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Genetic and Epigenetic Variation in the Human Genome

Analysis of Phenotypically Normal Individuals and Patients Affected with Brain Tumors

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Abstract
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Genetic and epigenetic variation is a key determinant of human diversity and has an impact on disease predisposition. Single nucleotide polymorphisms (SNPs) and copy number polymorphisms (CNPs) are the main forms of genetic variation. The challenge is to distinguish normal variations from disease-associated changes. Combination of genetic and epigenetic alterations, often together with an environmental component, can cause cancer. In paper I, we investigated possible alterations affecting the transcriptional regulation of PDGFRα in patients affected with central nervous system tumors by characterizing the haplotype combinations in the *PDGFRA* gene promoter. A specific over-representation of one haplotype (H2δ) in primitive neuroectodermal tumors and ependymomas was observed, suggesting a functional role for the ZNF148/PDGFRα pathway in the tumor pathogenesis. In paper II, 50 glioblastomas were analyzed for DNA copy number variation with a chromosome 22 tiling genomic array. While 20% of tumors displayed monosomy 22, copy number variations affecting a portion of chromosome 22 were found in 14% of cases. This implies the presence of genes involved in glioblastoma development on 22q. Paper III described the analysis of copy number variation of 37 ependymomas using the same array. We detected monosomy in 51.5% of the samples. In addition, we identified two overlapping germline deletions of 2.2 Mb and 320 kb (the latter designated as Ep CNP). In order to investigate whether Ep CNP was a common polymorphism in the normal population or had an association with ependymoma development, we constructed a high-resolution PCR product-based microarray covering this locus (paper IV). For this purpose, we developed a program called *Sequence Allocator*, which automates the process of array design. This approach allowed assessment of copy number variation within regions of segmental duplications. Our results revealed that gains or deletions were identical in size and encompassed 290 kb. Therefore, papers I-IV suggest that some SNPs and CNPs can be regarded as tumor-associated polymorphisms. Finally, paper V describes variation of DNA methylation among fully differentiated tissues by using an array covering ~9% of the human genome. Major changes in the overall methylation were also found in colorectal cancer cell lines lacking one or two DNA methyltransferases.

Keywords: genetic variation, epigenetics, brain tumor, array-CGH, glioblastoma, ependymoma, microarray, methylation

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Two roads diverged in a wood, and I -
I took the one less traveled by,
And that has made all the difference.

Robert Frost - The Road Not Taken

(Mountain Interval. New York: Henry Holt and Company, 1920)

To my parents,

for opening the doors to the world

and to Todd,

for sharing the love for cultural diversity

List of publications

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

I **De Bustos C**, Smits A, Strömberg B, Collins VP, Nister M, Afink G. *A PDGFRA promoter polymorphism, which disrupts the binding of ZNF148, is associated with primitive neuroectodermal tumours and ependymomas.* J Med Genet. 42(1):31-7, 2005.

II Diaz de Ståhl TD, Hartmann C, **de Bustos C**, Piotrowski A, Benetkiewicz M, Mantripragada KK, Tykwinski T, von Deimling A, Dumanski JP. *Chromosome 22 tiling-path array-CGH analysis identifies germ-line- and tumor-specific aberrations in patients with glioblastoma multiforme.* Genes Chromosomes Cancer. 44(2):161-9, 2005.

III Ammerlaan A*, **de Bustos C***, Ararou A, Buckley PG, Mantripragada KK, Verstegen M, Hulsebos T, Dumanski JP. *Localization of a Putative Low-Penetrance Ependymoma Susceptibility Locus to 22q11 Using a Chromosome 22 Tiling-path Genomic Microarray.* Genes Chromosomes Cancer. 43(4):329-38, 2005.

IV **de Bustos C**, de Ståhl TD, Piotrowski A, Mantripragada KK, Buckley PG, Darai E, Hansson C, Grigelionis G, Menzel U, Dumanski JP. *Analysis of copy number variation in normal human population within a region containing complex segmental duplications on 22q11 using high resolution array-CGH.* Genomics, 2006, *in press*.

V **de Bustos C*,** Ramos E*, Tran RT, Menzel U, Piotrowski A, Langford CL, Eichler EE, Hsu L, Henikoff S, Trask BJ* , Dumanski JP*. *Global DNA methylation profiling of chromosome 1 in differentiated human tissues and cell lines lacking DNMT1 and/or DNMT3B*. **Preliminary manuscript.**

* These authors contributed equally to this work.
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Related publications

i Buckley PG*, Mantripragada KK*, Benetkiewicz M, Tapia-Paez I, Diaz De Ståhl T, Rosenquist M, Ali H, Jarbo C, **De Bustos C**, Hirvela C, Sinder Wilen B, Fransson I, Thyr C, Johnsson BI, Bruder CE, Menzel U, Hergersberg M, Mandahl N, Blennow E, Wedell A, Beare DM, Collins JE, Dunham I, Albertson D, Pinkel D, Bastian BC, Faruqi AF, Lasken RS, Ichimura K, Collins VP, Dumanski JP. *A full-coverage, high-resolution human chromosome 22 genomic microarray for clinical and research applications.* Hum Mol Genet. 1;11(25):3221-9, 2002.

ii Mantripragada KK*, Buckley PG*, Benetkiewicz M**, **De Bustos C****, Hirvela C, Jarbo C, Bruder CE, Wensman H, Mathiesen T, Nyberg G, Papi L, Collins VP, Ichimura K, Evans G, Dumanski JP. *High-resolution profiling of an 11 Mb segment of human chromosome 22 in sporadic schwannoma using array-CGH.* Int J Oncol. 22(3):615-22, 2003.

iii de Ståhl TD, Hansson C, **de Bustos C**, Mantripragada KK, Piotrowski A, Benetkiewicz M, Jarbo C, Wiklund L, Mathiesen T, Nyberg G, Collins VP, Evans G, Ichimura K, Dumanski JP. *High-resolution array-CGH profiling of germline and tumor-specific copy number alterations on chromosome 22 in patients affected with schwannomas.* Hum Gen. 118(1):35-44, 2005.

iv Tran RK, Zilberman D, **de Bustos C**, Ditt RF, Henikoff JG, Lindroth AM, Delrow J, Boyle T, Kwong S, Bryson TD, Jacobsen SE, Henikoff S. *Chromatin and siRNA pathways cooperate to maintain DNA methylation of small transposable elements in Arabidopsis*. Genome Biol. 6(11):R90, 2005.

* and ** These authors contributed equally to this work

Contents

Abbreviations

APC	adenomatous polyposis of the colon
Array-CGH	array based comparative genomic hybridization
AT/RT	atypical teratoid / rhabdoid tumor
AXIN-1	axis inhibitor 1
BAC	bacterial artificial chromosome
β-catenin	cadherin-associated protein beta
CDKN2A	cyclin-dependent kinase inhibitor 2a
CGH	comparative genomic hybridization
CNP	copy number polymorphism
CNV	copy number variation
CNS	central nervous system
DCC	deleted in colorectal carcinoma
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EGFR	epidermal growth factor receptor
EMSA	electrophoretic mobility shift assay
ERBB	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog
GB	glioblastoma
HASH1	human achaete-scute homolog 1
HAT	histone acetyltransferase
HDAC	histone deacetylase
HIC	hypermethylated in cancer
hTERT	telomerase reverse transcriptase
i(17q)	isochromosome 17q
LARGE	acetylglucosaminyltransferase-like protein
LCL	lymphoblastoid cell line
LCR	low copy repeat
LOH	loss of heterozygosity
LOI	loss of imprinting
MDM2	mouse double minute 2 homolog
MGMT	O^6-Methylguanine-DNA methyltransferase
MYCN	v-myc avian myelocytomatosis viral-related oncogene
MYOD1	myogenic differentiation antigen 1
NAHR	non-allelic homologous recombination

NF1	neurofibromatosis 1
NF2	neurofibromatosis 2
NHEJ	non-homologous end joining
PAC	P1-derived artificial chromosome
PcG	polycomb group
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PNET	primitive neuroectodermal tumor
PNS	peripheral nervous system
PTCH	homolog of patched, drosophila
PTEN	phosphatase and tensin homolog
RB	retinoblastoma
REN^{KCDTD11}	potassium channel tetramerisation domain containing protein 11
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROMA	representational oligonucleotide microarray analysis
ROX/MNT	max-binding protein
RYR2	ryanodine receptor 2
SD	segmental duplication
SLC5A8	solute carrier family 5 (iodide transporter), member 8
SMARCB1/INI1	swi/snf-related, matrix-associated, actin-dependent regulator of chromatin, member of subfamily b
SMOH	homolog of Drosophila smoothened
SNP	single nucleotide polymorphism
SUFU	homolog of Drosophila suppressor of fused
SVZ	subventricular zone
THBS1	thrombospondin 1
TIMP3	tissue inhibitor of metalloproteinase 3
TOP3B	topoisomerase dna III beta
TSG	tumor suppressor gene
VNTR	variable number of tandem repeats
VZ	ventricular zone
WHO	world health organization
ZNF148	zinc finger protein 148

Introduction

GENETIC VARIATION

Genetic information is encoded by DNA molecules, which are inherited from cell to cell. However, it is not the only form of information which is distributed from one generation to the next one, since epigenetic information has been shown to be also inherited[1-3]. DNA is constituted by distinctive basic repeat elements designated as nucleotides, consisted of an organic base, a sugar (deoxyribose) and a phosphate group. The four possible organic bases in DNA are adenine (A), guanine (G), cytosine (C) and thymine (T). DNA consists of two long chains that create a double-stranded helix, where nucleotides are held together by hydrogen bonds. In the helix, only two specific complementary pairs are possible: A pairs with T through two hydrogen bonds, while G pairs with C through three hydrogen bonds. The information content of a gene is determined by the specific order of these deoxyribonucleotides[4].

DNA is structured into chromosomes within the human cell nucleus, although a minute amount of DNA can also be found in the mitochondria. Humans have a *chromosome set* of 23 chromosomes. The majority of human cells are constituted by two copies of the chromosome set, which is known as diploid set. However, sperm and egg cells (denominated germ cells) are different in that they have only one chromosome set, and are therefore haploid. Each chromosome set contains 22 autosomes and one sex chromosome, namely X or Y. To date, approximately 30.000 genes have been mapped to the human genome[5]. However, not every chromosome displays the same gene density. Chromosomes 1, 11, 17, 19, and 22 present the highest gene density, while chromosomes 4, 5, 8, 13, 18, and X present the lowest density[5].

The decoding of genetic information is achieved by transcription of intermediary RNA molecules from selected regions of the cellular DNA. Hence, a gene may be defined as a specific nucleotide sequence that is transcribed into RNA. A considerable amount of RNA molecules are translated into proteins. However, some transcription units specify only non-coding RNA. The translation process utilizes RNA molecules as templates, and it requires the interaction of messenger RNA (mRNA), transfer RNA (tRNAs), ri-

bosomes and proteins. Once translation is initiated, the elongation process involves the formation of a peptide bond between adjacent amino acids. After translation, a protein may be modified in various ways (post-transcriptional modifications). For example, phosphate groups, lipids or carbohydrates can be enzymatically added and attached covalently to the polypeptide chain. Gene transcription is regulated by DNA-protein and protein-protein interactions (genetic level), as well as by chromatin structure (epigenetic level). A structural gene may have a number of different response elements that can be activated in different cell types (tissue-specific) and in a spatio-temporal specific manner[4,6].

Genetic variation is present in the phenotypically normal population and also in individuals with a disease phenotype. It can be classified into two major groups, namely submicroscopic (less than ~3 Mb) and microscopic variation (more than ~3 Mb)[7]. While submicroscopic variation occurs quite frequently in both phenotypically normal and patient populations, microscopic variants are preferentially seen in the disease affected population. It is now evident that these two types of genetic variants create a large heterogeneity among individuals and it is believed that they most likely play an important role in human diversity and disease susceptibility[7]. Genetic variation occurring at a significant frequency in the population is usually termed as *polymorphism*. Within the group of submicroscopic variation, we can discern two major types of polymorphisms, designated as single nucleotide polymorphisms (SNPs) and copy number polymorphisms (CNPs).

Submicroscopic genetic variation

Single nucleotide variation

Single nucleotide variations are DNA sequence variants that occur when a single nucleotide (A, T, C, or G) is changed. A single nucleotide variation must occur in at least 1% of the population in order to be categorized as single nucleotide polymorphism or SNP. The majority of SNPs are assumed to have no effect on protein function. However, there are common SNPs that play an important role in the alteration of protein structure and regulation of its expression. These latter SNPs, designated as functional polymorphisms, can therefore be classified as producing; coding variation (where the amino-acid sequence of the encoded protein is altered), or regulatory variation (polymorphism in a non-coding region which affects the level or pattern of gene expression)[8]. We do not know much about the variability of gene regulation in the population, but it is believed to influence disease susceptibility to a certain degree[8].

Copy number variants

Copy number variants (CNVs) are submicroscopic structural DNA variations that affect large genomic sequences, involving gains or losses of several to hundreds of kilobases of genomic DNA when compared to a reference genome[7,9]. CNVs are the result of genomic deletions and duplications. Some of these inserted or duplicated sequences can further undergo different rearrangements, such as inversion. A CNV that occurs in more than 1% of the phenotypically normal population is known as a copy number polymorphism or CNP. CNPs have become the center of attention during the past two years, since they are believed to have an important functional effect on the evolution of the human genome and may be associated with disease predisposition. Several recently published studies have revealed a relatively high amount of CNVs in phenotypically normal individuals[10,11]. Sebat et al. identified 76 CNVs among 20 healthy individuals by means of representational oligonucleotide microarray analysis (ROMA)[12]. Secondly, Iafrate et al. studied both phenotypically normal controls and individuals with previously characterized chromosomal imbalances. Out of a total of 55 individuals, they identified 255 loci affected by CNVs, using a 1Mb-resolution BAC array[9]. Thirdly, Sharp et al. identified 160 CNVs in 47 normal individuals using a BAC microarray specifically developed to flank segmental duplications[13]. These three genome-wide studies were performed using array-CGH. Finally, Tuzun et al. followed a different strategy, by comparing fosmid paired-end sequences derived from a different individual's genome to the human genome reference sequence[14]. With this approach, 297 variants were identified, including 139 insertions, 102 deletions and 56 inversion breakpoints. Our laboratory was among the first to describe this type of genetic variation in the context of chromosome 22-related studies[11] (Paper II and III, see below).

CNVs have been associated with phenotypic variation and disease, and they can affect genes in several ways[7] (Figure 1). First, a change in gene copy number affecting an entire dosage-sensitive gene can cause a disease phenotype, while in the case of dosage-insensitive genes it could also lead to disease in the situation where a deletion exposes a recessive mutation in the non-deleted allele. Secondly, CNVs which affect only part of the gene can lead to a partial or complete decrease in the expression of the gene. Thirdly, CNVs located at a certain distance from a particular dosage-sensitive gene can mask a critical regulatory element which would lead to down-regulation of gene expression, while in the case of dosage-insensitive genes it could alter gene expression where a deletion exposes a functional SNP within a regulatory element[7].

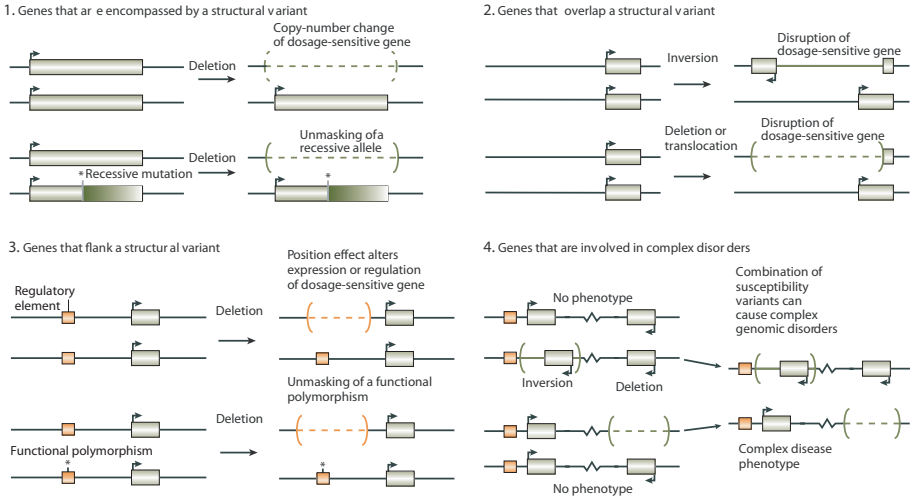


Figure 1. Different scenarios in which genes can be affected by copy number variations. Adapted by permission from Macmillan Publishers Ltd: [Nature Review Genetics] Structural variation in the human genome, copyright 2006[7].

Many CNVs have been suggested to arise due to presence of *segmental duplications* (SDs; also designated as low copy repeats, LCRs) in the intervening sequences, which mediate gene copy number losses or gains via non-allelic homologous recombination (NAHR) or non-homologous end joining (NHEJ)[15,16]. NAHR is a form of recombination in which crossover takes place between non-allelic sequences of a pair of chromosome homologs. It predominantly occurs in repeat-rich sequences, since the very high degree of sequence identity between the different repeats can facilitate abnormal pairing of non-allelic repeats[4]. NHEJ is a class of double strand break (DSB) repair mechanism that uses non-complementary sequences in a process that may or may not be error-free. SDs are structures with a high degree of sequence identity (>90%) and involve the movement of blocks (up to several hundred kb) of genomic sequence to one or more locations in the genome[17,18]. SDs account for ~5% of the total human genome[19]. They are subdivided into intra- or inter-chromosomal duplications and they can be a result of NAHR[16,18,20]. *Intra-chromosomal duplications* are usually large duplications with high degree of sequence identity (97.5-99%) and occurring within the same chromosome. Many are located within the proximal euchromatic regions of chromosomes[18]. Conversely, *inter-chromosomal duplications* are segments with 97.5% - 99.9% sequence identity that are distributed among nonhomologous chromosomes, and are biased to accumulate near heterochromatic regions of the genome (subtelomeric and pericentromeric regions)[18]. It is interesting to indicate that SDs can also be considered CNVs/CNPs in specific situations, since they can vary in copy number from

one individual to another, as a consequence of their repetitive nature[7]. SD-related NAHR mechanisms are found in several congenital genomic disorders, such as Williams-Beuren or DiGeorge syndrome[18].

Other submicroscopic variations

Variable number of tandem repeats (VNTRs) are polymorphisms belonging to the group of repetitive elements. In this case, what differs from individual to individual is not the specific sequence at a given locus (as in the case of SNPs), but rather, the number of times that a particular block of sequence is repeated at that locus. It arises because of instability in an array of tandem repeats. VNTR polymorphisms include microsatellite and minisatellite VNTRs. *Microsatellite DNA* are small arrays of tandem repeats originated from a simple sequence (usually less than 10bp) and can be detected by polyacrylamide gels. They account for ~2% of the genome. Analysis of these microsatellites serves as a conventional method for basic genotyping**.** *Minisatellite DNA* on the other hand, comprises of a collection of moderately sized arrays (9 to 65 bp) of tandemly repeated DNA sequences and can be detected by Southern blot hybridization or agarose gels. They are dispersed over considerable portions of the genome, but are often located at or close to telomeres. Hypervariable minisatellite DNA sequences are highly polymorphic, and are thought to be a hotspot for homologous recombination[21].

Restriction fragment length polymorphism (RFLP) is a genetic marker that can arise from SNPs or VNTR polymorphisms. In cases where it is derived from a single nucleotide variation, the alteration in sequence is such that the recognition site for a particular restriction enzyme is either added or eliminated. In situations where it arises through VNTR variations, the alteration affects the length of a restriction fragment, and that length will depend on the number of repetitive units.

Another category of submicroscopic structural variation is defined as *inversion*. As previously mentioned, copy number variation may comprise of duplicated sequences that are followed by an inversion event. However, inversion events can also occur independently in non-duplicated sequences. Essentially, a submicroscopic inversion is defined as a DNA sequence less than ~3 Mb, which is present in a reversed orientation[7]. Several studies have found inversion variants that do not have an effect in the phenotype of the parents that possess them, but appear to increase the risk for development of a disease phenotype in their offspring[22,23]. Similarly to CNVs, inversions seem to correlate with the presence of segmental duplications. Finally, an additional submicroscopic variation has been described, i.e. *segmental uniparental disomy*. This variation is defined as the presence in one diploid individual of a portion of a homologous chromosome pair where both alleles are derived solely from a single parent[7].

Microscopic genetic variation

Microscopic variants are DNA variations that affect genomic regions ranging from ~3 Mb to entire chromosomes and can be identified cytogenetically[7]. This microscopic variation is mostly occurring in individuals affected with an abnormal phenotype or disease, and are normally designated as chromosomal aberrations. Microscopic genetic variation can be further divided into numerical and structural variation. **Numerical variation** constitutes changes in the quantity of chromosomes in a cell, and they can be subdivided into ***i***) *polyploidy*, characterized by presence of a number of complete chromosome sets, ***ii***) *aneuploidy*, when one or several chromosomes are missing or have an extra copy, and ***iii***) *mixoploidy*, when one individual possesses two or several genetically different lineages[4]. Microscopic genetic **structural variation** affects the structure of chromosomes and are considered unbalanced or balanced if there is net gain or loss of chromosomal material, or not, respectively. Structural variations are comprised of ***i***) *balanced* and *unbalanced rearrangements* (e.g. translocations, inversions, interstitial and terminal deletions, insertions and duplications), ***ii***) *isochromosomes*, referring to chromosomes with two genetically identical arms, ***iii***) *double minutes* or *homogenously staining regions,* which are small fragments of extra-or intra-chromosomal amplified DNA and ***iv***) *fragile sites*, which define a small constriction in a chromosome[7].

A large part of the work described in this thesis has been focused in the study of genetic variation (SNPs and CNPs) in the normal population and among patients affected with cancer. Studies which were carried out in the past commonly considered any given genetic alteration detected in a tumor sample as the probable cause of tumor development or progression. However, we now understand that each phenotypically normal individual presents an inherent level of variation, and many of those variants are not directly related to a specific disease phenotype. Interestingly, it is also being considered that some of these variations might be protective of disease or could play an important role in positive selection. It seems hard to imagine that a copy number gain or loss affecting an entire gene could have no effect in any cell of the organism. However, it could provide a functional advantage to the cell and this could positively affect the individual. These advantages would proportionate better fitness, but also better protection against disease. For instance, it has been hypothesized that certain germline polymorphisms might predispose to secondary tumor formation, while other polymorphisms might protect against it[24]. Therefore, the possible role of CNPs in metastasis frequency should be further studied, and if true, this knowledge could be crucial for improvement of therapeutic strategies.

EPIGENETIC INFORMATION AND EPIGENETIC VARIATION

The genetic material or DNA is packaged inside the human cells nuclei together with specific complexes of proteins, forming chromatin[3]. The packaging of DNA has many different levels of organization. As a first level, 147 bp of super helical DNA is wrapped in two superhelical turns around a histone octamer, which is constituted by two histones of each variant H2A, H2B, H3 and H4. These complexes constituted by DNA and histones are designated nucleosomes, and they are connected to one another by short sequences of DNA of ~20 bp, where histone H1 binds (Figure 2). Regulatory proteins have partial access to DNA wrapped around the core histones, and obtain complete access when the DNA partly unwinds from the octamer[3]. As a second level of packaging organization, polynucleosomes are folded into a compact fibre with a diameter of ~30 nm. This scaffold is mainly established by the interaction of linker histones (different from core histones) of the H1 class and the DNA. As a third level, this chromatin fibre allows distant chromosomal regions to interact by forming loops[25]. Finally, specific regions of the genome may be folded in higher-level conformations that position them in distinctive areas of the nucleus, such as the nuclear envelope or the nucleolus[25].

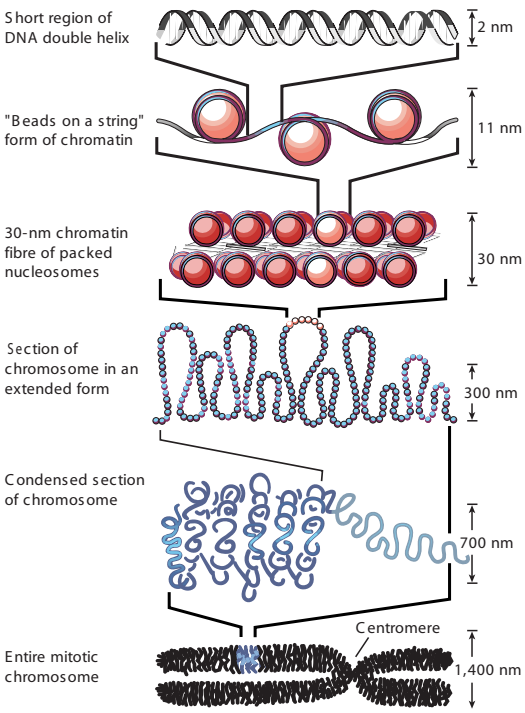


Figure 2. Different levels of chromatin packaging. Adapted by permission from Macmillan Publishers Ltd: [Nature] Controlling the double helix, Felsenfeld G. and Groudine M., copyright 2002[3].

The structure of chromatin reveals the transcriptional activity of specific regions of the human genome[3]. While transcriptionally inactive chromatin displays condensed conformation and is associated with late replication, transcriptionally active chromatin exhibits a more open conformation and replicates early in S phase. Human chromosomes can be therefore subdivided into euchromatic (actively transcribing DNA) and heterochromatic (non-transcribing DNA) regions. Heterochromatin can be further subdivided into facultative heterochromatin, corresponding to genomic regions that can return to euchromatic stage, and constitutive heterochromatin, corresponding to gene-poor areas that are constitutively condensed (e.g. centromeric regions). Heterochromatic regions are characterized by a high content in repetitive sequences and transposons. Independently of the DNA sequence information itself, states of chromatin structure are also inherited from cell to cell[3]. Epigenetics can be described as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence"[2].

Chromatin structure variation

Three different aspects are crucial in defining variation in chromatin structure: nucleosome remodeling, covalent modification of histones and replacement of core histones by histone variants[3].

Nucleosome remodeling. As mentioned above, regulatory factors have only partial access to the specific DNA sequences which are wrapped around the histone core forming the nucleosome, and require the help of protein complexes that coordinate the mobility of individual nucleosomes so that every DNA sequence can be exposed. This process in which nucleosomes are individually moved is called nucleosome remodeling. The groups of proteins responsible for this mobility are called chromatin-remodeling complexes (ex. members of the SWI/SNF family)[3]. Conversely, there is a set of large complexes encoded by the polycomb group (PcG) gene family (not part of the nucleosome remodeling), whose function is to silence in a stable manner, in combination with histone deacetylases (HDAC) and methyltransferases, different groups of genes such as the chromatin-remodeling families[26].

Covalent modification of histones. Histone proteins are some of the best evolutionarily conserved proteins, and consist of globular carboxy-terminal domains and amino-terminal tails formed of 20-35 residues which protrude from the nucleosome[27]. The globular domain contributes to the structure and stability of the nucleosome, while the histone tails control the folding of nucleosomal arrays into higher-order structures[28]. Histone tails can be affected by a variety of post-translational modifications, better known forms of modification being: acetylation, methylation, ubiquitination and phosphory-

lation of specific residues. The first two are the most extensively studied modifications. Acetylation and deacetylation is carried out by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. While histone acetylation favors transcriptional activity, histone deacetylation appears to repress transcription. On the other hand, histone methylation not only affects transcriptional activity, but it is also a key player in the regulation of DNA repair[29]. Histone methyltransferases are the enzymes which methylate the histone tails. To date, 24 sites of methylation have been found on histones. Mono- and di-methylation occurs in lysine and arginine side chains, whereas tri-methylation seems to affect only lysine residues. Heterochromatic and euchromatic regions are characterized by the presence of specific histone methylation marks. Consequently, both tri-methylated H3K9 and H4K20 are enriched in pericentric heterochromatin, tri-methylated H3K27 is enriched at the inactive X-chromosome, and euchromatin is associated to methylated H3K4 and H3K36 histones[29]. It is also remarkable that methylation of histones is not an irreversible process: demethylase LSD1 is capable of demethylating a specific lysine within histone H3[29]. Interestingly, each covalent modification can determine a different biological function depending on the combination of all the different modifications that are taking place in a specific chromatin region[27].

Histone variants. The core of the nucleosome consists of two H2A-H2B dimers and two H3-H4 dimers. These subunits are known as the replication-dependent histones. However, they are not the only histones present in the human nucleus. For different chromatin conformation stages, distinctive histone variants exist. For instance, there are two variants of H3: H3.3 and CENP-A. H3.3 is usually acetylated and therefore constitutes an epigenetic mark of transcriptionally active chromatin. It differs from H3 in 4 aminoacids, and in that it is deposited throughout the cell cycle[30]. CENP-A is localized solely in chromosome centromeres and it is necessary for chromosome segregation. In addition, H2A histone variants, designated as H2AZ and H2AX, have been shown to be associated with nucleosome stability and DNA repair, respectively[30]. Finally, macroH2A seems to be deposited exclusively in regions of the inactivate X chromosome.

DNA methylation

Methylation constitutes the most widespread covalent modification that affects DNA. Methylation in human cells solely affects the cytosine residues that are located 5´ to a guanosine, and they therefore become m^5C. It accounts for ~1% of total DNA bases and ~70%-80% of all CpG dinucleotides in the genome[2]. The amount of CpG dinucleotides is lower than the rest of dinucleotide combinations in the human genome[31]. This is because m^5C is chemically unstable and prone to deamination, which leads to conversion

into T. However, there are short stretches of DNA sequence that are rich in CpGs and usually unmethylated. These regions of DNA are named as CpG islands, and the majority of them are associated with gene promoters. There are many definitions of what a CpG island is, one of the most cited being the one described by Takai and Jones[32]. CpG island is considered a stretch of DNA of at least 500 bp in length (up to 4 kb), with a C+G content of >55% and an observed CpG over expected CpG ratio in excess of 0.65. These strict criteria lead to the exclusion of most Alu repetitive elements and therefore to a better association between CpG islands and genes[32]. Methylation of CpG islands can inhibit binding of transcription factors directly by altering the recognition sequence or indirectly by recruiting proteins with methyl binding domains and co-repressors. CpG methylation is therefore associated with transcriptional gene silencing.

There are several theories referring to the functionality of DNA methylation. Among these, two theories have gained the approval from the majority of scientists in the field. Firstly, methylation could have been developed as a defense system against foreign DNA, such as transposons and viruses[2,33,34]. Secondly, organisms could have created methylation as an approach to regulate gene expression and reduce background transcriptional noise, especially in complex genomes[2,35]. An example of the latter could be the silencing of cryptic promoters by DNA methylation[36].

In mammals, methylation of cytosines in CpG dinucleotides is catalyzed by DNA methyltransferases (DNMTs)[1,37]. Functional DNMTs are encoded by at least three different genes called *DNMT1*, *DNMT3A*, and *DNMT3B[1]*. Maintenance of DNA methylation is known to be carried out by DNMT1, with the cooperation of *de novo* methyltransferases DNMT3A and DNMT3B in complex genomic regions full of repetitive sequences[38]. While DNMT1 targets hemimethylated DNA, DNMT3A and 3B can target both unmethylated and hemimethylated DNA[39]. In addition, DNMT3L is a member of the DNMT3 family which doesn't manifest methyltransferase activity. Nonetheless, this protein seems to play an indirect role in methylation by the enhancement of DNMT3A and 3B methylation activity[40]. Therefore, methylation of DNA takes place through a complex interaction between DNMTs and DNA, and among different DNMTs.

Cross-talk among different levels of epigenetic and genetic variation

We can no longer look at a simple picture of genetic variation to understand differences in gene regulation. It is clear at this point that genetic variation is complex, with time- and location-specific regulation of gene transcriptional

activity being considered a consequence of a complex network of regulation levels affecting DNA and chromatin. In addition to DNA sequence variability which directly or indirectly affects gene regulation, the cell activity is further controlled by methylation of CpG islands. This can affect gene silencing, methylation of DNA sequences enriched with repeats and transposable elements, and modification of histone tails that also control gene regulation. The dynamic variation of chromatin structure also controls DNA replication, cell cycle progression, recombination events and DNA repair[27]. Thus, in order to obtain a complete harmony in every functioning cell of an organism, specific combinations of numerous genetic and epigenetic marks are required.

There are many different levels of cross-talk, one being the above mentioned "global" cross-talk between genetic and epigenetic variation. In addition, there are different levels of nucleosome cross-talk and they most likely have direct effects in the surrounding chromatin by changing the net charge of the histone tails and/or by attracting neighboring binding factors with specific functions[27]. The more complex level of nucleosome-DNA cross-talk allows a local effect on genes located in that specific chromatin region, and additionally a more global effect by establishing chromatin domains (euchromatin and heterochromatin) and larger chromatin regions, such as chromosomes. Once it was established that a number of different marks were required to control gene transcription activity, the interesting question is: which mark is established and which exerts its function first? For instance, Mutskov et al. suggested that histone modification was the primary event leading to gene silencing, while DNA hypermethylation appeared as a secondary event[41].

CENTRAL NERVOUS SYSTEM DEVELOPMENT AND DIFFERENTIATION

Neurons, ependymal cells, astrocytes and oligodendrocytes are the four major distinct cell types that form the adult central nervous system (CNS) in vertebrates. Many different hypotheses about the origin of these cells have been formulated during the years. At the moment, the identity of the individual progenitor cells that eventually give rise to a variety of cells in the CNS is still not known. Difficulties encountered in the identification of progenitor cells are related to the fact that particular populations of differentiated cells develop through numerous transition stages, which explains the different expression profiles that characterize those cells in diverse stages. Another difficulty is the lack of unique markers for different progenitor cells[42].

The first step in human development is called fertilization, where egg and sperm pronuclei fuse together to form the zygote, which is characterized by a diploid nucleus. In this very first stage, methylation patterns of paternal and maternal alleles are different. Subsequently, the zygote divides itself to form cells named blastomeres, forming the morula and thereafter blastocyst. At this moment of development, the zygote experiences genome-wide de-methylation. Interestingly, paternal de-methylation takes place somewhat earlier than maternal de-methylation. These cells are totipotent and still retain the capacity to differentiate into all possible cells that will form the entire organism. However, only some of the cells in the early embryo will eventually give rise to the mature organism. The rest will be involved in establishment of embryonic membranes and placenta. Implantation is the developmental stage where the blastocyst attaches to the uterine epithelium, and occurs around day 6 of human development. After implantation, paternal and maternal alleles experience widespread *de novo* methylation. However, not all cell types acquire methylation to the same extent. While somatic cells are heavily methylated, primordial germ cells continue relatively unmethylated until after gonadal differentiation. After this latter stage, the sperm genome experiences more methylation activity than the egg genome, and methylation patterns are sex-specific (e.g. imprinted loci). During the third week, the embryo gets organized in three germ layers in a process called gastrulation. Those three layers (endoderm, mesoderm and ectoderm), will eventually give rise to all the tissues of the organism. The latter will give rise to the epidermis and nervous system. The onset of organogenesis is defined by the development of the nervous system, which starts at the end of the third week of human development. The nervous system can be divided into the CNS and peripheral nervous system (PNS)[43].

All cells that form part of the CNS are originally derived from the early neuroepithelium (ectoderm cells) that constitutes the neural plate. Eventually, this neuroepithelial layer gives rise to the neural tube in a process called neurulation. Patterning of the neural tube begins at this stage of development, through cellular interactions that create organizing centers at the dorsal and ventral poles. The anterior end of the neural tube will form the future brain (forebrain, midbrain and hindbrain). The narrower caudal section will form the spinal cord. Cells originated in this stage, termed neural stem cells, will give rise to all major cell types within the CNS. These specialized neuroepithelial cells will promote the expression of genes important for cellular patterning, which are necessary for the regulation of size, complexity and histological organization of the forthcoming nervous system. This is achieved by the generation of signals, quite frequently in a concentration-dependent manner (morphogenesis). Genes involved in cellular patterning regulate the temporal and spatial distribution of cells, by specifying the identity of neuronal and glial subtypes in each developmental stage[42-44].

At this stage of development, there are several possible theories that have been formulated. The most accepted hypothesis is that neural stem cells first differentiate into two well-defined progenitor cells: neuronal-restricted progenitor cells that give rise to neurons, and the glial-restricted progenitor cells, which will further differentiate into glia[45-47]. A second hypothesis suggested is that one common neural progenitor cell could differentiate into oligodendrocytes and motor neurons, while astrocytes would arise from a different progenitor[48-50]. Interestingly, recent studies indicate yet another alternative scenario; where neural stem cells give rise to radial glial cells, as well as young neurons, in the ventricular zone (VZ) (Figure 3). Radial glial cells possess features consistent with late stages of differentiation, for example long radial processes, contact with blood vessels or elaborate cytoskeleton[51]. Yet, they are still considered primary precursor cells that give rise to neurons, astrocytes, ependymal cells and oligodendrocytes[52,53]. Radial glial cells have contacts with both ventricular and pial surface of the neural tube, and they appear to guide newly originated neurons towards the pial surface[42,51]. The second germinal zone is called the subventricular zone (SVZ) and it has been proposed to be the location where radial glial cells give rise to neurons in the adult brain[52]. Proliferation of SVZ cells continues throughout life[54].

Shortly after birth, many of the remaining neuroepithelial cells in the VZ become ependymal cells, perhaps through an intermediate step formation through radial glial cells. The ependymal cells in the adult nervous system are located in the luminal surface of the ventricular system and the central canal of the spinal cord. Cilia from the apical surface of the ependymal cells function as instrument for movement of cerebral spinal fluid. It has been indicated that ependymal cells do not divide after differentiation[53]. In the adult CNS, the SVZ decreases in size, similarly to the VZ, and mainly remains contiguous to the ependymal cell layer in the brain. Conversely, the mature regions of the spinal cord lack this SVZ region[42].

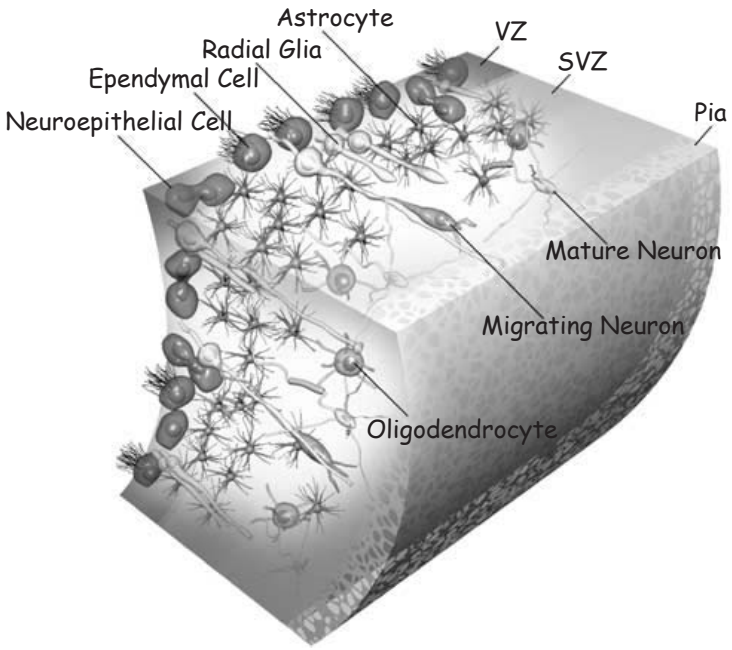


Figure 3. Early specification of neuroepithelial cells in the neocortex of the brain. VZ, ventricular zone; SVZ, subventricular zone. Adapted by permission from Macmillan Publishers Ltd: [Bone Marrow Transplant] Neural stem cells, Clarke et al., copyright 2003[42].

It is crucial to better understand the process of development and differentiation from totipotent stem cells to fully differentiated glial or neuronal cells, since many of the tumors that will be described throughout this thesis might arise from *i*) unrestricted stem cells, *ii*) progenitor cells that are restricted in different degrees or *iii*) fully differentiated cells. This should be taken into account when looking at the different genetic profiles and markers from brain tumors affecting either children or adults. It is reasonable to think that the odds of acquiring tumor-initiating genetic or epigenetic alterations for a specific cell are higher during the stages of development at which it replicates most rapidly[55]. Developing tissues at the embryonic stages are characterized by abundant presence of unrestricted and undifferentiated cells, which replicate incessantly. Accordingly, it seems plausible that pediatric tumors might be derived mainly from those unrestricted progenitor cells.

CANCER

Cancer is a large and heterogeneous group of diseases, which are the result of an accumulation of genetic and/or epigenetic alterations. These mutations can be of somatic or germline origin. In general, mutations in more than one gene are necessary for the development of a tumor. Tumors are usually classified in several degrees of malignancy and according to their histopathological characteristics. The most utilized classification in the study of central nervous system tumors is the World Health Organization (WHO) system[56,57]. Each tumor group within the WHO classification displays a distinct prognosis, clinical behavior and often also a separate genetic profile. As an example, brain tumors are classified in four different degrees by the WHO system. The most benign tumors are classified as grade I entities, and usually present a few genetic abnormalities. Grade IV tumors are, on the other hand, all the most malignant and genetically complex. The latter ones can develop de novo (e.g. primary glioblastoma) or as a consequence of progression events in tumors that initially appear as a low-grade lesion (e.g. secondary glioblastoma). There are two major types of genes that can be altered in human cancer: oncogenes and tumor-suppressor genes.

Oncogenes

Proto-oncogenes are genes whose normal activity is to control/promote cell proliferation. Mutations leading to a gain of function make oncogenes no longer capable of responding to normal regulatory signals and therefore contribute to tumor formation. A single hit in one allele is sufficient to produce an abnormal cellular stimulus. This hit can be of genetic or epigenetic origin, since it has been shown that several oncogenes are also activated by hypomethylation of the promoter region[58]. Oncogenes form a very heterogenous group of genes, but can be categorized into four major classes[59]: ***i)*** Type I oncogenes are growth factors, for example members of the PDGF family. Many cancer cells synthesize growth factors to which they are responsive, creating a positive feedback signaling loop; ***ii)*** Type II oncogenes are transmembrane receptor genes, such as *PDGFRα* and *ERBB1*; ***iii)*** Type III oncogenes are intracellular transducer genes (e.g. *Ras*); and ***iv)*** Type IV oncogenes are nuclear transcription factor genes, including *Myc* and *Gli*.

Tumor-suppressor genes

Products of tumor-suppressor genes (TSGs) normally inhibit cell growth, and therefore prevent the occurrence of cancer. The Knudson's two-hit hypothesis proposes that the requirements for tumor development are a mutation on each of the two alleles of a TSG[60]. In the case of individuals with one germline mutation, only one hit would be required for tumorigenesis. How-

ever, a few exceptions have been suggested since the formulation of the hypothesis in 1971 (Figure 4)[60]: ***i***) inactivation of the TSG by epigenetic changes of one or both alleles, often by methylation of CpG islands in the gene promoter region, and ***ii***) one-hit exception, mainly explained by haploinsufficiency, where a reduction of 50% in the level of gene function is sufficient to generate an abnormal cellular phenotype that leads to tumor formation, without inactivation of the second allele[61,62].

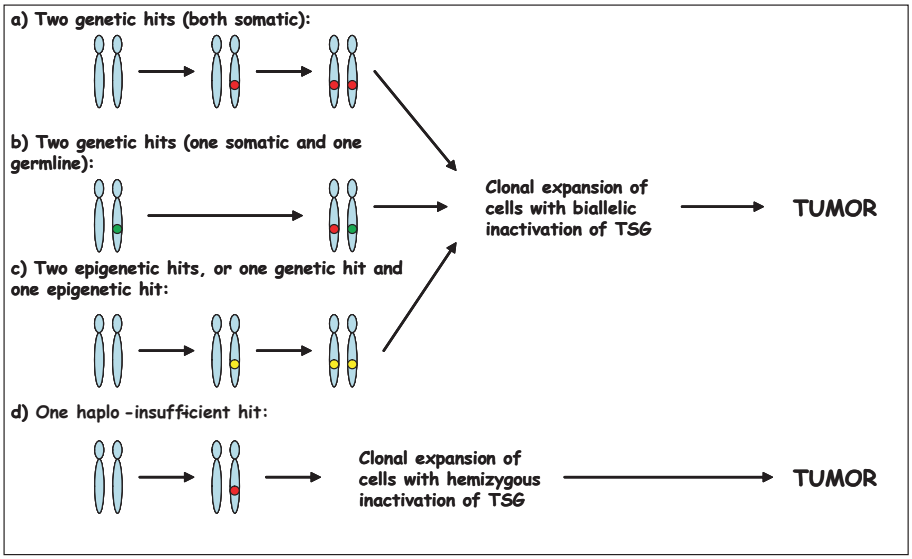


Figure 4. Graphical illustration of Knudson's two-hit hypothesis and exceptions to the model. Examples ***a*** and ***b*** represent inactivation of a tumor suppressor gene (TSG) by two genetic hits. In example ***a***, both mutations are acquired somatically. In example ***b***, the individual exhibits a germline mutation, and the second hit is a somatic mutation. The first exception to the model is illustrated by example ***c***, where a TSG can be inactivated either by a combination of one epigenetic alteration (hypermethylation of gene promoter) and one genetic change, or by two epigenetic hits. The second exception to the model is illustrated by example ***d***. In the case of some TSGs, inactivation of a single allele is sufficient for clonal expansion of preneoplastic cells and tumorigenesis (haplo-insufficiency).

During the last few years, many studies have focused on the analysis of epigenetic alterations in tumors, and this has led towards the discovery that promoter hypermethylation is at least as frequent as any other genetic alteration affecting classical TSGs[63]. Until now, hypermethylation of CpG islands in promoter regions of TSGs is the best studied epigenetic alteration in cancer, and its role in tumor development or progression can not be questioned, since this event has been found to occur in every type of human cancer. However, it is still not certain whether this event is a primary or secondary

event. It is generally believed that it can either be the primary cause of tumor development, or it can also appear as a secondary mark, being targeted by a primary epigenetic/genetic mark, such as a chromatin remodeling event. The occurrence of either situation would depend on the context and nature of the tumor. An example of methylation as the primary cause of tumor development is the O^6-Methylguanine-DNA methyltransferase (*MGMT*) gene, which is occasionally hypermethylated in pre-malignant lesions, and it is considered to predispose the tumor to mutations affecting crucial genes, such as p53[64]. It is also worth mentioning that in cases of familial cancer, genes with a one-hit germline mutation are frequently inactivated by a secondary event consisting of hypermethylation of the promoter of the wild-type allele[63]. But methylation changes not only affect the specific promoter regions of genes; global hypomethylation is a common feature in cancer, and it is considered to enhance genomic instability[65]. This global hypomethylation takes place predominantly in heterochromatic regions (e.g. repetitive sequences, transposon elements). Likewise, other epigenetic alterations in cancer have gained the attention of researchers, such as the silencing of TSGs by short double-stranded RNAs (dsRNA) with the absence of DNA methylation or by hypoacetylation of chromatin histones[66]. Chromatin memory is often disrupted during cancer progression[58]. Additionally, loss of imprinting (LOI) is an alteration that frequently occurs in cancer, for instance in Wilms tumors[67-69]. LOI can be defined as deregulation of germline-established parent-of-origin-specific gene silencing, which can be produced by silencing of the normally active allele, or activation of the normally silenced allele[58].

It is important to point out that germline mutations of a specific TSG usually only lead to a particular type of tumor in a specific tissue, even when that TSG plays a key role for all cells in the body, as is the case for e.g. the *Rb* gene[70]. But why do those genetic/epigenetic mutations affect the functionality of a TSG and lead to cancer in only one or a few tissues? When trying to understand this concept, it must be taken into account that a specific phenotype is the product of the genotype, epigenotype and the environment. Thus, even if the genotype is the same in all tissues (in the case of a germline mutation), the phenotype still depends most likely on other epigenetic alterations, such as those ones affecting the chromatin structure surrounding that gene, and on the environment of the tumor cell which determines which mutations are selected[55]. Consequently, we can conclude that the tumor phenotype is dependent on a combination of specific factors that can only be reached in very specific conditions, and only one or a few tissues will therefore comprise of a suitable cell environment, where all those conditions can be met.

The above hypothesis could also explain why phenotypically identical tumors (for example, astrocytic tumors that occur in children and in adults)

exhibit large differences in the altered genetic pathways leading to tumor development and progression. As previously mentioned above, pediatric tumors might develop from more unrestricted progenitor cells, whereas adult tumors are most likely derived from partially differentiated cells. As a result, the cell environments in these two cell settings are different, even if they belong to the same tissue. Therefore, since the cell environment is different, the genetic and epigenetic alterations required to form the specific combination of events that will lead to that particular tumor will most likely be also different between the unrestricted and more differentiated cells.

Finally, it should be mentioned that the dynamics of gene silencing mediated by genetic or epigenetic alterations are very different. In the case of a genetic alteration affecting a particular gene, silencing is the result of an instant blockade in the transcription of a gene from the mutant allele. However, in the case of epigenetic alterations, gene silencing does not take place instantaneously. It occurs after a gradual decrease in the production of functional protein, which is followed by chromatin conformation changes and other epigenetic alterations that finally lead to several degrees of gene silencing in individual cells of the tumor clone[63].

CENTRAL NERVOUS SYSTEM TUMORS

Tumors of the central nervous system (CNS) are classified based on their cell morphology, and the naming is given by similarities between the cancerous cell that constitutes the tumor and normal cells of the brain. As an example, ependymoma is composed of cells that morphologically resemble ependymal cells. CNS tumors can be divided in three distinct groups: A) gliomas (astrocytoma, oligodendroglioma and ependymoma), B) meningiomas and C) embryonal tumors (medulloblastoma, primitive neuroectodermal tumors (PNET) and Atypical teratoid/Rhabdoid tumor (AT/RT)). Brain tumors occur in children as well as in adults. Despite the similar clinical outcome and diagnostic pathology, many differences can be seen between pediatric and adult CNS tumors, especially at the genetic level.

Gliomas

Astrocytoma

Astrocytic tumors constitute the first subtype of gliomas. Neoplastic cells that give rise to these lesions resemble astrocytes. These tumors show different degrees of malignancy and they can be classified as pilocytic astrocytoma (grade I), low grade diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (grade IV)[56,57]. Low-grade gliomas are

often described as tumors with a relatively good prognosis. However, a considerable number of these cases develop into tumors with higher malignancy grades. The malignant progression events from benign precursor lesions to malignancy are frequently seen in various solid tumors, and reflect histological progressive stages that are characterized by specific genetic and epigenetic alterations affecting cancer genes in a sequential manner[55].

Pilocytic astrocytoma is classified as a WHO grade I astrocytic tumor, and is the most common glioma in children. It can be localized in the cerebellum and other regions of the brain, and does not usually progress into a high-grade astrocytoma[56,57]. However, there is a malignant subtype with monomorphous pilomyxoid pattern, which is associated with a bad prognosis[71]. Pilocytic astrocytomas are characterized by normal karyotypes and only a few infrequent genetic alterations have been found to date[72,73]. Therefore, there is a possibility that other non-genetic alterations might be involved in the generation and development of these low-grade tumors. Several studies have focused on the analysis of epigenetic alterations, and more specifically methylation changes, of particular genes in these tumors[74-78]. On one hand, several genes have been shown to be epigenetically inactivated by hypermethylation, such as *THBS1*, $p16^{INK4A}$ and *SLC5A8*[77,78]. On the other hand, the *MYOD1* gene was found to be hypomethylated in tumor tissue when compared to a panel of normal brain tissue DNAs[76]. This therefore suggests the potential role of tumor-suppressor gene methylation in the development of these tumors. Finally, pilocytic astrocytoma in the form of optic glioma occasionally develops in patients affected with Neurofibromatosis type 1 (NF1), which is an autosomal dominant disorder[79]. NF1-associated pilocytic astrocytomas display genetic alterations of the *NF1* locus (frequent loss of heterozygosity (LOH)), whereas the sporadic subtypes do not exhibit gene-copy loss, point mutations or methylation changes affecting this gene[80,81].

Diffuse astrocytomas are considered grade II tumors[56]. From the histopathological point of view, they do not exhibit necrosis or microvascular proliferation. The peak incidence is approximately 30-40 years of age. These tumors rarely metastasize. They can be located in cerebrum, brain stem or spinal cord. There are several genetic alterations found quite frequently in these tumors, such as gain on chromosome 7[82-84] or 8q[82]. p53, which is a transcription factor involved in the regulation of cell cycle progression and apoptosis, is frequently mutated in astrocytomas with several degrees of malignancy[50]. The inactivation of p53 constitutes an early event in astrocytoma formation and leads to chromosomal instability. Overexpression of PDGFRα without gene amplification is a frequent event in diffuse astrocytomas, and it has been associated with LOH on 17p[85]. When referring to epigenetic alterations, there are a few genes that have been found to by hy-

permethylated in diffuse astrocytomas, such as *MGMT, THBS1, TIMP-3* and *p16INK4A*[86].

Anaplastic astrocytoma is a grade III tumor which is histopathologically distinguished from grade IV astrocytomas by the absence of necrosis[87]. Anaplastic astrocytomas can be located in the cerebral hemispheres and other sites in the CNS. They belong to the category of high-grade or malignant astrocytomas and many of these tumors develop from astrocytomas grade II. Histopathologically, high-grade gliomas have usually poor prognosis. They share a few genetic alterations with diffuse astrocytoma, such as p53 mutations and gain of chromosome 7. However, anaplastic astrocytomas also present new markers of tumor progression such as LOH on chromosomes 19q (accompanied by mutations in *p19ARF*), 13q (*RB*1 alterations), 9p, 10q, 22q and 6[56,88]. These tumors will eventually develop into glioblastoma (WHO grade IV).

Glioblastoma is the most malignant astrocytoma (WHO grade IV), and it can be clinically divided into two groups: primary and secondary glioblastoma (Figure 5). Primary glioblastoma (GB1) appears de novo, with no signs of progression from less malignant astrocytomas, as in the case of secondary glioblastoma (GB2). These two groups present different molecular genetic alterations. On one hand, GB1 is characterized by LOH on chromosome 10, amplification (40%) and overexpression (60%) of *EGFR*, *p16* deletion (35%), *PTEN* mutation (36%), loss of *DCC* (23%) and *MDM2* amplification /overexpression (50%)[56,57]. On the other hand, GB2 is characterized by overexpression of the *PDGFR* gene (60%), *TP53* mutations (65%) and *DCC* loss of expression (50%)[56,57]. These tumors have the ability to infiltrate surrounding tissue, which is the main cause of malignancy. This may be due to the remarkably high incidence of *p53* mutations, which allow cells to migrate to different environments to avoid the process of apoptosis[50]. Methylation changes could be of importance in progression pathways towards malignancy. For example, the promoter of *MGMT* is hypermethylated in 75% and 36% of primary and secondary glioblastomas, respectively, and this alteration might suppress the functionality of this gene, which in turn is protection against carcinogenesis[89]. Different studies have detected hypermethylation of several tumor-suppressor genes in GB1, GB2, or both tumor forms[86,90]. However, the majority of methylation profiling studies have focused on a few cancer-associated genes, already associated with genetic alterations. With this approach, genes that are only inactivated by epigenetic alterations are most probably overlooked. Therefore, more general, and not gene-specific, approaches will be required in order to understand the complexity of the genetic and epigenetic network disrupted in cancer. Adult patients affected by GB2 are usually younger than patients affected with GB1[91]. Both groups share morphological characteristics and poor prognosis in adult pa-

tients. Glioblastomas arise more frequently in adults, even though there are cases of children affected by these brain tumors. Interestingly, the relative frequencies of different histological types vary considerably between children and adults, and presently available data on genetic alterations are mainly from brain tumors in adults. No consistent genetic markers have been found in children brain tumors[72,92-94].

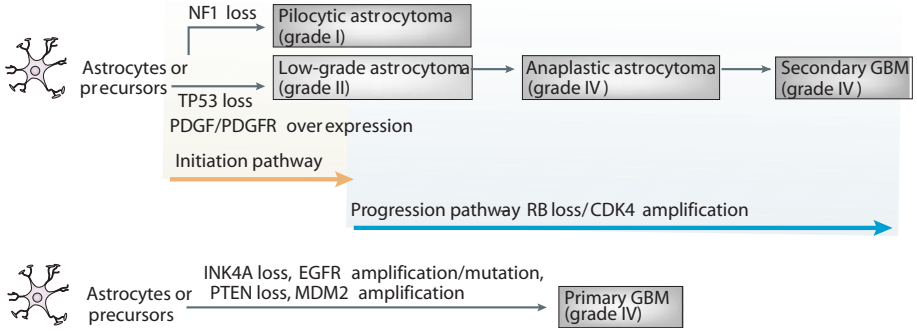


Figure 5. Development and progression pathways of primary and secondary glioblastoma. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] The Molecular and Genetic Basis of Neurological Tumours, Zhu Y and Parada LF., copyright 2002[50].

Oligodendroglial tumors

Oligodendroglioma is normally classified as grade II gliomas (WHO), although there is a more malignant subtype called **anaplastic oligodendroglioma** that is classified as grade III, and exhibits poor prognosis[56]. These tumors are composed of cells that resemble oligodendroglial cells. They occur more frequently in adults (average 42.6 years of age) than in children and they are localized in the cortex and white matter of the cerebral hemispheres[56]. Comparative genomic hybridization to metaphase chromosomes (metaphase-CGH) studies have revealed several tumor-specific aberrations frequently occurring in oligodendrogliomas, especially LOH on chromosomes 1 and 19q[95,96]. Other genetic alterations observed in oligodendrogliomas are infrequent mutation of *p53, EGFR* overexpression (without amplification of the gene), and co-expression of different ligands and receptors from the PDGF family[56,97,98]. A few genes, as for example *CDKN2A* and *SLC5A8*, have been shown to be frequently hypermethylated in oligodendrogliomas[76,77,99,100]. On the contrary, *MYOD1* is often hypomethylated in these tumors[76].

Oligoastrocytoma is classified as a grade II tumor, and it is constituted by cells resembling two different lineages: oligodendroglial and astrocytic cells.

The average age for onset of disease is 40 years. These tumors are usually located in the cerebral hemispheres[56]. They display similar genetic aberrations to oligodendrogliomas, as for example LOH on chromosomes 1 and 19q[101]. **Anaplastic oligoastrocytoma** is considered grade III by the WHO classification. Progression from oligoastrocytoma to anaplastic oligoastrocytoma is defined by genetic markers such as LOH on 9p, 10 and 11p, homozygous deletion of *CDKN2A*, mutations in *PTEN* and overexpression and/or amplification of *EGFR*[56].

Ependymal tumors

Ependymomas are a subset of gliomas that are composed of neoplastic cells resembling ependymal cells. They occur both in the spinal canal and the ventricular system. These tumors occur in patients of all ages. However, tumors located in the spinal canal (spinal ependymoma) develop more frequently in adults, while tumors from the ventricular system (intracranial ependymomas) occur generally in children. Vast majority of ependymomas are sporadic tumors, with only a few cases that have been described where several members of a family were affected with ependymoma and other related neuroectodermal tumors[56,102,103]. Several studies have reported genomic regions of allelic loss by different methods such as LOH microsatellite studies[102,104-107] or CGH[108-112]. Chromosome 22 aberrations are a common event affecting these tumors, with similar percentages of occurrence in intracranial and spinal ependymomas[104,105,110]. In spinal ependymomas, the remaining allele of the *NF2* gene has been found to be mutated in tumors with monosomy 22, which therefore presents it as a major tumor suppressor gene in the development of this type of ependymoma[104]. This is consistent with the fact that patients affected with Neurofibromatosis type 2 (NF2) can develop spinal ependymomas[113]. However, the *NF2* gene has not been found to be mutated in intracranial ependymoma, suggesting the existence of an alternative mechanism related to chromosome 22[104,114]. A candidate region in 22q11.2 defined by several studies[105,106,115] encompasses the *SMARCB1/INI1* tumor-suppressor gene which is involved in the development of atypical teratoid/rhabdoid tumor (AT/RT)[116,117]. However, mutations of this gene have not been observed in ependymomas[115,118]. Gain of chromosomes 1, 7q and 9p as well as loss of chromosome 17 are quite commonly found in these tumors[111,112]. Even if ependymoma is mainly a benign tumor (WHO grade II), there is also a malignant subtype (WHO grade III) designated as **anaplastic ependymoma,** which has a poor clinical prognosis[56]. On the other hand, there are two types of ependymal tumors that are classified as grade I, known as **myxopapillary ependymoma** and **subependymoma.** Further studies will be required in order to determine whether these four different subsets of ependymal tumors share genetic alterations or are entirely distinct entities.

Meningiomas

Meningiomas are composed of neoplastic cells that resemble meningothelial cells. Surgically treated meningiomas are usually benign neoplasms of the meningeal lining of the central nervous system and account for ~20% of all intracranial tumors[56]. The majority of meningioma cases are sporadic, although ~5% of all meningiomas are associated with NF2. This tumor is the second most common neoplasm in NF2 patients. The most consistent genetic alteration associated with both sporadic and NF2-associated meningiomas is deletion of chromosome 22[56]. However, only 50% of sporadic meningiomas exhibit aberrations of the *NF2* gene[119], suggesting the existence of another meningioma-related tumor suppressor gene on chromosome 22. In addition, deletions on chromosome 1 are the second major genetic aberration found in meningioma pathogenesis[56]. A recent study has identified four candidate loci on chromosome 1, which are most probably involved in development and/or progression of meningioma[120].

Embryonal tumors

Medulloblastoma

Medulloblastomas are highly malignant (WHO grade IV) tumors that affect mainly children and adolescents. As opposed to the rest of central nervous system tumors, they tend to metastasize, mainly along the pial lining. Medulloblastoma tumors are thought to develop from cells of the external germinal layer of the cerebellum which are supposedly disturbed during their normal differentiation into cells of the internal granular layer. In medulloblastomas, a few inconsistent karyotype abnormalities have been recognized, such as loss of 17p (through isochromosome 17q or unbalanced translocation)[121,122], chromosome 22[92,123] or chromosome 1[124,125], and amplification of *MYCN*, *ERBB1* or *hTERT*[126-129]. Isochromosome 17q [i(17q)] is involved in both development and progression of many different tumors, among them medulloblastoma[130]. The i(17q) breakpoint maps to 17p11.2, within a region with large (38–49 kb), palindromic, low-copy repeat sequences[130]. Considerable effort has been invested in the search for a putative tumor-suppressor gene in 17p13.3. To date, several genes mapping to this region have been found to be inactivated by deletions, point mutations or hypermethylation of promoter regions. *REN*^{KCTD11}, identified by Di Marcotullio et al, was mapped to 17p13.2, and was found to be a negative regulator of Gli function, which results in an alteration of the Hedgehog pathway[131]. *ROX/MNT* was mapped to 17p13.3 and displayed reduced expression in 43% of medulloblastomas in a study from Cvekl et al.[132]. Finally, *HIC-1*, also mapping to 17p13.3, is commonly hypermethylated in medulloblastomas[122,133,134]. Medulloblastoma, like many other central nervous system tumors, has been shown to express both PDGF receptors and ligands[135-138]. Moreover, PDGFRβ and members of

its downstream signaling pathway were shown to be involved in the establishment of metastases in patients with medulloblastoma[138,139]. Several studies have described *PTCH1* mutations and less frequent *SMOH*, *SUFU* and *PTCH2* mutations in sporadic medulloblastomas, suggesting a constitutively active *Sonic Hedgehog-PTCH* aberrant signaling pathway involved in these tumors[140-148]. Other genetic alterations involved in medulloblastoma development affect the *APC*, *β–catenin* and *AXIN1* genes, which are part of the WNT/Wingless signaling pathway[149-151]. Thus, medulloblastomas seem to be associated with disturbed developmental pathways. At present, no single suppressor gene loss can explain the majority of sporadic cases. Methylation changes have been reported for *RASSF1A, HIC-1* and *CASP8* in medulloblastoma[122,133,152-156].

PNET

Supratentorial primitive neuroectodermal tumors (PNETs) are very rare grade IV pediatric brain tumors that are located in the cerebrum, supra-sellar or pineal regions. They are constituted of undifferentiated or poorly differentiated neuroepithelial cells[56]. PNETs and medulloblastomas are histopathologically very similar, but share very few genetic aberrations[157]. Russo et al. observed loss of 4q, 9p, 14q and 19q in PNETs[158]. In addition, PNETs have been shown to express neurogenic basic helix-loop-helix genes, such as *NeuroD* and *HASH1*[159].

Atypical teratoid/rhabdoid tumor

Atypical teratoid/rhabdoid tumors (AT/RTs) are very malignant tumors (WHO grade IV) composed of neoplastic rhabdoid cells, with additional components of primitive neuroectodermal, mesenchymal and/or epithelial cells[56,157]. It is an extremely rare tumor that affects children usually younger than 5 years. Half of AT/RTs arise in the posterior fossa, while the rest develop in supratentorial, pineal, multifocal or spinal locations[56,157]. The majority of AT/RTs are characterized by inactivation of the *SMARCB1/INI1* gene[160]. These tumors display monosomy or partial deletion of chromosome band 22q11.2, as well as mutation of *INI1*[116,123,160-163]. This gene is a member of the chromatin-remodelling SWI/SNF multiprotein complex. This alteration seems to be the only recurrent genetic alteration in AT/RT neoplastic cells[164]. However, 15% of the tumors studied to date display no alterations of *SMARCB1*/*INI1*, neither at the DNA, RNA or protein level[160]. Methylation changes in the promoter region of this gene have not been found either[165]. Therefore, further studies will be required to determine whether any other epigenetic change in this region could account for the percentage of tumors with no genetic aberration found in *SMARCB1*/*INI1*. In addition, other alterations have been identified as being involved in, most likely, progression events in AT/RTs, such as loss of chromosome 19 in 43% of tumors (3 out of 7) studied[166].

PDGFRA IN CENTRAL NERVOUS SYSTEM TUMORS

Platelet Derived Growth Factors (PDGFs) is a family of ligands, which is composed of five members (AA, AB, BB, CC and DD) that bind to two different tyrosine kinase receptors; PDGF receptor α (PDGFRα) and receptor β (PDGFRβ) (Figure 6). These receptors are structurally similar and consist of an extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane domain, a kinase domain, and a carboxy-terminal tail[167]. The PDGFRα binds four of the five PDGF ligands (AA, AB, BB, CC)[168]. *PDGFRA*, the gene coding for PDGFRα, is located on human chromosome 4q11-q21 and contains 23 exons, spanning a region of 65 kb[169]. The first non-coding exon is followed by an intron of approximately 23 kb. The *PDGFRA* promoter region is highly conserved between mouse, rat and human, indicating the existence of important functional elements within this area[170]. PDGF is a critical determinant in embryonic development, and it plays a very important role in cellular proliferation, migration and survival[171,172]. During embryonic development, PDGF receptors and ligands are generally expressed in distinct but at the same time adjacent cell layers of the embryo (paracrine signaling), allowing for strict compartmentalization of the PDGF ligand activities[173,174]. PDGFRα expression occurs mainly during embryonic development, although it also takes place at lower levels in adult tissues, such as the brain[175,176].

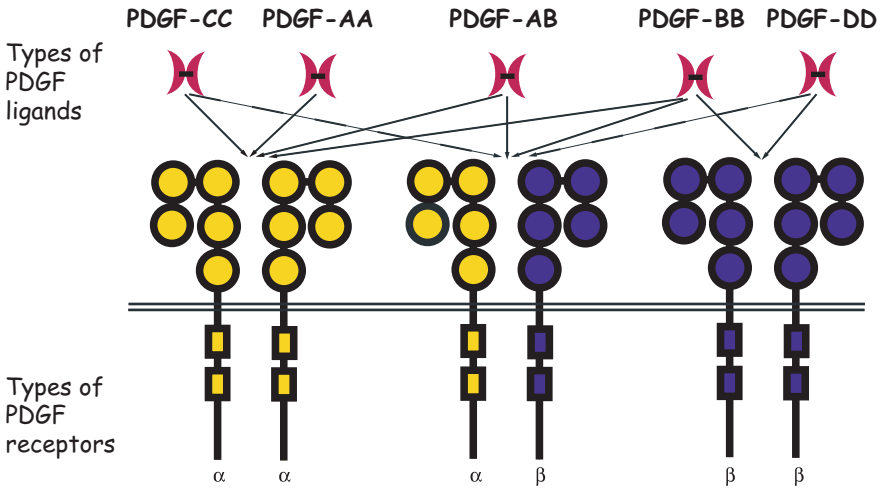


Figure 6. Structure of PDGF ligands and receptors, and illustration of binding specificity. Adapted from Fredriksson et al. 2004[168].

Different studies have underlined the importance of PDGFRα in brain tumorigenesis[177]. PDGFRα has been shown to be over-expressed or amplified in several brain tumors, for instance oligodendroglial tumors, ependymal tumors, high-grade astrocytomas and embryonal tumors[97,98,135,136,177,178]. The overexpression of the receptor is paralleled by a high level of PDGF ligand expression in the tumors, thus creating an autocrine PDGF loop[177,179].

Several studies in mouse models have suggested the correlation between deregulated PDGFRα expression and congenital neural tube defects (NTD). In one recent study, 5 different haplotypes were identified when screening for SNPs in the human *PDGFRA* promoter. Those haplotypes could be subdivided into two major groups denominated as H1 and H2 (α-δ)[180]. They studied the possible haplotype combinations in familial spina bifida patients, sporadic spina bifida patients and control individuals. H1 homozygotes were completely absent from the group of sporadic patients, whereas H1/H2α heterozygotes were overrepresented in the group of both sporadic and familial spina bifida patients, but strongly underrepresented in unrelated controls. After validating these results with promoter activity assays, they underlined a clear correlation between certain *PDGFRA* promoter haplotype combinations and the genesis of neural tube defects (NTD). A second study was performed in relation with coronary heart disease, in order to clarify whether there is any correlation between these promoter haplotype combinations and the specified disease. However, no significant correlation was found[181]. A third study reported a 2.2 fold higher risk of having an NTD-affected child in mothers with low promoter activity alleles (H1, H2β, H2γ) of *PDGFRA*[182]. These results are not in agreement with the figures reported by Joosten et al.[180], since this laboratory obtained a higher risk for both familial and sporadic NTDs in the case of high promoter activity alleles of *PDGFRA*. However, it must be noted that haplotype distributions from control populations in these three different studies were significantly different, especially for haplotypes H1 and H2α. Hence, this could reflect the existence of major differences in the distribution of PDGFRA promoter haplotypes among different populations. Thus, it seems necessary to obtain an appropriate control population of phenotypically normal individuals before analyzing a cohort of individuals with a disease phenotype from a specific population. From these studies we can also conclude that *PDGFRA* promoter characterization can therefore be a crucial step in order to establish the developmental mechanisms of particular diseases.

CHROMOSOME 22 IN CENTRAL NERVOUS SYSTEM TUMORS

The euchromatic part of chromosome 22 was sequenced in 1999, as a first step towards the sequencing of the entire human genome[183]. Chromosome 22 is the second smallest human chromosomes, accounting for approximately 1.5% of the human genome. Since it is an acrocentric chromosome, its short arm is composed of tandemly repeated ribosomal RNA genes and other tandem repeat sequences[183]. Its small size, together with the existence of a large number of human disorders associated with this chromosome, made it a test field for the human genome project.

Chromosome 22 deletions and point mutations account for the most common aberrations found in various central nervous system neoplasms, especially in ependymal tumors and AT/RTs. Additionally, several studies have reported different degrees of monosomy or partial allelic losses in 22q in several other brain tumors, such as astrocytic tumors (e.g. glioblastoma multiforme)[56,57]. At present, two major tumor suppressor genes on chromosome 22, *NF2* and *SMARCB1/INI1* gene, have been considered as possible candidates. Mutations of *NF2* are common in *NF2*-associated neurological tumors, for instance meningioma, schwannoma or ependymoma. This gene encodes a protein denominated Merlin that interacts with the actin-cytoskeleton, and acts as a tumor suppressor gene. *SMARCB1/INI1*, located centromeric to *NF2*, was found to be mutated in the majority of pediatric rhabdoid tumors[163], while failing to show any mutation in ependymal tumors[118]. This gene encodes for an invariant subunit of SWI/SNF chromatin remodeling complex, which plays an important role in chromatin remodeling and facilitates gene regulation. Interestingly, the variety of *SMARCB1/INI1* somatic mutations includes a high rate (40%) of homozygous deletions, and these are frequently associated with chromosomal translocations and other rearrangements of 22q11[184]. Hence, it has been recently proposed that inactivation of *SMARCB1* causes both selective growth advantage and genetic instability that are necessary for initiation and progression of different tumors; ie. rhabdoid tumors or epithelioid sarcomas[184,185].

While *SMARCB1* has been identified as the tumor suppressor gene involved mainly in all AT/RTs, *NF2* was found to be mutated, in addition to schwannomas and meningiomas, specifically in spinal cord ependymomas, but not in other subtypes of ependymal tumors. Two regions outside the *NF2* locus have been identified till now as feasible candidates for ependymal tumor initiation, by means of low-resolution loss of heterozygosity (LOH) studies. Those candidate regions are both located on chromosome 22; 22q11-q12 and 22q13[105,115]. Therefore, these specific areas might present a candidate tumor suppressor gene for a subgroup of ependymomas. Alternatively, chromo-

some 22 copy number gains and losses are relatively common in astrocytic tumors. Several studies have identified different chromosome 22 candidate regions for astrocytoma-related tumor suppressor genes or oncogenes[186-189]. It has been suggested that monosomy 22 correlates with the malignant transformation and progression of astrocytic tumors, since deletion of 22q increases in frequency with increasing malignancy grade of the tumor[190,191]. A recent study has estimated that chromosome 22 alterations in these tumors occur with a frequency of 5% in diffuse astrocytomas, 33% in anaplastic astrocytomas and 38% in glioblastomas [189].

Aims of the study

- To investigate a possible correlation between *PDGFRA* promoter-specific haplotypes and occurrence of different types of central nervous system tumors.

- To delineate the position of a candidate tumor suppressor gene locus in chromosome 22 for glioblastoma and ependymoma

- To develop a tool for high-resolution analysis of gene copy number in chromosomal regions which are rich in segmental duplications

- To evaluate size and incidence of a copy number polymorphism Ep CNP in the normal population.

- To investigate methylation changes affecting chromosome 1 among fully differentiated tissues

- To describe the effects of lack of DNA methyltransferases in global methylation patterns

Material and Methods

Cell culture and transfections (Paper I)

Transfection assay with promoter-luciferase plasmids is a method to analyze the activity of a specific gene promoter. *PDGFRA* promoter-luciferase plasmid and β-actin promoter-LacZ plasmids (used to determine the transfection efficiency) have been previously described[180].

The human medulloblastoma cell line D324med, and the mouse fibroblast cell line NIH3T3 were grown in Dulbecco´s modified Eagle medium. The primitive neuroectodermal tumor cell line PFSK-1 was grown in RPMI. DNA from the PDGFRA promoter-luciferase plasmids was isolated with a high Speed midi-prep kit from Qiagen. Cells were transfected using Fugene6 (Roche). Luciferase and β-galastosidase activities were detected in a luminometer.

Electrophoretic mobility shift assay (Paper I)

Electrophoretic mobility shift assay (EMSA) is one of several gel retardation assays, useful for the detection of specific interactions between DNA-binding proteins and their target sequences. Binding of protein to DNA target usually reduces the electrophoretic mobility of the target sequence, originating specific bands that correspond to different DNA-protein complexes[192].

Nuclear extracts from PFSK-1 cells are lysed in a sucrose buffer. In this step, the nuclei are kept intact. In a following step, cells are washed and suspended in a hypotonic buffer. Subsequently, addition of a hypertonic buffer (in a gradual manner) forces extraction of nucleoplasm into the buffer. However, the genomic DNA is kept intact, preserved by the well conserved nuclear envelop. A last step involving DNA centrifugation will separate the nuclear extracts from the nuclear envelop. Oligonucleotides probes for the EMSA are synthesized as individual oligonucleotides of typically less than 200 bp. They contain the precise recognition sequence for the segment of the *PDGFRA* gene promoter that includes the SNP in study. Next they are pairwise mixed and annealed using a decreasing temperature gradient. Subsequent incubation of nuclear extracts and the radioactively labeled oligonu-

cleotides allows specific binding, upon careful specification of binding buffer conditions. In order to avoid unspecific binding of proteins to the PDGFRA gene promoter oligonucleotides, poly-dIdC is added. Reactions are incubated for 20 minutes and subsequently separated on a 4% native polyacrylamide gel. In principle, if the oligonucleotide binds to a protein to form a complex, this will provoke a slower mobility on the gel than that of free oligonucleotides, due to the larger size of the complex. Pattern of bands are revealed upon autoradiography. The free probe will be observed as a band at the bottom of the gel.

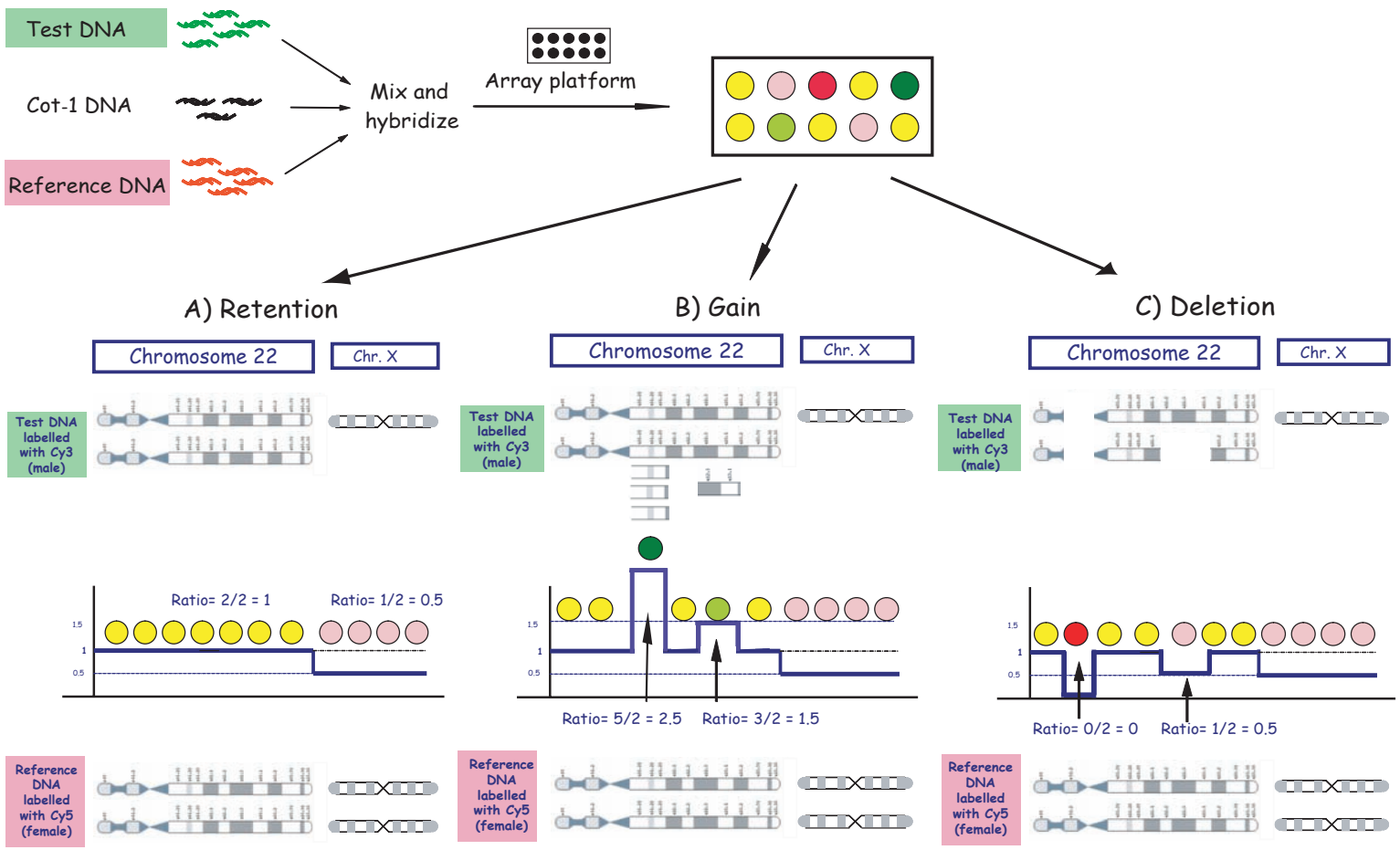
Microarray-based Comparative Genomic Hybridization (Papers II-V)

Array-based comparative genomic hybridization (array-CGH) has emerged as a technique that allows efficient detection of genomic losses and gains. The resolution of analysis is higher than in other conventional methods such as LOH studies (microsatellite analysis) or metaphase-CGH[193-196]. To date, numerous studies have demonstrated the high level of sensitivity, accuracy and reliability of analysis reached with this powerful technique[197-206]. In this method, test and reference genomic DNA samples are differentially labeled with different fluorochromes and co-hybridized to the array. The microarray platform can be made of genomic clones, cDNAs, PCR fragments or oligonucleotides[198,201,206,207]. The resolution of analysis depends on the size and distribution of measurement points in the array. In addition, the platform can be used in genome-wide, chromosome- or region-specific approaches[193,198,208]. The resulting ratio of fluorescence intensities on each measurement point is proportional to the ratio (test versus reference) of DNA copy number. Array-CGH is currently being utilized for many different applications. Gene dosage variation, gene expression, replication timing analysis and methylation profiling are some examples[203,206,209-211].

Paper II and III make use of a microarray comprising genomic clones from human 22q[193]. This array constructed in our group was the first minimal tiling path microarray that covered an entire human chromosome. Chromosome 22 array is composed of 460 clones, which results in an average resolution of 75kb. It covers 34.7 Mb, which represents approximately 1.1% of the human genome. It is also composed of 9 chromosome X controls and 31 autosomal controls. The microarray constructed in Paper IV was produced following a PCR-product based approach which was firstly introduced by Mantripragada et al.[198]. In this study, a high resolution microarray was developed to detect diagnostically significant deletions within the *NF2* gene. In order to avoid problems originated due to the presence of high repeat con-

tent, a strictly sequence-defined microarray was designed where all common repeats and redundant sequences were excluded. PCR fragments printed as target for array hybridization consisted of pools of PCR products, covering ~20 kb of genomic sequence. Paper V makes use of a microarray comprising genomic clones for chromosome 1 in a tiling fashion[120]. It is composed of 2,118 BACs/PACs which cover chromosome 1 with an average resolution of ~110 kb. As control clones, the array has 18 chromosome X clones.

Figure 7. Schematic illustration of array-CGH methodology. Test and reference DNA (male versus female in this example) are differentially labeled with fluorochromes (Cy3 and Cy5), mixed and co-precipitated with unlabeled Cot-1 DNA. Cot-1 DNA allows the blocking of repetitive sequences. Subsequently, the hybridization solution is added and the mixture is hybridized to a microarray that contains genomic clones such as BACs, PACs, fosmids or cosmids or PCR products. Hybridization time can vary from 16 to 72 hours. Following washing and scanning of slides, data is obtained through image analysis. Only chromosomes 22- and X-derived clones are represented in these graphs. Autosomal controls (not represented in these graphs) are also included in the chromosome 22 array (Papers II and III) and used in the normalization of all experiments. The Y-axis represents the normalized average fluorescence ratio for each data point on the array. On the X-axis, locations throughout chromosomes 22 and X are displayed. Chromosome X controls (on the right corner of every array-CGH profile) exhibit an average ratio of 0.5 (in light red), in accordance with hybridization of a male DNA (one copy) versus female-derived DNA (two copies). ***A*** shows a situation of retention in which all genomic clones corresponding to chromosome 22 display an average ratio of 1 (in yellow). ***B*** exhibits two different regions of copy number gain present in the test DNA. The most centromeric region shows an average ratio of 2.5, consistent with five copies of this locus, represented by a dark green color. The most telomeric region displays an average ratio of 1.5, consistent with three gene copies, and this is represented by a light green color. ***C*** shows a homozygous deletion of a centromeric region on chromosome 22 (average ratio close to 0, dark red color), as well as a hemizygous deletion displaying an average ratio of 0.5 (light red color) analogous to the ratio showed by chromosome X controls.


Test DNA
Cot-1 DNA
Reference DNA
Mix and hybridize
Array platform
A) Retention
B) Gain
C) Deletion
Chromosome 22
Chr. X
Test DNA labelled with Cy3 (male)
Reference DNA labelled with Cy5 (female)
Ratio= 2/2 = 1
Ratio= 1/2 = 0.5
Ratio= 5/2 = 2.5
Ratio= 3/2 = 1.5
Ratio= 0/2 = 0
Ratio= 1/2 = 0.5

Microsatellite analysis (Paper III)

Microsatellite polymorphisms are a type of genetic variation that enter in the category of VNTRs. The polymorphism is usually less than 100 bp and the repeat unit is normally four nucleotides or less. Sequences flanking a specific microsatellite locus are chosen for primer design, thus allowing PCR amplification of the entire locus, which will vary from allele to allele. Subsequently, PCR products are denatured to ensure right size, separated by size on a 6% acrylamide denaturing gel, and visualized by autoradiography.

Evolutionary analysis (Paper IV)

Comparative analysis among different species facilitates the understanding of the evolutionary origin of human DNA. In general, coding sequences are more conserved than non-coding sequences. Regulatory sequences can be subject to small alterations that might lead to changes in expression characteristics. Segmental duplications account for approximately 5% of the human genome[18]. However, they are not very well conserved throughout different species. The majority of them appear to have evolved in the primate lineage.

In order to determine whether a specific human locus replete of segmental duplications was conserved in other species, genomes from Gallus gallus (chicken), Mus Musculus (mouse), Canis familiaris (dog) and Pan troglodytes (chimpanzee) were compared against homo sapiens. For this purpose, the program PipMaker was used[212]. It aligns similar regions derived from two different DNA sequences. MultiPipMaker makes alignments among three or more DNA sequences[213]. Pair-wise sequence comparisons are easily observed by dot-plot analysis, which is offered also by PipMaker.

DNA preparation for methylation profiling (Paper V)

DNA derived from test and reference samples was digested with a methylation-sensitive restriction *HpaII* enzyme. After digestion, DNA was fractionated by ultracentrifugation, using 5% to 30% sucrose gradients. Fragments less than 2.5 kb were selected, confirmed on size by agarose gel electrophoresis and pooled together. Finally, selected DNA was precipitated with isopropanol.

Results and discussion

Paper I - A PDGFRA promoter polymorphism, which disrupts the binding of ZNF148, is associated with primitive neuroectodermal tumors and ependymomas.

A wide variety of CNS tumors exhibit paracrine and autocrine PDGF signaling, despite the fact that embryonal brain appears to show only paracrine signaling and that normal adult brain expression is limited to few neural progenitors. Accordingly, we decided to investigate possible alterations in the transcriptional regulation of PDGFRα by characterizing the different types of haplotype combinations in the *PDGFRA* gene promoter and by examining their possible association to different subsets of brain tumors. Defects in the transcriptional regulation of PDGFRα may lead to the aberrant high PDGFRα expression in these brain tumors. The panel of tumors consisted of 14 low grade astrocytomas, 14 mixed gliomas, 28 oligodendrogliomas, 30 ependymal tumors, 40 medulloblastomas and 12 PNETs. All five groups were compared against a panel of 91 normal controls derived from individuals with equivalent country of origin as the patients, in order to avoid differences in haplotype combination due to population-linked variance.

The PDGFRα promoter region displays eight different polymorphic sites, which give rise to five different promoter haplotypes, namely H1, H2α, H2β, H2γ and H2δ. The *PDGFRA* promoter was PCR amplified and sequenced for all samples. We found overrepresentation of one specific haplotype (H2δ) in the groups of PNETs and ependymomas, with a haplotype frequency distribution 10-fold and 6.5-fold higher than in the control group, respectively. In order to study the effect of the polymorphism on the promoter activity, transfection assays of haplotype-specific PDGFRA promoter-luciferase plasmids were performed in PNET (PFSK-1) and medulloblastoma (D324 Med) cell lines, as well as in mouse NIH3T3 fibroblasts. However, activity measurement differences were not statistically significant between different haplotypes. Subsequently, analysis of protein binding to the PDGFRA promoter was assessed by electrophoretic mobility shift assay (EMSA). The H2δ haplotype failed to bind to a specific protein derived from PFSK-1 nuclear extracts, while H2α haplotypes showed a distinct band, in-

dicative of protein binding. The program Genomatix MatInspector identified ZNF148 (also called ZBP-89) and NGFI-C as potential transcription factors binding to this region. Further analysis identified ZNF148 as the binding factor by demonstrating the appearance of a supershifted complex upon addition of a ZNF148 antibody. It remains to be established what role ZNF148 plays in regulation of PDGFRA promoter activity and tumorigenesis.

The results shown here suggest that specific PDGFRα promoter haplotypes may be associated with the development or progression of specific brain tumors such as PNET and ependymoma. These tumors might share the same or similar precursor cell population which could be regulated by ZNF148-dependent PDGFRα signaling. Furthermore, these findings indicate the potential importance of the zinc finger protein ZNF148 in the regulation of the PDGFRα promoter.

Paper II - Chromosome 22 tiling-path array-CGH analysis identifies germ-line- and tumor-specific aberrations in patients with glioblastoma multiforme

The rational of paper II was to analyze 50 glioblastoma tumors in order to investigate chromosome 22-associated abnormalities in both tumor-derived and constitutional DNA. For this purpose, we applied a chromosome 22 minimal-tiling-path genomic microarray. This array covered 34.7 Mb with an average resolution of 75 kb, representing 1.1% of the genome and 2.4% of known genes[193].

Out of 50 tumor samples, 18 tumors (36%) presented different gene copy-number alterations affecting chromosome 22 measurement points. Monosomy 22 was the most frequent genetic change, occurring in 10 tumors (20%). Four tumor-derived DNA samples (8%) displayed partial deletions along chromosome 22, which could be subsequently subdivided into 2 interstitial and 2 terminal deletions. Two putative tumor-suppressor loci, 11.1 and 3.08 Mb in size, were delineated by the distribution of overlapping hemizygous deletions. One tumor sample displayed fluorescence ratios in chromosome X consistent with the existence of three gene copies of this chromosome, and implying trisomy X. Most surprising was the identification of one glioblastoma sample that was characterized by a complex “amplifier genotype” picture, composed of several high- and low-copy amplicons. Interestingly, while some amplicons affected large regions containing a high number of genes, other amplicons involved chromosomal locations characterized by the presence of only one known gene (e.g. *LARGE* or *PDGFB* genes).

Finally, two germline regional gains were observed in both tumor and constitutional DNA from two glioblastoma patients. The first gain (250-300 kb in size) affected a locus which includes the *TOP3B* gene (topoisomerase DNA III beta), in addition to numerous immunoglobulin lambda variable-gene segments. This locus is flanked by two modules (A1 and A2) of an intra-chromosomal segmental duplication, which could have been involved in the generation of this gene copy number variation. TOP3B protein seems to be involved in the unlinking of parental strands at the final stage of DNA replication and/or in the dissociation of structures in mitotic cells that could lead to recombination[214]. The other 900 kb regional gain mapped to 22q13 and contained only one known protein coding gene, *TAFA protein 5* (*TAFA5*). This gene belongs to a novel family of proteins with similarity to chemokines and has brain-specific expression[215]. These two CNVs might be associated to glioblastoma development, since it was not found in a group of 45 phenotypically normal individuals screened using the same array platform. Therefore, this is a good example of CNVs which might not be selectively neutral, and which should probably be investigated as disease-predisposition factors.

Paper III - Localization of a Putative Low-Penetrance Ependymoma Susceptibility Locus to 22q11 Using a Chromosome 22 Tiling-path Genomic Microarray

Rearrangements involving chromosome 22 are the most common genetic aberrations in ependymal tumors[56]. In paper III we analyzed 37 ependymal tumors with the same microarray platform as in paper II, in order to fine map candidate regions comprising an ependymoma-related potential tumor suppressor gene. In our study, 51.5% of the samples displayed monosomy 22. This profile could be further divided into three categories. The first profile was characterized by complete loss of one copy of chromosome 22, which was found in 24% of the cases. The second profile, occurring in 21% of the cases, was consistent with analysis of a heterogeneous cell population, with a fraction of tumor cells most likely affected by loss of one copy of chromosome 22. Profile number three suggests detection of a heterogeneous population of cells constituted by a mixture of cells with loss of one or two copies of chromosome 22. Only two patients affected with ependymoma showed interstitial deletions, being both located on 22q11. These patients, P22 and P20, displayed 2.2 Mb and ~510 kb overlapping deletions, respectively. Deletions from those two patients were present in both tumor and peripheral blood-derived DNA. Subsequently, constitutional DNA from parents of P22 and P20 was hybridized using the same microarray, revealing same-size deletions in the mother of P22 and the father of P20. Finally, 42.5% of the

ependymomas in study exhibited no apparent DNA copy number alterations on chromosome 22.

In order to confirm the deletions detected in the tumor and constitutional DNA from ependymoma patients P22 and P20, and in their respective family members, as well as to narrow down the size of the smallest deletion (family P20), we performed microsatellite analysis. Constitutional peripheral blood-derived DNA samples from additional family members were also used in this analysis. Analysis of the constitutional DNA from P22 and family members confirmed the existence of a 2.2 Mb deletion in the mother of P22 and the patient. The alteration was found in neither the father nor the brothers of P22. Constitutional deletion of family 20 was confirmed in patient P20 and her father, as well as in the grandfather, paternal uncle and two sisters of the patient. Furthermore, the boundaries of the deletion in family 20 were narrowed down from 510 to 320 kb.

In summary, two samples (P22 and P20) showed an overlapping interstitial deletion in 22q11, thus defining a 320 kb candidate ependymoma region. However, these deletions were found in a few phenotypically normal family members. This finding confirms previous data suggesting that chromosome 22 may comprise a candidate tumor suppressor gene important for the development and/or progression of ependymomas, and narrows down the probable low-penetrance intracranial ependymoma susceptibility locus to 22q11.

Paper IV - Analysis of copy number variation in normal human population within a region containing complex segmental duplications on 22q11 using high resolution array-CGH

We decided to further validate the results from paper III, and investigate whether the deletion polymorphism named Ep CNP and found in the family of the ependymoma patient P20 was a common event in the normal population or could be associated with susceptibility for ependymoma development. Ep CNP is located in a chromosomal band (22q11) characterized by a high density of segmental duplications (SDs), which have been linked to different genomic diseases.

In order to study this locus with high resolution, we firstly developed a program designated as *Sequence Allocator*. This software designs primers for amplification of genomic regions that are used as targets for array construction. We constructed a PCR-product based microarray that consisted of both non-redundant fragments and PCR products representing duplicated se-

quences that are present four times (instead of two) in the human genome. This approach allowed us to asses copy number variation within regions of SDs with an average resolution of 11kb. We analyzed 26 peripheral blood- and 42 lymphoblastoid cell line (LCL)-derived DNA samples from normal controls, which resulted in the identification of two (7.7%) and three (7.1%) gains, respectively. Deletions of Ep CNP were only found in the LCL group (7.1%). All gains and deletions showed same size, which was determined to be 290 kb. Interestingly, in a total of 122 cases (the majority of which were normal controls) analyzed with different types of arrays in three recent studies[9,12,13], only two amplifications (1.6%) and no deletions were found in the Ep CNP locus. Our results point to a considerably higher frequency of gain and probably deletion of Ep CNP in the general population. The discrepancy of results, however, is likely due to the technical difficulties encountered during studies of a locus containing such a high content of redundant sequences. Furthermore, we found out that deletion of Ep CNP seems to be more common than gain in patients affected with neuroectodermal tumors. We detected six deletions and no gains in a total of 188 cases of ependymoma, schwannoma and pheochromocytoma previously analyzed in our chromosome 22 minimal tiling path array[216] (Paper III). Thus, we can hypothesize that gain of Ep CNP occurs more frequently in normal individuals and might thus be neutral or play a role in protection against disease. Analysis of evolutionary conservation in this locus between human and four different species (Gallus gallus, Mus Musculus, Canis familiaris and Pan troglodytes) revealed that the majority of duplication events leading to generation of these segmental duplications occurred most probably during great-ape evolution, since they were highly conserved in chimpanzee but not in chicken, mouse or dog. In this study we therefore demonstrated the ability of this state-of-the-art approach for detection of copy number variation within regions of pronounced genomic complexity.

Paper V - Global DNA methylation profiling of chromosome 1 in differentiated human tissues and cell lines lacking DNMT1 and/or DNMT3B

DNA methylation is a major epigenetic modification that plays a crucial role in many mammalian processes, such as gene expression, X-chromosome inactivation or genomic imprinting. DNA methyltransferase 1 (DNMT1) is the enzyme known to carry out maintenance of DNA methylation, with the cooperation of *de novo* methyltransferases DNMT3A and DNMT3B. Our aim was to apply a methylation profiling method in combination with genomic clone-based microarrays. For this purpose, we used a minimal tiling

path microarray, which covers human chromosome 1 (~9% of the genome) with an average resolution of ~100 kb, by employing ~2100 clones.

In this approach, DNA from test and reference samples was digested with the methylation-sensitive restriction *HpaII* (5´-CCGG-3´) enzyme. Methylation status influences the size distribution of the resulting fragments. After digestion, fragments shorter than 2.5 kb and longer than 80 bp were isolated by sucrose-gradient fractionation. Subsequently, test and reference DNA were competitively hybridized onto the genomic microarray.

HCT116 is a colorectal cancer cell line that has been extensively studied with regard to genetic and epigenetic alterations. Previously generated HCT116-derived knock-out cell lines of DNMT1 (1KO)[217] and DNMT3B (3BKO)[218] as well as a double knock-out cell line of both genes (DKO)[218] were compared against the parental HCT116 cell line, as a reference. Studies of global methylation in 1KO, 3BKO and DKO had revealed decrease of 20%, 3% and 95% decrease in overall methylation, respectively. Therefore, we were interested in detecting with high resolution the methylation changes that had occurred in chromosome 1, as a consequence of the lack of one or several DNMTs. We also aimed to analyze differences in methylation among fully differentiated human tissues. In order to avoid detection of individual-specific methylation changes, two panels of tissues derived from a female and a male individuals were selected for intra-individual tissue-specific methylation detection. All tissues derived from one individual were compared against spleen from the same individual. We profiled DNA from lung, heart, liver, spleen, four sections of the brain (cerebellum, medulla oblongata, occipital lobe and pons), and the testis/ovaries of the male/female, respectively.

Accordingly, extensive peaks of positive log2 ratios (presumed hypomethylation) were identified in 1KO and DKO when compared to the parental cell line HCT116. The pattern of methylation appeared to be similar in both lines, although peaks were more pronounced in DKO. 3BKO did not display positive log2 ratio peaks. Surprisingly, spikes of clones with negative fluorescence log2 ratios were detected in 1KO, 3BKO and DKO, in identical locations. Additionally, major differences were observed in some regions of chromosome 1 among studied tissues. In-depth analyses indicated that some deviant clones included genes with known differential expression in these tissues, such as presumed hypomethylation of the clone including the promoter and first exon of the cardiac-specific *RYR2* gene in heart tissue. Interestingly, different areas of the brain shared the methylation pattern from many deviant clones. In conclusion, the methodology employed in this paper for overall profiling of methylation alterations may be useful in global assessment of methylation abnormalities of chromosomal domains.

General discussion

This thesis reflects the importance of variation in the human genome among normal and disease-affected individuals. It is now understood that the global picture of human variation is very complex and deserves thorough study. **Papers I - IV** reveal the importance of SNP and CNP variation, interpreted in relation to predisposition and development of central nervous system tumors. **Paper V** pictures the utility of the platform for identification of changes in DNA methylation, among different normal tissues. This paper may be seen as an initial pilot study, in preparation for future analysis, when normal and cancer tissue is compared.

SNPs were believed, until recently, to be the major source of variation found in the human genome. To date, thousands of studies have correlated numerous SNPs with protection against disease, or predisposition to disease. **Paper I** describes an overrepresentation of one specific haplotype in the promoter region of a tyrosine kinase receptor (*PDGFRA*) in various groups of patients affected with CNS tumors, when compared to normal controls. PDGFRα is known to have an aberrant expression in many CNS tumors. For these types of studies, the right selection of the control group appears to be crucial. In our study, patients and individuals from the control group possessed the same nationality. Interestingly, other previously published studies that had characterized the same promoter region of the gene described different haplotype distributions in control individuals with different nationalities or ethnicities.

Other types of genetic variation have recently been discovered in the human genome; i.e. CNPs. Several recently published, important papers are clearly pointing to the fact that this type of submicroscopic variation is a heavily underestimated aspect of the human genome biology, with respect to both health and disease[9,11,12]. Cancer is now considered the result of a combination of different genetic and epigenetic alterations occurring in a specific cell environmental context. This fact makes scientists turn from the question of "are we looking in the right place?" to the question "are we looking in the right way?". In the beginning, majority of efforts were put in the search of a particular disease-specific gene. However, when dealing with a polygenic disease, the disease-associated candidate gene is, most of times, altered in a

small percentage of the patient population. It then became obvious that the problem consisted in that scientists were not obtaining a global picture.

Therefore, in order to have a glance at the global picture, we focused on two major points: study of other forms of variation and development of more high-throughput methodology. **Papers II - IV** denote the importance of studying the second type of genetic variation, CNP, in patients affected with CNS tumors and the significance of comparing these results to the size, location and frequency of CNPs encountered in the normal population. Moreover, a microarray platform was developed and applied in these studies in order to permit a more global approach. A good example of the application of array-CGH for study of CNPs and larger gene copy number aberrations is described in **paper III**. In a group of patients affected with ependymoma, chromosome 22 copy-number change profiles of DNA derived from tumor and peripheral blood from two young patients indicated the existence of two small germline deletions (2.2 Mb and 290 kb in size). Moreover, both deletions were shown to be inherited from the mother or father of the children. In one of the family cases, the deletion was also found in other family members. This raises the question as to whether these deletions are associated to tumor development. Do other members of these families have a higher risk for development of these tumors? Even if these tumors are sporadic, and they are not considered to be inherited, several family cases have been reported during the last decades, where several members of a family developed ependymoma[102,103]. It might be therefore possible that those deletions are associated with tumor predisposition, but only in combination with other multiple genetic and epigenetic factors. Consequently, this raises many questions that need further study.

Papers II - IV indicate also some limitations of our approach: ***i***) we are only looking at one form of genetic variation (CNP), ***ii***) we are looking at chromosome 22, which represents only 1% of the human genome and ***iii***) even if our resolution of analysis is ~75 kb, there are still many small CNPs that might escape our detection. Our laboratory has focused in the development of solutions to solve the problems indicated in the points above, and this thesis demonstrates some of those approaches in papers IV and V. **Paper IV** dealt with the problem of resolution (***iii***) by developing a PCR-based microarray with ~10 kb resolution that allows copy number change detection in simple and complex regions of the human genome, such as regions rich in SDs. This resolution has been further improved to the level of ~1 kb by spotting single PCR products in the array instead of pools of PCR products. In **Paper V**, we set out to investigate a different type of variation (DNA methylation changes) by using clone-based microarray platforms. This constitutes a primary step towards dealing with the problem indicated in point ***i***, since many other types of variation should be also addressed. The array used in

this study represented chromosome 1, which constitutes ~9 % of the human genome. Finally, point ***ii*** has recently been approached in our laboratory through the construction of a whole-genome minimal-tiling-path array composed of >32 500 BAC clones, which has been designated as the 32K array[208]. This array has an average resolution of <100 kb and covers approximately 99% of the sequenced fraction of the human genome.

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