows purification of both the PRLRi pAb immunoreactive protein itself as well as protein(s) associated with it, we further tested specificity of the PRLRi pAb using recombinant cytokeratin-8 and its known interacting partners, cytokeratin-18 and cytokeratin-19 (Fig. 5B). Western blotting analysis revealed that the PRLRi pAb specifically stained only cytokeratin-18, but not keratin-8 or keratin-19. Thus, the protein recognised by the PRLRi pAb in our panel of cancer cell lines is most likely cytokeratin-18.

**SPECIFICITY OF THE ANTIBODIES ASSESSED BY IHC ON CELL LINES**

The specificity of the selected antibodies was further evaluated using an IHC technique on formalin-fixed, paraffin-embedded sections exposed to high-pH buffer heat-induced epitope retrieval. The 1A2B1 and H-300 antibodies strongly stained paraffin-embedded CHO–PRLR cells (Fig. 6E and Q respectively), but not the parental CHO–K1 cells (Fig. 6F and R respectively) demonstrating that these antibodies are able to bind the long PRLR isoform in the IHC setting. Meanwhile, the 250448 antibody stained only the CHO–PRLR cells very weakly (Fig. 6I), and thus seemed not to be suitable for the IHC application at the conditions used. In correlation with the data obtained by western blotting, the B6.2 and U5 antibodies did not stain PRLR–transfected CHO cells (Fig. 6A and M respectively).

When our panel of human breast and colon cancer cell lines was analysed by IHC, the staining pattern reflected very well that obtained by western blotting. This is illustrated for the T47D and AU565 cells: B6.2 strongly stained AU565 cells, whereas hardly any staining was observed on T47D cells (Fig. 6D and C respectively). By contrast, 1A2B1 and H-300 strongly stained T47D cells (Fig. 6G and S respectively) and only weakly AU565 cells (Fig. 6H and T respectively). The immunoreactivity of 1A2B1 on T47D cells seems to localise to the cell membrane, while H-300 staining appeared to be primarily in the cytoplasm. The U5 antibody stained neither T47D nor AU565 cells (Fig. 6O and P respectively).

**IHC STAINING OF HUMAN PLACENTA**

Human placenta has previously been reported to express relatively high levels of the PRLR mRNA (Kline et al. 1999, Trott et al. 2003), and was therefore used to further check specificity of the selected antibodies by IHC on tissue sections. The mAb 1A2B1 stained decidual cells in early and term pregnancy (Fig. 7B and F respectively). Trophoblasts and glandular epithelium were not stained by this antibody (Fig. 7J and B respectively), indicating that the decidual cells are the only cell type in the placenta expressing significant levels of
the long PRLR isoform. Pre-incubation of the mAb 1A2B1 with rECD–PRLR completely abolished immunostaining indicating specificity of the signal (data not shown). With the H-300 antibody, a weak staining was apparent in decidual cells of the term placenta (Fig. 7G) and even weaker in the early placenta (Fig. 7C). The B6.2 antibody did not immunostain decidual cells, glandular epithelium or trophoblasts (Fig. 7A, E and I). The only cells stained with B6.2 in the first trimester and the term placenta sections were immune cells (Fig. 7M). These included polymorphonuclear granulocytes and monocytes/macrophages as based on their morphology. By contrast, immune cells were not stained by 1A2B1 and H-300 (Fig. 7N and O respectively), strongly supporting the notion that the protein(s) recognised by mAb B6.2 is not the long PRLR isoform. When placenta was immunostained with the PRLRi pAb, strong reactivity was observed in trophoblasts (Fig. 7L and P). In first trimester decidua, glandular epithelium but not decidual cells showed immunoreactivity (Fig. 7D), whereas widespread staining was observed in term decidua (Fig. 7H). Immune cells were not stained by the PRLRi pAb (Fig. 7P). These data show that the 1A2B1 mAb and PRLRi pAb stain different cell types, strongly indicating that the PRLR ECD epitope recognised by mAb 1A2B1 is not present in the protein(s) that binds the PRLRi pAb. The staining pattern of PRLRi pAb on placenta was similar to that reported for cytokeratin-18 (Heffter et al. 2001).

**IHC staining of human breast cancer sections**

The selected antibodies were further used for staining of full-size paraffin sections of human mammary carcinomas. In agreement with previous reports (Mertani et al. 1998, Bhatavdekar et al. 2000, Gill et al. 2001, Glasow et al. 2001), mAb B6.2 was found to stain cancer cells in eight out of ten cases of breast tumours. B6.2 immunoreactivity was most often heterogeneous with very strong staining intensity observed in six out of ten tumours (Fig. 8A). Normal adjacent breast epithelium that was available in eight of the samples also demonstrated B6.2 immunoreactivity with strong staining observed in four cases (Fig. 8E). With mAb 1A2B1, very weak immunoreactivity was detected in a subset of cancer cells in one of these mammary carcinoma samples (Fig. 8B). No significant immunostaining was observed in normal adjacent breast epithelium (Fig. 8F). Application of the H-300 antibody on the human breast carcinoma sections did not result in any significant staining of either cancer cells or normal adjacent epithelium (Fig. 8C and G). Moderately
to strongly, PRLRi pAb-immunostained cancer cells were observed in 70% of cases (Fig. 8D). The staining was most often heterogeneous with cancer cells showing negative, weak, moderate and strong staining in the same tumour section. Normal adjacent breast epithelium was present in 80% of the breast cancer samples. As illustrated in Fig. 8H, normal epithelium most often showed heterogeneous immunostaining with moderately to strongly positive cells observed next to apparently negative cells. Normal epithelium was found to be stained also in cases where all the cancer cells were negative. This staining pattern of PRLRi pAb correlated very well with that of cytokeratin-18, as previously described (Woelfle et al. 2004).

The 1A2B1 antibody was applied to paraffin sections of TMA blocks representing 160 human mammary carcinomas. A paraffin section of the CHO cells transfected with the long PRLR isoform was included in the experiment as positive control and showed strong immunoreactivity with the 1A2B1
mAb (data not shown). The vast majority of the breast cancer samples displayed no significant reactivity with the 1A2B1 antibody (97%), indicating that these tumours did not express PRLR at levels that could be detected with the applied method. Only four samples showed a weak immunoreactivity. Other clinical markers, ER, PR, the proliferation marker Ki67 and HER2 were analysed using the same TMA blocks. We detected the ER immunoreactivity in 82% of the samples, PR in 65%, Ki67 in 36% and HER2 in 11% of cases. Representative samples of 1A2B1, ER and HER2 immunostaining are shown in Fig. 9.

Discussion

In the present study, six commercially available anti-PRLR antibodies were characterised for the specificity of antigen recognition using Western blotting, immunoprecipitation and IHC techniques as summarised in Table 2. Two mAb, 1A2B1 and 250448, both raised against the extracellular domain of PRLR, seemed to stain specifically the human PRLR in these applications. They recognised a protein with a relative electrophoretic mobility of approximately 80 kDa expressed in CHO cells transfected with cDNA encoding the long isoform of PRLR and in T47D breast cancer cells. The 1A2B1 antibody stained both the N-glycosylated and non-glycosylated receptor, while mAb 250448 immunoreactivity seemed to be glycosylation dependent. In the IHC application, mAb 1A2B1 showed by far stronger staining than 250448, which may be explained by recognition of different epitopes by the two antibodies. It is possible that stronger staining could be obtained with mAb 250448 under other experimental conditions, for example by using enzymatic digestion for epitope retrieval on paraffin sections or by using cryosections.

PRL binding to the cell surface was found to correlate with expression of the long form of the PRLR, suggesting that this isoform is the major PRL-binding protein expressed in breast cancer and colon cancer cell lines examined in the present study. The T47D cells exhibited strong PRL binding and corresponding high levels of the long PRLR isoform expression detected by Western blot. All the other cancer cell lines demonstrated relatively weak PRL binding and markedly lower expression levels of the long PRLR isoform. In addition, we have examined expression of PRLR in a larger panel of human breast cancer cell lines. Only 1 out of 13 (BT-483) expressed PRLR at a level comparable with that of T47D, while in the other cell lines the signal intensity was at the border of the detection limit at the conditions used (Fig. 10).

The pAb H-300 was raised against the ICD of the long PRLR isoform. In addition to the 80 kDa protein recognised by the mAb 1A2B1 and 250448, the H-300 antibody recognised a 70 kDa protein in immunoprecipitation experiments, which may represent the ΔS1 isoform of PRLR that has the same ICD as the long isoform, but lacks a large part of ECD (Kline et al. 2002) and thus may not be recognised by the ECD-specific 1A2B1 and 250448.

The anti-rat PRLR U5 antibody did not work in any of the applications tested in the present study. To control the quality of the antibody batch, we tested it by Western blotting using lysates of the Nb2 rat lymphoma cells and human rECD-PRLR. The mAb U5 recognised at least three proteins (electrophoretic mobility of ~90, 60 and 40 kDa) in rat cell extracts, but failed to stain ECD PRLR. Meanwhile, 1A2B1 did not stain rat proteins, but recognised ECD PRLR (data not shown). Thus, lack of staining of human cells by U5 is

![Figure 9 Lack of PRLR overexpression in human breast cancer. Immunohistochemical analysis of PRLR (A), ER (B) and HER2 (C) in tissue microarray (TMA) slides comprising 160 cases of human mammary carcinomas. Pictures of representative samples are shown.](image-url)

Table 2 Properties of the antibodies used in the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6.2</td>
<td>Putative PRLR-interacting protein</td>
<td>WB, IP, IHC, FACS</td>
</tr>
<tr>
<td>1A2B1</td>
<td>Preferentially stain the long PRLR isoform</td>
<td>WB, IP, IHC, FACS, non-function blocking</td>
</tr>
<tr>
<td>250448</td>
<td>N-glycosylated form of PRLR</td>
<td>WB, IP, function blocking</td>
</tr>
<tr>
<td>U5</td>
<td>Rat PRL; no cross-reactivity with human PRLR</td>
<td>None for human PRLR</td>
</tr>
<tr>
<td>H-300</td>
<td>Long and presumably ΔS1 isoforms of human PRLR</td>
<td>WB, IP</td>
</tr>
<tr>
<td>Anti-PRLR intermediate isoform</td>
<td>Cross-reactivity with cytokeratin-18 in WB, IHC</td>
<td>Stain cytokeratin-18 in WB, IHC</td>
</tr>
</tbody>
</table>
most likely due to the lack of cross-reactivity with the PRLR of human origin. Although U5 was previously used to evaluate PRLR expression in human placenta (Maaskant et al. 1996) and human breast tumours (Touraine et al. 1998), our data suggest that this antibody does not recognise the human receptor at least under conditions used.

The PRLR isoform was raised against a peptide that is present in the ICD of the intermediate PRLR isoform, but is not found in any other isoforms of PRLR. Since the ECD of PRLR is described to be identical to that of the long PRLR isoform, it was expected that the PRLR protein will be simultaneously recognised by both ECD- and ICD-specific antibodies. As control, we have expressed the PRLR isoform generated by site-directed mutagenesis according to the sequence published by Kline et al. (1999) in CHO cells. This protein was equally well stained by both 1A2B1 and PRLR antibodies (data not shown). However, in human cancer cells, the mAb 1A2B1 did not recognise the PRLR isoform-specific splice junction, which would correspond to the unique nucleotide sequence around the PRLR isoform-specific splice junction, supporting our observation that PRLR is very rarely expressed in primary human breast cancer cells. Neither in cancer cell lines nor in human tissues, an overlap was observed between the staining obtained with the PRLR and the mAb 1A2B1. Taken together, these data indicate that the protein detected in our experiments by the PRLR isoform-specific splice junction, does not seem to be highly expressed in the analysed samples. It is worth noticing that we failed to find any entries in the expressed sequence tag (EST) database, which would correspond to the unique nucleotide sequence surrounding the PRLR isoform-specific splice junction, supporting our observation that PRLR is very rarely expressed in human tissues.

Here, we show that the PRLR isoform-specific splice junction reacts with cytokeratin-18. Cytokeratin-18 does not share significant overall sequence homology with PRLR isoform-specific splice junction, harbouring a stretch of amino acid residues that can be aligned with a peptide derived from cytokeratin-18 (Fig. 5C), which might account for the cross-reactivity. Cytokeratin-18 is highly expressed in human glandular epithelium as well as in a variety of breast, lung and colon carcinomas (Moll et al. 1982). In agreement with this described expression pattern, we detected the ‘PRLR isoform-specific splice junction protein’ immunoreactivity in multiple human cancer cell lines of different origin, but never in sarcomas, lymphomas or leukemias (data not shown). In the IHC application, the PRLR isoform-specific splice junction protein was found in cancer cell lines, breast tumours and placental trophoblasts with a staining pattern similar to that described in the literature for cytokeratin-18 (Heffler et al. 2001, Woelfle et al. 2004). We conclude that the PRLR isoform-specific splice junction protein observed in all our experiments is indeed cytokeratin-18.

The B6.2 mAb was raised against membranes of human metastatic breast cancer cells (Colcher et al. 1981) and characterised as the mAb against an N-glycopeptide present on PRLR (Banerjee et al. 1993). In this study, we demonstrate that the B6.2 epitope is not sensitive to PNGase F treatment and thus can not represent the glycosylation pattern of the PRLR protein. The B6.2 epitope cannot be immunoprecipitated by three other anti-PRLR antibodies (1A2B1, 250448 and H-300), whereas H-300 and 1A2B1 detected PRLR among the proteins pulled down with B6.2 under the conditions used. This indicates that B6.2 recognises a protein that is directly or indirectly associated with PRLR. The absence of the B6.2 antigen among the proteins pulled down with 1A2B1, 250448 and H-300 antibodies suggests that PRLR is not accessible when it is in the complex with the B6.2 antigen, or that only a minor subpopulation of PRLR is associated with the B6.2 antigen. The B6.2 epitope can be detected by flow cytometry on unfixed cancer cells (data not shown), and thus it may be colocalised with PRLR on a cell membrane and has an extracellular domain. It remains to be elucidated whether association of PRLR with the B6.2 antigen has functional implications. Banerjee et al. (1993) demonstrated that the B6.2 antibody blocked PRL-induced proliferation of T47D cells. However, we were not able to reproduce these data at the concentration of B6.2 up to 40 μg/ml, while mAb 250448 blocked PRL-induced T47D proliferation by 50% (data not shown), neither had we observed inhibition of the PRL-induced signalling by the B6.2 mAb. Meanwhile,

Figure 10 Western blot analysis of protein extracts from the indicated human breast cancer cell lines. PRLR expression was detected using the 1A2B1 mAb. β-Actin staining was used as loading control.
the PRLR signalling was drastically inhibited by the 250448 mAb.

Several studies have reported binding of B6.2 to cancer cells in 70–95% of human mammary carcinomas (Colcher et al. 1981, Hand et al. 1983, Mertani et al. 1998, Bhatavdekar et al. 2000, Gill et al. 2001, Glasow et al. 2001). Accordingly, we found the B6.2 immunoreactivity in eight out of ten human breast cancer samples. In agreement with the data generated by western blotting, the B6.2 immunoreactivity could not be detected in various cell types expressing significant PRLR levels (CHO–PRLR and decidual cells). The B6.2 antibody was selected as an antibody reactive with human mammary tumour cells and not with normal human tissues (Colcher et al. 1981, Hand et al. 1983). In the present study, B6.2 was found to bind not only to breast cancer cells, but also to normal adjacent breast epithelium and to a subset of immune cells present in human breast carcinomas and human placenta tissue samples. We have used heat-induced antigen retrieval to increase the sensitivity of the IHC method that might explain visualisation of the B6.2 immunoreactivity in cell types, where it was not previously detected.

The 1A2B1 antibody strongly stained the long form of PRLR in transfected cells in the IHC application and detected PRLR expression in T47D cells. Staining of human placenta revealed expression of the PRLR protein in decidual cells, but not in trophoblasts. This observation contradicts the data published by Maaskant et al. 1996, who reported expression of the PRLR mRNA in both cell types. In the latter study, in situ detection of PRLR mRNA was performed using mixed probes to sequences in the intracellular as well as the extracellular part of the receptor with possible detection of multiple PRLR isoforms. Taken together, these data suggest that human placenta trophoblasts may express other isoform(s) of PRLR, which are not recognised by the 1A2B1 antibody.

PRLR expression level in breast cancer cell lines seems to reflect very well that of primary breast cancer cells. The 160 cases of human breast carcinomas that were analysed by IHC in the present study were found to express low/undetectable levels of the long form of PRLR at the conditions used. The clinical material used in this study has been treated according to the standard procedure following the international guidelines. Therefore, overfixation of the material is not likely to account for the observed lack of the PRLR immunoreactivity. Furthermore, in the same TMA blocks, we detected expression of a number of commonly used clinical markers (ER, PR, the proliferation marker Ki67 and HER2) at the frequencies, which are generally reported for breast cancer samples. In agreement with our results, low expression level of the PRLR transcript in breast cancer cell lines and primary breast cancer cells has been reported (Ormandy et al. 1997, Touraine et al. 1998, Peirce & Chen 2001, Meng et al. 2004). Relative quantification of the PRLR mRNA performed by Touraine et al. revealed levels 30–100-fold below that found in T47D cells in 14 out of 15 breast cancer samples. As shown in Fig. 4, PRL stimulation did, however, induce intracellular signalling in breast cancer cell lines such as AU565 and MCF7, indicating that even very low expression level of the long form of PRLR is sufficient to mediate PRL responsiveness.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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