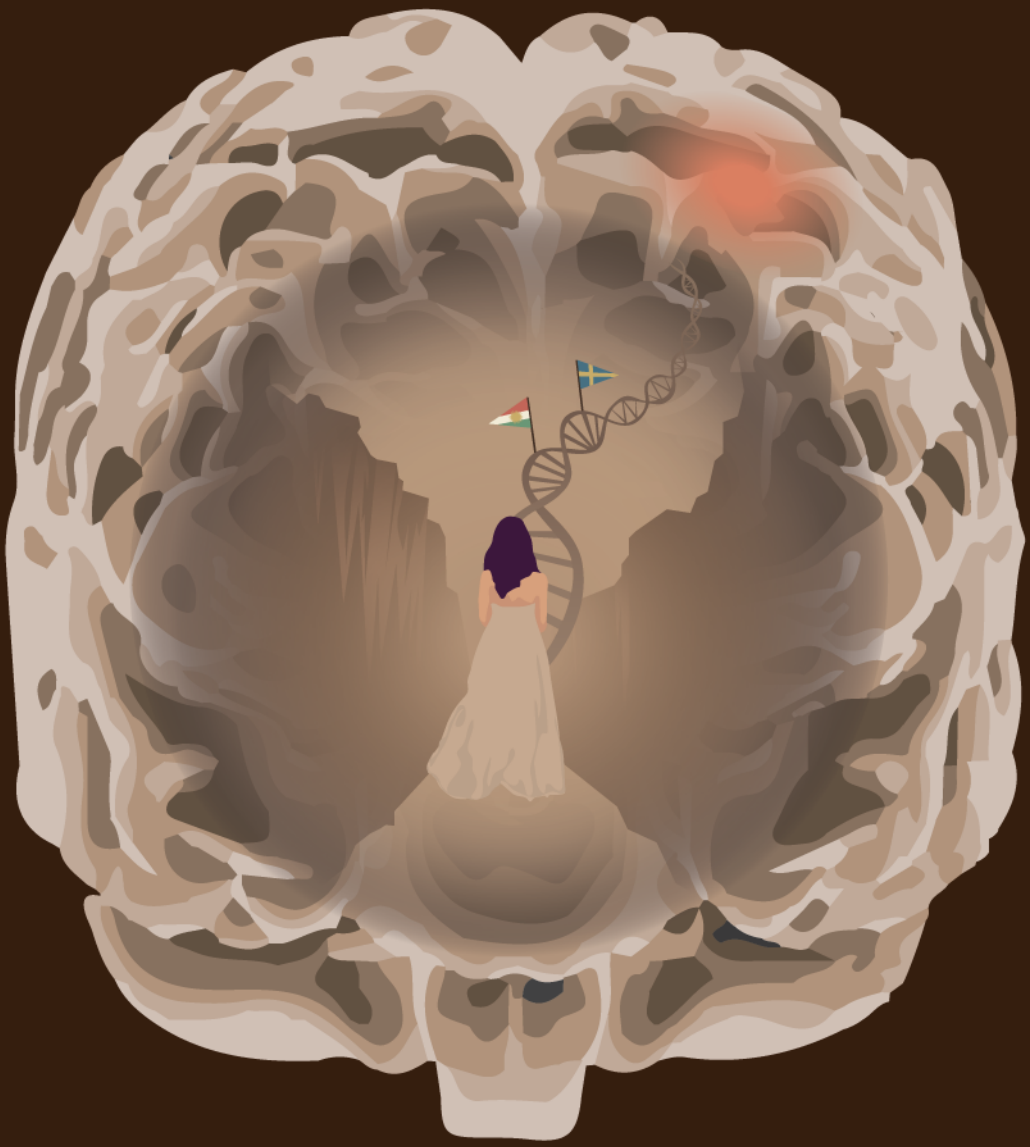

Genotype-Phenotype Studies in Brain Tumors

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Umeå University, Umeå 2013

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To Mum and Dad

Together

"one evening, a blind man,
a deaf man and a mute
sat together happy on a park bench.
The blind man saw with the eyes of the deaf man.
The deaf man listened with the ears of the blind man.
The mute understood both by reading their lips.
And all three together, simultaneously captured the scent of the flowers."

Sherko Bekas, 1940-2013

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Abbrevations

A	Adenine
AKT	v-akt murine thymoma viral oncogene homolog 1
ARF	Alternative reading frame
ASCAT	Allele-Specific Copy number Analysis of Tumors
ATM	Ataxia telangiectasia mutated
BRAF1	Breast cancer susceptibility gene 1-interacting protein
C	Cytosine
CCDC26	Coiled-coil domain containing 26
CEPH	Council on education for public health
CEU	Utah residents with ancestry from Northern Europe
CDK	Cycline dependent kinase
CDKN2A/B	Cycline dependent kinase inhibitor 2 A/B
CI	Confidence Interval
CNNE	Copy number neutral event
CNS	Central nervous system
CT	Camputed tomography
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
EPL	Early progenitor-like
ER	Estrogen receptor
ErbB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
FISH	Fluorescence in situ hybridization
G	Guanine
G-CIMP	Glioma CpG island methylator phenotype
GWAS	Genome-Wide Association Studies
Gy	Gray
HD	Homozygous deletion
IDH1	Isocitrate dehydrogenase 1
IHC	Immunohistrochemistry
LD	Linkage disequilibrium
LOH	Loss of heterozygosity
LRIG	Leucine-rich repeats and immunoglobulin-like domains
MAF	Major allele frequency
MET	Met proto-oncogene
MGMT	O^6-methylguanine DNA methyltransferase
MLLT10	Myeloid/lymphoid or mixed-lineage leukemia
MRI	Magnetic Resonance Imaging
NF1/2	Neurofibromatosis type ½
NB	Neuroblastic
OR	Odds ratio
PDGFR	Platelet derived growth factor receptor

PG	Preglioblastoma
PHLDB1	Pleckstrin homology-like domain family B member 1
PI3K	Phosphoinositide 3-kinase inhibitor
PR	Progesterone receptor
PTEN	Phosphate and tensin homolog
RB1	Retinoblastoma protein 1
RET	Ret proto-oncogene
RNA	Ribonucleic acid
RR	Relative risk
RTEL1	Regulator of telomere elongation helicase 1
RT-PCR	Reverse transcription polymerase chain reaction
SNP	Single nucleotide polymorphism
T	Thymine
TCGA	The cancer genome atlas
TERT	Telomerase reverse transcriptase
TMA	Tissue microarray
TP53	Tumor protein 53
VEGFR	Vascular endothelial growth factor
WHO	World health organization

Abstract

Meningioma and glioma are the most common primary brain tumors, but their etiologies are largely unknown. Although meningioma is usually benign, their intracranial location can lead to lethal consequences, and despite progress in surgery, radiotherapy, and chemotherapy the prognosis for patients with glioma remains poor. The only well-established environmental risk factor for meningioma and glioma is ionizing radiation, but this only accounts for a very small number of cases. Evidence for inherited predisposition to meningioma and glioma is provided by a number of rare inherited syndromes such as Li-Fraumeni syndrome and neurofibromatosis. However, collectively these diseases account for only a small proportion of the twofold increased risk of brain tumors seen in first-degree relatives for meningioma and glioma patients. It is very possible that much of the excess familial risk is a consequence of co-inheritance of multiple low-risk genetic variations. With this in mind, the aims of the studies in this thesis were to discover genetic risk variants influencing the probability of acquiring the disease and to identify the association between risk variants on the tumor phenotype. Thus these studies seek to contribute to a better understanding of the etiology of these diseases.

The genes involved in brain tumor progression were selected in a case-control study coordinated in Sweden, Denmark, and Finland as a part of the INTERPHONE study. To identify genetic variants influencing meningioma risk, a comprehensive tagging of the selected genes was performed. We identified nine risk variants in *EGF* (epidermal growth factor), *ERBB2* (epidermal growth factor receptor 2), and *LRIG2* (leucine-rich repeats and immunoglobulin-like domains 2) genes. However, these findings could not be confirmed in another larger independent dataset. In addition, the study identified a correlation between LRIG2 protein expression and ER (estrogen receptor) status when analyzed with different parameters. In a separate immunohistochemical (IHC) study with a larger sample of meningioma patients from Finland, the same correlation between LRIG2 and ER status was observed.

In an effort to explore the potential association between reported germline risk variants and somatic genetic events, matched tumor and blood samples from glioma patients were analyzed by SNP (single nucleotide polymorphism) array. The results identified correlations between *EGFR* (epidermal growth factor receptor) gene variants and loss of heterozygosity at the *EGFR* locus as well as homozygous deletion at the *CDKN2A/B* (cyclin-dependent kinase inhibitor 2A/B) locus. To further study the relationship

between germline risk variants and tumor phenotype, the same patient material was used and analyzed by three different techniques: SNP array, IHC, and fluorescence in situ hybridization (FISH). The results revealed *EGFR* risk variants effecting copy number variation of the *EGFR* gene and the expression of the IDH1 (isocitrate dehydrogenase 1), and p53. Further comparison between different techniques such as SNP array and FISH analysis revealed the difficulty in achieving consistent results with different techniques.

To summarize, the glioma studies show a link between genotype and phenotype where genetic risk variants in the *EGFR* gene were found to be associated with specific somatic aberrations. These associations are biologically interesting because EGFR is involved in multiple cellular processes including cell division, migration, adhesion, differentiation, and apoptosis. Additional studies of the direct functional role of these observations need to be conducted to elucidate the molecular mechanisms underlying the identified association between germline gene variants and somatic aberrations. For the meningioma studies, no significant risk variants influencing the disease were found but a correlation between LRIG2 and ER status was observed. This result suggests a potential role for the LRIG protein in the pathogenesis of meningioma, but more studies are needed to confirm this hypothesizes.

Populärvetenskaplig sammanfattning på svenska

Hjärntumörer är den elfte vanligaste cancerformen i Sverige där drygt 1 200 personer diagnostiseras varje år. Det finns flera olika hjärntumörer men meningiom som utgår från hjärnhinnorna, och gliom som uppkommer ur gliaceller i hjärnans stödjevävnad är bland de vanligaste tumörtyperna. Meningiom är i allmänhet godartade, medan gliom vanligtvis är elakartade. Det faktum att dessa tumörer är lokaliserade i hjärnan samt att de växer in i omkringliggande vävnad gör att det kan vara svårt att ge botande behandling med operation, strålning och cytostatika.

Orsaken till varför man får tumörer i hjärnan är fortfarande till stora delar okänd. Den enda väl etablerade riskfaktorn för meningiom och gliom är joniserande strålning. Ärftliga faktorer anses ha betydelse för en del fall av hjärntumörer och det finns sällsynta ärftliga syndrom såsom Li-Fraumeni och neurofibromatos som ger ökad risk för hjärntumörer. Dock står familjär ärftlighet och ärftliga syndrom för endast en liten del av orsaken till dessa tumörers uppkomst.

Människans arvmassa (DNA) är uppbyggd av baserna, adenin (A), tymin (T), cytosin (C) och guanin (G) och dessa baser binds parvis ihop till två dubbelsträngar som inrymmer alla våra gener. Vår arvmassa är nästan helt identisk och skiljer sig endast med 0.1% mellan olika individer och dessa olikheter brukar definieras som genetiska variationer som är fördelade över hela genomet. Om basparen på den givna platsen i arvsmassan är exempelvis C och G så kan det vara kombinerat antingen; CC (homozygot), GC (heterozygot) och GG (homozygot). Med detta i åtanke har det föreslagits att dessa nedärvda variationer kan påverka utveckling av sjukdom och hur man svarar på behandling. Syftet med denna avhandling har varit att upptäcka genetiska riskvarianter som påverkar sannolikheten att utveckla hjärntumörer samt att identifiera sambandet mellan nedärvda riskvarianter och genetiska förändringar i tumören.

Gener som man sedan tidigare vet är involverade i utvecklingen av hjärntumörer, analyserades i en studie samordnad i Sverige, Danmark, och Finland som en del av tidigare internationell fall- kontrollstudie (INTERPHONE). För att identifiera genetiska varianter som påverkar risk att få meningiom gjorde vi jämförelser mellan friska och sjuka individer. Vi identifierade nio riskvarianter i generna; EGF (epidermal growth factor), ERBB2 (epidermal growth factor receptor 2) och LRIG2 (leucine-rich repeats and immunoglobulin -like domains 2). Dock kunde inte dessa fynd

bekräftas i ett annat större oberoende dataset. Studien identifierade en korrelation mellan LRIG2 proteinuttryck och ER (östrogenreceptor) status vilket bekräftades i en separat studie med ett större antal meningiom patienter från Finland, där samma korrelation mellan LRIG2 och ER status observerades.

För att förstå sambandet mellan nedärvda riskvarianter och somatiska händelser i tumören, analyserades tumör och blodprover från samma gliompatienter. Resultaten påvisade ett samband mellan genetiska variationer i EGFR (epidermal growth factor receptor) genen och förlust av heterozygositet vid ett specifikt område av EGFR genen samt homozygot deletion vid ett specifikt område i CDKN2A/B (cyklinberoende kinashämmare 2A/B) genen. För att ytterligare undersöka förhållandet mellan riskvarianterna och deras effekt på tumören, analyserades samma patientmaterial med tre olika tekniker, SNP array, immunohistokemi (IHC), och fluorescens in situ hybridisering (FISH). Resultaten visade att riskvarianter inom EGFR genen bidrar till varierande kopieantal av EGFR genen och uttryck av proteinerna IDH1 (isocitrate dehydrogenas 1), och p53. Ytterligare jämförelser mellan SNP array och FISH analys visade att det är svårt att uppnå jämförbara resultat med olika tekniker.

Sammanfattningsvis, så visar studierna på gliom ett samband mellan nedärvda genetiska varianter och genetiska förändringar i tumören, där genetiska riskvarianter i EGFR genen är associerade med specifika somatiska avvikelser. Dessa förändringar är biologiskt intressant eftersom EGFR är inblandad i flera viktiga cellulära processer. Ytterligare studier av den direkta funktionella rollen för dessa iakttagelser bör genomföras för att klarlägga de molekylära mekanismerna bakom det identifierade sambandet. Studierna på meningiom påvisade inga signifikanta riskvarianter som påverkar sjukdomens utveckling, men ett samband mellan LRIG2 protein och östrogenstatus observerades. Detta resultat tyder på en potentiell roll för LRIG proteinet vid uppkomst och utveckling av meningiom, men ytterligare studier behövs för att bekräfta denna hypotes.

Original papers

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

I. Genetic variants in EGF, EGFR, ERBB2, LRIG2, LRIG3 and meningioma risk *(Manuscript)*. **Ghasimi S**, Wibom C, Brännström T, Haapasalo H, Eray M, Dobbins S, Henriksson R, Ahlbom A, Auvinen A, Collatz-Laier H, Feychting M, Johansen C, Kiuru A, Houlston R, Melin B, Andersson U.

II. Immunohistochemical analysis of LRIG proteins in meningiomas: correlation between estrogen receptor status and LRIG expression (J Neuro-oncol, 2012, 108(3):435-41). **Ghasimi S**, Haapasalo H, Eray M, Korhonen K, Brännström T, Hedman H, Andersson U.

III. EGFR gene variants are associated with specific somatic aberrations in glioma (PLoS One, 2012, 7(12):e47929). Wibom C, **Ghasimi S**, Van Loo P, Brännström T, Trygg J, Lau C, Henriksson R, Bergenheim T, Andersson U, Ryden P, Melin B.

IV. Genetic risk variants in the EGFR regions are associated with copy number variation in the EGFR gene as well as IDH1, and p53 protein expression *(Manuscript)*. **Ghasimi S**, Wibom C, Dahlin A, Brännström T, Golovleva I, Andersson U, Melin B.

Introduction

War on cancer was declared by US President Richard Nixon in the beginning of the 1970s with the hope of increasing the research needed to improve our understanding of cancer biology and to develop more effective cancer treatments (legislative.cancer.gov/history/phsa/1971). In those days, scientists were optimistic that they would identify a single gene or a handful of genes that would allow them to eliminate this disease. We now know that cancer comprises hundreds of unique diseases and is much more complex than originally thought. The variability occurring between tumors of the same type illustrates that there is a great heterogeneity even within a particular type of tumor, and this is a major obstacle facing current research efforts. It is very common that two patients having the same cancer, same diagnosis, and same treatment can have very different outcomes. This indicates that in-depth observation of the tumor itself is needed to develop personalized treatments. One way to approach this goal is to study the human genetic variations that can enable a more detailed understanding of the impact of genetics in cancer progression.

Deoxyribonucleic acid (DNA)

The complete set of genetic information, the genome, is encoded in the DNA sequences inside the cell nuclei. DNA strands consist of two long polymers made up of four different nucleotides. Each nucleotide is composed of the sugar molecule deoxyribose, a phosphate group, and one of the four nitrogen basis adenine (A), thymine (T), cytosine (C), and guanine (G) [1]. The nucleotides are ordered in a linear sequence in a predetermined way and the two molecules form complementary strands that are entwined to form a double helix in which A pairs with T and C pairs with G [2, 3]. In each cell, these large molecules are organized into structures called chromosomes. In humans, the complete genome is organized in 23 pairs of chromosomes. The portions of DNA that contain the genetic information are called genes, and these will be read and transcribed by a series of enzymes to form RNA from the DNA. The RNA, in turn, can be translated into amino acids to form proteins with different functions. This is the central dogma of molecular biology as stated by Francis Crick (Figure 1) [2].

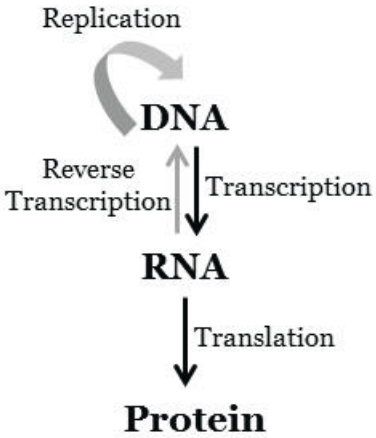

Figure 1: An illustration of the central dogma describing how the genetic material (DNA) can be copied (replicated) and how the genetic message can be transformed to protein (transcription and translation). There is no information flow from the proteins back to nucleic acid, but RNA can be transcribed back to DNA with help of reverse transcription. This figure is redrawn from the textbook Genetik written by Stefan Escher and Anssi Saura, 2009.

Sequence variation

The genomes of all humans differ by only 0.1%. The most abundant genetic variations are single nucleotide polymorphisms (SNPs). These are germline variations scattered throughout the genome in both coding and noncoding regions, and they occur approximately every 1,200 base pairs (bp) in the human genome [4]. An SNP is a single base locus in the genome that occurs in the population as different variants, for example, some individuals can have a cytosine base (C) at the locus and other individuals can have a guanine base (G), thymine base (T) or adenine base (A). The fact that the human genome contains one parental and one maternal copy of each gene means that we can inherit two alleles and observe a number of alternative forms. If the two alleles are the same, the genotypes of the individual will be homozygous (e.g., CC) and if the two alleles are different, the individual will be heterozygous (e.g., CG) for any given SNP locus (Figure 2). The frequency of different alleles can vary between different populations at specific locations in a chromosome, and the minor allele frequency (MAF) is the frequency of rare alleles in a specific population.

Variations in the genome occurs due to mutation events in the DNA sequence, either caused by copying errors during cell division or by the exposure to the environmental factors such as toxic compounds, radiation, or viruses. Variations that arise in the germline are passed on to the offspring while somatic mutations only affect the individual organism.

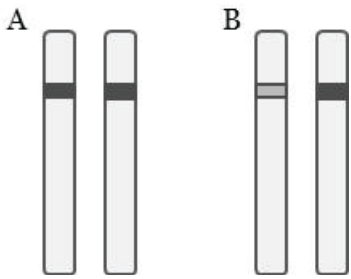

Figure 2: A simplified illustration of a chromosome locus with homozygous (A) and heterozygous (B) alleles.

Cancer genomes often show numerous DNA sequence changes, ranging in size from a single base change (point mutation) to insertions or deletions of large chromosomal fragments, and even whole genome duplications [5-7]. For this reason, genotypes in cancer are no longer limited to CC, GG, or CG, but also can be, e.g., C, GGG, CCG, or CGGG. One way to delineate genomic aberration in cancer genomes is using SNPs as markers in study approaches.

Genetic studies with genetic variants as markers

Cancer is a multifactorial disease that develops through the complex interaction of genetic factors such as copy number variation, epistatic interaction, and modifier effects as well as numerous environmental factors. Many types of genetic variants, including SNPs, are predisposing factors in many different multifactorial diseases, and numerous methods and approaches have been developed to study such complex disorders. Genetic variants have been widely applied as markers, and variant analysis for identifying disease genes commonly relies on one of two different approaches: linkage studies in family pedigrees and association studies, the candidate gene approach or genome wide association studies (GWAS). Association studies are generally performed to determine whether genetic variants increase the susceptibility to develop a specific trait, and these

studies require two groups of individuals: the cases (the group that displays the trait of interest) and the controls (the healthy group). Samples with a significant over or under representation of a variant between the groups will indicate a possible association between the marker and the investigated trait (Figure 2). However, it is very important that the groups resemble each other in all other aspects apart from the investigated trait otherwise misrepresentation of the study groups could lead to a phenomenon referred to as population stratification [8].

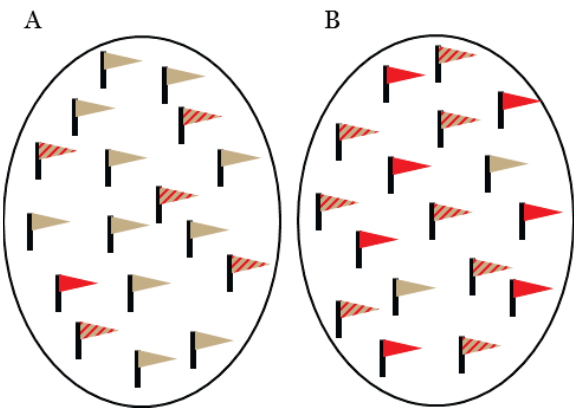

Figure 3: An illustration of an association study presenting the case group (A) having a genetic variant (the grey ("alleles") flags) over-represented compared to the control group (B).

Development of high-throughput genotyping platforms has provided new opportunities to efficiently scan the entire genome. GWAS is a powerful approach for identifying common, low-penetrance loci for multifactorial diseases without any prior knowledge of location or function. GWAS can provide general insights into the allelic architecture of cancer susceptibility, and it highlights the relevance of particular pathways in different cancers that can pinpoint disease mechanisms and suggest new drug targets. However, as mentioned previously, multifactorial diseases are complicated to map and the functional variants are difficult to identify. The reason for this could be that they are caused by multiple genetic factors with low effect sizes in combination with environmental features, or the causal alleles might also be rare and differ between individuals or among populations. In addition, the true functional variant might not be the one measured in the analysis but rather a nearby allele in linkage disequilibrium (LD) with the SNP [9]. LD is the association of alleles in different loci, not necessarily on

the same chromosome, and this can also be used in association studies to investigate a candidate gene using fewer SNPs because alleles in strong LD with each other will be inherited together [10].

Understanding genetic variation and its functional consequences is challenging, but such insights have the potential to provide a greater understanding of the cause of cancer as well as provide valuable information for the development of more effective treatments. Genetic information offers the ability to identify individuals with a high risk of developing certain cancers, and this predictive power can be used to develop guidelines for early detection and personalized treatment. The goal of such research would be to prevent the disease from ever developing or to at least provide screening methods that can detect the cancer early before it becomes more aggressive.

Brain tumors – The disease

There are two major types of brain tumors, *primary* and *secondary*. Primary brain tumors arise de novo in the brain and secondary brain tumors develop in another part of the body and then metastasize to the brain. Each type of tumor has its own biology, treatment, and prognosis. Even benign tumors can be lethal due to their location in the brain, the ability to infiltrate locally, and their tendency to transform to higher graded. A person with a brain tumor can experience various signs and symptoms. As the tumor grows the pressure on the surrounding brain tissue increases and this pressure can affect blood flow as well as damage brain cells or cause swelling of the brain. Common symptoms of a brain tumor include headaches and seizures and they might cause a person feel nauseated or vomit. Depending on the location of the tumor, there are other signs and symptoms such as changes in personality and loss of speech or problems with vision.

Epidemiology

Every year about 1,200 people in Sweden are diagnosed with a primary brain tumor. According to the World Health Organization (WHO) classification, primary brain tumors consist of 128 subgroups, but meningioma make up of approximately 20% and high-grade glioma make up 30% of all brain tumor cases in adults (http://www.cancercentrum.se). The incidence for meningioma is more common among females, with a female to male ratio of 2:1, and males are more likely to be diagnosed with glioma than females with a male to female ratio of 1.5:1. Brain tumors affect more Caucasians than those of African descent across all age groups [11] (www.cbtrus.org/2011). Differences between countries have also been found with a higher incidence, for example, in Australia, Canada, Denmark, Finland, New Zealand, and the US and a lower incidence, for example, in Rizal in the Philippines and Mumbai in India [12]. However, geographical variations have to be interpreted cautiously because the criteria and registration of brain tumors is not always consistent.

Morphological classification

The central nervous system (CNS) is lined with a set of three membranes – the dura, the arachnoid, and the pia mater – collectively referred to as the meninges. The dura is the outermost membrane and also the thickest and most durable. The arachnoid is the middle membrane. The pia mater is the innermost as well as the most delicate membrane and adheres to the CNS surface. Meningioma is a tumor arising from the meninges, developing from

the arachnoid cap cells forming the outer layer of the arachnoid membranes. This tumor type is classified histologically according to WHO criteria (Table 1) [13] where the majority of meningioma is benign (grade 1). Benign meningioma has a variety of histological subtypes, but these do not differ in behavior or prognosis. The most common subtypes are meningothelial, fibrous, and transitional meningioma. Other subtypes include psammomatous, angiomatous, microcystic, secretory lymphoplasmocyte, rich, and metaplastic meningioma. Atypical meningioma (grade II) is characterized by increased mitotic activity. Brain invasion is an independent criterion for atypical meningioma. Malignant and anaplastic (grade III) meningioma show a male predominance [14], and it has been suggested that these observations might be related to higher proliferation indices in male patients [15].

Table 1: Simplified schedule of the most common subtypes of meningioma and glioma classifications according to the 2007 WHO classification.

	WHO designation Glioma	Grade	WHO designation Meningioma	Grade
	Astrocytic tumors			
Secondary Glioblastoma	Pilocytic astrocytoma	I	Meningothelial	I
			Fibrous	
	Diffuse astrocytoma	II	Transitional	
	Anaplastic astrocytoma	III	Psammomatous	
Primary Glioblastoma	Glioblastoma	IV	Angiomatous	
			Microcystic	
	Oligodendroglial tumors			
	Oligodendroglioma	II	Secretory lymphoplasmocyte	
	Anaplastic oligodendroglioma	III	Metaplastic	
	Oligoastrocytoma	II	Atypical	II
	Anaplastic oligoastrocytoma	III	Anaplastic	III

Glioma was first classified in 1979 and updated in 2007 according to the WHO classification [13]. Grade I lesions are benign with a slow proliferation rate and include pilocytic astrocytoma. Grade II tumors are also a slow

growth rate with a high degree of cellular differentiation, however these tumors are prone to malignant progression, including diffuse astrocytoma, oligodendroglioma, and oligoastrocytoma. Grade III tumors are characterized by a higher cellular density, atypia, mitotic cells and the group include anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma. The most malignant and frequent subtype, grade IV tumors, display microvascular proliferations and necrosis in addition to grade II features and this subtype group include glioblastoma, which can either be primary or secondary (Table 1) [13, 16].

Diagnostics and treatment

Contrast-enhanced CT (computed tomography) and MRI (magnetic resonance imaging) are the most common methods to diagnose brain tumors, however, there is no special feature in MRI that differentiates benign meningioma from malignant. The first-line treatment for both meningioma and malignant glioma is surgery. For meningioma, depending on the tumor location, complete resection is usually the goal [17]. The most reliable predictive factor of meningioma recurrence is the extent of surgical resection [18]. Due to the infiltrative nature, it is virtually impossible to surgically resect a malignant glioma tumor completely. Nevertheless, surgical resection has been shown to have a significant impact on survival [19]. Reduction of the tumor mass improves perfusion due to lower interstitial pressure, and this in turn enhances delivery of chemotherapeutic agents and improves oxygenation that facilitates radiotherapy. Surgery is also necessary to obtain material for histopathological diagnosis.

Three to four weeks after surgery, patients with high-grade glioma are currently treated with concomitant radiotherapy of 2 Gray (Gy) per fraction given daily, five days a week, for a total dose of 60 Gy and with Temozolomide (an alkylating/methylating agent that alkylates the DNA, usually the N-7 or -6 position of guanine, and leads to DNA damage and cell death) [20]. This is followed by adjuvant Temozolomide alone. Patients expressing the 0-6-methylguanine-DNA-methyltransferase (MGMT) enzyme have a poorer response to Temozolomide, but in other patients the MGMT gene is silenced through methylation and this promotes Temozolomide response [21]. There are phase II trials showing that a combination of Bevacizumab (a monoclonal antibody against vascular endothelial growth factor (VEGF) and Irinotecan (an inhibitor of topoisomerase I) is an effective treatment for recurrent malignant glioma [22, 23]. Results from these studies have led to investigations into new therapeutic strategies where

Bevacizumab and Irinotecan are combined as a first-line treatment, but these trials are still ongoing.

About one third of meningioma cannot be totally resected due to location, large size, or proximity to critical structures. Radiotherapy-based treatment is considered the primary treatment option for patients with critically located tumors [24]. In tumors treated by surgical resection, radiotherapy has become an important management tool that can be used both as an adjunct to surgery or as treatment against recurrence [25]. Postoperative radiotherapy is considered standard care regarding atypical and anaplastic meningioma. Chemotherapy is rarely used in treatment of meningioma and is mainly only applied in very aggressive cases.

Meningioma and glioma – The etiology

The etiology of brain tumors is still not very well understood. It is estimated that about 5% of the cases are strongly hereditary. However, most of the genetic abnormalities that effect brain tumors are not hereditary but instead result from somatic mutations occurring throughout the lifetime. Acquired somatic mutations can be due to internal factors (such as hormones) or external factors (such as ionizing irradiation). Most tumors evolve through multiple changes resulting from environmental factors and/or a combination of hereditary and environmental factors, but there is still much that is unknown about the cause and nature of these tumors.

Familial aggregation

Studies have revealed a twofold increase risk for first-degree relatives developing meningioma or glioma [26, 27]. A segregation analysis has shown that familial aggregation could explain only 5% of all glioma cases and that a recessive model fits about 2% of all cases [28]. However, demonstration of familial aggregation does not necessarily prove a genetic etiology since families share common environments though aggregation is often among the first indicators that genetic susceptibility might play a part in the pathogenesis of a complex disease. One study showed that the familial aggregation of glioma was best fit by a multifactorial inheritance model [29] suggesting that a multifactorial inheritance pattern might account for some brain tumors.

Genetic syndromes

There is strong evidence that some inherited genes influence the risk of developing a meningioma or glioma. Meningioma can originate spontaneously or be part of hereditary syndromes such as NF2, Li-Fraumeni, Turcot syndrome, Gardener, von Hippel-Lindau syndrome, Cowden syndrome, Gorlin syndrome, and multiple endocrine neoplasia type I (MEN I) [30]. These syndromes are rare and can only account for a small portion of all brain tumor cases [31, 32]. Syndromes associated with glioma are Li-Fraumeni Turcot syndrome, retinoblastoma, neurofibromatosis type 1 and type 2 (NF1 and NF2) [11, 33]. All these syndromes provide an important starting point for identifying candidate genes and pathways for glioma and meningioma genesis.

Ionizing irradiation

The only well-established exogenous risk factor for developing meningioma and glioma is ionizing irradiation. The effects of ionizing radiation after the exposure of the Japanese population to atomic bomb irradiation in Nagasaki and Hiroshima have been studied in cohorts of survivors [34-36]. These cohorts all have an increased incidence of all brain tumor types, including meningioma and glioma. However, the first study describing an association between ionizing radiation and brain tumor risk was a study on Israeli children treated to the scalp with radiation therapy for tinea capitis. These children were treated with doses from 1 Gy to 6 Gy and follow-up indicated an increased incidence of meningioma (relative risk (RR) 9.5; 95% CI 3.5–25.7) and glioma (RR 2.6; 95% CI 0.8–8.6) and for both tumor types the risk was dose-dependent and elevated after a latency of ≥ 30 years [37-39]. Studies have also shown increased incidence of glioma in children that received prophylactic central nervous system (CNS) irradiation for acute lymphoblastic leukemia [11].

Mobile phone

An extensive debate concerning risk factors for malignant brain tumors lies in the use of mobile phones. An international study (INTERPHONE) comprised 2,409 meningioma and 2,708 glioma cases with matched controls from 13 countries [40]. This study showed no association between meningioma risk or glioma risk and the use of mobile phones, even after 10 years of usage. A reduced odds ratio (OR) was found for regular mobile phone users, but it has been speculated that this reduction in risk could be due to recall bias among the participants or methodological limitations of the study [41]. Several other studies, however, showed an association between mobile phone use and risk of developing several types of tumors, including glioma [42-45]. A recent long-term Danish cohort study showed no association between mobile phone use and brain tumors [46]. The current consensus of evidence is that mobile phone use does not increase the risk of developing a brain tumor. However, with the exponential increase in the duration of use of mobile devices it is important to continue the investigations to allow for a latent period of several decades in the development of tumors.

Allergies and infections

Allergies have been associated with decreased risk for glioma and there is some evidence for an association with meningioma [47-53]. However, whether the underlying cause of the association is due to the host immune system, external agents, or to the medication against asthma and allergies has not been clarified [33]. In most studies the levels of IgE, an objective assessment tool for allergic conditions, was inversely correlated with the risk of glioma [54-56].

Occupations

Determining a relation between a certain occupation and the risk for brain tumors is difficult because in most professional areas workers are exposed to more than one possible risk factor [12] and, therefore, it is hard to determine what the total risk is for all these factors combined. A case-control study from Germany found no significant associations between brain tumors and occupational risk factors in workers in the chemical, agricultural, transport, electric/electronic, construction, and metalworking industries [44]. Another study noted a higher incidence of glioma in professionals in the fields of information technology, farming, finance, medicine, and management and a decreased incidence in childcare workers [57]. Despite numerous studies, no consistent risks have been isolated for any chemical or group of workers apart from those in the petrochemical and oil industry. In these circumstances, no specific agent has been identified and the possibility of multiple exposures has to be considered. A possible association between glioma and physiological stress was also investigated, and this study found that major life events in a 5-year period prior to diagnosis constituted a risk factor for glioma [58]. Studies trying to find a connection between socio-demographic variables and glioma found inconclusive results [59].

Hormonal status and exogenous hormones

Meningioma and glioma incidence varies according to gender, and an etiologic role for hormones (both exogenous and endogenous) has been hypothesized. A review of the current knowledge about hormonal status and meningioma and glioma, published in 2010, included 15 articles concerning these tumor types, and the final conclusion of the study was that female sex

hormones are associated with an increased risk of meningioma and a decreased risk of glioma [60]. However, in the EPIC study no correlation was observed between female hormones and glioma [61]. Associations between hormones and meningioma risk have been suggested by a number of findings, including an increased incidence with the presence of estrogen, progesterone, and androgen receptors in some meningioma. Associations between breast cancer and meningioma have also been suggested. In addition, studies have indicated that meningioma often change in size during the luteal phase of the menstrual cycle and pregnancy and have shown the regression of multiple meningioma in patients following cessation of estrogen agonist [62-69].

Meningioma and Glioma - The biology

Molecular genetics for meningioma

The most common genetic changes observed in meningioma involve the neurofibromatosis 2 (*NF2*) gene on chromosome 22.q12.2 [92]. *NF2* mutations are found in up to 60% of all sporadic meningioma [93], and most mutations are small insertions, deletions, or nonsense mutations affecting splice sites. The protein product of the *NF2* gene is called merlin, and *NF2* mutations associated with meningioma usually result in a truncated merlin protein [94, 95]. The frequency of *NF2* mutations is similar in WHO grade I and II meningioma suggesting that these mutations represent important initiation events rather than affecting tumor progression [96]. In contrast, differences in the frequency of *NF2* alterations have been observed based on variant histology, and higher rates are seen in fibroblastic, transitional, and psammomatous than in meningothelial or secretory grade I meningioma [96-98]. This suggests that *NF2* alterations play a role in the mesenchyme-like phenotype of meningioma.

Although the relevant candidate genes have yet to be identified, chromosomal alterations, including losses of *1p*, *6q*, 9p, *14q*, and *18q* have been associated with atypical or anaplastic histology. Gains or amplifications involving *1q*, *9q*, *12q*, *15q*, *17q*, and *20q* have similarly been associated with higher grade of meningioma [99-101]. Alterations on *9p21* have been found to represent losses of the tumor suppressor genes *CDKN2A* (*p16 INK4a*), *p14ARF*, and *CDKN2B* (*p15 INK4b*) [102], and significantly shorter survival is seen in patients with anaplastic meningioma carrying *9p21* deletions [103].

Molecular genetics for Glioma (Grade II and Grade III)

Approximately 50%–80% of grade II and grade III astrocytoma, as well as oligodendroglioma and mixed oligoastrocytoma, have mutations in the *IDH1* or *IDH2* genes [104-107]. These patients tend to be young individuals with better survival compared to individuals with glioma and wild-type *IDH* genes [104]. The most common genetic characteristic of oligodendroglioma (70%) and mixed oligodendroglioma (50%) is co-deletion of chromosome arms 1p and 19q [108] that result from a 1;19 translocation [109, 110]. The majority of diffuse astrocytoma arise because of early concomitant mutations in *IDH1* and *TP53* [106, 111], and this provides evidence that these

alterations are among the earliest genetic abnormalities in the development of low-grade astrocytoma [106, 112]. In a comprehensive study of astrocytoma, three distinct subclasses of low-grade astrocytoma were proposed within the proneural subtype, including neuroblastic (NB), early progenitor-like (EPL), and preglioblastoma (PG) [113]. *IDH* mutations were common in the NB and EPL subclasses [104, 113]. Point mutations in *TP53* and strong nuclear p53 staining were detected in the EPL subclass, but they were absent in the NB subclass. In contrast, pre-regulation of platelet derived growth factor receptor (PDGFR) expression and phosphatase and tensin homolog (PTEN) methylation was observed in EPL [113] and a gain of *8q* was seen in both the EPL and NB subclasses [113]. The PG subclass was characterized by wild-type *IDH* and also showed a more similar molecular profile to the primary glioblastoma including an association with *EGFR* amplification, a loss of *PTEN*, and increased activity of the PI3K/AKT pathway [113].

Molecular genetics for Glioblastoma

Studies in the 1990s revealed two major pathways of glioma progression characterized by EGFR amplification and TP53 alteration [114, 115]. In 2008, The Cancer Genome Atlas (TCGA) described a comprehensive study of the molecular characteristics of glioblastoma. This confirmed the previous findings but also added some new genes that had been identified. The study revealed that glioblastomas frequently acquire gains of chromosomes 7 and 19, losses of chromosomes 10 and 13, *EGFR* amplification, *PTEN* mutation, *CDKN2A/B* deletion, *TP53* mutation, *NF1* mutation, *PDGFRA1* mutation, and *MDM2* amplification [116]. Additional studies have found *IDH1* and *IDH2* mutations at a low frequency in glioblastoma [116, 117].

Because glioblastoma have a heterogeneous appearance, four subtypes have been described including the proneural, mesenchymal [116-118], neural, and classical subtypes [117]. The classical subtype is defined by gain of chromosome 7 and loss of chromosome 10, and these tumors often have *EGFR* amplification and *PTEN* mutations [117, 118]. *TP53* mutations are rarely found in the classical subtype [117]. The mesenchymal subtype acquires low levels of NF1 expression and deletion and/or *NF1* point mutations [117, 119]. Genes in the tumor necrosis factor pathway are highly expressed in the mesenchymal subtype, which might explain the higher overall frequency of necrosis in tumors of the mesenchymal subtype [117]. The neural subtype is not as clearly defined as the others due to its intermediate expression pattern that falls between the mesenchymal and proneural subtypes [120] but similarly to the classical subtype, it often features *EGFR* amplification [117].

The proneural subtype makes up the majority of secondary glioblastoma. Younger ages of onset and longer survival times for patients with proneural glioblastoma have been reported [83, 117, 118]. Studies have shown that the primary molecular genetic characteristics of the proneural subtype are point mutations in *IDH1* and *IDH2* and alterations of *PDGFRA* [113, 117, 119]. Amplification of *PDGFRA* is seen in all molecular subtypes, but it is detected at a higher rate in the proneural subtype [117].

Loss of heterozygosity of *17p* and *TP53* mutations is more frequent in the proneural subtypes while loss of chromosome 10 and gain of chromosome 7 is much less common in the proneural subtype [117]. Glioma CpG Island Hypermethylation (G-CIMP) has been observed in many cancers and also reported in glioma [83, 121, 122], and promoter methylation seems to be more frequent in secondary glioblastoma [111, 123]. Studies have also shown hypermethylation of the MGMT promoter in all glioma subtypes [123, 124] regardless of G-CIMP status.

Genetics and association studies

Because SNPs determine attributes such as how a person looks or develops a disease, it is suggested that genetic variants might help to elucidate cancer etiology, predict disease occurrence, and predict response to chemotherapy. Unlikely lifestyle exposure, SNPs do not change during the process of carcinogenesis and, therefore, could be suitable biomarkers.

In 2009, GWAS identified five genetic variants associated with glioma risk [70, 71]. These risk variants were located in independent regions within or near telomerase reverse transcriptase *(TERT)* on chromosome 5p15.33, coiled-coil domain containing 26 (*CCDC26)* on chromosome 8q24, *CDKN2B* on chromosome 9p21.3, pleckstrin homology-like domain containing 26 *(PHLDB1)* on chromosome 11q23, and regulator of telomerase elongation helicase 1 *(RTEL1)* on chromosome 20q13.3. Two additional risk variants within *EGFR* on chromosome 7p11.2 [72-74] have been identified. More recently, one GWAS-based study identified a risk variant, rs7837822, in the polyadenylation site of *TP53* that is associated with glioma [75]. A GWAS showed that a genetic variant, in the polyadenylation site of TP53 (rs78378222) being associated with glioma [75]. After the initial GWAS efforts have been made by resequencing and fine mapping to explore the genomic area. The genetic variant of TP53 has been validated by a fine mapping study, observing association both for glioblastoma and other gliomas [76]. Studies based on RNA transcripts have suggested that the rs78378222[C] variant giving an impaired termination and polyadenylation of the *TP53* transcript [77]. However, no studies have yet published of correlation to protein expression. An imputation effort combined with next generation sequencing observed the genetic variant rs55705857, near *CCDC26*, that are associated with oligodendroglioma with co-deletion of 1p and 19q [78] as well as with glioma with *IDH* mutations [74]., within 8q24.21, near CCDC26, being associated to IDH mutated tumors and the histopathological subtype oligodendroglioma. Nearly 40% of the patients with oligodendroglial tumors and glioma with *IDH* mutations carried this risk variant [79]. This genetic SNP is completely conserved throughout mammalian evolution [79] and resides within the CCDC26 locus which has been associated in several inflammatory pathways [80]. Genetic risk variant, within the 11q23.3, at PHLDB1 locus has also been associated to IDH mutated tumors [81]. As the PHLDB1 (11q23.3) and CCDC26 (8q24) genetic variants have been strongly associated with IDH1 and 2 mutation [82], it is likely that these SNPs may also be associated with a hypermethylated phenotype [83], indicating an interaction between germline variants and the acquisition of specific somatic alterations. A genetic tagging study analyzed

the association between a genetic variant, rs2736100, located at the TERT locus with telomere length and attrition rate at age 50 and 60 in 900 individuals. The results revealed an association between the risk variant and telomere length especially at older age [84], indicating that TERT genotypes may have higher impact of disease at older age. In addition, another study showed a strong association with glioma at higher age regardless of glioma subtype [85]. While the genetic risk variants in *CCDC2* and *PHLDB1* strongly have been associated with low-grade glioma [86, 87], risk variants in *RTEL1* and TERT have been associated with high-grade glioma [71, 87]. In addition, CDKN2A/B has been linked to low-grade glioma and oligodendroglioma [78, 87]. A germline genetic variant, rs4947986, within *EGFR* intron/exon boundary 7 has been associated with glioma risk [73]. Moreover, a large GWAS identified two germline variants in *EGFR* gene being associated with glioma risk. One risk variant, rs11979158, located in *EGFR* intron 1 and another risk variant, rs2252586, within a telomeric region to *EGFR* were observed [74] and both risk variants were significant regardless of tumor grade.

The associations between genetic risk variants and meningioma phenotype have not yet been studied to the same extent as in glioma. However, there are studies that have identified susceptibility loci for meningioma. In the large INTERPHONE study, genetic variants and meningioma risk were investigated and 12 risk variants were identified in DNA repair genes [88]. An association between meningioma risk and three variants in the gene that encodes breast cancer susceptibility gene 1-interacting protein 1 (BRIP1) at chromosome 17q22 was found. The *BRIP1* gene is involved in the repair of DNA double-strand breaks, and defects in this gene have been linked to breast cancer susceptibility [89]. Association between four variants in the *ATM* gene, a member of the phosphatidylinositol-3 kinase family involved in DNA break repair, and meningioma risk have been seen [90]. A recently published GWAS identified a variant at chromosome 10p12.31 near *MLLT10* that is associated with meningioma risk [91].

Genes in the present association studies

Epidermal growth factor receptor (EGFR), and its ligand (EGF)

The epidermal growth factor receptor *(EGFR)* gene, located on chromosome 7p12, encodes a transmembrane glycoprotein on the cell surface. The EGFR protein belongs to the ErbB family of growth factor receptors that also includes ErbB2, ErbB3, and ErbB4 [125]. Binding of a ligand such as epidermal growth factor (EGF) to EGFR leads to dimerization of the receptors in the ErbB family – either as homo-dimers (two receptors of the same type) or hetero-dimers (two different receptors from the ErbB family). After dimerization, the EGFR becomes activated and internalized and initiates a cascade of downstream signaling molecules that further activate different pathways leading to transcription in the nucleus and, finally, to cell proliferation. This pathway is an important regulator of cell growth, survival, proliferation, and differentiation [125].

Studies have shown that wild-type *EGFR* is amplified, overexpressed, and/or mutated in approximately 30%–60% of glioblastoma patients [116, 117]. It has also been noticed that the amplification is often associated with up regulated expression of the deletion mutant *EGFR* variant III form (*EGFRvIII*). This unique genetic variant consists of an in-frame deletion of 267 amino acids in the extracellular domain of the *EGFR* gene (exons 2–7) leading to a truncated protein. EGFRvIII receptors lack the extracellular domain and are unable to bind a ligand, but they remain constitutively active [126] and are associated with a poor survival of glioblastoma patients [127, 128]. However, the prognostic role of *EGFR/EGFRvIII* in the glioblastoma setting is not clear. Some studies showed no effect of *EGFR* amplification on patient survival, and other studies have reported a poor prognosis in younger patients and in those with anaplastic glioma [129, 130]. More specifically, immunohistochemical and reverse transcriptase polymerase chain reaction (RT-PCR) analyses revealed that an increase of constitutively active *EGFRvIII* mutant expression occurs in approximately 30%–60% of glioblastoma patients, but no expression of this mutant is observed in the normal adult brain or any other tissues [127, 128, 131, 132]. Studies have also found overexpression of both EGFR and ErbB2 receptors [133, 134] and the ligand EGF [134, 135] in meningioma.

Leucine-rich repeats and immunoglobulin-like domain (LRIG)

The protein family, leucine-rich repeats and immunoglobulin-like domain (LRIG) involves three members: LRIG1, LRIG2, and LRIG3. These proteins consist of 15 leucine-rich repeats, three immunoglobulin-like domains, a transmembrane domain and a cytosolic tail whereas they are similar in the extracellular domain (Figure 3). This structure suggests a function involving protein-protein interaction. All members of this protein family have shown expression to variable degree in all tissues analyzed [136-138] and it has been suggested that subcellular localization of the LRIG protein may be biologically important [139, 140].

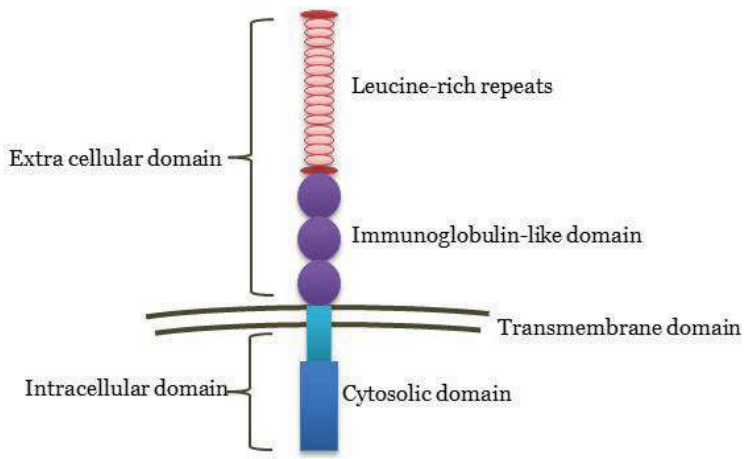

Figure 3: Schematic structure of an LRIG protein. The extracellular part of the protein is the conserved region and there is great variation in the intracellular part. This illustration is based on a study by Holmlund et al. 2004 [138].

LRIG1, the gene located at chromosome 3p14 [137] have been shown to down regulate the EGFR family members, the MET receptor, and RET receptor signaling [141-143] and it has been suggested as a tumor suppressor gene where its expression has been linked with a good prognosis and a better patient survival in epithelial cancers [144-147]. *LRIG2* is located at chromosome 1p13 [138] and *LRIG3* at chromosome 12q13 [136]. LRIG2 and LRIG3 expressed in the perinuclear area of astrocytoma have been associated with better patient survival [148], while LRIG2 expressed in the cytoplasmic area in oligodendroglioma and uterine cervical carcinomas have

been associated with poor survival [144, 149]. Interestingly, a newly published study revealed that *Lrig2*-deficient mice had an increased spontaneous mortality, transiently reduced growth rate, and protection against *PDGFB*-induced glioma suggesting that *LRIG2* promotes glioma and regulates growth factor signaling in a manner distinct from that of *Lrig1* [150].

Cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B)

The CDKN2A/CDKN2B gene products (p16INK4A and p15INK4B, respectively) are involved in cell cycle control by inhibiting the CDK4 and preventing the activation of cyklin-dependent kinase inhibitors (CDK) kinases by cyclin D, since the CDK4 / cyclin D1 complex phosphorylates the RB1 protein and activates genes involved in the G1 to S transition (Figure 4) [151]. However, when p16INK4a binds to CDK4 it inhibits the CDK4/cyclin D1 complex and thus inhibits the G1 to S transition [151]. Therefore, loss of normal RB1 function might result from altered expression from any of the p16INK4a/CDK4 / RB1 pathways. Studies have shown that *CDKN2A* and *CDKN2B* often are homozygous deleted in glioblastoma [152]. A pilot project of TCGA showed mostly in primary glioblastoma that 78% of the genetic alterations in the RB1 signaling pathway were homozygous deletions in *p16INK4a* [116]. Alterations in the p16INK4a/CDK4/RB1 pathway seem to be rare in oligodendroglioma, but frequent (65%) in anaplastic oligodendroglioma [153].

TP53

The *TP53* gene encodes protein 53 (p53) that plays a role in several cellular processes, including the cell cycle, cellular response to DNA damage, cell death, and cell differentiation [154]. Following DNA damage, TP53 is activated and the p53 protein induces transcription of genes such as *p21* [151, 155]. Mutations in *TP53* have commonly been detected in astrocytoma [156]. *TP53* mutations occur more often in secondary glioblastoma than in primary glioblastoma [157, 158], and it has been suggested that *TP53* mutations in glioblastoma can occur through different mechanisms [157].

Isocitrate dehydrogenase 1

The isocitrate dehydrogenase 1 (*IDH1)* gene at 2q33 encodes the isocitrate dehydrogenase 1 (IDH1) enzyme [159] that catalyzes the decarboxylation of isocitrate into α-ketoglutarate thereby reducing NADP to NADPH. Mutations

of *IDH1* are almost exclusively heterozygous missense mutations at codon 132. The most common mutation is a single base transition substitution of arginine for histidine, the so-called R132H mutation, that accounts for about 90% of all mutations [160]. This mutation results in a gain of function that allows the enzyme to directly catalyze α-ketoglutarate to (R)-2-hydroxyglutarate (Figure 4) [161]. Recent comprehensive DNA sequencing analyses of primary glioblastoma tumors [152] also identified somatic mutations in *IDH1* that occur in 12% of all glioblastoma patients. *IDH1* mutations are enriched in secondary glioblastoma cases and in younger individuals and are coincident with increased patient survival [104, 162, 163]. Higher *IDH1* mutation rates are seen in grade II and III astrocytoma and oligodendroglioma [162, 163] suggesting that *IDH1* mutations generally occur in the progressive form of glioma rather than in de novo glioblastoma. Mutations in the related *IDH2* gene are less frequent and are generally non-overlapping with tumors containing *IDH1* mutations [104, 163]. A study found that all glioma with 1p/19q co-deletion were associated with *IDH1* or *IDH2* mutations [164]. This, and the fact that *IDH1* mutations are predominantly seen in secondary glioblastoma, suggests that *IDH1* mutation might be a precursor to subsequent progression of glioma.

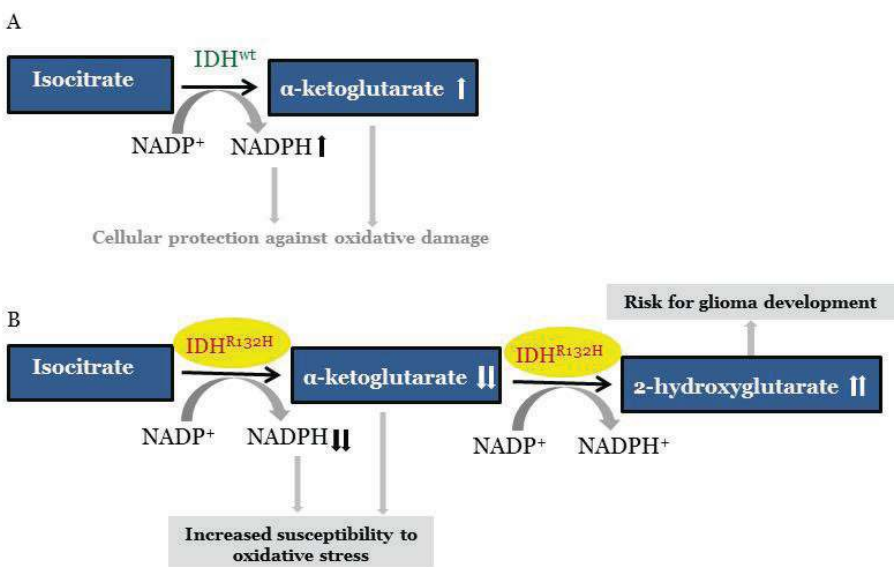

Figure 4: Illustration of normal (wild-type) IDH activity (A) versus mutant IDH activity in glioma tumorigenesis (B). This figure is redrawn and simplified from Nikiforova et al 2011 [165].

Aims of the present studies

The overall aim of this thesis was to investigate the influence that genetic risk variants have on the risk of developing brain tumors and their effects on the tumor phenotype. The specific aims of the individual manuscripts are given below.

Paper I

- Identify genetic variants in selected genes (*EGF*, *EGFR*, *ERBB2*, *LRIG2*, and *LRIG3*) related to meningioma risk
- Investigate the potential association between the significant risk variants in *LRIG2* with LRIG2 protein expression and other parameters
- Investigate the association between LRIG protein expression and gender, tumor grade, progesterone receptor (PR) status, and estrogen receptor (ER) status.

Paper II

- Characterize the protein expression of LRIG1, LRIG2, and LRIG3 in meningioma
- Evaluate possible associations between LRIG proteins and meningioma histological subtypes, gender, PR status, and ER status.

Paper III

- Investigate if reported germline risk variants are associated with somatic aberration of glioma tumors

Paper IV

Investigate the association between reported risk variants and their effect on glioma tumor phenotype.

Patients and Materials

Paper I

Paper I was based on three population studies conducted in Sweden, Denmark, and Finland that have previously been included as part of the INTERPHONE study* [40]. Blood samples from 382 meningioma cases and a total of 1,135 matched controls to each case in terms of age, sex, and region of residence, with a mean age of 53 years and 52 years, respectively, were used in the study. Seventy-seven of the Finnish patients were genotyped, and for 44 of these patients paraffin-embedded brain tumor tissues were available for immunohistochemical analysis. This made it possible to investigate the interaction between genotype and phenotype.

In addition, for validation of our significant results an independent German dataset based on 961 meningioma cases and 811 controls, with a mean age of 60 years for both groups, was used [91].

Paper II

In paper II, we used tumor samples from a previously presented study [67] based on patients who underwent surgery for intracranial meningioma at the Tampere University Hospital in 1989-1999. A total of 409 cases were included in this study, consisting of 324 female and 85 male with a median age of 59 years at diagnosis. The tumor material consisted mainly of Grade I tumors and only 22 cases with Grade II tumors whereas 10 of these cases were recurrent tumors.

Paper III and IV

Papers III and IV were based on glioma patients diagnosed at Umeå University Hospital from 1995 to 2008. A total of 197 patients were diagnosed, but only 108 of the patients with matched blood and tumor samples were available where 95 of these patients were included in Papers III and 91 patients in IV. Of the 95 patients in Paper III, 81 patients with sufficient material were further analyzed. For paper IV all the 91 patients remained for further analysis. A total of 70 identical patients were used in both studies (Figure 5).

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* The INTERPHONE study is an international set of case-control studies focusing on the association between mobile phone use and brain tumor risk. The materials were collected between 2000 and 2004 to determine whether mobile phone use increases the risk of brain tumor and if radio frequency emitted by mobile phones is tumorigenic. This study was a collaboration between 16 centers from 13 countries: Australia, Canada, Denmark, Finland, France, Germany, Israel, Italy, Japan, New Zealand, Norway, Sweden, and the UK [39].

For validation of the significance of our findings, we used an independent dataset available from TCGA. Matched tumor and blood samples from 334 glioblastoma patients was downloaded, however, only 300 were eligible for use as a validation set due to sample mix-ups and failed probes.

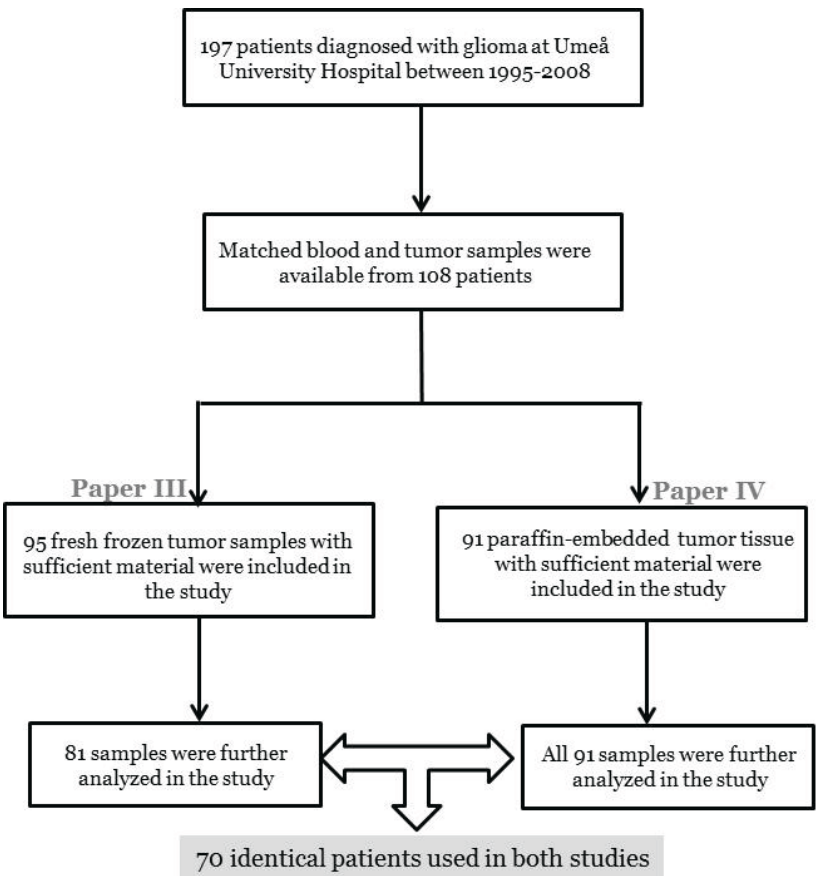

Figure 5: Flow chart for inclusion of patients in Paper III and Paper IV.

Ethics

The use and handling of blood samples, tissue samples, and patient data in Papers I, III, and IV were approved by the Ethical Committee of Umeå University. Paper II was based on a collaboration with a Finnish group and the use of tumor samples in that study was approved by the Ethical Committee of Tampere University Hospital.

Methods

Immunohistochemistry (I, II and IV)

Immunohistochemistry (IHC) is a method used to detect and locate proteins in tissue sections based on the specific binding of antibodies. Sections of formalin-fixed, paraffin-embedded tissue specimens are obtained and mounted on a glass slide that is subsequently incubated with a primary antibody specific to the protein of interest. Thereafter, a secondary antibody that is specific to the primary antibody is applied. The secondary antibody is conjugated to a fluorescent dye that is visible under UV light. This technique is an effective way to detect where a given protein is located within the tissue examined. However, it is important that the primary antibody is well validated because the success of IHC depends on being able to show that the staining corresponds to the protein of interest.

Tissue Microarray (II)

Tissue microarray (TMA) technology allows rapid visualization of molecular targets in thousands of tissue specimens at the same time. TMA consists of cylindrical core samples from up to one thousand fixed and paraffin-embedded tissue samples arrayed at high density on a block. Up to 300 consecutive sections can be cut from each TMA and probed with detection reagents. This allows the same control tissues to be placed directly on the actual study slide to help improve the specificity and sensitivity of IHC. In addition, reproducibility of the staining reaction, as well as the speed and reliability of the interpretation, is improved because all the tissues are on the same slide. TMA requires less tissue per assay than traditional whole-section analysis but might not provide the entire tissue profile. This can be a problem in certain heterogeneous cancers where small cores might not be representative of the entire tumor.

Single nucleotide polymorphism selection and genotyping (II)

SNPs were selected from the dbSNP, HapMap, and SNPper databases. Haploview software was used for identification of tag SNPs and LD for covering genes of interest with a setting of minimum r^2 of 0.9 and a minor allele frequency of 5% in a HapMap CEPH (CEU: Utah residents with ancestry from Northern Europe) were used. For SNPs that were used as a surrogate marker for validation in another independent data set, a minimum r^2 of 0.8 were assessed. R^2 is an important measurement showing correlation between two SNPs. A high r^2 indicates a high correlation, at the maximum r^2

of 1 means that the two SNPs are in perfect LD with each other, and most likely to be inherited together. An advantage with Haploview software is that it can accurately infer haplotypes and estimate recombination probabilities between adjacent loci, however, its disadvantages is computationally intensive when considering recombination for a large number of loci.

For genotyping, DNA was extracted using conversational methods and quantified using PicoGreen whereas genotype validation was performed on 14 family trios with available HapMap consortium. For internal concordance, 90 samples including SNPs genotyped were tested twice. In addition, three separate DNA samples were analyzed in quadruplicate on each plate that was used, for monitor the analytical variability.

SNP array (III)

DNA was extracted from blood samples and corresponding brain tumor tissue, and this was used as the substrate for genotyping with Illumina HumanOmni1-Quad BeadChips. The DNA was amplified, fragmented, and annealed to locus-specific bead types during the hybridization step. One bead type corresponded to each allele per SNP locus. After hybridization, allelic specificity was conferred by enzymatic base extraction and products were subsequently fluorescently stained. The great advantage with this technique is that it allowed us to determine both the copy number status and the genotype of more than a million SNPs. Because this technique has been primarily used for genetic association studies, it is also useful for analyzing gains and losses of genetic material in human tumors. The disadvantage of the technique could be that even if it covers the entire genome it might not cover genes of interest at a dense level, especially genes that might not yet be well studied.

Fluorescence in situ hybridization (IV)

Fluorescence in situ hybridization (FISH) is used for detecting and locating chromosomal abnormalities. The basic elements are a DNA probe and a target sequence. Prior to hybridization, the DNA probe is labeled by incorporation of a fluorophore. The labeled probe and the target DNA are denatured to yield single-strand DNA sequences and combined to allow the annealing of complementary DNA sequences. In the final step the signals are evaluated by fluorescence microscopy.

This technique makes it possible to study chromosomal aberrations in non-dividing cells, which is useful for the visualization of chromosomal aberrations directly. However, the FISH technique also has its limitations

because it cannot detect loss of heterozygosity or chromosomal rearrangements.

Statistics

Association analysis between SNPs and meningioma risk in **Paper I** was performed using logistic regression analysis. For each genotype SNP, odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were estimated by comparison to individuals homozygous for the common allele in logistic regression models.

Association between genetic risk variant, gender, tumor grade, ER status, and PR status with LRIG1, LRIG2, and LRIG3 protein expression was evaluated using Fisher's exact test and X^2-test depending on the number of categories. This was applied in both **Paper I** and **Paper II**, and a *p*-value < 0.05 was considered significant.

In **Paper II**, the subcellular distribution of LRIG proteins was evaluated using the Kruskal–Wallis test (continuous variables), and the results were presented in bare plots. Fischer's exact test and X^2-test (categorical variables) were used for comparison between protein expression and other parameters such as gender and histological subgroups.

In **Paper II**, the tumor cell content and tumor cell ploidy was estimated with the Allele-Specific Copy Number (ASCAT) algorithm [166]. Correction for multiple testing was used in this study. The HumanOmni1 BeadChip contains about 1 million SNPs scattered throughout the entire genome, and permutation was used to correct the results because of the large number of SNPs tested. In this test, the SNPs are randomly assigned to a group and then *p*-values are calculated. The lowest *p*-value in each group is saved and the procedure is repeated a number of times (in this study, a total of hundred times). The lowest *p*-values will be plotted on a graph with a normal distribution curve, and the mean value will be the lowest average *p*-value expected with a random division of the groups. A cut-off of 99% is used, and this gives a 0.5% false positive rate in the random test [167].

In **Paper IV**, Fisher's exact test and X^2-test (depending on the numbers of categories) were used for evaluating the association between different markers and genetic risk variants. The cut-off for significance was $p < 0.05$.

Results and Discussions

Meningioma – Paper I and II

Risk variants associated with meningioma

To identify genetic variants influencing meningioma risk 382 meningioma cases were compared with 1135 controls, in **Paper I**. Genes associated with brain tumor progression were selected (*EGF*, *EGFR*, *ERBB2*, *LRIG2*, and *LRIG3*) and analyzed for determination of association with risk of meningioma. Results revealed a total of nine significant genetic variants being associated with meningioma risk where six SNPs were observed in the *EGF* gene, one SNP in the *ERBB2* gene, and two SNPs in the *LRIG2* gene. For minimizing false positive findings, the significant genetic risk variants in *EGF* and *LRIG2* were validated in another independent German case-control data set. The risk variant in the *ERBB2* gene could not be validated in the independent data set due to the r^2 for the surrogate marker was < 0.8. However, none of the observations found in **Paper I** could be confirmed in the independent data set. Studies have previously identified statistically significant associations with genetic variants in other genes, e.g. *BRIP1* and *ATM,* and meningioma risk [88, 90]. Moreover, same independent data set used for validation in **Paper I** recently published an identified variant at 10p12.31 near *MLLT10* associated with meningioma risk [91]. The reason for our finding could not be confirmed in the independent data set could be due to different reasons. First, both studies were case-control studies. However, they were different in terms of approach where our study was focused on candidate genes and the independent data set on GWAS. Different approach studies in turn led to use of different methods where the SNP array used for GWAS did not cover the whole variability in the genes we had in our study, instead surrogate markers were used with an r^2 of ≥ 0.8. This could be the weakness of the GWAS as it is not fully dense at the specific regions of interest and may underestimate risk variants. Second, an important explanation could be the frequency of genotypes which varies noticeably in different populations usually by different ethnic and different geographical regions [168] where it has clearly been shown that differences in allele frequency are most prominent in the western parts of Finland and some differences in different parts of the Nordic countries have been found [169]. Third, the statistical power estimates are closely linked to the sample size, as the larger sample size improves greatly. However, with all this in mind, it is important to emphasize that robust genetic risk variants appear regardless of the study design or the differences in populations, and this has been clearly shown in glioma studies where candidate gene analysis, pathway analysis of

genes, and GWAS all found associations with the same risk variants in the same genes [72-74].

LRIG protein expression in meningioma

The human LRIG protein family haven been studied in many different tumor types and it has been suggested that the subcellular localization of these proteins might be biologically important [139, 140], and associated with poor or better survival [144, 149]. Since the expression of LRIG 1, LRIG2, and LRIG3 proteins had not been studied in meningioma, in **Paper II** the characterization and distribution of these proteins in meningioma were investigated. LRIG1 and LRIG2 showed expression in the cytoplasm, perinuclear region, and nucleus while LRIG3 only showed expression in the cytoplasm. Sporadic immunoreactivity in several compartments within individual cells was observed. Expression of the LRIG proteins was compared to gender and histological meningioma subtypes. The results revealed a significant association with LRIG1 and LRIG2 being expressed in the cytoplasm, most frequent in the benign subtypes (fibrous and transitional) suggesting that the LRIG proteins may not have a clear role in malignant progression from grade I to grade II meningioma.

Moreover, in **Paper I** IHC staining of the LRIG2 protein in 44 meningioma tumor samples from the Finnish patients was analyzed. Expression of LRIG2 was compared to the genetic variant associated with meningioma risk and no association was found indicating that the *LRIG2* SNP might not influence meningioma development. Because of the small sample size and too few subtypes of meningioma in **Paper I**, only tumor grade (I and II) was compared to LRIG2 protein expression. No significant association was found. Because of the small sample size and too few subtypes in **Paper II**, only tumor grade (I and II) was compared to LRIG2 protein expression. No significant association was found.

Estrogen and meningioma

Since meningioma is twice as common in women as it is in men it has been suggested that there is an association between hormones and meningioma risk [61, 170, 171]. Recently, LRIG1 has shown to be an estrogen-regulated growth suppressor in breast cancer and with this in mind, we investigated if there is a relationship between LRIG1, LRIG2, LRIG3 protein expression and

ER status. In **Paper I**, only *LRIG2* could be analyzed as it was the only protein expression data available. Results revealed a significant correlation between ER status and cytoplasmic LRIG2 expression ($p < 0.001$). In **Paper II** the expression of LRIG1, LRIG2, and LRIG3 proteins was compared with ER status. LRIG1 expressed in the cytoplasmic and nuclear region showed a significant association with ER status ($p = 0.003$ and 0.004, respectively). LRIG2 showed similar observation as in **Paper I**, cytoplasmic LRIG2 expression being correlation with ER status ($p = 0.006$), suggesting that LRIG proteins might have a potential role in the pathogenesis of meningioma, although more studies are needed to confirm this hypothesis.

Glioma – Paper III and IV

Correlation between genetic risk variants and tumor genomic instability

Several loci associated with glioma risk have been identified previously [172], but it is important to further analyze the functianl effect of these risk variants to provide a better understanding of glioma biology. In **Paper III**, to explore if reported genetic risk variants (Table 2) are associated with genomic instability, matched blood and tumor samples from 95 glioma patients were analyzed. Using the ASCAT algorithm, accounting for non-aberrant cell admixture and tumor aneuploidy, 81 glioma patients were obtaind for the ASCAT profile. Frequency of defined somatic events (normal, loss, increased copy number, loss of heterozygosity (LOH), copy number neutral events (CNNE), homozygous deletion (HD), amplification, and simultaneous LOH and increased copy number) over the whole genome and determined regions were tested. Results revealed genetic variant, rs17172430, in the risk group *(G)* displaying somatic aberrations as 59 region/event combinations were observed. This finding was validated in another independent data set, TCGA, and two events were additionally confirmed. The events were both HD within 9p21.3 region correlated to rs17172430 ($p = 0.0264$ and 0.0210, respectively). This identified locus located in the *EGFR* gene could be indirectly linked to the genome instability as *EGFR* acts as an early activator of transcription in the RAS signaling pathway (MAPK mediates RAS-induced chromosome instability).

Germline risk variants correlated with somatic aberrations and protein expressions

Genetic germline risk variants located in different genes have been associated with specific tumor alteration [82] and glioma subtypes [173]. Since matched tumor and blood samples were available in **Papers III** and **IV,** correlation between previously identified germline risk variants (Table 2) and somatic genetic events in genes of interest were detected by ASCAT algorithm. In **Paper III**, 35 events were found to be significantly more expressed in the risk group. Out of these 35 events observed in our data set 4 of the events were also confirmed in the TCGA data set. One event was LOH in the *EGFR* gene, associated with the risk variant rs17172430 located in *EGFR (p* = 0.0455*)*. Three of the events were HD in the *CDKN2A/CDKN2B* genes, which were associated with two different genetic variants, rs17172430 and rs11979158, both located in the *EGFR* gene *(p* = 0.0267 and 0.0117, respectively*)*. The risk variant, rs17172430, in *EGFR* were homozygous in 60 samples where 19 of these displayed LOH at the *EGFR* locus while 35 cases displayed HD at the *CDKN2A* locus and 14 samples displayed a combination of both HD at *CDKN2A/B* and LOH at the *EGFR* locus.

In **Paper IV,** to better understand the correlation between reported risk variants (Table 2) with somatic aberrations and protein expression a total of 91 glioma patients were detected for different markers, *EGFR* amplification, *1p/19q* co-deletion and protein expression of p53, IDH1 and Ki-67, analyzed by means of IHC, FISH, and ASCAT algorithm. The Results revealed four correlations between three different risk variants with four different phenotypes. One risk variant, rs17172430, located at the *EGFR* gene showed an association with somatic aberration in *EGFR* (*p* = 0.017) where the risk allele (*G*) was mostly linked to gain of chromosome 7 (42.5%) and of *EGFR* amplification (37.0%). Same risk variant, rs17172430, also showed correlation with p53 protein expression where the risk allele (*G*) displayed mostly weak-moderate expression (71.4%) and strong expression (23.8%) of (*p* = 0.036). The second risk variant, rs2252586, was also located at the *EGFR* gene and it showed association with IDH1 expression (*p* = 0.003) where the risk allele (A) was present in 42.1% of the cases. The third risk variant, rs498872, located at the *PHLDB1* gene showed association with loss of *1p*, however, this event seem not to be related to the risk allele (*G*) (*p* = 0.022).

Taken together, findings in **Paper III** and **Paper IV**, suggests that risk variants in the *EGFR* gene may have a driving effect on glioma progression. In addition, the observed association of risk variants with specific molecular

genetics may suggest the risk variants to be involved in the development of primary glioblastoma.

Table 2: Previous reported risk variants investigated in Paper III and Paper IV

Risk variant	Chromosome	Gene	Major allele	Risk allele	References	
rs2736100	5	TERT	A	C	Shete et al. 2009	**Study III** and **study IV**
rs2252586	7	EGFR	G	A	Sanson et al. 2011	**Study III** and **study IV**
rs6969537	7	EGFR	G	G	Schwartzbaum et al. 2010	**Study III** and **study IV**
rs17172430	7	EGFR	G	G	Andersson et al. 2010	**Study III** and **study IV**
rs11979158	7	EGFR	A	A	Sanson et al. 2011	**Study III** and **study IV**
rs4947979	7	EGFR	A	A	Andersson et al. 2010	**Study III** and **study IV**
rs4295627	8	CCDC26	A	C	Shete et al. 2009	**Study III**
rs6470745	8	CCDC26	A	G	Shete et al. 2009	**Study IV**
rs1412829	9	CDKN2B	A	G	Wrensch et al. 2009	**Study III** and **study IV**
rs4977756	9	CDKN2A/B	A	G	Shete et al. 2009	**Study III** and **study IV**
rs498872	11	PHLDB1	G	A	Shete et al. 2009	**Study III** and **study IV**
rs55705857	11	PHLDB1	A	G	Jenkins et al. 2012	**Study IV**
rs78378222	17	TP53	A	C	Stacey et al 2011	**Study IV**
rs1476278	17	ERBB2	A	G	Andersson et al. 2010	**Study III**
rs2952155	17	ERBB2	G	A	Andersson et al. 2010	**Study III**
rs6010620	20	RTEL1	G	G	Shete et al. 2009 and Wrensch et al. 2009	**Study III** and **study IV**

SNP array versus FISH

Because *EGFR* amplification and *1p/19q* co-deletion is clinically interesting, we focused on these regions and their results using both SNP array using the ASCAT algorithm and FISH methods. A total of 59 identical patients in **Paper III** and **Paper IV** were available for comparison of the two methods. Results revealed that it is difficult to obtain similar results for *1p/19q* co-deletion. FISH analysis found co-deletion in seven patients while ASCAT algorithm found no co-deletions. However, a slightly better agreement was observed for *EGFR* amplifications between the methods. FISH analysis detected 19 glioblastoma patients displaying *EGFR* amplification. Out of these 19 patients 18 identical patients were available in the ASCAT data set where the results could differ depending how the samples were adjusted (Table 3). The strength of ASCAT algorithm is to account for aneuploidy tumor cell content. For samples with a whole-genome duplication, in the ASCAT dataset, with a ploidy above 2.8 were adjusted and divided with 2 as they seem to have undergone whole-genome duplication. In addition, if we only investigated same part detected via FISH probe compared to the whole *EGFR* gene different results could be observed. The disagreement between the methods could be due to many reasons. First, the weakness of ASCAT is that it assume that samples are from same clone so even if it estimate the tumor content it will ignore the tumor heterogeneity which is the major

complexity of glioma. Second, different parts of the tumor samples have been used for each method which also indicated the heterogeneity of the glioma. Third, except for adjusting of data which affects the results also when two methods are compared it is important that same definition and threshold for the events will be used which can be critical. Since in **Paper III** *EGFR* is considered as amplification when the ratio is above 4 while in **Paper IV**, *EGFR* amplification is considered when the ratio is above 2. These observations indicates that a better understanding of interpretation and management of data is needed which can affect the final results being observed.

Table 3: Summarized results for detection of *EGFR* amplification in 19 glioblastoma patients, using the SNP array (ASCAT algorithm) and FISH analysis.

	FISH		ASCAT			
	Number of cells (%)	Available in	Adjusted		Unadjusted	
Patients	amplified in EGFR	ASCAT dataset	Gene	Probe	Gene	Probe
1	90%	x	x	x	x	x
2	80%	x				
3	100%	x	x	x	x	x
4	100%	x	x	x	x	x
5	85%	x				
6	100%	x			x	x
7	85%	x	x	x	x	x
8	100%	x	x	x	x	x
9	91%	x				
10	97%					
11	35%	x				
12	69%	x	x	x	x	x
13	40%	x				
14	100%	x				
15	100%	x			x	x
16	90%	x				
17	100%	x				
18	100%	x				
19	100%	x	x	x	x	x

Conclusions

Based on the results from the studies included in this thesis, the following conclusions can be made:

- Genetic variants in *EGF*, *ERBB2*, and *LRIG2* showed no association with meningioma risk. This study clearly shows that for identification of a true and robust genetic variant affecting disease, replications of findings are very important.
- Cytoplasmic expression of LRIG1 and LRIG2 showed association with benign meningioma subtypes suggesting that the LRIG protein might not have a clear role in malignant progression from grade I to grade II.
- LRIG1 and LRIG2 expressed in the cytoplasm showed an association with ER status.
- Genetic risk variants in *EGFR* are associated with somatic aberrations, such as LOH of *EGFR*, HD of *CDKN2A/B*.
- Genetic risk variants in *EGFR* showed association with additional phenotypes, *EGFR* aberration, p53, and IDH1 protein expression. These findings and the findings in Paper III indicate a potential functional effect of the germline *EGFR* variants on glioma progression which needs to be further investigated.
- SNP array could possibly be used as a complementary technique to FISH analysis for detection of amplification of the *EGFR* gene, however further investigations are needed rule out the definitions of the threshold and events between the techniques.

During the past decades, significant progress has been made in understanding the origin, biology, and genetics of brain tumors and studies have identified genetic risk variants associated with the risk of brain tumor progression. The genetic risk variants have also been correlated to specific histologic and molecular subtypes of the tumors. Studies have found a correlation between tumor subtype and the presence of risk variants suggesting that SNPs could be used to support diagnosis. In cases where biopsy is difficult, a blood test might be used in combination with other clinical parameters such as PET-MR imaging. However, the sensitivity of such a single germline marker is still not sufficient for diagnosis and this

highlights the need for ongoing research efforts. Taken together, the numerous genetic variants in genes associated with risk of developing brain tumors such as meningioma and glioma provide insights that have the potential to provide a greater understanding of cancer biology and suggest potential targets for therapeutic and preventive strategies

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“There will come a time when you believe everything is finished. That will be the beginning.”

Louis L’Amour

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