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ORIGINAL ARTICLE

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Abstract
In the present study the expression of LRIG1 (leucine rich repeats and immunoglobin-like domains 1) and its relation to
EGFR (epidermal growth factor receptor) was examined in tumour samples and adjacent non-neoplastic tissues from
30 patients with colorectal cancer. The *LRIG1* gene, at chromosome 3p14, encodes an intergral membrane protein, which
counteracts signalling by receptor tyrosine kinases belonging to the ERBB (epidermal growth factor receptor) family. *LRIG1*
is expressed in all tissues and organs analysed to date, including breast, brain, skin, kidney, spleen and colon.
Overexpression of EGFR is seen in 70 – 90% of colorectal cancers, and is associated with a poor survival. Western blot
analysis showed LRIG1 upregulation in 43% and downregulation in 43% of the colorectal cancers compared to adjacent
non-neoplastic tissue. No correlation was evident between LRIG1, analysed by Western Blot and the expression of EGFR
analysed by immunohistochemistry. FISH (fluoroscence in situ hybridisation) analysis showed increased *LRIG1* copy
number in one of nine tumours. Four colorectal cancer cell lines demonstrated two *LRIG1* gene copies. In conclusion, there
was a great heterogeneity in the expression of the LRIG1 protein in colorectal cancer, which was not related to gene dosage
of the *LRIG1* gene. Further studies can be of interest to evaluate whether alteration in LRIG1 expression in colorectal
cancer is of biological or clinical significance.

The human gene *LRIG1* at chromosome 3p14
encodes a protein with extracellular or lumenal
leucine-rich repeats and immunoglobulin-like do-
mains, a transmembrane part, and a cytoplasmic tail
[1–3]. LRIG1 antagonises EGFR-mediated signal-
ling by enhancing ubiquitinylation and degradation
of the receptor [4,5]. EGFR (ERBB1) is expressed
in many colon carcinomas and is associated with
poor survival [6–8]. Previous studies have shown
that response to EGFR targeted treatments does not
correlate to immunohistochemical EGFR expression
[9] and therefore other EGFR regulating proteins
are important to study. In squamous cell carcinoma
of the skin, patients with tumours underexpressing
LRIG1 show worse survival [10]. Since LRIG1 is a
potential suppressor of EGFR driven colon cancer
growth, we found it of interest to explore *LRIG1*
gene copy number and the expression of LRIG1

protein in sporadic colon cancer. The *LRIG1* locus
was analysed by fluorescence in situ hybridisation
(FISH) and the protein expression was analysed by
Western blot analysis.

Material and methods

Patients and sample preparation

Tumour samples and non-neoplastic colon tissues
were collected during 2002 from ten patients
with colorectal cancer (group A). In group A,
samples of the tumour and adjacent non-neoplastic
colon tissue were frozen in liquid nitrogen imme-
diately after excision and stored at $-80°C$. The
other adjacent parts of the tissue samples were
fixed in formalin, paraffin embedded and used for
routine morphological examination and tumour
grading.

Fresh frozen and formalin fixed paraffin embedded cancer tissue and surrounding normal colorectal tissue from patients undergoing surgery for colorectal cancer has been collected at the Department of Pathology since 1987. From this collection we randomly sampled 20 tumors diagnosed from 1989 to 1998 (Group B). The colorectal tumours were of different grading and had a lot of fresh frozen tumour available for the western blot analysis. Group B was used to expand the analysis using a larger number of samples for Western blot analysis of LRIG1 in fresh frozen samples and immunohistochemical analysis of the EGFR in formalin fixed tissue. Clinical characteristics of the patients are presented in Table I. The ethical committee of Umeå University approved the study. None of the patients had received any treatment prior to specimen collection.

Cell lines

To avoid the bias of interference by stroma and/or healthy colon cells, *LRIG1* gene copy number was evaluated in four cell lines derived from colon cancer tumours. The colorectal cancer cell lines LOVO, SW480, HT29 and DLD-1 were obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were cultivated in Dulbecco's modified Eagle's medium, supplemented with 10% v/v fetal bovine serum and 50 μg/ml gentamicin from Invitrogen AB (Täby, Sweden). The FISH slides were prepared according to standard procedures.

Table I. Clinical data of the 30 colorectal cancer patients in the study.

	n (%)
Age, years	
Medium	70
Range	45–89
Sex	
Female	19 (63)
Male	11 (37)
Stage	
Dukes A	6 (20)
Dukes B	16 (53)
Dukes C	8 (27)
Site	
Rectum and Sigmoid colon	13 (43)
Colon	17 (57)
Histological subtype/grade	
Adenocarcinoma	27
Mucinous adenocarcinoma	3
Well differentiated	2
Moderately differentiated	23
Poorly differentiated	2
Deceased	16 (53)

FISH

FISH was performed according to standard techniques as described in a previous study [11]. In each sample, two independent investigators scored *LRIG1* signals. At least 100, and if possible 200 nuclei were counted, depending on the quality of the sample. The presence of three or more signals in more than 20% of the nuclei was the criteria for scoring an increased copy number of *LRIG1*.

Western blot analysis

Western blot was performed as previously described [11]. The primary antibodies used were LRIG1-151 [2] and rabbit anti-actin (Sigma-Aldrich, St. Louis, MO, USA). The samples were stepwise diluted by 50% in three steps. The results were analysed visually and an apparent change between tumour and non-neoplastic tissue of at least 50% was considered as a significant difference (Figure 2). Attempts to establish a reliable EGFR western blot method did not result in a method stable enough for analysis.

Immunohistochemistry

A considerable effort has been made to achieve an immunohistochemical analysis of LRIG1. In colorectal cancer, unlike other tumours, a persistent background staining made the analysis unreliable, and therefore LRIG1 Western blot was performed instead. Immunohistochemical analysis of the EGFR was performed on formalin fixed and paraffin embedded colorectal cancer specimens according to routine procedures. For this study, 4 μm sections were placed into a completely automated immunostaining machine, Benchmark LT, Ventana Medical Systems Inc., Tucson, AZ, USA). The processes in the machine include deparaffinization, protease treatment (Protease 1), EGFR antibody reaction (mouse monoclonal 3C6, prediluted 1:200), and avidin-biotin blockage. Antibody reaction was demonstrated using iVEIW kit with DAB (diaminobenzidine) as chromogen and hematoxylin for counterstaining according the manufacturer's protocol. All reagents were from Ventana Medical System, Inc. All slides were independently evaluated by two investigators and intraobserver disagreements (<10%) were reviewed a third time followed by a conclusive judgement. One of the investigators was an experienced pathologist (R.P), Staining was scored as follows: 0, no membranous staining in any tumor cells; 1+, membranous staining in more than 10% of the tumor cells with any intensity.

Results

LRIG1 gene copy number in fresh frozen colorectal cancer tissues

To evaluate the number of LRIG1 gene copies, FISH was performed on cell nuclei from colorectal cancer samples (Group A, n = 10; Figure 1). Nine tissue samples were successfully evaluated; a tenth sample was of poor quality and analysis failed. An increased copy number of LRIG1 in more than 20% of the nuclei was found in one tumour, showing three to five LRIG1 signals per nucleus. Normal signal pattern corresponding to two copies per nucleus was detected in 8 of 9 tumours. The colorectal cancer cell lines LOVO, SW480, HT29, and DLD-1 were also examined by FISH and demonstrated two LRIG1 signals per nucleus corresponding to normal gene copy numbers.

Western blot analysis of LRIG1 expression in colorectal cancer samples and matched non-neoplastic tissue

Western blot analysis was performed on samples from both group A and B (Table II, Figure 2, n = 30). In 13/30 (43%) tumours the LRIG1 protein was downregulated in the tumour compared to matched non-neoplastic tissues. In the same number of tumours (13/30) the LRIG1 protein was upregulated. In 4/30 (13%), no significant difference in LRIG1 expression was evident.

Immunohistochemical EGFR expression in colorectal cancer samples

Immunohistochemical analysis was performed on the 20 available samples of formalin fixed and paraffin embedded colorectal cancer tissue (Table II, Figure 3). In 13/20 samples (65%) a positive staining for the EGFR was found. Negative staining was found in 7/20 (35%) samples. No significant correlation between LRIG1 expression determined by Western blotting and the immunohistochemical EGFR expression was seen.

Discussion

The present study revealed a great heterogeneity in the expression of LRIG1 in colorectal cancer. FISH analysis showed chromosomal abnormalities at the 3p14 locus in only one tumour, with slightly increased copy number. This was in contrast to a recent report concerning LRIG1 in breast cancer, where a significant (40%) proportion of the tumours showed increased copy number of the LRIG1 locus [11]. In this study, chromosomal aberrations at 3p14 do not appear to be common events in colorectal carcinoma. This is also supported by the analysis of four colon cancer cell lines, which did not show any changes in LRIG1 copy number.

Of the 30 tumours examined by Western blotting, up- or downregulation of LRIG1 was evident in 26 cases, with equal numbers of tumours over- and underexpressing the protein, respectively. The differences in LRIG1 protein expression was not due to chromosomal loss or gain and is therefore due to other mechanisms. In a study of LRIG1 in squamous cell carcinoma of the skin [10], a correlation between increased LRIG1 expression and a more favourable prognosis has been shown. In the present study, the small number of tumours investigated precluded analysis of clinical outcome.

LRIG1 has recently been shown to downregulate the ERBB receptors [4,5], including the EGFR, a receptor of major clinical importance in the pathogenesis of colorectal cancer [7]. The EGFR is a well-established factor of importance in colorectal cancer progression both as a predictor of outcome and as the target of new drugs [12]. Moreover, the
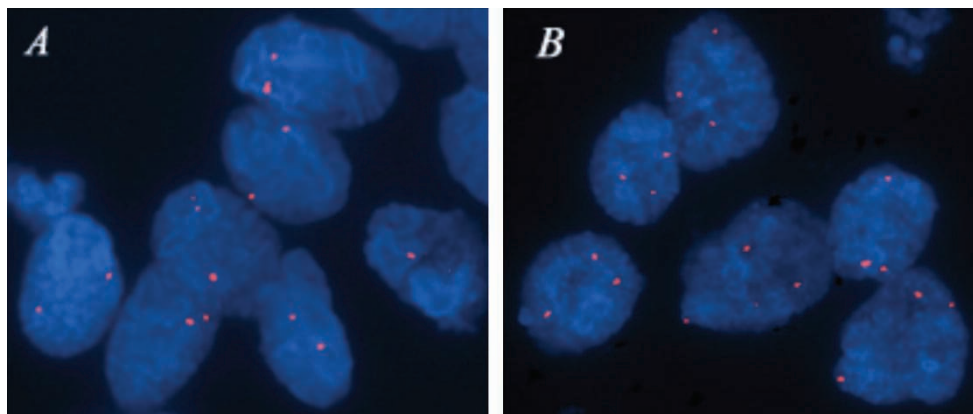

Figure 1. FISH analyses of LRIG1 locus in colorectal cancer cells from two patients. (A) Nuclei showing normal gene copy number (two copies per nucleus) of the LRIG1 gene. (B) Nuclei showing increased gene copy number (more than two copies per nucleus) of the LRIG1 gene at 3p14. Analysis was performed with a specific LRIG1 probe (red).

Table II. Crosstable of LRIG1 Western blot (WB) results and immunohistochemical (IHC) EGFR scores in 30 colorectal cancer samples and matching adjacent non-neoplastic tissue.

	EGFR IHC Positive[a]	EGFR IHC Negative	EGFR IHC Missing	Total
LRIG1 WB Downregulated#	8	1	4	13
LRIG1 WB Upregulated#	5★	4	4	13
LRIG1 WB Equal¤	0	2	2	4
Total	13	7	10	30

=Tumour compared to normal tissue, where a visual change of at least 50% was considered significant.
¤ =No visible change between tumour and normal tissue.
[a] =Positive immunostaining in at least 10% of cells.
★ =The tumour with *LRIG1* increased copy number was one of the tumours in this group.
Missing =No paraffin embedded tissue available for IHC analysis.

observation that response to anti-EGFR treatment is independent of immunohistochemical EGFR expression levels makes EGFR regulation studies of current importance [9]. As LRIG1 downregulates the ERBB receptors by receptor ubiquitinylation, immunohistochemical analysis of EGFR was performed to see if there was a correlation between LRIG1 Western blot levels and immunohistochemical EGFR levels. In this report, LRIG1 expression did not correlate to EGFR protein expression. This is in contrast to our previous report of LRIG1 in breast cancer, where a correlation between LRIG1 and EGFR RNA levels was evident [11]. From this perspective the relationship between LRIG1 and EGFR could be suggested to be tissue specific and not evident for all tumour types. However, determination of the actual impact of LRIG1 on EGFR levels would require more detailed biochemical analyses and therefore this study cannot exclude a correlation that could be detected by other methods. Moreover, LRIG1 also has regulatory effects on ERBB2, 3 and 4, and such potential interactions were not investigated in this study [5]. In conclusion, the LRIG1 protein was up- or downregulated in colorectal cancer without evident impact on EGFR protein levels.
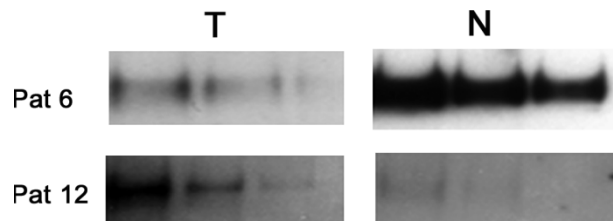

Figure 2. Example of Western blot analyses of LRIG1 in samples from two colorectal cancer patients. For comparison, the samples were stepwise diluted by 50% in three steps. Tumours (T) versus matched non-neoplastic tissue (N) from the same patient. Pat 6 was considered underexpressing the LRIG1 protein in the tumour compared to adjacent non-neoplastic tissue and Pat 12 was considered as overexpressing the LRIG1 protein in the tumour compared to non-neoplastic tissue.
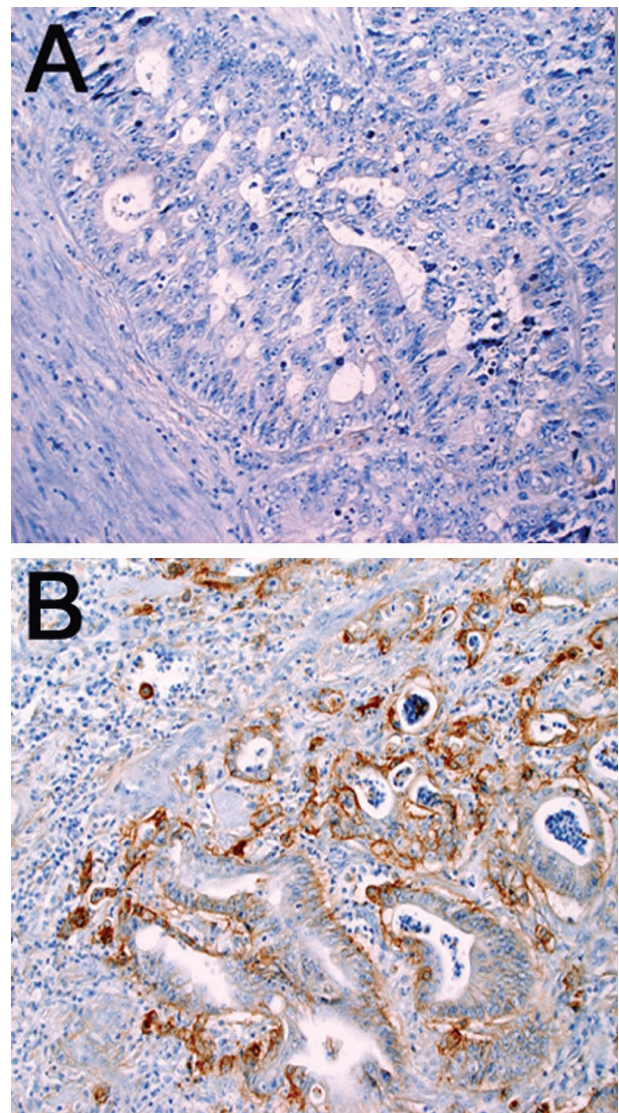

Figure 3. Immunohistochemical analyses of EGFR in primary colorectal tumors. (A) Negative staining in colorectal cancer cells and surrounding tissues. (B) Positive membranous staining in more than 10% of colorectal cancer cells (original magnification ×20), and mostly negative staining in surrounding tissues.

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