Co-expressed peptide receptors in breast cancer as a molecular basis for in vivo multireceptor tumour targeting

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Abstract. Breast cancers can express different types of peptide receptors such as somatostatin, vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP) and NPY(Y_1) receptors. The aim of this in vitro study was to evaluate which is the most appropriate peptide receptor or peptide receptor combination for in vivo diagnostic and therapeutic targeting of breast cancers. Seventy-seven primary breast cancers and 15 breast cancer lymph node metastases were investigated in vitro for their expression of somatostatin, $VPAC_1$, GRP and NPY(Y_1) receptors using in vitro receptor autoradiography on successive tissue sections with ^{125}I-[Tyr^3]-octreotide, ^{125}I-VIP, ^{125}I-[Tyr^4]-bombesin and ^{125}I-[Leu^{31},Pro^{34}]-PYY respectively. This study identified two groups of tumours: a group of 68 tumours (88%) with at least one receptor expressed at high density (>2,000 dpm/mg tissue) that may provide a strong predictive value for successful in vivo targeting, and a group of nine tumours (12%) with no receptors or only a low density of them (<2,000 dpm/mg tissue). In the group with high receptor density, 50 of the 68 tumours (74%) expressed GRP receptors, 45 (66%) expressed NPY(Y_1) receptors, 25 (37%) expressed $VPAC_1$ receptors and 14 (21%) expressed somatostatin receptors. Mean density was 9,819±530 dpm/mg tissue for GRP receptors, 9,135±579 dpm/mg for NPY(Y_1) receptors, 4,337±528 dpm/mg for somatostatin receptors and 3,437±306 dpm/mg for $VPAC_1$ receptors. It is of note that tumours expressing NPY(Y_1) or GRP receptors, or both, were found in 63/68 (93%) cases. Lymph node metastases showed a similar receptor profile to the corresponding primary tumour. This in vitro study strongly suggests that the combination of radiolabelled GRP and Y_1 analogues should allow targeting of breast carcinomas and their lymph node metastases for in vivo peptide receptor scintigraphy and radiotherapy.

Keywords: Peptide receptors – Breast cancer targeting – NPY receptors – GRP receptors – Receptor co-expression

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Introduction

Peptide receptors expressed in human cancers can be targeted in vivo with radiolabelled ligands either for diagnostic scintigraphy or for targeted peptide radiotherapy. The best example is that of somatostatin and somatostatin receptors [1, 2]. It has, however, been shown that the chances of success of somatostatin receptor scintigraphy [3, 4, 5, 6], of intraoperative tumour detection using Octreoscan [7] and of somatostatin-receptor targeted radiotherapy [2, 4, 8, 9] are greatest for those tumour types expressing somatostatin receptors at a high density.

Breast cancers can express somatostatin receptors. The latter are found in vitro in 50%–70% of the tumours [10, 11, 12], sst_{2A} being the predominant receptor subtype, as shown by receptor protein measurements from either receptor binding [13] or immunohistochemistry [14]. However, many breast cancers do not have a high somatostatin receptor density [11, 14]. Moreover, breast cancers are characterized by a strong somatostatin receptor heterogeneity in tumour samples in vitro in at least 50% of the somatostatin receptor-positive cases, with regions of high density adjacent to regions virtually devoid of somatostatin receptors [11, 14, 15]. Successful scintigraphic detection of breast cancer, both primary and metastatic, has been reported with Octreoscan; however, the percentage of positive cases varies between 50% and 94% [12, 16, 17, 18, 19]. A well-controlled study by van Eijck et al. [12] showed 70% somatostatin receptor posi-

tivity in breast cancers diagnosed at mammography, while another well-controlled study by Albérini et al. [19] found 50% positivity. Other scintigraphic studies, without concomitant in vitro receptor confirmation, have reported up to a 94% incidence of positive cases [17, 18]; such figures may represent an overestimation since non-tumoural breast tissue has also been shown to be positive on scintigraphy in 15% of cases [2]. Despite these encouraging data, up to now in vivo somatostatin receptor scintigraphy has not become a recognized tool for the routine diagnosis or radiotherapeutic somatostatin receptor targeting of breast cancers. This is probably due in large part to the insufficient amount and heterogeneous distribution of somatostatin receptors expressed by some of these tumours.

Breast cancers can express other peptide receptors than those for somatostatin. VIP receptors of the $VPAC_1$ subtype have been shown in vitro to be expressed in the majority of breast cancers and metastases [20, 21, 22]. Gastrin-releasing peptide (GPR) receptors have been found in high density in more than two-thirds of these cancers with in vitro methods [23, 24]. Recently, NPY(Y_1) receptors were also shown to be abundantly expressed in vitro in breast cancers [25]. Individually, however, none of these receptors is expressed in 100% of cases in sufficiently high density and with sufficient homogeneity to permit tumour identification and therapy of all cancer sites in all patients. Although it should theoretically be possible to combine several of the above-mentioned peptides for optimal in vivo targeting of primaries and metastases, there are presently no in vitro studies indicating which peptides would be worth combining in such a cocktail.

The aim of this study was to quantitatively compare the tissue distribution and the protein expression of four peptide receptors, namely somatostatin, VIP, GRP and NPY(Y_1) receptors, in successive sections of breast carcinomas, using receptor autoradiography. Our goal was to identify which peptide receptor or receptor combination was expressed most frequently and in highest density in breast cancers, in order to recommend to nuclear physicians the optimal peptide combination for detection and therapeutic targeting of primary tumours and metastases.

Materials and methods

Breast cancer samples from 77 patients were included in this study. They consisted of 77 primary tumours as well as 15 lymph node metastases from four of these patients. The diagnosis was reviewed and formulated by use of cryostat sections, according to the WHO guidelines stated by Tavassoli [26]. Of 77 patients, 58 (75%) were found to have an invasive ductal carcinoma. Histological evaluation identified a great majority of cases (n=45) with intermediate grade (G2), while nine cases had a low grade (G1) and four cases a high grade (G3), according to a modified Bloom-Richardson grading method [26]. There were nine invasive lobular carcinomas and two ductal carcinomas in situ, as well as three mucinous, two medullary, two apocrine and one tubular carcinoma. Using in vitro receptor autoradiography in successive sections of each tumour case, we compared the expression of somatostatin receptors (octreotide binding), VIP receptors, GRP receptors and NPY receptors of the Y_1 subtype.

Knowing from previous studies that many of these receptors are expressed heterogeneously within the breast cancers, care was taken to evaluate the receptor status on large tumour samples (one sample per tumour) in order to obtain more representative results. The mean surface area of the 77 investigated tumour sections was 138±66 mm^2. Somatostatin receptor autoradiography was performed as demonstrated previously using ^{125}I-[Tyr^3]-octreotide (2,000 Ci/mmol, Anawa) as tracer, in order to identify the binding sites recognized by octreotide and Octreoscan [11, 27]. VIP receptor autoradiography was performed with ^{125}I-VIP (2,000 Ci/mmol, Anawa) as radioligand [21]. For every single tumour, the $VPAC_1$ or $VPAC_2$ receptor subtype expression was assessed using the $VPAC_1$-selective analogue [K^{15},R^{16},L^{27}]VIP(1–7)/GRF(8–27) and the $VPAC_2$-selective analogue Ro25-1553 for selective displacement, as reported previously [21]. For GRP receptors, ^{125}I-[Tyr^4]-bombesin was used as ligand [23]. For NPY(Y_1) receptor autoradiography, ^{125}I-[Leu^{31},Pro^{34}]-PYY was used as a selective Y_1 ligand [25].

In all cases, the autoradiographs were quantified using a computer-assisted image processing system, as described previously [27]. Tissue standards for iodinated compounds (Amersham, Aylesbury, UK) were used for this purpose. A tissue was defined as receptor-positive when the absorbance measured in the total binding section was at least twice that of the non-specific binding section. In addition, by setting a cut-off point for receptor density at 2,000 dpm/mg tissue, we arbitrarily distinguished two groups of tumours: those with high receptor density (>2,000 dpm/mg tissue), that is likely to represent a clinically relevant positivity, in particular for radiotherapy, and those with low receptor density (<2,000 dpm/mg tissue) or no receptors at all. This cut-off point was based on our previous experience with somatostatin receptors [27, 28, 29].

Results

Table 1 shows the various primary breast tumour carcinomas grouped according to their receptor profile. The very strict selection of strongly receptor-positive tumours, i.e. those with a receptor density of >2,000 dpm/mg tissue, as mentioned above, should permit more conclusive assessments to be made regarding potential clinical implications for the corresponding peptide receptor group. To provide full information, Table 1 reports not only the receptor density values for high-density receptor specimens but also those for specimens with a low receptor density.

All 77 primary tumour samples showed expression of at least one of the four peptide receptors. However, nine cases did not express any of the four receptors in high density. Thus, as many as 68/77 (88%) cancers had at least one peptide receptor expressed in high density (Table 1). In these 68 cases, the receptor type most frequently expressed was the GRP receptor (50/68 cases, 74%), followed by the NPY(Y_1) receptor (45/68, 66%). A high

Table 1. Receptor profiles of the 68 primary breast cancers with at least one receptor expressed at high density (>2,000 dpm/mg tissue). Receptor densities are shown within parentheses for those cases with low receptor densities (<2,000 dpm/mg tissue). Receptor density values are expressed as mean±SEM ($n \geq 3$).

Receptor profile	No. of tumours	Somatostatin-R	VIP-R	GRP-R	NPY(Y_1)-R
4 receptors					
SS-R VIP-R GRP-R NPY(Y_1)-R	*n*=4	+ 5,616±1,023	+ 4,004±750	+ 8,383±2,644	+ 11,258±546
3 receptors					
SS-R VIP-R NPY(Y_1)-R	*n*=1	+ 3,390	+ 6,287	(183)	+ 12,452
SS-R VIP-R GRP-R	*n*=2	+ 2,391; 5,674	+ 2,237; 2,132	+ 12,014; 13,323	(161, *n*=1)
SS-R GRP-R NPY(Y_1)-R	*n*=5	+ 4,308±967	(984±113, *n*=5)	+ 12,274±846	+ 10,325±985
VIP-R GRP-R NPY(Y_1)-R	*n*=9	(1,002±219, *n*=8)	+ 3,413±462	+ 7,564±1,151	+ 9,256±1,434
2 receptors					
GRP-R NPY(Y_1)-R	*n*=14	(1,077±208, *n*=11)	(1,174±116, *n*=14)	+ 11,053±731	+ 9,751±1,156
VIP-R NPY(Y_1)-R	*n*=4	(126; 32,5, *n*=2)	+ 3,736±996	(1,411, *n*=1)	+ 5,531±1,542
SS-R GRP-R	*n*=2	+ 2,062; 3,194	(1,380, *n*=1)	+ 11,131; 8,263	(133; 1,494)
1 receptor					
GRP-R	*n*=14	(505±174, *n*=7)	(645±155, *n*=12)	+ 9,179±1,209	(530±223, *n*=8)
NPY(Y_1)-R	*n*=8	(309±73, *n*=4)	(865±158, *n*=6)	(679±408, *n*=4)	+ 7,503±1,375
VIP-R	*n*=5	(314±58, *n*=5)	+ 2,719±489	(149±32, *n*=3)	(345±184, *n*=3)
Total	*n*=68	14/68 4,337±528	25/68 3,437±306	50/68 9,819±530	45/68 9,135±579

density of VIP receptors was found much less frequently (25/68, 37%), although VIP receptors (high and low density) were found in the majority of the 68 tumours tested (63/68, 93%). In all cases, the VIP receptors were of the $VPAC_1$ type, with a high affinity for [K^{15},R^{16},L^{27}]VIP(1–7)/GRP(8–27) but no affinity for Ro25-1553. A high density of somatostatin receptors identified with ^{125}I-[Tyr^3]-octreotide was found in a minority of cases (14/68, 21%) whereas the total number of tumours with somatostatin receptors (high or low density) in this series amounted to 51/68 (75%). Interestingly, as many as 41 (60%) of the 68 tumours co-expressed two to four receptor types. A further important observation was that the number of tumours expressing a high density of GRP or NPY(Y_1) receptors, or both, was as high as 63/68 (93%) in the group of high receptor density specimens or 63/77 (82%) in the whole group of tested primary breast cancers. The other interesting result is related to the mean values of receptor density for each receptor type: highest mean density values, 9,819±530 dpm/mg tissue, were found for the 50 GRP receptor-positive cases, while the 45 NPY(Y_1) receptor-positive cases

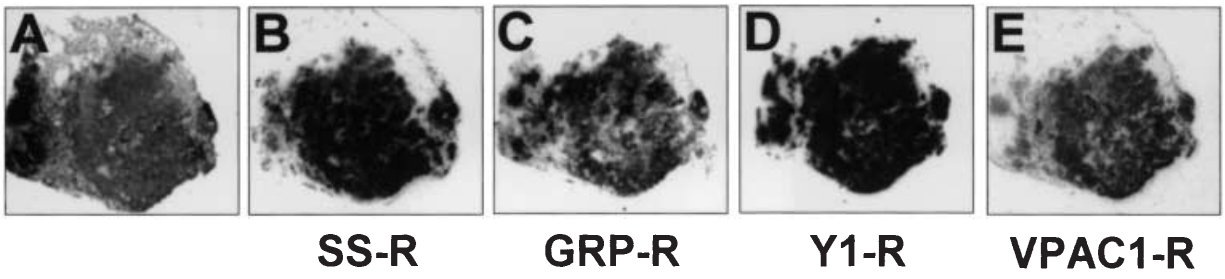

Fig. 1A–E. Breast cancer with four peptide receptors expressed homogeneously in the whole tumour sample. **A** Haematoxylin-eosin stained section. *Bar* =1 mm. **B** Autoradiograph showing total binding of ^{125}I-[Tyr^3]-octreotide. **C** Autoradiograph showing total binding of ^{125}I-[Tyr^4]-bombesin. **D** Autoradiograph showing total binding of ^{125}I-[Leu^{31},Pro^{34}]-PYY. **E** Autoradiograph showing total binding of ^{125}I-VIP. All four receptors are expressed in high density in the whole tumour sample. In all four cases, non-specific binding was found to be negligible

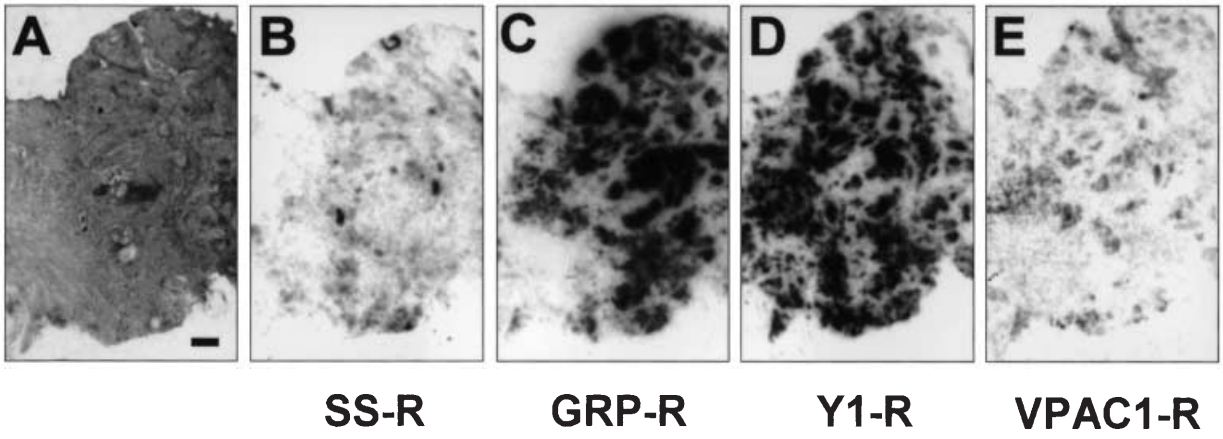

Fig. 2A–E. The most frequently found receptor profile in breast carcinomas: high density of GRP receptors and NPY(Y_1). **A** Haematoxylin-eosin stained section. *Bar* =1 mm. **B** Autoradiograph showing total binding of ^{125}I-[Tyr^3]-octreotide. **C** Autoradiograph showing total binding of ^{125}I-[Tyr^4]-bombesin. **D** Autoradiograph showing total binding of ^{125}I-[Leu^{31},Pro^{34}]-PYY. **E** Autoradiograph showing total binding of ^{125}I-VIP. NPY(Y_1) and GRP receptors are expressed in high density in the whole tumour. Somatostatin receptors and VIP are expressed in low density only

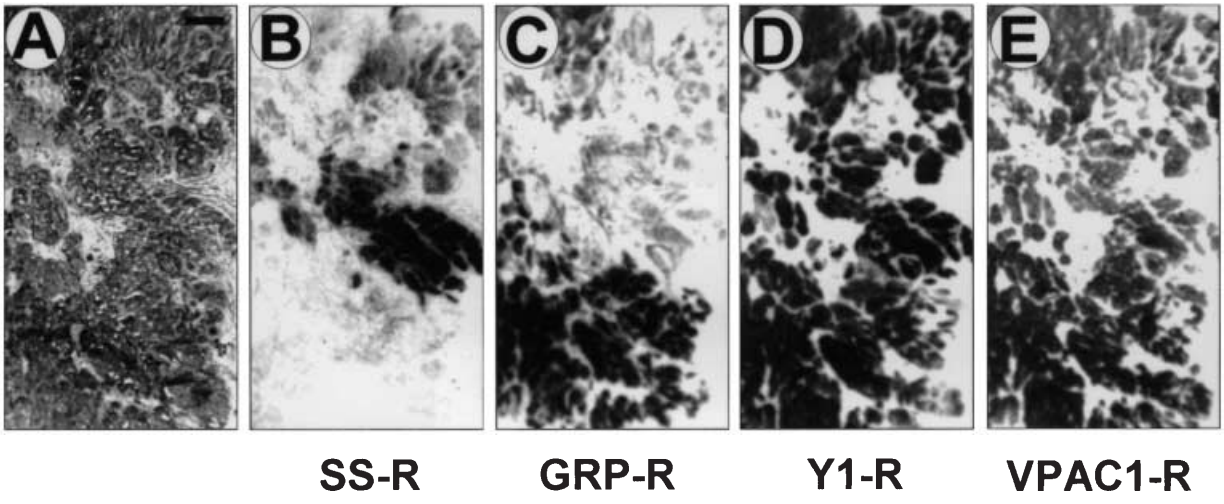

Fig. 3A–E. Breast carcinoma with four peptide receptors expressed in a distinct topography. **A** Haematoxylin-eosin stained section. *Bar* =1 mm. **B** Autoradiograph showing total binding of ^{125}I-[Tyr^3]-octreotide. **C** Autoradiograph showing total binding of ^{125}I-[Tyr^4]-bombesin. **D** Autoradiograph showing total binding of ^{125}I-[Leu^{31},Pro^{34}]-PYY. **E** Autoradiograph showing total binding of ^{125}I-VIP. Somatostatin and GRP receptors are heterogeneously and complementarily expressed while NPY(Y_1) and VIP receptors are homogeneously expressed

Table 2. Examples comparing the peptide receptor profile in individual primary tumours (PT) with their lymph node metastases (LN) in four cases of breast carcinoma

Case	Somatostatin-R	VIP-R	GRP-R	NPY(Y_1)-R
No. 1				
PT	0	914	0	14,766
LN	156	4,293	364	12,621
No. 2				
PT	3,288	786	14,741	10,723
LN1	4,557	1,475	16,231	13,266
LN2	4,144	2,600	12,824	12,510
No. 3				
PT	0	1,428	13,849	10,267
LN1	0	2,360	15,697	10,724
LN2	0	2,329	1,121	10,687
LN3	0	1,058	1,628	5,823
LN4	105	2,730	2,588	11,215
LN5	122	942	7,150	10,158
LN6	85	1,119	775	11,652
LN7	246	1,038	9,693	6,037
LN8	131	1,231	13,911	10,608
LN9	0	1,225	1,217	9,207
No. 4				
PT	0	623	12,622	17,663
LN1	967	3,739	0	15,464
LN2	1,316	4,975	343	14,074
LN3	3,697	1,193	796	13,746

Numbers represent receptor density values expressed in dpm/mg tissues

showed a mean density of 9,135±579 dpm/mg tissue. Much lower densities were found in the 14 somatostatin receptor-positive cases (4,337±528 dpm/mg tissue) and in the 25 $VPAC_1$ receptor-positive cases (3,437± 306 dpm/mg tissue). A typical example of a tumour with all four receptors expressed in high density is seen in Fig. 1. Figure 2 shows the receptor profile found most frequently in this breast cancer series, namely a high density of GRP receptors combined with NPY(Y_1) receptors. No correlation was found between the tumour type or tumour grade and the peptide receptor status. In particular, tumours with grade 1 or grade 3 were not found to have a receptor status (profile, density) distinct from those with grade 2.

Topographical heterogeneity of somatostatin receptors was found in 9/25 cases (36%) having a high receptor density, of $VPAC_1$ receptors in 2/25 cases (8%), of GRP receptors in 14/50 cases (28%) and of NPY(Y_1) in 12/45 cases (27%). Importantly, in all but two cases with heterogeneous receptor distribution, the area with low or no receptor content for a given receptor was positive for at least one of the other tested receptors. Figure 3 shows an example of heterogeneous distribution for somatostatin and GRP receptors, while NPY(Y_1) and $VPAC_1$ receptors were expressed in the whole tumour sample.

The 9/77 tumours having only low receptor density had the following receptor profile: seven cases had somatostatin receptors (mean density: 668±280 dpm/mg tissue), all nine had $VPAC_1$ receptors (707±101 dpm/mg tissue); five had GRP receptors (690±267 dpm/mg tissue) and eight had NPY(Y_1) receptors (566±207 dpm/mg tissue).

Table 2 shows the receptor profile in four breast cancer patients and compares the respective receptor density in their primary tumours and in the various axillary lymph node metastases. Although there are marked variations in density, the peptide receptor profile found in the primary tumour is the same as in nodal metastases in three of the four patients: Case no. 1 has an NPY(Y_1) receptor profile both in the primary tumour and in the metastasis. Case no. 2 expresses somatostatin, GRP and NPY(Y_1) receptors in the primary tumour and in the two metastases. Case no. 3 has a GRP and NPY(Y_1) receptor profile, with lower levels of $VPAC_1$ receptors, in the primary tumour and the majority of the metastases. Only case no. 4 has an inconsistent profile differing in the primary tumour and in the metastases. An example of a comparable receptor profile in a primary tumour and its metastasis is shown in Fig. 4.

Discussion

This in vitro receptor autoradiography study shows that among several peptide receptor candidates expressed in breast carcinomas, some may be more suitable than others for potential in vivo clinical use in nuclear medicine, based on their incidence, density and tumour distribution in vitro. Among the four peptide receptors tested, this study shows that GRP and NPY(Y_1) receptors are those peptide receptors most frequently expressed in high density in resectable primary breast cancers, as they are found, alone or together, in more than 82% of these cancers. Lymph node metastases, as far as could be evaluated in this study, appear to have a similar peptide receptor profile to their corresponding primary tumours. We do not, however, know the receptor profile of distant metastases (bone metastases) in patients with advanced, nonresectable metastatic breast cancer.

The present in vitro determination of four different peptide receptors in human breast cancers is a predictive study that could lead to a novel approach to improve and optimize breast cancer targeting in vivo with radiopeptides. According to the present in vitro data, the simultaneous application of radiolabelled GRP (or bombesin) and NPY(Y_1) receptor-selective analogues in vivo should permit scintigraphic visualisation of a large number of breast cancers. The present study was primarily based on tumours from patients with resectable primary breast cancers, and it is this group among the breast can-

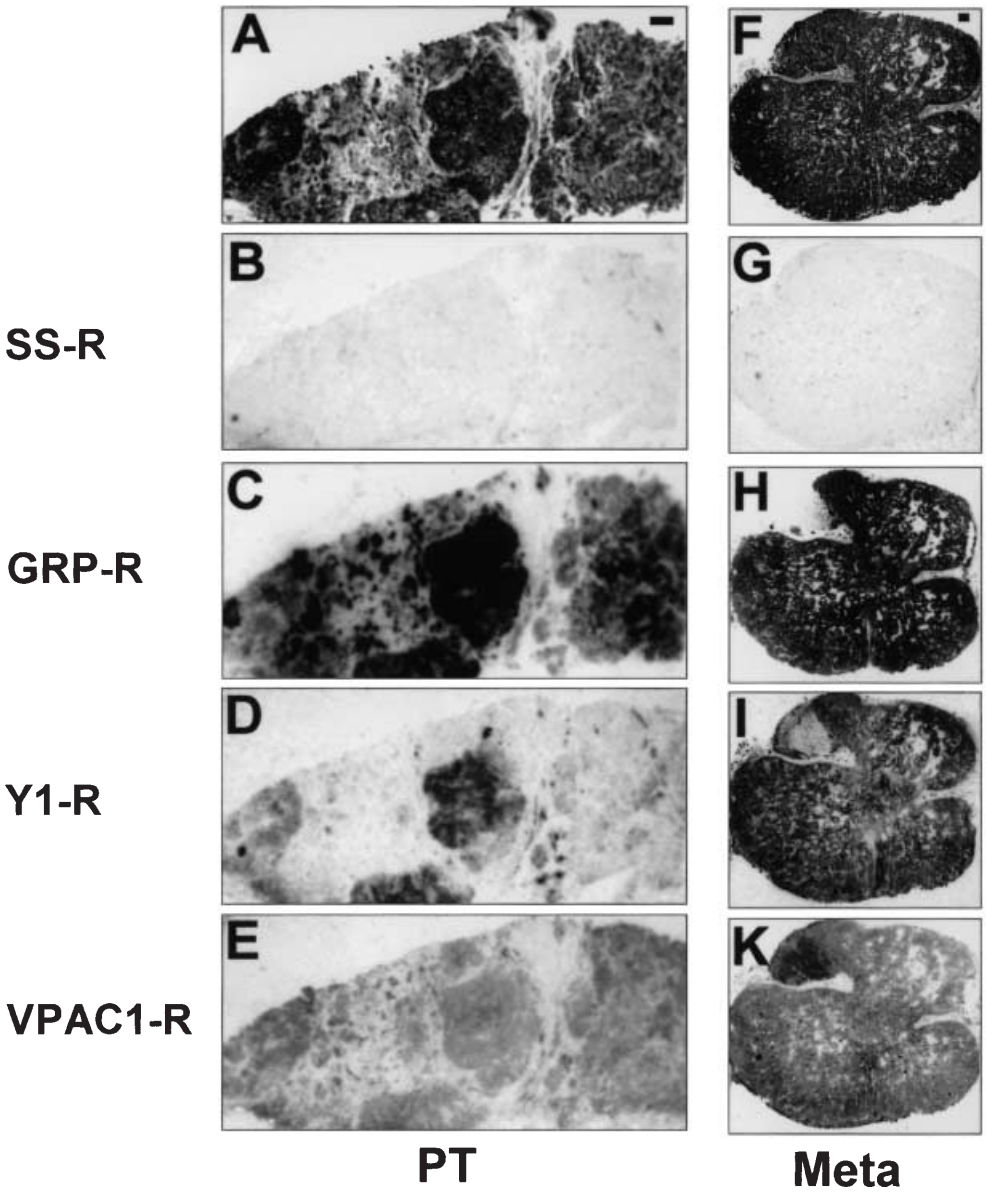

Fig. 4A–K. Receptor profile in a primary tumour (*PT, left*) and its lymph node metastasis (*Meta, right*). **A, F** Haematoxylin-eosin stained sections. *Bars* =1 mm. **B, G** Autoradiographs showing total binding of ^{125}I-[Tyr^3]-octreotide. **C, H** Autoradiographs showing total binding of ^{125}I-[Tyr^4]-bombesin. **D, I** Autoradiographs showing total binding of ^{125}I-[Leu^{31},Pro^{34}]-PYY. **E, K** Autoradiographs showing total binding of ^{125}I-VIP. While somatostatin receptors are absent, the three others are strongly expressed in the primary tumour and in its metastasis, with GRP receptors found in the highest density. Interestingly, NPY(Y_1) receptors are found heterogeneously in the primary tumour, while the entire lymph node metastasis is NPY(Y_1) receptor positive

cer population that should represent the first selection for in vivo scanning, given the high chance of tumour detection. However, once the in vivo proof of principle has been obtained in this group of patients, it will be essential also to scan patients with advanced metastatic breast cancers, including recurrent and/or hormone-resistant breast cancers, to gain in vivo information on the receptor status in this category of patients, not available from the present in vitro study. In such cases, GRP/NPY scintigraphy may represent a tool to assess whether the peptide receptor expression of the primary tumour, lymph node metastases and bone metastases is comparable in a given breast cancer patient and, in this regard, may help clarify the extent to which breast cancer is a systemic disease [30, 31]. Furthermore, it is worth considering that the presence of a high receptor density may permit detection of lymph node metastases harbouring only very small areas of tumour cells. Would, for instance, sentinel lymph nodes [32] be adequately visualized through in vivo targeting of these two receptors?

Based on the strict selection of tumours with a high density of receptors, the receptor density in more than 82% of these tumours should be sufficiently high also to permit targeted peptide radiotherapy with the same analogues. Indeed, the density of GRP and/or NPY(Y_1) receptors expressed in these breast cancers equals or even exceeds the number of somatostatin receptors usually expressed by neuroendocrine tumours [27, 28]. The latter have been shown to be the most suitable candidates for somatostatin radiotherapy [8, 9, 33]. As is the case for somatostatin receptor radiotherapy [2], an inclusion criterion for GRP/NPY receptor radiotherapy should be strongly GRP/NPY receptor-positive scintigraphy of the tumour in that patient.

Radioactivity background in the thorax and breast should not be problematic, as neither GRP nor NPY(Y_1) receptors are expressed in the lung. NPY(Y_2) rather than NPY(Y_1) receptors are preferentially expressed in the normal breast [25]; GRP receptors are, however, frequently found in the normal breast [23]. The presence of these GRP receptors in normal breast does not appear to seriously hamper the detection of primary breast tumours, probably due to the much lower cellularity of the glandular tissue per organ volume compared with tumour tissue. Indeed, GRP receptor scintigraphy was recently initiated with a technetium-99m labelled bombesin analogue and shown to successfully visualize breast cancers [34], even though in normal volunteers the normal breast gave a weak positive signal [35].

It is questionable whether it would be worth applying three or four radiopeptides concomitantly, namely octreotide and/or VIP in combination with the GRP and NPY(Y_1) analogues. On the one hand, this would certainly allow detection of a higher proportion of breast cancers, including perhaps even the small percentage (12%) of tumours with low receptor densities only. A significant drawback, on the other hand, would be the increased radioactivity background of the three to four concomitantly applied radioligands. While somatostatin receptor scintigraphy does not show a strong background over the thoracic area owing to a lack of sst_2 receptors in normal breasts and lungs, VIP receptor scintigraphy is characterized by a strong lung uptake based on $VPAC_1$ receptors in this tissue [21]. Masking by these lung $VPAC_1$ receptors may prevent the detection of a positive breast cancer in this area, while the $VPAC_1$ receptors expressed in the normal breast will probably play only a minor role in this respect [21]. Furthermore, adding somatostatin to GRP and NPY(Y_1) analogues for scintigraphy may not be a sufficiently great advantage in terms of increased tumour signal, given the generally lower incidence and density of somatostatin receptors compared with GRP and NPY(Y_1) receptors in breast cancers. Based on the above arguments, the combination of GRP and NPY(Y_1) appears preferable.

Prerequisites for a clinical trial are the development of NPY(Y_1) radiopharmaceuticals and the optimization of the GRP radiopharmaceuticals presently available. ^{99m}Tc-labelled NPY analogues selective for Y_2 have recently been synthesized [36]. Moreover, novel and specific Y_1 ligands have recently been reported [37] that need to be linked to chelators for radioimaging. While several radioligands specific for GRP receptors have been synthesized [38, 39, 40], only the ^{99m}Tc-labelled bombesin analogue RP527 has been used to target human tumours up to now [34, 35]. The present study may therefore trigger and motivate the initiation of new preclinical and clinical studies in these directions.

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