

# ErbB 1-4 Expression Alterations in Primary Colorectal Cancers and their Corresponding Metastases

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**Abstract.** *Background:* EGFR (epidermal growth factor receptor) targeted therapies are important new tools in colorectal cancer treatment. EGFR analysis of the primary tumour was previously recommended to identify patients who will benefit from the EGFR targeted therapy. Previous studies have displayed diverging results regarding the expression of EGFR in the primary tumour compared to the metastases. The present study was performed to investigate whether EGFR and ErbB2-4 expression differed between 64 primary tumours and their corresponding metastases. *Patients and Methods:* EGFR and ErbB2-4 expression were analysed in the primary tumour and in the corresponding metastases using immunohistochemistry (IHC). *Results:* In 49/64 samples (76%), the primary tumours were EGFR positive; in 33% (16/49) of EGFR positive samples, the tumours lost the EGFR expression in the metastasis compared to the primary tumour. From the primary tumours, 15/64 (23%) were negative and 5 of these (33%) developed EGFR expression in the metastasis. ErbB2, ErbB3, and ErbB4 expression was evident in 54%, 67%, and 81%, respectively. There was no significant difference between ErbB2, ErbB3, and ErbB4 expression in primary tumours and metastases. The co-expression of the ErbB family members was also analysed, with a significant increase of ErbB3/ErbB4 co-expression in late stage tumours. *Conclusion:* The EGFR expression was lost in 33% of metastasising primary colorectal cancer tumours, a finding that agrees with at least one previous study. Thus, the present results clearly implicate the need for EGFR analysis of both the primary tumour and metastases to accurately determine EGFR status when considering the use of EGFR targeted therapies.

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The epidermal growth factor receptor (EGFR, ErbB1), a tyrosine kinase receptor identified in the early 1960's, is a member of the ErbB growth factor receptor family. Overexpression of EGFR has been correlated to a worse prognosis in several tumour types, including breast (1) and gynaecological cancers (2, 3). In colorectal cancer, the EGFR is expressed in 72-82% of the primary tumours as assessed by immunohistochemistry. A correlation between EGFR expression in colorectal cancer and the outcome of the patients has previously been proposed (4, 5). Several agents blocking the EGFR activity have been developed. In clinical practice, monoclonal antibodies (mAbs) – such as cetuximab and the tyrosine kinase inhibitors gefitinib and erlotinib – have received the most attention and evaluation. In colorectal cancer, studies have suggested survival benefit for patients receiving cetuximab as a supplement to chemotherapy, compared to cetuximab or chemotherapy alone (6). Although an optimal method has yet to be identified, immunohistochemical analysis of the primary tumour was previously recommended to identify those patients who most likely will benefit from the EGFR targeted treatment. However, previously published studies, although with diverging results, show the heterogeneity in expression between the primary tumour and the metastases (7- 11).

The HER family members ErbB2, 3 and 4 have only been investigated in a few non-conclusive studies in colorectal cancer (12-14). This study evaluates 64 colorectal cancer tumours with corresponding metastases for EGFR and ErbB2-4 expression.

## Patients and Methods

*Patient material.* This study is based on tissue material from primary colorectal cancers and their corresponding lymph node and distant metastases. The material was identified by searching the computerised patient record database at the Department of Clinical Pathology, University Hospital, Umeå, Sweden from 1982-2000. The tissue material consists of biopsies or surgically removed tissue from primary colorectal tumours and their corresponding distant metastases. In a few cases, tissue from distant metastases was

collected before diagnosis of the primary tumour (range 3-6 months) and sometimes after initial primary diagnosis (range 3 months-7 years). The samples were collected during a period when informed consent was not yet mandatory, but the study was approved by the local ethics committee. Clinico-pathological data were collected from patient records, including pathology reports. Table I illustrates the clinical data. No patient had received anti-EGFR treatment.

**Immunohistochemistry EGFR and ErbB2.** CRC specimens were fixed with formalin and embedded with paraffin using routine procedures. For this study, 4 µm sections were placed into a completely automated immunostaining machine (Benchmark LT, Ventana Medical Systems Inc., Tucson, AZ, USA). The immunostaining machine performed several treatments: deparaffinisation, protease treatment (Protease 1), EGFR antibody reaction (mouse monoclonal 3C6, dilution 1:200), and avidin-biotin blockage. For ErbB2 the antibody was mouse monoclonal Pathway Her2, clone CB11, prediluted. Antibody reaction was demonstrated using iVEIW kit with DAB (diaminobenzidine) as chromogen and hematoxylin for counterstaining according to the manufacturer's protocol. All reagents were from Ventana Medical System, Inc. (Tucson, AZ, USA). In each staining machine run, a positive control section was included. A series of samples were excluded from the analysis since they did not contain enough cells.

**Immunohistochemistry ErbB3 and ErbB4.** Manual staining of the sections was performed for ErbB3 and ErbB4. Endogenous peroxidase activity was blocked by adding 3% H<sub>2</sub>O<sub>2</sub> in methanol. Slides were placed in a 10 mM citrate buffer (pH 6.0) and pre-treated in a microwave oven at 900 W and then cooled for 20 minutes. Next, the slides were blocked by incubation with a non-immune serum blocking solution (Zymed Laboratories San Francisco, CA, USA). Blocked slides were incubated with anti-ErbB3 (1:30 dilution) (rabbit anti-ErbB3, Nova Castra Laboratories Ltd, Newcastle, UK) or anti-ErbB4 (1:50 dilution) (rabbit anti-ErbB4, Lab Vision Corporation, Fremont, CA, USA) antibodies for 1 h at room temperature (ErbB4) or overnight (ErbB3). Subsequently, the slides were washed and incubated with biotinylated secondary antibody (Zymed Laboratories, CA, USA, Vector Laboratories, UK) and HRP-streptavidin (Zymed Laboratories). The staining was developed using 3, 3'-diaminobenzidine (Vector Laboratories). The sections were counterstained with Mayer's hematoxylin. In each staining run, a negative/blank control was included.

**EGFR/ErbB2.** EGFR and ErbB2 were stained mainly at the cell membrane. All slides were independently reviewed twice, and intraobserver disagreements (<10%) were reviewed a third time followed by a conclusive judgment. Evaluation of the antibody was performed using a four-graded scale: 0 negative, 1+ weak staining intensity, 2+ intermediate staining intensity, and 3+ strong staining intensity (Figure 1). The sample was determined as ErbB2 positive if at least 10% of the tumour cells were immunostained (15) and EGFR positive if at least 1% of the tumour cells were stained (7).

**ErbB3/ErbB4.** The intensity of ErbB3/ErbB4 staining was graded using a 0-2 scale: 0 negative, 1+ weak staining, 2+ strong intensity (9) (Figure 2). ErbB3 and ErbB4 staining was mostly cytoplasmic but with membranous staining in some areas, often in the basal part of the

cells. A slight background staining was present in samples stained for ErbB4, and staining intensity above the background level was considered. To be determined as a positive sample, at least 10% of the tumour cells had to be immunostained (13).

**Statistical analysis.** Statistical analyses were performed using the Wilcoxon's paired signed rank test and Chi-square test utilising SPSS 13.0 for Windows (SPSS Inc., Chicago, USA). All primary tumours were analysed according to the defined stage at first diagnosis, although all patients eventually became stage IV patients.

## Results

**EGFR expression in primary colorectal tumours and corresponding metastasis.** In 49 of 64 (76%) analysed primary tumours, immunostaining for EGFR was evident. In both the primary tumour and metastasis a similar expression was evident in 26/64 (40%), and 16/49 (33%) of the tumour cells had lost the expression in the corresponding metastasis. EGFR negative primary tumours was evident in 15/64 (23%) of the tumours, and among these 5/15 (33%) had developed EGFR expression in the corresponding metastasis. Accordingly, 10/15 (67%) maintained their EGFR negative status. EGFR expression in early (Stage I-II) and late stage (Stage III) tumours at time of primary tumour surgery did not significantly differ (Table II).

**ErbB2 expression in primary colorectal tumours compared to corresponding metastasis.** The primary tumours were positively immunostained for ErbB2 in 27/50 (54%) of the cases. Among these, 8/27 (30%) had lost expression in the correlated metastasis, 4/27 (15%) had increased expression, and 15/27 (55%) had a similar expression at both sites. ErbB2 negative primary tumours were evident in 23/50 (46%); of these, 4/23 (17%) had developed ErbB2 expression in the metastasis. Accordingly, most cases (19/23, 83%) maintained their ErbB2 negative status.

**ErbB3 and ErbB4 expression in primary colorectal tumour and corresponding metastasis.** ErbB3 and ErbB4 immunostaining in examined primary tumours were positive in 32/48 (67%) and 42/52 (81%), respectively, and no significant difference in expression between primary tumour and metastasis was evident. There was no significant correlation between early/late stage tumour and ErbB3-4 expression. Co-expression between the different receptors is shown in Table II; a significant higher amount of tumours co-expressing ErbB3 and ErbB4 was evident in late stage tumours ( $p=0.025$ ) (Table II).

## Discussion

To further investigate the ErbB protein family and its expression in metastatic colorectal cancer, the expression of EGFR and ErbB2-4 was evaluated in 64 primary tumours and their corresponding metastases. In this study, more than

Table I. Clinical characteristics of the 64 colorectal cancer patients included in the study.

	N (%)		N (%)
Age, years		Primary tumour	
Median	63	Colon	37 (58)
Range	36-83	Rectum	27 (42)
Gender		Metastasis	
Female	28 (43)	Liver	30 (46.9)
Male	36 (56)	Skin	6 (9.4)
Stage		Lung	8 (12.5)
Stage I	3 (4.7)	CNS	2 (3.1)
Stage II	18 (28.1)	Bone	6 (9.4)
Stage III	28 (43.8)	Scar	10 (15.6)
Primary tumour characteristics		Peritoneum	1 (1.6)
Adenocarcinoma	52 (81.3)	Gallbladder	1 (1.6)
Mucinous	12 (18.8)	Survival months	
Grade 1-2	52 (81.3)	Median	29
Grade 3	7 (10.9)	Range	1-154
Missing value	5 (7.8)		

one-third of the cases (33%) lost EGFR expression in the metastasis, and among the EGFR negative primary tumours one third displayed EGFR positive metastases compared to their corresponding primary tumour. Similar changes, although not as pronounced, were seen with regard to ErbB2.

The activated EGFR is well known as a potent regulator of cell adhesion, differentiation, apoptosis and metastasis (16), often overexpressed in colorectal cancer (4). Three previous studies of the immunohistochemical expression of EGFR in primary tumours and metastasis in colorectal cancer have shown diverging results. The presented results were similar to the observations by Scartozzi, Bralet and McKay (7, 8, 9), all studies that found a significant loss of EGFR expression in the metastatic sites (27-60%); however, they are in disagreement with studies by Italiano *et al.* and Bibeau *et al.*, who found no significant differences (10, 11). Bibeau *et al.*, using the tissue microarray technique, showed a significant underestimation of the EGFR positivity when comparing EGFR expression levels in tissue sections vs. tissue microarrays.

The discrepancy in results between the studies could be due to methodological issues, such as the antibodies used for identifying the expression and handling of tissues. Thus, the data could be hampered by the fact that the EGFR is sensitive to sample age and the way samples are prepared and fixed (17). The tumour samples in this study were collected over a long period (17 years) and might have been prepared according to different protocols, causing differences in immunostaining between the primary tumour and the usually later collected metastasis. However, in this study, the period between sampling of the primary tumour and

Table II. Expression of ErbB1-4 in 64 colorectal cancer primary tumours and coexpression data between the ErbB receptors in early (Stage I-II) and late stage (Stage III-IV) tumours.

Positive Immunoassaying/ Coexpression	Early stage I+II n (%)*	Late stage III-IV n (%)**	P-value***	All# N (%)
ErbB1	16/21 (76%)	33/43 (77%)	0.96	49/64 (76%)
ErbB2	6/15 (40%)	21/35 (60%)	0.19	27/50 (54%)
ErbB3	9/16 (56%)	23/32 (72%)	0.17	32/48 (67%)
ErbB4	13/18 (72%)	29/34 (85%)	0.32	42/52 (81%)
ErbB1+ErbB2	6/15 (40%)	18/36 (50%)	0.51	24/51 (47%)
ErbB1+ErbB3	10/20 (50%)	22/34 (65%)	0.28	32/54 (59%)
ErbB1+ErbB4	9/19 (47%)	22/36 (61%)	0.32	31/55 (56%)
ErbB2+ErbB3	5/14 (36%)	15/27 (55%)	0.23	20/41 (49%)
ErbB2+ErbB4	5/14 (36%)	16/34 (47%)	0.32	21/48 (44%)
ErbB3+ErbB4	10/19 (53%)	24/29 (83%)	0.025	34/48 (71%)

\*/\*\*The column shows the Stage I+II\* and Stage III+IV\*\* tumour groups and the proportion of positive tumours. The variability in the denominator due to the number of tumours with reliable results for the different ErbB receptors. #All analysed samples. \*\*\*Chi-square analysis between expression in early and late stage tumours.

corresponding metastasis tissue is in general rather short in comparison to overall collection study time range, a condition that may limit this technical problem. The antibodies used in this study react both to the phosphorylated and the non-phosphorylated EGFR, an important factor when comparing results from different studies. In all four studies, the same antibody was used, but in the other three studies the samples were collected during a shorter period (4-10 years). Also, malignant tumours and metastases are highly heterogeneous, and the expression of a receptor in the analysed sample or biopsy does not always represent the expression in the non-adjacent areas.

Nevertheless, altogether the results demonstrated the necessity of new methods to identify patients that might benefit the most from being treated with EGFR targeted antibodies. It has been suggested that tumours with an increased EGFR copy number, as determined by FISH, respond better to cetuximab treatment than tumours without an increased copy number (18). This method might be a better tool for determining clinically significant EGFR over-expression. Moreover, with the available methods for analysing EGFR, patients with EGFR defined negative tumours displayed significant clinical responses to treatment with cetuximab (19). This demonstrates the complexity in correlating EGFR expression to the effects of anti-EGFR therapy.

Previous studies have shown that ErbB2 expression in colorectal cancer is present in a wide range (20-89%) of tumours with no evident correlation to the patient's prognosis (20, 21). The disagreement to the results of this study might be differences in handling of the biopsies, storage time and the fact that other antibodies were used (22, 23).

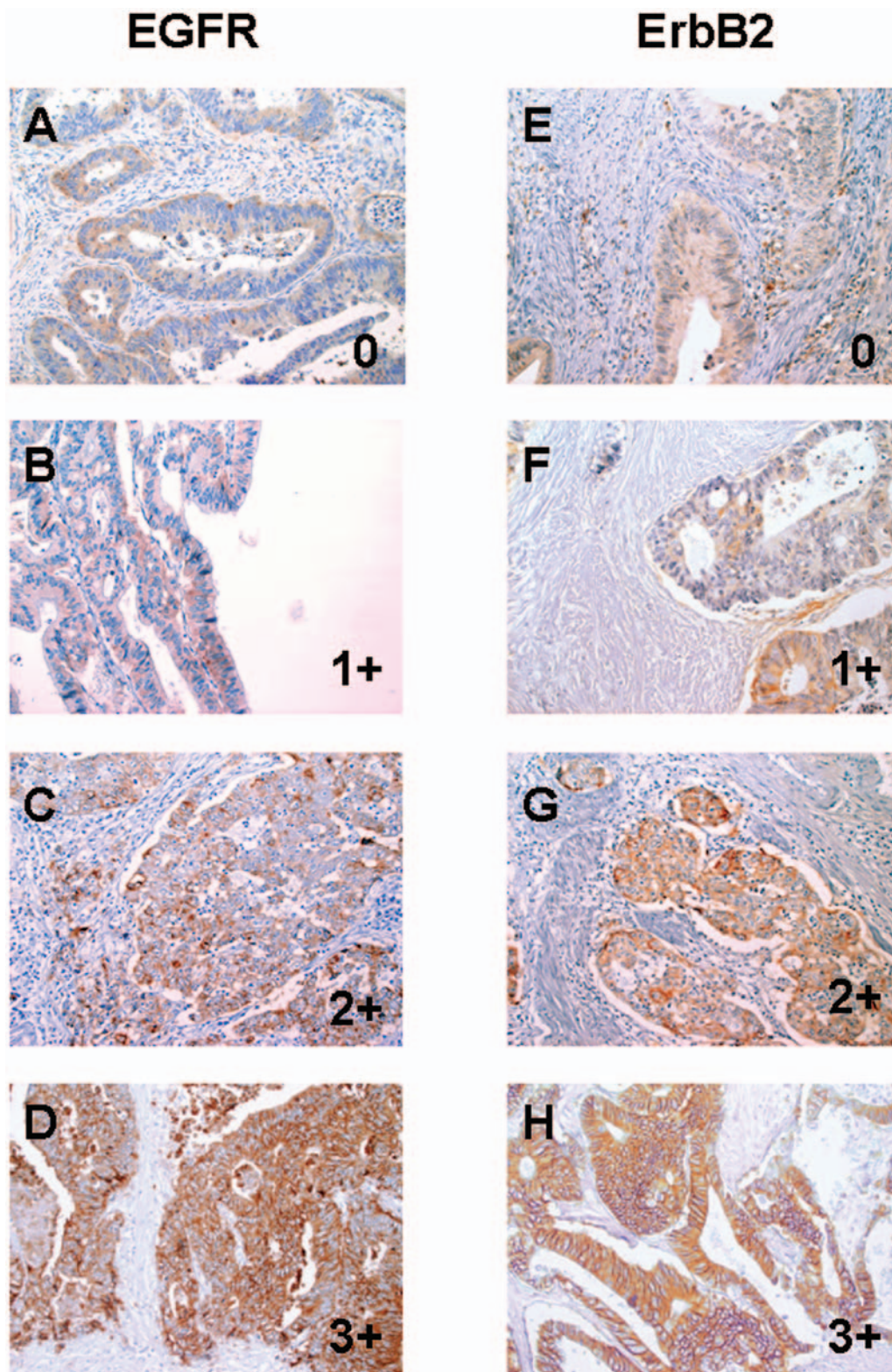


Figure 1. Immunohistochemical staining for EGFR (A-D) and ErbB2 (E-H) expression in primary colorectal cancers at 20X objective magnification: A) Negative EGFR (0), B) Weak EGFR staining in >1% of the tumour cells (1+), C) Intermediate EGFR staining (2+), and D) Strong EGFR staining (3+). E) Negative ErbB2 (0), F) Weak ErbB2 staining in >10% of the tumour cells (1+), G) Intermediate ErbB2 staining (2+), and H) Strong ErbB2 staining (3+). Mostly membranous staining pattern was observed.

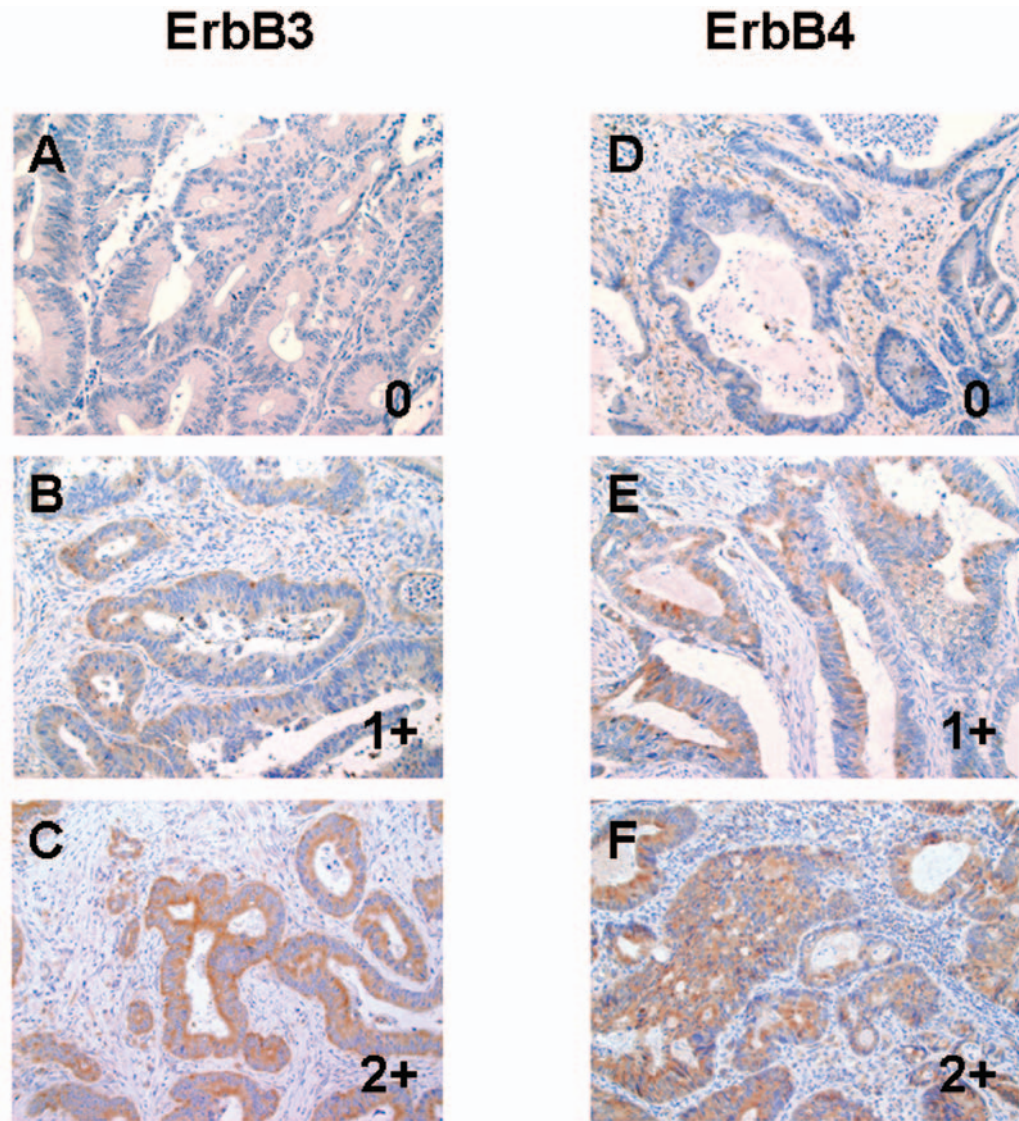


Figure 2. Immunohistochemical staining for ErbB3 and ErbB4 expression in primary colorectal cancers at 20X objective magnification: A) Negative ErbB3 (0), B) Weak ErbB3 staining (1+), and C) Strong ErbB3 staining (2+). D) Negative ErbB4 (0), E) Weak ErbB4 staining (1+), and F) Strong ErbB4 staining (2+). For both antibodies both cytoplasmic and membranous staining pattern was observed. Especially for ErbB4 a slight background staining was present and therefore only staining intensity above the background levels was considered.

In a study by Lee *et al.*, co-expression of ErbB2 and ErbB4 correlated to survival (12), but in the present study no significant co-expression of the two receptors was shown. Nevertheless, in this study, ErbB2 positive cases lost the expression in 30% of corresponding metastasis, in concordance with the EGFR results.

Considering that few previous studies have investigated the ErbB3 and ErbB4 receptors in colorectal cancer, the consistency in ErbB3 and ErbB4 expression in primary tumour and metastases is an interesting observation that emphasizes the differences between the ErbB family

members. To the authors knowledge, the significant co-expression of ErbB3 and ErbB4 in late stage tumours has not been previously reported. It is based on a rather small set of samples but might indicate the importance of these proteins in the progression of colorectal cancers.

The finding of a loss of about 30% of the EGFR expression in colorectal cancer metastases compared to the primary tumour show the heterogenous biology of metastatic malignancies. There is a need for further characterisation of the metastases of primary tumours in order to make the correct decision of metastatic treatment when using protein specific antibodies.

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