EGFR, HER2 and HER3 expression in primary colorectal carcinomas and corresponding metastases: Implications for targeted radionuclide therapy

Q. WEI[1-3], Y. SHUI[2], S. ZHENG[2], K. WESTER[3], H. NORDGREN[4], P. NYGREN[5], B. GLIMELIUS[5,6] and J. CARLSSON[3]

[1]Department of Radiation Oncology, the Second Affiliated Hospital; [2]Cancer Institute (National Ministry of Education Key Laboratory of Cancer Prevention and Intervention), Zhejiang University School of Medicine, Hangzhou 310009, P.R. China; [3]Unit of Biomedical Radiation Sciences, Department of Oncology, Radiology and Clinical Immunology, Rudbeck Laboratory; [4]Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University Hospital; [5]Unit of Oncology, Department of Oncology, Radiology and Clinical Immunology, Uppsala University Hospital, SE-751 85 Uppsala; [6]Department of Oncology and Pathology, Karolinska Institute, SE-171 77 Stockholm, Sweden

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Abstract. Members of the epidermal growth factor receptor, EGFR, family are interesting as targets for radionuclide therapy using targeting agents labeled with α- or ß-emitting radionuclides, especially when EGFR-positive colorectal carcinomas, CRC, are resistant to EGFR inhibiting agents like cetuximab and various tyrosine kinase inhibitors. The expression of EGFR, HER2 and HER3 was therefore analyzed in CRC samples from primary tumors, corresponding lymph node metastases and, in a few cases, liver metastases. The expression of HER2 and EGFR was scored from immunohistochemical preparations using the HercepTest criteria 0, 1+, 2+ or 3+ for cellular membrane staining while HER3 expression was scored as no, weak or strong cytoplasm staining. Material from 60 patients was analyzed. The number of EGFR 2+ or 3+ positive primary tumors was 16 out of 56 (29%) and for lymph node metastases 8 out of 56 (14%) whereas only one out of nine (11%) liver metastases were positive. Thus, there was lower EGFR positivity in the metastases. Only one among 53 patients was strongly HER2 positive and this in both the primary tumor and the metastasis. Eight out of 49 primary tumors (16%) were strongly HER3 positive and the corresponding numbers for lymph node metastases were 9 out of 49 (18%) and for liver metastases 2 out of 9 (22%). The observed number of strongly EGFR positive cases was somewhat low but EGFR might be, for the cases with high EGFR expression in metastases, a target for radionuclide therapy. HER2 seems not to be of such interest due to rare expression, neither HER3 due to mainly expression in the cytoplasm. The requirements for successful EGFR targeted radionuclide therapy are discussed, as well as patient inclusion criteria related to radionuclide therapy.

Correspondence to: Dr Jörgen Carlsson, Unit of Biomedical Radiation Sciences, Department of Oncology, Radiology and Clinical Immunology, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden
E-mail: jorgen.carlsson@bms.uu.se

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Introduction

There is, so far, no successful curative treatment for the majority of patients with disseminated colorectal carcinoma (CRC). The disease represents the third most frequent malignancy worldwide, with about one million new cases diagnosed yearly (1). The incidence and mortality rates of CRC are higher in western than in eastern countries. However, the incidence rates have increased rapidly in some previously low incidence areas, such as Shanghai, China (2). The majority of CRC patients are diagnosed with resectable localized disease. However, even after potentially curative surgical excisions, about half of the patients will develop local recurrences or distant metastases, which in most cases will be fatal. Combinations of irinotecan, oxaliplatin, and 5-fluorouracil with leucovorin have some efficacy, but the prognosis remains poor with median survival of ~18-20 months (3,4). This has led to the hope that receptor-mediated tumor targeted therapy with radionuclides could improve the response and survival rates.

For receptor targeted radionuclide therapy to be an effective complement or alternative to chemotherapy, it is necessary that the disseminated tumor cells and metastases express the target structure to a similar extent as the corresponding primary tumors. When the target for radionuclide therapy is a growth factor receptor in the epidermal

growth factor receptor (EGFR) family, there are several indications that when the expression is high in the primary tumor, it is often also high in the metastases (5). The reason for this is probably that the receptor expressing tumor cells are dependent on the growth stimulation from the growth factor-receptor interactions. If tumor cells, e.g. due to genomic instability, lose the receptor expression they might also lose their growth advantage and be overgrown by tumor cells with high receptor expression (5).

The EGFR family members are EGFR (ErbB-1/HER1), HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). They have an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular domain with protein-tyrosine kinase activity. However, HER3 has no intrinsic tyrosine kinase activity but is instead signaling via dimerisation with any of the other receptors. EGF and five other ligands bind to EGFR and neuregulins (NRG) are the ligands for HER3 and HER4. HER2 has, so far, no known ligand but is activated via dimerisation with any of the other receptors (6,7). Overexpression of EGFR and HER2 has often been associated with malignant transformation. It has been stated that EGFR is often overexpressed in primary CRC and associated with poor survival (8,9). EGFR positive cells have also been detected in peripheral blood from colon cancer patients (10,11). No expression of the mutated EGFRvIII receptor has, to our knowledge, so far been found in CRC (5,12).

Targeted therapy using non-radioactive agents is a clinical reality for tumors expressing high levels of EGFR or HER2 although resistance has been reported (6,7,13-15). Both EGFR and HER2 seem to be good targets also for radionuclide based tumor therapy and whether this is the case for colorectal carcinomas is the subject of this study. It remains to be determined whether also HER3 and HER4 receptors are suitable for radionuclide-targeted therapy. One problem seems to be that HER3 and HER4, in immunohistochemical staining, often is in the cytoplasm. This staining pattern is not understood and it cannot be excluded that there is also a fraction of the receptors in the cellular membrane since both HER3 and HER4 contain transmembrane regions. Cytoplasm staining of HER3 has been reported for several tumor types such as pharyngeal, esophageal and base of tongue tumors as well as for CRC (16-19), However, some membrane HER3 staining has also been reported for CRC (19,20). The role of HER4 for tumor growth is not clear and HER4 was not analyzed in this study.

EGFR-family targeted radionuclide therapy is mainly planned to target the native receptors and the effect of radiation is probably not, when the dose is high, dependent on whether the targeting agent interferes with intracellular signaling cascades. The cell killing properties of ionizing radiation are well known and treatment induced resistance has, to our knowledge, not been reported. With this background we investigated the expression of EGFR, HER2 and HER3 in CRC with immunohistochemical methods.

Materials and methods

Patients and samples. Sixty patients with primary node positive CRC who were diagnosed and treated in the Second Affiliated Hospital, Zhejiang University School of Medicine, in the year of 2005, were enrolled under approval of the Institutional Review Board. Primary tumor and lymph node metastasis samples were taken from all patients while liver metastases were taken from only 10 patients. The patient and tumor characteristics are shown in Table I. Briefly, the tissues were fixed in 4% buffered formalin, processed and embedded in paraffin. Sections, 4-μm, were cut and deparaffinized in xylene and hydrated through graded concentrations of ethanol to distilled water.

Table I. Tumor and patient characteristics (n=60).

Characteristics	Patients n (%)
Tumor type	
Typical adenocarcinoma	50 (83)
Mucinous adenocarcinoma	9 (15)
Signet-ring cell carcinoma	1 (2)
Differentiation of typical adenocarcinomas	
Low	4 (8)
Moderate	20 (40)
High	26 (52)
T-stage at initial diagnosis	
T2	4 (7)
T3	22 (37)
T4	34 (57)
Primary tumor location	
Cecum	1 (2)
Colon	23 (38)
Sigmoid	10 (17)
Rectum	26 (43)
Primary tumor size, cm	4.0 (range: 2.0-9.0)
Gender	
Male	42
Female	18
Age at diagnosis, years	59 (range: 25-89)
Metastatic sites analyzed	
Lymph node	60
Liver	10

EGFR-staining. EGFR was assessed by immunohistochemistry using a streptavidin-biotin complex technique as previously described (16,17). After deparaffinization of the sections, endogenous peroxidase was blocked in 0.3% H_2O_2 in PBS for 20 min. Then, enzymatic antigen retrieval was done in 0.05% protease K (code no. S3020, Dako, Glostrup, Denmark) in PBS for 10 min at room temperature. The slides were preincubated in PBS for 10 min. The primary mouse monoclonal antibody directed against EGF receptor (clone

Table II. EGFR-scores for primary colorectal carcinomas, CRC, and the corresponding lymph node metastases (n=56).

Primary tumors, EGFR-scores	Lymph node metastases, EGFR-scores			
	0	1+	2+	3+
0	27	2	0	0
1+	3	6	2	0
2+	2	2	4	0
3+	2	4	0	2

The scoring was based on a scale where 0 corresponded to completely negative staining, 1+ corresponded to faint perceptible staining of the tumor cell membranes, 2+ corresponded to moderate staining of the entire tumor cell membranes and 3+ was strong circumferential staining of the entire tumor cell membranes creating a fishnet pattern.

31G7, Zymed Labs, South San Francisco, CA, USA) receptor were diluted 1:100, and incubated overnight at 4˚C. The secondary biotinylated antibodies (goat anti-mouse, Dako) and the peroxidase-labeled streptavidin-biotin complex (Dako) were diluted 1:200 and incubated for 30 min at room temperature. All slides were developed in 0.05% diaminobenzidine (Sigma, St. Louis, MO, USA) for 5 min and counterstained in Harris haematoxylin (Sigma). Finally, the slides were dehydrated through graded alcohol to xylene and mounted in organic mounting medium (Pertex®, Histolab, Gothenburg, Sweden).

HER2 staining. The HER2 staining was made as previously described (16,17). After deparaffinization, the sections were incubated in methanol and hydrogen peroxide for 30 min quenching endogenous peroxidase. Antigen retrieval was done in a water bath at 95-98˚C, pH 6.0 for 40 min. Thereafter the glasses were cooled at room temperature and then washed in distilled water. Immunohistochemical staining was performed using the Elite ABC kit (Vectastain, Vector Laboratories, Burlingame, CA, USA). Blocking serum was applied for 15 min and followed by incubation with rabbit anti-human c-erbB-2 oncoprotein (code no. A 0485, Dako) diluted 1:350. Sections were then incubated with the biotinylated secondary antibody and were visualized by using the peroxidase substrate 3-amino-9-ethyl-carbazole (AEC) (Sigma A-5754) as chromogen. Finally, the sections were counterstained with Mayer's haematoxylin and mounted with Aquamount (BDH Ltd., Poole, UK).

HER3 staining. The HER3 staining was made as previously described (16,17). After deparaffinization, the sections were incubated in methanol and hydrogen peroxide for 30 min quenching endogenous peroxidase. Antigen retrieval was done in a pressure chamber at 125˚C, pH 9.0 for 4 min. Thereafter the glasses were cooled at room temperature and washed in distilled water. Immunohistochemical stainings were performed using the Elite ABC kit (Vectastain, Vector Laboratories). Blocking serum was applied for 15 min and followed by incubation with the monoclonal antibody MAB4021 (Chemicon, Temecula, CA) diluted 1:1000. Sections were then incubated with a biotinylated secondary antibody and were visualized by using the peroxidase substrate 3-amino-9-ethyl-carbazole (AEC) (Sigma A-5754) as chromogen. Finally, the sections were counterstained with Mayer's haematoxylin and mounted with Aquamount (BDH Ltd.).

EGFR and HER2-scores. The HER2 expression was scored using the HercepTest scoring criterion. The HER2-score was based on a scale where 0 corresponded to tumor cells that were completely negative, 1+ corresponded to faint perceptible staining of the tumor cell membranes, 2+ corresponded to moderate staining of the entire tumor cell membranes and 3+ was strong circumferential staining of the entire tumor cell membranes creating a fishnet pattern. The Canadian and the Dako HercepTest guidelines (21) that require >10% of the tumor cells to be stained were applied. Cytoplasmic staining was considered non-specific and was not included in the scoring. As positive controls we used in house positive control tissue sections as well as positive control sections supplied by Dako. As negative controls we used normal tissue, which are expected not to express HER2, e.g. connective tissue seen in the same sections as the tumor cells. In the metastases sections we used lymphocytes and the surrounding capsule of the lymph nodes as negative internal controls. The expression pattern of EGFR is similar to that of HER2 and EGFR expression was therefore evaluated using the same scoring criterion as for HER2. As EGFR positive controls we used in house positive control skin tissue sections. As negative controls we used connective tissue seen in the same sections as the tumor cells. In the metastases sections we used lymphocytes and the surrounding capsule of the lymph nodes as negative internal controls.

HER3 evaluation. The HER3 staining was evaluated as negative, weak or strong (16,17). Negative corresponded to tumor cells that were not at all stained, weak corresponded to faint staining of the tumor cytoplasm with/without stained granules and strong corresponded to intensive tumor granular cytoplasm staining. As positive controls we used normal laryngeal epithelium. Positive reference staining can also be found at website: www.proteinatlas.org. Negative controls were connective tissue in the same sections as the tumor cells. In the metastases sections lymphocytes and the surrounding capsule of the lymph nodes served as negative internal controls.

Excluded cases. Cases were only excluded when tumor cells could not be found in either the section from the primary tumor or in the section from the corresponding metastasis. Sixty patients were originally included and samples from 56, 55 and 49 patients were evaluated for EGFR, HER2 and HER3, respectively.

Results

EGFR expression in primary tumor and lymph node metastasis. The fraction of EGFR expressing primary tumors was somewhat low, see Table II. Only 16 out of 56 (29%) primary

Table III. Major results from the EGFR-score analyses.

EGFR-score characteristics	Fraction	Percentage
Primary tumors with 2+ or 3+	16/56	29%
Lymph node metastases with 2+ or 3+	8/56	14%
Patients who had 0 or 1+ in primary tumors and changed to 2+ or 3+ in lymph node metastases	2/56	4%
Patients who had 2+ or 3+ in primary tumors and changed to 0 or 1+ in lymph node metastases	10/56	18%

Table IV. HER2-scores for the analyzed primary CRC and the corresponding lymph node metastases (n=55).

Primary tumors, HER2-scores	Lymph node metastases, HER2-scores			
	0	1+	2+	3+
0	51	0	0	0
1+	2	1	0	0
2+	0	0	0	0
3+	0	0	0	1

The scoring was based on a scale where 0 corresponded to completely negative staining, 1+ corresponded to faint perceptible staining of the tumor cell membranes, 2+ corresponded to moderate staining of the entire tumor cell membranes and 3+ was strong circumferential staining of the entire tumor cell membranes creating a fishnet pattern.

Table V. HER3-scores for the analyzed primary CRC and the corresponding lymph node metastases (n=49).

Primary tumors, HER3-scores	Lymph node metastases, HER3-scores		
	0	W	S
0	29	4	1
W	3	4	0
S	0	0	8

0, no stained cells; W, weak cellular staining of cytoplasm with or without weak stained granules; S, strong granular cytoplasmic staining.

tumors had 2 or 3+ scores. The EGFR expression in the corresponding lymph node metastases was even lower with only 8 (14%) being positive. As can be seen in Table III, two cases increased from 0-1+ in the primary tumors to 2+ - 3+ in the lymph node metastases. The opposite change, down-regulation in metastases, was observed in 10 cases.

HER2 expression in primary tumor and lymph node metastasis. HER2 was strongly expressed in only one primary tumor and in this case also in the corresponding metastasis (Table IV). No other strongly HER2 stained samples were found.

HER3 expression in primary tumor and lymph node metastasis. Of 49, 15 (31%) of the primary tumors expressed HER3 and strongly in 8 (16%). Lymph node expression was slightly higher, 17 out of 49 (35%) and strongly in 9 (18%). Overall there was fairly good correspondence in HER3 expression between primary tumors and lymph node metastases (Table V).

EGFR, HER2 and HER3 expression in liver metastases. The expression of EGFR, HER2 and HER3 was analyzed in liver metastases from 10 patients (Table VI) and these receptors were also analyzed in primary tumors and lymph node metastases from the same patients. Samples from three patients were strongly EGFR expressing (2+) in the primary tumors and in two of the corresponding lymph node metastases, but none of them strongly expressed EGFR in the liver metastases. Only one liver metastasis expressed EGFR strongly while the corresponding primary tumor and lymph node metastasis samples did not. None of these samples were HER2 positive. Two patients expressed HER3 strongly in the primary tumors and lymph node metastases while only one of them strongly expressed HER3 in the liver metastases.

Morphological appearance. Typical immunohistochemical stainings are shown in Fig. 1. The EGFR staining of CRC tissue (primary tumor, lymph node metastasis and liver metastasis; a, b and c, respectively) all showed mosaic or fishnet staining patterns, typical for cell membrane staining. The normal liver tissue, shown in d, had a weak EGFR cell membrane staining in the hepatocytes. Strong HER2 cell membrane stainings from the only HER2 positive case (primary tumor and lymph node metastasis) are seen in Fig. 1e and f. The HER3 stainings had a granular pattern in the cytoplasm and this was seen in primary tumors (g) as well as in the lymph node (h) and liver metastases (i). Thus, HER3 did not have a cell membrane staining pattern.

Discussion

EGFR expression in CRC. There is a surprisingly wide span between previously published levels on EGFR-expression in

Table VI. EGFR, HER2 and HER3 scores from primary tumors, lymph node metastases and liver metastases (n=10).

	EGFR			HER2			HER3		
Sample	PT	LNM	LM	PT	LNM	LM	PT	LNM	LM
1	0	0	0	0	0	0	0	-	0
2	0	0	2+	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	1+	1+	0	0	0	0	S	S	S
5	0	0	0	0	0	0	0	0	0
6	1+	1+	0	0	0	0	0	0	0
7	2+	1+	-	0	0	0	0	0	0
8	2+	2+	1+	0	0	0	0	0	0
9	0	0	0	0	0	0	S	S	W
10	2+	2+	1+	0	0	0	0	0	-

PT, primary tumor; LNM, lymph node metastases; LM, liver metastases. The scoring was based on the same criteria as described in the Tables IV and V. -, not possible to evaluate due to lack of tumor cells in the section.

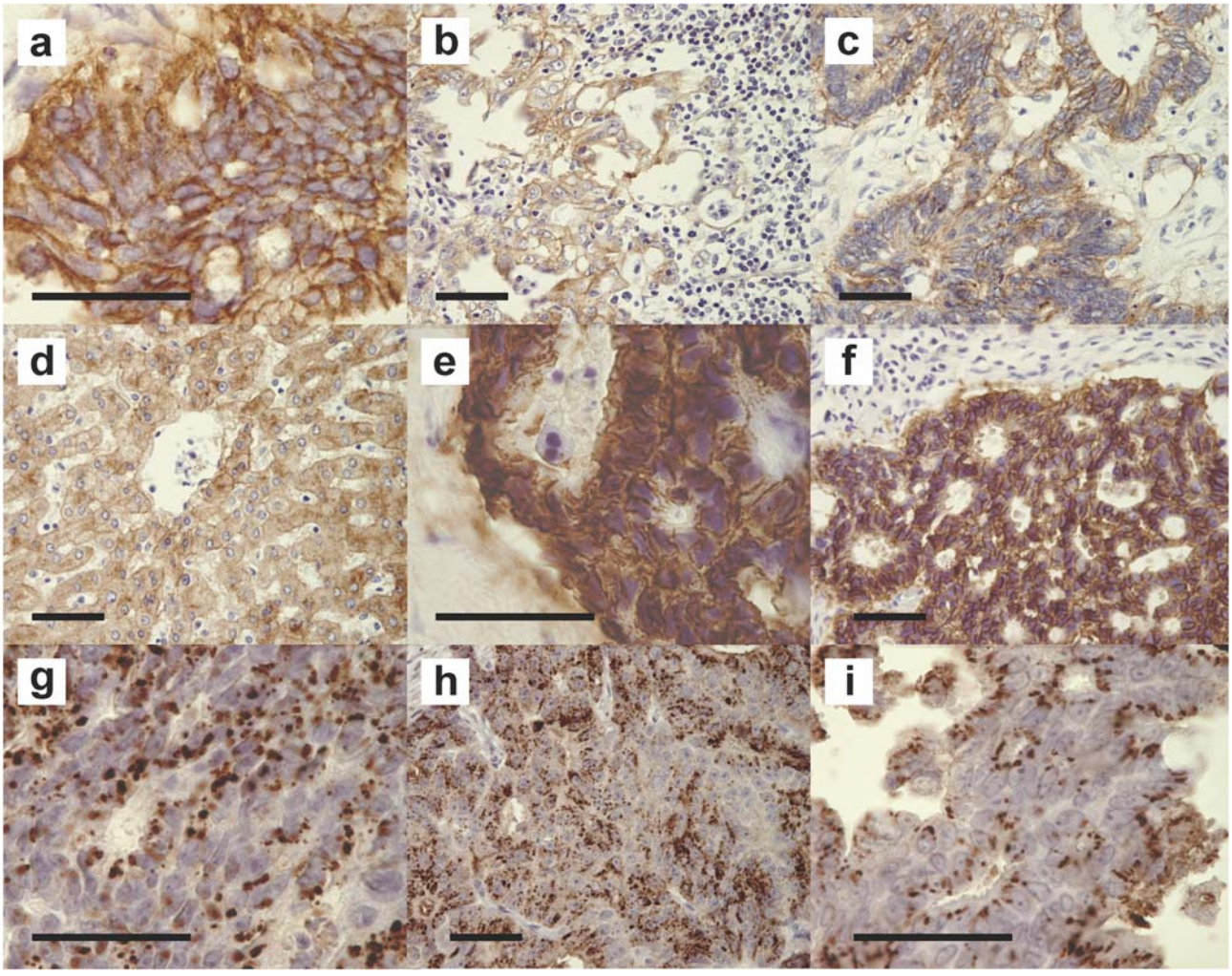

Figure 1. Typical brown IHC EGFR-staining of sections from a primary colorectal carcinoma cancer (a) and the corresponding lymph node (b) and liver (c) metastasis. Note the homogeneous membrane staining of virtually all tumor cells. EGFR staining of apparently normal liver tissue (taken some distance from a liver metastasis) is shown in (d). Typical brown IHC HER2-stainings of sections from the only HER2 positive primary colorectal carcinoma cancer (e) and the corresponding lymph node metastasis (f). Immunohistochemical HER3-stainings (brown) of sections from a primary colorectal carcinoma cancer (g) and the corresponding lymph node (h) and liver metastases (i).

primary tumors of CRC. The fraction of tumor cells being EGFR-positive varied in the range 20-95% (12,22-35). The variations in expression of EGFR in lymph node (28,30,33) and liver metastases (29-35) were similarly high. Our values for the Hangzhou patients are in the lower part of the ranges. Independent of low or high values there is, in the literature, often a rather good concordance on EGFR expression between primary tumors and corresponding metastases, whether lymph node or liver metastases are considered (5,28,30-32). However, our results indicate lower EGFR positivity in the metastases. Downregulation in metastases have recently been indicated also in other studies (20,33-35).

HER2 expression in CRC. HER2 has been reported to be expressed in primary colorectal carcinomas but the levels vary within an extremely wide range, 3-82% (22,23,25,36-42). In our study the HER2 expression was nearly zero (one positive case out of 55). In a report by Knosel *et al* (39) summarizing 10 previously published CRC studies during 1994-2001, including 1007 patient cases, more than half of the cases were HER2 positive. HER2 expression in metastases has been less studied but reported to be in the range 36-54% (28,38,39) with our present study as an exception. Thus, the lack of HER2 expression in our material is striking, and is reasonably not due to errors in the analysis since we had well documented positive and negative controls and an extensive experience in HER2 analyses. For example, the obtained HER2 results are low in comparison to the results from other tumor types studied at our laboratory (applying the same IHC techniques and scoring criteria), e.g. breast (43) and urinary bladder (44,45). The level is actually as low as in squamous carcinomas, e.g. laryngeal (16), esophageal (17) and oral cavity and base of tongue (18) cancers. The variations in CRC data are probably mainly due to variations in the scoring criteria (cut off levels and lack of discrimination between low expression and overexpression). Considering only our data, HER2 is not of interest for targeted radionuclide therapy.

HER3 expression in CRC. HER3 has previously been reported to be expressed in 36-89% of CRC (5,23,25,46-48). In our study, the fraction strongly HER3 expressing cases were only 16 and 18% for primary tumors and lymph node metastases, respectively. If also weak staining is included, the fractions increased to ~31 and 35%. Cytoplasm staining was observed in our CRC samples and such HER3 staining has previously also been observed in other types of cancer (16-19). Membrane HER3 staining of CRC cells has, to our knowledge, only been reported in two studies (19,20). The human protein atlas shows mainly cytoplasm HER3 staining but also some cases with nuclear staining (http://www.proteinatlas.org). Clear cell membrane HER3 staining, as for EGFR and HER2, cannot be found in the atlas. HER3 has a transmembrane region and it is possible that HER3 most often is situated in intracellular membranes and only visiting the outer cell membrane occasionally. Since outer cell membrane localization is necessary for targeted radionuclide therapy using macromolecules and peptides, HER3 seems not an interesting target for radionuclide therapy.

EGFR expression in normal tissues. We only discuss EGFR here since HER2 and HER3 seems not to be candidates for targeted radionuclide therapy of CRC. The expression of EGFR in normal tissues has been characterized many years ago (49,50). Distributions of EGFR in various tissues can also be found at the human protein atlas (http://www.proteinatlas.org) showing that EGFR is expressed in skin, liver, digestive tract and reproductive organs. EGFR staining in liver is shown in Fig. 1d. EGFR is attractive as target mainly when the uptake of radioactivity is higher in the tumor, and studied metastases, than in most normal tissues. This can, in the patient setting, best be studied through nuclear medicine techniques such as PET (positron emission tomography) or SPECT (single photon emission tomography) applying tracer amounts of the targeting agent labeled with radionuclides suitable for imaging, e.g. ^{68}Ga (PET) and ^{111}In (SPECT). Such methods provide information on whether metastases can be targeted or not and give information about the uptake in normal tissues (51).

Reasons for variations in EGFR expression. The reported variations in EGFR expression, in tumors and metastases, between different studies must, to a large part, be due to variations in the applied cut off levels and lack of discrimination between low expression and overexpression. In some studies, EGFR positivity was scored positive if at least half of all cells in the tumor were stained while in other studies positivity was scored if only one or a few EGFR positive tumor cells were found. There are also differences in IHC retrieval techniques between the laboratories. Variations are probably also due to different patient inclusion criteria and etiological differences.

EGFR targeted radionuclide therapy. In spite of the large variations in EGFR expression between different studies and a possible decreased expression in metastases, there is a reasonably good chance that EGFR targeted radionuclide therapy can be of value for selected patients, i.e. those with verified EGFR expression in metastases. EGFR targeted radionuclide therapy could actually be feasible for the subgroup of patients with EGFR expression and which are resistant to therapy with substances that interfere with downstream EGFR signaling (e.g. cetuximab and various tyrosine kinase inhibitors) due to K-Ras (52-57) or other mutations (58). EGFR expression has been reported to be independent of K-Ras mutations (59). As long as the EGFR is expressed, toxic radionuclides coupled to an EGFR-binding substance could be of therapy value.

Inclusion criteria. We do not discuss 'conventional' patient inclusion criteria such as age and health status, only those aspects that are unique for targeted radionuclide therapy. It is important to decide how the immunohistochemical techniques and scoring systems (cut off level) should be designed to allow for relevant patient inclusion. It seems not possible to exactly judge which criteria that is most relevant for CRC since the EGFR positive tumor cells, whether few or many, might be the most malignant and those that form disseminated disease. For example, increased EGFR expression has been reported in the invasive margin of CRC (60), which indicates that a low cut-off could be reasonable. The HercepTest applied in a standardized way for analysis of HER2 in breast cancer

(21) can serve as a good example and we suggest therefore 10% cut-off for 2+ and 3+ EGFR expression and that the morphological scoring criteria should be identical to those in the HercepTest. The retrieval and staining procedures described in Materials and methods of this study can be considered for standardization. Another important inclusion criteria for therapy is of course that the uptake of the targeting agent is low in normal tissues and high in the tumor and studied metastases, as analyzed by imaging techniques.

Design of targeting agents. The design of suitable radiolabeled EGFR-binding agents with high binding to EGFR-positive CRC cells and low uptake in critical normal tissues is a challenge. However, there is hope for a favorable development since new knowledge is continuously emerging about biodistribution, pharmacokinetics and cellular processing of different types of targeting agents and the research on molecular design of new agents is rapidly expanding. The development of peptides and small proteins, e.g. affibody molecules, is one strategy (61,62). The area of antibody engineering is also rapidly developing and various forms of antibody fragments are developed such as minimal recognizing units, single chain fragments, scFv, and dimeric scFv (63). Liposomes containing toxic radionuclides and conjugated with targeting agents might be of special interest for killing of disseminated tumor cells that remain in the systemic circulation (64). Suitable radionuclides for therapy, that are commercially available, are the ß-emitters ^{177}Lu and ^{90}Y while the α-emitter ^{211}At is a future candidate (51,65,66).

To conclude, EGFR might be a target for radionuclide therapy in CRC patients with EGFR positive metastases. HER2 and HER3 seem not to be of such interest, at least based on the present results.

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