





# Expression and prognostic value of LRIG1 and the EGF-receptor family in renal cell and prostate cancer

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*Days up and down they come  
Like rain on a conga drum  
Forget most, remember some  
But don't turn none away  
Everything is not enough,  
And nothing is too much to bear  
Where you've been is good and gone  
All you keep is the getting there  
Well, to live's to fly, both low and high  
So shake the dust off of your wings  
And the sleep out of your eyes*

*Townes van Zandt - To live is to fly*



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## Abstract

The epidermal growth factor receptor (EGFR) family consists of four (EGFR, ErbB2, ErbB3, and ErbB4) receptor tyrosine kinases (RTK) whose signalling is important for physiological and malignant cellular functions such as proliferation, survival, migration, and differentiation. EGFR and ErbB2 in particular are established oncogenes in many solid tumours and are targets for anti-cancer treatment. LRIG1 (leucine-rich repeats and immunoglobulin-like domains-1) is a protein that negatively regulates the EGFR-family, and other RTKs and is a proposed tumour suppressor. This thesis examines the expression of the EGFR-family members and LRIG1 in renal cell carcinoma (RCC) and in prostate cancer (PC).

In RCC, up-regulation of EGFR was shown for all RCC types analysed: clear cell (ccRCC), papillary (pRCC), and chromophobe (chRCC). ErbB2 was down-regulated in ccRCC. ErbB3 expression was low in non-neoplastic kidney and not significantly altered in RCC. ErbB4 was strongly down-regulated in the vast majority of RCCs of all types. LRIG1 was down-regulated in ccRCC. No prognostic value was found for any of these factors in RCC. In prostate cancer cells, LRIG1 was shown to be up-regulated by androgen stimulation and suppressed the growth of prostate cancer cells. In prostate cancer, the expression and prognostic value of LRIG1 was investigated in two patient series, one with untreated patients and one with patients who had undergone prostatectomy. In the untreated patient series, LRIG1 correlated with malignancy grade (Gleason score) and poor outcome for patients (both cancer specific and overall survival), being an independent prognostic factor. In contrast, in the series of patients who had undergone prostatectomy, LRIG1 expression correlated with a good outcome (overall survival).

Thus in RCC, there were alterations in gene-expression of the EGFR-family members and LRIG1 between kidney cortex and RCC and between the RCC types. Despite few associations with clinical factors, these alterations are likely to be of biological importance. In prostate cancer LRIG1 was up-regulated by androgen stimulation and inhibited cell proliferation. LRIG1 expression had prognostic value in prostate cancer, maybe as a secondary marker of androgen receptor activation or because of growth inhibition of prostate cancer cells. Contradicting findings in untreated patients and patients treated with prostatectomy poses the question of whether the prognostic value of LRIG1 and other markers vary depending on the specific biological and clinical circumstances in the materials studied.

## List of papers

This thesis is based on the results from the following papers, which are referred to in the text by their Roman numerals.

- I.** LRIG1 and epidermal growth factor receptor in renal cell carcinoma: a quantitative RT-PCR and immunohistochemical analysis.  
**Marcus Thomasson**, Håkan Hedman, Dongsheng Guo, Börje Ljungberg and Roger Henriksson. *British Journal of Cancer* 2003;89(7): 1285–1289.
- II.** ErbB4 is downregulated in Renal Cell Carcinoma: A quantitative RT-PCR and immunohistochemical analysis of the epidermal growth factor receptor family.  
**Marcus Thomasson**, Håkan Hedman, Teemu T. Junttila, Klaus Elenius, Börje Ljungberg and Roger Henriksson. *Acta Oncologica* 2004;43(5): 453-459.
- III.** Differences and similarities in the expression of the epidermal growth factor receptor family and the proposed tumor suppressor LRIG1 in types of renal cell carcinoma.  
**Marcus Thomasson**, Håkan Hedman, Börje Ljungberg and Roger Henriksson. 2009, Submitted manuscript.
- IV.** LRIG1 and the liar paradox in prostate cancer: A study of the expression and clinical significance of LRIG1 in prostate cancer.  
**Marcus Thomasson**, Wang Baofeng, Peter Hammarsten, Anna Dahlman, Jenny Liao Persson, Andreas Josefsson, Pär Stattin, Roger Henriksson, Anders Bergh and, Håkan Hedman. 2009, Submitted manuscript.

No permission was needed for reprint of Paper **I** and **II**

# Populärvetenskaplig sammanfattning på svenska

Tillväxtsignalering har en viktig roll i utvecklingen av cancer. Felaktig tillväxtsignalering kan bero på förändrat uttryck av signalämnen eller receptorer för signalämnen eller förändrad känslighet hos dessa receptorer eller felaktiga receptorer som signalerar utan signalämne.

I denna avhandling undersöks uttrycket och betydelsen av en familj av tillväxtsignaleringsreceptorer, EGFR (epidermal growth factor receptor)-familjen och en naturligt förekommande hämmare av dessa receptorer, LRIG1 (leucine-rich repeats and immunoglobulin-like domains protein 1), i njur- och prostatacancer. EGFR-familjen består av fyra olika receptorer, EGFR, ErbB2, ErbB3 och ErbB4, där framförallt EGFR och ErbB2 är kända tillväxtstimulerande faktorer och mål för behandling i flera cancerformer.

Njurcancer är en sjukdom som drabbar ungefär 900 personer årligen och orsakar ca 600 dödsfall per år i Sverige. Njurcancer kan vid tidig upptäckt botas med operation. Vid spridd sjukdom kan man idag trots tillskott av nya behandlingsmetoder endast sakta ned sjukdomsförloppet och lindra besvär. Njurcancer delas upp i undergrupper efter mikroskopiskt utseende där de olika grupperna skiljer sig åt vad gäller underliggande genetiska förändringar. De vanligaste undergrupperna är klarcellig, papillär och kromofob njurcancer, vilka är de undergrupper som undersökts i denna avhandling.

Prostatacancer är den vanligaste cancerformen och orsaken till cancerdöd bland svenska män. Nästan 10000 svenska män drabbas varje år av sjukdomen. Prostatacancer är oftast beroende av hormonsignalering från manligt könshormon för sin överlevnad och tillväxt. Hormonbehandling, genom kirurgisk eller kemisk kastrering, är en vanlig behandling av spridd prostatacancer. Efter en tids kastrationsbehandling blir prostatacancer ofta kastrationsresistent d.v.s. får förmågan att tillväxa med hjälp av mycket små mängder hormon och tillväxer trots kastrationsbehandling.

I njurcancer fann vi att en sedan tidigare beskriven ökning av uttrycket av EGFR i tumörer jämfört med njurvävnad förekom i alla undersökta undergrupper av njurcancer. Uttrycket av ErbB2 var lägre i klarcellig njurcancer jämfört med i njurvävnad. Detta var oväntat eftersom ErbB2 i många andra cancersorter är en kraftigt tumörstimulerande faktor. Uttrycket av ErbB3 var lågt i både tumörer och njurvävnad. Uttrycket av ErbB4 var mycket lågt i tumörer jämfört med njurvävnad och detta kan tyda på att

signalering från ErbB4 kan ha egenskaper som är hindrande för uppkomsten eller tillväxten av njurcancer. Uttrycket av LRIG1 var minskat i klarcellig njurcancer. Vi hade hoppats att kunna finna samband mellan uttrycket av dessa faktorer och hur det går för patienterna med ett visst uttryck av dessa faktorer, men något sådant samband kunde inte ses.

I prostatacancer verkar uttrycket av LRIG1 vara högre i tumörer än i prostatavävnad. Våra försök visar att stimulering med manligt könshormon ökar uttrycket av LRIG1 i prostatacancerceller. Ökat uttryck av LRIG1 kan också hämma tillväxten av prostatacancerceller i odlingsförsök. Vi undersökte sambandet mellan uttryck av LRIG1 i tumörer och hur det gick för patienter i två olika kliniska material. Ett äldre svenskt material, med patienter som ej fått någon tidig behandling utan följts och fått symptomlindrande behandling först när det utvecklats metastaser och ett nyare material från U.S.A. där samtliga patienter opererat bort prostatakörteln. I materialet med patienter som inte behandlats från början verkade det gå sämre för de patienter vars tumörer hade högt uttryck av LRIG1. I materialet med opererade patienter var det tvärt om och det gick bättre för de patienter vars tumörer hade högt uttryck av LRIG1. Vi har föreslagit att dessa till synes motsägelsefulla resultat beror på att effekten av LRIG1 uttryck varierar beroende de omständigheterna kring patienternas sjukdom. Hos obehandlade patienter tror vi att LRIG1 framförallt är en markör för den stimulering med manligt könshormon som tumören utsätts för och att denna stimulering är starkare än den tillväxthämmande effekten av LRIG1. Hos de opererade patienterna där mängden av eventuellt kvarvarande tumören är mycket liten, blir förutsättningarna för tumörtillväxt annorlunda, och att den tillväxthämmande effekten av LRIG1 då får en större betydelse.

Sammanfattningsvis har vi påvisat att uttrycket av EGFR, ErbB2, ErbB4 och LRIG1 skiljer sig mellan icke tumöromvandlad njurvävnad och njurcancer. Uttrycket av EGFR-familjens medlemmar och LRIG1 i tumörer kunde ej användas för att förutse förloppet för patienter men är sannolikt viktiga för njurcancers biologi. I prostatacancer visar vi att LRIG1 regleras av stimulering av manligt könshormon och kan minska tillväxthastigheten hos prostatacancerceller. Uttrycket av LRIG1 i prostatacancer kan relateras till hur det går för patienter men denna samvariation skiljer sig mellan obehandlade och opererade patienter. Det finns flera möjliga förklaringar till en sådan skillnad och dessa bör undersökas mer i framtiden.

## Abbreviations

AR – Androgen receptor

BTC – Betacelluline, an EGFR and ErbB4 ligand

CI – Confidence interval (95%)

CBL – Casitas B-lineage Lymphoma gene, an E3 ligase

EGF – Epidermal growth factor

EGFR - (ErbB1, HER1) – Epidermal growth factor receptor

ErbB - Erythroblastic leukemia viral oncogene homolog

Erk – Extracellularly regulated kinase, a seronine/threonine protein kinase  
(same as MAPK)

FGF – Fibroblast growth factor

HB-EGF - Heparin-binding EGF-like growth factor

HER – Human EGF receptor

IHC – Immunohistochemistry

IR – Immunoreactivity

LRIG - Leucine-rich repeats and immunoglobulin-like domains

MAPK – Mitogen-activated protein kinase (same as ERK)

MET - mesenchymal-epithelial transition factor, a RTK

mTor – mamalian target of rapamycin, a seronine/threonine protein kinase

neu - neuro/glioblastoma derived oncogene homolog, name of ErbB2 in rats

NRG – Neuregulin, a family of ligands of the EGFR-family

PI3K – Phosphatidylinositol-3 kinase

PLC- $\gamma$  - phospholipase C-gamma

PSA – Prostate specific antigen

RCC- Renal cell carcinoma

ccRCC – clear cell RCC (also called conventional RCC)

pRCC – papillary RCC

chRCC – chromophobe RCC

RET – Rearranged during transfection, a RTK

RR – Relative risk

RTK – receptor tyrosine kinase

TGF- $\alpha$  – Transforming growth factor alpha

TUR-P – Trans-urethral resection of the prostate

# Introduction

Cancer is a class of diseases that arise from cells of the patient's own body. These cells have somehow acquired genomic changes that alter the properties of the cells. The genomic changes in most types of cancer accumulate by an evolutionary process driven by cellular growth advantages. There are six hallmark traits that a cell or cell population exhibit to be considered a cancer [1]: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. The acquisition of these traits is a multi-step process with a gradual gain or loss of properties leading to a more malignant phenotype and eventually a fully developed cancer.

There are many types of genes that can be involved in this process. One group of genes that are often involved in the genesis and progression of cancer encodes receptor tyrosine kinases (RTK). A family of RTKs, the epidermal growth factor (EGF) receptor (EGFR) family, play a role in several types of cancer. Leucine-rich and immunoglobulin-like domains protein 1 (LRIG1) is a negative regulator of the EGFR-family. This thesis focuses on the role of the EGFR-family and LRIG1 in renal cell carcinoma and prostate cancer.

## Receptor tyrosine kinases

The human RTK family is a group of 58 cell surface receptors for various growth factors, cytokines and hormones. Upon binding of extra-cellular ligand, these receptors undergo structural changes enabling them to bind and phosphorylate tyrosine residues on other proteins, changes that start a signal transduction cascade, from the cell surface to internal structures such as the nucleus. The signals transmitted by these receptors regulate such cellular processes as metabolism, proliferation, differentiation and survival. Since these processes are important for the development of cancer it is hardly surprising that deregulation of RTK signalling is often found in cancer [2].

## The epidermal growth factor receptor family

The EGFR- family is named after its first described member, the EGFR (also called ErbB1 or HER1). EGFR, one of the first described proto-oncogenes, is a human homolog of the avian erythroblastic leukemia viral gene (v-erb-b) [3-4]. The EGFR-family consists of four members, EGFR, ErbB2 (Neu,

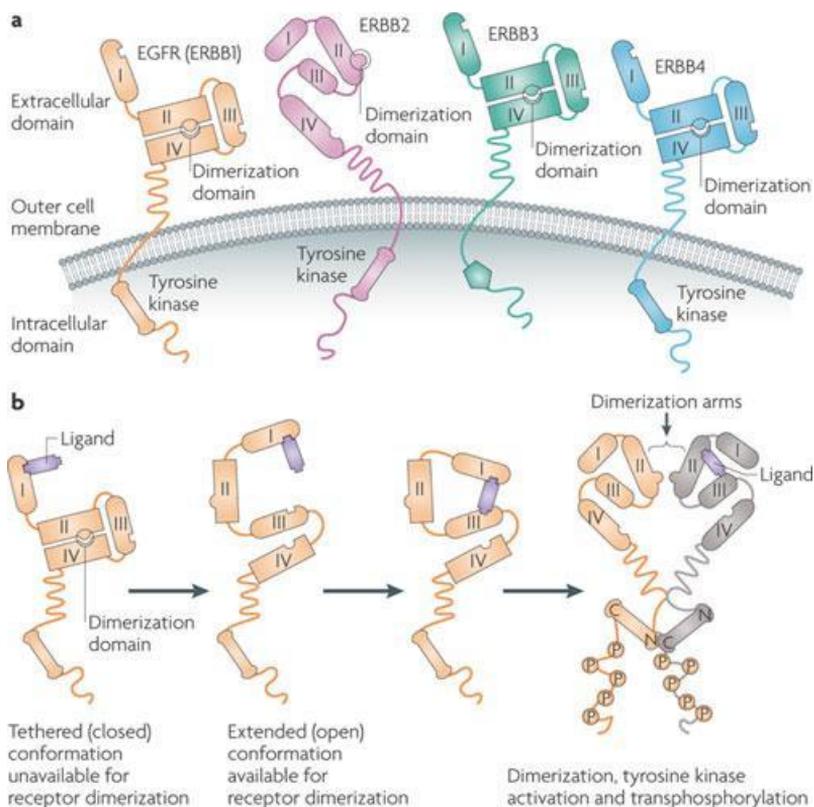
HER2), ErbB3 (HER3), and ErbB4 (HER4). They all share a similar structure with most RTKs. That is, they are glycoproteins with an extra cellular ligand binding domain a hydrophobic trans-membrane part, and a cytoplasmic part that contains the tyrosine kinase domain [5]. A schematic representation of their structure taken from [6] is shown in **Figure 1a**.

The EGFR-family members have distinct properties that are important for their signalling function [5]. ErbB2 does not bind any known ligand but exerts signalling function as a preferred dimerisation partner of the other family members [7-8]. ErbB3 is devoid of intrinsic kinase activity but is an important signalling molecule when dimerised with other members of the EGFR-family [9]. The ErbB4 receptor has four isoforms, with two variants in the extracellular juxtamembrane region (JM-a and JM-b) and two variants in the cytoplasmic region (CYT-1 and CYT-2) [10]. JM-a, one of the juxtamembrane variants is cleaved by tumour necrosis factor alpha converting enzyme (TACE, also called ADAM17), whereas JM-b is not [11-12]. After cleavage and translocation the intracellular part may affect the nucleus [13]. The cytoplasmic variants differ in their ability to couple to phosphoinositide 3-kinase (PI3-K) and effectuate signalling through that pathway [14].

The EGFR-family members have at least eleven ligands (**Table I**). These ligands are produced as transmembrane precursors in the signalling cell and are processed and released by proteolysis. The EGF, amphiregulin, and transforming growth factor alpha (TGF- $\alpha$ ) are ligands specific for the EGFR, whereas betacellulin, heparin-binding EGF (HB-EGF), and epiregulin bind both EGFR and ErbB4. Another family of ligands the neuregulins (NRG), comprises NRG-1-4. NRG-1 and NRG-2 bind to both ErbB3 and ErbB4, whereas, NRG-3 and NRG-4 bind specifically to ErbB4 [15-16]. As mentioned above, ErbB2 lacks a specific known ligand.

**Table I Ligands of the EGFR-family**

<b>Ligand</b>	<b>Receptor interaction</b>			
	<b>EGFR</b>	<b>ErbB2</b>	<b>ErbB3</b>	<b>ErbB4</b>
<b>EGF</b>	+	-	-	-
<b>TGF-<math>\alpha</math></b>	+	-	-	-
<b>Amphiregulin</b>	+	-	-	-
<b>Betacellulin</b>	+	-	-	+
<b>HB-EGF</b>	+	-	-	+
<b>Epiregulin</b>	+	-	-	+
<b>Neuregulin 1</b>	-	-	+	+
<b>Neuregulin 2</b>	-	-	+	+
<b>Neuregulin 3</b>	-	-	-	+
<b>Neuregulin 4</b>	-	-	-	+



**Figure 1 a | Simplified structures of the four members of the EGFR-family.** Each receptor is composed of three functional domains: an extracellular domain responsible for ligand binding, the  $\alpha$ -helical transmembrane segment; and the intracellular domain, containing the protein tyrosine kinase domain as well as motifs and residues that mediate interactions with intracellular signalling molecules[17]. Epidermal growth factor (EGFR), ErbB3 and ErbB4 exist in a tethered ('closed') conformation in which the dimerisation domain is not available to interact with partner ErbB in the absence of ligand [18]. There is no known ligand for ErbB2; this receptor exists in an active extended ('open') conformation and may be permanently available for dimerisation [17-18] **b | Conceptualization of the receptor conformational change upon ligand binding.** Ligand binding to ErbB receptors seems to induce a conformational change in the folded structure of the molecule that exposes the dimerisation domain; this step is required for dimer formation and functional activation of EGFR, ErbB3 and ErbB4 [18-21]. The kinase domain interaction is asymmetric, with the amino-terminal lobe of one tyrosine kinase interacting with the carboxy-terminal lobe of the other. ErbB2 lacks a specific ligand but exerts its function through interaction with the other EGFR family members. From Baselga and Swain 2009 [6], with kind permission of the Nature publishing group.

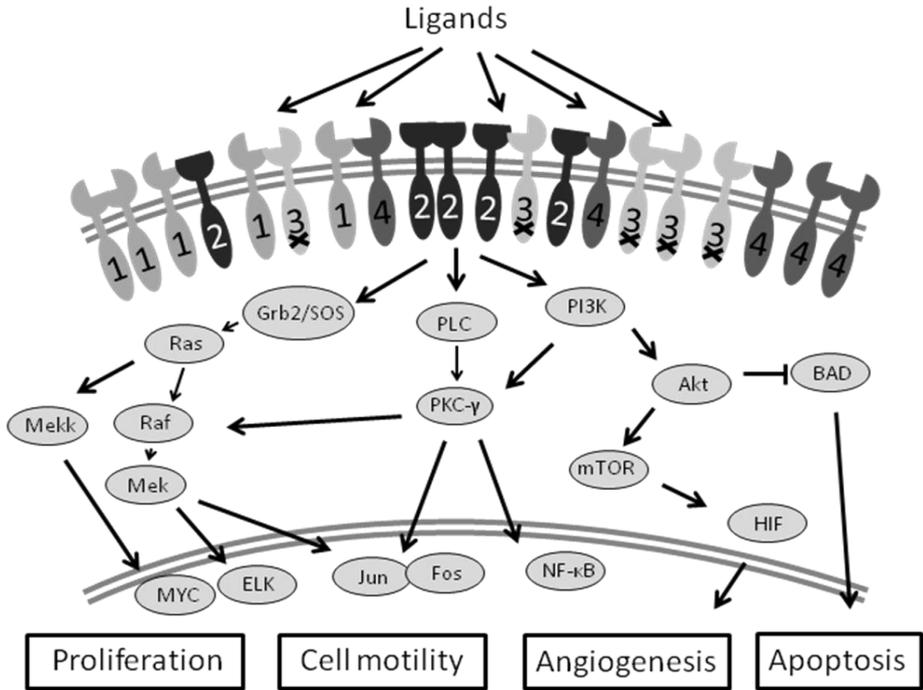
Most of the ligands, with the exception of EGF, are thought to primarily act over short distances as autocrine or paracrine factors. Upon binding of ligand the receptor goes through a conformational change that makes it prone to bind and form dimers. These dimers can be either homo-dimers,

with two receptors of the same kind or hetero-dimers, with two different EGFR-family members. Schematic representation of the dimerisation process is shown in **Figure 1b**. The type of dimer that is formed is to some extent governed both by the type of ligand and the affinity of the receptor for the other partners of receptor complexes, and what population of potentially active ErbB receptor dimers are present in the cells [22].

Downstream of receptor activation, the EGFR-family mainly activates three distinct signalling pathways, the mitogen-activated protein kinase (MAPK) pathway, the PI3K pathway, and the phospholipase C- $\gamma$  (PLC $\gamma$ ) pathway [5]. The MAPK pathway is a signalling cascade activated by phosphorylation of proteins leading to the activation of transcription factors such as MYC, ELK, FOS and JUN and translation of proteins enhancing cell proliferation. The PI3K pathway involving down-stream protein such as AKT and mTOR also affects cell proliferation as well as increases the expression of anti-apoptotic factors and inhibits the pro-apoptotic protein BAD. The PLC $\gamma$  pathway is important for regulation of cell motility through re-modulation of the cytoskeleton. The EGFR-family members can also activate other signalling pathways to a lesser extent and may do so to a larger extent under pathological conditions such as over-expression of the receptors [23] (**Figure 2**).

After ligand-binding and receptor activation, EGFR (ErbB1) receptor complexes are internalised through formation of vesicles, which become early endosomes [24]. This endocytosis can be either clathrin dependent or clathrin independent [25]. Under certain physiological conditions (low ligand concentrations and moderate expression levels of EGFR), clathrin mediated endocytosis is likely the most common [26]. Receptor dimers involving ErbB2 are less prone to internalisation [26]. Internalisation of ErbB3 and ErbB4 has been poorly studied but data suggest that internalisation is less important for the signal termination than in EGFR [26]. Early endosomes are either targeted for lysosomal fusion or recycled back to the cell surface. The fates of the vesicles are determined by the nature of the receptor ligand complex due to differences in pH stability. As the endosome matures pH drops and less stable complexes dissociates leading to recycling while more stable complexes are routed for lysosomal degradation [27]. EGFR homodimers are more stable than dimers with ErbB2, ErbB3 and ErbB4 [28]. Receptor dimers formed by EGF is more stable than those formed by TGF- $\alpha$ , making TGF- $\alpha$  a more potent mitogen than EGF, as a larger portion of receptors complexes are recycled to the surface and therefore available for renewed activation [5]. Receptors continue to signal after internalisation through RAS activation as long as the receptor complex is intact. This internal signalling can activate the MAPK and PI3K pathways

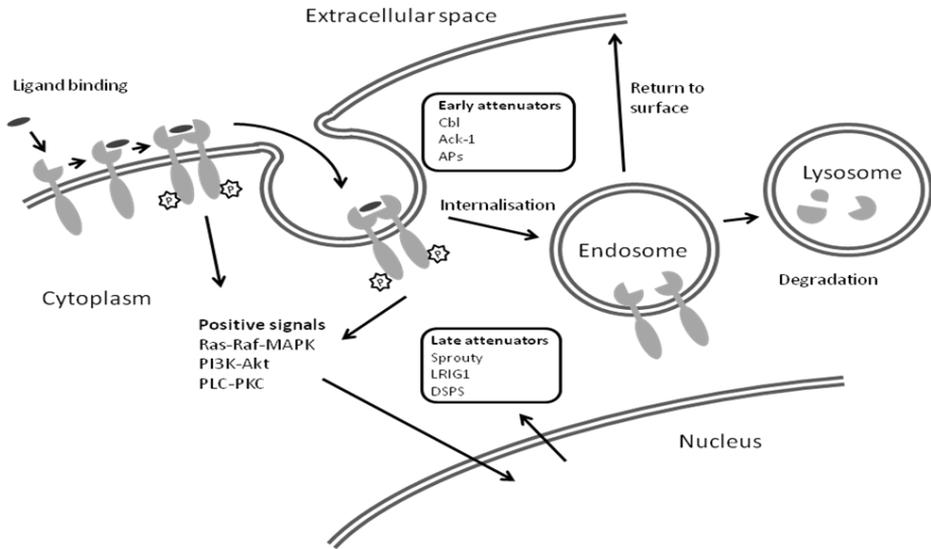
although the PLC- $\gamma$  pathway can only be activated at the plasma membrane [29-30].



**Figure 2 | A highly simplified illustration of the three most important downstream signalling pathways of the EGFR-family.** The Ras/Raf/Mek pathway primarily activates gene transcription factors promoting cellular proliferation. The PLC/PKC pathway is primarily involved in regulating cell motility. The PI3K pathway primarily promotes survival signalling through inhibition of apoptosis, but can also stimulate proliferation and regulates pro-angiogenic factors and protein synthesis through mTOR.

### Negative regulation of EGFR-family signalling

As the signalling of the EGFR-family is physiologically important it is highly regulated. There are several known regulatory mechanisms, some known as early attenuators are continually expressed while others, known as late attenuators, are transcriptionally up-regulated upon activation of receptor signalling. This kind of negative regulation, activated by signalling, is commonly known as negative feedback loops and is a common feature in the regulation of RTKs. [31]. LRIG1 is a late attenuator, as will be discussed in a later section. An overview of these mechanisms is shown in **Figure 3**.



**Figure 3 | Schematic illustration of EGFR-family signaling and receptor complex internalization.** After ligand binding, the receptors dimerise and activate each other by phosphorylation. Attenuation of the signalling is achieved both by continually expressed proteins involved in endocytosis and cytoskeleton rearrangement (early attenuators), and transcription-dependent negative regulatory pathways (late attenuators) which are expressed after activation of transcription factors in the nucleus. DSPs, dual-specificity phosphatases; APs, clathrin-associated proteins.

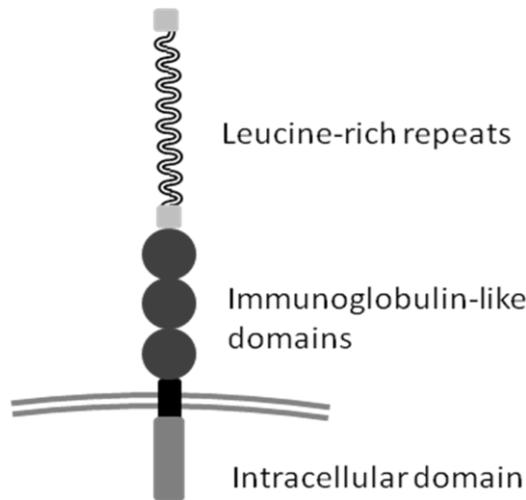
Of the early attenuators the most studied is the E3 ubiquitin ligase c-Cbl which regulates EGFR. The protein c-Cbl binds to a phospho-tyrosine site on EGFR and attaches ubiquitin to the receptor [32-33] which promotes internalisation of the receptor into endosomes [34]. In the endosome, the E3 ligase activity of c-Cbl leads to continued ubiquitination targeting the receptor for lysosomal degradation [35]. Experiments with cells that do not express c-Cbl have shown that internalisation occurs even in the absence of c-Cbl, but that c-Cbl is necessary for the sorting to the late endosome/lysosome [36]. This regulatory pathway is well conserved through evolution with Cbl orthologs being present in both nematodes (*Sli-1*) and insects (Cbl), asserting a similar regulation on the orthologs of EGFR, *Let-23* and *DER*, respectively. ErbB2, ErbB3 and ErbB4 are under physiological conditions not ubiquitinated by Cbl [37]. Another E3 ligase, Nrdp1, has been shown to interact with ErbB3 and ErbB4 [38-39]. Nrdp1 interacts with both ligand-stimulated and unstimulated forms of these receptors, inducing delocalisation of the receptors to intracellular compartment, a possible mechanism for ErbB3 regulation analogous to that of Cbl in EGFR degradation [40].

In addition to LRIG1 there are several other known late attenuators of EGFR-family signalling. RALT (Mig-6/Gene 33/ERRFI1) is a multi-adaptor protein with many interactive domains that binds to all EGFR-family members, after ligand activation and inhibits their tyrosine kinase activity [41]. RALT is induced by activation of the Ras/ERK/MAPK pathway, downstream of receptor activation. Although RALT inhibits all EGFR-family members it acts differently on different dimers. ErbB4 signalling is rapidly and strongly inhibited, while EGFR signalling is less strongly inhibited. ErbB2/ErbB3 dimers are also inhibited by RALT but at a later time [41]. RALT knockout mice exhibit hyper-activation of EGFR-family members, sustained signalling via the ERK pathway, with hyper-proliferation of keratinocytes [42]. The mutant mice develop spontaneous tumours in various organs and are sensitive to chemical induced tumorigenesis of the skin. FRS2 $\beta$  (SNT-2/FRS3) is a docking/scaffold adaptor protein. It binds to the EGFR regardless of ligand binding status and prevents autophosphorylation [43]. This is in contrast to its family member FRS2 $\alpha$  which mediates FGF-signalling, a function that FRS2 $\beta$  can compensate for in FRS2 $\alpha$  knockout mouse fibroblasts. SOCS3/SOCS4/SOCS5 are members of the SOCS protein family which was originally identified as feedback inhibitors of cytokine signalling pathways. These three members can be induced by EGF stimulation. The SOCS proteins down-regulate EGFR by binding the receptor and proteins (such as Elongin B and C) leading to the formation of a large complex leading to the ubiquitination of the receptor [44]. The SOCS proteins may also affect EGFR signalling due to the fact that they compete for their binding site with STAT3, a mediator of EGF signalling through the Ras/Erk/MAPK-pathway [44].

The members of the Sprouty family have been suggested as EGFR inhibitors but recent data suggest a more complicated role. Sprouty was identified in *Drosophila* as an inhibitor of RTKs, including the insect equivalents of fibroblast growth factor receptor (FGFR) [45] and *Drosophila* EGFR (DER) [46]. Spry2 is the one of four mammalian members of the Sprouty family that shows most homology with the ancestral gene and it is an inhibitor of FGFR, VEGFR and MET [47-48]. The N-terminal part of Spry2 interacts with several downstream components of the RAS/ERK/MAPK-pathway and is capable of inhibiting these [49]. Despite this, experiments over-expressing Spry2 in cells has lead to increased activation of the MAPK-pathway. This is due to the C-terminal part of Spry2 binding Cbl, decreasing down-regulation of EGFR by this protein. It has been proposed that this dual function of Spry2 might be important in fine tuning EGFR-signalling [31].

## The LRIG family

The leucine-rich repeats and immunoglobulin-like domains (LRIG) protein family is a relatively newly characterised family of trans-membrane proteins [50]. The family consists of three proteins, LRIG1, LRIG2, and LRIG3 that share a similar structure with an extra-cellular part with a leucine-rich repeats domain, three immunoglobulin-like domains, and an intracellular domain (**Figure 4**). This structure suggests a function involving protein-protein interaction. All members of the LRIG-family are expressed to variable degrees in all tissues analysed [50-52].



**Figure 4 | Schematic structure of an LRIG protein.** The 15 leucine-rich repeats and three immunoglobulin-like domains, suggest functions involving protein-protein interactions. The extracellular part of the protein is the evolutionary most conserved region. Variation is greater in the intracellular part. It is the intracellular part of LRIG1 that interacts with c-Cbl.

## LRIG1

LRIG1 was first discovered and cloned in mice and named LIG-1 [53]. It was renamed to Lrig1 as the gene name LIG-1 was already reserved for DNA ligase 1. The human cDNA for LRIG1 was cloned in 2001 in a search for a human equivalent of the *Drosophila* EGFR inhibitor Kekk-1 [51]. Although Kekk-1 and LRIG1 are now known not to be true orthologs they do show similarities in structure and function [51]. The LRIG1 gene, located on chromosome 3p14, has an open reading frame of 1093 amino acids and encodes a protein of an apparent weight of 143 kD. Expression of LRIG1 mRNA has been found to varying degrees in all investigated human tissues with strong expression in brain, lung, and kidney [54].

Immunohistochemical analysis of LRIG1 protein showed strong expression in glandular epithelium [54]. LRIG1 has been shown to be a regulator of the EGFR-family members, MET, and RET RTKs. LRIG1 expression is increased upon EGF stimulation and it interacts physically with all members of the EGFR-family and increases their down-regulation rate [55-56]. In the case of EGFR, to some degree the down-regulation is achieved by recruitment of the E3 ubiquitin ligase, c-Cbl, which targets the EGFR-LRIG1 complex for lysosomal degradation by tagging the EGFR/LRIG1 complex with ubiquitin [55-56]. This is likely not the only mechanism by which LRIG1 regulates EGFR as a soluble construct of the extra cellular part of LRIG1 can inhibit EGFR activation and EGFR dependent cell growth [57]. The down regulation of MET by LRIG1 is also in part accomplished by receptor degradation but this mechanism is Cbl independent [58]. The ability of LRIG1 to inhibit signalling by the RET-receptor does not depend on receptor degradation but does seem to involve inhibition of binding of RET ligand, Glial cell line-derived neurotrophic factor (GDNF), and recruitment of RET to lipid rafts in the cell membrane, an important step in RET activation [59]. The LRIG1 gene has been ablated in mice [60]. The LRIG1 deficient mice were born at expected Mendelian ratios with no evidence of congenital deformities with normal growth, behaviour and fertility. The most notable phenotype of LRIG1 deficient mice was the development of skin abnormalities on their tails, facial areas and ears at three or four weeks of age. Histological examination showed epidermal hyperplasia, hyperkeratosis with parakeratosis, neutrophil influx and subcorneal pustules in a pattern reminiscent of psoriasis [60].

LRIG1 is down-regulated in several cancer cell lines [61]. In breast cancer, LRIG1 expression was found to be decreased in ErbB2 positive tumours and suppression increased proliferation of ErbB2 positive cell lines [62]. Another study found in breast cancer an increased copy number at chromosome band 3p14, the gene locus of LRIG1 [63]. This increase in copy number is more common in cells with increased copy number of ErbB2 but its biological significance is unknown [64]. In squamous cell carcinomas of the skin and cervix, low LRIG1 expression correlates with poor survival in patients [65-66].

### **LRIG2 and 3**

LRIG2 was characterised in 2004 [52]. The gene for LRIG2 is located on chromosome 1p13 and has an open reading frame of 1065 amino acids, encoding a protein of an approximate weight of 132 kD. Treating LRIG2 with N-glycosidase F reduces the apparent weight to 107 kD, indicating that LRIG2 is a glycoprotein with N-linked oligosaccharides. LRIG2 has a

structure similar to LRIG1 and an amino acid sequence that is 47% identical to LRIG1 with the strongest conservation in regions in the extracellular, trans-membrane, and the membrane proximal part of the cytoplasmic tail. The membrane distal part of the cytoplasmic tail shows no significant homologies to LRIG1. All investigated human tissues express LRIG2 mRNA to varying degrees. The highest LRIG2 mRNA expression has been seen in uterus, ovary, and prostate, and low expression was seen in heart, skeletal muscle and bladder. LRIG2 protein expression in cells in vivo has been seen in the plasma membranes and the cytoplasm of the cells (sometimes cytoplasmic expression is perinuclear, concentrated around but not in the nucleus). Although the molecular function of the LRIG2 protein is not known, due to its structural similarities with LRIG1 it has been speculated that it may interact with RTKs. The clinical relevance of LRIG2 in cancer is still uncertain, two studies have shown correlations between LRIG2 protein expression and prognosis for brain tumour patients. In one study [67], perinuclear expression of LRIG2 protein in astrocytic tumours correlated with better survival for patients; in another study [68] cytoplasmic expression in oligodendrogliomas correlated with a shorter survival. Although deletion of 1p is a known favourable prognostic factor in oligodendrogliomas no correlation between LRIG2 protein expression and loss of 1p could be found in this study [68].

LRIG3 was characterised in 2004 [50]. The gene for LRIG3 is located at chromosome 12q13.2. The encoded protein has a structure highly similar to LRIG1 and LRIG2. The amino acid sequence of LRIG3 is 47% identical to LRIG1 and 54% identical to LRIG2. The similarities in sequence with the other LRIG-family members is highest in the extracellular, trans-membrane, and the membrane proximal part of the cytoplasmic tail, yet the membrane distal part of the cytoplasmic tail shows no significant similarities to LRIG1 or LRIG2. All investigated human tissues express LRIG3 mRNA to varying degrees. The highest LRIG3 mRNA expression has been seen in stomach, skin, and thyroid, and low expression was seen in heart and blood [50].

### **The EGFR-family in cancers other than RCC and prostate cancer**

The EGFR-family in RCC and prostate cancer is discussed under the headings of RCC and prostate cancer. Both over-expression and structural alterations of the EGFR are frequent in malignant human solid tumours. Over-expression has been reported in bladder cancer, breast cancer, colon cancer, head-and-neck cancer, non-small cell lung cancer, prostate cancer, and renal cell carcinoma [69-70]. Gliomas, particularly glioblastomas, have a high incidence of both gene amplification [71] and mutations that result in

constitutively active EGFR [72]. Mutations of EGFR have also been found in lung, ovary and breast cancer [72]. Expression of EGFR has been investigated as a prognostic marker in several cancers with varying results [70]. Treatments directed at the EGFR are in clinical use. Cetuximab is an EGFR antibody that is used to treat metastatic colon cancer in tumours that do not exhibit mutations in K-RAS, and in squamous head-and-neck cancer. Small molecule inhibitors of the EGFR, erlotinib and gefitinib, are used to treat non-small cell lung cancer, but have primarily shown effect in tumours exhibiting activating mutations in the catalytic domain of EGFR [73].

Although ErbB2 does not bind any known ligand [16] it can by forming hetero-dimers with other EGFR-family members trigger very potent downstream signalling that promote mitosis and cell survival. ErbB2 is over-expressed in many human cancers including breast, pancreas [74], colon [75], oesophagus [76], endometrial [77] and cervical cancer [78]. In some of these cancers it has shown potential as a prognostic marker. In breast cancer, ErbB2 is of especially great biological and clinical importance. In clinical materials, 25-30 % of breast cancers over-express ErbB2, most often because of gene amplification [79]. It is a strong marker of poor prognosis [79] and breast cancer patients are routinely tested for expression and gene amplification of ErbB2. The monoclonal ErbB2 antibody trastuzumab is used to treat women with tumours showing gene amplification both in the adjuvant [80-81] and palliative setting [82-83].

Over-expression of ErbB3 has been described in several cancers including breast, lung, ovarian, colon, gastric, pancreatic, and head and neck cancers [84]. ErbB3 also has clear but moderate transforming ability in several cell lines. Variable results on the prognostic value of expression ErbB3 have been found in cancers of the breast and colon, but evidence for correlation with poor prognosis is documented in ovarian, prostate, pancreatic and oral cancers [84]. In bladder cancer, one study showed a correlation between expression of ErbB3 and good prognosis and an inverse correlation with ErbB2 expression [85]. Since ErbB3 lacks tyrosine kinase activity of its own (see above), it exerts its effects in conjunction with other EGFR-family members. The dimerisation of ErbB2 and ErbB3 is believed to have the strongest oncogenic effect of all ErbB dimers [8]. The lack of tyrosine kinase activity has made ErbB3 less attractive as a treatment target but recent reports have pointed out the mechanistic importance of ErbB3 in the effects of EGFR and ErbB2 inhibiting therapies and perhaps it plays a key role in acquired resistance to those drugs [86-89].

Data concerning the role of ErbB4 in cancer is contradictory. There is data that support that ErbB4 mediates differentiating signals in some cells [90].

Some studies indicate a role in tumorigenesis for ErbB4. Over-expression of ErbB4 together with other EGFR-family members and ligand induces the ability for tumour formation in mouse fibroblast [91], and inhibits apoptosis in rat pheochromocytoma cells [92]. ErbB4 is over-expressed in thyroid cancer and has been found to be associated with increased proliferation in lung cancer [93] and decreased survival in childhood medulloblastoma when co-expressed with ErbB2 [94]. Intriguingly, it has recently been described that 19% of examined melanomas express tyrosine kinase activating mutations in ErbB4 [95]. The cancer where ErbB4 is most studied is breast cancer. ErbB4 signalling is believed to have an important role in differentiation of normal breast epithelium [96]. Although a majority of studies indicate an association between ErbB4 expressions and good prognosis and other good prognostic markers (such as estrogen receptor expression), some studies show no prognostic value or even an association with poor prognosis [97]. The isoforms of ErbB4 expressed (see above) [10] may also be important for the biological effects and prognostic value of ErbB4 in breast cancer. Recently much interest has been given to the effects of cleaved ErbB4 fragments that are translocated to the nucleus and perinuclear space but data are somewhat contradictory and there is some controversy regarding what method should be used to evaluate expression of these fragments. Perinuclear expression of cleaved ErbB4 has been associated with poor prognosis [98] as well as with differentiation and good prognosis [99].

## **Renal cell carcinoma**

Renal cell carcinoma (RCC) is a heterogeneous group of tumours originating from the kidney cortex that constitutes around 3% of all adult malignancy in Sweden. Around 900 new cases are diagnosed each year in Sweden and about 600 deaths are caused by RCC each year [100]. Men are affected more than women, with a ratio of about 3:2. Most cases occur late in the patient's life, in the sixth or seventh decade of life [100]. There are several types of RCC that are characterized by their histopathological appearance.

### **RCC types**

Clear cell (conventional) RCC (ccRCC), the most common of the RCCs, accounts for about 75% of RCC cases [101-103]. The histopathological appearance of ccRCC is a tumour comprised primarily of cells with clear cytoplasm in routine sections although foci of cells with eosinophilic cytoplasm can be seen. There is a characteristic branching vasculature and commonly solid and cystic architectural patterns [104]. Papillary RCC (pRCC), as the name suggests, is characterized by papillary structures. The

cells of these structures can be of different size and have variable cytoplasmic staining [104]. Chromophobe RCC (chRCC) has a morphology of pale or eosinophilic cytoplasm containing many microvesicles. Collecting duct carcinoma, comprising <1% of RCC, has a variable appearance, but a morphology of irregular channels lined with highly atypical epithelium is the most accepted description [104]. About 4-5% of RCC samples do not fit into any of the categories described above and are referred to as unclassified RCC [104].

## **Aetiology and genetic alterations in RCC**

Aetiological studies have shown associations between RCC and tobacco smoking and obesity [105-106]. Hypertension seems to be associated with RCC: this might be due to anti-hypertensive medication but more likely it is due to the hypertension itself [107-108]. The hereditary syndromes associated with the RCC types are interesting examples, but they comprise only 2-5% of all RCC and the vast majority of cases are sporadic [109].

In ccRCC, loss of genetic material of chromosome 3p is the most common gross genetic change. The vast majority of both hereditary and sporadic cases of ccRCC show deletion or inactivation of the *von Hippel-Lindau* (VHL) gene located on chromosome 3p22, either by mutation or hypermethylation [110-112]. The product of the VHL gene is a protein that under normal conditions (normoxia) binds to and induces the degradation of the transcription factor Hypoxia inducible factor (HIF). Defects in VHL protein leads to enhanced levels of HIF which elicits a response that is normally induced by hypoxia, with transcription of many genes, particularly many involved in angiogenesis [113].

In pRCC tumours, trisomies of chromosome 3q, 7, 12, 16, 17, and 20 and loss of the Y chromosome are common [104]. These changes in a RCC tumour suggest pRCC even in cases where the histological appearance is not typical. There are histopathologically different sub-types of pRCC [102]. One subgroup, sometimes called type 1 pRCC, is connected to hereditary papillary renal carcinoma (HPRC), which is caused by a mutation in the MET-receptor. As discussed above, MET is regulated by LRIG1. Similarly, many sporadic tumours with similar appearance display either mutations in *MET* or duplication of the gene by trisomies of chromosome 7. Another subgroup, called type 2 pRCC, is seen in both sporadic cases and in patients with hereditary leiomyomatosis RCC (HLRCC). The latter is a hereditary cancer syndrome caused by a mutation of *fumarate hydratase* (FH) that encodes a protein involved in the Krebs cycle [103].

Tumours classified as chRCC is thought to originate from type B intercalated cells of renal collecting ducts [102]. Chromosomal changes in chRCC typically include monosomy of multiple chromosomes (1, 2, 6, 10, 13, 17, and 21) [104]. The hereditary syndrome *Birth-Hogg-Dubé* (BHD) is connected to chRCC. The BDG gene has also been found mutated in sporadic cases of chRCC [103]. The function of folliculin, the protein encoded by the *BHD* gene is unknown.

## **Symptoms and diagnosis of RCC**

RCC typically presents with one or more of a classical triad of symptoms: hematuria, flank pain, and abdominal mass. The full triad of symptoms are now uncommon and found in less than 10% of all cases. Nonspecific symptoms such as weight loss, fever, malais, hypercalcemia, anemia or, erythrocytosis are common. About 50% of the RCC patients in developed countries are diagnosed incidentally upon unrelated radiological examination [114-115].

## **Treatment of RCC**

Although the RCC types differ both in appearance and genetically they have until recently often not been separated in most clinical studies. Importantly, the treatment has not differed between the types of RCC. The cornerstone of RCC treatment is surgery, open radical nephrectomy [116], but even in localised tumours surgical treatment is curative only in 50-60% of cases [117]. RCCs have shown to respond poorly to conventional cytostatic drugs and radiation [118]. Immunomodulating therapies with interferons and interleukins have long been the most common treatment of metastasised RCC despite modest effects [119-121]. Recently, new treatment modalities such as multi kinase inhibitors (e.g. sorafenib and sunitinib) and mTOR inhibitors (e.g. temsirolimus) and anti-angiogenic treatment (e.g. bevacizumab) have shown clinical benefits and improvement in overall survival in patients with metastatic RCC [122]. These treatments have primarily been tested in patients with ccRCC and knowledge of their efficacy in other RCC types is limited [123-126]. Not all patients benefit from these new drugs that are expensive and often have substantial side effects. Therefore, it is important to find molecular markers that predict which patients will benefit from these treatments.

## **Prostate cancer**

Prostate cancer is the most common cancer among males in the Western world. In Sweden, almost 10,000 cases are diagnosed every year [100]. The

incidence has been rising during the last 30 years with a marked rise in the early 1990s [127]. Prostate cancer is the leading cause of cancer death in Sweden with about 2,500 deaths per year.

The clinical outcome of prostate cancer is highly variable: some patients have a rapidly progressing disease, but the majority of patients have slow progression and long expected survival.

### **Risk factors and aetiology of prostate cancer**

Prostate cancer is mainly a disease of the elderly and the mean age of diagnosis is 75 years [128]. The incidence of histological prostate cancer in autopsy materials increases with age, from 5-14% in men in their 50s to 40-80% in men in their 90s [129]. Prevalence of microscopic lesions at autopsy is similar around the world but the incidence of clinical cancer varies greatly. For instance clinical prostate cancer has been uncommon in Japanese men but second generation Japanese immigrants to the USA have an incidence approaching Americans of European decent [130-131]. This has led to the conclusion that environmental factors play a significant role in the development of clinical prostate cancer. Diet in particular is a likely factor. Increased risks from high intake of animal fat and protective effects of phyto-estrogens from certain vegetables, particularly soy, have been suggested. Ethnic background is also important with some groups having higher incidence, such as Americans of African decent. Some families have increased risk of prostate cancer, about 10% of all prostate cancer is defined as being hereditary [132]. Only in very few families with high prostate cancer incidence can the increased risk be linked to a specific gene [133]. The difficulty identifying specific genes or gene loci involved in hereditary prostate cancer suggests involvement of many low penetrant genes.

Prostate cancer is usually a multifocal disease; on average three different tumours are found in the prostate at the time of diagnosis [134]. This could be seen as an indication that there is a general dysfunction in the prostate of prostate cancer patients. The normal prostate is regulated by androgen and depend on androgen for development, growth and function [135]. The prostate epithelium is primarily not directly regulated by androgen but indirectly by surrounding androgen receptor (AR) positive stromal cells that produce paracrine factors, including EGF [136]. In the transformation to a malignant phenotype, AR signalling shifts from paracrine to an autocrine mode, where AR signalling in the tumour cells directly activates growth factor production [137]. This direct stimulation is often due to gene fusions where androgen regulatory elements regulate oncogenic transcription factors, causing overexpression of oncogenes [138]. Fusion of the androgen

regulated gene *TMPRSS2* and members of the ETS gene family of transcription factors is seen in around 50% of prostate cancers [139].

## **Symptoms and diagnosis of prostate cancer**

Diagnosis of prostate cancer is made either by identifying symptom, primarily lower urinary tract obstruction or a palpable mass detected by digital rectal examination, or by finding an elevated levels of Prostate-specific antigen (PSA) in a blood test. Bone pain from metastasis is a common first symptom in advanced cases.

PSA is a protein that is produced specifically by the prostate and can be detected in the blood. Although it is a marker of prostate disease, it can indicate non-cancer issues. Moderate increase in PSA can also be caused by prostatitis or benign prostate hyperplasia (BPH). A PSA level of >10 ng/mL corresponds to a 66% chance of a positive finding of prostate cancer in a subsequent biopsy. PSA in the range of 4-10 ng/mL correspond to a positive biopsy rate of 22%. High levels of PSA >100 ng/mL is almost always associated with metastatic spread of prostate cancer. By introducing PSA testing, prostate cancer can be detected earlier and in more patients than before. Consequently, this test has resulted in an increased incidence of diagnosed prostate cancer in Western countries. A problem with PSA testing, however, is that many of the patients who are detected would never develop clinical symptoms. PSA screening, although very common particularly in the USA, is still controversial. In two large studies of the possible benefits of PSA-screening, one showed no detectable survival benefit from PSA-screening [140] and the other showed a small survival benefit, but to save one life, 1,410 men would have to be screened and 48 additional patients treated [141]. It should be noted that in the first study, a large portion of control patients was tested for PSA outside the study. A recent case-control study showed that PSA testing did not meet the formal requirements of a screening test [142].

On suspicion of prostate cancer, a trans-rectal biopsy is usually performed. Biopsy can confirm existence of cancer, but it can also be used to evaluate the grade of the disease and to a lesser degree also the extent of the disease. There are currently no non-invasive imaging technique that accurately detect prostate cancer, so biopsies have to be taken from areas in the prostate where cancer occurrence is most common, primarily the peripheral zone. This and the fact that a standard biopsy series samples only a small portion (1/1000) of the prostate can produce false negative results and under-grading. Grading of the of prostate cancer is done according to the Gleason score system [143], which is still one of strongest predictors of outcome

[144]. The differentiation of the prostate cancer cells are scored on a scale from 1 to 5, where 5 is the lowest degree of differentiation. The scores of the most common area and the second most common area are then added creating a scale between 2 and 10.

This system is highly predictive of outcome for the patient, especially in patients with low ( $\leq 5$ ) and high (8-10) Gleason score. The majority of patients (>70%) has a Gleason score of 6 or 7; however, and this group has highly variable outcome, which shows the need for additional prognostic markers. The only prognostic marker that is stronger than Gleason score is the stage of the disease. Stage is determined by how far the cancer has spread. Stage is classified according to the TNM-classification. Staging is performed by digital rectal examination, radio-nuclide bone scan, computer tomography (CT), magnetic resonance imaging (MRI), and in some cases through surgical removal of lymph nodes.

The most important distinction made when treating prostate cancer is whether the cancer is localised or is metastasised. Localised disease can be either truly localised (no spread outside the fibrous capsule) or locally advanced (spread outside the fibrous capsule but without metastasis).

Treatment with curative intention is possible only in patients with localised disease. In locally advanced tumours, curative treatment is often preceded by staging operation and/or neoadjuvant hormone treatment. Curative treatment of prostate cancer is performed either by surgical prostatectomy or radiation therapy [145]. For low risk patients, “watchful waiting”, also called active surveillance is also an option [146]. The radiation therapy can be either external beam radiotherapy, brachy therapy or a combination of the two. In the case of metastasised disease the cornerstone of the palliative treatment is castration, either surgical or chemical [147]. Unfortunately prostate cancer becomes with time castration resistant and progresses even with very low levels of hormones [148-149]. Further palliative treatment can then be given systemically or locally. Local treatment is usually external radiation therapy against bone metastases. Systemic therapies include radioactive isotope therapy or cytostatic treatment. New treatments such as anti-angiogenic treatments and growth receptor targeted therapies are currently being tested in clinical trials.

### **The EGFR-family in prostate cancer**

EGFR is the most thoroughly studied EGFR-family member in prostate cancer. High EGFR expression as determined by IHC is associated with high tumour grades, high tumour stages and increased risk for PSA recurrence

after surgery [150-152]. EGFR expression is more common in tumours that are resistant to castration [153-154]. Anti-EGFR treatment has shown some promising results in animal models [155], but so far clinical studies have not found any significant benefits [156-158]. Both EGFR and its ligands are up-regulated by androgen stimulation [159-160].

ErbB2 expression in primary prostate cancer has been reported at divergent rates [153-154, 161], but in most studies it is expressed in a minority of cases. It is reported that ErbB2 expression is more common in castration resistant than in non castration resistant prostate cancer [154]. Expression of ErbB2 has been linked to worse survival in patients with castration resistant prostate cancer [153-154], but one study has shown correlation to better survival [162]. One study showed that high expression correlated with increased relapse rate and worse survival in patients who received radiotherapy [161]. Treatment with the ErbB2 antibody trastuzumab showed no significant benefit for patients with castration resistant prostate cancer [163].

ErbB3 expression has been shown to be increased in prostate cancer compared to normal tissue in some studies [84], but no change has been reported in other studies [164]. A difference in expression between castration sensitive and castration resistant prostate cancer is also controversial [153, 162, 164].

Studies show that a minority of prostate cancer samples express ErbB4 [153, 162]. There are studies that the most common prostate cancer cell lines lack expression of ErbB4 while expressing the other EGFR-family members [165], but the expression of ErbB4 in these cell lines has also been reported [166]. Constitutively active ErbB4 inhibits colony formation in prostate cancer cell lines but this seems only to be true for the Cyt-1 isoform [167-168]. One study showed a borderline significant relation between ErbB4 expression and better survival in castration resistant prostate cancer [153]. The importance of ErbB3 and ErbB4 in prostate cancer is still unclear as studies in the field are contradictory.

## **Aims**

As the EGFR-family members have important biological roles and prognostic value in several other cancers we wanted to investigate their role in renal cell carcinoma. We also wanted to investigate the potential role of the newly discovered EGFR-family inhibitor LRIG1 in RCC.

Expression and prognostic value of EGFR had previously been investigated in RCC but ErbB2, ErbB3, ErbB4 and LRIG1 had never been thoroughly investigated.

Our first studies revealed alterations of expression of LRIG1 (**Paper I**), ErbB2 and ErbB4 (**Paper II**) in RCC compared to kidney cortex. These studies were performed in a rather small material; to further investigate these alterations we analysed expression of the EGFR-family and LRIG1 in a larger material. In this study we wanted to investigate possible relationships of the gene expressions with clinical factors, such as stage, grade, and survival, and investigate differences in expression between the different types of RCC.

In prostate cancer, the expression of EGFR-family members have been investigated previously so here we concentrated on expression of LRIG1 and relationship of that expression with clinical factors.

### **Specific aims:**

To investigate the expression and clinical significance of EGFR-family members and LRIG1 in RCC.

To investigate differences in expression of the EGFR-family members and LRIG1 between RCC types.

To investigate the expression and clinical significance of LRIG1 in prostate cancer.

# Materials and methods

## Patients

### Limited RCC patient material (Paper I and II)

Specimens from RCC were obtained from 31 patients (**Table II**) who underwent nephrectomy at the Department of Urology, Umeå University Hospital between 1986 and 1998. Of these tumours, 18 were ccRCC, ten pRCC, and three chRCC. In eight of the patients (five with ccRCC and three with pRCC), we analysed matched specimens of corresponding kidney cortex. RNA was prepared from tissue samples by mechanical disruption in TRIzol reagent (Gibco-BRL, Rockville, MD), followed by chloroform extraction and ethanol precipitation according to the manufacturer's instructions. These samples were the first set from which RNA was extracted out of a larger material (see below). From the eight patients with matched samples of tumour and kidney cortex, sections of the formalin-fixed paraffin-embedded tissue were used for immunohistochemistry (IHC).

**Table II**                      **Limited RCC material**

		N
Total number of patients		31
Sex	male/female	17/14
Age in years	median (range)	64 (36-85)
Tumour diameter in mm	median (range)	80 (30-250)
Tumour stage (TNM)	I	7
	II	5
	III	10
	IV	9
Tumour grade(Skinner)	1	0
	2	5
	3	21
	4	5
Metastasis at neprectomy		9
Dead from disease		16
Follow up time in months	median (range)	44 (1-139)

### Extended RCC patient material (Paper III)

Specimens from RCC were obtained from 104 patients who underwent nephrectomy at the Department of Urology, Umeå University Hospital, between the years 1986 and 1999 (Table II). All patients from the limited RCC material were also included in this study but all of those samples were

re-analysed. The tumours included 81 ccRCC, 15 pRCC, seven chRCC, and one unclassified carcinoma. In addition, specimens of histological verified non-neoplastic kidney cortex were obtained from 27 of the nephrectomised kidneys. RNA was prepared from tissue samples in the same manner as in **Paper I** and **Paper II** (see above).

**Table III**                      **Extended RCC material**

		N
Number of patients	Total	104
Sex	Male/female	56/48
Age in years	Median (Range)	65 (25-85)
Tumour diameter in mm	Median (Range)	80 (30-250)
Tumour stage (TNM)	I	26
	II	15
	III	30
	IV	33
Tumour grade(Skinner)	1	4
	2	14
	3	60
	4	26
Metastasis at nephrectomy		35
Dead from disease		57
Follow up time in months	median (range)	29 (0-139)

### **Swedish prostate cancer patient material (Paper IV)**

This material consisted of samples and clinical data from patients diagnosed with prostate cancer at transurethral resection of the prostate (TUR-P) for micturition difficulties. The samples were formalin-fixed and paraffin-embedded and graded according to the Gleason system [169]. The samples were collected at the Central Hospital, Västerås, Sweden, between 1975 and 1991, i.e., before serum prostate specific antigen (PSA) was used in Sweden as a diagnostic tool. Examination for metastases was undertaken using a bone scan shortly after the transurethral resection. The patients had not received any anti-cancer treatment before TUR-P. The study included 355 patients of which 256 were followed by watchful waiting (the standard treatment in Sweden at the time) and 99 patients received palliative treatment immediately after TUR-P. At onset of symptoms from metastatic disease the patients received palliative treatment with androgen ablation therapy, in some cases together with radiation therapy or oestrogen treatment according to treatment practices at that time. Data on Gleason score, TNM-stage, and follow-up time is presented in **Table III**. Cause of death was retrieved from medical records. Median survival was 5.2 years. As of August 2003, 29 patients (8.2%) were alive, 137 patients (38.6%) had died

from prostate cancer, and 184 patients (51.8%) had died from other causes. In five cases, cause of death was undeterminable or unknown and these cases were excluded from the prostate cancer-specific survival analysis. Tissue micro arrays were constructed as previously described [170]. The tissue micro arrays contained up to eight (median five) samples of tumour tissue (cores with a diameter of 0.6 mm) and up to four samples (median four) of non-malignant tissue from each patient [171].

**Table IV Swedish prostate cancer material**

		N
Total number of patients		355
Age in years	median (range)	74 (51-95)
T- stage	T1	172
	T2	92
	T3	72
	T4	11
	missing	6
N-stage	No	
	N1	
	NX	355
M-stage	Mo	232
	M1	41
	Mx	82
Gleason score (GS)	4	3
	5	58
	6	102
	7	68
	8	46
	9	59
	10	19
Treatment (of any kind)		99
Expectance (no treatment)		256
Deceased		326
Dead from disease		137
Follow up time in years	median (range)	5.2 (0-21.1)

### U.S. prostate cancer patient material (Paper IV)

This material consisted of cases included in a TMA acquired from the Cooperative Prostate Cancer Tissue Resource (CPCTR TMA1) [172]. This material consisted of prostate tissue of 299 American patients who had undergone prostatectomy at various centres in 1991 and 1992. Samples from six patients could not be evaluated for LRIG1 staining, leaving 293 patients. The TMA included two samples from each patient. Clinical data including

Gleason score, TNM-stage, and follow-up time for these patients is summarized in **Table V**. Information about prostate cancer specific survival was not available for these patients. For 183 patients, information on PSA recurrence was available; of these, 41 had PSA recurrence, 72 had no reported recurrence, and 70 had never been disease free after prostatectomy; i.e., surgery was not radical.

**Table V U.S. prostate cancer material**

		N
Total number of patients		293
Age in years	median (range)	66 (42-79)
T- stage	T1	1
	T2	183
	T3	114
	T4	1
	missing	
N-stage	No	263
	N1	9
	NX	21
M-stage	M0	112
	M1	0
	Mx	181
Gleason score (GS)	4	0
	5	14
	6	80
	7	166
	8	20
	9	13
	10	0
Treatment (Prostatectomy)		293
Deceased		69
Dead from disease		Unknown
Follow up time in years	median (range)	10.2 (0.17-14.2)

### Real time RT-PCR

Real time reverse transcription (RT)-polymerase chain reaction (PCR) is a technique that quantitatively assesses gene expression by measuring mRNA levels. A reverse transcriptase enzyme can convert mRNA to cDNA which is then amplified by PCR that produces a specific DNA-sequence determined by the specific primers. The initial amount of cDNA is quantifiable by various methods that release a measurable dye released with every DNA replication; this is usually done by a specific probe added to the reaction. The number of PCR cycles before the amount of dye becomes measurable is inversely proportional to the initial amount of cDNA (hence real time).

It is difficult to exactly correlate the amount of mRNA to the amount of cells or tissue that it was extracted from. Therefore, to compare different samples it is common to standardize the expression of the gene of interest to one or more genes that is believed to be relatively constant in the cells or tissues of interest, these genes are called reference genes. These reference genes are often housekeeping genes with functions common to most cells. In most cases in this thesis, 18S rRNA was used as reference gene. When analysing the isoforms of ErbB4 (**Paper II**),  $\beta$ -actin was used as the reference gene as that was the standard procedure in the lab in Turku, Finland, where this analysis was performed. When analysing LRIG1 expression in LNCaP cells (**Paper IV**), GAPDH was used as reference gene as the analysis was performed on cDNA from mRNA, thus precluding 18S rRNA as a reference gene. The RT-PCR in this thesis was performed according to methods previously developed in our group [51, 173].

## **Immunohistochemistry**

Immunohistochemistry (IHC) is used to detect and localize proteins in a tissue section by using specific binding of antibodies. A section of tissue is mounted on a glass and incubated with an antibody (primary antibody) to the protein of interest. Thereafter, a secondary antibody that is specific to the type of primary antibody is applied. This antibody is conjugated to a staining agent (in our studies, the enzyme peroxidase). After this, the section is stained by incubation with a substrate that is converted to an insoluble dye by the peroxidase activity. Staining now appears where the protein is localised. This method is relatively simple and can be performed on samples that have been preserved in paraffin for several years. To optimize the IHC, several washing steps are used and it may be necessary to prepare the sections with chemical or thermal treatment; unfortunately this preparation can increase the risk of unspecific binding of the antibodies. Therefore good negative controls are essential for reliable results.

Evaluation of IHC when performed manually can be subjective. To minimize the risk of this, all evaluations were done independently by two investigators. Results were then compared for reproducibility. In the case of the TMA evaluations (**Paper IV**), one of the evaluators was an experienced pathologist (A. Bergh).

## Results and Discussion

### EGFR is up-regulated in RCC compared to kidney cortex

In our first study (**Paper I**), six out of eight tumours showed increased EGFR mRNA expression compared to non-neoplastic cortex from the same kidney. Protein expression measured as immunoreactivity by IHC was greater in four tumour samples, equal in three, and lower in one tumour sample compared to non-neoplastic kidney cortex from the same patient. In our larger study (**Paper III**), EGFR mRNA expression was significantly higher in 104 RCC tumours compared to 27 samples of kidney cortex ( $P < 0.001$ ). EGFR expression was not significantly different between the RCC types ( $P = 0.718$ ). Individual analysis of RCC types showed a significant increased of EGFR expression in ccRCC ( $P < 0.001$ ) and pRCC ( $P = 0.016$ ). The increase in chRCC was not significant ( $P = 0.257$ ) although the mRNA expression levels in this RCC type were comparable to the levels in ccRCC and pRCC. The lack of statistical significance was likely due to the small number of chRCC samples analysed.

The interest for the expression and function of EGFR in RCC is not new. There have been several reports that have indicated an increase of EGFR protein or mRNA in RCC [174-176]. The role of over-expression of the EGFR ligand TGF- $\alpha$  in RCC is also well studied [177-178], especially in ccRCC, where loss of VHL function causes over-expression of TGF- $\alpha$  [179]. Our results regarding EGFR in RCC are, therefore, not novel but confirm previous reports that EGFR is over-expressed in RCC. To our knowledge our study (**Paper III**) is the first to show that the up-regulation is present in all of the most common RCC types.

Several studies have compared EGFR expression to clinical outcome of RCC patients [180-185]. Some studies have not found correlation with patient outcome [184-185]. Others have found correlation between EGFR expression and poor prognosis [180, 182-183]. One showed correlation between EGFR expression and good prognosis [181]. It should be noted that the studies that showed prognostic value of EGFR expression were all performed with IHC and several differentiated between different cellular localisations of the staining to find correlation. It is possible that mRNA expression is not as prognostic as protein expression, and especially localised protein expression. It is known that mRNA and protein expression do not always correlate [186-188]; for instance, it is possible that a high mRNA expression could indicate high protein turnover and not high protein expression. This does not seem to be the case in our RCC studies, however. The IR in the IHC of **Paper I** and

**Paper II** indicate that the mRNA and protein levels of the EGFR-family members and LRIG1 are related. Western-blot analysis on protein samples from limited number of RCC tumours from the patients in **Paper III** also indicated that mRNA and protein expression levels correlated (data not shown).

Thus, it seems likely that EGFR has an important biological role in RCC and ccRCC in particular but how big that role is yet to be determined. Single therapy with EGFR inhibitors has not shown impressive results in treatment of RCC [189]. Therapies with broader inhibition of several tyrosine kinases have shown better results [122]. It is likely that angiogenesis inhibition is important for the effect of these drugs but a contribution from inhibition of EGFR and its downstream targets cannot be ruled out.

### **ErbB2 is down-regulated in ccRCC**

Our results showed a down-regulation of ErbB2 in ccRCC. Down-regulation of ErbB2 has previously been suggested in some articles [174, 190], however, these studies were relatively small. Also some other small studies have shown conflicting results [176, 191].

In our second study (**Paper II**), all 31 RCC examined showed ErbB2 mRNA expression that was lower than the mean mRNA expression in non-neoplastic kidney cortex. In ccRCC, five out of five tumour samples showed down-regulation of ErbB2 mRNA expression compared to matched non-neoplastic kidney cortex by at least a factor of four. This down-regulation of ErbB2 was confirmed in the later study (**Paper III**), but there it was found to be restricted to ccRCC ( $p = 0.001$ ) and the difference between RCC types was significant ( $p < 0.001$ ). No correlation between ErbB2 mRNA expression and patient outcome was found.

Clinical trials with lapatinib, a dual EGFR/ErbB2 tyrosine kinase inhibitor have been performed without showing any significant benefit [192]. This study was designed before the publication of our article (**Paper II**) and shows the importance of this kind of basic descriptive science, as patients could have avoided inclusion in a trial with a drug with little possibility of improving on the results of pure EGFR inhibitors.

### **ErbB3 expression is low in RCC and kidney cortex**

Until now the expression of ErbB3 has not been systematically investigated in RCC before. In a report testing an antibody against ErbB3, no detectable ErbB3 protein was shown in six RCC samples [193].

Our studies showed that expression of *ErbB3* was low in both non-neoplastic kidney cortex and RCC samples of different types. In material from patients with matched samples, both up- and down-regulation in tumour compared to the non-neoplastic kidney were seen (**Paper II**). In the second study (**Paper III**), no significant difference in *ErbB3* mRNA expression was found between tumour and kidney cortex. The RCC types did not differ significantly in their expression of *ErBB3* mRNA.

In light of this, it was surprising that there was a significant inverse correlation between *ErbB3* mRNA expression levels and tumour grade (Spearman correlation coefficient = -0.287,  $p = 0.009$ ) and tumour size (Spearman correlation coefficient = -0.244,  $p = 0.027$ ) in ccRCC. The finding that *ErbB3* mRNA had a negative correlation with tumour grade and stage despite the very low expression levels was interesting. Is it possible that ErbB3 expression somehow is detrimental to growth of ccRCC cells? There is not enough data at this time to make such a statement but one could speculate about possible mechanisms. When ErbB3 dimerises with ErbB2, it induces a potent downstream signal transduction cascade (this signal is commonly very oncogenic, through induction of mitosis). As ErbB2 also is down-regulated in ccRCC it could be that this signalling is somehow detrimental to ccRCC cells. There are several known examples of cell lines where activation of members of the EGFR-family under specific conditions lead to apoptosis [194]. But if there is such a mechanism, why are *ErbB2* levels down-regulated but not correlated to stage or grade and the opposite is true for *ErbB3*? Due to this question and small difference in ErbB3 expression, we did not want to over-interpret these findings and we have not stressed them in the article (**Paper III**). To elucidate the role of ErbB2/ErbB3 signalling in RCC one could for example stimulate RCC cell lines with neuregulin 1 and 2 or transduce RCC cells with ErbB2/ErbB3. Regrettably, such experiments were beyond the scope and time limit of the present thesis.

### **ErbB4 expression is down-regulated in RCC**

In our first study of ErbB4 expression (**Paper II**), it was readily apparent that ErbB4 expression was lower in RCC than in kidney cortex. *ErbB4* expression was very low in 17 of 18 ccRCC and in all ten pRCC. In 14 of the total 31 RCC tumours *ErbB4* mRNA was not detected at all. The chRCC also showed *ErbB4* down-regulation but less so than the other RCC types. To determine if the reduced *ErbB4* levels were isoform specific, RT-PCR for the different isoforms was performed. In both kidney cortex and in the tumours that expressed *ErbB4* the juxtamebrane variant JM-a predominated and JM-b expression was very low or absent. Of the cytoplasmic variants, the

expression of CYT-1 was higher than that of CYT-2, in both kidney cortex and in the tumours that expressed *ErbB4*. The ratio of the isoforms was thus not significantly altered in the few tumours where *ErbB4* could be detected.

In our second study of *ErbB4* expression (**Paper III**), *ErbB4* again was markedly lower in all RCC types than in kidney cortex ( $p < 0.001$ ). Although, chRCC again appeared to have slightly higher ErbB4 expression there was no significant difference of *ErbB4* expression between RCC types. No significant correlation between *ErbB4* mRNA expression, tumour stage or patient outcome was found. There was a non significant trend towards higher *ErbB4* expression in grade 1 tumours but this is highly uncertain since there were only four grade 1 tumours. The large and consistent down-regulation of ErbB4 suggests that it is important in RCC. The consistent and often almost complete down-regulation of *ErbB4* makes it difficult to detect correlations between *ErbB4* expression and clinical parameters and patient survival. In addition, if the silencing of the signalling ErbB4 mediates is very important in RCC, tumours that still express ErbB4 might have other mutations inhibiting this pathway.

Since a missing receptor is not a good therapeutic target it would be highly interesting to investigate what mechanisms are involved in making ErbB4 signalling detrimental for RCC. It is possible that down-stream mediators of this signalling could be potential targets for therapy. To investigate this possibility transducing RCC cell lines with ErbB4 and studying effects on cells and activation of downstream mediators would be highly interesting. As noted above constitutively active ErbB4 of the Cyt-1 isoform inhibits colony formation in prostate cancer cell lines [167-168]. Interestingly Cyt-1 was the more common of the two cytoplasmic variants of ErbB4 in kidney (**Paper II**).

### **LRIG1 is down-regulated in ccRCC**

In our first study (**Paper I**), LRIG1 expression appeared down-regulated in the majority of tumours and was decreased in six of eight tumours compared with kidney cortex tissue from the same patient. At the time of this study, the interaction between EGFR and LRIG1 was not established but suspected. Because of this we related the *LRIG1* and *EGFR* levels in tumour and kidney to each other. The ratio of *EGFR/LRIG1* was high, especially in the ccRCC samples. A non-significant trend towards lower *LRIG1* expression in tumours of grade 4 ( $P = 0.088$ ) was noticed. IHC showed that staining intensity for LRIG1 was less pronounced in tumours than in the kidney cortex tissue. **Paper I** was the first publication to describe down-regulation

of LRIG1 in cancer. In the larger RCC study (**Paper III**), *LRIG1* expression was found to be down-regulated only in ccRCC ( $p = 0.020$ ). There was no indication of decreased mRNA expression levels of *LRIG1* in pRCC or chRCC. The ratio of *EGFR/LRIG1* was greater in tumour compared to kidney cortex ( $p < 0.001$ ). The *EGFR/LRIG1* ratio was significantly higher in ccRCC compared to kidney cortex, pRCC and chRCC ( $p < 0.001$ ,  $= 0.002$ , and  $0.001$  respectively). No significant association between *LRIG1* expression levels and tumour stage or grade or patient survival was found.

To further investigate the function of LRIG1 in RCC, we tried to transduce RCC cells with viral vectors expressing LRIG1. Despite that these vectors induced LRIG1 expression in COS-7 cells, none of the transduced RCC cell lines expressed detectable levels of LRIG1 despite incorporation of vector and expression of co-expressed selection markers (data not shown). This could indicate, but does not prove, that there is a negative selection against LRIG1 expression in RCC cells. This line of investigation would have been interesting to pursue but as of yet we have not had time to do so.

The down-regulation of LRIG1 in ccRCC is likely to be important for tumour biology. In ccRCC, EGFR-signalling is increased both by up-regulation of the receptor and autocrine stimulation with TGF- $\alpha$  (a result of VHL mutation, see above, EGFR in RCC section) and down-regulation of LRIG1 will further increase intensity and duration of the EGFR-signalling and promote tumour growth. Could LRIG1 then be a target for therapy in RCC? To increase LRIG1 expression in tumour cells would likely require gene-therapy, a method not yet perfected. As mentioned earlier, a soluble part of LRIG1s ectodomain [57] can inhibit EGFR and EFGR mediated cell growth, but such a molecule is still large and hard to deliver. It is not likely that LRIG1 therapy would be more effective in RCC than other therapies targeting EGFR alone in RCC (see Introduction). Thus interest in LRIG1 expression in RCC is more biological than therapeutic.

### **LRIG1 in prostate cancer**

Previous gene expression results that were retrieved from the Oncomine database ([www.oncomine.org](http://www.oncomine.org)) [195-196] indicated that LRIG1 mRNA was up-regulated in prostate cancer tissue compared to non-neoplastic prostate tissue [197-198]. In our IHC study of prostate cancer (**Paper IV**), there was a stronger staining in prostate tumours compared with non-neoplastic epithelium in the U.S. material ( $p = 0.018$ ). There was a similar trend in the Swedish TMA material, but it was not statistically significant ( $p = 0.074$ ).

Thus, both LRIG1 mRNA and protein seems to be up-regulated in prostate cancer compared to non-neoplastic prostate.

In the Swedish TMA material, the mean LRIG1 IR in neoplastic cells correlated with Gleason score (Spearman correlation = 0.212,  $p < 0.001$ ) and local tumour stage (Spearman correlation = 0.199,  $p < 0.001$ ). The correlation of LRIG1 IR with Gleason score was not linear as the highest mean LRIG1 scores were seen in tumours with a Gleason score of 8. LRIG1 mean IR was significantly higher in tumours with a Gleason score of 8 than in those with Gleason scores 4, 5, 7 and 9 ( $p = 0.033$ ,  $< 0.001$ ,  $< 0.001$  and  $0.028$  respectively). In addition, the LRIG1 IR in tumour correlated with, tumour cell proliferation, vascular density, and phosphorylated EGFR.

In the U.S. TMA material, LRIG1 IR did not correlate significantly with Gleason score or tumour stage. The staining of the U.S. material was stronger than that of the Swedish material. The reason for this is not clear. It is of course possible that this difference in IR was due to differences in protein expression between the Swedish and U.S materials. However, it is also possible that small difference in the staining procedures or differences in tissue fixation might have influenced the staining. Thus as more samples were classified with strong IR a statistical difference in the U.S series could be harder to detect.

### **LRIG1 expression is induced by androgen stimulation in prostate cancer cells**

Since LRIG1 expression in the Swedish material correlated with Gleason score, we asked this question: is LRIG1 regulated by androgen signalling? There are some previous microarray gene expression data that indicate that LRIG1 is regulated by androgen [199]. To confirm this, LNCaP prostate cancer cells were stimulated with the synthetic androgen R1881. After twelve hours, R1881 treated cells expressed 155% of *LRIG1* mRNA compared to untreated control cells ( $p < 0.05$ ). This shows that LRIG1 expression is indeed induced by androgen stimulation.

### **LRIG1 expression reduces proliferation of prostate cancer cells**

Effects of LRIG1 induction were studied in prostate cancer cells. PC3 cells were used for this experiment since they express very low levels of endogenous LRIG1 [61]. PC3 cells that were transduced with control vector expressed barely detectable levels of LRIG1 mRNA, whereas cells transduced with LRIG1 vector expressed higher *LRIG1* levels. The expression level was

not super-physiological but in the lower range of un-manipulated LNCaP cells. The cells transduced with LRIG1 grew significantly slower than the control cells. After five days of culture the number of LRIG1 transduced cells was 21 % ( $p < 0.001$ ) lower than the number of the cells transduced with control vector. Thus, LRIG1 can inhibit proliferation of prostate cancer cells and may have a tumour suppressing effect in prostate cancer.

### **Relationships between LRIG1 and survival in prostate cancer**

In the Swedish TMA material, the prostate cancer-specific survival for the patients who had received no treatment before onset of metastasis was worse for patients with a mean tumour LRIG1 score of  $>1$  than for those with a mean LRIG1 score of  $\leq 1$  ( $p = 0.001$ ). The difference in median survival was 7.8 years (10.4 versus 18.2 years). High LRIG1 mean expression was an independent risk factor when compared with the known risk factors of Gleason score, T-score, and metastasis at diagnosis (RR= 2.25 CI 1.38-3.69,  $P = 0.001$ ). Further supporting the notion that LRIG1 expression had prognostic value independent of Gleason score was the finding that it was a marker for poor prognosis in patients with tumours with a Gleason score of 6 only ( $P = 0.009$ ). Overall survival also showed worse prognosis for patients with tumours with mean tumour LRIG1 IR score of  $>1$  ( $p = 0.001$ ).

In the U.S. material, overall survival was better for patients with tumours with mean tumour LRIG1 IR score of  $>1$ . Information about prostate cancer-specific survival was not available in the U.S. material. Kaplan-Meier analysis of PSA recurrence was performed but showed no significant correlation with LRIG1 expression. Overall survival is not an optimal endpoint in prostate cancer, since only a minority of the total deaths among the patients are likely to be due to the cancer. This is even more so in a material such as the U.S. patient series as these patients received treatment with curative intention. Nevertheless, patients with a mean tumour LRIG1 score of  $>1$  had a better overall survival than those with a mean tumour LRIG1 score of  $\leq 1$  ( $p = 0.029$ ), a finding that was not seen in the Swedish material where survival rates was better for patients with an LRIG1 score of  $>1$  (see above).

These opposing findings pose some questions. In the Swedish material similar correlations were noted for the prostate cancer-specific and overall survival with regard to their correlation with LRIG1, strengthening the findings. One explanation for the finding that LRIG1 expression in the Swedish material correlated with factors indicating poor prognosis (such as stage, grade and cell proliferation) and was an independent prognostic

marker for poor outcome could be that LRIG1 expression in prostate cancer reflects androgen receptor activation and/or EGFR signalling. Thus, under certain circumstances - e.g., as seen in the Swedish material the tumour promoting effects of androgen receptor and/or EGFR activation may outweigh the tumour suppressing effect of LRIG1. The U.S. patient series was treated with prostatectomy and thus, having smaller tumour loads after surgery and was likely, although this is purely speculation, subject to earlier and more aggressive hormone treatment and possibly neo-adjuvant hormone treatment before surgery. Here, instead, the tumour suppressing effect of LRIG1 may have outweighed the tumour promoting effects of its inducers, i.e., androgen receptor and/or EGFR signalling.

### **LRIG1 and the liar paradox**

In **Paper IV**, we propose that the role for LRIG1 in PC, where a known tumour stimulant such as AR activation also increases the expression of a potential tumour suppressor, can be viewed as a variant of the liar paradox [200]. Liar paradoxes are types of self-referring statements where the connected propositions may both be true although inconsistent with each other. In biological signalling networks, a simple form of such a paradox is represented by a chain of events where (statement 1) A induces B, and (statement 2) B inhibits A. In such a case, the level of A cannot be decided by analysing B alone. High levels of B could both imply high levels of A, as a result of statement 1 or low levels of A, as a result of statement 2. One solution to this type of paradox in biological systems is regarding the truth as time dependent, making results unpredictable before analysis and highly dependent on conditions [200]. LRIG1's ability to inhibit the proliferation of prostate cancer cells is in line with the prognostic effect of LRIG1 shown in the U.S. material. LRIG1's induction by AR and/or EGFR is in line with the prognostic effect in the Swedish material. In this situation, LRIG1 would become a secondary marker for AR/EGFR activation in more aggressive tumours. Which of these roles, the up-regulation of LRIG1 by AR stimulation or the growth inhibiting ability of LRIG1, exert the greatest effects may thus depend on the clinical and biological circumstances of patients.

To establish whether our findings regarding the prognostic values of LRIG1 are clinically relevant, it will have to be validated in other clinical materials. To become a clinically useful prognostic marker the factors responsible for divergent correlation in our two study materials will need to be explained. To this end, similar studies in one or more well defined clinical materials will have to be performed. Since LRIG1 is induced by androgen, it would be interesting to investigate the expression and prognostic value of LRIG1 in castration resistant prostate cancer. Markers that are prognostic for worse

outcome in untreated patients and predictive for better outcome after treatment are known, but to be clinically useful a clear mechanism for the difference has to be provided.

### **Concluding words on LRIG1 in prostate cancer**

To further investigate the role of LRIG1 in prostate cancer, we are conducting an experiment with transgenic mice. We are crossbreeding animals with defective LRIG1 genes with transgenic mice who spontaneously develop prostate cancer, TRAMP-mice [201]. The aim of this study is to determine if LRIG1 plays a functional role in the development of prostate cancer in these mice. Results from this experiment were not available at the time this thesis went to press.

In summary, LRIG1 expression was increased in prostate cancer compared to non-neoplastic prostate. LRIG1 expression was induced by androgen stimulation of prostate cancer cells. LRIG1 expression inhibits the proliferation of prostate cancer cells. In a material of untreated patients LRIG1 expression was associated with poor survival; in another material with patients who had undergone prostatectomy, it was associated with better survival. Although, data from the material with untreated patients is stronger, it is possible that the predictive value of LRIG1 depends on clinical circumstances.

## Conclusions

The previously shown up-regulation of EGFR in RCC was shown to be present in all studied RCC types (ccRCC, pRCC, and chRCC).

ErbB2 gene expression was down-regulated in ccRCC.

ErbB3 expression was low and did not differ significantly between kidney cortex and RCC.

ErbB4 expression was markedly down-regulated in all studied RCC types.

LRIG1 gene expression was down-regulated in ccRCC.

The gene expressions of the EGFR-family members and LRIG1 were not prognostic markers in RCC.

LRIG1 expression was induced by androgen stimulation and inhibited proliferation of prostate cancer cells.

The predictive value of LRIG1 expression for patients treated with prostatectomy differed from the prognostic value in untreated prostate cancer patients. This difference poses questions concerning the biology of LRIG1 and its role in prostate cancer.

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*Just when every ray of hope was gone  
I should have known that you would come along  
I can't believe I ever doubted you  
My old friend the blues*

*Steve Earle- My old friend the blues*