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The Identification and Characterisation of LRIG Gene Family and Its Expression in Astrocytic Tumours

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To my wife and my son

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ABSTRACT

Gliomas are the most common primary brain tumours, and their capacity to invade surrounding normal brain prevents complete removal of the tumour. Malignant glioma has still a poor prognosis. However, with the rapid development of molecular biology our understanding about glioma has increased dramatically. Among known growth factors, EGF and its receptor are frequently amplified and over expressed in malignant glioma. Therefore, it is of interest to find approaches to hamper the activity of EGF/EGFR. The aim of this thesis was to identify and characterize human analogues to a recently identified gene in Drosophilia, kekkon-1, which negatively regulates the activity of Drosophilia EGF receptor.

In the first part, we set up a quantitative real-time RT-PCR assay, which showed good linearity, reproducibility and uniformity. We analyzed the expression of the most commonly used reference genes, and showed that 18S was the most reliable endogenous reference gene in this study.

In the second part, we cloned, identified, and sequenced a gene family, which we named leucine-rich repeats and immunoglobulin–like domains family (LRIG). The LRIG gene family had three vertebrate paralogs and one homolog in ascidiacea. The proteins encoded by human LRIG genes shared an overall structure with a signal peptide, 15 tandems leucine-rich repeats with N- and C-terminal flanking regions followed by 3 immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tail. Northern blot showed the mRNA sizes to be 5.5 kb for LRIG1, 4.8 kb for LRIG2, and 5.1 kb for LRIG3. LRIG1-3 mRNAs were detected in all human and mouse tissues analyzed, however, at various levels. FISH and BLAST analysis showed that LRIG1 was located at 3p14, LRIG2 at 1q13, and LRIG3 at 12q13. LRIG1 was shown to be down-regulated in several cancer cell lines and proposed to be a tumour suppressor gene.

In the third part, we analysed the expression of LRIG gene family in human astrocytic tumours. LRIG1-3 mRNAs were detected in all human glioma cell lines, in primary tumour tissues and control-matched normal brain tissues, at various levels. Subcellular localizations of LRIG1-GFP fusion proteins were visualized in nuclear, perinuclear, and cytoplasmic compartment. According to the predicted protein sequences, short peptides were synthesized and used to raise antibodies in rabbits. The antibodies were used for immunohistochemical analysis of LRIG1-3 in 404 human astrocytic tumours in a tissue micro array. The pattern of immunoreactivity of LRIG1-3 was heterogeneous with staining in nuclear, perinuclear and cytoplasmic compartment of positive tumour cells. Perinuclear staining of LRIG1-3 displayed a significant inverse correlation with WHO grade and especially positive LRIG3 perinuclear and cytoplasmic staining correlated with a low proliferation index. The LRIGs correlated with survival, and LRIG3 perinuclear staining was in addition to tumour grade an independent prognostic factor.

The results suggest that LRIGs may play a role in normal tissue, and may be of importance in the pathogenesis and prognosis of tumours. The exact function of LRIG1-3 remains to be established.

Keyword: astrocytoma, brain, EGFR, LRIG, leucine-rich repeat, real-time RT-PCR

LIST OF PAPERS

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

I. **Guo D**, Henriksson R, Hedman H. (2002) The iCycler iQ detection system for evaluating reference gene expression in normal human tissue**.** ***Bio-Rad tech note*** : 2804

II. Nilsson J, Vallbo C, **Guo D**, Golovleva I, Hallberg B, Henriksson R, Hedman H. (2001) Cloning, characterization, and expression of human LIG1. ***Biochem Biophys Res Commun***, 284(5): 1155-1161.

III. Hedman H, Nilsson J, **Guo D**, Henriksson R. (2002) Is LRIG1 a tumour suppressor gene at chromosome 3p14.3? ***Acta Oncol*****,** 41(4): 352-4.

IV. Holmlund C, Nilsson J, **Guo D**, Starefeldt A, Golovleva I, Henriksson R, Hedman H. (2004) Characterization and tissue-specific expression of human LRIG2. ***Gene***, 332: 35-43.

V. **Guo D**, Holmlund C, Henriksson R, Hedman H. (2004) The LRIG gene family has three vertebrate paralogs widely expressed in human and mouse tissues and a homolog in ascidiacea. ***Genomics***, 84(1): 157-165.

VI. **Guo D**, Hedman H, Nilsson J, Haapasalo H, Raheem O, Bergenheim T, Henriksson R. The expression of leucine-rich repeats and immunoglobulin-like domains family (LRIG1-3) in human astrocytic tumours. Manuscript

ABBREVIATIONS

ARF	Alternate open reading frame
CDK4	Cyclin-dependent kinase 4
CNS:	Central nervous system
Ct	Threshold cycle
EGFR	Epidermal growth factor receptor
GAC1	Glioblastoma amplification on chromosome 1
GFAP	Glial fibrillary acidic protein
GBM	Glioblastoma multiforme
LGI1	Leucine-rich repeat glioma-inactivated gene 1
LRR	Leucine-rich repeat
LRIG	Leucine-rich repeats and immunoglobulin-like domains
LOH	Loss of heterozygosity
MDM2	Murine double minute 2
MMAC1	Mutated in multiple advanced cancers
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PIP3	Phosphatidylinositol-3,4,5 triphosphate
PKB	Protein kinase B
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma susceptibility gene
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
TP53	Tumour protein 53
TSG	Tumour suppressor gene
TMA	Tissue micro array

INTRODUCTION

Glioma

Gliomas are the most common primary brain tumours, accounting for more than 40% of all central nervous system (CNS) neoplasms. Gliomas consist of a wide range of different types of glial neoplasms that vary in their location within the CNS, age and gender distribution, growth potential, extent of invasiveness, morphological features, tendency for progression and clinical course (68). The capacity of malignant glioma to invade surrounding normal brain prevents complete removal during surgery. The diagnosis of malignant glioma will most often imply an unexpected disaster for the patient and his/her relatives. Therefore, it is of utmost importance to look for new approaches to treat this group of tumours.

Aetiology

X ray-irradiation: The occurrence of brain tumours after high dose exposure to ionizing radiation is well established. Children who received CNS radiation during treatment for leukaemia have developed excess of glioma (109; 140). The association with low dose exposure is controversial. In one study, prenatal exposure from diagnostic radiography has been correlated to an excess of paediatric brain tumours (13). **Inheritance:** Some gliomas have a relatively clear genetic character, particularly those that occur in association with neurofibromatosis 1 and 2, tuberous sclerosis, Li-Fraumeni syndrome and Turcot syndrome type I. The familial occurrence of gliomas has also been investigated in several epidemiologic studies, showing that familial aggregation of gliomas usually occurs in the absence of these known tumour syndromes (89; 90; 105; 127). **Chemical carcinogens:** N-Nitroso compounds (NOCs) are suspected of playing a role in brain tumour development based on evidence from animal studies. NOCs and their precursors are ubiquitous in the environment of our modern industrialized society (59), and they are also among the most potent experimental carcinogens. **Electric and magnetic fields:** Epidemiologic studies showing an increased brain tumour risk associated with electricity-related occupations have been reported, and especially with

regard to astrocytomas (13). More recently, several researches have focused on the use of cellular phones. It seems that ipsilateral use of an analogue cellular phone yielded a significantly increased risk for malignant brain tumours (51-53). However, this issue is still controversial, and further studies are needed.

Several studies concerning the relation between glioma incidence and head trauma, alcohol, tobacco, ABO blood type, and infectious agents have been made, but without any conclusive results so far.

Classification, grading system and its limitation

The most widely used current classification of human glioma is that of the World Health Organization, revised in 2000 (68)**.** This classification is based on characterization of the presumed cell origin of the tumour and the histopathological grade of aggressiveness. It divides diffuse gliomas into astrocytic tumours, oligodendrogliomas, and oligoastrocytomas. Significant indicators of anaplasia in gliomas include nuclear atypia, mitotic activity, cellularity, vascular proliferation, and necrosis. Based on these indicators, the grading system divides the gliomas into grade I, II, III, and IV (Table 1).

Table 1 Comparison of the World Health Organization and St Anne/Mayo grading system of astrocytomas

WHO grade	**WHO designation**	**St Anne/Mayo**	
		Designation	Histological Criteria
I	Pilocytic astrocytoma		
II	Diffuse astrocytoma	Astrocytoma grade 2	One criterion, usually nuclear atypia
III	Anaplastic astrocytoma	Astrocytoma grade 3	Two criteria, usually nuclear atypia and mitotic activity
IV	Glioblastoma multiforme (GBM)	Astrocytoma grade 4	Three criteria: nuclear atypia, mitoses, endothelia proliferation and/or necrosis

In general, tumour grading is based on the area showing the highest degree of anaplasia with the assumption that this tumour cell population determines the course of disease. This system is a practical and effective approach to brain tumour analysis. In the vast majority of cases, it provides

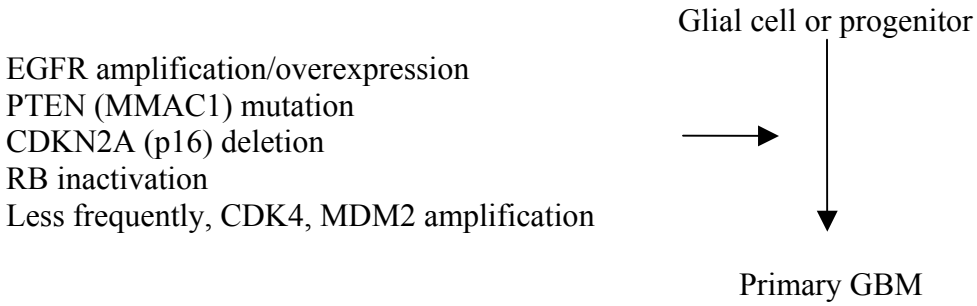
a mean for grouping tumours into specific and relevant categories. However, there are some limitations to consider when using the WHO classification and grading system.

The WHO classification is based on visual criteria alone. It is, therefore, a somewhat subjective classification system, which allows considerable interobserver variation in the diagnosis (26; 41). It is not uncommon that different neuropathologists will make different diagnosis on the same tumour sample. The classification defines tumours according to the cell type, which the tumour cells mostly resemble histologically. The cell of origin of the majority of brain tumours is still unknown, as no pre-malignant states have been recognized. In some tumours, cells may be so atypical that it is difficult to compare them with any normal cell type. The advances in neuroradiology and parallel improvements in stereotactic and surgical techniques now permit the biopsy of most neoplastic and non-neoplastic lesions in CNS. The neuropathologists, may thus, be expected to make a diagnosis on the basis of very small and fragmented biopsies, and it is of importance in this context to emphasize the heterogeneity of brain tumour pathology. Further, cytotoxic or radiation therapy before histological diagnosis makes classification and malignancy grading extremely difficult or impossible. In a significant number of cases, therefore, standard histological classification is not effective.

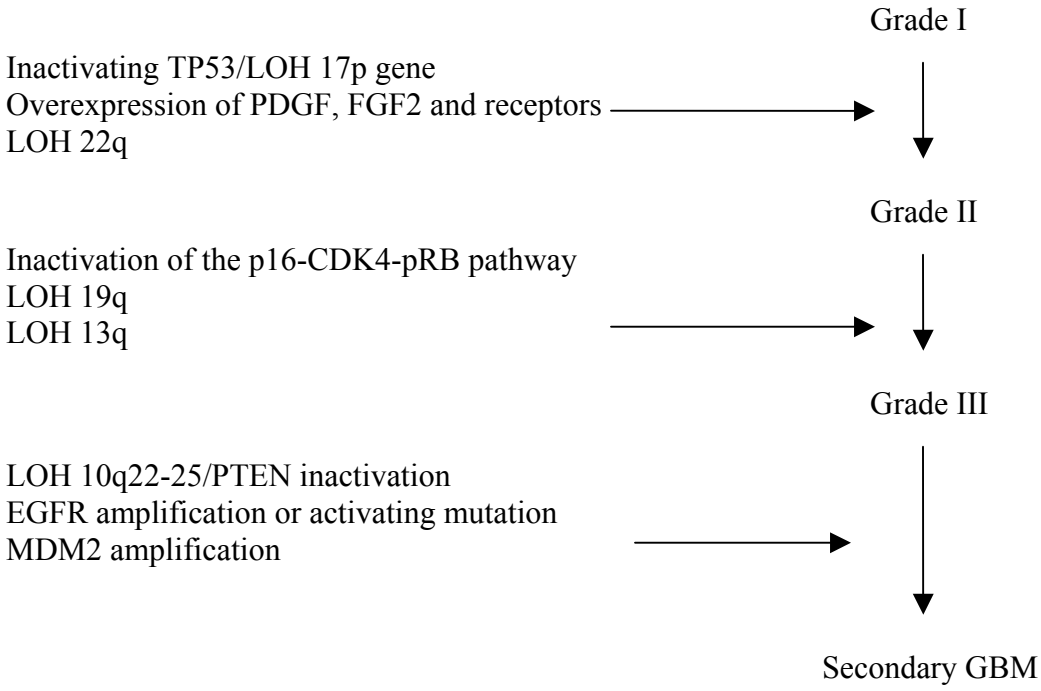
On the other hand, a number of recent reports have indicated that gene expression based classification of malignant gliomas correlates better than previous used parameters with survival (42; 104; 131), recurrence (58), and treatment outcome (23).

Primary and secondary glioblastoma

Glioblastoma arising de novo without evidence of a less malignant precursor lesion are termed primary glioblastoma. It usually develops in older patients with a mean age of 55 years old. The primary glioblastoma typically shows EGFR overexpression, PTEN (MMAC1) mutation, CDKN2A (p16) locus deletion, and less frequently, MDM2 amplification.



Glioblastoma evolving from a previous lower grade astrocytoma are defined as secondary glioblastoma. Secondary glioblastoma develops in younger patients (<45 years) and often contains TP53 mutation and overexpression of PDGF and PDGF receptors.



To summarize, from histopathological point of view, primary GBM are indistinguishable from secondary GBM, however, the two types of tumours exhibit distinct genetic alterations and occur in different age groups (42; 68; 69). The concept that primary and secondary glioblastoma are two different diseases has been accepted (68).

Treatment

The orthodox concept for the treatment of glioma is to combine surgical tumour resection with radiotherapy, sometimes followed by chemotherapy. Recently, an approach of using temozolomide concomitant with radiotherapy has been shown to increase two years survival from 8% (only post-operative radiotherapy) to 29% in the group of patients treated with the combination (122). Other approaches under development are immunotherapy (37), gene therapy (44), and stereotactic guided laser-induced interstitial thermotherapy (79).

Prognosis

Despite the best currently available treatments, patients diagnosed with malignant glioma still have a poor prognosis. Typical median ranges of survival are more than 5 years for diffuse astrocytomas WHO grade II, 2-5 years for anaplastic astrocytomas WHO grade III, and around 1 year for the majority of patients with WHO grade IV tumours after diagnosis (68). Clinical factors have been identified that are useful for assessing individual glioma prognosis, such as patient age, tumour grade, tumour type, the volume of tumour remaining after surgery, general condition, the use of radiotherapy, and some neuroradiological characteristics, such as necrosis in imaging studies (68). As previously indicated, with the development of molecular biology, it is of obvious importance to find new biological tumour markers that predict the outcome of each individual patient in order to apply an earlier individualised therapy.

Molecular Biology

The discovery that specific genes regulate the proliferation of cells by stimulatory and inhibitory signals, and that defects in these signal pathways are associated with cancer, has revolutionized our understanding of the mechanisms behind tumourigenesis. Glioma is thought to be the consequence of the accumulation of genetic changes that confer uncontrolled growth on a glial cell. Several reviews have discussed the chromosome alterations and genetic changes in glioma (68; 106; 120;

137). Some chromosomal alterations seem to play particular important roles in the genesis and progression of gliomas (137), such as loss of heterozygosity (LOH) at 1p, 9p, 10p, 10q, 13q, 17p, 19q, and 22q, EGFR amplification at chromosome 7, homozygous deletions of CDKN2A/B at 9p and DMBT1 at 10q, and mutations of PTEN at 10q and TP53 at 17p.

In the following parts, three relevant topics will be discussed: biomarkers of interest for human glioma, growth suppressors inactivated in human glioma and growth factors activated in human glioma.

Biomarkers of interest for human glioma

Glioma biomarkers can principally be divided into four main groups (13). The first group includes markers of lineage and differentiation. These markers identify the presumed tumour cell of origin. The most commonly used differentiation marker is glial fibrillary acidic protein (GFAP). GFAP is a major component of the normal glial cytoskeleton, and it is usually present in perikaryon and cytoplasmic processes. It is used to identified astrocytes and tumours of astrocytic origin, however, it should be emphasized that GFAP can also be found in other CNS tumours and in peripheral nervous system. OLIG1 and OLIG2 are newly identified oligodendrocyte lineage specific genes. OLIG2 protein is restricted to oligodendrocytes and their progenitors in normal human brain (84; 145), and OLIG2 was initially proposed to be a specific marker for oligodendroglial tumour cells (91). Immunohistochemical analysis, however, shows that OLIG2 is highly expressed in all diffuse gliomas (84). OLIG2 protein expression is present but inconsistent and generally lower in most other brain tumours and is absent in non-neuroectodermal tumours. The expression heterogeneity of OLIG2 in astrocytomas precludes immunohistochemical classification of individual gliomas by OLIG2 alone.

The second group includes markers of growth and proliferation of glioma cell, such as DNA labelling by Bromodeoxyuridine, Ki-67/MIB-1 and proliferating cell nuclear antigen (PCNA). PCNA is an auxiliary protein of DNA polymerase-σ necessary for DNA replication, and its expression is the highest in G1/S cell cycle phase. PCNA labelling has been performed in several series of brain neoplasms (67; 118; 126). Ki-67 is a nuclear antigen that is expressed in proliferating

cells (116), but not in the resting cells (G0 phase). MIB-1, a monoclonal antibody against Ki-67, thus, stains cells that are actively proliferating. McKeevor et al (92) concluded that Ki-67 as measured by the MIB-1 monoclonal antibody was a superior predictor of survival in glioma compared to other markers of proliferation, such as PCNA or bromodeoxyuridine labelling. The superiority of Ki-67 over the other proliferation markers may be because Ki-67 is expressed throughout most of the cell cycle, thereby allowing the detection of a greater proportion of proliferating cells. In addition, results obtained with anti-Ki-67 antibody are less variable than those obtained with anti-PCNA (67).

The third group consists of markers of invasion, such as neural cell adhesion molecules (NCAM), urokinase plasminogen activator (uPA), Urokinase plasmiongen activator receptor (uPAR), tissue-type plasminogen activator (tPA) (112), protease (80) and Cathepsin B (75).

The fourth group includes markers that depend on loss or gain of genetic material representing pathogenetic mechanisms that involve alterations in gene copy number or function, which provide tumour cells with a growth and survival advantage. Such genes include the pro-oncogene EGFR and the tumour suppressor genes TP53 and RB, which are further discussed later in this thesis.

Growth suppressors inactivated in human glioma

Among the most common genetic changes in glioma are losses in the function of growth suppressing factors, which result in disturbance of normal growth control and finally in tumourigenesis.

Retinoblastoma susceptibility gene (RB1, alias RB) is located on chromosome 13q14. The RB gene is mutated in hereditary retinoblastoma, and it was the first tumour suppressor gene to be cloned (78). It encodes a nucleoprotein (pRB) that plays a key role in the regulation of the cell cycle where it controls the G1–S transition. pRB inhibits the E2F family of transcription factors, which activate transcription of genes required for DNA replication. Mitogenic stimuli trigger phosphorylation of pRB by cyclin D- and cyclin E-dependent kinases, which relieve the inhibitory activity of pRB by disrupting its association with E2F. The activity of the cyclin D/cyclin-dependent kinases complex is in turn controlled by inhibiting proteins such as p16 (CDKN2A). Thus, both the

loss of p16 and pRB function can result in dysregulated cell proliferation (93). In addition to its central role in cell cycle regulation, pRB has been shown to participate in the DNA-damage response, apoptosis, differentiation, and senescence.

Mutations in the RB pathway are common in gliomas (147) and LOH at the RB locus itself has been found in 25–45% of glioblastomas and in about 25% of anaplastic astrocytomas (55; 60). Another RB gene silencing mechanism, promoter region methylation, is also commonly found in brain tumours (45).

Tumour protein 53 (TP53) is located at 17p13.1, and encodes a DNA-binding nuclear protein, p53. p53 contains DNA-binding, oligomerization, and transcription activation domains. It is postulated to bind as a tetramer to a p53-binding site and plays an essential role as a gatekeeper of cell cycle, thereby protecting the integrity of the genome. p53 is transcriptional activator of $p21^{Cip1}$, a potent CDK inhibitor, and regulates the transition from G0 to G1 through $p21^{Cip1}$, which expression results in G1 cell cycle arrest (35). The cycle progression in G_2 is also inhibited (129). p53 regulates the response of cells to DNA damage by causing cell cycle arrest or apoptosis, thereby functions as a tumour suppressor (5). Murine double minute 2 (MDM2) protein can bind to p53 and interferes with p53 function. MDM2 functions as an ubiquitin ligase and targets p53 for degradation. The ARF gene from INK4α locus encodes a protein $p14^{ARF}$, which can prevent MDM2-mediated downregulation of p53 (16).

p53 is one of the most frequently inactivated proteins in glioma. In most instances, mutation of one p53 allele is followed by loss of the remaining wild-type allele, resulting in cells completely devoid of functional p53 protein (20). A significant consequence of the loss of normal p53 activity is increased genome instability, which accelerates cancer transformation. LOH of 17p or TP53 mutations have been reported in glioma by many authors (19; 48; 62). Alteration of the TP53 gene seems often to be an early event in the tumourigenesis of glioma (137). However, p53 mutations can also appear late in the malignant progression towards GBM (137).

Phosphatase and tensin homolog (PTEN) tumour suppressor gene is also known as mutated in multiple advanced cancers (MMAC1) and TGF-β-regulated and epithelial cell-enriched phosphatase

(TEP-1). PTEN was identified in 1997 as a gene that is frequently disrupted in GBMs and other tumours (82). PTEN encodes a dual lipid-protein tyrosine phosphatase with an enlarged core catalytic domain that primarily binds phosphoinositides but also can accommodate peptide substrates (77). PTEN dephosphorylates the second messenger molecule phosphatidylinositol-3,4,5 triphosphate (PIP_3) to phosphatidylinositol-3,4 diphosphate (PIP_2) (100).

PTEN gene is frequently inactivated in high grade gliomas through mutation or homozygous deletion, but this is rare in low grade glioma (34; 85). Loss of PTEN function raises intracellular PIP_3 levels and lead to excessive phosphorylation and activation of protein kinase B (PKB, also known as Akt). PKB acts as a proto-oncogene that makes cell refractory to apoptotic stimuli and promotes cell proliferation and uncontrolled stimulation of growth (21). PTEN has been linked not only to tumour growth, but also to the tumour's ability to invade normal brain tissue. Restoring PTEN expression in tumour cells strongly reduced both the invasive capacity and the growth of the glioma cells (24; 88). In another study, the absence of PTEN was associated with increased tumour malignancy, whereas increased PTEN was a positive prognostic factor for patients survival (113).

p16 was described in 1993 and it was demonstrated that p16 binds to CDK4 and inhibits the catalytic activity of the cyclin D/CDK4enzymes. p16 seems to act in a regulatory feedback circuit with CDK4, D-type cyclins and retinoblastoma protein. The gene is located on chromosome 9p21 (64) and encodes the protein $p16^{INK4}$ (inhibitor of cyclin-dependent kinase 4) (128), which is a growth suppressor. Now, four members of the INK4 family have been identified ($p15^{INK4b}$, $P16^{INK4a}$, $p18^{INK4c}$, $P19^{INK4d}$). There are many reviews that describe particular aspects of INK4 family (10; 111). Briefly, the RB/CDK4/$p16^{INK4a}$/$p15^{INK4b}$ pathway plays a crucial regulatory role in cell cycle progression. Inactivation of the $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ genes has frequently been observed in a variety of human tumours, including malignant gliomas, and is largely due to homozygous deletion or to hypermethylation of CpG islands in the promoter region (62; 101; 111).

Growth factors activated in human glioma

PDGF and PDGFR: platelet-derived growth factor family of growth factors consists of five disulfide-bonded homo- and heterodimeric isoforms: PDGF-AA, -BB, -AB,-CC and –DD. The

different isoforms bind with different affinity to the two structurally related tyrosine kinase receptors PDGFRα and β (54). The PDGFRα and PDGFRβ genes map to chromosome 4q11-12 and 5q33-35, respectively. PDGFRα binds the PDGF-A, -B, and -C isoforms with high affinity and PDGFRβ binds the PDGF-B and –D isoforms.

The biological effect of respective PDGF isoform is dependent on the cell type stimulated, receptor types, and presence of other growth factors. The normal functions include stimulation of cell proliferation, promotion of cell survival, and differentiation (6; 15; 76).

In glioma, co-expression of PDGF and its receptors is often observed, establishing an autocrine loop that contributes to proliferation and also to tumour progression (56; 86). The PDGFRα is amplified in 5-8% of the glioblastomas. It has also been shown that retrovirus-mediated expression of PDGF-B in the brain of neonatal mice results in the formation of astrocytomas (132). Treatment of glioblastoma cell lines with CT52923, a potent selective small molecule antagonist of the PDGFR, inhibit PDGFR autophosphorylation, block the Ras/Raf/MAPK proliferative pathways or the PI3-K/Akt survival pathway and cause a reversion of the transformed phenotype (86).

EGFR: The epidermal growth factor receptor (EGFR) is a well studied, versatile signal transducer that has been well conserved during evolution (144). It functions in a wide range of cellular processes including cell fate determination, proliferation, migration, and apoptosis (144). EGFR belongs to the ErbB family of receptor tyrosine kinases (RTKs). The family includes four members: EGFR (also termed ErbB1/human epidermal growth factor receptor 1 (HER1)), ErbB2 (neu/or HER2), ErbB3 (HER3), and ErbB4 (HER4) (144).

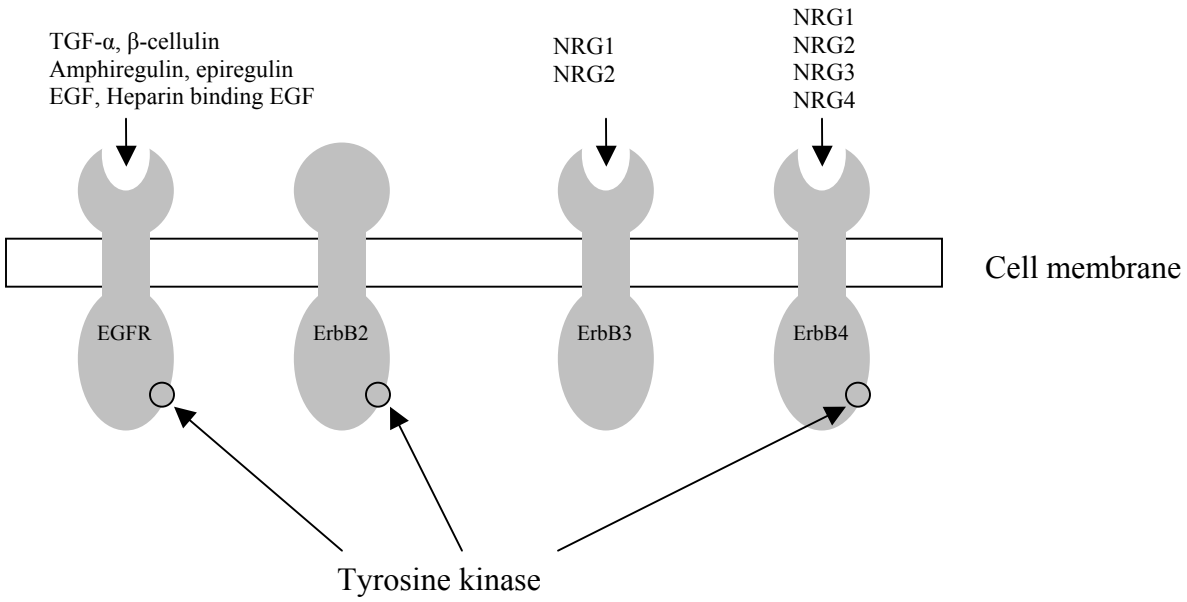


Figure 1. The ErbB family members and their ligands

The ErbB receptors are transmembrane proteins with an extracellular ligand binding domain, a single membrane spanning segment, and an intracellular protein tyrosine kinase domain (133). The unstimulated EGFR is at the plasma membrane predominantly in a monomeric form. Upon ligand binding, receptors form homo- or heterodimeric complexes resulting in autophosphorylation of their cytoplasmic part. This creates docking sites for intracellular adapter molecules and molecules with intrinsic signalling function, which in turn activates an array of signal pathways. The most intensively studied EGFR-coupled signal pathways are the Ras/Raf/MAP kinase pathway, the PI3-K (phosphatidylinositol 3-kinase pathway), and the PLCγ pathway (66; 144).

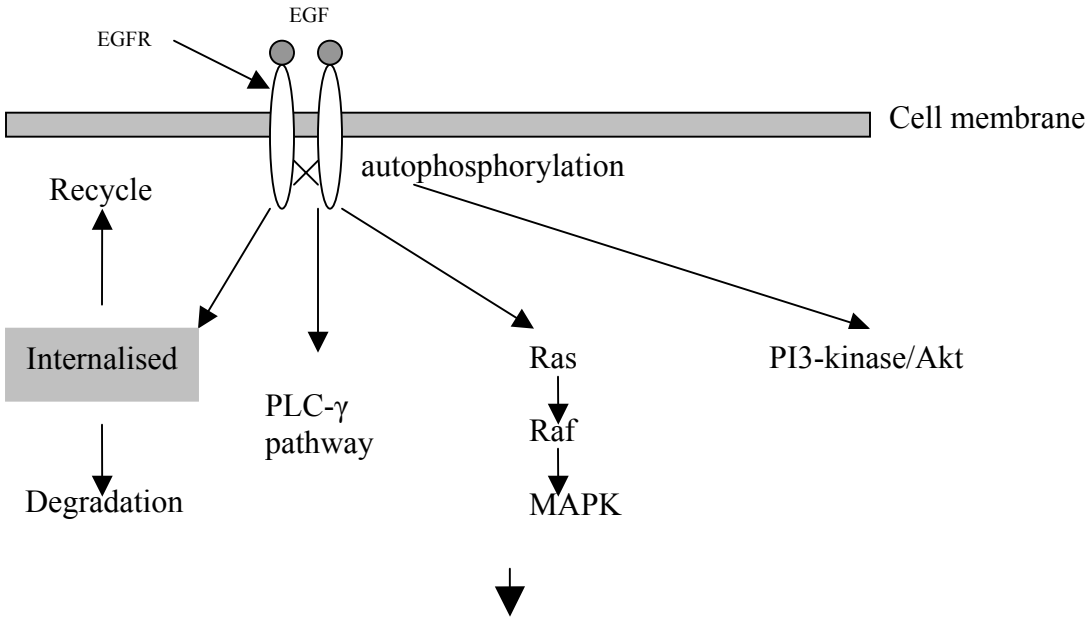


Figure 2. EGFR signal pathways

The EGFR function is dysregulated in several malignant disorders including high grade astrocytomas. The mechanisms involve EGF/EGFR autocrine loop and ligand-independent activation due to overexpression and/or activating mutations (144), which lead to inhibition of apoptosis, cellular proliferation, promotion of angiogenesis, and metastasis. The EGFR gene is the most frequently amplified gene in GBMs (4; 83). While the gene encoding EGFR maps to chromosome 7, the amplified genes are typically present as double-minute extra-chromosomal elements. Amplification is found in approximately 50% of GBMs and a small percentage of anaplastic astrocytomas express high levels of EGFR. These observations suggest that EGFR overexpression and /or gene alteration is a late event in glioma genesis and is frequently observed in primary or de novo GBM. Gene amplification and mutation commonly lead to co-expression of normal and mutated EGFR proteins, including the expression of the most common EGFR mutation in GBM, EGFRvIII (1). EGFRvIII has a deletion in the extracellular domain, which constitutively activates receptor with transforming potential.

It has to be emphasized that here also exist other growth factors that may be of importance in the stimulation of glioma growth. Such as, insulin-like growth factors (IGF) and IGF receptors, fibroblast growth factor (FGF) and FGF receptor.

Negative regulation of EGFR

Inhibitory pathways are important for terminating and fine-tuning EGFR activity. It is of interest to look more closely at the endogenous negative ErbB receptor regulation pathways, the mechanism by which tumours could overcome these pathways, and further whether these pathways might be employed in the treatment of patients.

***Receptor degradation*:** Receptor degradation is one of the primary mechanisms by which cells negatively regulate growth factor receptor activity. In general, ligand stimulation results in EGFR localization to plasma membrane clathrin-coated pits, then internalization of coated pits and delivery to endosomes, and sorting of endosomal receptors for trafficking back to the cell surface or to lysosomes for degradation (125). The Cbl family of E3 ubiquitin ligase are important negative regulators of activated tyrosine kinase-coupled receptors (81). Cbl proteins evolutionarily conserved, facilitate the ubiquitinylation of activated tyrosine kinases and other signalling proteins, which facilitate their lysosomal degradation, whereas polyubiquitin tag promotes proteasomal degradation (33). Overexpression of Cbl in cells augments EGF-stimulated receptor ubiquitylation and degradation (81).

Naturally occurring negative modulators of EGFR: A mechanism by which cells negatively regulate ErbB receptors is through the use of modulator proteins that physically interact with receptors to determine their response to ligand binding.

Herstatin: Herstatin is the product of an alternatively spliced human ErbB2 that lacks the transmembrane and cytoplasmic domains (32). Herstatin binds with high affinity to EGFR and ErbB2, and thereby inhibits ErbB receptor homo- and heterodimer formation and subsequent tyrosine phosphorylation (9).

Mig-6 was identified in a yeast two-hybrid screen with the kinase active domain of the EGFR as bait (50). Upon EGF stimulation Mig-6 binds to the EGFR, involving a highly acidic region between amino acids 985-995. This interaction is kinase activity-dependent. Mig-6 overexpression results in reduced activation of the mitogen activated protein kinase ERK2 in response to EGF stimulation, but not in response to FGF or PDGF stimulation. Mig-6 enhances EGF induced EGFR internalisation without affecting the rate of receptor degradation. The induction of Mig-6 mRNA expression in response to EGF, but not FGF, indicates the existence of a negative regulatory feedback loop. Consistent with these findings, a possible role as tumour suppressor is indicated by Mig-6-mediated inhibition of EGFR overexpression-induced transformation of Rat1cells (50).

Some other negative regulator or modulators such as SIRPα1 (65), Gangliosides (94) are also under investigation. The negative EGFR regulator in Drosophila, Kekkon-1, and its human counterparts will be discussed below.

Here I would like to introduce an inhibitor in potato, named potato carboxypeptidase inhibitor (PCI) (17)**.** PCI, a 39-amino acid protease inhibitor with three disulfide bridges, is an antagonist of human EGF. It competes with EGF for binding to EGFR and inhibited EGFR activation and cell proliferation induced by this growth factor. PCI also has been shown to suppress the growth of several human pancreatic adenocarcinoma cell lines, both in vitro and in nude mice. PCI has a special disulfide scaffold called a T-knot that is also present in several growth factors including EGF and transforming growth factor alpha (17; 125).

The development of artificial ErbB inhibitors: Owing to the important role of the ErbB receptor activation in tumour growth and progression, the development of ErbB inhibitors has been a subject of intense interest.

Four strategies for targeting the EGFR are at different stages of experimental and clinical development. This include: 1) monoclonal antibodies directed towards the EGFR are in clinical trial and one, Cetuximab recently has received approval for the treatment of colorectal cancer, 2) inhibition of the receptor tyrosine kinase (RTK) domain by small-molecule inhibitors is being developed, 3) inhibition of receptor trafficking to the cell membrane (143), and 4) inhibition of

EGFR synthesis through anti-sense oligonucleotides. To date, monoclonal antibodies and synthetic inhibitors of tyrosine kinase have taken centre stage (30).

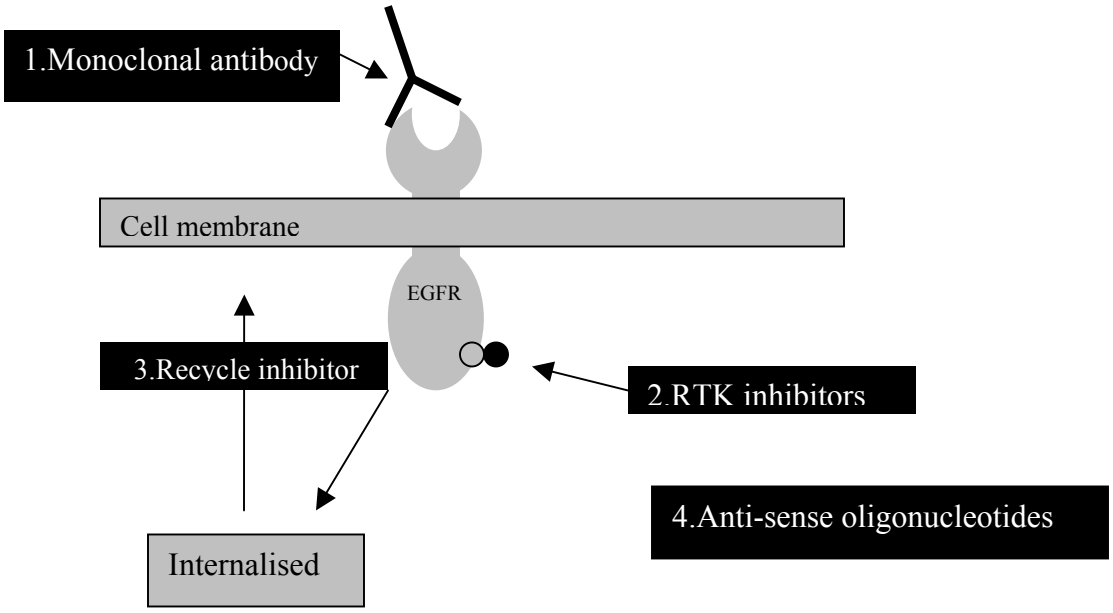


Figure 3. Four strategies for targeting the EGFR

Monoclonal antibodies bind to the extracellular domain of the EGFR and inhibit ligand binding to the receptor. After binding to the EGFR, the monoclonal antibody induces receptor dimerization and causes down regulation with subsequent inhibition of down-stream signalling (11). Cetuximab (IMC-C225, Erbitux ImClone Systems Inc, New York, NY, USA), ABX-EGF (Abgenics, San Francisco, CA, USA), and EMD 72000 are monoclonal antibodies directed against the EGFR that are currently in clinical trials. Another class of monoclonal antibodies consists of bispecific antibodies that can bind the EGFR and an immunologic effecter cell. Examples of this class of agents include M26.1, MDX-447, and H22-EGF. These agents have shown promising activity in early clinical trials (30; 36).

Small molecular receptor tyrosine kinase inhibitors compete with ATP for the intracellular catalytic site of the EGFR. In contrast to the monoclonal antibodies, this class of agents do not down-regulate

EGFR expression. Receptor tyrosine kinase inhibitors differ with respect to reversibility of inhibition and specificity to the EGFR vs. other RTK. Based on these differences, four different classes of RTK inhibitors can be defined and these include: 1) reversible EGFR inhibitors (e.g. gefitinib and erlonitib), 2) irreversible EGFR inhibitors (e.g. EKB-569), 3) reversible dual-ErbB inhibitors (e.g. GW2016), and 4) irreversible pan-ErbB inhibitors (e.g. CI-1033). So far, gefitinib is the only RTK inhibitor that has been approved for clinical use in the treatment of non-small cell lung cancer.

Leucine-rich repeat superfamily

Leucine-rich repeat (LRR) motif has been studied and reviewed in several reports (63; 71; 72). It is a protein motif that is characterized by a periodic distribution of hydrophobic amino acid, especially leucine residues, separated by more hydrophilic residues. The LRRs are generally 20-29 residues long and contain a conserved 11-residue segment with the consensus sequence $LxxLxLxx^{N}/_{C}xL$ (where x can be any amino acid and L is a leucine, valine, isoleucine or phenylalanine). The average repeat length is 24 amino acids. The number of repeats in LRR protein varies a lot. Chaoptin has most tandem repeats 41, while Rev has just one repeat. In extracellular proteins, LRR blocks are generally flanked on either side by cysteine-rich regions, which are referred to as N- and C-terminal cysteine-rich flanking regions.

LRR containing proteins comprise a number of intracellular and extracellular proteins with diverse functions (22; 61). On July 2003 (NCBI 34 version of the human genome), there were 252 genes containing the motif leucine-rich repeat. The proteins containing the repeat are thought to be involved in protein-protein interactions. In particular, LRRs are common motifs in the extracellular region of transmembrane proteins and also in secreted proteins that are involved in ligand-receptor interactions or in cell adhesion. The LRR superfamily contains also several families of signal-transducing receptors.

LRR containing proteins in glioma and other tumours

Decorin, a small Leucine-rich proteoglycan, has a role in modulating matrix assembly (139), cell proliferation and migration (141). Most of the biological functions of decorin are mediated by the protein core's unique organization of 10 tandems LRR, which fold into an arch-shaped structure whose concave surface is well suited to bind both globular and non-globular proteins as well as metal ions.

Decorin is a ligand for the EGFR (61), and binding to the EGFR causes receptor dimerization and autophosphorylation. This interaction triggers a signal cascade leading to activation of MAPK, and mobilization of intracellular calcium. However, it also results in up-regulation of p21 (a potent inhibitor of CDK), and ultimately to growth suppression (27; 96). Decorin expression is markedly suppressed in most transformed cells derived from primary malignant tumours (27).

Decorin has been also shown to cause a functional inactivation of the oncogenic ErbB2 protein in breast carcinoma cells (114). Upon de novo expression of decorin, the ErbB2 protein is reduced by approximately 40%, whereas its degree of tyrosyl phosphorylation is almost completely abrogated. Both co-culture experiments and experiments with recombinant decorin demonstrate an initial induction of ErbB2 tyrosine kinase, followed by a profound and long-lasting down-regulation of its activity. This leads to growth inhibition and differentiation of mammary tumour cells and a concurrent suppression of their tumourigenic potential in vivo. These decorin-mediated effects appear to involve the activation of ErbB4 (114), which in turn would block the phosphorylation of heterodimers containing either ErbB2 or ErbB3. The results provide an explanation for the heightened decorin levels around invasive carcinomas and suggest that decorin expression may represent a host response in defence of neoplastic cells enriched in ErbB2. Upon transgenic expression of decorin in mice, tumour cells with diverse histogenetic backgrounds revert to their normal phenotype and it has been proposed that decorin might represent a natural antagonist to the growing cancer cells (115). Interestingly, it has been shown that decorine-induced inhibition of TGF-β release by glioma cells significantly enhances anti-glioma immune response (98; 121).

Leucine-rich repeat-glioma-inactivated gene 1 (LGI1) was identified and located at 10q24 by means of breakpoint cloning in a glioblastomas cell line (25). Northern blotting and RT-PCR studies revealed reduced levels of LGI1 mRNA in glioma cell lines and malignant gliomas compared with normal brain tissue (25). The coincident loss of LGI1 expression with loss of chromosome 10 suggested that it might be important in malignant progression of gliomas. Immunohistochemistry study demonstrated a gradual down regulation of the LGI1 gene product with increasing grade of malignancy, thus LGI1 was suggested to be a tumour suppressor gene in gliomas (14). Recently, it was further demonstrated that LGI1 significantly suppresses cell proliferation and invasion of glioma cells through the EEK1/2 pathway (73; 74).

Glioblastomas amplification on chromosome 1 (GAC1) is transmembrane protein (2), located at chromosome band 1q32.1. Analysis of GAC1 expression in human adult tissues revealed that GAC1 mRNA is 3.5 kb transcript in size, with highest expression in brain. The chromosomal band 1q32 has been repeatedly shown to contain amplified sequences by comparative genomic hybridization analysis of gliomas (97), and GAC1 has also been shown to be amplified and over expressed in malignant glioma (2) or coamplified with MDM4 (110). The functional significance of GAC1 amplification in malignant gliomas is still controversial (110).

The SLIT family (SLIT1-3) consists of large extracellular matrix-secreted and membrane-associated glycoproteins, which belongs to the LRR superfamily. The SLITs (SLIT1-3) are ligands for the repulsive guidance receptors of the ROBO gene family (57). The SLIT-ROBO interactions mediate repulsive cues on axons and growth cones during neural development. SLIT2 (38) maps to 4p15.2 and encodes a human ortholog of the Drosophila Slit protein. It was demonstrated that SLIT2 is frequently inactivated by promoter region CpG island hypermethylation in gliomas (28) and may, thus, be a good candidate for a glioma tumour suppressor gene.

The NTRK family: Neurotrophic tyrosine kinase receptor family includes NTRK1 (TRKA), NTRK2 (TRKB) and NTRK3 (TRKC). Neurotrophins regulate the proliferation, differentiation and survival of the developing neurons in the central nervous system via NTRK family. All NTRK proteins contain three tandems LRR followed by two immunoglobulin-like domains in their extracellular part (107). Wang and coworkers (136) described the expression of NTRK family

members in normal brain and glioma by immunohistochemical analysis. In normal brain, TRKA, B, C immunoreactivity was found in neurons and some week staining was also seen in astrocytes. No TRK expression was seen on oligodendrocytes. In tumour samples, TRKA, B, and C immunoreactivity was observed exclusively in specimens from astrocytic gliomas but not in oligodendrogliomas. TRKA, B, C may be expressed in a linage-restricted manner, thereby distinguishing between astrocytes and oligodendrocytes. TRK expression appears to be increased in activated neoplastic astrocytes (136). In the peritumour brain tissue, only neurons showed immunoreactivity of TRKA and TRKB (134). High levels of immunoreactivity were present in the lower grade astrocytoma, whereas in the advanced malignant forms the immunoreactivity was very weak. This suggested that TRKA and TRKB are involved in the early stage of glioma pathogenesis, and may respond to signals that elicit glial proliferation, and thus contribute to progression toward malignancy (134).

The SLITRK family (SLITRK 1-6) was identified as neuronal transmembrane proteins that control neurite outgrowth (7; 8). Structurally, they are characterized by two leucine-rich repeat (LRR) domains located amino-terminally to the transmembrane domain. The LRR domains in the SLITRK family proteins are similar to the SLIT family described above. Six members of the family have been identified and their expression was detected mainly expressed in brain, but the expression profile of each SLITRK was unique. SLITRK genes are also differentially expressed in different brain tumours (8). The results propose that the human SLITRK genes can be useful molecular indicators for various brain tumours.

RATIONALE FOR THE PRESENT THESIS

The transmembrane protein, kekkon-1 (kek-1), has been shown to act as a modulator in a negative feedback loop and down-regulation of Drosophilia epidermal growth factor receptor (3; 40). Insect kek-1 is capable of binding to and suppressing the signal functions also of mammalian ErbB receptor family member (39). Similar membrane glycoprotein was also described in mouse brain (124). The present study was undertaken in order to identify and characterize human analogues to Kek-1 and to elucidate their potential role in the pathogenesis of tumours.

SPECIFIC AIMS OF THE PRESENT STUDY

1. To identify, clone, and characterize human and mouse counterparts of the Drosophilia kekkon-1.

2. To set up quantitative real-time RT-PCR assay for analyzing LRIG gene expression.

3. To study the expression of LRIG1-3 in human and mouse tissues.

4. To study the expression of LRIG1-3 in human astrocytic tumours.

MATERIAL AND METHODS

RNA samples (I, II, III, IV, V, VI)

Human and mouse total RNA from the following organs was purchased from the indicated suppliers: human liver, spleen, ovary, kidney, testicle, thymus, pancreas, colon, small intestine, adrenal, placenta, stomach, bladder, and cervix (Ambion, Inc.); human heart, trachea, salivary gland, prostate, skeletal muscle, mammary gland, prostate, and uterus (BD Biosciences Clontech, Palo Alto, CA, USA); human brain and lung (Origene Technologies, Inc.); human thyroid and skin (Stratagene, La Jolla, CA, USA); mouse brain, liver, embryo, heart, lung, spleen, ovary, kidney, testicle, and thymus (Ambion,Inc.). Blood was obtained from three volunteers at the University and total RNA was isolated using the RNAqueous Blood Kit (Ambion, Inc.) and then pooled. In vitro-transcribed RNA of human LRIG1, LRIG2, and LRIG3 were produced as previously described (119). Total RNA from cell lines was prepared using the RNAqueous kit (Ambion Inc., Austin, TX). Fresh frozen tumour tissues and matched specimen of grossly normal brain tissues available from 6 patients were used for RNA extraction. All RNA samples were DNase treated using the DNAfree kit (Ambion Inc.) prior to the quantitative real-time RT-PCR analysis.

Cell lines (III, VI)

The human glioma cell lines U-118 MG, U-138 MG, and U-343 MG, the prostatic carcinoma cell lines PC-3 and DU-145, the colorectal adenocarcinoma cell lines HT-29 and DLD-1, rat C6 glioma cell line, and African green monkey kidney cell line Cos-7 were obtained from the American Type Culture Collection (Manassas, VA). The glioma cell lines U-105 MG and U-251 MG were kindly provided by Dr. M. Nister (Uppsala University, Uppsala, Sweden). The lung cancer cell line U1690 and breast carcinoma cell line ZR-75 were kindly provided by Dr. J. Bergh (Uppsala University, Sweden), and the lung mesothelioma cell line P31 by Dr B. Sandström (FOI, Umeå, Sweden). The glioma cell line SF-767 by the UCSF Neurosurgery Tissue Bank (San Francisco, CA). All cell lines

were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 µg/ml gentamycin from Life Technologies (Täby, Sweden).

Clinical material (VI)

Brain tumour samples and clinical data were obtained from patients who underwent surgery at the Tampere university hospital, Tempere, Finland during 1983-2001. Four hundred and four astrocytic tumour samples were included in this study. The preparation of astrocytic tumour tissue micro array sections have previously been described (49). The diameter of the tumour tissue core in the micro array block was 600 µm. These 404 tumours include 46 Grade I, 51 Grade II, 46 Grade III, and 261 Grade IV according to the WHO classification system. Between 1% and 3% of samples in the TMA sections were lost during the section preparation and immunostaining process. Overall survival analysis included178 patients treated similarly.

Quantitative RT-PCR, primers, and probes (I, II, III, IV, V, VI)

Quantitative real-time reverse transcription- (RT-) PCR was performed using an iCycler iQ system (Bio-Rad, Hercules, CA). Primers and probes for hLRIG1, hLRIG2, hLRIG3, mlrig2, mlrig3, hGAPDH, h18S rRNA were synthesized by Scandinavian Gene Synthesis AB (Köping, Sweden). Mouse Lrig1 primers and probe were designed and synthesized by Applied Biosystems' (Stockholm, Sweden) Assays-by-Design service. Primers and probe for β-actin was purchased from PE Biosystems (Stockholm, Sweden). Relative mRNA quantification or absolute mRNA quantification was performed by comparing the threshold cycle values (Ct) for the samples, with standard curves generated with plasmid DNA containing cloned cDNA-fragments of respective gene, or in vitro transcribed RNA of respective gene.

cDNA isolation, and sequencing (II, IV, V)

Human and mouse brain Rapid-Screen cDNA library (Origene Technologies, Inc., Rockville, MD) were used to isolate human LRIG1, LRIG2, LRIG3 and mouse LRIG3 cDNA. The libraries were screened by real-time PCR. Plasmid DNA was prepared from positive bacterial colonies and the inserted cDNAs were sequenced by using DYEnamic ET dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and on a 377 DNA sequencer (Applied Biosystems, Foster City, CA). Each fragment was sequenced at least two times.

Bioinformatic analysis (II, IV, V)

The signal peptide was predicted by using web server http://www.cbs.dtu.dk/services/SignalP/ as described by Bendtsen JD (12), the extracellular domains were predicted by using Pfam 6.1 http://www.sanger.ac.uk/Software/Pfam/search.shtml, the transmembrane domain was predicted by using TMHMM 2.0 http://www.cbs.dtu.dk/services/TMHMM/, potential N-linked glycosylation sites and phosphorylation sites were predicted by using Prosite http://www.expasy.org/prosite/, BLAST search was done by web server http://www.ncbi.nlm.nih.gov/BLAST/, phylogenetic tree analysis was done by using software DNAstar MegAlign module1.16.

Confocal fluorescence laser microscopy (IV, VI)

Confocal fluorescence laser microscopy was performed essentially as previously described (103). Briefly, C6, COS-7, U-118 MG and U-251 MG cells were seeded on cover slips and transfected with pLRIG1-GFP. Forty-eight hours post transfection, the cells were fixed and labelled with tetramethylrhodamine-conjugated wheat germ agglutinin (WGA) and analyzed by using a Leica TCS SP2 laser scanning confocal microscope with an oil immersion objective. GFP and tetramethylrhodamine fluorescence images were acquired sequentially in each optical plane by using 488-nm argon and 543-nm helium–neon lasers, respectively.

FISH analysis (II, IV)

FISH was performed on metaphases of a healthy blood donor. Chromosomal preparations were made from cultured lymphocytes from peripheral blood by conventional methods. Slides were incubated in 2 x SSC, dehydrated in ethanol, denatured in 70% formamide/2 x SSC and dehydrated in ethanol. Plasmid DNA with inserted genomic fragments was labelled with digoxigenin-11-dUTP (Boehringer–Mannheim GmbH, Mannheim, Germany) by nick translation. 50–100 ng of probe was used for hybridization in a moist chamber at 37°C for 14–16 h. Probes were detected with rhodamine anti-digoxigenin antibodies (Boehringer–Mannheim GmbH). Analysis was performed using a Leica microscope (DMLB) equipped with filter wheel containing DAPI, Texas Red, FITC, and Quad filters. Digital images were captured and stored using MacProbe 4.0 software (PSI, Perceptive Scientific International Ltd.).

Northern blotting (II, IV, V)

A human poly A RNA blot (Origene Technologies) was probed with ^{32}P-labeled probes that were made using the StripEZ PCR-kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions, with the templates LRIG1, LRIG2, LRIG3, GAPDH and β-actin. Hybridization was performed in UltraHyb (Ambion, Inc., Austin, TX) at 42°C for 2–7 h and then the membrane was washed twice with 0.1 x SSC at 42°C for 15 min. Bound probe was visualized using the GS-525 Molecular Imaging System (Bio-Rad, CA) or exposed to X-ray film for 25 min to 14 hours thereafter developed.

Antibodies (III, VI)

The rabbit antibody: anti-LRIG1-151, anti-LRIG2 and anti-LRIG3 rabbit antibody against the synthetic peptide: EPGGSDQEHSPHHQC for LRIG1, CFDFSRTRNIQDGSEGT for LRIG2, and CDLDSGSEEDGKERTD for LRIG3 were raised in a rabbit. The serum was affinity-purified using UltraLink columns coupled with the corresponding peptides and elute with ActiSep elution medium

from Sterogene Bioseparations (Carlsbad, CA). The anti-LRIG antibodies were produced by AgriSera (Vännäs, Sweden). Horseradish perioxidase-conjugated donkey anti-rabbit immunoglobulin antibodies were purchased from Amersham Biosciences (Uppsala, Sweden).

Immunohistochemistry (VI)

The TMA paraffin blocks were cut in 4-µm-thick sections and treated as previously described (103), except that antigen retrieval was performed in 10 mM citrate buffer, pH 7. The primary rabbit antibodies were diluted in PBS containing 10 mg/ml bovine serum albumin and the slides were incubated with anti-LRIG1 (0.5 µg/ml) overnight at 4°C, anti-LRIG2 (1 µg/ml) at 37°C for one hour, anti-LRIG3 (2.2 µg/ml) overnight at 4°C. Sections were thereafter labelled, counterstained and mounted as described (103).

The sections were evaluated using a microscope with 40x objective. For cytoplasmic immunostaining, four staining intensities were recorded: 0, no or very low intensity; 1, weak intensity; 2, moderate intensity; and 3, strong intensity. Nuclear and perinuclear immunostaining were scored as 0, negative, less than 10% of cell stained or 1, positive, 10% or more of cells stained.

Cell proliferation was analyzed by a mouse monoclonal antibody MIB-1 recognizing the Ki-67 antigen, the proliferation index was reported as the percentage of immunopositive nuclei as described previously (146).

Statistical analysis (VI)

All statistical analyses were performed with SPSS 11.5 (SPSS Inc, Chicago, IL) for Windows software. Significance of associations and differences between groups were determined using Chi-square, Mann-Whitney, and Kruskal-Wallis tests. Multivariate analysis was performed using stepwise Cox-multivariate analysis to evaluate the predictive power of each variable independently of the others. Survival was demonstrated by Kaplan-Meier method, defined as the time between

operation and the patient's death or last official contact. Comparison between study groups was performed with the log-rank test. The significance level was set at $p<0.05$.

Ethical considerations

The studies were approved by the local ethical committees in Umeå, Sweden, and Tampere, Finland.

RESULTS AND DISCUSSION

1. The linearity and uniformity of real-time RT-PCR and expression of reference genes (I)

In our work, we first set up a quantitative real-time RT-PCR method for mRNA quantification. To evaluate this method, standard curves were generated with the iCycler iQ software by plotting the log of the DNA copy number against respective threshold cycles (Ct). The standard curves, covering 4-7 orders of magnitude of template concentrations, showed good linearity, with a correlation coefficient of 0.998 for each reference gene. The coefficient of variation at each concentration was <2% (0.24-1.94%), showing high uniformity of the real-time RT-PCR detection system. After evaluation of the real-time RT-PCR method, we analyzed three commonly used reference genes: GAPDH mRNA, β-actin mRNA, and 18S rRNA from ten normal human tissues. GAPDH and β-actin mRNA expression varied up to 26 and 38 fold among the different tissues. ANOVA analysis showed statistically significant difference in GAPDH and β-actin levels ($p<0.001$), while 18S rRNA expression level was relatively constant among the analyzed tissues ($p=0.2457$).

In our opinion, 18S rRNA appeared to be the best choice of reference gene for RNA quantification studies.

Proper selection of internal control gene is important when performing quantitative gene expression studies. GAPDH is one of the most commonly used control genes, although it has been documented that GAPDH mRNA levels are not always constant as shown in our studies and also by others (142; 146). Some authors routinely use β-actin as reference gene, while others do not support the idea to use β-actin (117). Alternatively, rRNA, which is the bulk of a cellular RNA, can be used as reference gene. We (I) and others (43) have found 18S rRNA to be superior over GAPDH and β-actin as internal control gene. To minimize the impact of variability in the expression of single reference genes, a geometric averaging of a set of reference genes has been recently proposed for normalising gene expression data (135). Further, it is important to stress that there is no universal reference gene

suitable for all experimental analyses, however, an optimal reference gene is one that is not influenced by the experimental condition.

2. The cloning and identification of LRIG gene family members LRIG1-3 (II, IV, V)

To identify human proteins, which may function as modulators of growth factor receptor-mediated signalling, we looked for human genes with similarities to Drosophila Kekkon-1. A previous report (124) had identified mouse Lrig1 (previously Lig-1) (GenBank accession no. NM_008377) with an overall structure similar to Kekkon-1. By using BLAST, we identified a human expressed sequence tag (EST) clone (AA293029) with high homology to mouse Lrig1. This EST sequence was then used to design probe and primers for real-time PCR as described above (**I**), which was used to screen a human brain cDNA library. This resulted in a clone with high homology to mouse Lrig1 without the start codon. The remaining 59 bp-coding region including the start codon was amplified by RT-PCR and sequenced from both glioma and brain RNA, giving identical nucleotide sequences. The combined sequence of 4763 nucleotides, covering the complete open reading frame and the 39-untranslated region, has been deposited in GenBank and given the accession no. AF381545 (**II**).

In a BLAST search for human LRIG1 homologs, we identified a human mRNA sequence (GenBank accession no. AB018349). This sequence had been deposited into GenBank as a brain mRNA for KIAA0806 protein. We cloned a corresponding cDNA from the same human brain cDNA library and the obtained clone had a nucleotide sequence spanning nucleotides 124–3519 of AB018349, including the complete coding sequence of KIAA0806. This corresponding gene and protein has been named LRIG2 (**IV**).

In order to find all possible human LRIG1 and LRIG2 homologs, we used BLAST to explore the human genome sequences in the GenBank and Ensembl databases. This search revealed two homologous predicted mRNA sequences (Ensembl ENSG00000170388 and GenBank accession no. NM_153377). These two sequences were apparently not derived from LRIG1 or LRIG2. Neither of the two predicted mRNAs appeared to encode full-length LRIG polypeptides. Primers and probe for real-time PCR were designed and used to screen the human brain cDNA library. A cDNA that had a

nucleotide sequence including both of the predicted mRNA sequences was identified, cloned and sequenced, and it appeared to encode a full-length LRIG polypeptide (GenBank accession no. AY505340) and was named LRIG3 (**V**).

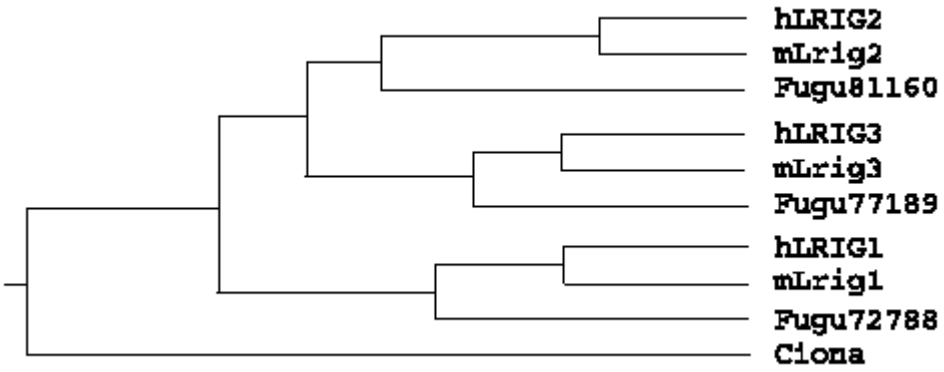
We also cloned mouse Lrig3 cDNA from a mouse brain cDNA library (**V**) and deposited it in GenBank (Accession no. AY505341). A BLAST search identified mouse Lrig2 (Accession no. XM_194114). So far, we have identified three human and three mouse LRIG gene family members.

3. Evolutionary history of the LRIG gene family (V)

To gain insight into the distribution and evolution of the LRIG gene family, we used BLAST to search for LRIG homologs in the sequenced genomes of human (Homo sapiens), mouse (Mus musculus), rat (Rattus norvegicus), puffer fish (Fugu rubripes), and an ascidian tunicate (Ciona intestinalis). For the alignment, amino acid sequences from the highly conserved IgC2.2 domain and the membrane proximal part of the cytoplasmic tail of LRIG1 were selected and used to search the different translated genomic sequences for homologous polypeptides (tBLASTn). In the human genome, both the IgC2.2 and the cytoplasmic sequences yielded three hits, which represented the LRIG1–3 genes at chromosomes 3p14, 1p13, and 12q13, respectively (**II, IV, V**). In the mouse genome, three hits were also recorded, representing the Lrig1–3 genes at chromosomes 6D2, 3F2+2, and 10D2, respectively. Similarly, in rat, three hits were recorded, at chromosomes 4q34, 2q34, and 7q22. This suggests that in mammals, the LRIG gene family is restricted to three paralogs, present in human, mouse, and rat (**V**).

The occurrence of LRIG homologs in the puffer fish F. Rubripes (**V**) was analysed by using the same conserved LRIG amino acid sequences for alignment and BLAST. Three hits were obtained in the Fugu genome and these hits were within the predicted Fugu genes SINFRUP00000072788, SINFRUP00000081160, and SINFRUP00000077189. Alignment with mammalian LRIG polypeptides revealed that the predicted Fugu polypeptides were homologous to the mammalian LRIG polypeptides along their entire lengths, however, they were lacking the parts corresponding to the N-terminal parts of mammalian LRIG polypeptides. In C. intestinalis, the IgC2.2 sequence yielded one hit, within the predicted gene 77.18.1. The corresponding predicted polypeptide was

homologous to mammalian LRIG polypeptides along its entire length but it was lacking the parts corresponding to the N- and C-terminal parts of mammalian LRIG polypeptides. A phylogenetic analysis of the LRIG polypeptides from human, mouse, Fugu, and Ciona revealed apparent paralogs of human LRIG1–3 in mouse and Fugu, while the Ciona polypeptide was more distantly related (**V**).



From the phylogenetic analysis it was apparent that the primordial LRIG gene underwent at least two gene duplications before mammals and teleosts diverged, resulting in the three modern-day LRIG paralogs found in human, mouse, and fish (**V**). The finding of an apparent LRIG gene in Ciona showed, on the other hand, that an ancestral LRIG gene was present in Chordata before urochordates and euchordates diverged, approximately 500 Myr ago. This follows a trend, which is that gene families with several vertebrate paralogs often have only one homolog in the urochordate C. intestinalis (31). The available data, thus, suggest an evolutionary history in which the ancestral LRIG gene was present in the deuterostome lineage and that the primordial gene underwent two duplications in the vertebrate lineage after the urochordate–vertebrate split and before the teleost–mammalian split.

4. Gene structure and genomic localization of the human and mouse LRIG genes (II, IV, V)

To identify the gene structures and genomic localizations of the LRIG genes, we performed FISH and BLAST analyses. By FISH analyses, human LRIG1 was shown to be located at 3p14 and human LRIG2 at 1p13 (**II, IV**). By BLAST analysis, LRIG3 gene was shown to be located at 12q13 (**V**).

The mouse Lrig loci were located in syntenic regions corresponding to the human LRIG loci, i.e., at mouse chromosomes 6D2, 3F2+2, and 10D2, respectively. The human LRIG1–3 genes and the mouse Lrig1–3 genes were composed of between 18 and 20 exons, of which the first 17 showed the same exon/intron structure. Human LRIG1 and LRIG2 and mouse Lrig1–3 contained a Kozak consensus sequence a/gxxATGg surrounding the translation initiation codon. The corresponding Kozak-like sequence in human LRIG3 was gxxATGa.

A literature study revealed that LRIG1-3 genes are localized on chromosome bands known to be altered in various human cancers (70). LRIG1 is located at chromosome 3p14, in an area where two other possible tumour suppressor genes have been suggested, one at 3p12 and FHIT at 3p14.2 (87; 138). This support our proposal that LRIG1 might act as tumour suppressor genes (**III**) in the genomic region 3p12–14 (130). LRIG2 is located at chromosome 1p13. Loss of the short arm of chromosome 1 is together with loss of heterozygosity of the long arm of chromosome 19, the most common genetic alteration found in oligodendrogliomas (68). Thus, LRIG2 could also be a potential tumour suppressor gene. LRIG3 is located on chromosome 12q13, a region that is amplified in a subset of glioblastomas (108). A specific molecular subtype of glioblastoma, which is characterized by up-regulation of contiguous genes on chromosome 12q13–q15, was recently described (95). Therefore, it is important to emphasize the potential role of LRIG1-3 in brain tumours.

5. Protein domain organization and their biological functions (II, III, V, VI)

The protein domain organization was predicted to be unique among LRR containing proteins with a signal peptide, 15 tandem leucine-rich repeats flanked by cysteine-rich N-flanking and C-flanking regions followed by three immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tail. (See figure below)

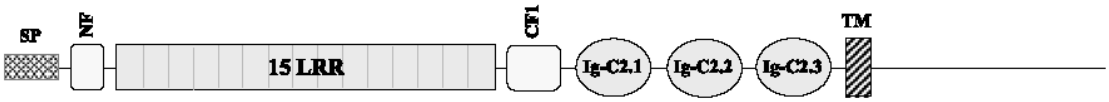


Figure 4. Domain organization of a LRIG protein

The LRIG gene family belongs to LRR superfamily. There are 252 proteins containing the leucine-rich repeats registered on the July 2003, NCBI 34 version of the human genome.

Because of the role of EGFR in cell growth and proliferation, its activity is tightly regulated. Attenuation of EGFR signalling can occur in a number of ways including receptor downregulation. In a separate study, our group has in collaboration with Yarden's group been able to show that LRIG1 hampers the activity of ErbB1-4 (47) by enhancing ubiquitylation of these receptors. The interactions between LRIG1 and the ErbB- receptors involve both the LRR domain and the immunoglobulin-like domains.

The LRR containing proteins are thought to be involved in various protein-protein interactions and some of LRR-containing proteins have been identified and demonstrated to play roles in pathogenesis of brain tumour (For more detail, please see Introduction). In short, GAC1, is amplified and over expressed in malignant glioma (2). Northern blotting and RT-PCR studies revealed reduced levels of LGI1 mRNA in glioma cell lines and malignant gliomas compared to normal brain tissue. An immunohistochemical study demonstrated a gradual down regulation of the LGI1 gene product with increasing grade of malignancy (14), as seen in our studies on glioma (**VI**). LGI1 was supposed to be a tumour suppressor gene in gliomas as we proposed for the LRIG family (**II, III, IV, and V**). Decorin, a biological ligand for EGFR (61), inhibits TGF-β released by glioma cells, which significantly enhances anti-glioma immune response in vivo (121). Other LRR containing protein family, such as SLIT family (28; 29) , NTRK (136) family and SLITRK (8) family have been studied and are supposed to play important roles in brain tumours. Thus, the LRR containing proteins, including LRIGs, seem to have important functions in the normal brain as well as in the pathogenesis of brain tumours. It is important to emphasize that LRIG1-3 belongs to a specific gene family with a domain organization that differs from the other LRR containing proteins, especially in the extracellular part, which contains 15 tandem LRR followed by 3 immunoglobulin-like domains. However, the exact function can obviously not be obtained directly from protein domain studies, and further work and analyses are needed.

6. The expression of LRIG1-3 in normal human and mouse tissue (II, III, V)

To examine the transcript size and tissue expression of the human LRIG genes, Northern blot analyses were performed. The sizes of LRIG1-3 mRNA were 5.5 kb, 4.8 kb and 5.1 kb, respectively.

To evaluate relative expression levels in a quantitative and more sensitive manner, we analyzed RNA samples from 26 human tissues and 10 mouse tissues by quantitative real-time RT-PCR. LRIG1-3 transcripts were detected at various levels in all tissues analyzed. The widespread tissue expression of the LRIG genes could reflect that the LRIG proteins have functions that are integral to most organs and many cell types. Notably, the expression patterns of the three LRIG paralogs were not identical. The highest expression was, for example, for LRIG1 in liver, brain, stomach, small intestine, and skeletal muscle; for LRIG2 in uterus, ovary, and skin; and for LRIG3 in stomach, thyroid, and skin. This showed that the expression of the three LRIG paralogs was differentially regulated. Differential regulation of expression could reflect differences in the functions of the LRIG paralogs, however, conclusions about functions cannot be drawn from expression data alone. A redundant function of the LRIG paralogs is also possible, which in fact is supported by the relatively mild phenotype of the reported Lrig1 gene knockout mouse strain (123).

The relative expression levels of respective LRIG ortholog appeared to differ between human and mouse, which could reflect either experimental artefacts or true species differences. Experimental artefacts due to RNA sampling are not unlikely, since the RNA samples were obtained from different commercial suppliers who could have used different protocols and procedures for their RNA isolation. There might also be individual and developmental differences in the expression of respective LRIG genes, which was not evaluated in the present study. Furthermore, our results differs from a previous report (124), in which, it was shown that mouse Lrig1 expression was predominantly in brain, and lower expression was detected in the thymus and heart. However, no expression was detected in the kidney, liver, lung, or small intestine (124). In brain the expression seems to be restricted to a small subset of glial cells, such as Bergmann glial cells of the cerebellum and glial cells in the nerve fibre layer of the olfactory bulb as found by Northern blot and in situ hybridization analyses (124). Recently, it was reported that LRIG1 was strongly expressed in the

epidermal basal cells and outer root sheath cells of hair follicles in normal human skin (123). One reason for the differences obtained might be due to that quantitative real-time RT-PCR method we used is more sensitive than Northern blotting and in situ hybridization used in the other study.

7. Subcellular localization of LRIG1-3 (IV, VI)

Eukaryotic cells have various subcellular organelles with their unique biochemical environments and specific functions. The subcellular location, thus, plays a crucial role for the function of a protein (99). In this work, we studied the subcellular location of LRIG-GFP fusion proteins in different cells and especially in glioma cells by confocal fluorescence laser microscopy (**VI**). LRIG1-GFP fusion protein was visualized and found in nuclear, perinuclear, cytoplasmic compartment and cell membrane. Immunoreactivity for LRIG1-3 could also be seen in nuclear, perinuclear and cytoplasmic compartment of positive glioma cells, showing a granular or a diffuse pattern (**VI**).

The subcellular localization of LRIG proteins might be biologically a significant, especially with regard to perinuclear localisation. Some reports indicate that the presence of perinuclear staining may reflect increases in protein synthesis, and processing, and thus increased function, or degradation. On the other hand, a functional protein usually travels across several subcellular locations to fulfil its function or to be modified or regulated before it is transported to proper subcellular location. PKC (46), for example, is a family of isozymes that translocate to new intracellular sites on activation. Subcellular mislocalization of specific protein may also imply mutation, such as, NPC1 and NPC2 in Niemann–Pick type C (NPC) disease (18). One candidate tumour suppressor, decorin (102), has been found to build up perinuclear aggregates and to be destroyed in ovarian tumour cell. Thus it is important to emphasize that LRIG can be localised to different subcellular compartments, and therefore, we found it of interest to elucidate the subcellular distribution of LRIG studies in human tissues and tumours.

8. The expression of LRIGs in cancer cell lines and human glioma (III, VI)

In paper **III**, LRIG1 gene expression was examined in different tumour cell lines and corresponding normal tissues by real-time RT-PCR. In many tumour cell lines, LRIG1 expression appeared absent

or was down regulated compared to corresponding normal tissues. These results as well as the observation that LRIG1 is down-regulated in renal cell carcinoma (130) indicate that LRIG1 could be tumour suppressor gene in human. This motion was supported by newly published results from a collaborative study between our group and Gur and co-workers (47) in which it was shown that LRIG1 hampers the activity of ErbB1-4. Moreover, LRIG proteins share overall structure of the drosophila cell surface protein Kekkon-1 which functions in Drosophila as an inhibitor of EGFR-mediated signalling. However, further studies are justified to clear out the significance of these observations made in cell lines and to elucidate the explicit role of LRIG1 as a potential negative regulator of oncogenesis.

In order to evaluate if glioma cells express these genes, we analyzed RNA from 6 glioma cell lines by quantitative RT-PCR. All three LRIG1-3 mRNAs were expressed in the glioma cells, however, at various levels. We also investigated whether the LRIG mRNAs were expressed in 6 primary gliomas, and whether the levels were altered in primary tumours compared to normal brain tissues from the same patients. The expression of LRIG1-3 mRNA was seen in all tumour specimens analyzed. Although there was a relatively large variation of expression in the tumour tissues compared to the normal brain specimens, we were not able to find any consistent difference in expression level of LRIG1-3 between tumour tissues and their corresponding normal brain tissues. The results showed that there was a considerable heterogeneity in the expression of LRIG1-3 in the gliomas compared to normal tissues.

To further study the expression of LRIG1-3 in human astrocytic tumours, 404 cases on a TMA were included in an immunohistochemical study (**VI**). Immunoreactivity for LRIG1-3 could be seen in nuclear, perinuclear, and cytoplasmic compartments of positive tumour cells, showing a granular or a diffuse appearance. The perinuclear staining of LRIG1-3 had a significant inverse correlation with WHO grade ($p<0.001$, $p<0.003$, $p<0.001$, respectively). Thus, the presence of perinuclear staining of LRIG proteins indicated low or less malignant WHO grade. LRIG3 cytoplasmic staining was also significantly inversely correlated with WHO grade ($p<0.003$). High-grade tumours had relatively low immunoreactivity of LRIG3 in their cytoplasm compared to low-grade tumours. In fact, few glioblastoma (grade IV) tumours displayed a positive LRIG staining, which was especially evident

for LRIG3. Similar observation has been seen for LGI1 with a gradual down-regulation with increasing grade of the malignancy (14).

The relationship between LRIG1-3 and proliferation index (Ki-67 index) in the whole material of 404 tumours was evaluated. LRIG3 perinuclear staining was in accordance with the results above significantly related to proliferation index; tumour samples with LRIG3 perinuclear staining ($p<0.05$) and cytoplasmic staining ($p<0.05$) demonstrated lower proliferation index. However, cytoplasmic staining of LRIG 2 was associated with a relative higher proliferation index. This difference is hard to explain, but the observation could be due to technical issues or may suggest real differences in function between the various LRIGs.

Overall survival was analyzed in 178 patients for whom survival data was available (WHO grade II: 22 cases; grade III: 21 cases; grade IV: 135 cases). We did not include grade I cases in our analysis because there were too few patients (n=6) and only one patient had died. Perinuclear staining of LRIG2 and LRIG3 showed a significant correlation with survival, with a better survival of those patients with perinuclear staining of the glioma cells ($p<0.01$ and $p<0.0001$, respectively). Perinuclear staining of LRIG1 demonstrated the same trend, however, this relation was not statistically significant ($p= 0.1034$). Neither overall staining, nor cytoplasmic or nuclear staining of the LRIGs displayed any significant relationship to survival. Most importantly, by Cox forward stepwise regression analysis, LRIG3 perinuclear staining was found to be in addition to histological grade an independent prognostic factor.

This is the first investigation to evaluate the expression of the LRIG gene family in brain tumours. All of LRIG1-3 were shown to be expressed at the RNA and protein levels in astrocytic tumours, however, with a large heterogeneity in expression levels and at various subcellular locations. The immunohistochemical staining pattern of 404 analyzed astrocytic tumours displayed a correlation between perinuclear expression of LRIG1-3 and histopathological grade, proliferation index, and survival rate. The histopathological and clinical significance of these results need to be further evaluated.

CONCLUSIONS

A quantitative real-time RT-PCR method was established, which showed good linearity and reproducibility. The housekeeping gene 18S was used as the reference gene for the further analyses.

The LRIG gene family had three vertebrate paralogs widely expressed in human and mouse tissues and a homolog in ascidiacea. The LRIG proteins had a signal peptide, 15 tandem leucine-rich repeats with N- and C- cysteine-rich flanking regions followed by 3 immunoglobulin-like domains, a transmembrane domain and a cytoplasmic tail.

Human LRIG1 was shown to be located at chromosome 3p14, LRIG2 at 1p13, and LRIG3 at 12q13. All these gene regions are known to be altered in various tumours, including brain tumours.

LRIG1-3 mRNA were detected in all human and mouse tissues analysed suggesting that the LRIG proteins may have universal roles in human tissues.

The LRIG proteins were expressed in various subcellular locations; nuclear, perinuclear and cytoplasmic compartments.

In astrocytic tumours, perinuclear staining of LRIG1-3 and cytoplasmic staining of LRIG3 showed a significant inverse association with the WHO grade. Perinuclear staining of LRIG3 indicated lower proliferation index of the tumour. Perinuclear staining of LRIG2-3 showed better prognosis than other cases, and LRIG3 perinuclear staining was in addition to histopathological grade an independent prognostic factor.

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