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# Immunocytochemical localisation of plasma membrane GHRH receptors in human tumours using a novel anti-peptide antibody

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ABSTRACT

Antagonists of growth hormone releasing hormone (GHRH) directly inhibit the growth of a variety of human neoplasms. However, the plasma membrane receptor mediating these effects has not been immunocytochemically visualised in primary tumour cells. Given that previous attempts using an antibody to the amino-terminal region did not result in the visualisation of plasma membrane receptors, we have developed and characterised an anti-peptide antibody to the carboxy-terminal region 403–422 of the human pituitary GHRH receptor. This sequence is identical to residues 339–358 of splice variant 1 (SV1) of tumoural GHRH receptors. Specificity of the antibody was demonstrated by (1) immunocytochemical staining of GHRH receptor-transfected cells, (2) detection of a broad glycosylated protein band migrating at $M_r$ 50,000–60,000 in Western blots of membranes from human pituitary, and (3) abolition of tissue immunostaining by preadsorbtion of the antibody with its immunising peptide. The distribution of GHRH receptors was investigated in 69 formalin-fixed, paraffin-embedded human tumours showing that GHRH receptors were frequently expressed in breast, ovarian and prostate carcinomas. Immunoreactive GHRH receptors were clearly confined to the plasma membrane and uniformly present on nearly all tumour cells. In Western blots of membranes prepared from human tumours, the anti-GHRH receptor antibody detected a non-glycosylated protein band migrating at $M_r$ 40,000, which corresponds to the expected molecular weight of splice variant 1 of tumoural GHRH receptors. Together, our findings provide direct evidence for the presence of GHRH receptor protein on the plasma membrane of primary human tumour cells. The GHRH receptor visualisation could be of value for a rapid immunohistochemical identification of those tumours which could be a target for diagnostic or therapeutic intervention using GHRH analogues.


## 1. Introduction

Growth hormone releasing hormone (GHRH) is a hypothalamic peptide that upon binding to specific G protein-coupled receptors stimulates the synthesis and secretion of growth hormone (GH) from the anterior pituitary gland.[1,2] GH in turn induces hepatic production of insulin-like growth factor (IGF-I), which is a known mitogen for various cell types.[1,3] GHRH antagonists have been shown to effectively inhibit *in vivo* growth of a number of experimental human cancers such as

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prostate, mammary, and ovarian cancers, renal carcinomas, small-cell lung cancer (SCLC) and non-SCLC, pancreatic and colorectal carcinomas, and malignant glioblastomas.[4–11] The antitumour effects of GHRH antagonists were initially thought to be exerted only indirectly through inhibition of the pituitary GH/hepatic IGF-I axis.[3] However, it was later shown that GHRH antagonists also inhibit the proliferation of various human cultured cancer cell lines *in vitro*.[5,8–12] Such direct antiproliferative action would have to be mediated through specific membrane-bound GHRH receptors on the tumour cells.

Recent evidence suggests that various experimental cancers express splice variants of GHRH receptors.[6,13,14] Among these tumoural forms of GHRH receptors, the splice variant 1 (SV1) displays the greatest similarity to the pituitary GHRH receptor and is predominantly detected in human tumors.[12,13,15] SV1 and the pituitary GHRH receptor differ only in the first three exons, encoding a part of the extracellular domain of the receptor that in SV1 has been replaced by a fragment of intron 3, which has a new putative in-frame start codon.[13] Consequently, the amino-terminal domain of SV1 is considerably shorter and does not contain the putative glycosylation site.[13] SV1-transfected 3T3 cells and tumours expressing SV1 mRNA display high affinity binding sites for the GHRH antagonist JV-1-42 suggesting that SV1 encodes a fully functional GHRH receptor.[6,12,15]

GHRH is a member of the superfamily of structurally related peptide hormones that includes vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), secretin, and glucagons.[1] Given the structural similarity between VIP and GHRH as well as considerable homology between their cognate receptor proteins, GHRH antagonists may interact with VIP/PACAP receptors which are known to be highly abundant in human normal and neoplastic tissues.[9,11,16] It is therefore crucial to demonstrate the presence of GHRH receptor protein on the plasma membrane of human tumour cells.

Although earlier studies have reported the generation and characterisation of GHRH receptor antibodies, immunoreactive GHRH receptors have not been visualised in extra-pituitary tissues.[17,18] More recently tumoural GHRH receptors were detected in Western blots of crude membrane extracts from tumour cell lines using antibodies developed to the amino-terminal region of SV1.[19–21] In an effort to visualise GHRH receptors directly in the plasma membrane of primary tumour cells, we have now generated and characterised an antibody directed to the carboxy-terminal sequence of the human pituitary GHRH receptor. This sequence is identical in the pituitary-type GHRH receptor and SV1 of tumoural GHRH receptors. We have also developed an immunohistochemical protocol that allows efficient detection of GHRH receptors in formalin-fixed, paraffin-embedded human tissues. The generation of this novel antibody enabled us to visualise plasma membrane GHRH receptors directly in a variety of human malignancies.

## 2. Materials and methods

### 2.1. Patients, tumours and tissue preparation

Sixty-nine tumour specimens were retrieved from the archives of the Department of Pathology of the Otto-von-Guericke-University Magdeburg. All tissue specimens had been fixed in formalin and embedded in paraffin. The following tumours were investigated: breast carcinoma ($n = 8$); ovarian carcinoma ($n = 10$); prostate cancer ($n = 6$); thyroid carcinoma ($n = 6$); carcinoid ($n = 15$); pancreatic insulinoma ($n = 8$); growth hormone-producing pituitary adenoma ($n = 4$); pheochromocytoma ($n = 4$); glioblastoma ($n = 4$) and meningioma ($n = 4$). In addition, fresh tumour specimens including GH-producing pituitary adenoma ($n = 2$) and breast carcinoma ($n = 4$) as well as adjacent non-cancerous tissues ($n = 2$) were immediately frozen in liquid $N_2$ and stored at −70 °C until Western blot analysis.

### 2.2. Generation and purification of anti-GHRH receptor antibodies

Polyclonal anti-peptide antibodies were generated against the carboxy-terminal tail of the human pituitary GHRH receptor. The identity of the peptide was RTRAKWTTPSRSAAKVLTSM which corresponds to residues 403–422 of the human pituitary GHRH receptor. This sequence is identical to residues 339–358 of SV1 of tumoural GHRH receptors. The peptide was synthesised, purified and coupled to keyhole limpet hemocyanin as described. The conjugates were mixed 1:1 with Freund's adjuvant and injected into groups of three rabbits 6 for GHRH receptor antisera production. Animals were injected at 4 week intervals, and serum was obtained 2 weeks after immunisations beginning with the second injection.

### 2.3. Immuno-dot blot analysis

The specificity of the antisera as well as possible cross-reactivity with VIP/PACAP receptors was initially tested using immuno-dot blot analysis as described. Serial dilutions of the unconjugated peptides corresponding to the carboxy-terminal sequences of the GHRH, $VPAC_1$, $VPAC_2$ and $PAC_1$ receptors were blotted onto nitrocellulose membranes. The identities of the peptides were: TRVSPGARRSSSFQAEVSLV, which corresponds to residues 438–457 of the human $VPAC_1$ receptor; LQFHRGSRAQSFLQTETSVI, which corresponds to residues 419–438 of the human $VPAC_2$ receptor; LSKSSSQIRMSGLPADNLAT, which corresponds to residues 506–525 of the human $PAC_1$ receptor and RTRAKWTTPSRSAAKVLTSM, which corresponds to residues 403–422 of the human GHRH receptor. Membranes were then incubated with the antisera at dilutions ranging from 1:1000 to 1:20,000 for 60 min at room temperature.[16,22] Blots were developed using the enhanced chemiluminescence method (Amersham, Braunschweig, Germany). For subsequent analysis, antibodies were affinity purified against their immunising peptides using the Sulfo-Link coupling gel according to the instructions of the manufacturer (Pierce, Rockford, IL).

### 2.4. Immunocytochemistry

A plasmid encoding the pituitary-type GHRH receptor was obtained from www.cdna.org (UMR cDNA Resource Center, Rolla, MO). Human embryonic kidney 293 (HEK-293) cells were transiently transfected with either GHRH receptor or empty vector (MOCK). Cells were grown on coverslips for 48 h, fixed

and incubated with 1 μg/ml anti-GHRH receptor antibody {9017} followed by cyanin 3.18-conjugated secondary antibodies (Amersham, Braunschweig, Germany). Specimens were mounted and examined using a Leica TCS-NT laser scanning confocal microscope as described.[16,22–24]

### 2.5. Western blot analysis

Membranes were prepared from fresh tumour specimens. Tissues were lysed in homogenisation buffer (5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 10 mM Tris–HCl, pH 7.6 containing 1 mM phenylmethylsulfonylfluoride, 1 μM pepstatin, 10 μg/ml leupeptin and 2 μg/ml aprotinin), and membranes were pelleted at 20,000 × g for 30 min at 4 °C. Membranes were then dissolved in lysis buffer (150 mM NaCl, 5 mM EDTA, 3 mM EGTA, 20 mM Hepes, pH 7.4 containing 4 mg/ml dodecyl-beta-maltoside and proteinase inhibitors as above). Subsequently, either these crude membrane preparations or partially-purified glycoproteins were subjected to SDS polyacrylamide gel electrophoresis. For enrichment of glycoproteins, membrane preparations were incubated with 150 μl wheat germ lectin agarose beads (Amersham) for 90 min at 4 °C. Beads were washed five times in lysis buffer, and adsorbed glycoproteins were eluted with SDS-sample buffer for 20 min at 60 °C. Samples were then size separated on 10% SDS polyacrylamide gels and immunoblotted onto nitrocellulose.[16,22–24] Blots were incubated with 1 μg/ml anti-GHRH receptor antibody {9017} followed by peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection (Amersham). For adsorption controls, antibodies were preincubated with 10 μg/ml of their cognate peptides for 2 h at room temperature.[16,22–24]

### 2.6. Immunohistochemistry

Seven micrometers paraffin sections were cut, floated onto positively charged slides and immunohistochemically stained as described.[16,22,24] Briefly, sections were dewaxed, microwaved in 10 mM citric acid (pH 6.0) for 20 min at 600 W and subsequently incubated with 2 μg/ml anti-GHRH receptor antibody {9017} overnight at 4 °C. Staining of primary antibody was detected using biotinylated goat anti-rabbit IgG followed by an incubation with avidin-biotinylated peroxidase solution. Tissue was then rinsed and stained with 3,3′-diaminobenzidine-glucose oxidase for 15 min. Cell nuclei were lightly counterstained with hematoxylin. For immunohistochemical controls, the primary antibody was either omitted, replaced by preimmune serum or adsorbed with several concentrations ranging from 1 to 10 μg/ml of homologous or heterologous peptides for 2 h at room temperature. A tumour known to stain positively was included in each batch of staining as a positive control.

### 2.7. Assessment of staining patterns

All slides were evaluated by the same investigator. The presence or absence of staining and the depth of colour were noted, as well as the number of cells showing a positive reaction and whether or not the staining was localised to the plasma membrane. Tumours were only categorised as positive when they exhibited a moderate to strong plasma membrane staining in the majority of tumour cells which was easily visible with a low-power objective.

## 3. Results

### 3.1. Characterisation of GHRH receptor antibodies

Specificity and selectivity of the antisera was initially monitored using immuno-dot blot analysis. After four booster injections, all three anti-GHRH antisera developed a titer against their immunising peptide. As shown in Fig. 1, the antiserum {9017} specifically detected quantities as low as 50 ng of its cognate peptide but did not detect the peptides corresponding to the carboxy-terminal regions of the VIP/PACAP receptor subtypes $VPAC_1$, $VPAC_2$, $PAC_1$. Antisera were affinity purified and further characterised using immunofluorescent staining of transfected cells. When HEK-293 cells were transiently transfected with a plasmid encoding the pituitary GHRH receptor or empty vector (MOCK) and stained with anti-GHRH receptor antibody {9017}, prominent immunofluorescence was only detected in cells expressing the GHRH receptor but not in MOCK-transfected cells (Fig. 2). In HEK 293 cells, immunoreactive GHRH receptors were detected at the plasma membrane as well as throughout the cytosol (Fig. 2). GHRH receptor antisera were then tested for possible cross-reactivity with other proteins present in human tissues using Western blot analysis. When glycoproteins enriched from membranes of GH-producing pituitary adenoma were subjected to Western blot analysis, the GHRH receptor antiserum {9017} revealed two immunoreactive bands migrating in the range of $M_r$ 50,000 to 60,000 (Fig. 3A). These bands may represent differently glycosylated forms of the pituitary GHRH receptor. In addition, a

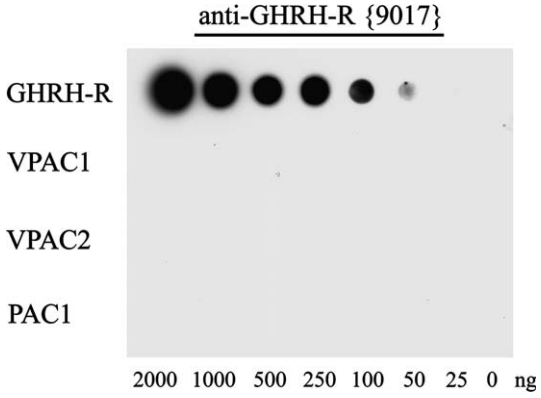


**Fig. 1 – Immuno-dot blot analysis of the specificity of anti-GHRH receptor antibodies. Serial dilutions (0–2000 ng) of the peptides corresponding to the carboxy-terminal regions of the GHRH, $VPAC_1$, $VPAC_2$ and $PAC_1$ receptors were blotted onto nitrocellulose membranes and incubated with anti-GHRH receptor antiserum {9017} at a dilution of 1:5000. Membranes were developed using the enhanced chemiluminescence method. Note that the anti-GHRH receptor antiserum selectively detected the peptide corresponding to its cognate receptor but did not detect the peptides corresponding to closely related VIP/PACAP receptor subtypes. *GHRH-R*, GHRH receptor.**

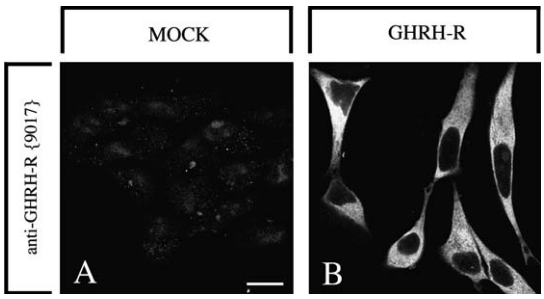


**Fig. 2 – Characterisation of anti-GHRH receptor antibodies by immunofluorescent staining of transfected cells. HEK-293 cells transiently transfected with a plasmid encoding the pituitary GHRH receptor (*GHRH-R*) or empty vector (*MOCK*). Cells were fixed and immunofluorescently stained with anti-GHRH receptor antibody {9017}. Note that prominent immunofluorescence was only detected on GHRH receptor-transfected cells. Representative results from one of three independent experiments are shown. Scale bar, *A* = *B* = 20 μm.**

higher molecular weight band was detected at $M_r$ 120,000. This band may correspond to a dimeric form of the receptor. All immunoreactive bands were completely abolished by preadsorbtion of the antibody with 10 μg/ml of its immunising peptide (Fig. 3A). When glycoproteins enriched from membranes of human breast carcinomas were subjected to Western blot analysis, the GHRH receptor antiserum {9017} did not detect any immunoreactive band suggesting that these tumours did not contain the glycosylated pituitary-form of GHRH receptors (Fig. 3B). However, when crude membrane preparations from human breast carcinoma were subjected to Western blot analysis, the GHRH receptor antiserum {9017} revealed a major immunoreactive band migrating at $M_r$ 40,000 (Fig. 3B). Given that the deglycosylated pituitary GHRH receptor migrates at $M_r$ 45,000,[2] it is very likely that the non-glycosylated protein band migrating at $M_r$ 40,000 in human breast cancer corresponds to SV1 of tumoural GHRH receptors. This immunoreactive band was also completely abolished by preadsorbtion of the antibody with 10 μg/ml of its immunising peptide (Fig. 3B).When the expression of the GHRH receptors was examined in breast cancer tissue and non-cancerous breast tissue, non-glycosylated forms of the receptor were selectively detected in neoplastic but not in the adjacent normal tissue (Fig. 3C).

### 3.2. *GHRH receptor immunohistochemical staining in human tumours*

The anti-GHRH receptor antibodies were then subjected to immunohistochemical staining of human tissues. Initial experiments showed that heat-induced epitope retrieval is required for efficient immunohistochemical staining of paraffin-embedded tissue (not shown). All three antisera yielded similar staining patterns with prominent immunoreactivity predominantly localised to the plasma membrane of the tumour cells (Fig. 4). Immunostaining for each antiserum was completely abolished by preadsorbtion with 10 μg/ml of the immunising peptide (Fig. 4). The anti-GHRH receptor antibody {9017} was then subjected to immunohistochemical staining of 69 human tumours. The prevalence of GHRH receptors in human tumours is summarised in Table 1. Immunoreactive GHRH receptors were frequently detected in human endocrine-related cancers including breast (50%), ovarian (80%) and prostate (75%) carcinoma (Fig. 4). GHRH receptors were

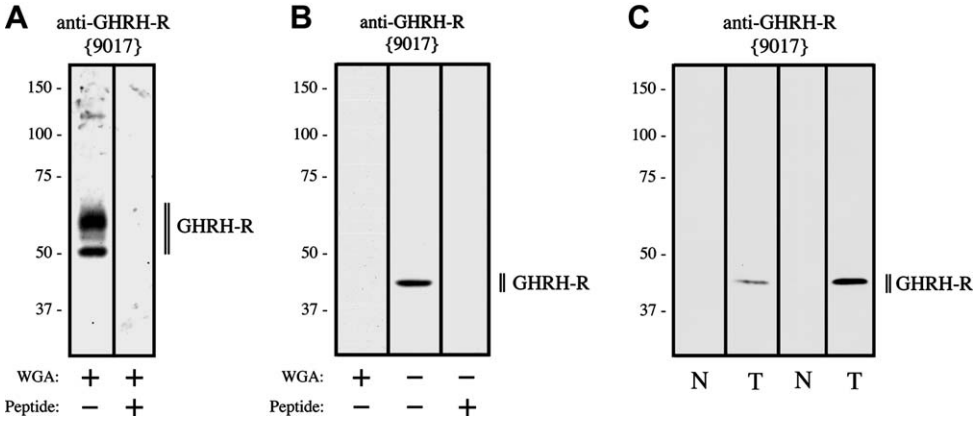


**Fig. 3 – Western blot analysis of the specificity of anti-GHRH antibodies in human tissues. *A*, glycoproteins were enriched from membranes of a GH-producing pituitary adenoma using wheat germ lectin agarose beads (*WGA* +). Glycoproteins were then separated on a 10% SDS–polyacrylamide gel, blotted onto nitrocellulose and incubated with 1 μg/ml anti-GHRH receptor antibody {9017} in the absence (*Peptide* –) or presence (*Peptide* +) of 10 μg/ml peptide antigen. *B*, glycoproteins were enriched from membranes of a breast carcinoma using wheat germ lectin agarose beads. Glycoproteins (*WGA* +) or crude membrane extracts (*WGA* –) were then separated on a 10% SDS–polyacrylamide gel, blotted onto nitrocellulose and incubated with 1 μg/ml anti-GHRH receptor antibody {9017} in the absence (*Peptide* –) or presence (*Peptide* +) of 10 μg/ml peptide antigen. *C*, crude membrane extracts from two breast carcinomas (*T*) and adjacent non-cancerous tissues (*N*) were then separated on a 10% SDS–polyacrylamide gel, blotted onto nitrocellulose and incubated with 1 μg/ml anti-GHRH receptor antibody {9017}. Blots were developed using enhanced chemiluminescence. Representative results from one of three independent experiments are shown. *GHRH-R*, GHRH receptor; *Ordinate*, migration of protein molecular weight markers ($M_r \times 10^{-3}$).**

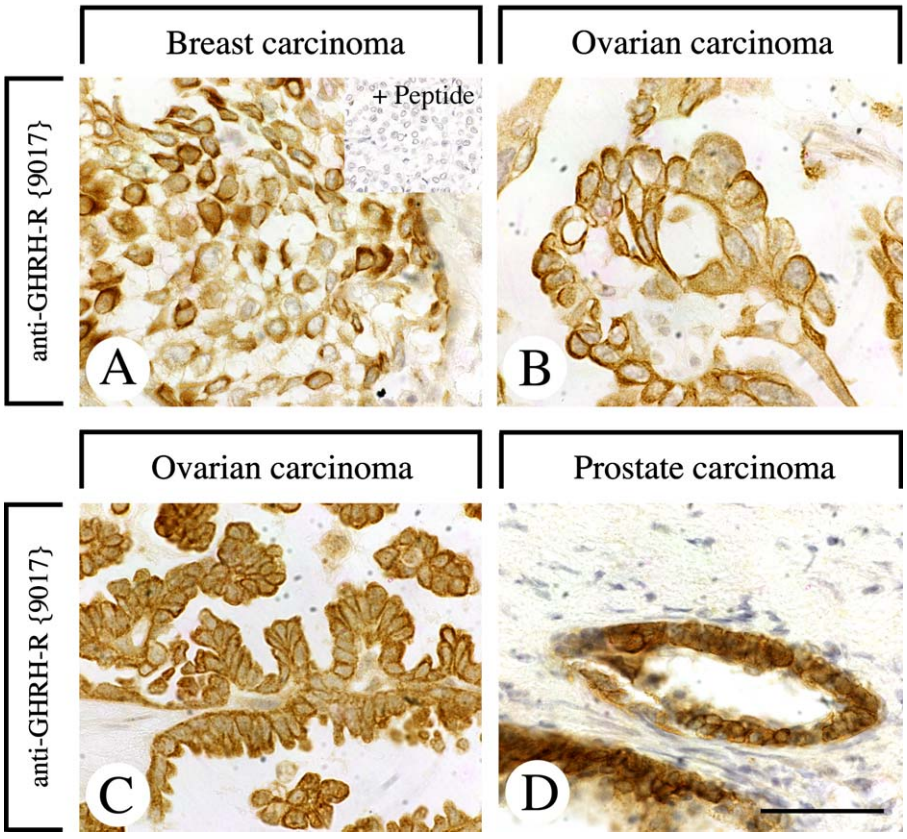


**Fig. 4 – GHRH receptor immunohistochemical staining in human endocrine-related cancers. GHRH receptor immunohistochemical staining in breast carcinoma (*A*), two ovarian carcinomas (*B*,*C*) and prostate carcinoma (*D*). Sections were dewaxed, microwaved in citric acid and incubated with affinity-purified anti-GHRH receptor antibody {9017} at a concentration of 2 μg/ml. Sections were then sequentially treated with biotinylated anti-rabbit IgG and AB solution. Sections were then developed in DAB–glucose oxidase and lightly counterstained with hematoxylin. For adsorption controls, primary antibody was incubated with 10 μg/ml of the peptide used for immunisations. Note that GHRH receptor immunoreactivity was predominantly localised at the level of the plasma membrane. *Inset*, peptide adsorption control. Representative results from one of three independent experiments are shown. Scale bar, 100 μm.**

also commonly expressed in pituitary adenoma (75%) and meningioma (100%). A highly abundant expression of GHRH receptors was also evident in glioblastoma (100%). In addition, GHRH receptors were present at the plasma membrane of neuronal cell bodies and processes adjacent to glioblastoma (not shown). In contrast, GHRH receptors were not detected in neuroendocrine tumours including carcinoid or insulinoma. GHRH receptors were also not detected in pheochromocytoma or thyroid carcinoma.

**Table 1 – Prevalence of GHRH receptors in human tumours**

| Tumour type (*n*) | GHRH-R *n* (%) |
|---|---|
| Breast cancer, ductal invasive (8) | 4 (50) |
| Ovarian carcinoma (10) | 8 (80) |
| Prostate cancer (6) | 6 (75) |
| Thyroid cancer (6) | 0 |
| Carcinoid (15) | 0 |
| Pancreatic insulinoma (8) | 0 |
| Pituitary adenoma, growth hormone-producing (4) | 3 (75) |
| Pheochromocytoma (4) | 0 |
| Glioblastoma multiforme (4) | 4 (100) |
| Meningioma (4) | 4 (100) |

## 4. Discussion

In an effort to visualise tumoural GHRH receptor protein in human primary tumours, we generated antibodies that exert selective specificity for the carboxy-terminal region of the GHRH receptor. We show that the cytoplasmic tail of the GHRH receptor can serve as an epitope for the generation of antisera that effectively stain formalin-fixed, paraffin-embedded human tissues. Several lines of evidence indicate that these antibodies specifically detect the GHRH receptor and do not crossreact. First, in immuno-dot blot assays the anti-GHRH receptor antisera selectively detected the cytoplasmic tail of the GHRH receptor but not peptides corresponding to the carboxy-terminal regions of the closely related VIP/PACAP receptors. Second, immunocytochemical staining of HEK 293 cells yielded prominent immunofluorescence only in GHRH receptor-transfected cells but not untransfected cells or cells transfected with empty vector.

Immunoreactive GHRH receptors were detected at the plasma membrane as well as throughout the cytosol. This staining pattern is identical to the GHRH receptor distribution previously reported by others for heterologous cells.[2,25] Third, in Western blots of glycoproteins enriched from GH-producing pituitary adenoma specimens, which are known to express high levels of GHRH receptors, the anti-GHRH receptor antibody detected two broad glycosylated bands migrating in the range of $M_r$ 50,000–60,000. The size and appearance of these bands is identical to pituitary GHRH receptors previously detected by photo affinity labelling.[2,25] In Western blots of membranes from human breast carcinoma, the anti-GHRH receptor antibody detected a single non-glycosylated band migrating at $M_r$ 40,000, which corresponds to the expected molecular weight of SV1 of tumoural GHRH receptors. This band was only detected in neoplastic but not in adjacent normal tissue. Fourth, tissue immunostaining of the GHRH receptor antibody was predominantly confined to the plasma membrane of the tumour cells and completely abolished by preadsorbtion with homologous but not heterologous peptides. Finally, it should be noted that three of three GHRH receptor antisera gave similar results.

GHRH antagonists effectively inhibit the growth of a number of experimental tumours including prostate, mammary, and ovarian cancers.[4,5,7–11,26] *In vivo* and *in vitro* studies revealed that in addition to their inhibition of the pituitary GH/hepatic IGF-I axis GHRH antagonists act directly on cancer cells and strongly inhibit their proliferation.[4,5,7–11,26] In some cells, proliferation is induced by the stimulatory action of local GHRH, which implies that GHRH may function as an autocrine/paracrine growth factor for various tumours such as prostate and ovarian cancer.[6,27] However, the specific membrane-bound GHRH receptor on the tumour cells which mediates these effects has not been visualised yet. Recent evidence suggests that various experimental cancers express splice variants of the GHRH receptor.[6,12,13,15,27] Among these tumoural forms of GHRH receptors, SV1 displays the greatest similarity to the pituitary GHRH receptor and is predominantly detected in human tumors.[6,12,13,15,27] Interestingly, heterologous expression of SV1 in 3T3 fibroblasts has recently been shown to activate cell proliferation responses to GHRH analogs.[12,15]

Earlier studies have reported the generation and characterisation of GHRH receptor antibodies. However, immunoreactive GHRH receptors have not been visualised in extrapituitary tissues.[17,18] More recently, tumoural GHRH receptors were detected in Western blots of crude membrane extracts from tumour cell lines using antibodies developed to the amino-terminal region of SV1.[19–21] Based on our experience with a variety of other G protein-coupled receptors,[16,22–24] we generated and characterised an antibody directed to the carboxy-terminal sequences of the GHRH receptor. Using this antibody, we provide direct evidence for the presence of GHRH receptor protein on the plasma membrane of primary human tumour cells. The availability of these novel GHRH receptor antibodies will facilitate further basic morphological investigation of GHRH receptor expression in human tumors and normal human tissues. The immunohistochemical GHRH receptor evaluation offers several major advantages. This method can analyse GHRH receptors in routinely processed archival paraffin-embedded material of any diagnostic pathology centre. It requires only an immunopathological laboratory to perform the test which can be carried out without costly and time-consuming receptor autoradiography. The immunohistochemical evaluation of the cellular GHRH receptor status of a given tumour specimen can be accomplished in less than 24 h. RT-PCR can be accomplished in a similar short time. However, RT-PCR is based on total RNA isolation from a fresh tumour sample and therefore would not only detect GHRH receptor transcripts originating from tumour cells but also from fibrocytes, lymphocytes or other non-malignant cells.

In conclusion, we have generated and extensively characterised anti-GHRH receptor antibodies. Using these antibodies we provide the first demonstration of plasma membrane GHRH receptors in a variety of human formalin-fixed, paraffin-embedded tumour tissues. In Western blots, a predominant protein band migrating at $M_r$ 40,000 which most likely corresponds to SV1 of tumoural GHRH receptors was selectively detected in neoplastic but not in adjacent normal tissue. Overexpression of GHRH receptor splice variants in some of the most frequently occurring human malignancies may provide the molecular basis for diagnostic and/or therapeutic intervention using GHRH analogues.

## Conflict of interest statement

None declared.

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