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Studies of LRIG1 and the ERBB receptor family in breast and colorectal cancer

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Ju mer man tänker, desto mer inser man att det inte finns något enkelt svar.

Nalle Puh

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Abstract

The *LRIG1* gene (leucine-rich repeats and immunoglobulin like domains-1) at chromosome 3p14 is a proposed tumour suppressor gene whose gene product negatively regulates various receptor tyrosine kinases. This function has been the basis for classifying *LRIG1* as a potential tumour suppressor gene (TSG). The ERBB receptor family is important in malignant cellular functions such as proliferation, survival, adhesion, migration and differentiation. In breast cancer, amplification of the *ERBB2* proto-oncogene is an important negative prognostic factor. The epidermal growth factor receptor (EGFR/ERBB1), is expressed in colorectal cancer and has been correlated to a worse prognosis. Until recently, immunohistochemical analysis of EGFR expression was used to select patients suitable for treatment with EGFR targeted antibodies.

This thesis characterizes LRIG1 in breast and colorectal cancer to gain further knowledge of the gene and its expression. Also, the EGFR expression in metastases and the invasive margin of colorectal cancers was investigated to correlate changes to clinical factors. Breast cancer samples and matched normal tissues were evaluated for LRIG1 and the ERBB receptors at gene, RNA and protein levels. An increase in copy number of the *LRIG1* gene was evident. Also, increased *LRIG1* copy number was associated with high levels of *ERBB2* mRNA. Another set of breast cancer tumours were analysed for *LRIG1* by FISH analysis. The results were coherent with the previous results. To further analyze the correlation to *ERBB2*, tumours with *LRIG1* increased copy number were analysed for *ERBB2*. The data showed that 89% of tumours with increased *LRIG1* copy number were either *ERBB2* amplified or had an increased copy number of *ERBB2*.

To investigate *LRIG1* and the EGFR in colorectal cancer, the gene and protein expression was analysed by several methods in tumours and corresponding normal tissues. There were no significant changes at gene level found, but at the protein level, both over- and under expression were seen. No evident correlation between LRIG1 and EGFR expression was detected.

The ERBB receptor family expression in colorectal cancer tumours and corresponding metastases was investigated to explore if the expression was altered in the metastatic lesion. The results showed that the EGFR expression was lost in the corresponding metastases in 33% of the tumours and that the same percentage of tumours gained expression in the metastases. Co-expression of the ERBB family members was also analysed; there was a significant increase of ERBB3/ERBB4 co-expression in late stage tumours. EGFR expression at the invasive margin of colorectal cancers was analysed to clarify whether expression correlated to the patient's prognosis. Significant correlation to survival and the presence of budding was seen.

In conclusion, 34% of the breast cancer tumours studied had an increased copy number of *LRIG1* with a significant co-incidental increase in *ERBB2* copy number. This raises the question of a functional correlation between *LRIG1* and *ERBB2*, a finding that might be of clinical importance. The studies of EGFR and the ERBB receptors in colorectal cancer reflect the heterogeneity of EGFR expression in tumours. In addition, these findings suggest that survival of the patients correlates to an increasing EGFR expression at the invasive margin.

Keywords: Breast cancer, Colorectal cancer, LRIG1, EGFR, ERBB2, Invasive margin.

List of papers

This thesis is based on the following papers, which are referred to in the text by roman numbers**.**

I. Increased copy number at 3p14 in breast cancer.

Ljuslinder I, Malmer B, Golovleva I, Thomasson M, Grankvist K, Höckenström T, Emdin S, Jonsson Y, Hedman H, Henriksson R

Breast Cancer Res. 2005;7(5):R719-27

II. Co-incidental increase in gene copy number of *ERBB2* and *LRIG1* in breast cancer

Ljuslinder I Golovleva I, Grankvist K, Henriksson R, Malmer B, Hedman H

Breast Cancer Research 2009, 11:403

III. LRIG1 expression in colorectal cancer.

Ljuslinder I, Golovleva I, Palmqvist R, Oberg A, Stenling R, Jonsson Y, Hedman H, Henriksson R, Malmer B.

Acta Oncol. 2007;46(8):1118-22

IV. ERBB 1-4 expression alterations in primary colorectal cancers and their corresponding metastases

Ljuslinder I, Malmer B, Isaksson-Mettävainio M, Öberg Å, Henriksson R, Stenling R, Palmqvist R

Anticancer Research, 29:5, 2009, pp. 1489-1494

V. Increasing EGFR expression at the invasive margin is a negative prognostic factor in colorectal cancer

Ljuslinder I, Malmer B, Öberg Å, Henriksson M, Palmqvist R

Submitted Manuscript.

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Abbreviations

CGH	Comparative genomic hybridization
Cbl	Casitas b-cell lymphoma
CRC	Colorectal cancer
DCIS	Ductal cancer in situ
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERBB	v-erb-b1 erythroblastic leukemia viral oncogene homolog
FAP	Familial adenomatous polyposis syndrome
FISH	Fluoroscense in situ hybridization
HNPCC	Hereditary nonpolyposis colorectal cancer
HRT	Hormone replacement therapy
IHC	Immunohistochemistry
LCIS	Lobular cancer in situ
LRIG	Leucine-rich repeats and immunoglobulin-like domains
MMR	Mismatch repair
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-OH kinase (PI 3-kinase)
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RT-PCR	Reverse transcriptase PCR
SNP	Single nucleotide polymorphism
TSG	Tumour suppressor gene

Introduction

Breast cancer

Epidemiology and Demographic factors

Breast cancer, a major cause of cancer death among women, is diagnosed in approximately 1000 000 women worldwide annually. In Sweden, approximately 7000 cases of breast cancer are diagnosed every year (National Board of Health and Welfare 2007) and the disease is the most common cause of death in women aged 40-44 years. At diagnosis, the medium age is 63 years and the risk of disease increases with age. The incidence and mortality in European countries is shown below (Figure 1).

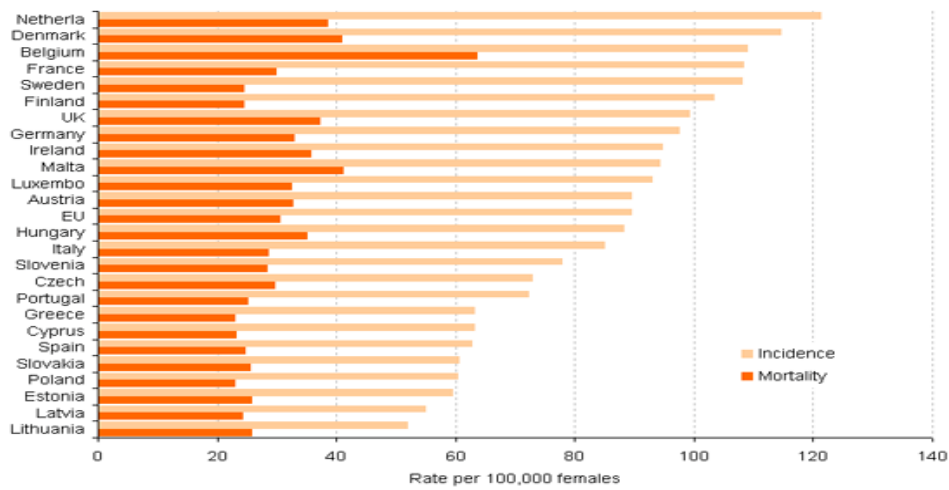

Figure 1. Reprinted with permission from Cancer Research UK, April 2009
http://info.cancerresearchuk.org/cancerstats/types/breast/incidence/?a=5441

Molecular biology of breast cancer

Over the last few decades, the genes that contribute to initiation and clinical progression of breast cancer have been a central area of research (Sjoblom et al. 2006, Wood et al. 2007). Identification of germline mutation of *BRCA1* and *BRCA2* (Futreal et al. 1994, Wooster et al. 1994) have been important findings in hereditary breast cancer. In sporadic breast cancer, a tumorogenic multi-step accumulation of mutations is believed to be the basis of the disease. Moreover, activation of onocogenes due to mutations is probably an early event in sporadic tumours, followed by non-mutational

inactivation of tumour suppressor genes (Polyak 2007). In malignant tumours, gene amplifications are presumed to contain proto-oncogenes and deletions to contain tumour suppressor genes.

Gene amplifications

Amplification means a cellular process characterized by the production of multiple copies of a particular gene. There are several chromosomal regions amplified or duplicated in breast cancer that can cover large regions of DNA or be limited to a single locus (Table 1).

Table 1. Genes frequently amplified in breast cancer

Region	Gene	Described by
1q22	*RAB25*	(Cheng et al. 2004)
8p11-12	*FGFR1*	(McLeskey et al. 1994)
8q24	*MYC*	(Escot et al. 1986)
11q13	*Int-2/FGF-3*	(Naidu et al. 2001)
11q13	*CyclinD1*	(Lammie et al. 1991)
12q14-15	*MDM2*	(McCann et al. 1995)
17q21	*ERBB2*	(Slamon et al. 1987)
20q13	*STK6/ZNF217*	(Ginestier et al. 2006)

The *ERBB2* gene at *17q 21* encodes a transmembrane tyrosine kinase receptor that is a member of the type 1 family, which also includes *ERBB1* (EGFR), *ERBB3* and *ERBB4*. FISH (Fluoroscence in situ hybridisation) analysis shows *ERBB2* amplification in 15-25% of breast cancer tumours and the amplification is associated to a poorer prognosis (Slamon et al. 1989). Moreover, several genes are co-amplified and co-overexpressed with *ERBB2* (Bertucci et al. 2004) and studies have shown that *ERBB2*-amplified breast cancers represent a specific molecular subtype (Sorlie et al. 2003). Amplifications of *FGFR-1* and *MYC* at 8p12 and 8q24 have been reported to correlate to a significantly worse outcome (Letessier et al. 2006) (Al-Kuraya et al. 2004).

Gene mutations and losses

In breast cancer, many human genes contain several point mutations, small duplications, deletions and insertions. Breast cancer often shows losses of genomic regions thus implying that these areas might actually harbour a tumour suppressor gene. Previously, the method of choice to screen for deleted regions has been "Loss of heterozygosity" (LOH) using microsatellite markers, but now more accurate methods, such as for example oligonucleotide array-based SNP genotyping (SNP array), are being used.

Oncogenes: Proto-oncogenes are genes that often promote cell division, but when one allele of the gene is altered by mutation or amplification, it can induce proliferation and uncontrolled cell growth, ultimately causing the formation of a tumour. Oncogenes are thus the mutant forms of proto-oncogenes. In breast cancer, especially lobular cancers, the *PI3CA* is often mutated (Buttitta et al. 2006).

Tumour suppressor genes: *TP53,* one of the powerful TSGs, plays an important role in the cell, acting as a transcription factor and as a detector and controller of cellular damage. The *TP53* gene is mutated in approximately 20-40% of breast cancers (Langerod et al. 2007) and correlates to a worse outcome (Overgaard et al. 2000). Furthermore, *PTEN* and *FHIT* are other TSGs frequently mutated in breast cancer (Ingvarsson et al. 2001, Saal et al. 2005).

Hereditary breast cancer

Inherited factors contribute to a major part of all breast cancer with hereditary forms of breast cancer directly responsible for 5% to 10% of all breast cancers diagnosed in women aged 45 or younger. Hereditary breast cancer is characterized by young age of onset, a high prevalence of bilateral breast cancer and presence of tumours in several family members (Lynch et al. 2008). The genes are transmitted in an autosomal dominant manner (Easton et al. 1993) and germline mutations in the *BRCA1* gene, located at 17q21, is associated with a 50% risk of breast cancer by the age of 50 and with an 85% lifetime risk in high-risk families (Antoniou et al. 2003, Begg et al. 2008). The *BRCA2* gene, at chromosome 13, associates to early-onset breast cancer, ovarian cancer and in rare cases male breast cancer (Wooster et al. 1994).

Risk factors

Hormonal and reproductive risk factors

A late menarche reduces the breast cancer risk, but length of menstrual cycles and regularity has not consistently been related to increased risk (Kelsey et al. 1993). Childbearing is a breast cancer risk protector, with a risk reduction of 25% in women who have at least one full-term pregnancy (Key et al. 2001). Moreover, the number of pregnancies and a young age at first full-term pregnancy provide increasing protection (Ewertz et al. 1990). There has been some controversy concerning the risk influence of breastfeeding, but some research has shown a lower cancer risk in women who breastfeed their infants (Lord et al. 2008) and that the effect of breastfeeding seems to be higher in younger women (Kelsey et al. 1993).

Obesity: After menopause, adipose tissue is the major source for steroids and obese women indeed have higher estrogen levels. An EPIC (European Prospective Investigation of Cancer) publication noted that obese women (BMI > 30) had a 31% higher risk for breast cancer compared to women with BMI < 25. Also, general obesity was a significant predictor of breast cancer risk in postmenopausal women not taking exogenous hormones, whereas waist-hip ratio or waist circumference were not related to excess risk when adjusted for BMI. On the other hand, obesity was not a risk factor among premenopausal women (Lahmann et al, 2004).

Estrogen: Estrogen promotes the proliferation of mammalian epithelial cells with existing mutations (Henderson & Feigelson 2000). In 2002, a pooled analysis of all nine prospective studies available on endogen estrogen levels and breast cancer risk (Key et al. 2002) showed a positive association between estrogen levels in blood and breast cancer risk.

Progesterone: During the last decade, progesterone and the nuclear progesterone receptor (PR) have been intensely studied to find out the correlation to receptor-positive breast cancer. Studies have provided evidence that cultured breast cancer cells exposed to progestin initiates cell cycle progression and that PR signalling is important for cell cycle regulation in breast cancer (Lange et al. 2008).

*Hormone replacement therapy (HRT)***:** The Women's health initiative was a placebo-controlled trial that evaluated the overall health effects of HRT (Chlebowski et al. 2009). In the study, a 24% risk increase for invasive breast cancer was evident when patients were treated with estrogen + progesteron compared to placebo. In contrast, no increase in breast cancer risk was shown in the group treated with estrogen alone compared to placebo. Based on the data of the WHI study, most professional societies recommend minimizing exposure when HRT is used for treatment of menopausal symptoms.

Table 2. Established risk factors for breast cancer

Factor	Relative risk	High Risk group
Age	>10	Elderly
Geographical location	5	Developed country
Age at menarche	3	Menarche before age 11
Age at menopause	2	Menopause after age 54
Age at first full pregnancy	3	First child in early 40´s
Family history	>2	Breast cancer in first degree relative when young
Previous benign disease	4-5	Atypical hyperplasia
Cancer in other breast	>4	
Diet	1.5	High intake of saturated fat
Body weight		
Premenopausal	0.7	Body Mass Index > 35
Postmenopausal	2	Body Mass Index > 35
Alcohol consumption	1.3	Excessive intake
Taking exogenous hormones		
Oral contraceptives	1.24	Current use
Hormone replacement therapy	1.35	Use for > 10 years

ABC of breast diseases, McPherson et al., BMJ. 2000 Sep 9;321 (7261):624-8.

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*Other risk factors***:** Table 2 summarizes the most important risk factors in breast cancer. Other factors suggested to be important in the development of breast cancer include oral contraceptives, spontaneous and induced abortion, physical activity, height, alcohol consumption and dietary factors (such as fruit intake) (McPherson et al. 2000).

Symptoms/Diagnostics

Breast cancer is most commonly detected during mammography screening programs or during self-examination. The symptoms may include a palpable lump, dimpling of the skin, change in size or shape of the breast, retraction of overlying skin, inversion of the nipple or a discoloration of the skin of the breast. To evaluate the breast, a mammogram, often in combination with ultrasound, is performed. In selected cases, a Magnetic Resonance Imaging (MRI) can be helpful. If breast cancer is suspected, the next step is a biopsy to confirm the diagnosis and to evaluate tumour characteristics.

Staging

Breast cancer is classified according to the UICC/ICD-10 system (Table 3).

Table 3. Breast cancer TNM classification

		Stage	Primary tumour (T)*	Regional lymph node status (L)	Distant metastasis (M)
T- Tumour		**0**	Tis	N0	M0
T1	Tumour ≤ 2 cm	**I**	T1	N0	M0
T2	Tumour ≥ 2 cm but ≥ 5 cm		T0	N1	M0
T3	Tumour ≥ 5 cm	**IIA**	T1	N1	M0
T4	Tumour of any size with direct extension to chest wall or skin		T2	N0	M0
N- Lymph node		**IIB**	T2	N1	M0
N0	No cancer in regional node		T3	N0	M0
N1	Regional movable metastasis	**III A**	T0	N2	M0
N2	Non-movable regional metastases		T1	N2	M0
N3	Cancer in the internal mammary lymph nodes		T2	N2	M0
M- Metastasis			T3	N1/N2	M0
M0	No distant metastases	**III B**	T4	Any N	M0
M1	Distant metastases	**III C**	Any T	N3	M0
		IV	Any T	Any N	M1

Criteria for staging breast tumours according to the UICC ICD-10 TNM classification.

*Size measurements are for the tumour's greatest dimension**.**

Histology

Noninvasive tumours that arise in the ducts or lobules of the breast are called ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). The majority of breast cancers are called invasive breast cancers as they have invaded through the wall of the ducts or lobules. Subgroups of breast cancer include ductal, lobular, medullary, tubular and metaplastic tumours.

Molecular classes

Gene-expression profiling studies of breast cancer have resulted in new knowledge but also the need for new classifications. Four molecular breast cancer classes have been proposed. The first class, basal-like tumours, are ER-negative, PR–negative and *ERBB2*-negative tumours, also called a "triple-negative" tumour. The other class is called luminal-A cancers, tumours that are mostly ER-positive and histologically low-grade. The third class is called the luminal-B cancers, often high-grade tumours, mostly ER-positive but sometimes expressing low levels of the hormone receptors. The last group is called the ERBB2-positive tumours, *ERBB2* amplified and with a high expression of the ERBB2 protein (Perou et al. 2000).

Prognostic factors

The prognostic factors recommended by the St. Gallen guidelines are lymph node status, tumour size, histological grade (Nottingham histologic grade or Elston-Ellis grade), vascular invasion, age, ER status and *ERBB2* status (Goldhirsch et al. 2007).

Nottingham histologic grade (NHG): The histologic grading system used today was first introduced by Elston and Ellis (Elston & Ellis 1991). Using NHG three features are analysed as follows: the degree of tubule formation, variation in the mitotic rate and the size and shape of nuclei. Each feature is given a score of 1-3. The sum of scores from the three features according to NHG is as follows: Grade I, well differentiated, 3-5 points; grade II, moderately differentiated, 6-7 points; grade III, poorly differentiated, 8-9 points.

Ki-67: The Ki-67 protein, a cellular marker for proliferation, may be used as a possible prognostic factor in breast cancer. The protein was recently reviewed as a prognostic factor in a meta-analysis involving 12155 patients (de Azambuja et al. 2007). An association between over-expression of Ki-67 and a worse outcome in early breast cancer was seen for the entire study population and in subgroup analysis for node negative, node positive and untreated patients. In Sweden, a national quality assurance program has been performed and Ki-67 is regarded as an independent risk factor in tumours of grade I-II. However, due to the strong correlation between grade III and Ki-67 expression (i.e., mitotic index), Ki-67 is not regarded as an independent risk factor in grade III tumours.

ERBB2: The determination of *ERBB2* over-expression/amplification in the diagnosis of early breast cancer is a routine procedure in Sweden (Haglund et al. 2005). The current practice is to screen tumour samples by immunohistochemistry (IHC). If the tumour tests 2-3+, the tissue is retested by FISH or other in situ hybridization tests.
A FISH ratio > 2 when dividing copies of *ERBB2* and the chromosome 17 centromere classifies the tumour as *ERBB2* amplified (Carlson et al. 2006).

Treatment

Operable breast cancer is initially treated by breast-conserving surgery or mastectomy. Most patients with tumours less than 5 cm in diameter and with no signs of lymph node involvement in the preoperative diagnostic procedure are offered a sentinel node biopsy. An axillary dissection is performed for larger tumours, multifocal disease, positive sentinel node and preoperatively-known lymph node metastases.

To minimize the risk of loco-regional relapse after breast conservative surgery and in patients undergoing mastectomy with lymph node positive disease, adjuvant radiotherapy is given for 5–6 weeks. Newly published data from the overview process of the EBCTCSG in Oxford has shown that the 70% reduction in loco-regional relapse after radiotherapy in a 15 year follow-up results in an absolute overall survival gain of 5-7% (Clarke et al. 2005). From the overview analyses, combined with data from single large adjuvant trials, it has been shown that adjuvant endocrine treatment in estrogen receptor positive disease results in a 25-35% reduction of recurrence and death in

breast cancer irrespective of menopausal status. Adjuvant chemotherapy with CMF (cyclophosfamide, methotrexate, 5-FU) gives approximately the same reductions although the effect is more evident in pre- and perimenopausal women. The addition of antracyklines as well as antracykline-taxane combinations have been shown to further increase this reduction of recurrence and death in breast cancer by 10 and 20%, respectively (Lopez-Tarruella & Martin 2009). Trastuzumab is a monoclonal antibody directed against the extracellular domain of the ERBB2-receptor. Several randomized trials of trastuzumab treatment in patients with *ERBB2*-overexpression have shown a breast cancer death reduction of 3-6% when added to standard chemo-endocrine therapy and radiotherapy (Hall & Cameron 2009). Thus, trastuzumab treatment is now recommended as standard treatment for these patients.

The main factors that determine postoperative adjuvant treatment are based on the above mentioned data: endocrine responsiveness, *ERBB2*-status and risk of relapse based on the St. Gallen risk factors.

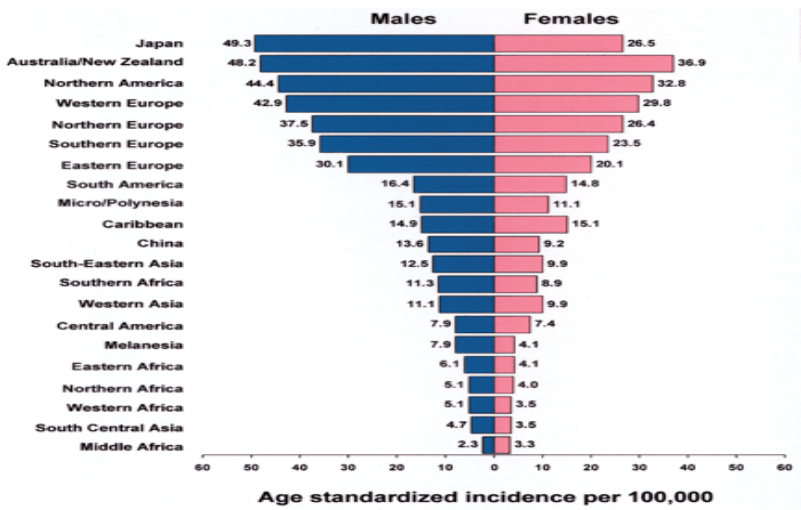
In advanced disease, endocrine responsiveness, *ERBB2* status, metastatic site and rate of progression together with factors such as age and co-morbidity as well as quality of life aspects are used to determine an individual treatment plan. In general, when possible, endocrine treatment is always considered as first line treatment, with the exception of *ERBB2*-amplified disease where trastuzumab in combination with endocrine or cytotoxic therapy is recommended. Chemotherapy will be considered for patients with an endocrine-nonsensitive, aggressive disease with for example advanced liver metastases and after failure on one or more endocrine treatments.

Colorectal cancer

Epidemiology and Demographic factors

Colorectal cancer (CRC) is one of the most common malignancies in Europe and worldwide (Ferlay et al. 2007). Every year there are 5300 new cases in Sweden and 2000 die of the disease (National Board of Health and Welfare 2007). Global incidence rates are shown in Table 2.

Figure 2. Age-standardized Incidence Rates for Colorectal Cancer



Age-standardized Incidence Rates for Colorectal Cancer. Data shown per 100,000.
Global Cancer Statistics, 2002, Parkin et al., CA Cancer J Clin. 2005 Mar-Apr;55(2):74-108.
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Molecular biology of colorectal cancer

The pathogenesis of colorectal cancer is complex and includes hereditary and environmental factors and the underlying factors have been intensely studied in the last decades. In approximately 10% of patients there is a clear familial association, but the majority are sporadic cancers. In the malignant process resulting in a colorectal cancer tumour, both TSGs and oncogenes are involved (Hisamuddin & Yang 2006). In 1990, a genetic model for the stepwise formation of CRC from normal colonic tissues was introduced by Fearon and Vogelstein (Fearon & Vogelstein 1990). In short, they proposed that the tumours are due to mutations in genes that regulate the repair of

DNA damage and cell proliferation, that mutations must occur in more than one gene, and that the order of the mutations influences the formation of CRC. An activated oncogene can lead to increased cell growth and contribute to tumour development. The TSG adenomatous polyposis coli (APC) is a gene that when germline mutated and inactivated is associated with FAP (familial adenomatous polyposis) and also attributes to approximately 70% of sporadic CRC (Miyaki et al. 1994). However, there are many other genes involved in the carcinogenic process (Cheah 2009) (Strimpakos et al. 2009) (Table 4).

Table 4. Main genetic abnormalities of CRC

Altered gene	Frequency	Normal function	Clinical significance
APC	70-80%	Tumour suppressor	Mutations linked to FAP and sporadic CRC
Src	80%	Oncogene	Overexpression/mutation leads to Akt/PI3K over activation
MSH2, MSH6, MLH1 and other MMR	15% of sporadic CRC 49% of HNPCC	DNA MMR	Mutations linked to HNPCC (Lynch syndrome). Microsatellite unstable tumours (MSI) related to poor response to adjuvant CT but also to a better prognosis
TP53 (p53)	50-60%	Tumour suppressor	Mutations associated to poor prognosis
KRAS	40-50%	Oncogene	Overexpressed mutations leads to resistance to EGFR mAbs
BRAF	5-12%	Oncogene	Mutations lead to resistance to EGFR mAbs
PTEN	18-40%	Tumour suppressor	Loss of activity related to poor response to EGFR mAbs

Abbreviations: APC, adenomatous polyposis coli; CRC, colorectal cancer; CT, chemotherapy; EGFR, epidermal growth factor receptor; FAP, familiar adenomatous polyposis; HNPCC, hereditary non-polyposis colorectal carcinoma; mAbs, monoclonal antibodies; MMR, mismatch repair; MSI, microsatellite instability.
Pharmacogenetics and biomarkers in colorectal cancer, Strimpakos et al, Pharmacogenomics J. 2009 Apr 21.
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In colorectal carcinogenesis, there are two major groups of genomic instability: Chromosomal instability (CIN) is present in 65-70% of CRCs and Microsatellite instability (MSI) is present in 15-50% of CRCs (Miyazaki et al. 1999). In a recent meta-analysis by Walther et al., CIN was associated with a worse prognosis (Walther et al. 2008). Yet another meta-analysis, investigating the prognostic importance of MSI in 7500 patients, found that MSI+ tumours had a better prognosis (Popat et al. 2005).

Hereditary colorectal cancer

In about 25% of CRC, there is a familial background and in 15% there is a strong family background (Lynch & de la Chapelle 2003). FAP is an autosomal dominant disease characterized by the appearance of multiple adenomatous polyps in the gastrointestinal tract in affected patients often at an early age. Untreated patients can develop colorectal cancer in early adulthood. The disease results from germline mutations in the gene encoding APC (Miyoshi et al. 1992). The other most common hereditary form of CRC is hereditary non-polyposis colorectal carcinoma (HNPCC or Lynch syndrome), constituting 5% of all CRC cases. HNPCC is an autosomal dominant disorder caused primarily by mutations in mismatch repair (MMR) genes (Barrow et al. 2008). The minimal criterion of HNPCC is if colorectal carcinoma is diagnosed in at least three first-degree relatives belonging to two or more successive generations and the age of onset should be less than 50 years in at least one patient. Moreover, patients with HNPCC also have an increased risk for several other malignancies such as gastric cancer, ovarian cancer and tumours from the urinary tract (Ericson et al. 2004). Patients with HNPCC need to be included in surveillance programs at a young age, as studies have shown that genetic anticipation (i.e., an earlier age at onset in successive generations) is a common feature of the disease (Nilbert et al. 2009).

Risk factors

Colorectal cancer is a multi-factorial disease including environmental and life-style factors and genetic components. Studies have shown that Western countries tend to have a higher incidence than Asian and African countries (Parkin et al. 2005) and that the incidence of colorectal malignancy is slightly higher in males than in females. Clearly, age is important; the incidence peaks in the seventh decade (National Board of Health and Welfare 2007). Moreover, a high-fat and low-fibre intake may have an etiologic role and it has been suggested that a high-fibre intake may be protective against colorectal cancer (Howe et al. 1992). Some data suggest that diets with a high content of red meat increases the risk of distal colon and rectal cancers (Larsson & Wolk 2006). Obesity and family history are other risk factors for colorectal cancer (Park et al. 2009). Excess alcohol drinkers have a 2-fold increased risk of developing

colorectal carcinoma and smoking increases the risk, especially when initiated at a younger age (Omata et al. 2009). Also, there is a significant risk-increase for CRC in patients with inflammatory Crohn's disease and Ulcerous colitis (Rubio & Befrits 2008).

Symptoms/Diagnostics

The most important symptoms leading to diagnose are bleeding, change in bowel habits, occult bleeding, abdominal pain, malaise and pelvic pain. The patient is investigated by physical examination and/or recto-colonoscopy or colonic x-ray and faeces haemoglobin. When colorectal cancer is suspected, biopsies are taken.

Staging

Table 5. Colorectal cancer staging

Staging Colorectal cancer			
Stage	**Tumour maximum penetration**	**Regional lymph node metastasis**	**Distant metastasis**
0	Tis	N0	M0
I	T1 or T2	N0	M0
II	T3	N0	M0
III	Any T	N1-N2	M0
	T4	N0	M0
IV	Any T	Any N	M1

TNM classification: Tis = carcinoma in situ; T1 = submucosa; T2 = muscularis propria; T3 = penetrates all layers (for rectal cancer, includes perirectal tissue); T4 = adjacent organs or peritoneum. N0 = none; N1 = 1 to 3 regional nodes; N2 = ≥ 4 regional nodes; N3 = apical or vascular trunk nodes. M0 = none; M1 = present.

Redrawn and adapted from the Merck manual online

The Dukes staging system, introduced in the 1930s, was originally intended for rectal cancers and has been modified in several ways (Table 5). There is a strong correlation between stage and 5-year survival rate in patients with colorectal cancer. For Stage I/Dukes A, the 5-year survival rate following surgical resection exceeds 90%. For Stage II/Dukes B, the 5-year survival rate is 82% following resection, with or without adjuvant therapy. For Stage III/Dukes C, the 5-year survival rate is 59% following resection and adjuvant chemotherapy. For Stage IV/Dukes D, the 5-year survival rate is poor (approximately 8%) (O'Connell et al. 2004).

Histology

Most colorectal cancers are adenocarcinoma, mucinous or non-mucinous.

Prognostic factors

Tumour extent, lymph node status, and the assessment of lymphatic and venous invasion are still the most important morphological prognostic factors (Zlobec and Lugli, 2008). Tumour grade, based on the percentage of gland formation, is another important prognostic factor (Compton, 2003). Obstruction and perforation significantly worsen the survival and some studies claim them as important determinants of prognosis independent of Dukes' stage (Wolmark et al., 1983). Moreover, MSI, CIN and budding have been suggested as additional prognostic factors, but they are not presently used in clinical practice. Another prognostic factor is carcinoembryonic antigen (CEA), where preoperative serum levels have been reported as a predictive factor of recurrence after curative surgery in CRC patients, particularly for cases classified as stage II or stage III (Takagawa et al. 2008).

Treatment

Surgery

In the 1980s, Heald developed Total Mesorectal Excision (TME)(Heald & Ryall 1986). Using the TME technique, the dissection is performed outside the mesorectal fascia, preventing spill of tumour cells from the primary tumour and lymph nodes of the mesorectum. Since TME was introduced combined with radiotherapy, the recurrence rates have been significantly reduced (Kapiteijn et al. 2001). In colon cancer, the abdomen is explored and if the tumour is determined as resectable, a left or right hemicolectomy with end-to-end anastomosis is performed. When the patient is suffering from familial polyposis with multiple colonic polyps, a total colonic resection is most often performed.

When the disease has metastasised to the liver, resection of a limited number of liver metastases can be performed in selected patients (Vigano et al. 2008). Moreover, in some cases, a combination chemotherapy regimen can induce down-sizing of liver metastasis, making surgery with curative intention possible (Nordlinger et al. 2009).

In rectal cancer, radiotherapy is used in addition to surgery to decrease risk of local recurrence. Patients with a resectable cancer without overgrowth to other organs are usually given short-course preoperative radiotherapy 5x5 Grey and prompt surgery. Sometimes a prolonged radiotherapy course for 5-6 weeks, often combined with chemotherapy, is preferable (Glimelius et al. 2003).

Systemic therapy

Studies have demonstrated a survival advantage for patients with Dukes stage C colon cancer who receive adjuvant chemotherapy. For many years, the standard adjuvant therapy in colon cancer was 5-FU/leucovorin, but later studies have shown an even greater benefit when adding oxaliplatin to 5-FU/leucovorin (de Gramont et al. 2000, Andre et al. 2004). There is still much debate of the value of adjuvant therapy in Dukes B colorectal cancer. At present, it is not recommended as standard therapy although adjuvant therapy is sometimes given to some Dukes B patients with high risk tumours such as T4 tumours, tumours with vascular invasion and obstructive tumours (Figueredo et al. 2008). In metastatic colorectal cancer treatment, great improvement of survival length has been made with standard treatment combinations including both irinotecan and oxaliplatin combined with 5-FU (Douillard et al. 2000) (Falcone et al. 2007). Moreover, monoclonal antibodies such as bevacizumab (targeted against vascular endothelial growth factor, VEGF) and cetuximab (targeted against EGFR) have shown activity in combination with oxaliplatin, irinotecan, 5-FU, and leucovorin (Hurwitz 2004, Fakih 2009). On the other hand, a significant worse outcome for patients receiving the combination of cetuximab and bevacizumab has been reported in a recent study (Tol et al. 2009).

The invasive margin and EGFR in colorectal cancer

The molecular events at the invasive margin of malignant tumours are an important area of research. Cells at the invasive margin have been shown to be low-proliferating, achieving new abilities needed to invade and metastasize. The process called epithelial-mesenchymal transition (EMT), where differentiated epithelial mucosal cells gradually become invasive phenotypes (Kang & Massague 2004) is induced by epidermal growth factor (EGF), transforming growth factor β (TGFβ), hepatocyte growth factor (HGF), insulin growth factor and metalloproteinases (MMPs) (Huber et al. 2005). Moreover,

recent studies have provided evidence that an epithelial cell that has undergone EMT is less sensitive to treatment with EGFR inhibitors than other epithelial tumour cells (Barr et al. 2008) (Buck et al. 2007). The cells also lose cell-cell interactions and cell adhesion, thus leading to a possibility for the cells to disseminate (Thiery 2002).

EGFR immunohistochemical expression has been investigated in many studies, but only a few studies focus on the invasive margin. Goldstein et al., studying 102 stage IV colorectal cancer patients, reported a worse outcome in patients with high EGFR levels in the deepest layers of the tumour (Goldstein & Armin 2001). Other studies, such as Italiano et al. who investigated 80 stage IV patients, found an increase of EGFR expression at the invasive margin, but without correlating the observation to clinical findings (Italiano et al. 2005). Buckley et al. studied different antibodies for EGFR IHC analysis and found higher EGFR expression levels at the advancing edge. When investigating the invasive margin, several studies have observed the phenomenon of budding, small clusters of malignant cells close to the invasive margin but still separated from the tumour. Moreover, patients with budding tumours have been shown to have a worse outcome (Park et al. 2005a, Park et al. 2005b, Kanazawa et al. 2008, Nakamura et al. 2008). Thus, a new staging system has been proposed that includes the presence of budding in the risk analysis (Shinto et al. 2005).

In the processes at the invasive margin where cancer cells invade and infiltrate the healthy surrounding tissues, formation of membrane protrusions (invadopodia) are essential. EGF has been reported to stimulate the formation of invadopodia, providing the cells both ventral invasion movement and ability to degrade the extracellular matrix (Yamaguchi et al. 2005).

In colorectal cancer, several signalling pathways are of importance, but one of the most studied is the Wnt pathway, leading to nuclear translocation of B-catenin and activation of transcription. Several research groups have shown that the EGFR down-regulates E-cadherin and drives Wnt pathway signalling through the transcriptional repressor Slug (Conacci-Sorrell et al. 2003). When focusing on the invasive margin, a *reduced* expression of E-cadherin at the invasive front has been reported (Brabletz et al. 2001).

ERBB receptor tyrosine kinases (RTK)

The interconnected signalling pathways that controls cell growth and differentiation in human cells are often altered in cancer. Studies have shown that these pathways play a crucial role in cancer development, progression, and response to different therapies. In the last few decades, a great effort has been made to find and correct the alterations that lead to cancer. Moreover, signalling molecules are now used as drug targets, and a multitude of new targeted drugs are under development.

ERBB family signalling pathways

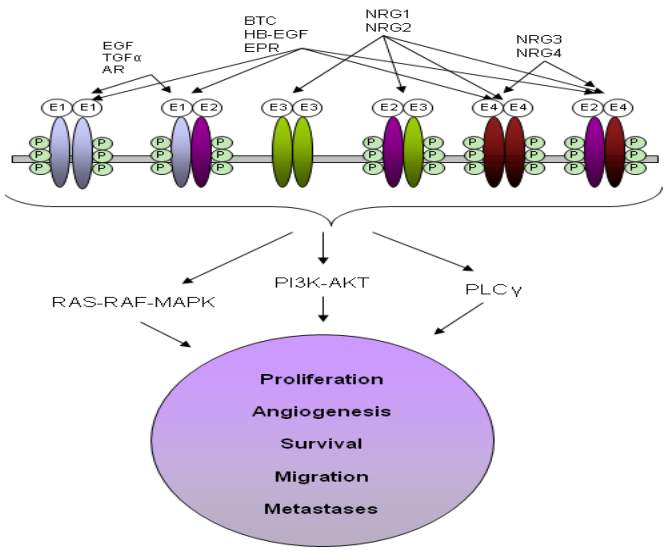

Figure 3. Mechanisms of action of the ERBB receptors in tumour cells. Activation results in receptor dimerization, tyrosine kinase activation and transphosphorylation (P). The phosphorylated ERBB receptors are able to interact with different signalling molecules that transmit the signals into the cell. The Ras-Raf-mitogen-activated protein kinase (MAPK), PI3 kinase/Akt, and PLC γ pathways are major signalling routes for the ERBB receptors. EGF: epidermal growth factor; TGF-α: transforming growth factor-α; AR: amphiregulin; BTC: betacellulin; HB-EGF: heparin binding-EGF; EPR: epiregulin; NRG: neuregulin. *Redrawn and adapted from "Epidermal growth factor receptor signalling in cancer" Gene. 2006 Jan 17; 366(1):2-16*

ERBB1-EGFR

The EGFR signalling pathway is an important regulator of growth, survival, proliferation and differentiation in mammalian cells (Ullrich et al. 1984, Yarden & Sliwkowski 2001). The *ERBB1* gene is located on chromosome 7 and encodes the EGFR, a 170 kDa glycoprotein consisting of an extracellular ligand-binding domain, a trans-membrane region, and an intracellular domain with tyrosine kinase activity. Multiple ligands bind to and activate the EGFR (Figure 3). Ligand binding to the

EGFR results in receptor homo- or heterodimerization at the cell surface, followed by internalization of the dimerized receptor.

The EGFR is over-expressed in the majority of solid tumours, including breast cancer (Dai et al. 2009), head-and-neck cancer (Grandis & Tweardy 1993), non–small-cell lung cancer (Rusch et al. 1993), renal cancer (Stumm et al. 1996) and ovarian cancer (Lafky et al. 2008). The impact of EGFR expression on prognosis has been investigated in many studies in different tumour types such as in astrocytomas and oligodendrogliomas where a significantly decreased overall survival was seen in patients with high EGFR protein expression (Andersson et al. 2004). In colorectal cancer, the receptor is over-expressed in 40-80% of tumours and has been proposed as a negative prognostic factor, although the data are quite contradictory. However, EGFR expression has been associated to tumour grade, stage (Steele et al. 1990, Kluftinger et al. 1992) and overall survival (Mayer et al. 1993). Nevertheless, in a meta-analysis by Nicholson et al., it was reported as a more modest prognostic indicator, correlating to poor survival rates in 52% (13/25) of the studies included in the meta-analysis (Nicholson et al. 2001).

A number of EGFR-targeted therapies have been developed, including the monoclonal antibody cetuximab, a therapy that has shown promising results in the clinical setting (Cunningham et al. 2004). As the EGFR targeted treatments are mostly used in patients with metastatic disease, a few studies have investigated the differences in EGFR expression in the primary tumour vs. the metastases (McKay et al. 2002, Scartozzi et al. 2004, Italiano et al. 2005, Bralet et al. 2007). These studies have shown both that a significant fraction of the primary tumour *lost* the EGFR expression in the metastases and data where the expression was *the same* in both primary tumour and corresponding metastasis.

A mutation analysis of the *K-RAS* gene is since recently performed before initiating cetuximab treatment as patients with K-RAS mutations do not respond to anti-EGFR treatment (Karapetis et al. 2008). The impact of EGFR over-expression in breast cancer has been intensely studied and meta-analysis has shown a modest correlation to prognosis (Klijn et al. 1992, Tsutsui et al. 2002).

ERBB2

The *ERBB2* gene is situated at chromosome 17, encoding a 185 kDa protein (Schechter et al. 1984). The gene product does not bind directly to any known ligand, but associates to ligand-activated forms of the other ERBB family members (Cho et al. 2003) (Figure 3). *ERBB2* is over-expressed in many human malignancies and triggers downstream signalling cascades controlling proliferation, cell survival and apoptosis (Pegram & Slamon 2000). Over-expression has been shown in tumours in the breast (see below), cervix (Soh et al. 2002), colon (Antonacopoulou et al. 2008), endometrium (Konecny et al. 2009), esophageus (Dreilich et al. 2006), lung (Cebollero Presmanes et al. 2008) and pancreas (Safran et al. 2001).

In breast cancer, the *ERBB2* gene is amplified in 25-30% of cases, resulting in an over-expression of the ERBB2 protein and a worse prognosis (Slamon et al. 1987). The monoclonal antibody (mAb) trastuzumab targets the ERBB2 receptor and the treatment significantly reduces the risk of relapse when used in the adjuvant setting (Pegram & Slamon 2000, Romond et al. 2005). Moreover, trastuzumab plays an important role in the treatment of *ERBB2* positive metastatic breast cancer patients (Cobleigh et al. 1999, Vogel et al. 2002). Unfortunately, primary or acquired resistance to the antibody makes many patients with ERBB2 over-expressing breast cancers non-responders to trastuzumab given as single agent therapy. Suggested potential mechanisms for trastuzumab resistance are inactivation or loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Nagata et al. 2004) and activation of other tyrosine kinase receptors, such as the insulin-like growth factor receptor (IGF-1R)(Lu et al. 2001). Another studied mechanism of resistance is accumulation of the truncated forms of the ERBB2 receptor (p95HER2) lacking the extracellular trastuzumab-binding domain (Scaltriti et al. 2007). Thus there are efficient treatments in this subgroup of patients, but resistance to the treatments is an important issue that has not been fully investigated.

ERBB3

The *ERBB3* gene is located at chromosome 12 (Plowman et al. 1990), encoding a 148-kDa trans-membrane polypeptide protein (Kraus et al. 1989). Ligands for the receptor are the neuregulins, but as the intracellular domain is not capable of acting as a kinase on its own, it enhances the kinase activity of other ERBB family members (Schaefer et al. 2006) (Figure 3).

Studies of ERBB3 expression in primary cancers have shown expression in tumours derived from ovary, prostate, brain, retina, melanocytes, colon, pancreas, stomach, oral cavity and lung (Sithanandam & Anderson 2008). Moreover, studies show that ERBB3 expression can stimulate tumour growth in breast cancer (Way & Lin 2005, Karamouzis et al. 2007). In colorectal cancer cell lines, ERBB2 and ERBB3 generally show high expression, activation and dimer formation (Venkateswarlu et al. 2002). In one study, high ERBB3 expression in human colorectal cancer correlated to a poor clinical outcome (Kapitanovic et al. 2000), but some studies report a *better* prognosis in ERBB3 over-expressing bladder cancer patients (Memon et al. 2006).

There is recent evidence that ERBB3 expression levels influence resistance to EGFR inhibitors targeting EGFR or ERBB2 in several tumour types, including head and neck, pancreas and colorectal cancer (Buck et al. 2006, Erjala et al. 2006, Sergina et al. 2007). The increased ERBB3 levels in many human cancers initially created hope that it might be a useful therapeutic target, but due to its impaired kinase activity, a more modulatory role was assumed. However, the new awareness that cross-talk among the ERBB receptors is as a central feature of cancer cells makes ERBB3 once again more interesting as a therapeutic target. In fact, there are new data reporting that the effect of trastuzumab treatment in breast cancer is mediated by disrupting the oncogenic ligand-independent ERBB2/ERBB3/PI3K complex, resulting in rapid inhibition of AKT signalling and cell proliferation (Junttila et al. 2009).

ERBB4

The *ERBB4* gene is located at chromosome 2 (Zimonjic et al. 1995), encoding a 180 kDa glycoprotein (Plowman et al. 1993). Although ERBB4 is a ligand-activated kinase, ERBB4 is cleaved by membrane-associated proteases and the activated intracellular kinase domain moves into the cell (Ni et al. 2001, Carpenter 2003) (Figure 3).

The role of ERBB4 in cancer is poorly understood with data showing that it is expressed in high levels in several cancers such as thyroid (Haugen et al. 1996), ovarian (Furger et al. 1998), breast cancer (Sundvall et al. 2008), ependymoma and medulloblastoma (Gilbertson et al. 1997, Gilbertson et al. 2002). Thomasson et al., noted that in renal cancer *ERBB4* mRNA and protein were down-regulated compared to normal tissues (Thomasson et al. 2004).

ERBB4 expression levels and potential effect on clinical outcome is another much debated question (Pawlowski et al. 2000, Barnes et al. 2005). Over-expression of ERBB1 and ERBB2 contributes as negative prognostic factors, but for ERBB4 the opposite might be the case. There are observations suggesting that ERBB4 activation might inhibit cell proliferation and promote apoptosis (Karamouzis et al. 2007), yet ERBB4 has been shown to increase proliferation in lung cancer (Starr et al. 2006). There are also data that ERBB4 signalling is important in the differentiation process in the mammary gland (Jones et al. 1999). Other data show that ERBB4 expression is associated with favourable prognostic factors, such as estrogen receptor expression (Witton et al. 2003). Moreover, sub-cellular localization may also play a role in the evaluation of the prognostic significance of ERBB4. It has been reported that an ERBB4 localization in the nuclei is associated with shorter survival when compared to localization of ERBB4 at the cell surface (Junttila et al. 2005) and one explanation to the contradictory data could be that four structurally and functionally different isoforms are generated from the ERBB4 gene by alternative splicing (Junttila et al. 2000). Interestingly, in breast cancer, tumours with high ERBB2 expression together with concomitant high ERBB4 levels have a more favourable outcome and lower recurrence rates compared to breast cancers that express ERBB2 without simultaneous ERBB4 expression (Barnes et al. 2005).

The LRIG family

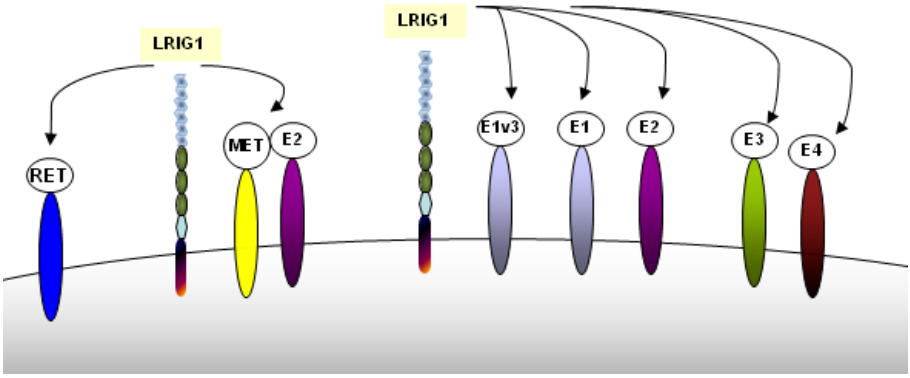

Figure 4. The LRIG1 protein is a membrane protein with fifteen contiguous leucine-rich repeats (LRR), followed by three C2-type immunoglobuline-like domains (Ig), a trans-membrane domain and a cytoplasmatic tail. LRIG1 interacts with ERBB1, ERBB2, ERBB3 and ERBB4 and induces their ubiquitinylation, followed by degradation of receptor complexes. LRIG1 also interacts with EGFRvIII and the RET receptor and regulates MET-ERBB2 synergism. E1v3=EGFRvIII, E1=ERBB1, E2=ERBB2, E3=ERBB3, E4=ERBB4, MET= hepatocyte growth factor receptor, RET= "rearranged during transfection"

LRIG1 (Leucine-rich repeats and immunoglobulin-like domains-1)

There are many different pathways for EGFR signalling, and in mammalian cells the LIG/LRIG family of trans-membrane proteins containing both leucine-rich repeats (LRRs) and immunoglobuline-like (Ig) domains represent such a pathway (Suzuki et al. 1996, Nilsson et al. 2001, Nilsson et al., 2003). The interest in the LRIG family was aroused when research showed a structural similarity between the LRIG proteins and the EGFR-down-regulating kekkon family in *Drosophila melanogaster* (Ghiglione et al. 2003). This observation resulted in a hypothesis that human LRIG1 might also be an inhibitor of EGFR-mediated signalling and therefore act as a tumour suppressor (Hedman et al. 2002).

The *LRIG1* gene is located at chromosome 3p14 and in mammalian cells the LRIG1 protein consists of 15 LRR, three Ig-like domains, a trans-membrane domain and a cytoplasmatic tail (Figure 4). *In vivo* data show that LRIG1 opposes tumour cell proliferation, motility and invasion, emphasising that *LRIG1* is indeed a tumour suppressor gene (Hedman et al. 2002). Moreover, studies have shown that LRIG1 downregulates EGFR, ERBB2, 3 and 4 expression by promoting receptor ubiquitinylation and degradation, often involving cbl (casitas b-cell lymphoma, an E3

ubiquitin ligase) (Gur et al. 2004, Laederich et al. 2004). EGFRvIII, the mutated and constitutively phosphorylated and active form of the EGF receptor, interacts with LRIG1 thus opposing EGFRvIII oncogenic activity. In astrocytomas, EGFRvIII (+) tumours are considered more aggressive and often resistant to treatment, and at least one study shows that expression of LRIG1 inhibits growth, motility and invasion in the tumour (Stutz et al. 2008).

The MET receptor tyrosine kinase (RTK) is over-expressed in a variety of human tumours and the expression correlates to poor patient prognosis (Birchmeier et al. 2003). The MET receptor interacts with LRIG1 which leads to destabilization of MET. Furthermore, LRIG1 opposes MET synergy with ERBB2 in driving cellular invasion (Shattuck et al. 2007). Another RTK that is negatively regulated by LRIG1 is RET, emphasizing the role of LRIG1 in multiple RTKs (Ledda et al. 2008).

Goldoni et al. has demonstrated that the ectodomain of LRIG1 (LRIG1ecto) inhibits both ligand-dependent and ligand-independent EGFR activation. Thus, cells that do not express detectable levels of EGFR fail to respond to soluble LRIG1 (Goldoni et al. 2007). Furthermore, the sub-cellular localization seems to be important for LRIG1 function, and LRIG proteins exhibit differential sub-cellular distribution in various cells and tissues. Moreover, in astrocytic tumours, peri-nuclear localisation of the LRIG proteins predicts better patient survival (Guo et al. 2006, Yi et al. 2009). LRIG1 is under-expressed in renal cell carcinoma (Thomasson et al. 2003), squamous cell carcinoma (Tanemura et al. 2005) and cervical carcinoma (Lindstrom et al. 2008). Also, recent data indicate that LRIG1 is a regulator of the quiescent state of epidermal stem cells (Jensen & Watt 2006).

In a study in breast cancer, Miller et al., have shown that the LRIG1 protein levels are significantly suppressed in ERBB2 positive breast tumours. Knockdown of endogenous LRIG1 in ERBB2-over-expressing breast cancer cell lines elevates ERBB2 and stimulates cellular proliferation. Moreover, when LRIG1 is expressed the trends are reversed (Miller et al. 2008).

LRIG2 and LRIG3

Recent studies have provided new knowledge of the structure and function of LRIG2 and LRIG3. The *LRIG2* gene is localized to chromosome 1p13 and the protein has a molecular weight of 107 kDa. Analysis has showed the most prominent *LRIG*2 RNA expression in skin, uterus, ovary, kidney, brain, small intestine, adrenal gland and stomach. LRIG2 is expressed both at the cell surface and in the cytoplasm. The absolute levels of *LRIG2* mRNA are relatively high, and the highest expression is found in skin, uterus and ovary (Holmlund et al. 2004). Yi et al., investigating 63 oligodendroglial tumours for the expression of the LRIG proteins, showed that cytoplasmic LRIG2 expression was an independent prognostic factor associated with poor patient survival (Yi et al. 2009). The LRIG3 gene is located at chromosome 12q14.1 and the protein has a molecular weight of approximately 120 kDa (Guo et al. 2004). A study of LRIG1, 2 and 3 in psoriatic epidermis reported that the LRIG protein expression patterns were altered compared to normal skin. These results indicate that the LRIG proteins might have a role in psoriasis (Karlsson et al. 2008).

Aims of the thesis

The first aim was to investigate the *LRIG1* gene and protein in breast and colorectal cancer using several methods, to uncover new knowledge of how the gene is expressed, and to learn more about the clinical importance of LRIG1 in cancer.

The second aim was to further analyse the finding of increased copy number of *LRIG1* and *ERBB2* in breast cancer and correlate the results to clinico-pathological factors.

The third aim was to investigate ERBB 1-4 expression changes in colorectal cancer between primary tumour and the corresponding metastases. This aim included examining whether changes in expression correlated to clinical data.

The last aim was to find out how the EGFR is expressed at the invasive margin of colorectal cancer tumours. We wanted to investigate a large patient material to find out whether expression changes are frequent and whether there is a correlation to clinical factors and prognosis.

Material

Material Breast cancer

Breast cancer - Paper I and II

Previously collected (1986 to 1995) frozen samples from 70 patients were first included. During 2002, tumour samples and non-neoplastic breast tissue were collected from another nine breast cancer patients. In the latter group of nine, a sample of the tumour and a piece of the adjacent non-neoplastic breast tissue were collected immediately after excision: one part of each sample was frozen in liquid nitrogen and stored at -80°C and another part was stored in RNAlater. The other adjacent parts of the tissue samples were fixed in formalin, paraffin embedded and used for routine morphological examination and tumour grading. In six cases the FISH analysis failed, leaving 73 patients in total in the study (Table 6). None of the patients had received any treatment before specimen collection. The study was approved by the local ethics committee.

Table 6. Clinical data of the 73 breast cancer samples paper I and II

	N	% of total		N	% of total
Tumour type			**Estrogen receptor**		
Ductal	58	79	Positive	56	77
Lobular	9	12	Negative	17	23
Mixed group	4	5	**Progesteron receptor**		
Medullar	2	4	Positive	50	68
Axillar lymph node			Negative	23	32
Negative	40	55	**Deceased**	41	56
Positive	33	45			

Material Colorectal cancer

Colorectal cancer - Paper III

Since 1987, the Department of Pathology has collected fresh-frozen, formalin-fixed and paraffin-embedded cancer tissue and surrounding normal colorectal tissue from patients undergoing surgery for colorectal cancer. From this collection, we randomly sampled 20 tumours diagnosed between 1989 and 1998. The colorectal tumours were of different grades and had sufficient fresh-frozen tumour needed for Western blot analysis. During 2002, tumour samples and non-neoplastic adjacent colon tissues were collected from ten patients with colorectal cancer. Samples of the tumour and non-neoplastic colorectal tissue were frozen in liquid nitrogen immediately after excision and stored at -80°. The other parts of the tissue samples were fixed in formalin, paraffin-embedded and used for routine morphological examination and tumour grading. The ethical committee of Umeå University approved the study. None of the patients had received any treatment before specimen collection.

Table 7. Clinical data of the 30 colorectal cancer patients in paper III

Age, years	**N**	**% of total**
Medium	70	
Range	45-89	
Sex		
Female	19	63
Male	11	37
Stage		
Stage I	6	20
Stage II	16	53
Stage III	8	27
Site		
Rectum	13	43
Colon	17	57
Histological subtype/grade		
Adenocarcinoma	27	90
Mucinous adenocarcinoma	3	10
Well differentiated	2	7
Moderately differentiated	23	77
Poorly differentiated	2	7
Deceased	16	53

Colorectal cancer - Paper IV

The tissue material of 64 paraffin-embedded colorectal cancers with corresponding lymph node and distant metastases was collected during 1982-2000. There were a few cases where samples from distant metastases were collected before diagnosis of the primary tumour (range 3-6 months) and after 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. No patient had received anti-EGFR treatment.

Table 8. Clinical data of the 64 colorectal cancer patients in paper IV.

Age, years	**N**	**% of total**
Median	63	
Range	36-83	
Sex		
Female	28	43
Male	36	56
Stage at diagnosis		
Stage I	3	4.7
Stage II	18	28.1
Stage III	28	43.8
Stage IV	15	23
Histologic subtype/grade		
Adenocarcinoma	52	81.3
Mucinous	12	18.8
High/Moderately differentiated	52	81.3
Poorly differentiated	7	10.9
Missing Value	5	7.8
Primary tumour		
Rectum	27	42
Colon	37	58
Metastasis		
Liver	30	46.9
Skin	6	9.4
Lung	8	12.5
CNS	2	3.1
Bone	6	9.4
Scar	10	15.6
Peritoneum	1	1.6
Gallbladder	1	1.6
Survival months		
Median	29	
Range	1-154	

Colorectal cancer – Paper V

Tissue was sampled from 464 patients diagnosed with colorectal cancer between 1995 and 2003 at the department of Surgery, Umeå University Hospital, Sweden. In 57 cases, the specimen was excluded due to lack of internal positive control and 21 due to inadequate tissue availability; this left 386 patients for final analyses (See Figure 1, paper V). The study was approved by the local ethics committee. Table 9 illustrates the clinical data. No patient had received anti-EGFR treatment.

Table 9. Clinical data of the 386 colorectal cancer patients in paper V

Age, years	**N**	**% of total**
Medium	71%	
Range	36-90	
Sex		
Female	171	44
Male	215	56
Stage		
Stage I	56	15
Stage II	149	39
Stage III	85	22
Stage IV	90	23
Missing value	5	1.2
Site		
Rectum	140	36
Colon	246	64
Histological subtype/grade		
Adenocarcinoma	336	87
Mucinous adenocarcinoma	50	13
Well differentiated	5	4
Well-Moderately differentiated	179	46
Moderately-Poor differentiated	156	40
Poorly differentiated	38	10
Deceased	240	62

Cell lines

Breast cancer paper I and Colorectal cancer paper II

The breast cancer cell lines MDA-MB-231, MDA-MB-415 and HS 578T and the colorectal cancer cell lines LOVO, SW480, HT29 and DLD-1 were obtained from American type culture collection (Manassas, VA, USA) and the breast cancer cell line ZR-75-1 was kindly provided by Dr. J. Bergh (Uppsala University, Sweden). The cancer cell lines were cultured in Dulbecco's modified Eagles medium, supplemented with

10% w/v foetal bovine serum and 50 μg/ml gentamicin from Invitrogen AB (Täby, Sweden). The immortalized epithelial mammary cell line hTERT-HME1 was obtained from BD Bioscencies Clontech (Stockholm, Sweden) and cultured according to the manufacturer's instructions using media and supplements from Clonetics, Bio Whittaker (Walkersville, MD, USA).

Methods

FISH- *Fluorescence in situ hybridization*

FISH is a molecular technique that is used to detect and localize specific DNA sequences on target chromosomes. Fluorescent probes bind to regions on chromosomes to which they have a high degree of sequence similarity. A fluorescence microscope is used to visualise whether the probe binds to the target chromosomes. The method is very accurate, especially when used in cell-lines. In our studies, primary human malignant tumour tissue that included a mixture of cancer cells and healthy stroma cells was used. This is particularly important when investigating low-grade amplification, as in our study. One disadvantage with the method, however, is that relatively large amounts of tumour cells are needed for the analysis.

FISH- ***LRIG1 Paper I and II***

The frozen breast cancer samples were disintegrated in methanol: acetic acid solution on ice. The nuclei were collected from the tissues by passing through a nylon mesh (pore size, 70 μm) and then centrifuged. Thereafter, the nuclei were washed in methanol:acetic acid solution 2-3 times and FISH slides were prepared by adding the cell suspension on glass slides. When air-dried, the slides were immediately used or stored at -20°C. FISH-slides were incubated in KCl for 20 minutes at 37°C before hybridization and then fixed in Carnoy's solution. Next, the FISH slides were treated with RNAase and then washed in 2 × SSC (saline sodium citrate). Finally, slides were incubated for 10 minutes in a solution containing pepsin, and this was followed by incubation in PBS and stepwise dehydration in alcohol.

The FISH probe was the BAC clone 751k5 (Invitrogen, Carlsbad, USA) containing the *LRIG1* gene. The DNA was labelled using nick translation (Spectrum Orange, Abbot

Diagnostics, Wiesbaden-Delkenheim, Germany). The probe was incubated with Cot-1 DNA 1 h at 37°C and applied to a slide. Target DNA and the probe were denatured simultaneously at 72°C and then the slides were incubated overnight in a humidity chamber at 37°C. The next day post-hybridisation washing and nuclear counterstaining with DAPI was done. Centromere probe for chromosome 3 was used along with the *LRIG1* specific probe. *LRIG1* gene signals were counted in at least 100 nuclei by two investigators. The criteria for scoring an increased number of *LRIG1* were three or more signals in more than 20% of the nuclei.

ERBB2

ERBB2 FISH analysis was performed according to routine clinical procedures (Carlson et al. 2006).

Western Blot

This method can detect specific proteins in a protein mixture and can provide information about the size of the protein. It is important to have a high-quality antibody directed towards the studied protein. The advantage with the method is that it is quick and inexpensive. In our studies, a mixture of malignant and healthy cells was used, so the results might in part represent the expression in the healthy stroma always included in a tumour. One disadvantage is that if a protein is degraded quickly, Western blotting is not possible to use to detect the protein.

Western Blot - *LRIG1 paper I and III*

Briefly, cell lysates, protein concentrates and immunoprecipitated material were incubated in LDS (lithium dodecyl sulfate) followed by electrophoresis in a TRIS-acetate NuPAGE gradient gel. Next, the proteins were transferred to polyvinylidene difluoride membranes by using a Mini-Gel blot module. Non-specific binding was blocked using milk and the membranes were then incubated with the primary and secondary antibodies. The primary antibodies used were LRIG1-151 and rabbit anti-actin (Sigma-Aldrich, St. Louis, MO, USA). The visualization was performed using ECL-plus (Amersham Biosciences, New Jersey, USA). In paper 3, the Western blot was performed as described above and the same antibodies were used. Several attempts to

establish a reliable EGFR Western blot method did not result in a method stable enough for our analyses.

Immunohistochemistry (IHC)

This method localizes proteins in a tissue section using antibodies that bind specifically to their antigens. IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue. Also, the method is quite simple and can be used in tissue samples many years after the collection. One well-known disadvantage with IHC is problems with unspecific binding of the antibodies.

Immunohistochemistry LRIG1

To scan larger archive patient materials for changes in LRIG1 expression, several attempts were made to achieve an immunohistochemical analysis of LRIG1 in breast and colorectal cancer.

What follows is a description of the LRIG1 IHC protocol. Blocks of formalin-fixed and paraffin-embedded tissues were cut in 5 μm-thick sections and deparaffinised using standard methods. Endogenous peroxidase activity was blocked with peroxidase in methanol and the slides were washed in PBS. The samples were merged in citrate buffer (pH 6.0 or 7.3) and treated with microwaves at several different time periods (1x5 min, 2x5 min). Next, the samples were pre-incubated with goat serum to block unspecific binding followed by incubation with primary antibodies (LRIG1, a polyclonal rabbit antibody diluted 1:500 to 1:150, in a variety of different concentrations). Sections were washed in PBS and the secondary goat antibody was applied followed by a new PBS wash. The sections were incubated with a DAB substrate kit and rinsed. Finally, the glasses were counterstained with Mayer staining, rinsed in water and mounted. In each staining run, a negative/blank control was included. In colorectal and breast cancer, unlike in other tumours, the LRIG1 stainings showed strong staining in all tumour cells without variability, a condition that made the analysis difficult to interpret.

Immunohistochemistry - *EGFR and ERBB2 paper III, IV and V*

Analysis of EGFR expression was performed on formalin-fixed and paraffin-embedded colorectal cancer samples according to routine procedures. Briefly, 4 mm sections were placed into an automated immunostaining machine. Antibody reaction was demonstrated using an iVEIW kit with DAB (diaminobenzidine) and chromogen and hematoxylin for counterstaining (*Paper III and IV*, EGFR antibody mouse monoclonal 3C6, Ventana, Tucson, AZ, USA, *Paper V*, EGFR antibody monoclonal E30, DAKO, Denmark). For the ERBB2 analysis, the antibody used was a mouse monoclonal Pathway Her2, clone CB11. Otherwise, the analysis was performed as described above.

Immunohistochemistry - *ERBB3 and ERBB4 paper IV*

Manual staining of the sections was performed for ERBB3 and ERBB4. Endogenous peroxidase activity was blocked and the slides were placed in a citrate buffer (pH 6.0) and pre-treated in a microwave oven at 900 W and then cooled. Next, the slides were blocked with a non-immune serum blocking solution followed by incubation with ERBB3 or ERBB4 antibodies at room temperature (rabbit anti–ERBB3, Nova Castra Laboratories Ltd, Newcastle, UK and rabbit anti-ERBB4, Lab Vision Corporation, Fremont, CA, USA). The slides were washed and incubated with biotinylated secondary antibody and HRP-streptavidin. Finally, the sections were counterstained with Mayer's hematoxylin and in each staining run a negative/blank control was included.

RT-PCR –*Real Time Quantitative Reverse Transcription PCR*

The method is a development of PCR technology that enables reliable detection and measurement of products generated during each cycle of the PCR process. This technique became possible after introduction of an oligonucleotide probe, which was designed to hybridize within the target sequence. Cleavage of the probe by the Taq polymerase is used to monitor the increase in fluorescence during PCR.

RT-PCR - *LRIG1 and ERBB2 paper I and III*

RNA was prepared from the tissue samples using the RNAqueous kit (Ambion inc, Austin, Texas, USA) as previously described by Nilsson et al. (2001). Real-time quantitative reverse transcription RT–PCR was performed using an iCycler (Bio-Rad, Hercules, CA, USA). The samples were run in triplicates and relative quantification was

performed by comparing the threshold cycle values (Ct) for the samples to standard curves. The housekeeping gene 18S rRNA levels were used to normalize the EGFR and LRIG1 values in respective RNA samples.

Methods used but not included in Papers I-V

PCR - *Polymerase chain reaction*

PCR is a method that amplifies small amounts of DNA. It is fast and sensitive, but requires information of the target sequence, and when the PCR reaction involves amplification of a single DNA sequence, the final product will be a mixture of very similar, but not completely identical DNA sequences.

PCR - *LRIG1 and ERBB2*

Quantitative real-time PCR analysis was used to scan for amplifications and/or duplications of *LRIG1* in a larger breast cancer patient material (kindly provided by O. Stål, Linköping). In some cases, where the *LRIG1* copy number was quite high (more than 5 copies), levels were high enough to be detected by the PCR methods used, but in most tumours with increased *LRIG1* copy number, the *LRIG1* DNA levels were not high enough to reach cut-off (Gunnarsson et al. 2003). Moreover, the mixture by healthy stroma cells in the DNA suspension might have obscured detection of possible *LRIG1* increased copy numbers in the tumour cells. We also tried to set up a method to screen our 78 tumours for *ERBB2* amplification by PCR, and in 80% of cases the results were in accordance with the *ERBB2* FISH results, but as approximately 20% mismatched, the method was excluded. In both the analysis of *LRIG1* and *ERBB2*, an iCycler (Bio-Rad, Hercules, CA, USA) was used. The samples were run in quadruplicates, and relative quantification was performed by comparing the threshold cycle values (Ct) for the samples to standard curves. The *APP* gene levels were used to normalize the *ERBB2* and *LRIG1* values and a doubling of the *LRIG1*/*APP* or *ERBB2*/*APP* copy number ratio was considered as amplification.

SNP array - *Single Nucleotide Polymorphism*

Genomic copy number alterations and allelic imbalances are common events in cancer development. In recent years, arrays focusing on single nucleotide polymorphism (SNP) genotyping have been developed. The array we used is called 370CNV BeadChip (Illumina®) and allows for combined DNA copy number (SNP-CGH) and loss-of-heterozygosity (LOH) analysis at high resolution. One drawback with the method is the lack of known SNPs in certain areas of the genome.

SNP array - ***LRIG1***

To gain further knowledge whether it is the entire *LRIG1* gene or a minor part that is duplicated in breast cancer, we analysed *LRIG1* in 5 tumours with increased copy number for *LRIG1* by arrays as previously described (Peiffer et al. 2006). Unfortunately, the numbers of known SNPs surrounding and within the *LRIG1* gene are very few and in the region of 4000 bp (the entire gene is approximately 120 000 bp), where our FISH probe is localized, there were only two SNPs. Thus, no copy number variations of the *LRIG1* locus were detected in all five tumours. Moreover, as for the PCR results, the data could be obscured by the influence of healthy stroma.

CGH - *Comparative Genomic Hybridization*

CGH is a molecular method to analyse copy number changes in a sample of DNA. In this method, normal human metaphase preparations are hybridized with fluorescently labelled tumour and normal DNA. When analysing the chromosomes, regional differences in the fluorescence ratio of gain and losses compared to the control DNA can be detected and can identify abnormal gains and losses of the genomic regions. A disadvantage is that CGH does not detect chromosomal aberrations that do not change the copy number and the results depend on the presence of at least 50% tumour cells.

CGH- ***LRIG1***

We performed CGH array analysis as previously described (Kirchhoff et al. 1998) in two patient samples with the highest copy numbers of *LRIG1*. In both tumours, a significantly higher DNA content was detected at the peri-centromeric region 3p14 where *LRIG1* is located. Unfortunately, in peri-centromeric areas, the chromosomal

instability and highly repetitive DNA sequences can prevent accurate and consistent hybridization measurements. For this reason, centromeric and telomeric sites are often excluded from CGH analysis (Speicher et al. 1993). Thus, as CGH results from these areas can be less reliable, we chose not to use these observations in our publications.

Statistical analysis

Paper IV and V

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). In paper V, Fisher's exact test was used for the cross-tabulations. The exact linear-by-linear association test was performed to test the linear association between two ordinal scale variables. The Cox proportional hazard model was used for multivariate survival analyses. Kaplan-Meier survival analysis was used to estimate the cancer-specific survival and comparisons between groups were performed with the log-rank test. SPSS statistical software version 13.0 and 15.0 was used for the statistical analyses; *p*-values <0.05 were considered as statistically significant.

Results and Discussion

LRIG1, ERBB1 and ERBB2 in breast cancer

When initiating this project, *LRIG1* was a newly discovered gene and very little was known about the function of the gene product and how it was expressed in different tumour types. Therefore, one aim of the thesis was to pinpoint which methods were the most suitable to investigate LRIG1; of course, another aim was to see whether expression levels were correlated to clinical parameters. During the last five years, studies have shown that LRIG1 down-regulates all of the ERBB receptors by ubiquitinylation and degradation of the receptors. Other studies have shown that LRIG1 also negatively regulates the MET and RET receptors (Shattuck et al. 2007, Ledda et al. 2008).

First, we examined 30 breast cancer tumours using FISH analysis (Paper I). As *LRIG1* is situated at 3p14, a genomic area with common deletions (Maitra et al. 2001), the expected result was LOH when investigating the locus using FISH analysis. Unexpectedly, increased copy number of the gene in 39% (11/28) of the tumours was evident. Analysis using complementary probes was performed in a smaller subset of tumours, showing no aneuplody of chromosome 3, 18 or the X chromosome (Figure 5).

These results were interesting and challenged the idea of *LRIG1* as a tumour suppressor gene as TSGs in many cases are deleted in malignant tumours. However, there are other studies where amplification of 3p14 in malignant tumours have been described (Hyman et al. 2002, Thomas et al. 2008). Moreover, 3p14 high grade amplification in melanoma cell lines was shown by Jönsson et al. (Jonsson et al. 2007). On the other hand, in the in situ study by Esseghir et al., the authors could not detect increased LRIG1 levels in breast cancer tumours or any correlation to clinico-pathological data. However, LRIG1 transcripts were only detected in 15% of tumours, showing the heterogeneity in different breast cancer materials and the difficulties in comparing the data from different methods (Esseghir et al. 2006).

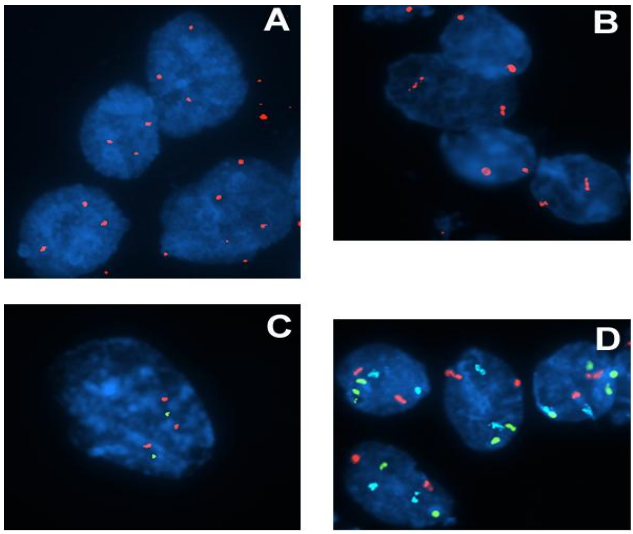

Figure 5. FISH analysis of breast cancer nuclei. A) Analysis with a specific *LRIG1* (red) probe showing increased gene copy number (more than two copies) of the *LRIG1* gene at 3p14. B) Analysis with a specific CEP3 (centromeric probe for chromosome 3, red). Showing no additional chromosome 3. C) Analysis with a specific 3p subtelomeric probe (green) and *LRIG1* (red) mixture showing increased gene copy number of the *LRIG1* gene but only two copies of the 3p arm. D) Analysis with a mixture of probes for CEP3 (red), X chromosome (green) and chromosome 18 (blue), showing no aneuploidy for these chromosomes.
Increased copy number at 3p14 in breast cancer. Ljuslinder et al., Breast Cancer Res. 2005;7 (5):R719-27.
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In an attempt to verify the data and locate the exact area of duplication, conventional CGH analysis was performed in two patients. This method showed a clear tendency towards higher DNA levels at the 3p14 locus, a finding that supported the FISH data (data not shown). A disadvantage is that the peri-centromeric 3p14 locus is difficult to analyse by CGH array due to repetitive elements in the area. A SNP (Single Nucleotide Polymorphism) array was also performed in five tumours with increased *LRIG1* copy number, but the relatively small amount of known SNPs in the *LRIG1* gene made it impossible to define the size of the area increased in copy number. Moreover, we analysed 50 breast cancer tumour samples using a 3p CGH array, but as the results were technically questionable, the data from the CGH arrays could not be used (data not shown).

The subgroup of nine breast tumours collected in 2002 was investigated by Western blot analysis to compare levels in primary tumours and adjacent healthy breast tissues. In four out of nine tumours, LRIG1 levels were higher in the malignant tumour than in

the healthy tissues. On the other hand, in a later and larger similar study by Miller et al., a statistically significant down-regulation of the LRIG1 protein was evident in breast tumours (n=67) vs. normal breast samples (n=42) (Miller et al. 2008). This illustrates the importance of a larger number of samples when investigating new genes and proteins as the results greatly differed in these two small patient populations. The differences might be due to mere chance, the different patient populations or by factors in the methods used. As the Miller study analysed a larger subset of tumours by Western blot and with a diversity of laboratory methods, it is more likely that LRIG1 protein levels are decreased in the tumour and not increased as in the few samples analysed in our study.

We also performed RT-PCR analysis for *LRIG1, ERBB1* and *ERBB2*, showing that *LRIG1* mRNA was over-expressed in 2/7 tumours and under-expressed in 2/7 tumours. In two out of three cases, tumours with *LRIG1* increased copy number had a significantly higher *LRIG1* mRNA level in the tumour than in the matched healthy tissue. The results from the *ERBB1* analysis showed that in the four tumours with increased *LRIG1* copy number, all showed significantly lower expression of *ERBB1* in the tumour than in the corresponding healthy tissue. This emphasizes the assumption of LRIG1 as a down-regulator of ERBB1. On the other hand, two of the tumours *without* increased *LRIG1* copy number had a lower *ERBB1* expression in the matched non-neoplastic tissue. Notably, three tumours with increased copy number of *LRIG1* showed a significantly higher expression of *ERBB2* than their matched normal controls. Of course, this was an important observation considering the impact of ERBB2 signalling in breast cancer risk evaluation and treatment (Slamon et al. 1987).

At this point, we tried to find laboratory methods to screen a larger breast cancer material for changes in LRIG1 levels and to correlate to clinical parameters. Unfortunately, as described in the methods section, in breast and colorectal cancer, an adequate quality of immunohistochemical analysis was not achieved. Moreover, *LRIG1* PCR analysis was performed in another larger material (data nor shown), but as the gene is often duplicated (3-5 copies) and not amplified (> 7 copies), the results only showed significant *LRIG1* DNA increase in the tumours with the most copies. The *ERBB2* PCR results (in the same samples), on the other hand, showed *ERBB2* amplification in a majority of *ERBB2* FISH-positive tumours, but as the concordance

was not 100%, the method was unsuitable for screening breast cancer samples for *LRIG1* and *ERBB2* gene duplications and/or amplifications.

As the data from Miller et al. was somewhat contradictory to our observations, another 45 tumours were analysed by FISH for *LRIG1*, thus constituting a patient cohort of 73 patients altogether (Table 6). Moreover, 18 tumours with *LRIG1* increased copy number were examined using FISH for *ERBB2*. The results were coherent with our first data, showing increased copy number of *LRIG1* in 34% (25/73) of the tumours, but in addition also demonstrating increased *ERBB2* copy number in 16 out of 18 (89%) analysed tumours with increased *LRIG1* copy number (Paper II). These results tell us that in most cases, breast cancer tumours with increased *LRIG1* copy number also have a co-incidental increase in *ERBB2* copy number. To strengthen these observations, the Miller's study also showed a clear functional correlation between *LRIG1* and *ERBB2* at the protein level when investigated by Western blot analysis. In other words, there seems to be a connection between LRIG1 and ERBB2 both at the genomic and the protein level, indicating a possible importance in the clinical setting. In our study, the patient cohort was too small to detect a correlation to clinical parameters, but as *ERBB2* amplification is a negative prognostic factor, the increase of *LRIG1* is most likely also a negative prognostic co-factor**.** One might speculate that the altered *LRIG1* gene, resulting in lower protein levels, is an advantage for the tumour in one way or another.

So why has not the increase in *LRIG1* gene copy number in breast cancer been discovered before? One explanation, as previously shown, is that the region where *LRIG1* is situated is a common area of LOH, but there are few data on gain at the 3p14 locus (Hyman et al. 2002, Jonsson et al. 2007, Thomas et al. 2008). LOH has previously been the major tool when trying to find TSGs, but it is not an option when searching for oncogenes. Thus, in a LOH study by Isola et al., an investigation of *ERBB2* positive breast cancer tumours resulted in a 3p14 LOH in 33% of tumours investigated (Isola et al. 1999). This report thus contrasts to the increase in copy number in the same percentage when analysed for *LRIG1* by FISH. Another explanation is that the area where LRIG1 is situated is close to the centromere, an area that is difficult to analyse by CGH and SNP arrays due to chromosomal instability and repetitive elements.

How does an increase in *LRIG1* gene copy number decrease the protein and mRNA protein levels? Recently, new data analyzing the impact of gene copy number variations on protein expression has been published. In a study of pilocytic astrocytomas, a tandem duplication of the *BRAF* gene results in an oncogenic fusion gene and an altered protein product (Jones et al. 2008). Furthermore, another possible mechanism is that one allele of the gene is lost and the other is amplified with loss of function, as shown in genes located in LOH areas in paediatric gliomas (Wong et al. 2006). On the other hand, although LRIG1 functionally interacts with ERBB2, other genes in the nearby area might be of greater importance. *EphA3*, for example, is such a candidate gene. The Eph RTK family members are widely overexpressed in human cancers, enhancing tumorogenesis, angiogenesis and metastasis (Surawska et al. 2004). The *EphA3* gene is located at 3p11.2, quite close to 3p14. Although the molecular function of *EphA3* is still not clear, it might be another gene of importance and co-duplicated with *ERBB2*.

In conclusion, the finding of increased copy number of *LRIG1* in *ERBB2* positive breast cancer is a new finding and points at a possible functional role for LRIG1 in this subgroup of patients.

LRIG1 and the ERBB receptors in colorectal cancer

In colorectal cancer, the EGFR is an important growth factor receptor involved in tumour progression but also the target for several new treatments (Vokes & Chu 2006). As LRIG1 was assumed to be a negative regulator of ERBB receptor signalling, it was of importance for us to investigate LRIG1 at both genomic, mRNA and protein levels in colorectal cancer.

Ten tumours with corresponding non-neoplastic tissues were analysed for *LRIG1* using FISH. In one out of nine tumours (one failed analysis), there was an increased copy number and in the other eight tumours a normal signal pattern was seen. Moreover, four colorectal cancer cell lines were investigated and demonstrated normal gene copy numbers (Paper III).

As previously mentioned, an effort was made to establish a reliable immunohistochemical method to analyse protein expression. Unfortunately, this effort was not successful. In both breast and colorectal cancer, all tumour cells were stained without any variability. Thus, expanding the material to 30 tumours, Western blot analysis was used to compare protein expression levels in tumours and matched healthy tissues. The results demonstrated a down-regulation of LRIG1 in 13/30 (43%) of the tumours and an up-regulation in an equal number of samples. The heterogeneity of LRIG1 expression in colorectal cancer is at this stage an unverified observation, and considering the small patient number it was not possible to relate the findings to clinical data. However, in other studies of cervical cancer and squamous cell cancer of the skin, a correlation between LRIG1 levels and a better outcome has been shown (Tanemura et al. 2005, Lindstrom et al. 2008), but in the present study no survival analysis was possible due to the small numbers of tumours analysed. In the next step, EGFR immunohistochemistry was performed and in 13/20 (65%) of the tumours analysed, positivity for the EGF receptor was evident. When comparing the results from the LRIG1 Western blots and the EGFR IHC results, no evident correlation between the protein levels was detected. The reason for this could be due to methodological issues or that the down-regulating effect of LRIG1 is overridden by other regulatory events in colorectal cancer.

In the last decade, treatments targeting the EGFR have been developed and a multitude of studies have shown benefits for patients with colorectal cancer in the metastatic setting (Cunningham et al. 2004). At first, IHC analysis of the EGFR was used to select patients for EGFR targeted therapies, but quite soon reports were published showing that there was a weak connection between EGFR IHC levels and response to the treatments (Chung et al. 2005). As the treatments are mostly used in patients with stage IV disease, we decided to investigate the differences in ERBB1-4 expression in 64 primary tumours and their corresponding metastases (Paper IV). The results showed that in 49/64 samples (76%) the primary tumours were EGFR positive and that 33% lost the EGFR expression in the corresponding metastasis. The primary tumour was negative in 15/64 (23%) and out of these five (33%) developed EGFR positivity in the metastasis. During the time the study was performed, five other similar studies were published. In our study, as well as in the studies by Scartozzi, Bralet and McKay (McKay et al. 2002, Scartozzi et al. 2004, Bralet et al. 2007), a significant number of patients lost the EGFR expression in the metastases (27-60%), but they are inconsistent with the results by Italiano et al. (Italiano et al. 2005) and Bibeau et al., (Bibeau et al. 2006) where no conclusive differences were evident.

How can the divergence in results be explained? First, the age and handling of the tissue samples has been shown to be important when analysing the EGFR by IHC (Atkins et al. 2004). Second, the procedure of IHC analysis is also important, as shown by Bibeau et al. where a significant underestimation of the EGFR positivity was evident when comparing EGFR expression levels in tissue sections vs. tissue microarrays (Bibeau et al. 2006). Moreover, the heterogeneity of the tumours and "bias" due to which individual analyses the samples might also influence the results. Nevertheless, EGFR expression seems to be lost in the metastases in a considerable number of colorectal cancer patients, an observation that most likely has an impact when treating patients with anti-EGFR therapies.

We also found it of interest to evaluate the expression of ERBB 2-4 by IHC, as there are few reports on expression of the other ERBB family members in colorectal cancer. Data show ERBB2 expression in 11-89% of tumours, but without significant prognostic impact (Nakae et al. 1993) (Kluftinger et al. 1992). In our study, ERBB2 staining was positive in 27/50 (54%) of the cases and 30% of these lost the expression

in the metastases. In the ERBB2 negative group, 4/23 (17%) developed ERBB2 expression in the metastasis.

When analysing the other two ERBB receptors, positive ERBB3 and ERBB4 staining was evident in 32/48 tumours (67%) and in 42/52 tumours (81%), respectively. Expression in the primary tumours compared to the expression in the metastases was unchanged in most cases and no correlation between early/late stage tumours and ERBB3/ERBB4 was seen compared to the data from other studies. There are few data on co-expression between the ERBB receptors in colorectal cancer, although recent data show the importance of the cross talk between the four receptors (Wieduwilt & Moasser 2008). Analysis of the co-expression between the different ERBB receptors detected a significant higher amount of tumours co-expressing ERBB3 and ERBB4 in late stage tumours (p=0.025). Some data suggest that co-expression of ERBB2 and ERBB4 correlates to a poorer survival (Lee et al. 2002), but in our material no such correlation was evident.

The invasive margin of malignant tumours has been the scope of an increasing number of studies during the recent years (Christofori 2006). When analysing the tumours in Paper IV, we noticed a tendency towards a higher EGFR expression in the deepest layers of the tumours. Thus, as EGFR has been proposed as being a co-actor in the molecular processes at the invasive margin, we decided to investigate the EGFR expression by IHC in 463 colorectal cancer patients (Paper V). EGFR scores in the central part of the tumour and the invasive margin were evaluated. The results showed that EGFR was positive in the central area in 176/386 (46%) and at the invasive margin in 222/386 (57%). Statistical analysis of the group of tumours that had a higher expression level at the invasive margin (97/286; 34%) detected a significant worse outcome in this subgroup of patients (n=273, p=0.013). A complementary multivariate analysis (adjusted for age, gender, localisation, grade, stage and tumour type) confirmed a lower survival rate in the same subgroup of patients (p=0.029, RR=1.53).

As budding is a negative prognostic factor in colorectal cancer (Ohtsuki et al. 2008), it was included in the analysis. Notably, a significant correlation was evident between EGFR expression at the invasive front and the presence of budding (p=0.0001). Previous studies in smaller patient materials have noticed an increased EGFR

expression at the deepest layers and buddings of colorectal cancer tumours. For example, Goldstein et al. noted that stage IV colorectal cancer tumours tended towards a negative prognostic impact of a high EGFR expression at the invasive margin (Goldstein & Armin 2001). Italiano et al., and Buckley et al., briefly commented on a higher EGFR expression at the invasive front, but as these studies had other scopes, the observations were not correlated to clinical findings (Italiano et al. 2005, Buckley & Kakar 2007). These data together with the results from our study suggest a prognostic value of analyzing EGFR expression at the invasive margin (Figure 6).

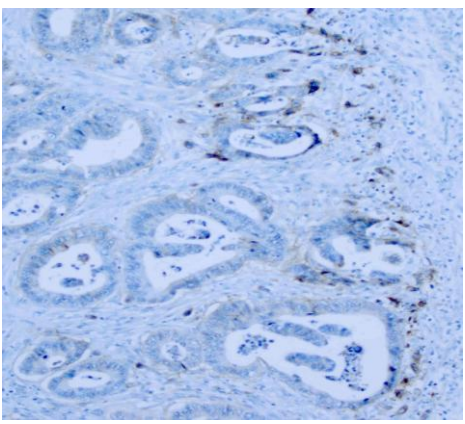

Figure 6. Immunohistochemical staining of the EGFR in colorectal cancer. This case illustrates a low expression in the central area (left part of the figure) and a high expression at the invasive margin as well as in tumour budding (right part of the figure).

Nevertheless, adding budding to the multivariate analysis obliterated the significant effect of EGFR expression on survival, thus stressing the close relationship between these two phenomena at the invasive margin of a colorectal tumour (Figure 6). In addition, over-expression of EGFR at the invasive front correlates well to the knowledge of molecular processes as down regulation of E-cadherin with concomitant EGFR and B-catenin over-expression, resulting in EMT. As EMT is important when discussing resistance to anti-EGFR therapies, these results represent another piece of the puzzle when aiming for methods to find the subset of patients that most likely benefit from EGFR-targeted therapies.

Conclusions

There was an increased copy number of *LRIG1* at 3p14 in 34% of the breast cancer tumours. In a majority of tumours with increased *LRIG1* copy numbers, there was a co-incidental increase in *ERBB2* copy number, suggesting a relationship between these two genetic events in breast cancer.

In colorectal cancer, LRIG1 was both under and over-expressed compared to matched non-neoplastic tissues without evident correlation to EGFR levels. The *LRIG1* gene copy number appeared to be normal in most human colorectal cancer.

In the investigated colorectal cancer tumours, a third of EGFR positive primary tumours lost the EGFR expression in the metastases, and conversely, a third of EGFR negative samples developed EGFR expression in the metastases. ERBB2, ERBB3, and ERBB4 expression was detected in 50-80% of the tumours, with a significant increase of ERBB3/ERBB4 co-expression in late stage tumours.

EGFR was over-expressed at the invasive margin in a significant number of colorectal cancer tumours. There was a correlation between increased EGFR expression at the invasive margin and a worse prognosis. Moreover, there was a close correlation between the presence of budding and EGFR expression levels.

Future directions

The *LRIG1* gene is recently discovered and there is much more to explore concerning the function and importance of the gene. It would be of interest to investigate a larger subset of breast cancer patients with increased *LRIG1* copy number with respect to clinical data, *ERBB2* status and response to therapy. Moreover, functional studies with breast cancer cell-lines that investigate the impact of LRIG1 levels on response to anti-ERBB2 and EGFR therapies are planned. The ERBB receptor family and the cross-talk between the receptors are issues that need to be clarified further in future studies. It would be valuable to correlate EGFR expression levels in buddings and the invasive margin to K-RAS status and the effect of anti-EGFR targeted therapies.

Populärvetenskaplig sammanfattning på svenska

Tjock- och ändtarmscancer och bröstcancer är några av de vanligaste tumörsjukdomarna i Sverige. Sjukdomarna utvecklas genom omvandling av friska celler till cancerceller. En del är känt kring utveckling av cancer men de exakta mekanismerna är fortfarande till stor okända. Tillväxtfaktorer och deras receptorer finns ofta i stora mängder, dvs överuttrycks, i många olika cancerformer. Epidermal growth factor receptor (EGFR) är en sådan tillväxtfaktorreceptor som är överuttryckt vid många olika tumörformer, däribland bröstcancer och tjocktarmscancer. Man vet att detta i vissa fall ger en sämre prognos för patienten, men man har också utvecklat nya målstyrda behandlingar som är riktade mot EGFR. EGFR tillhör en grupp av receptorer som kallas ERBB1(EGFR) samt ERBB2, ERBB3 och ERBB4. De andra medlemmarna i ERBB familjen finns också uttryckta i många tumörer och deras kliniska betydelse är till stor del fortfarande okända. I bröstcancer analyseras tumörer för ERBB2 eftersom man har sett att det är en viktig prognostisk faktor och det finns effektiva behandlingar med antikroppar riktad mot receptorn.

På onkologens forskningslaboratorium har man sedan några år tillbaka arbetat med en gen som heter *LRIG1*. *LRIG1* är en gen som kodar för ett protein som hämmar effekten av ERBB receptorerna. Man vet också att genen är belägen på kromosom 3p14.

I denna avhandling har vi undersökt bröstcancertumörer samt tarmcancer med avseende på LRIG1 och ERBB familjen. Syftet var att lära sig mer om LRIG1 och ERBB familjen samt även att undersöka vilka metoder som passar bäst att använda.

Vi har i bröstcancer upptäckt att *LRIG1* finns i ökad mängd på kromosomnivå samt att man ser en samvariation med förhöjda nivåer av *ERBB2*. I tarmcancer har vi sett att *LRIG1* genen tycks finnas i normalmängd på kromosomnivå, men att LRIG1 proteinet förekommer i både höga och låga mängder.

Vi har även undersökt hur EGFR uttrycks i tarmcancer när sjukdomen är spridd i kroppen. I studien jämförde vi uttrycket i den primära tumören med uttrycket i dottertumören. Här fann vi att en avsevärd andel av tumörerna, ca 33 %, förlorar sitt

EGFR uttryck när sjukdomen sprider sig. Det här är viktig information när man skall välja den målstyrda behandlingen till patienten. Vi tittade också på hur de andra ERBB proteinerna uttrycktes i tarmcancer och fann att ett samtidigt uttryck av två av receptorerna var vanligare i mer avancerade tumörer.

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