Thesis for the Master's degree in Molecular Biosciences

Analysis of potential master keys in colorectal, testicular and malignant peripheral nerve sheath tumors

by

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60 study points

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Summary

Cancer of the large bowel may be looked upon as a model of stepwise tumor development, as it has morphological distinct benign and malignant stages paralleling a preferential order of genetic and epigenetic aberrations. Despite being a well-investigated disease, close to half of the patients diagnosed with colorectal cancer die within 5 years. Testicular cancer on the other hand, which is the most common cancer among young males, represents a model for successful cancer treatment, due to its effective response to chemotherapy. The malignant tumors are in the triploid range but the underlying molecular changes initiating and promoting cancer in the male germ cell lineage remain mostly unknown. Malignant peripheral nerve sheath tumors are rare malignancies, which exhibit highly complex genomes. The disease is aggressive, with surgery as the only consensus for therapy. These three cancer types develop from three distinct germ layers, and changes common among them may be important for cancer in general.

In the present thesis, we aimed to establish whether factors upstream of central signaling pathways, like the MAPK-, and PI3K-AKT-pathways[1], are commonly altered in the abovementioned malignancies, and possibly also mutually exclusive, hence qualifying as master keys in cancer. *KRAS, BRAF, PIK3CA,* and *PTEN* were investigated for mutations by direct sequencing, whereas methylation-specific PCR was utilized to determine the promoter methylation status of *RASSF1A*.

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[1] See Appendix I for complete list of abbreviations and gene symbols/names approved by the Human Gene Nomenclature Committee; http://gene.ucl.ac.uk/nomenclature/

In colorectal cancer, 80 % of the tumors examined were altered in one or more of the genes analyzed, and we identified a number of mutations in *PTEN*, previously not reported for this cancer type. Additionally, these mutations were associated with mutations in *BRAF* and microsatellite unstable tumors, and inversely associated with mutations in *TP53*.

In testicular germ cell tumors only few mutations were identified, all in seminomas, whereas several non-seminomas contained promoter methylation of *RASSF1A*. Thus, our findings are in line with the view that gene promoter methylation is a more common mechanism than gene point mutations are during the development of this cancer type. However, large sequence changes such as chromosomal amplifications and deletions are also commonly found.

Also among the malignant peripheral nerve sheath tumors, were few mutations found. Strikingly, however, about half of the tumors harbored promoter methylation of *RASSF1A*. Only few studies address promoter hypermethylation as a mechanism to inactivate genes in this malignancy, thus it remains to be seen if this is a more general mechanism in the development of the disease.

Mutually exclusive events are found in key signaling pathways, suggesting that such alterations will have the same functional effect in the pathway. Here we have shown that mutations in *KRAS* and *BRAF* are inversely associated, implying that the mutations are equivalent when it comes to the tumorigenic effect. Furthermore, mutations in *PTEN* are also found to be inversely associated with alterations in *KRAS*, as well as with mutations in *TP53*. Promoter methylation of *RASSF1A* has previously been shown to be mutually exclusive to *KRAS* mutations. Our findings suggest the opposite, as we observed concomitant gene promoter methylation and mutations in the respective genes.

The MAPK- and PI3K-AKT pathways can be referred to as master keys, as they are found frequently altered in cancer. Indeed, we show that colorectal carcinomas are commonly altered in approximately 80% in one or more of the five genes analyzed from these two pathways. Furthermore, we support the hypothesis that the mechanism for development in TGCTs is gene promoter methylation, and finally, we have shown that RASSF1A is a factor with master key features in-as-much as it is altered in a significant portion of all three tumor diseases. However, further investigations needs to be done before conclusions can be drawn about how commonly the MAPK and PI3K-AKT pathways are affected, through which upstream factors and by which mechanisms this occurs in TGCT and MPNST.

Introduction to cancer biology

Already 400 years BC the Greek physician Hippocrates, also entitled “the father of medicine”, described several types of cancer. This terminology includes a class of diseases characterized by uncontrolled division of cells that have escaped from normal regulatory mechanisms, which in turn give rise to tumors, or neoplasms. A neoplasia describes growth disturbance, and can either be benign or malignant. When the tumor cells have a slow growth rate, remain localized to their tissue of origin and do not spread to other sites in the body, the tumor is said to be benign. However, “cancer” generally refers to malignant tumors, which have severe defects in regulation of cell proliferation, invade surrounding tissue, and are able to spread to other organs to form secondary tumors, a process called metastasizing. Several lines of evidence indicate that tumorigenesis is a multi-step process and that these steps reflect genetic and epigenetic changes that drive the progressive transformation of normal cells into malignant cancers.

Boveri was the first to propose a mechanistic basis to Mendel’s theories {Boveri T, 1914 83 /id}. Based on his work on sea urchin eggs in the beginning of the 20th century, he discovered that the individual chromosomes carry different information, and he also described chromosomal abnormalities in neoplastic cells. Despite of rudimentary techniques and restricted knowledge of cell biology and genetics, Boveri managed to formulate what is now known as the somatic mutation theory of cancer. This theory implies that an acquired genetic change in a single somatic cell is the origin of a neoplasia, and this still holds the central stage in cancer research today [1].

In 1976, Nowell proposed that tumor development proceeds by a process called clonal selection, in accordance to darwinian evolution [2,3]. He stated that primary tumors are of the same unicellular origin, developed from the progeny of a single genetically unstable cell. As a result of this genetic instability, mutant cells are produced, and whereas nearly all of them are eliminated, occasionally one has gained an additional selective advantage and becomes the precursor of a new predominant population of clones that inherits the genetic and epigenetic alterations (see figure 1). A variety of experimental evidence supports the theory of monoclonality and clonal selection, making it the current dogma for tumor progression. However, there are also some findings suggesting a polyclonal model, for instance in breast cancer [4].

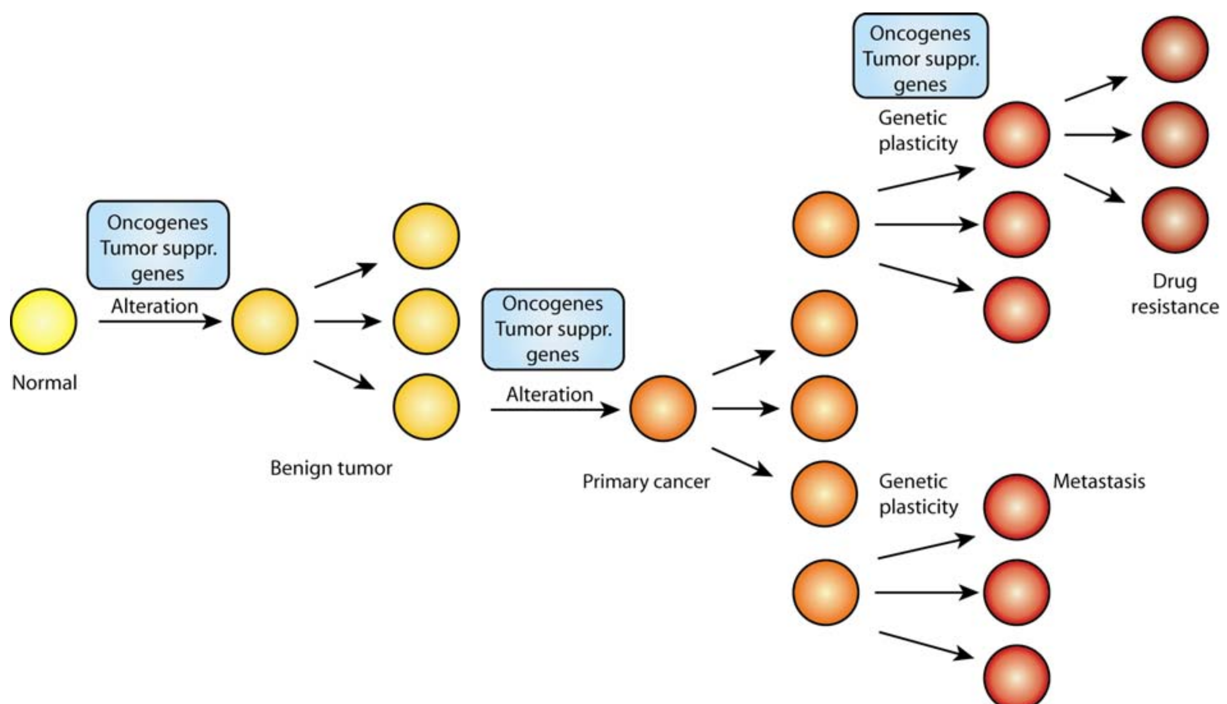

Figure 1: Clonal selection. The classical view of cancer is that it arises through a series of genetic and/or epigenetic alterations, activating or inactivating oncogenes and tumor suppressor genes, respectively. As a result of the alterations, a monoclonal population of tumor cells acquires selective growth advantages on the expense of other cells. Modified after Feinberg *et al*, ref. [5].

Ten to twenty percentages of cancers are classified as familial cancers due to familial clustering, however, the hereditary components are unknown (see figure 2). In contrast, although the inherited cancer syndromes cause approximately 5% of all cancers [6], molecular analyses of the affected genes have led to the discovery of new pathways and processes relevant to carcinogenesis. A major principle of cancer research is that the genes predisposing to the disease are usually somatically or epigenetically altered in sporadic cancer types as well. Numerous risk factors of cancer are known. In the cases having a sporadic origin (~ 90%), the interaction with environmental carcinogens including tobacco smoke, alcohol, diet, pollutants, and radiation as well as infectious agents, can in a combination with genetic and acquired susceptibility, be important [7-9]. Polymorphic variants of genes inactivated in inherited cancer syndromes may also predispose to sporadic cancer, in addition to ethnic background, gender, age, nutrition and preexisting health [10]. Cumulative lifetime exposure to carcinogens as well as accumulation of somatic mutations in aged cells might explain the increasing incidence of cancer with age.

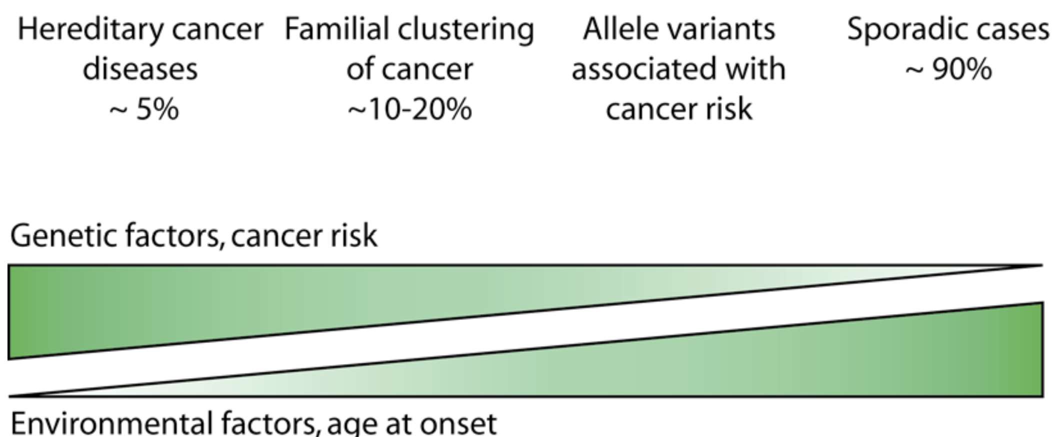

Figure 2: The genetic and environmental contribution to cancer. Approximately 5% of all cancer cases are of hereditary origin, thus the genetic factors play a significant role. The majority of cancers cases are sporadic; the age of onset is generally higher than in the hereditary cases, and environmental factors are believed to be the main cause of the disease.

Cell cycle

The most basic function of the cell cycle is to replicate the vast amount of DNA in the genomes, and then segregate the copies precisely into two genetically identical daughter cells [11]. The eukaryotic cell cycle is generally divided into four phases (see figure 3). In the Gap_1 (G_1) phase the cells monitor the extra- and intracellular signals that determine whether the cells will commit to replication and division or, alternatively, to exit the cell cycle into a quiescent stage, G_0. The cells can reside in G_0 for days or weeks, until conditions are favorable for resuming proliferation, or they may remain in G_0 indefinitely like nerve cells do. Once the cells have committed to DNA replication by passing the Restriction point, (R) (see further down), the decision is irreversible. Following the G_1 is the S-phase in which the DNA is synthesized, and G_2, a growth phase. When the cells have doubled their mass of proteins and organelles, they enter the mitotic (M) phase where the actual segregation of DNA take place, followed by cell division [12]. The cell cycle transitions are regulated by protein complexes consisting of cyclin-dependent kinases (CDKs) and cyclins. The activity of these protein complexes is controlled by phosphorylation, dephosphorylation and interactions with CDK inhibitory proteins (CKIs), like $p16^{INK4A}$ and $p27^{KIP1}$ [13]. In addition to the normal cell cycle components, there are regulatory processes termed checkpoints. These ensure proper replication of the DNA, as well as assembly of the mitotic spindle and completion of cell division. The abovementioned R-point in the G_1/M transition is one of them. Cells containing damaged DNA are prevented from entering S-phase, and instead they enter cell cycle arrest. If the damage cannot be repaired, the cells undergo apoptosis, which prevents expansion of cells with inappropriate DNA, leading to mutations and carcinogenesis. The G_2/M-checkpoint ensures correct separation of chromosomes between the two daughter cells by monitoring the

binding of chromosomes to microtubules [11]. The RB1- and TP53-network are important in regulation of the cell cycle, and at least one of them is believed to be inactivated in most, if not all, human tumors. This will be discussed further on page 25.

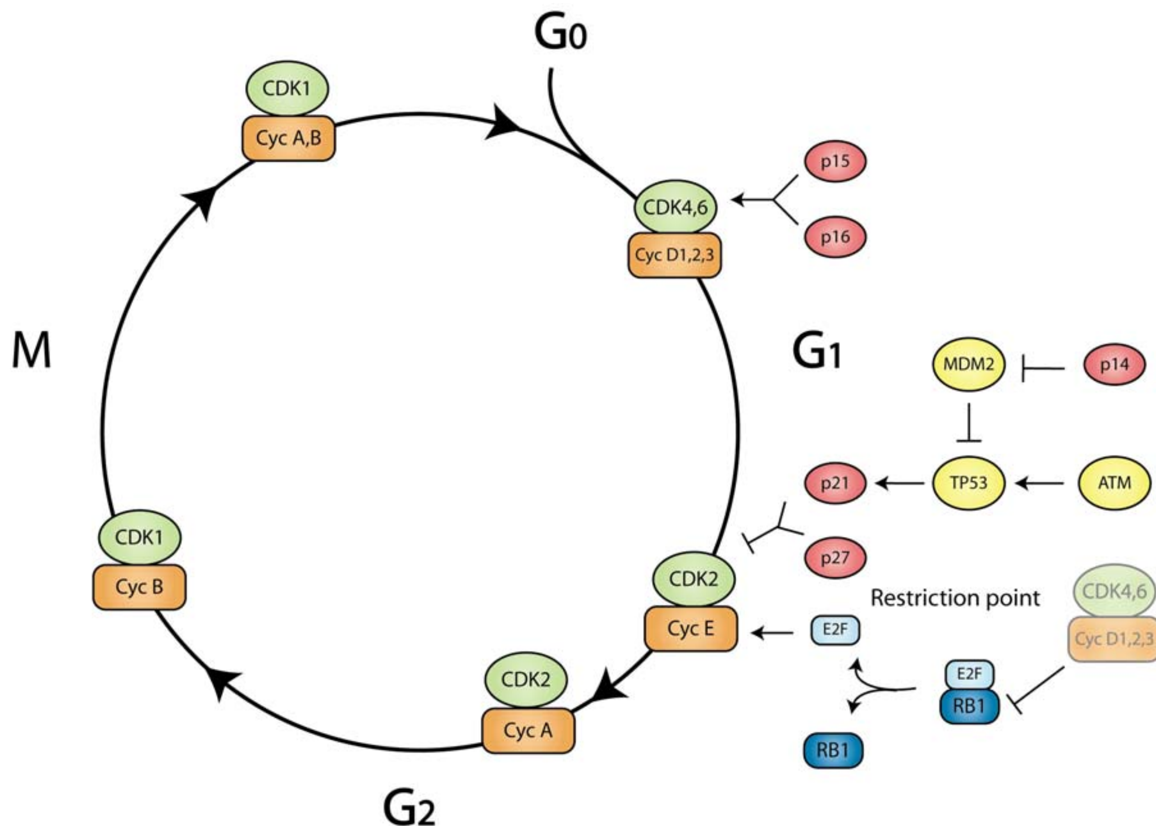

Figure 3: The principle of the cell cycle and the main components involved.

Milestones of cancer genetics and epigenetics

Tracing the historical landmarks of cancer genetics and epigenetics from the transmission of traits proposed by Mendel to the present time, gives us a possibility to appreciate how far we have come in cancer research, but also makes us realize how challenging the translation of this knowledge into advanced medicine is. However, one of the most powerful tools in the fight against cancer is now within our grasp – the complete sequences of the human and mouse genomes. Figure 4 illustrates important achievements in this field.

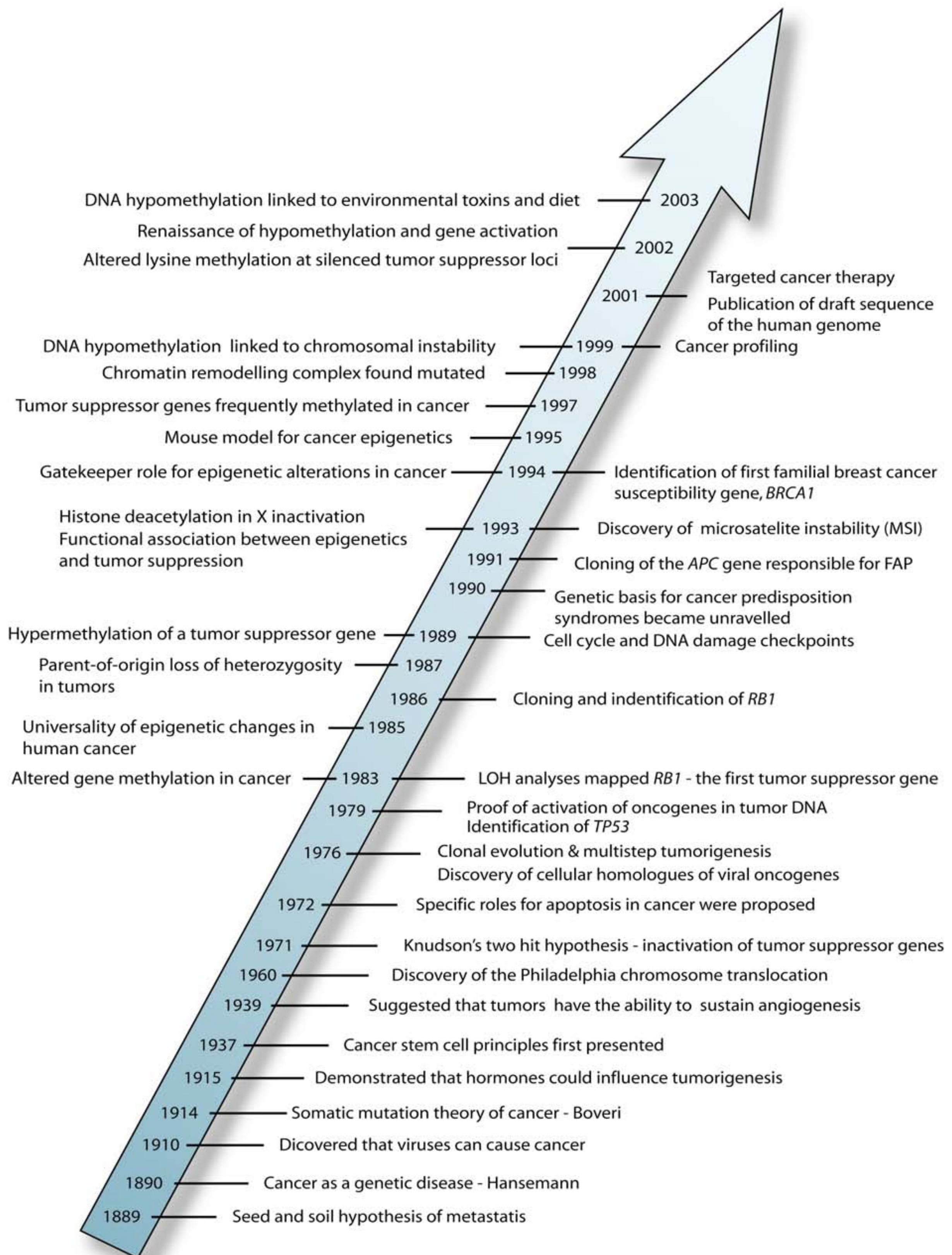

Figure 4: Timeline of milestones in cancer genetics and epigenetics. Nature Milestones [2], [14,15]

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[2] http://www.nature.com/milestones/milecancer/timeline.html

Genetics

Oncogenes

Boveri postulated the existence of "growth stimulatory chromosomes" [14]. The concept of the gene had not yet been introduced, but this prediction clearly foresaw the Nobel-prize-winning discovery of cellular proto-oncogenes by Varmus and Bishop in the 1970s [16]. These highly conserved genes are present in all normal cells, in which they have important regulatory functions. Proto-oncogenes typically encode proteins involved in positive control of the cell cycle, such as growth factor receptors, signal transduction proteins and transcription factors. When altered, they tend to relax control mechanisms increasing the rate of growth and division, and can thereby transform a normal cell into a neoplastic cell. The alteration of the normal functioning proto-oncogenes into an over-active oncogene can be quantitative (*e.g.* a point mutation or deletion) or qualitative (by gene amplification or chromosome rearrangement). Either way, the alterations are dominant, meaning that mutation in one allele is sufficient to transform a proto-oncogene into an oncogene.

Tumor suppressor genes

Knudson discovered the first tumor suppressor gene in the early 1970s when he carried out an epidemiological study of retinoblastoma development in children [17]. He postulated that "two hits" are required for the complete inactivation of anti-oncogenes, later renamed to tumor suppressor genes. In 1983 Cavenee and colleges used the principle of loss of heterozygosity (LOH) analysis to test Knudson's hypothesis and to map the *RB1*-gene [18]. Even though the discovery of tumor suppressor genes arose from examination of a single disease, it turned out to be of universal relevance. Tumor suppressor genes (TSGs) are a class of genes that oppose the effect of oncogenes, and block tumor development. Because their

cancer-preventive effects usually require the presence of only one functional allele, the TSG typically act recessive, as postulated in Knudson's "two hit hypothesis". Both alleles have to be affected, usually achieved by inactivation of one allele by a vast chromosomal event, (such as loss of a chromosome arm or an entire chromosome,) coupled with an intragenic mutation or epigenetic inactivation of the other allele [19,20]. However, there are several known examples of haploinsuffiency – in which functional loss of only one allele confers a selective advantage for tumor growth [21] One well defined example involves the Cdk inhibitor *p27* in mice [22]. The numbers of haploinsufficient tumor suppressors are increasing, and genes like *TP53, ARF* and *PTEN* have also shown such effects, particularly when combined with collaborating mutations affecting additional oncogenes or tumor suppressors [23]. The protein products of TSGs are typically involved in monitoring replication of DNA, progress of the cell cycle, and in promoting repair of damaged DNA, hence reduced expression or inactivation contributes to malignancy.

DNA repair genes

The integrity and survival of a cell is dependent on genome stability. Mammalian cells therefore have highly conserved DNA damage sensor mechanisms, which can activate several cellular responses that determine cell fate like DNA repair and apoptosis [24]. These checkpoints are encoded by DNA repair genes, a type of TSGs that keep the genetic alterations in the cell at a minimum. Hence inactivation of these genes can lead to a higher mutation rate in the genome. As with the other TSGs, both alleles of the DNA repair genes have to be inactivated to cause a physiological effect. One of the known repair systems of particular relevance for colorectal tumorigenesis is the mismatch repair (MMR) system. In human cells, *MSH2, MSH3*, and *MSH6* among others, participate in the process where base-base mismatches caused by errors of DNA polymerases, is recognized [25]. The repair system

degrades the section of error in the newly synthesized strand and gives the polymerase another chance to generate a strand free of errors. In the absence of MMR, base-base mismatches remain uncorrected resulting in a mutator phenotype that is accompanied by microsatellite instability and eventually, cancer (see page 40).

Epigenetics

Epigenetic inheritance is defined as cellular information other than the DNA sequence itself, that is heritable during cell division [15]. Since the discovery of its relevance in cancer in 1983 [26,27], this field has gradually gained more scientific attention, resulting in a better understanding of specific epigenetic mechanisms and their role in cancer. In general, there are three main types of epigenetic modifications: DNA methylation, histone modification and chromatin remodellation. For the brevity of this thesis, only DNA methylation will be introduced here.

DNA methylation

The best-known epigenetic modification is DNA methylation, which in general is associated with transcriptional inactivation. Methylation is transfer of a methyl group ($-CH_3$), using the S-adenosyl-L-methionine (SAM) as a donor, to the C-5 position in a cytosine ring. The conversion of cytosine to 5-methylcytosine is catalyzed by a family of DNA methyl transferases [28]. Methylation occur most frequently at cytosines located 5' to a guanine, in a so-called CpG site. DNA stretches of typically 0.5-4 kb, with a GC content of more than 55%, constitute CpG islands which contain approximately 20% of all CpG dinucleotides in the genome [28]. Such CpG islands can be found in about half of all mammalian genes and are frequently associated with regulatory sequences, like the promoter. CpG islands usually do

not undergo methylation due to protective boundary elements, whereas CpG dinucleotides not associated with CpG-islands are heavily methylated [28,29]. Methyl-DNA-binding domain proteins and histone deacetylases specifically recognize the modified sequences and attract large multi-protein complexes that can change the chromatin conformation from an open to a closed state, and hence lead to gene silencing [30].

DNA methylation in the normal cell

DNA methylation levels change in a highly orchestrated way during mammalian development. A well-regulated process around the gastrulation stage of embryogenesis establishes the global methylation patterns characteristic of genomic DNA of adult somatic tissues. During cleavage of the zygote, a wave of genome-wide demethylation removes the epigenetic modification, so that the DNA of the blastocyst is highly hypomethylated. Before implantation, a wave of global *de novo*-methylation re-establishes an overall methylation pattern, which is maintained through life. After gastrulation, this pattern does not change substantially. In the normal cell, DNA methylation can be found in imprinted genes, x-chromosome genes in women, germ-line specific, and tissue-specific genes (see figure 5) [31]. Intragenomic parasitic sequences and repetitive elements are also heavily methylated in normal cells.

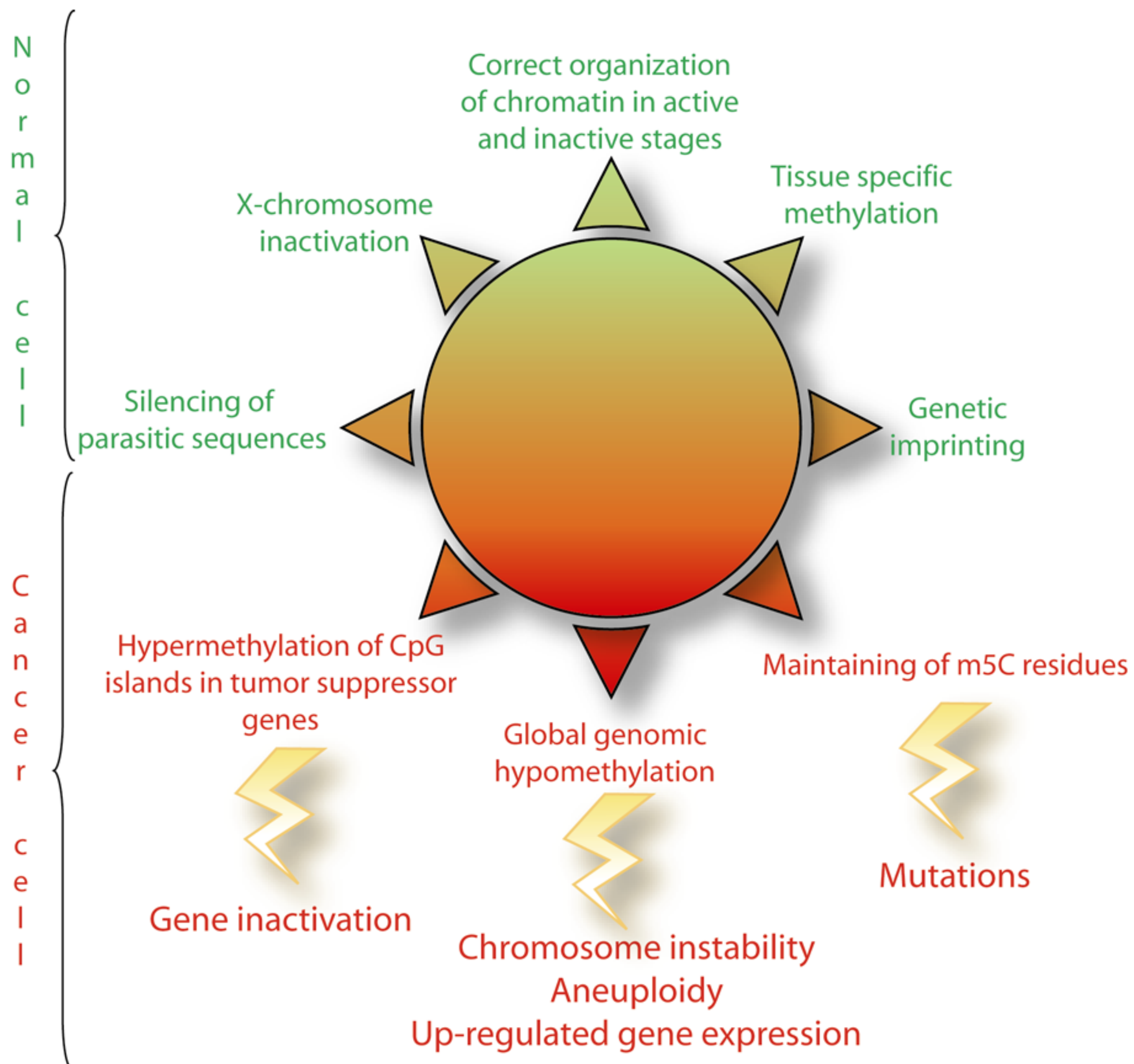

Figure 5: The influence of DNA methylation in normal cells and cancer cells. Modified after Esteller *et al*, ref. [32].

DNA methylation and cancer

Tumor development is typically characterized by a number of epigenetic alterations (see figure 5), including a general hypomethylation across the genome as well as gene specific hypermethylation. Hypomethylation, meaning loss of DNA methylation in CpG dinucleotides, was the first epigenetic abnormality to be identified [26]. Recent studies have demonstrated that this hypomethylation can increase genomic instability by removing the methylation of repetitive and parasitic sequences, hence activating oncogenes [33].

Although hypomethylation was the first epigenetic change identified in cancer, more attention has been focused on hypermethylation. In 1986, Baylin and co-workers found site-specific hypermethylation of calcitonine, with consequent silencing of calcitonine expression [15]. However, the first link between tumor suppressor genes and hypermethylation was made with the retinoblastoma-gene, *RB1* [19,20]. In the wake of that discovery, the potential impact of inactivation of tumor suppressor genes in cancer development by DNA hypermethylation was realized. The aberrant methylation of TSGs appears to occur early in tumor development and increases progressively, eventually leading to a malignant phenotype. In summary, cancer is today considered to be both a genetic and epigenetic disease.

Hallmarks of cancer

Cancer is a disease involving continually changes in the genome. In a review from 2000, Hanahan and Weinberg argue that the complexity in the development of the disorder can be narrowed down to 6 essential alterations in cell physiology [34]. To be able to grow at the expense of other cells, cancer cells must acquire some capabilities that give them selective growth advantages, including self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. In addition, it has recently been pointed out that stem cell capabilities as well as impaired cell adhesion also are central characteristics of tumor cells. (For review: [35])

Self-sufficiency in growth signals

Growth signals are required for the proliferation of all cells. Tumor cells generate many of their own signals, and thereby reduce their dependence on stimulation from their normal tissue microenvironment. Three molecular strategies for achieving this include alteration of extracellular growth signals, altered activity of cell surface receptors that transduce the growth-stimulatory signals or modifications of components in the downstream intracellular pathways that receive and translate the signals into action [34].

Insensitivity to anti-growth signals

To maintain tissue homeostasis and cellular quiescence, multiple anti-proliferative signals are in action. These signals force cells into G_0, the quiescent state, or induce them to differentiate so they are unable to proliferate further. In order to evade this, disruption of a component in the RB-pathway is an option. Many anti-proliferative signals are directed through the RB1-protein and two of its relatives, p107 and p130. Hypo-phosphorylated RB1 binds to and inhibits the E2F-transcription factors that, when unbound, control the expression of genes necessary for progression from G_1 to S-phase in the cell cycle. Altering components in the pathway or phosphorylaton of RB1 itself, releases the E2Fs, and hence the cell is insensitive to anti-growth signals [36].

Evasion of apoptosis

It is believed that all cells harbor a latent program for cell death where they die without damaging their neighbors; the cell shrinks and becomes engulfed by nearby cells. Activation of the intracellular cell death pathway is usually triggered in an all-or-none fashion [11]. Once the cell reaches a critical point along the path to destruction, it cannot turn back. The balance

between proliferation and apoptosis are carefully supervised to maintain the proper number and types of cells. Apoptosis is also a mechanism to remove cells with severe DNA damage and can be triggered by a variety of stimuli, from overexpression of oncogenes, withdrawal of essential survival signals, and activation of death signals. The machinery can be divided into sensors and effectors where the sensors are responsible for monitoring the extra- and intracellular environment for harmful conditions. These signals regulate the effectors, which start the cell death program. Mutation of the tumor suppressor gene *TP53* is one of the most frequent alterations observed in human tumors. An aberration in this protein can affect the ability of sensors to regulate the effectors, since several signals are funneled in part via TP53 [37]. It is expected that virtually all cancer cells harbor alterations that makes them capable of evading apoptosis [34].

Limitless replicative potential

The central dogma in cell biology stated that normal human cells could replicate indefinitely. It was not until Hayflick in the 1960s demonstrated that cells in culture could only divide a limited number of times, approximately 50, the dogma was changed, and the phenomenon termed cell senescence. The limited proliferation capacity reflects a progressive shortening of the cell's telomeres, the repetitive DNA sequences and associated proteins that cap the ends of each chromosome [11]. Fifty to hundred telomeric base pairs tend to be lost from the chromosome end each cell cycle. Unprotected chromosomes make end-to-end fusions with cell death as an expected outcome. Human somatic cells have turned off the enzyme that normally maintains the telomeres, *telomerase*. In order to gain a limitless replicative potential and to transform, the cancer cells have to up-regulate the telomerase activity, or circumvent the cell-cycle checkpoints [34].

Sustained angiogenesis

All human cells require a secure, controlled supply of oxygen. Since the diffusion of oxygen through tissues is limited, a vascular system has evolved to make sure that all cells are within the maximum distance of a capillary blood vessel [38]. Once a new tissue is formed, the growth of new blood vessels – the process of angiogenesis – is carefully regulated. Angiogenesis is subject to a complex control system with proangiogenic and antiangiogenic factors and normally, there is a strict regulation of the physiologic balance between these stimulatory and inhibitory signals. In order for tumors to reach a significant size, they have to acquire angiogenetic abilities, which is usually done by activating an angiogenic switch by changing the balance of inducers and inhibitors. One way to achieve this involves alteration of gene transcription. The ability to induce and sustain angiogenesis is found to be an early event in tumor development [39], and is therefore an attractive therapeutic target. Remarkable and very promising tumor growth suppression has been observed in animal models using several antiangiogenic approaches [38].

Tissue invasion and metastasis

Metastasis, the spreading of cancer cells in the organism, is the most lethal aspect of cancer development. There are many factors playing a crucial role in this complex process, including somatic mutations, epigenetic modulation, interaction with normal stroma, and environmental stimuli. Additionally, recent evidence implies a significant role for germline polymorphisms in cancer progression [40]. The capability for invasion and metastasis enables cancer cells to escape from the primary tumor, enter blood- or lymph vessels, and colonize somewhere else where nutrients and space is not a limiting factor. A strategy to make this possible often involves changing the physical coupling of cells to their microenvironment. Several classes of proteins become altered in this process; cell-cell adhesion molecules that mediate cell-to-cell

interactions and integrines which link cells to the extracellular matrix, are the most frequently changed. One commonly seen alteration involves the cell-to- cell adhesion molecule, E-cadherin, encoded by the *CDH1* gene. This protein is usually embedded in two adjacent plasma membranes to bind epithelial cells together. When tumors have made this gene or protein inactive, it favors metastasis by specifically contributing to local invasiveness [11].

Cancer critical genes and the signal transduction pathways they are part of

The genes selected for the present study are important components of signaling pathways frequently altered in cancer. The next few pages will give a short description of some of the central pathways, as well as a description of the selected genes and their role in cancer development. Figure 6 illustrates a general overview of the pathways to be described.

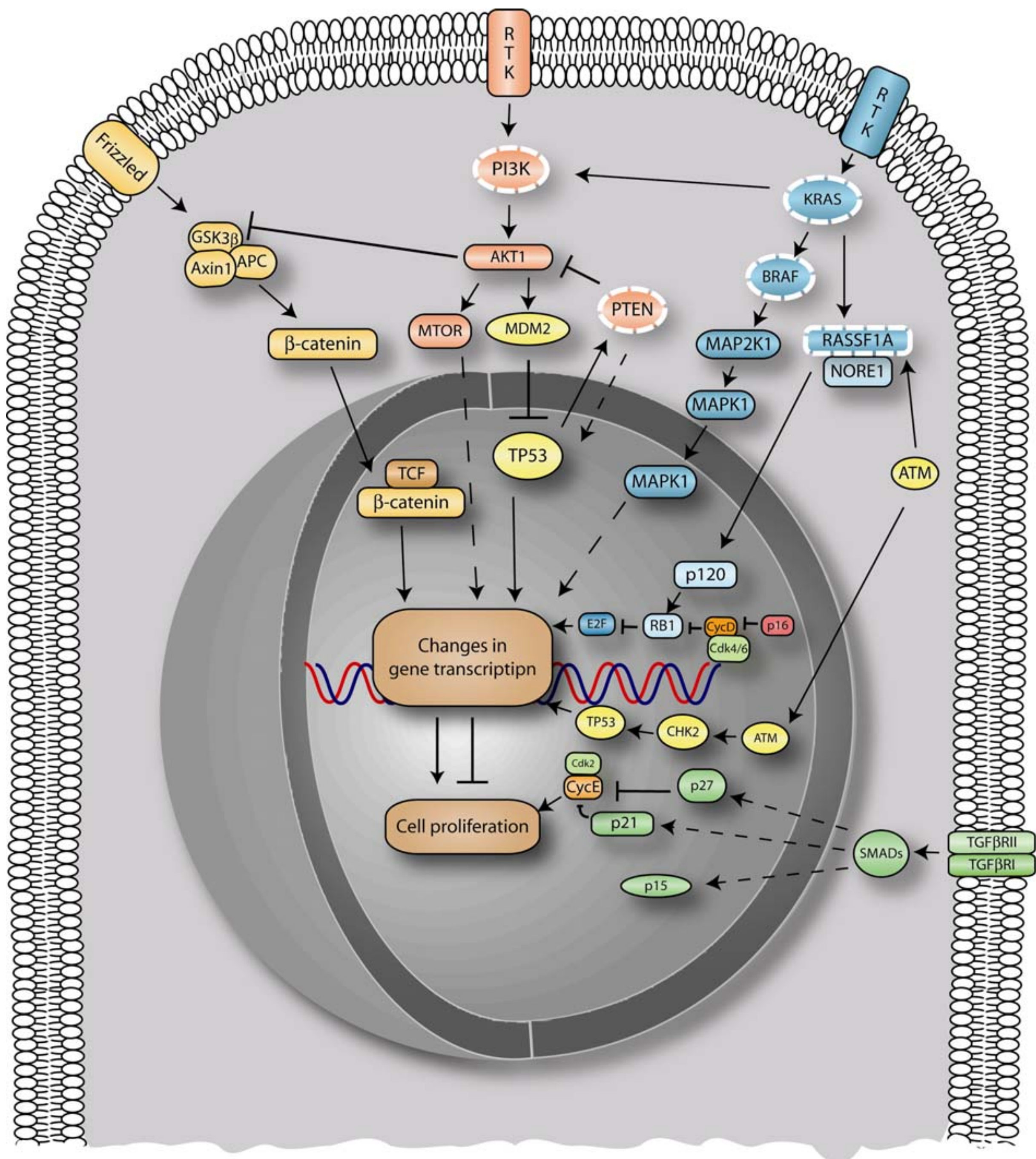

Figure 6: Cancer critical pathways, a general overview. The figure illustrates the WNT, receptor tyrosine kinase/MAPK, PI3K/AKT, and TGF-β signaling pathways. Genes investigated in this thesis, are marked with a dashed, white circle.

Signaling pathways important for homeostasis are often deregulated in cancer. In the last decade, several genes responsible for the origin of various cancer types have been discovered, their mutations identified, and the pathways through which they act characterized. Studies

have shown that the mutations within one pathway sometimes obey an “exclusivity principle”. That is, only one or a few genes in a pathway are generally mutated in any single tumor, since the functional effect of each mutation is similar [41]. The discovery and delineation of these highly conserved signal transduction pathways represent major scientific success stories as they have huge practical implications. The fact that defects in a relatively small number of pathways underlies various tumor types suggest that targeted therapeutics will be effective against a broad range of cancers.

The RB1 and TP53 pathways

Virtually all DNA viruses that cause tumors in experimental animals or humans, encode proteins that inactivate both *RB1* and TP53 [41]. (See page 23 for overview figure). Why have these two been singled out as targets for inactivation? In part, it may be impossible for a tumor of epithelial origin to form, unless the TP53 and *RB1* tumor suppressor gene pathways are non-functioning. This is supported by numerous studies, reporting that these two pathways are altered in a large fraction of cancers [41]. *RB1* and *TP53* are central to several processes and alterations can disrupt normal growth control in response to environmental signals, inactivate cell cycle checkpoints that otherwise limit cell division, or induce apoptosis in response to DNA damage or oncogene activation [42]. The control of G_1- progression and S-phase initiation in the cell cycle is often disrupted in cancer cells. Components of the transcription factor protein family, E2F, normally bind to specific DNA sequences in the promoters of many genes encoding S-phase entry proteins. E2F function is controlled mainly by the interaction with RB1-protein, which is an inhibitor of cell cycle progression. During G_1, RB1 binds to E2F and blocks the transcription of S-phase genes. When cells are stimulated to divide by extracellular signals, active G_1-Cdk accumulates and phosphorylates RB1, thereby reducing its affinity for E2F. When the RB1 dissociates, it allows E2F to

activate S-phase gene expression (Figure 3) [11]. This is also what happens when RB1 or some of the other components in the pathway become altered.

The TP53 pathway is composed of a network of genes and their products, which respond to a variety of stress signals that have an impact on cellular homeostatic mechanisms. An activating stress signal is transmitted to the TP53 protein, which is working as a transcription factor. Thereby it initiates a program of cell cycle arrest, cellular senescence and/or apoptosis [43]. Because TP53 normally interacts with another protein, MDM2, it is highly unstable and present at very small concentrations. MDM2 acts as an ubiquitin ligase, and targets TP53 for degradation by the proteasome. A DNA damage in the genome will activate protein kinases that phosphorylate TP53 and thereby reduce its binding to MDM2. This decreases TP53 degradation, and subsequently enhances its ability to stimulate gene transcription [11]. The transcriptional network of TP53-responsive genes produces proteins that interact with a large number of other signal transduction pathways, resulting in many positive and negative autoregulatory feedback loops that act upon the TP53 response [43]. A point mutation in the DNA binding domain that inactivates the ability of TP53 to bind to its recognition sequence is the most common way to disrupt the TP53 pathway. However, there are several other ways that achieves the same effect, including amplification of the *MDM2* gene.

The canonical Wnt signaling pathway

Wnt-proteins are molecules released from the surface of signaling cells, which act as local mediators to control many aspects of normal development. (See page 23 for overview figure.) They are among others required for maintenance of adult tissue, hence disturbances in Wnt signaling can promote both human degenerative diseases and cancer [44]. The Wnt-proteins act on target cells by binding to a complex consisting of transmembrane cell-surface receptors

of the Frizzled family and low-density lipoprotein receptor-related protein, LRP. They transduce signals to several intracellular proteins, including the signaling protein Dishevelled. The Dishevelled-dependent pathway regulates proteolysis of the protein β-catenin, encoded by the *CTNNB1* gene, which functions both in cell-cell adhesion and as a latent gene regulatory protein. In the absence of Wnt signaling, most of the β-catenin is located at cell-cell adherens junctions, and those molecules that are not, are rapidly degraded in the cytoplasm [11]. The proteasome-mediated degradation is controlled by a complex containing the serine/threonine kinase glycogen synthase-3β (GSK-3β), the tumor suppressor protein adenomatous polyposis coli (APC) and the scaffold protein Axin. GSK-3β phosphorylates β-catenin thereby marking it for ubiquitylation, APC helps promote degradation by increasing the affinity of the degradation complex for β-catenin, whereas Axin holds the protein complex together. When cells receive Wnt signals, phosphorylation and degradation of β-catenin is inhibited, and as a result, it accumulates in the cytoplasm and nucleus [45]. Nuclear β-catenin, now working as a coactivator, interacts with transcription factors such as lymphoid enhancer-binding factor 1/T-cell specific transcription factor (LEF1/TCF) that induce the transcription of Wnt target genes. Among the genes activated is *MYC*, which protein product is a strong stimulator of cell growth and proliferation, *JUN*, and *CCND1*[11]. Also a large number of the components in the Wnt signal transduction pathway itself are found to be targets of Wnt, hence providing feedback control during signaling [44]. Mutations of the *APC* gene occur in 60-80% of human colorectal cancers [46]. The effect is inhibition of the protein's ability to bind to β-catenin, so that, even in the absence of Wnt signaling, it accumulates in the nucleus and induce transcription of *MYC* and the other Wnt target genes. As a consequence, uncontrolled cell proliferation promotes the development of cancer [11]. Also other components in the cytoplasmic complex may be mutated in addition to or exclusively to APC, including β-catenin and Axin [47].

Receptor tyrosine kinase signaling

The signals conveyed by soluble growth factors, hormones and cytokines, required for survival and proliferation, are transmitted into the cell by transmembrane proteins with tyrosine kinase activity. (For overview figure see page 24.) The receptor tyrosine kinases are currently classified into 19 structural subfamilies, each dedicated to bind its complementary family of protein ligands [48]. Binding of a signal protein activates the intracellular tyrosine kinase domain of the receptor and makes it undergo receptor dimerization and autophosphorylation (See figure 7). This recruit adaptor proteins like GRB2, SHC1 and the guanine nucleotide exchange factor (see below) SOS1, which further interact with and activate various downstream effectors. An important component of the growth factor-RTK signaling pathway is the small GTP-binding protein, KRAS, which can activate several downstream cascades. The growth factor-RTK-RAS signaling pathway is one of the most frequently targeted genetic pathways in human cancers (~30%) [49], probably because activating mutations can make the cancer cells independent of exogenous growth factors [11].

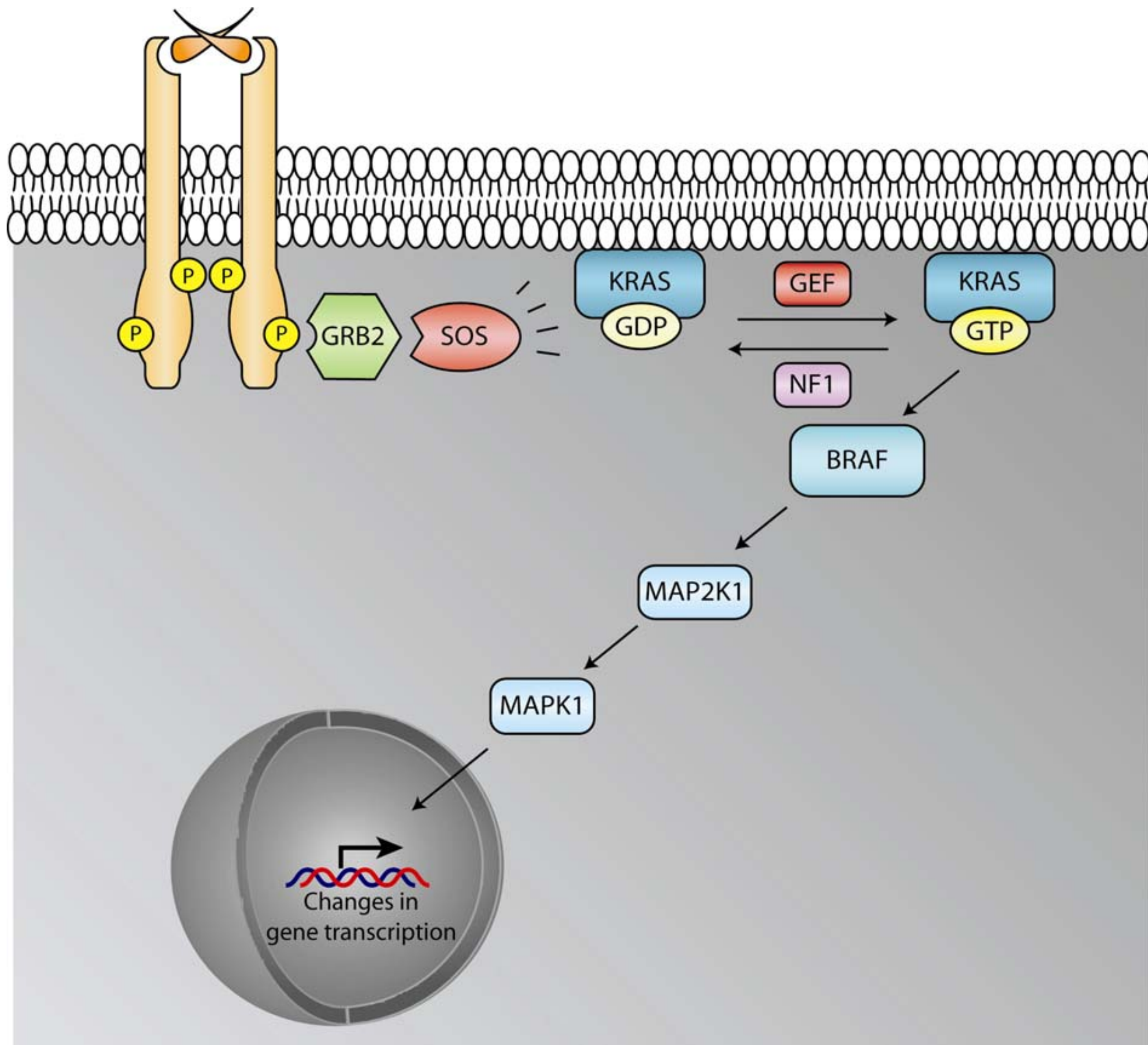

Figure 7: The MAPK-pathway. Ligand binding to receptor tyrosine kinases (RTKs) results in dimerization and autophosphorylation. This recruits the adaptor-protein GRB2 and the guanine nucleotide exchange factor SOS1, which activates KRAS. By associating with its RAS-binding domain, KRAS activates BRAF, which in turn activates the MAP-kinase-cascade, resulting in changes in gene expression.

KRAS

The *ras* gene family encodes highly related proteins involved in signal transduction. They belong to a family of membrane-bound GTP-ases cycling between to stages, a GDP-bound and a GTP-bound state. The cycle is controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), driving formation of active GTP-Ras and

inactive GDP-Ras, respectively [11]. Activating mutations are found in a wide range of human diseases, and represent some of the most frequently mutated oncogenes in cancer, indicating that the proteins have a powerful transforming potential. [50] The best-known effectors of RAS is the RAF-family, which serves to regulate the mitogen-activated protein kinase (MAPK) cascade activated by growth factors (See figure 7) [50].

KRAS mutations in cancer

KRAS, located on chromosome 12p, is the *ras* gene where the majority of transforming mutations is found, typically in codon G12, G13 and Q61 [51]. Point mutations in these hot spots produce a protein unable to hydrolyze its bound GTP, thereby bringing it into a perpetually active state. This leads to excessive and inappropriate signaling, promoting cellular transformation. The spectrum of tumor types showing *KRAS* mutations is broad, including pancreatic cancer (72-90%) [52], colorectal cancer (32-57%) [52,53], and testicular cancer (9-12%) [54]. KRAS serves as a node for a wide range of signal transduction pathways including the MAPK, PI3K-AKT, JNK and CDC42-RAC1-RHO pathways [55].

BRAF

BRAF encodes one out of three cytoplasmic serine/threonine kinases in the RAF-family, and is normally regulated by mitogens like growth factors, cytokines and hormones, which bind to and activate RTKs and G-protein-coupled receptors on the cell surface [56]. These receptors activate RAS, which in turn activates BRAF by binding to its RAS-binding domain. BRAF activates a second protein kinase, MAP2K1, which subsequently activates a third protein kinase, called MAPK1. This pathway, working through MAPK1, regulates gene expression, cytoskeletal rearrangement and other kinases, which in turn control senescence, apoptosis, proliferation, differentiation and motility [57]. By contrast, oncogenic *BRAF* is constitutively

active, thereby stimulating the MAPK-pathway and hence cell growth, independently of mitogenic signals.

BRAF mutations in cancer

Most of the mutations found in *BRAF* are encompassed in exon 11 and 15, which represent a glycine rich loop and a kinase domain, respectively. The alterations seems to involve many of the residues that stabilize the interaction between these two regions [57]. Occurring in 90% of the cases, V600E, substituting a valine residue by a glutamic acid, is the most common mutation in *BRAF* [58]. The V600 mutation, residing in exon 15, can also be converted to other amino acids, although at very low frequencies. Mutations in this codon overcome the need for a RAS-dependent step since insertion of an acidic residue at position V600 is believed to render a negative charge to the amino acid which mimics activation loop phosphorylation of T599/S602 in wild type *BRAF*, thus causing it to be constitutively active [59]. *BRAF* mutations are common in many cancers, but there is a trend towards the occurrence of mutations in cancer types known to be mutated in *RAS*, including malignant melanoma, colorectal cancer and borderline ovarian cancers [59]. However, concomitant mutations are extremely rare, suggesting that mutations of *BRAF* and *RAS* are mutually exclusive genetic events and that activation of the MAP-kinase signaling pathway can be achieved by mutations at various levels in the cascade [57] [60].

RASSF1A

The *RASSF1* gene was first discovered as recently as in 2000 [61] [62]. By differential promoter usage and splicing, it encodes seven transcripts, whereupon two isoforms, A and C, are ubiquitously expressed. They are members of a new group of *RAS* effectors that is thought to regulate cell proliferation and apoptosis [63]. During the last few years, inactivation of

TSGs has been shown to be as commonly caused by epigenetic events as by mutation/deletion events. *RASSF1A* falls in to this category. Chromosome band 3p21.3, containing the *RASSF1*, is found to frequently undergo loss of heterozygosity in tumors, and if the remaining band is methylated [61], *RASSF1A* obey Knudson's two hit model for inactivation of TSGs. A part of RASSF1 is homologous to the RAS-effector protein NORE1, and encodes a RAS-association domain (see figure 8).

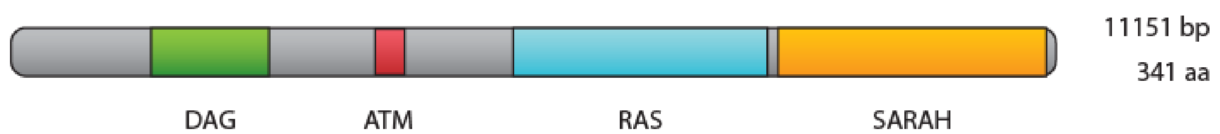

Figure 8: The structural domains of *RASSF1A*.
Abbreviations: bp, base pair; aa, amino acids; DAG, phorbol ester/diacylglycerol-type binding domain; RAS, RAS association binding domain; SARAH, SAV/RASSF1/HPO interaction domain; ATM, putative ATM phosphorylation site.

Several studies have begun to investigate the biochemical function of RASSF1A, but we are still far from understanding its true role. Apparently it is involved in several growth regulatory and apoptotic pathways, (See figure 9). Data indicate that binding of RASSF1A to GTP-bound RAS may require heterodimerization with NORE1, and that RASSF1A itself can bind RAS only weakly, if at all [64]. Recent research indicate that activation of *KRAS* and inactivation of *RASSF1A* and *NORE1* may be a mutually exclusive event in colorectal and pancreatic cancers, providing alternative ways of affecting RAS signaling [65]. The pro-apoptotic kinase MST1 is found to bind both RASSF1A and NORE1, indicating that this complex formation mediate the apoptotic effect of activated KRAS [66]. Shivakumur and co-workers have reported that RASSF1A can induce cell cycle arrest by engaging the RB-family cell cycle checkpoint both directly and indirectly. RASSF1A inhibited accumulation of cyclin D1 and the growth arrest could be relieved by ectopic expression of cyclin D1 cDNA. This

implies that RASSF1A modulates cell cycle progression through pathways regulating accumulation of cyclin D1 protein [67]. Recently, several groups have reported that RASSF1A might regulate mitotic progression due to the findings that it is a microtubule-binding protein [68]. It has also been reported that it can regulate mitosis by inhibiting the anaphase promoting complex (APC) through CDC20 and induces G_2/M arrest at pro-metaphase [69]. Thus, RASSF1A function as a tumor suppressor through controlling apoptosis and mitotic cell division.

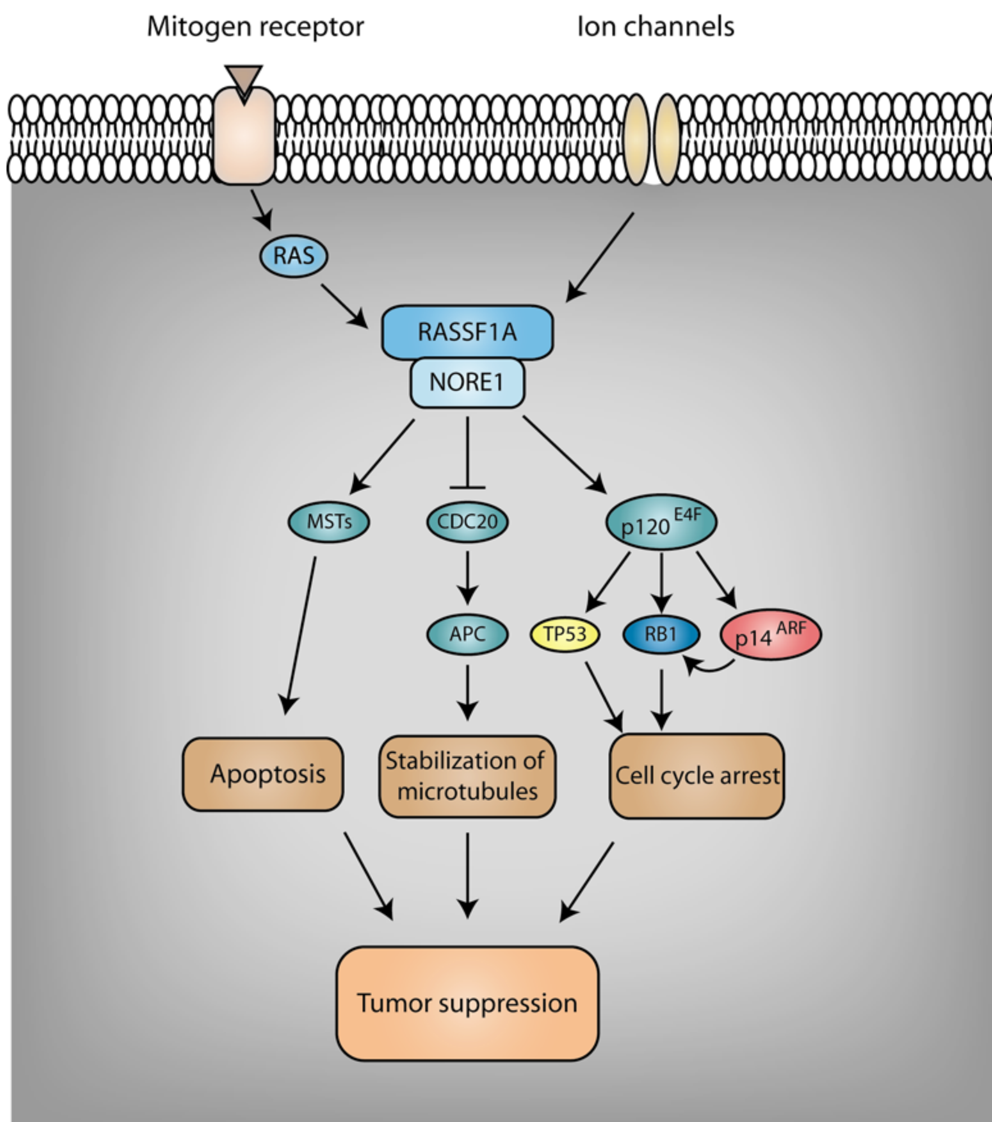

Figure 9: Summary of the putative RASSF1A network. RASSF1A can regulate apoptosis, the microtubule network and cell cycle progression by interaction with NORE1 and recruitment of various effectors. Modified after Pfeifer *et al* and Agathanggelou *et al*, ref. [68,70]

RASSF1A in cancer

Mutations of *RASSF1A* are rarely found in tumors. However, silencing of *RASSF1A* by promoter hypermethylation is a common event associated with a wide range of human malignancies including lung cancer, breast cancer, renal cell carcinomas, ovarian, gastric and bladder cancers [63].

The phosphatidylinositol- 3-kinase – AKT pathway

The phosphatidylinositol 3-kinase (PI3K) pathway regulates various cellular processes, such as proliferation, cell growth, cytoskeletal rearrangements, and apoptosis. (See page 23 for overview figure). The PI3Ks are members of a large and complex family containing three classes consisting of multiple subunits and isoforms. The proteins are heterodimeric lipid kinases composed of regulatory and catalytic subunit variants encoded by separate genes and alternative splicing [71]. Class IA PI3Ks are activated by receptor tyrosine kinases or by binding of active RAS to the p110 catalytic subunit. The primary consequence of activating PI3K is generation of the second messenger phosphatidylinositol 3,4,5 triphosphate (PIP_3), using phosphatidylinositol 3,4 diphosphate (PIP_2) as a substrate (see figure 10) [72]. Frequent genetic aberrations in the PI3K-pathway have been detected in several types of human cancers [73]. AKT1, (also known as PKB), a serine/threonine kinase, which acts downstream of PI3K, regulate various biological processes, including those involved in tumor development and progression [71]. AKT1 is recruited to the plasma membrane, and becomes activated through direct contact with a pleckstrin-homology (PH) domain with PIP_3. Also other components in the pathway are involved in tumorigenesis. Amplifications as well as deletions of short nucleotide sequences of the *PI3K* locus have resulted in elevated levels of lipid kinase activity. This implies that PI3K functions as an oncogene [74]. Alterations in the *PIK3CA* gene have been reported in a variety of cancer types, including cancers of the breast, brain,

liver, stomach and the lungs [71]. The phosphatase PTEN, which is also altered in a wide range of tumor types, counteracts the kinase activity of PI3K by dephosphorylation, thereby suppressing growth [75]. (See also page 37). The clinical evidence of PI3K-pathway deregulation in various cancers and the identification of downstream kinases involved in mediating the effects of PI3K (AKT1, MTOR, PDK1, ILK) provide present and potential targets of therapy [72].

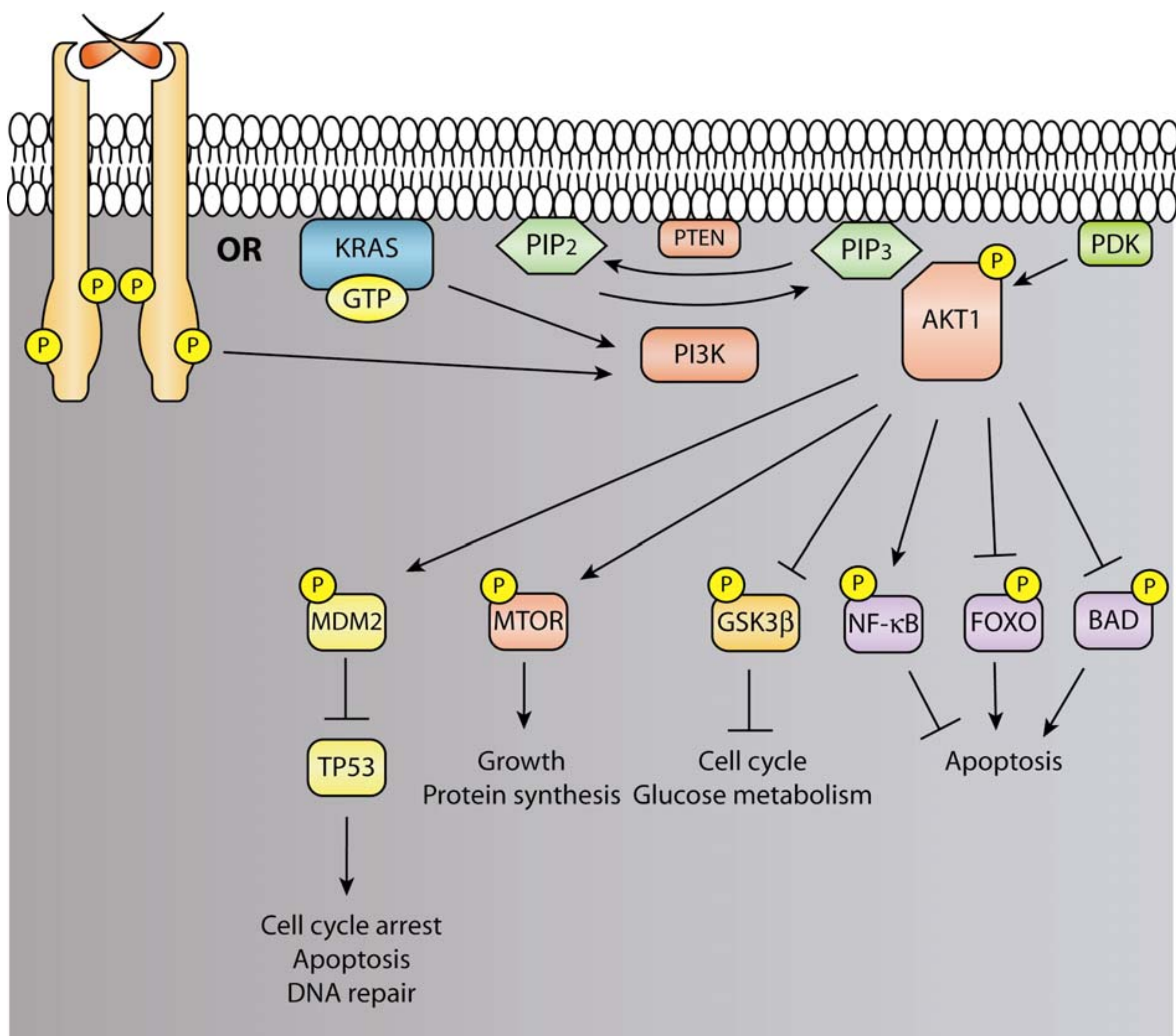

Figure 10: The PI3K – AKT signaling network. Ligand binding to receptor tyrosine kinases or activated KRAS activates PI3K, which phosphorylates PIP_2 converting it to PIP_3. This action can be reversed by dephosphorylation carried out by PTEN. Through recruitment and interaction with PIP_3 and phosphorylation by PDK, AKT1 becomes activated and mediates the activation and inhibition of several targets, resulting in cellular growth and survival through various mechanisms.

PIK3CA

PIK3CA, which encodes the catalytic p110α subunit of PI3K, is located on chromosome 3, and encodes 20 exons [76].

PIK3CA mutations in cancer

Gene amplifications, deletions, and more recently, somatic missense mutations of *PIK3CA* have been reported in 15% of human cancers. Especially cancers of the liver, breast, and colon is found to have a high rate of these mutations, with the approximate frequencies of 36, 26 and 25%, respectively [71]. Mutations can be found spread throughout the whole sequence of the gene, but are primarily clustered in the helical and kinase domain, located to exon 9 and 20. The most frequently altered residues are the glutamates E542 and E545, most often substituted by lysine, and histidine H1047, which becomes substituted by an arginine. These three hot spots are highly conserved in evolution, and are therefore likely to play a significant role for the functioning of the gene [71]. Indeed, at all three sites, the mutations cause gain of enzymatic function [77]. In addition, studies have shown strong evidence for the oncogenic potential of the mutant proteins, which can induce oncogenic transformation when expressed in primary chicken-embryo fibroblasts and NIH3T cells [73,78]

PTEN

Phosphatase and tensin homologue deleted on chromosome ten (*PTEN*), also known as *MMAC1*, encodes a dual specificity phosphatase. The gene product dephosphorylates tyrosine and serine/threonine residues on both proteins and lipids [79], and is found both in the cytoplasm and the nucleus, functioning as a tumor suppressor [80,81].

PTEN in cancer

Several studies demonstrate high levels of *PTEN* mutations or deletions in various human cancers, including brain, bladder, breast, prostate and endometrial cancers, as well as in the cancer predisposition syndrome Cowden disease. This makes *PTEN* the second most frequently mutated tumor suppressor gene after TP53 [82] [83]. The major task for PTEN is regulation of PIP_3 levels. By dephosphorylating PIP_3, it inhibits the growth factor signals transduced through PI3K. The loss of PTEN results in increased concentrations of PIP_3, which in turn activates several signaling molecules, including AKT1, MTOR, phospahtidylinositol dependent kinases (PDKs) as well as the small GTPases RAC1 and CDC42 [82]. In other words, *PTEN* is exerting its tumor suppressor function through negative regulation of the PI3K-AKT pathway, which also interacts with other signaling cascades like TGFβ/SMAD and the WNT/β-catenin pathways. It has been shown that *PIK3CA* and *PTEN* can be mutually exclusive, suggesting that tumorigenic signaling through the PI3K- AKT1 pathway can occur either through activation of *PIK3CA* or by inactivation of the tumor suppressor *PTEN* [71]. It has been proposed that PTEN-controlled pathways are involved in regulation of stem cell renewal, cell fate determination, and cell and organ size by controlling cell cycle checkpoints and hormones. However, more studies are required to find the exact and complete biological function of PTEN [82].

TGF-β signaling pathway

Transforming Growth Factor β (TGF-β) signaling is regulating a diverse set of cellular processes, including cell proliferation, differentiation, apoptosis, and specification of developmental fate [84]. TGF-β superfamily members signal through receptor serine/threonine kinases and intracellular Smad-proteins. Additionally, several intracellular proteins that mediate signals through receptor tyrosine kinases, cytokine receptors and G-

coupled receptors also participate in the TGF-β signaling network, however, the canonical Smad pathway is the most commonly mentioned in regard to TGF-β. (See page 23 for overview figure.) It is evolutionary conserved, and of great importance for the precise implementation of the tissue- and organ-patterning programs during development [85]. TGF-β binding induces the formation of a serine/threonine kinase complex consisting of TGF-β receptor type II (TGFβRII) and receptor type I (TGFβRI.) The constitutively active TGFβRII kinase phosporylates TGFβRI within a regulatory region, resulting in its activation. Then, TGFβRI phosphorylates receptor-activated (R)- SMADs. The phosphorylated (R)- SMADs form complexes with the common mediator SMAD4, enter the nucleus where they bind to DNA and interact with transcription factors to regulate gene expression [86]. The importance of the TGF-β pathway as a growth repressor has been demonstrated by the disruption of the signaling components in human cancers, contributing to tumorigenesis. Mutations have been observed both in TGF-β receptor family and the SMAD-proteins [84].

Development of solid tumors

The tissues of the adult vertebrate body are generated from three germ layers established during gastrulation. Some cells cohere to create an epithelial sheath facing the external environment, the ectoderm, which is a precursor to the epidermis and the nervous system. A part of the sheath becomes tucked into the interior to form endoderm, the precursor of the gut and the internal organs, such as lungs and liver. A third group of cells move into the space between the endoderm and the ectoderm, and makes up the mesoderm, which gives rise to muscles and connective tissue (See figure 11) [11].

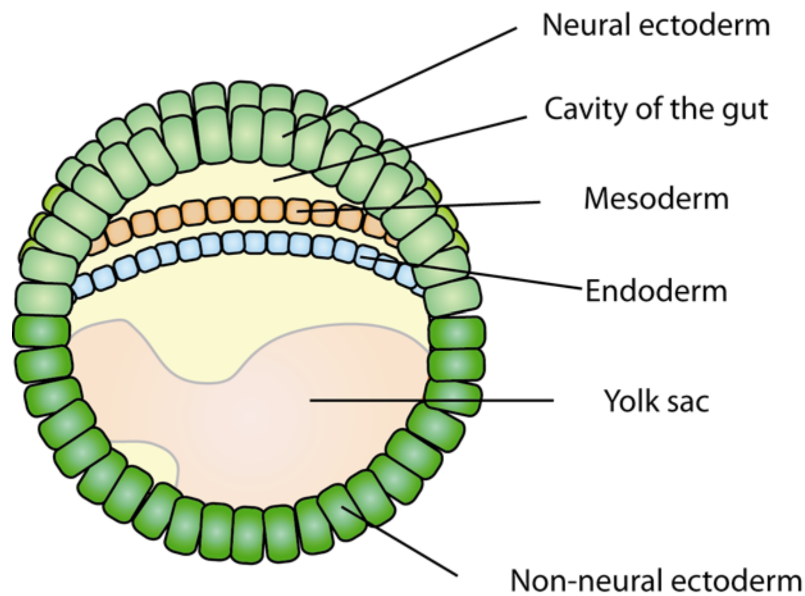

Figure 11: Gastrulation. Development of ectoderm, endoderm and mesoderm, which eventually will make up distinct tissues. Modified after Alberts *et al*, ref. [11].

Cancers are classified according to the tissue and cell type from which they arise. The most common cancer types arise from the epithelial cells, are termed carcinomas and include breast, prostate, lung, and colon cancer. Cancers arising from connective tissue or muscles are termed sarcomas [87]. The classifications of central neurological tumors are based on their predominant cell types, e.g. astrocytoma is composed primarily of astrocytes and oligodendroglioma primarily of oligodendrocytes. In the peripheral nervous system, most of the tumors are termed neurofibromas [55].

Development of the germ cell lineage is independent of the three germ layers. A group of cancers termed germ cell tumors are believed to arise from primordial germ cells. They represent a heterogeneous group of neoplasms, which arise in gonads, both in the ovaries and the testes. Additionally, this tumors can in rare cases be found in various extragonadal sites along the midline of the body [88].

Colorectal cancer

Colorectal cancer (CRC) is one of the most common cancer types, and represents the fourth most frequent cause of cancers deaths, worldwide [89,90]. The adenoma-carcinoma sequence is the basis for the development, and several underlying molecular changes have largely been identified. New information in this field has led to the development of targeted therapeutic options, which are being tested in clinical trials [90].

Epidemiology

The World Health Organization (WHO) has estimated that every year, nearly one million people worldwide develop colorectal cancer, of which almost 50 percent die within 5 years [91]. Norway has the highest incident of colorectal cancer among the Nordic countries, with approximately 3500 new cases each year[3]. This cancer is less common in developing than in developed countries, and in the latter group, colorectal cancer is the second most common tumor, with a lifetime incidence of 5%. However the incidence and mortality are now decreasing [92]. Most cases of colorectal cancer are sporadic, and genetic, epigenetic, and environmental factors are all believed to play a role both in the initiation and the progression. About 10-20% of all patients with this cancer are estimated to have a familiar risk without fulfilling the criteria for hereditary colorectal cancer [93], whereas 5-10% develop in the setting of defined hereditary cancer syndromes. High-penetrance mutations confer predisposition to hereditary non-polyposis colorectal cancer (HNPCC), which involves mutations in mismatch-repair genes, and to familial adenomatous polyposis coli (FAP), which involves mutations in the *APC* (Adenomatous Polyposis Coli) TSG. Patients with chronic inflammatory bowel diseases like Ulcerative colitis and Crohn’s colitis or hamartomatous

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[3] The Norwegian Cancer Registry’s website: http://www.kreftregisteret.no/

polyposis syndromes, including Peutz-Jeghers syndrome and Cowden syndrome, also have an elevated risk of developing colorectal cancer [90].

The adenoma-carcinoma sequence

The well-defined sequence of events during colorectal cancer development, suggested by Muto and co-workers in 1975 [94] – from aberrant crypt foci to benign adenomas, further on to carcinoma *in situ,* and finally to metastatic carcinomas – reflects the stepwise accumulation of histological and concurrent genetic and epigenetic changes (See figure 12). It might take several decades to develop these tumors, however, as a result of the relative ease with which the various stages can be observed, colorectal cancer provides an excellent model for cancer development. It has been found that somatic mutations occur, at least to some extent, in a predictable order during the sequence. The earliest genetic mutations detected in precursor lesions of sporadic colorectal cancer, as well as in carcinomas, are *APC* and *KRAS*, which is found to be mutated in 60-80% and 50% of the carcinomas, respectively [46].

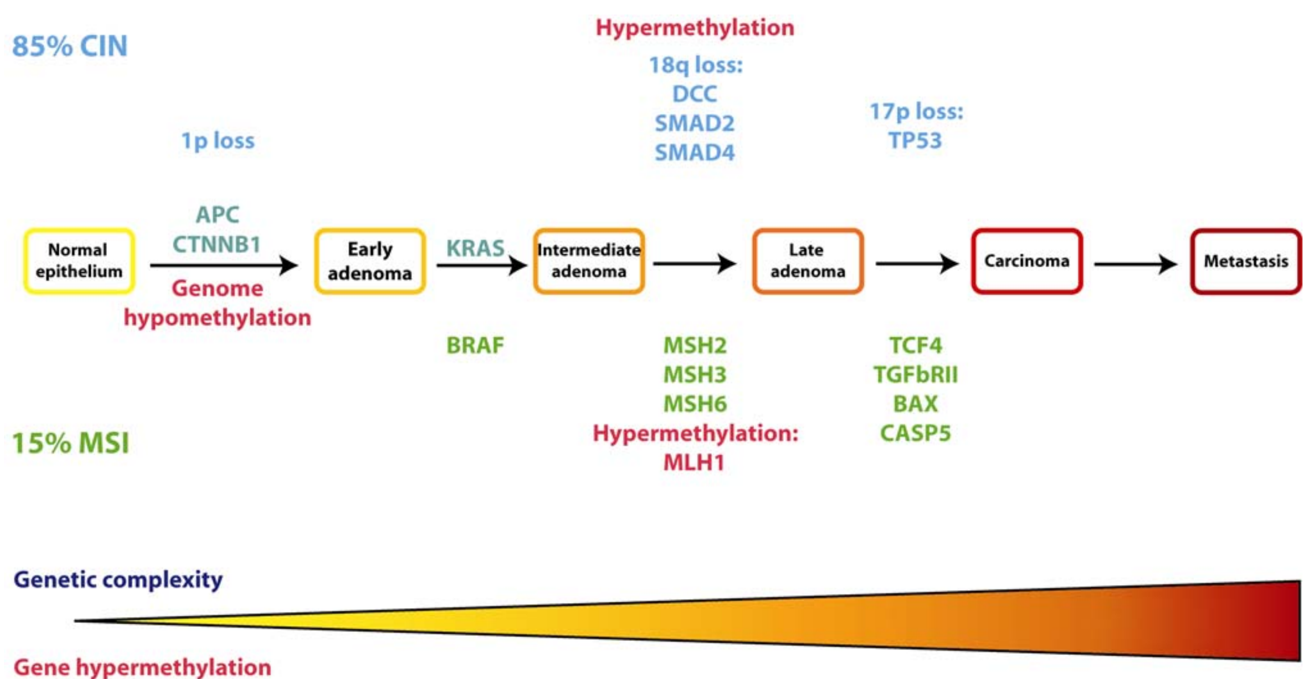


Figure 12: The adenoma-carcinoma sequence; genetic and epigenetic characteristics of the microsatellite instable (MSI) and the chromosomal instable (CIN) pathways in colorectal cancer.

Colorectal carcinomas can be classified into two main phenotypes: the microsatellite instability (MSI) and the chromosomal instability (CIN) phenotype [95]. CIN is responsible for approximately 85% of all sporadic colorectal cancers in addition to FAP tumors, and has been defined to have an accelerated rate of gains and losses of whole or large portions of chromosomes in the context of continuous growth [96], resulting in aneuploid tumors. More than 100 genes are expected to cause chromosomal instability when mutated, including genes that are involved in telomere metabolism, chromatid cohesion, spindle assembly and dynamics, cell-cycle regulation, DNA repair and checkpoint controls [97]. However, the actual mechanism(s) responsible for the genomic/chromosomal instability has only been identified in a small subset of tumors [98]. The CIN tumors are usually located in the left side of the colon, and the patients have poorer prognosis than do patients with MSI tumors [99]. The second group, those with MSI account for in approximately 15% of sporadic colorectal cancers, and additionally, tumors of the HNPCC spectrum follow this pathway. Tumors with the MSI phenotype is characterized by mutations or epigenetic changes in genes that maintain genetic stability, the mismatch repair genes [93], and is recognized by frameshift mutations in microsatellite repeats located throughout the genome. The MSI found in HNPCC is associated with defective DNA mismatch repair proteins caused by germline mutations in the one of the three main genes: *MLH1, MSH2* and *MSH6*. In contrast to HNPCC, the cause of MSI in sporadic colorectal cancer is frequently biallelic or hemiallelic methylation of the promoter sequence of *MLH1*, thus silencing the gene and causing reduced or lost protein expression [100]. The tumors displaying MSI are usually found on the right side of the colon, and are most often diploid.

Also a third phenotype, CpG island methylator phenotype (CIMP), has been suggested [101]. In this model, a cancer specific methylation pattern has been indicated, where CIMP positive

tumors display frequent methylation of several loci. These tumors show some overlap with the MSI phenotype and are associated with a proximal location within the colon. The CIMP negative tumors on the other hand, hardly show any cancer specific methylation. The CIMP model has been extensively discussed in the literature, since some studies support the original findings [102,103], whereas others do not [102,104].

Testicular germ cell tumors

Ninety-eight percent of testicular cancers are of germ cell origin, and hence are called testicular germ cell tumors (TGCTs). These tumors are comprised of: teratomas and yolk sac tumors of newborn and infants, seminomatous and non-seminomatous tumors of adolescents and young adults, and spermatocytic seminomas of elderly men. The various groups of tumors originate from germ cells at different stages of development, and are clinically, epidemiologically and genetically distinct [105]. The entity of seminomas and non-seminomas of young men is by far the most common form, and also the focus of this thesis.

Epidemiology

TGCTs account for 60% of all malignancies diagnosed in men between 20-40 years of age. The incidence has doubled the last 40 years, and an annual increase of 3-6% have been reported for Caucasian populations [88], however, the reason for this rapid increase is not known. The highest incidence is seen in people of Northern European decent, with the Scandinavian countries ranking high on the list. In Norway there is approximately 250 new cases each year[4]. In contrast, people from Africa or Asia have a low incidence of TGCTs, and since the differences persist after migration, it is suggested that the interaction between

[4] The Norwegian Cancer Registry's website: http://www.kreftregisteret.no/

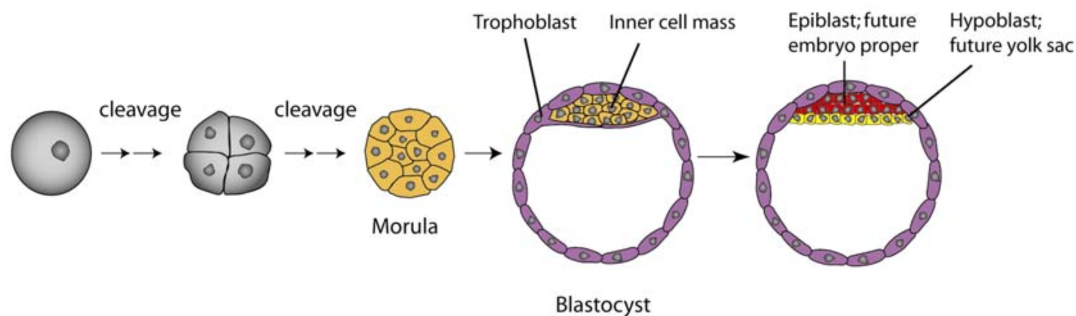
genetic and environmental factors is important [106]. The cause of testicular cancer is still not elucidated, but there are several predisposing factors known. Chryptorchidism (undescendend testis), gonadal dysgenesis, a family history of TGCT, and various syndromes with abnormal testicular development, including Klinefelter's syndrome (XXY), are associated with elevated risk of germ cell tumors. Patients with a history of TGCT also have increased risk of contralateral relapse [105]. Despite the increasing incidence, the mortality rate for TGCT patients has declined, the overall 5-year survival for all stages being approximately 80% [107]. This is due to major advances in treatment, including surgery, radiotherapy and chemotherapy. The overall goal for treatment is tumor-free survival for any TGCT patient, and this has already been reached for >99% of the patients with early-stage tumor, as well as for the majority of patients with advanced disease [107]. The high cure rate has brought attention also to the long-term effects of treatment, and data from these studies are now shaping treatment strategies that aim to keep clinical interventions at a minimum [106]. The main concerns are malignancies in other organs, cardiovascular disease and fertility, the latter being especially important because of the early on-set age of the disease. Overall, since TGCT has a high sensitivity to chemotherapeutic agents, it is regarded as the "model of a curable neoplasm."

Histopathology

TGCTs are classified into two subgroups: the seminomas and the non-seminomas, which are approximately equally common. These subgroups are both thought to develop through a non-invasive stage termed intratubular germ-cell neoplasia (ITGCN), or carcinoma *in situ*, which is suggested to initiate during fetal life from a primordial germ cell (PGC) [108]. The seminomas are in many ways resembling the ITGCNs, whereas non-seminomas include various tumor subgroups. They develop through a pluripotent embryonal carcinoma stage, which may further differentiate into various extra-embryonic tissues, like yolk sac tumors and

choriocarcinomas or into somatically differentiated teratomas. In many respects, this germ cell tumor development resembles the differentiation seen in early embryogenesis (see figure 13) [109].

a) Embryogenesis



b) Testicular tumorigenesis

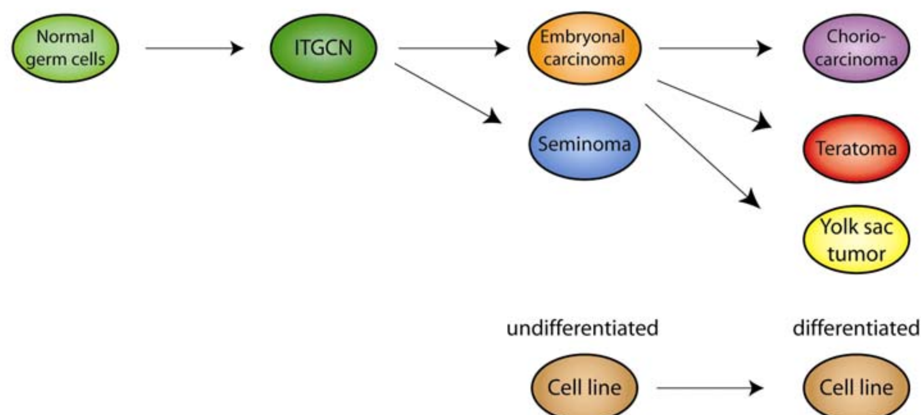


Figure 13: The association between A) embryogenesis and B) testicular cancer tumorigenesis. Abbreviations: ITGCN, intratubular germ-cell neoplasia. From Skotheim *et al*, ref. [109].

Genetic and epigenetic changes

Genome amplification is an early event in the development of germ cell tumors and TGCTs are in general aneuploid. ITGCNs and seminomas typically have hypertriploid genomes [110], whereas the non-seminomas usually are hypotriploid [111] [112,113]. A complex pattern of chromosome gains and losses has been identified, both in the seminomas and the non-seminomas. Overall, relative loss of chromosomes 4, 5, 11, 13, 18 and Y, and gain of

chromosomes 7, 8, 12 (see below) and X is found [88,113]. Karyotypic analyses have identified a specific marker, isochromosome 12p, consisting of two fused 12p-arms [114]. This chromosomal aberration have been found in virtually all testicular tumors, and identified in all histological subtypes, as well as in some ITGCNs [115], however the latter is debated. TGCTs without isochromosome 12p, still show amplifications of 12p sequences, indicating an early event of crucial importance for the development of cancer [88] Gene expression profiling has showed that various genes are overexpressed or altered in these tumors, including *GRB7*, *JUP* and *FHIT* [116,117] [118]. Only few mutated genes have been identified in TGCTs and then only at low frequencies. An activating mutation in codon 816 of *KIT*, which is a tyrosine kinase receptor, have been shown to be associated with development of bilateral TGCTs, and it is the first genetic event demonstrated to have a role in the initiation of testicular tumors [119]. The transcription factor *POU5F1* (alias OCT3/4), which is usually involved in regulation of pluripotency and characteristic of primordial germ cells, is found in ITGCN, seminomas and embryonal carcinomas [120], thereby representing a new diagnostic marker for undifferentiated TGCT. Epigenetic changes have also been identified in germ cell tumors like a genome wide hypomethylation, consistent with what is observed in other cancer types, and a regional hypermethylation. In general, seminomas show scarcely CpG methylation whereas the non-seminomas show CpG island methylation levels similar to other solid tumors [121].

Malignant peripheral nerve sheath tumors

Epidemiology

Malignant peripheral nerve sheath tumors (MPNSTs) are highly invasive, soft tissue sarcomas that arise within the peripheral nerves and frequently metastasize. Half of all MPNSTs are sporadic cases whereas the rest arise in individuals with the autosomal, dominant genetic disorder neurofibromatosis 1 (NF1). MPNSTs occur in 2-5% of NF1 patients, compared to an incidence of 0.001% in the general population [122]. The MPNSTs are usually resistant to conventional radiation and chemotherapy, and their location around the nerves prevents complete surgical resection, thus the 5-year survival rate ranges from 34-52% [55].

Neurofibromatosis 1

NF1, also known as von Recklinghausen disease, is caused by mutations in the *NF1*-gene, located on chromosome arm 17q. A wide variety of mutations have been found, but no frequently recurring mutation has yet been identified. The gene is large, spanning 350 kb genomic DNA, containing 60 exons, and it encodes the protein Neurofibromin [123]. Part of the protein shares sequence homology to the GTPase activator protein family that negatively interact with RAS oncogenes to regulate cell growth and differentiation, (See page 28). The GAP activity of Neurofibromin, in addition to *NF1*mutations in benign neurofibromas and malignant tumors associated with NF1, have led to the classification of NF1 as a tumor suppressor [124]. Individuals affected with NF1 harbor an increased risk of developing both benign and malignant tumors, supporting the classification of NF1 as a cancer predisposing syndrome. The prevalence of clinically diagnosed NF1 patients range from 1/2000 to 1/5000 in various population-based studies [123].

NF1 is characterized by a wide pattern of phenotypic features. In 1987, the National Institute

of Health (NIH) defined a set of diagnostic critera for NF1, where the patient ought to have two or more of the following features: neurofibromas, six or more café au lait spots, freckling of armpit or groin, optic glioma, Lisch nodules, distinctive bony lesions and a first degree relative with NF1 [125].

The hallmark of NF1, development of multiple neurofibromas, have two characteristic features: they invariably arise within peripheral nerves, and they are heterogeneous tumors that contain every cell type present in normal peripheral nerves, including Schwann-cells, perineural cells, fibroblasts and infiltrating mast cells. Dermal and plexiform are the two main types of neurofibromas in the setting of NF1. The first spontaneously cease growth and rarely progress to malignancy, whereas 5% of the latter undergo malignant transformation. There are indications suggesting that dermal neurofibromas might arise from a mature Schwann-cell lineage, whereas plexiform neurofibromas probably are derived from embryonic Schwann-cell lineages (Figure 14) [55]. In patients harboring an inherited *NF1*-mutation, Schwann-cells are thought to be the primary target for a second hit mutation [126].

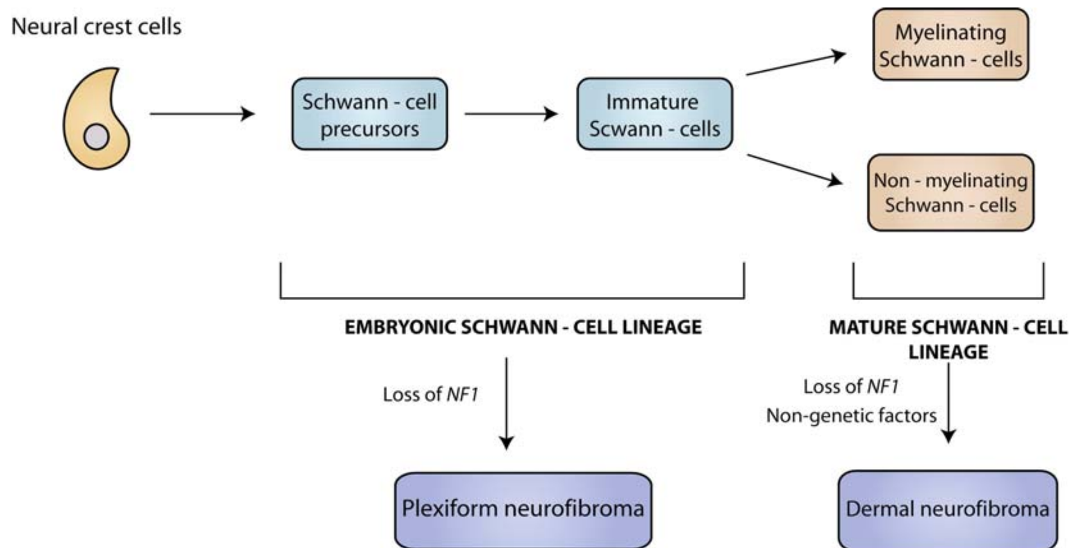


Figure 14: Development of Schwann cells and neurofibromas. MPNSTs usually develop from plexiform neurofibromas, which are thought to arise from embryonic schwann-cell lineages. Modified after Zhu *et al*, ref. [55]

The progression to malignancy – Neurofibromas to MPNST

The genetic changes underlying the malignant transformation of plexiform neurofibromas are not completely understood. However, there is an indication of a multistep process including several genetic, and possibly also epigenetic, alterations. Loss of Neurofibromin is believed to be the earliest event leading to tumor formation as patients inherit a mutated *NF1*-allele and loose the second copy in MPNST cells [127]. However, previous studies have demonstrated that loss of *NF1*-gene expression resulting in increased RAS activation alone is not sufficient for MPNST development [122]. Another early alteration in MPNST development is the expression of epidermal growth factor receptor (EGFR). Whereas EGFR is not expressed in normal human Schwann-cells, the protein is detected in primary MPNSTs, cell lines and subpopulations of neurofibroma Schwann cells [128]. MPNSTs exhibit complex karyotypes without a consistent cytogenetic pattern [129] [130] and comparative genomic hybridization (CGH) has been used to analyze gains and losses of DNA sequences and to map the copy number to the different chromosomes. The most frequent copy number changes observed in

MPNST are gains of 17q which might reflect amplification of Topoisomerase IIα (TOP2A) [131], and loss of 9p, 13q and 17p [130,132,133]. Based on CGH data and other studies reporting loss of heterozygosity, the tumor suppressor genes *p16^{INK4A}*, *RB1*, and *TP53* have been suggested targets for the deletions. TP53 is frequently mutated in various tumor types. Whereas expression in normal cells is usually low due to the short half-life of the protein, studies of sarcomas have detected expression of TP53 in MPNST and no or low levels of the protein in neurofibromas [134,135], which indicates an importance for TP53 in tumor progression. A few *TP53* mutations have been reported for MPNSTs [136-138], but biallelic inactivation seems to be rare [139]. The *CDKN2A* locus encodes the protein products p16^{INK4A} and p14^{ARF}, both important in negative regulation of the cell cycle through their interaction with the RB1 and TP53 pathway, respectively [13]. Studies of *p16^{INK4A}* have revealed gene alteration in 50-75% of the MPNSTs analyzed [140,141] However, alterations in neurofibromas are not reported, emphasizing the importance of disrupted *p16^{INK4A}* in the progression of the MPNSTs. Data from our group, [142], implies that the complete absence of p16^{INK4A} is sufficient for activation of the cell cycle in most MPNSTs, consequently it is not necessary to alter other central components of the cycle to further stimulate tumor proliferation. In addition, genetic alterations of *RB1* and amplification of *CDK4* have been detected in MPNSTs that lack alterations of p16^{INK4A} [143], indicating that the RB1-mediated G1/S-checkpoint pathway might be crucial. Furthermore, loss of expression of p27^{KIP1}, another component of the RB1-pathway, has been identified in most MPNSTs (91%) compared to only 6% of the neurofibroma [135]. A recent global gene expression profile study found that among others, *TWIST1* is over-expressed in MPNSTs. This gene, which inhibits expression of p14^{ARF} thereby bypassing TP53 induced apoptosis, might turn out to be an attractive therapeutic target for novel MPNST treatment strategies. Stable suppression of TWIST1 increases sensitivity to chemotherapeutic agents [127] and reducing TWIST1

expression in MPNST cells can inhibit chemotaxis, a key component of the metastatic process. The protein therefore seems to be necessary for tumor cell migration [127].

Master keys in cancer

Master keys in cancer biology can be defined at the level of single genes, gene families, signaling pathways, and cellular processes. Genes that are commonly altered across various cancer types, resulting in dysfunction of the encoded products, may be looked upon as master keys in tumor development. The tumor suppressor *TP53* is an excellent example of such a master key, as it has an influence on several signaling pathways and cellular processes, comprising the downstream TP53 network. Mutations in *TP53* are found in more than 50% of all solid tumors. Furthermore, by systematic mutational analysis of any gene family in any human cancer type, specific classes of proteins with particular importance in cancer may be identified. This is well exemplified by the "tyrosine kinome", where a systematic approach showed that at least 30% of all colorectal cancers had one or more mutated tyrosine kinase [144]. In the normal cell, the members of the kinome and the phosphatome, kinases and phosphatases, respectively, have important functions by activating and deactivating proteins. A pathway can represent a master key if mutually exclusive mutations among its factors, resulting in a similar functional consequence, are frequently found. The commonly altered canonical WNT signaling illustrates this, often through mutated *APC*, but alternatively through epigenetic or genetic changes of other components [47,145,146]. Although the molecular biology of cancer is too complex to be understood by looking at single factors, the analysis and identification of single gene master keys may aid in unraveling the molecular understanding of the tangled networks in which they normally participate, and how alterations at this step influence cancer development and progression.

Aims of the study

In the present study we hypothesized that key factors upstream of central signaling pathways may be commonly altered in cancer. In the epithelial cancers of the large bowel, alterations have previously been described for the five genes analyzed, although not in the same clinical series, and only limited knowledge exists with regard to the involvement of these genes in two other cancer models, arising in the peripheral nerves and in the testis. Furthermore, information across the three diseases remains unknown.

The aims of this study were two-fold:

1. To identify the frequencies and type of alteration of genes known to be upstream components in cancer critical pathways, in tumors that arise from cells originating from distinct embryonal germ layers. This includes colorectal-, testicular-, and malignant peripheral nerve sheath tumors, from the endoderm, primordial germ cells, and neuroectoderm, respectively.

2. To find out if alterations in genes belonging to the same pathway are mutually exclusive events, and whether or not the alterations are generally found across the three selected cancer models.

Materials

Colorectal cancer

From an unselected, prospective series collected from 7 hospitals in the South-East region of Norway during 1987-1989, 66 carcinomas from 65 individual were included in the present study [147]. The series are evaluated to contain a mean number of 84% neoplastic cells. Of the tumors, 36 were characterized as MSS and 30 as MSI. One of the tumors was from a patient with hereditary non-polyposis colorectal cancer (HNPCC), whereas the rest of the cases were sporadic. See Appendix IV for gender, age, localization and MSI status.

Testicular cancer

Primary tumor samples were obtained from 46 Norwegian patients diagnosed TGCT and 4 diagnosed ITGCN. The frozen tumor sample from each patient was sliced and three sections were stained with hematoxylin and eosin in order to estimate the fraction of neoplastic cells versus normal cells. Across the sample set, an average of 75% neoplastic tissue was observed (range: 30 to 100%). All frozen tumor samples were classified according to WHO recommendations. Nineteen of the tumors were seminomas, and 27 were non-seminomas. See appendix IV for histopathological status. The non-seminoma group included 10 embryonal carcinomas, 8 teratomas, 6 yolk sac tumors, in addition to 3 tumors in which the exact histological subgroups could not be determined.

Malignant peripheral nerve sheath tumors

Thirty-two MPNSTs, including 12 from Swedish patients, 10 from Norwegian patients and 10 from Dutch patients, were submitted to mutation and methylation analyses. Of the patients, 21 had a known family history of neurofibromatosis1 (NF1). See appendix IV, for gender and NF1-status.

Methods

DNA isolation

DNA from fresh-frozen tumors taken from the colorectal carcinomas, TGCT and the MPNST patients had previously been isolated using a 340 A Nucleic Acid Extractor (Applied Biosystems). The standard phenol/chloroform extraction principle was applied [148], which presents DNA of high quality and yield.

Mutation analysis

PCR prior to DNA sequencing

In 1971, Kleppe and co-workers initially presented a method named repair replication to replicate short synthetic DNA by the use of DNA polymerases [149]. However, Mullis has been acknowledged for inventing the technique of Polymerase Chain Reaction (PCR) in the 1980s [150]. PCR uses the naturally occurring enzyme polymerase, to amplify a DNA fragment in a chain reaction. The reaction consists of three steps, denaturation, primer annealing, and elongation, that are repeated over and over, resulting in an exponentially accumulation of the target DNA. Prior to DNA sequencing, PCR products from the individual genes and exons were generated. A total of 25µl PCR-mix was prepared from: 2 ng template, 1x PCR buffer (containing 1.5 mM $MgCl_2$) (Qiagen, GmbH, Hilden, Germany), 0-0.5 µl 25 mM $MgCl_2$, 0.6 pmol of each primer (Medprobe AS, Oslo, Norway), 0.2 mM of each of the four dNTPs (Takara Bio Inc), and 0.03 U Hotstar Taq Polymerase (Qiagen). The total volume was adjusted by adding MQ water free of inorganic ions, organic material, bacterias and other

particles. For primer sequences see Appendix II.

PTEN

For mutation analysis of *PTEN*, we used primers designed by an in-house collaborative lab [151]. However, we optimized the reaction by using a multiplex PCR kit (Qiagen), which allowed the nine exons to be amplified simultaneously in one PCR (See fig 15). A total of 25 µl PCR-mix was prepared, including 2 ng template, 1x Multiplex PCR Mastermix (containing buffer, 1.5 mM $MgCl_2$, nucleotides, and enzyme), 0.048 pmol of each of the nine primer pairs, and adjusted the final volume with MQ-H_2O.

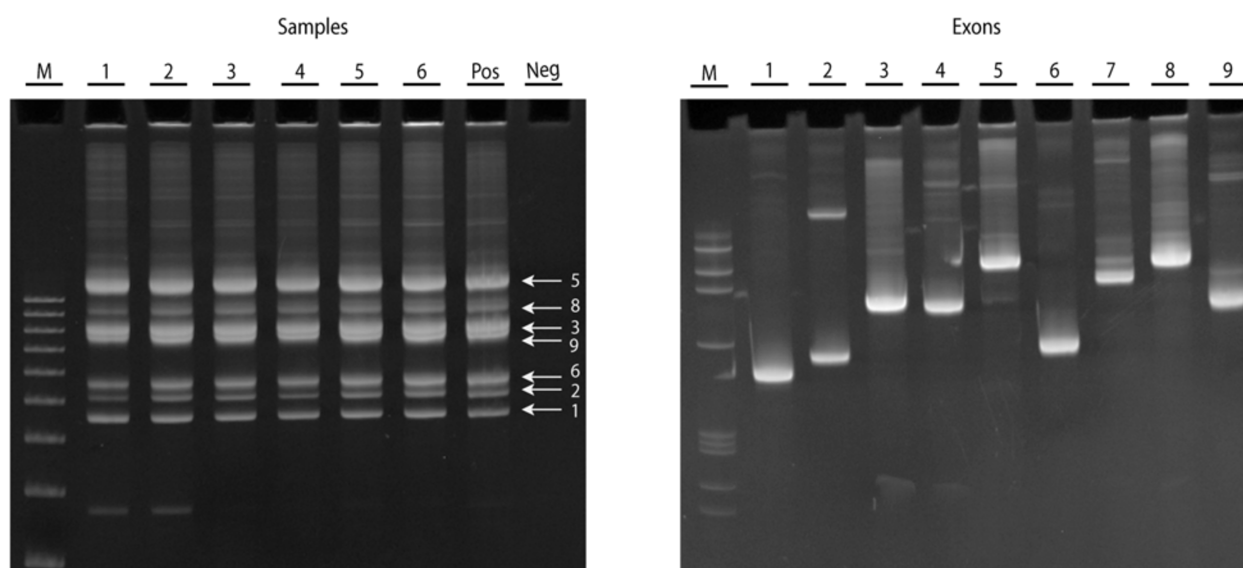


Figure 15: PCR products of the nine *PTEN* exons. The left panel is showing all exons simultaneously amplified by a multiplex PCR kit in six distinct samples. The right panel is showing the individual bands for each exon amplified in one sample, demonstrating that all exons are present, even though only six to eight bands are distinguishable in the left panel (white arrows). (It should be pointed out that the multiplex PCR reaction is not optimized for running single exons.) Abbreviations: M, marker (left: 100 base pair marker, right: φx marker); pos, positive control (containing DNA from normal blood); neg, negative control (containing water as template).

PIK3CA, KRAS and *BRAF*

The primers for *PIK3CA* exon 20 and *KRAS* exon 2 and 3, were designed using the primer-design program Oligo, whereas the primer pairs for *BRAF* and *PIK3CA* exon 9, were found in the literature [59] [152].

PCR programs

A Robocycler Gradient 96 (Stratagene, La Jolla, CA, USA) and an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) machine were used to carry out the PCRs. All of the programs started with 15 minutes at 95°C to activate the enzyme, followed by 35 cycles of 30-45 seconds of denaturation, 30-60 seconds at a specific annealing temperature, 30-60 seconds of elongation at 72°C, and a final extension at 72°C for 8 minutes. (See table x, Appendix II, for the various conditions for each gene.) The PCR products were separated by electrophoresis at 200 V for approximately 20 minutes using either a 2 % agarosegel (BioRad Laboratories Inc., CA, USA) with ethidium bromide (Mercury Continental Lab Products Inc., CA, USA, Sigma Chemical Co., St.Louis, MO, USA) added, or a 7,5 % polyacrylamide gel stained with ethidium bromide for 2 minutes. A Gene Genius (Syngene, Cambridge, UK) with UV-light was utilized to take photographs of the gels.

PCR product purification

A post-PCR purification step is required prior to the sequencing reaction, since leftover primers or free dNTPs may lead to background and noise in the sequencing electropherogram. Two different methods were used for purification, a column based approach utilizing the S-300 HR MicroSpin columns (Amersham Bioscience, Buckinghamshire, UK) and an enzymatic purification using ExoSAP-IT (GE Healthcare, USB corporation, Ohio, USA).

The columns are pre-packed with Sephacryl S-300 HR resin and equilibrated in TE buffer

(pH 7.6). The resin was resuspended by vortexing, the cap loosened and the bottom closure was removed. The columns were placed in a 1.5 ml Eppendorf-tube for support, and pre-spinned at 3000 rpm in an Eppendorf Centrifuge 5415 D (Eppendorf) for 1 minute. The columns were transferred to new Eppendorf-tubes, the caps were removed, and 20 µl PCR-product was applied to the top centre of the resin, followed by centrifugation at 3000 rpm for two minutes. The purified samples were collected in the bottom of the Eppendorf-tubes.

The ExoSAP-IT purification is a rapid and efficient protocol, which utilizes two enzymes, Exonuclease I and Shrimp Alkaline Phosphatase. One and a half µl ExoSAP-IT (Amersham Bioscience) was added directly to 10 µl PCR-product. The mix was then incubated 15 minutes at 37°C, followed by an inactivation step of 15 minutes at 80 °C on an Eppendorf Mastercycler Gradient PCR machine.

See table 1, page 62, for the various applications on the different machines.

Sequence reaction

In 1969, Atkinson and co-workers showed that the inhibitory activity of dideoxythymidin (ddTTP) on DNA polymerase I depends on its incorporation into the growing oligonucleotide chain in the place of a thymidylic acid (dTTP). In contrast to the deoxyribonucleotide, which contains a hydroxyl group at the carbon in the 3rd position, the dideoxynucleotides contain an H atom, thus obstructing the chain from being extended further [153]. In the wake of that discovery, Gilbert, together with graduate student Maxam, and Sanger in 1977 independently developed new techniques for rapid DNA sequencing [154] [155]. The methods devised by Gilbert and Sanger laid the foundation for the techniques utilized today. So, in 1985, when Hood invented the first automatic sequencer, he improved the existing Sanger' method of

enzymatic sequencing, which had become the laboratory standard [156]. In automating the process, Hood simultaneously modified both the chemistry and the process of collecting the data.

The standard sequence reaction is similar to the PCR reaction for replicating DNA. The reaction mix included the purified PCR product as a template, 0.15 pmol of one of the primers (either the forward or the reverse), BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), BigDye Terminator v1.1 5x Sequencing Buffer (Applied Biosystems) and MQ-H_2O. The reaction was run in the presence of dNTPs and a small fraction of ddNTPs. The ddNTPs are chemically modified with fluorescent labels, which emit light at specific wavelengths when exposed to the light from a laser beam. This provides a way of visualizing the individual DNA bases. When a ddNTP is incorporated instead of a regular dNTP, the extension reaction stops because of the H-atom. This means that every strand is of various lengths, dependent on when the polymerase incorporated a ddNTP. Starting with denaturation at 96°C for two minutes, followed by 25 cycles of 15 seconds denaturation at 96°C, 5 seconds of annealing at 50°C and 4 minutes elongation at 60°C, the sequence reaction was carried out on a Robocycler Gradient 96 PCR machine.

Sequence product purification

After the sequencing reactions, and prior to separation on a DNA sequencer, the mixture of strands has to be purified. This was also done in two distinct ways, one method based on ethanol precipitation and one based on gel filtration.

Ethanol precipitation

To precipitate the sequence product, 2 µl 3 M sodium acetate CH_3COONa (pH 4.6) and 50 µl 96% ethanol C_2H_5OH was added, followed by 30 minutes precipitation at room temperature.

The samples were spun at full speed (2250g) for 45 minutes on an Eppendorf Centrifuge 5804 (Eppendorf). The sample-tubes were opened, turned up side down on a paper and spun for 1 minute at 700g to remove the supernatant. Hundred and fifty µl 70% ethanol was added, and the samples were spun 25 minutes at full speed, prior to a repeat of turning the sample-tubes up side down on a paper and spinning for 1 minute at 700g. Finally, 5 µl loading buffer consisting of 4 µl deionised formamide CH_3NO (Merck, Darmstadt, Germany) and 1 µl Blue Dextran/EDTA (Applied Biosystems, Foster City, CA, USA) was added to the samples followed by denaturation of the products for 3 minutes at 95°C.

Gel filtration

Using the gel filtration method, dry Sephadex G-50 Superfine (Amersham Bioscience) was loaded on to a 96-well Multiscreen HV plate (Millipore), using a Column Loader 45 µl plate (Millipore). Three hundred µl MQ-H_2O was added to each well and the Multiscreen plate was incubated at room temperature for 2 hours for swelling of the resin. The Sephadex-powder and water makes a gel-column that is able to separate molecules according to size. Small molecules, like unincorporated dye terminators and excess primers, will diffuse into the pores, and their passage through the column is delayed. Molecules of larger size, like the sequence product, will pass directly trough the column. After swelling, the Multiscreen Plate was placed on top of an empty, old 96-well Optical Reaction Plate (Applied Biosystems), and centrifuged for 5 minutes at 910 g to pack the columns. To pre-rinse the columns, 150 µl MQ-H_2O was added to each well, prior to another round of centrifugation for 5 minutes at 910 g. Ten µl MQ-H_2O and 10 µl sequence reaction product was loaded on to each column, followed by centrifugation for 6 minutes at 910 g. The samples were collected in the wells of a new 96-well Optical Reaction Plate and the plate was immediately covered with a 3100 Genetic Analyzer Plate Septa (Applied Biosystems). No additional denaturation step is necessary prior

to sequencing, since the polymer contains urea.

DNA sequencing

After the sequence reaction and purification, the various fluorescent labeled fragments, all of different length, have to be separated. This was done either on a polyacrylamide gel using an ABI PRISM 377 machine or in a polymer-filled capillary array utilizing an ABI PRISM 3730. Both machines are capable of separating molecules by gel electrophoresis in which the length differ by no more than one base. At the bottom of the gel or the capillary array, the fragments are passing a laser beam that excites the fluorescent molecule, which in turn, emits light. The light is collected simultaneously and focused by lenses into a spectrograph. Based on the wavelength, the spectrograph separates the light across a charge coupled device (CCD) camera. Since each base has a distinct fluorochrome, the data collection software can read and interpret the fluorescence data and display them as electropherograms.

The ABI PRISM 377 DNA Sequencer is the most time and work consuming machine, mainly because a 4.5% polyacrylamide gel has to be prepared. Fifty ml solution was made of 18.0 g Urea (BioRad), 5.63 ml 40% Acrylamide/Bis solution 29:1 (BioRad), 20 ml MQ-H_2O, and 0.5 g Amberlite MB-150 (Sigma). The mix was filtered by utilizing a Steritop Filter (Millipore) and vacuum, which also removes gas and prevents bubble formation in the gel. Ten ml 5x TBE-buffer (Trisma Base, Boric acid, 0.5 EDTA, MQ-H_2O) was added, and the volume adjusted by MQ-H_2O. To polymerize the gel, 125 µl 20% ammonium persulfate (APS) (BioRad) and 30 µl TEMED (BioRad) were added, where-upon the gel-solution was injected between clean 36 cm glass plates, separated by two 0.2 mm gel spacers. On the top, a 36 lane, 0.2 mm Sharks tooth comb was inserted. The gel polymerized in room temperature for 1.5 hours prior to application. One and a half µl of each sample was loaded on the gel, and

were run at 51°C for 6.5-7 hours.

The ABI PRISM 3730 DNA Sequencer is fully automatic and hence very effective in contrast to the 377. The 96-well Optical Reaction Plate containing the samples, was put in a 96-well Plate Base, and placed in the machine. Instead of a polyacrylamide gel, the capillary array is filled with POP7 polymer (Applied Biosystems). The samples were run at 60°C for 2 hours.

Approximately 80% of the *BRAF* mutations analyses were run using the 377 DNA sequencer, whereas the rest of the analyses, including the other 3 genes, were run on the 3730 DNA sequencer.

All the samples were analyzed using the DNA Sequencing Analysis software (Applied Biosystems.)

Table 1: The various purification methods applied prior to DNA sequencing.

ABI PRISM DNA SEQUENCER	**377**	**3730**
Purification of PCR product:	S-300 HR Microspin Columns	ExoSapIT
Sequence reaction (µl):		
MQ-H_2O	8.50	6.25
Big Dye buffer	0.00	1.50
Big Dye Mix	6.00	1.00
Primer	0.50	0.25
PCR-product	5.00	1.00
Total (µl):	20.00	10.0
Purification of sequence product:	Ethanol precipitation	Sephadex G-50 Superfine

The electropherograms have been manually read, and all positive findings have been verified by a second independent run, as well as by another member of our group.

Methylation assay

The principle of Methylation-Specific PCR (MSP)

A bisulphite treatment reaction for deamination of cytosine derivatives was initially described in 1970 by Shapiro *et al* [157]. However, Frommer and Clark were the first to present sodium bisulphite ($NaHSO_3$) treatment as a way to distinguish between 5-methylcytosines and cytosines in the 1990s. It is a reaction where unmethylated cytosines are deaminated and converted to uracils, which then replicate as thymines during PCR, while the methylated cytosines remains unchanged (figure 16) [158,159].

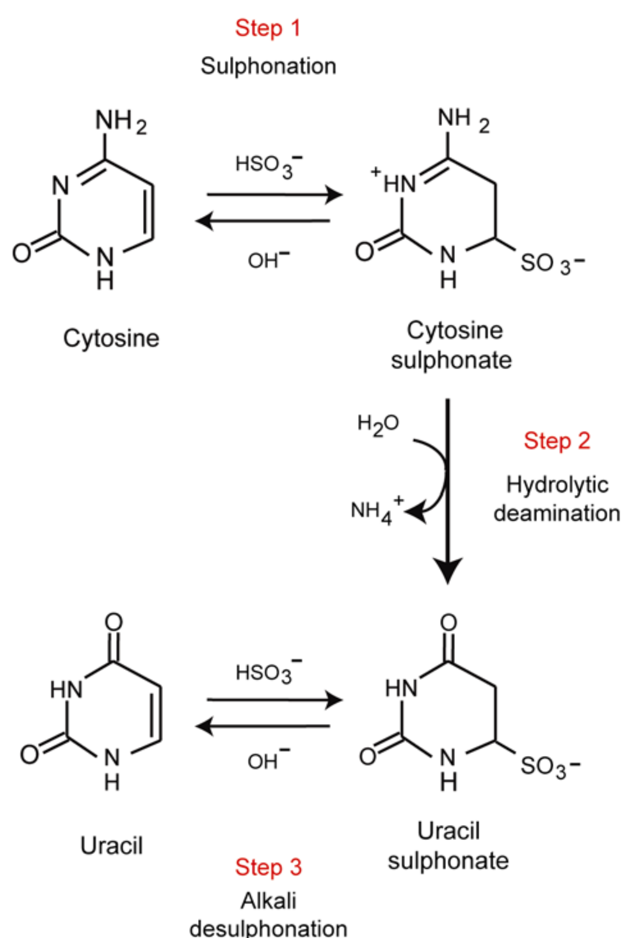


Figure 16: The stepwise conversion of unmethylated cytosine to uracil by sodium bisulphite treatment. From Clark *et al*, ref. [159].

In 1996, Herman and co-workers introduced MSP as a method to analyze the DNA methylation status of CpG sites. The technique consists of two parts; 1) the sodium bisulphite treatment of the DNA, and 2) revealing of the differences in the sequences induced by the bisulphate using PCR. After the treatment, the DNA strands will no longer be complementary, thereby requiring primer sets specific for unmethylated and methylated DNA [160]. MSP is the most widely used methylation assay today, since it is simple, quick, cost effective, and at the same time sensitive, as it can detect one methylated allele in a pool of 1000 unmethylated alleles [28,160]. In the present thesis we used MSP to assess methylation status of *RASSF1A*.

Bisulphite treatment of DNA

One point three µg DNA was diluted in a total volume of 50 µl in eppendorf-tubes by adding MQ-H_2O. Five point seven µl 3M sodium hydroxide (NaOH) (Merck) was added to each sample, prior to incubation for 10-15 minutes at 37°C to denature the DNA. This is an important step, as only cytosines located in single strands are available for modification [30]. Next, 33 µl 20mM hydroquinone (Sigma-Aldrich Inc., St. Louis, MO, USA) was added. This is an anti-oxidant and prevents oxidative damage to the DNA caused by the bisulphite treatment. Finally, 530 µl 4.3M Sodium Bisulphite (pH 5.00) (Sigma-Aldrich) was added to each sample prior to incubation for 16-17 hours (over night) at 50°C. The next day, 1 ml resin from DNA Wizard clean-up kit (Promega Ltd., Southampton, UK), which binds the DNA prior to the clean-up procedure, was added. After mixing, the samples were loaded onto filtered columns mounted on a vacuum manifold. The vacuum causes the liquid to pass down through the filters, and the columns were re-filled with 80% isopropanol (Merck, Darmstadt, Germany) to rinse the modified DNA. When the isopropanol had passed through the filters, they were placed in the old eppendorf-tubes and centrifuged for 2 minutes at 12700 rpm to remove the remaining alcohol. Each filter was then placed in a new eppendorf-tube, and the

DNA was eluted by adding 50 µl MQ-H_2O and spun for 1 minute at 12700 rpm. This step was repeated once, thus the DNA was dissolved in 100 µl. In order to complete the transformation from C to U, 11.4 µl 3M NaOH was added to each tube, prior to incubation for 15 minutes at 37°C. To visualize the DNA pellet during precipitation, 2 µl glycogen (5 mg/ml) (Ambion Ltd., Huntingdon, Cambridgeshire, UK) was mixed in. Nine hundred µl cold 100% ethanol and 42 µl 7.5 M ammonium acetate ($AcNH_4$) (Sigma-Aldrich) were added to precipitate the DNA at -80°C over night. In a pre-cooled centrifuge (Eppendorf) the samples were spun for 30 minutes at 13000 rpm in 4 °C. The supernatant was removed, 1 ml cold 70% ethanol added to the pellet and centrifuged for 15-30 minutes at 13000 rpm in 4 °C.
In order to remove all the ethanol, the supernatant was again discarded, the lids were opened and the samples heated to 50 °C for less than 1 minute to let the rest of the ethanol evaporate. Finally, the bisulphite treated DNA was dissolved in 30 µl MQ-H_2O.

Methylation specific PCR (MSP) of RASSF1A

The PCR mix consisted of 2 ng bisulphite treated DNA, 1x Qiagen PCR buffer (containing 1.5 mM $MgCl_2$), 1.6 pmol of each of the primers (Medprobe), 0.2 mM of each of the four dNTPs (Takara Bio Inc., Shiga, Japan), and 0.04 U Hotstar Taq Polymerase (Qiagen). The total volume was adjusted to 25 µl by adding MQ-H_2O. The primers used for the *RASSF1A* MSP were designed by Koul and co-workers [161]. A Robocycler Gradient 96 (Stratagene) was used to carry out the MSP, starting with 15 minutes at 95°C to activate the enzyme, followed by 35 cycles of 30 seconds of denaturation at 95°C, 30 seconds annealing at 62°C, 30 seconds of elongation at 72°C, and a final extension at 72°C for 7 minutes. (See also Appendix II.) The PCR products were separated by electrophoresis at 200 V for approximately 30-40 minutes using a 2 % agarosegel (BioRad) with ethidium bromide (Mercury, Sigma) added. A Gene Genius (Syngene) with UV-light was utilized to take

photographs of the gels. Bisulphite converted DNA from normal blood was used as a positive control for the unmethylated MSP reaction, whereas human placenta DNA treated *in* vitro with SssI methyltransferase (New England Biolabs Inc., Beverly, MA, USA) was used as a positive control for the methylated templates. This sample should be methylated in all CpG-sites. The positive *RASSF1A* samples were visually scored relative to the intensity of the positive control as either weakly methylated (including less intense samples than the positive control) or heavily methylated (including samples with equal or higher intensities than the positive control.) Samples displaying a band from the unmethylated reaction but not displaying any band from the methylated reaction were scored as unmethylated. For this thesis, only those samples scored as heavily methylated were classified as methylated, whereas the samples scored as weakly methylated were classified as unmethylated. This conservative way of classifying methylated samples limits the number of false positives. The author and another group member, Post. Doc. Guro E. Lind, have independently performed all visual scorings, and all results were verified by a second round of analysis. The methylation analyses of testicular germ cell cancers were performed by Guro E. Lind.

Statistics

General references: Altman, 1999 [162] and More & McCabe, 2002 [163].

The Chi squared (χ^2) test

The Chi squared (χ^2) statistic is a measure of how much the observed outcomes diverge from the expected outcome given that the H_0-hypthesis (no association between the variables) is true. If the expected outcome and the observed outcome are very diverse, a large value of χ^2 will result. The further away the observed outcomes are from the expected, the less likely it is that the null hypothesis is true, hence rejecting it.

The formula for the test statistic is:

$\chi^2 = \Sigma \frac{(O-E)^2}{E}$, where O denotes the observed frequency and E the expected frequency. The degree of freedom in Chi squared (χ^2) tests is: $df = (r-1)(k-1)$, where r denote the number of rows, and k denotes the number of columns.

The Fisher's exact test

An alternative approach for tables with small expected frequencies is the Fisher's exact test, which is based on the same principle as the Chi squared test. It can only be applied to 2 x 2 tables, but is preferred over the Chi squared test as it is more accurate.

All contingency 2x2 tables in the present thesis were analyzed using Fisher's exact test, where the degree of freedom is 1. Three x 2 tables were analyzed by the Chi squared (χ^2) test. All P values are derived from two tailed statistical tests using the SPSS 11.5 software, and values less than or equal to 0.05 (5%) were considered statistically significant.

Mutation and methylation frequencies of 5 cancer relevant genes

Hundred and forty-eight samples from three cancer types were analyzed for mutations in *KRAS, BRAF, PIK3CA,* and *PTEN,* and simultaneously for promoter methylation of *RASSF1A*. The overall alteration-frequencies for each gene in colorectal carcinomas, TGCTs and MPNSTs are summarized in table 2. The type of mutation and subsequent amino acid substitution are shown for the individual genes per sample in, appendix III. Figure 17 and 18 illustrates the mutation spectra, whereas figure 19 shows promoter methylation of *RASSF1A*.

Table 2: **Mutation and methylation frequencies in colorectal carcinomas, testicular cancer and malignant peripheral nerve sheath tumors.**

Gene	CRC	TGCT	MPNST
KRAS	26/66 (40%)	2/47 (4%)	0/32 (0%)
BRAF	14/65 (22%)	1/48 (2%)	1/32 (3%)
RASSF1A	18/60 (30%)	8/49 (16%)	14/30 (47%)
PIK3CA	15/64 (23%)	0/46 (0%)	1/31 (3%)
PTEN	12/63 (19%)	0/47 (0%)	0/32 (0%)

RASSF1A was analyzed for promoter methylation, whereas the rest of the genes were analyzed for mutations. Abbreviations: CRC, colorectal carcinomas; TGCT, testicular germ cell tumor; MPNST, malignant peripheral nerve sheath tumor.

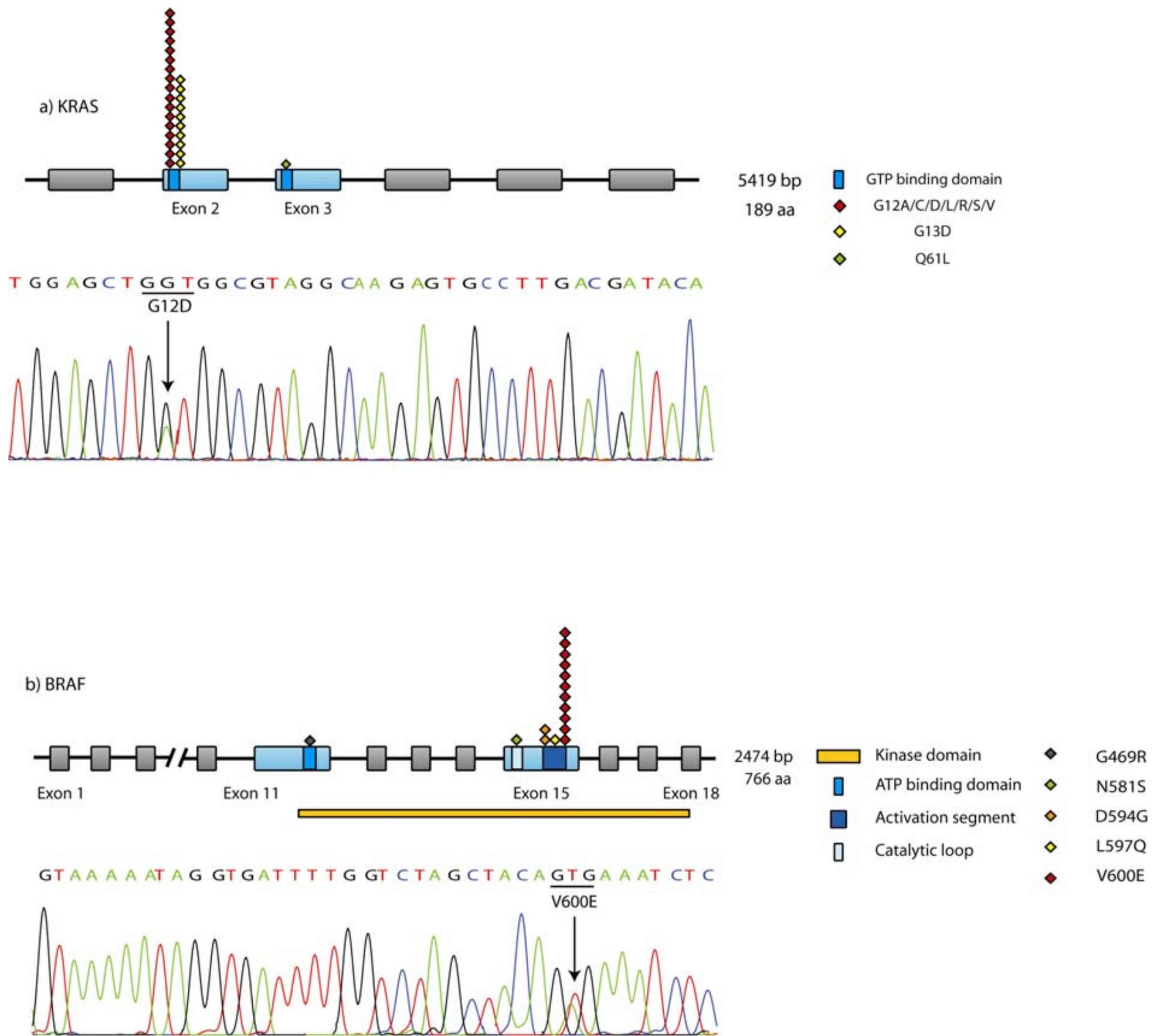


Figure 17: Mutation spectra and electropherograms for *KRAS* and *BRAF* across tumor types. The figure illustrates the genetic position and type of mutations detected in colorectal carcinomas, testicular germ cell tumors and malignant peripheral nerve sheath tumors for a) ***KRAS*** and b) ***BRAF***.

Abbreviations: aa, amino acid; bp, base pairs; A, Alanine; C, Cysteine; D, Aspartic acid; E, Glutamic acid; G, glycine; L, Leucine; N, Asparagine; Q, Glutamine; R, Arginine; S, Serine; V, valine.

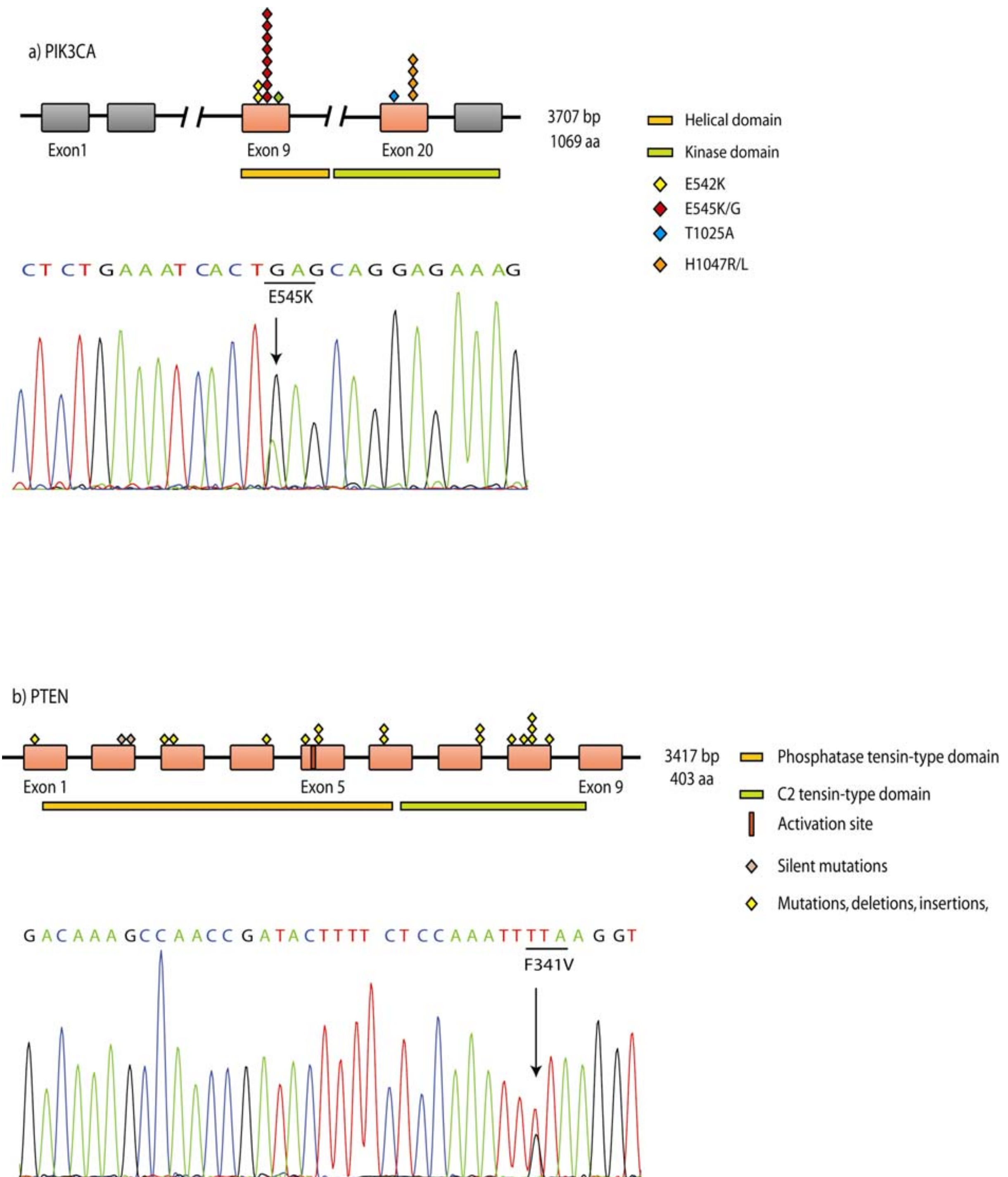


Figure 18: Mutation spectra and electropherogram for *PIK3CA* and *PTEN* across tumor types. The figure illustrates the genetic position and type of mutations detected in colorectal carcinomas, testicular germ cell tumors and malignant peripheral nerve sheath tumors for a) ***PIK3CA***, and b) ***PTEN***.

Abbreviations: aa, amino acid; bp, base pairs; A, Alanine; E, Glutamic acid; G, glycine; H, Histidine; K, Lysine; L, Leucine; R, Arginine; T, Threonine.

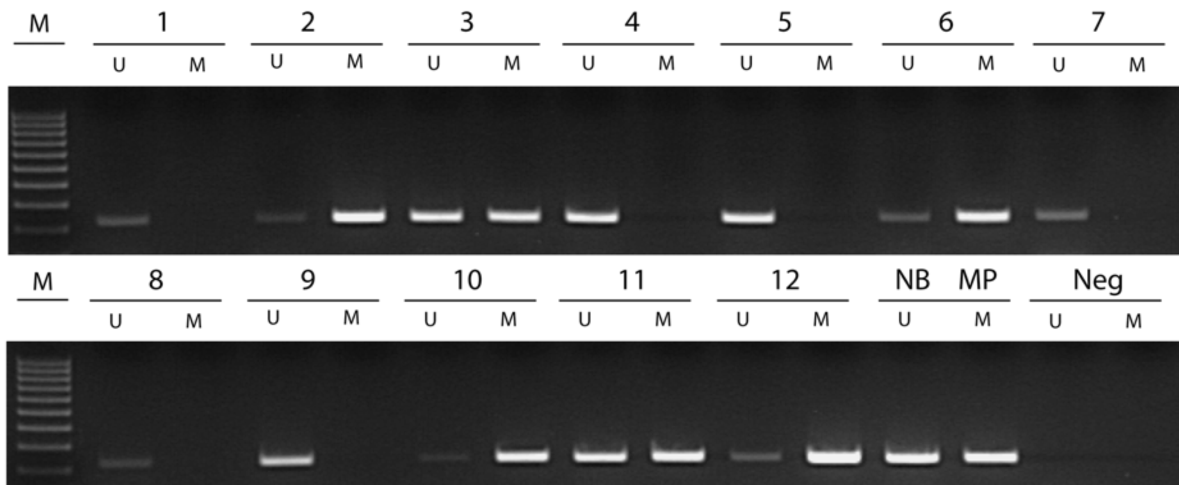


Figure 19: Promoter hypermethylation of *RASSF1A* in malignant peripheral nerve sheath tumors. The methylation was evaluated by methylation-specific PCR (MSP). A visible product in lanes termed U, indicates the presence of unmethylated alleles, whereas a product in lanes termed M, indicates the presence of methylated alleles. Abbreviations: NB, normal blood (positive control for unmethylated samples); MP, methylated placenta (positive control for methylated samples); M, marker (100 base pair marker); Neg, negative control (containing water as template); U, lane for unmethylated MSP product; M, lane for methylated MSP product.

Colorectal carcinomas

In total, 80% of all the colorectal tumors, both MSI and MSS, were altered in one or more of the genes analyzed, and the observed changes were evenly distributed among the genes. The detailed results of the mutation and methylation analyses in colorectal tumors, in addition to comparisons with other known tumor genetics are summarized in table 3, whereas comparisons with clinical and pathological features of the same tumors are summarized in table 5.

Table 3: Mutation and methylation of the individual genes, compared with the other genes analyzed, as well as to mutation status of *TP53* and *APC*.

	KRAS		***BRAF***		***RASSF1A***		***PIK3CA***		***PTEN***	
	Wt	Mut	Wt	Mut	U	M	Wt	Mut	Wt	Mut
KRAS										
Wt	-	-	26	14	25	11	32	7	26	25
Mut	-	-	25	0	17	7	17	8	12	0
P value			**<0.001**		NS		NS		**0.002**	
BRAF										
Wt	26	25	-	-	31	15	38	12	44	6
Mut	14	0	-	-	11	3	11	3	7	6
P value	**<0.001**				NS		NS		**0.012**	
RASSF1A										
Unmethylated	25	17	31	11	-	-	32	10	34	7
Methylated	11	7	15	3	-	-	13	5	13	5
P value	NS		NS				NS		NS	
PIK3CA										
Wt	32	17	38	11	32	13	-	-	39	9
Mut	7	8	12	3	10	5	-	-	12	3
P value	NS		NS		NS				NS	
PTEN										
Wt	26	12	44	7	34	13	39	12	-	-
Mut	25	0	6	6	7	5	9	3	-	-
P value	**0.002**		**0.012**		NS		NS			
TP53										
Wt	18	13	24	6	19	9	23	7	23	7
Mut	11	7	17	1	10	5	14	3	17	0
P value	NS		NS		NS		NS		**0.039**	
APC										
Wt	17	18	30	4	19	13	25	9	30	4
Mut	23	8	21	10	23	5	24	6	21	8
P value	**0.045**		0.069		0.089		NS		NS	

Comparisons of different groups were tested with Fisher's exact test or Chi squared test. Abbreviations: Wt, wild type; Mut, mutation; U, unmethylated; M, methylated; NS, not significant.

Inverse associations were found between mutations in *KRAS* versus *BRAF,* and also for *KRAS* versus *PTEN,* (*P* = <0.001 and *P* = 0.002, respectively). Additionally, mutations in *BRAF* and *PTEN* were associated (*P* = 0.012). Mutations in *TP53* and *APC*, previously analyzed in the same sample series, were compared to the results of the genes investigated here. Twenty-six tumors had *KRAS* mutations and eighteen of these harbored wild type *APC* (*P* = 0.045). Methylation of *RASSF1A* was frequently found together with wild type *APC* (P = 0.089), whereas mutations in *BRAF* displayed a trend towards the opposite (P = 0.069). Tumors with

mutated *PTEN* were typically found in those with wild type status of TP53 ($P = 0.039$).

Table 4: Mutations in *PIK3CA* compared with mutations in *KRAS* or *BRAF* in the same tumor.

	BRAF/KRAS	
	wt	mut
PIK3CA		
wt	21	28
mut	4	11
	NS	

Abbreviations; wt, wild type; mut, mutation

No significant association was found between mutations in *PIK3CA* and mutations in either *KRAS* or *BRAF* (table 4).

Table 5: Mutation and methylation frequencies of the analyzed genes, compared with the patients' clinicopathological features.

	KRAS		*BRAF*		*RASSF1A*		*PIK3CA*		*PTEN*		**TOTAL**	
	Wt	Mut	Wt	Mut	U	M	Wt	Mut	Wt	Mut	Wt	Mut
Individuals												
No	40/66	26/66	51/65	14/65	42/60	18/60	49/64	15/64	51/63	12/63	10/63	53/63
Sex												
Male	19	15	31	3	22	8	25	8	29	4	8	24
Female	21	11	20	11	20	10	24	7	22	8	2	29
P value	NS		**0.014**		NS		NS		NS		0.082	
Age (years)												
<68	18	8	21	5	17	7	22	4	23	3	5	19
≥68	22	18	30	9	25	11	27	11	28	9	5	34
P value	NS		NS		NS		NS		NS		NS	
Site												
Right side	17	7	15	9	20	3	20	4	14	9	3	20
Left side	14	10	21	2	13	9	15	8	21	2	5	19
Rectum	9	9	15	3	9	6	14	3	16	1	2	14
P value	NS		**0.047**		0.078		NS		**0.009**		NS	
MSI status												
MSI	22	8	19	11	22	7	24	6	20	9	4	25
MSS	18	18	32	3	20	11	25	9	31	3	6	28
P value	0.077		**0.008**		NS		NS		**0.05**		NS	
Ploidy status												
Diploid	28	14	31	11	27	12	32	10	30	11	7	33
Aneuploid	12	12	20	3	15	6	17	5	21	1	3	20
P value	NS		NS		NS		NS		**0.043**		NS	
Histological grade												
Poorly differentiated	11	4	9	5	10	3	9	5	10	4	1	13
Moderately differentiated	28	19	38	9	29	14	38	8	37	8	8	37
Well differentiated	1	3	4	0	3	1	2	2	4	0	1	3
P value	NS		0.09		NS		NS		NS		NS	

Comparisons of different groups were tested with Fisher's exact test or Chi squared test. Abbreviations: MSI, microsatellite instability; MSS, microsatellite stable; Wt, wild type; Mut, mutation; U, unmethylated; M, methylated; NS, not significant. The column termed TOTAL is the overall status of each tumor: the wild type tumors harbor no alterations, whereas the tumors scored as mutated are altered in one or more of the five genes analyzed.

Mutations in *BRAF* and *PTEN* were more common in MSI tumors (11/30, $P = 0.008$ and 9/29, $P = 0.05$, respectively) as well as in tumors with a right-sided location in the colon (9/24, $P = 0.047$ and 9/23, $P = 0.004$, respectively). Additionally, *PTEN* mutations were typically found in tumors with diploid genomes. We saw a tendency of more mutations in some genes in females than in males, but this was only statistically significant for *BRAF* ($P = 0.014$). Moreover, methylation of *RASSF1A* was more frequent in tumors of the left side of the colon and the rectum, but this observation was not statistically significant (P = 0.078). There were no statistical significant associations between changes in any of the genes and age or histological grade.

Testicular germ cell tumors

In total, 22% of all TGCT samples were altered in one or more of the genes analyzed. In fact, all changes were seen in RTK signaling through alterations of *KRAS, BRAF,* and *RASSF1A* (See Appendix III for details).

Mutations in *KRAS* (n=2), *BRAF* (n=1), and *PTEN* (n=1), were found in four individual tumors, all in the seminomatous subgroup of TGCTs (See table 6). In contrast, all cases with hypermethylation of the *RASSF1A* promoter were non-seminomas (table 6 and 7).

Table 6: Mutation and methylation frequencies of individual genes in the histological subtypes of TGCT.

Gene	TGCT		
	Seminoma	Non-Seminoma	ITGCN
KRAS	2/16	0/27	0/4
BRAF	1/17	0/27	0/4
RASSF1A	0/18	8/27	0/4
PIK3CA	0/15	0/27	0/4
PTEN	0/16	0/27	0/4

RASSF1A was analyzed for promoter methylation, whereas the rest of the genes were analyzed for mutations.
Abbreviations: TGCT, testicular germ cell tumors; ITGCN, intratubular germ-cell neoplasia

Table 7: Distribution of methylation in the none-seminomatous sub-group of TGCTs.

Classification	RASSF1A
Embroynal carinoma	0
Teratoma	2
Yolk sac tumor	4
Mixed/combined	2
Total	8

A gene methylation profiling study of TGCTs was performed in parallel to this study [164]. Among the 16 genes analyzed, 6 were methylated in two or more of the tumors, showing

RASSF1A as one of the most frequently methylated genes among the non-seminomatous TGCTs (figure 20).

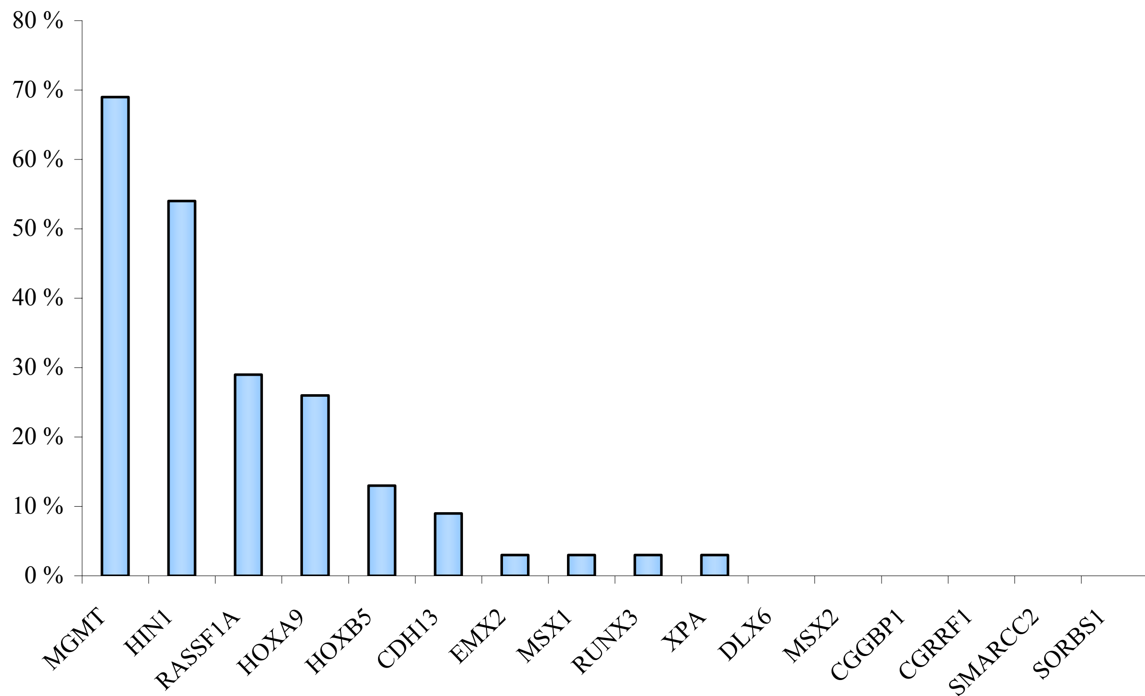


Figure 20: Methylation percentages of target genes in non-seminomatous testicular germ cell tumors (TGCTs). Data from Lind *et al*, ref. [164]

Malignant peripheral nerve sheath tumors

In total, 50% of the MPNSTs were altered, mainly through promoter methylation of *RASSF1A*. Mutation and methylation frequencies for the individual genes in MPNSTs are summarized in table 8. *RASSF1A* was found hypermethylated in 14/30 cases (~ 47%), and this type of change seemed to be more common in cases with the hereditary disease NF1 than among the sporadic cases, although not statistically significant. On the other hand, *BRAF* and *PIK3CA* were mutated in only one tumor each and no mutations were seen in *KRAS* or *PTEN* in MPNSTs.

Table 8: Mutation and methylation frequencies of individual genes in malignant peripheral nerve sheath tumors taken from patients with and without the disease Neurofibromatosis, who are carriers of germ line *NF1* mutations.

Gene	MPNST	
	NF1 patients	Sporadic cases
KRAS	0/21	0/11
BRAF	0/21	1/11
RASSF1A	11/20	3/10
PIK3CA	1/19	0/11
PTEN	0/21	0/11

RASSF1A was analyzed for promoter methylation, whereas the rest of the genes were analyzed for mutations. Abbreviations: MPNST, malignant peripheral nerve sheath tumors.

No associations were found among the changes in the MPNSTs and NF1-status or gender.

Genetic complexity

The MPNST are typically genetic complex, but show a wide range of complexity. This is illustrated in figure 21, summarizing the genomic changes in 30 MPNSTs as assessed by chromosomal CGH in our laboratory (not part of the present study, data unpublished). The methylation of *RASSF1A* is evenly distributed among the samples, and thereby seemingly independent of the genetic complexity, which is also shown in figure 21.

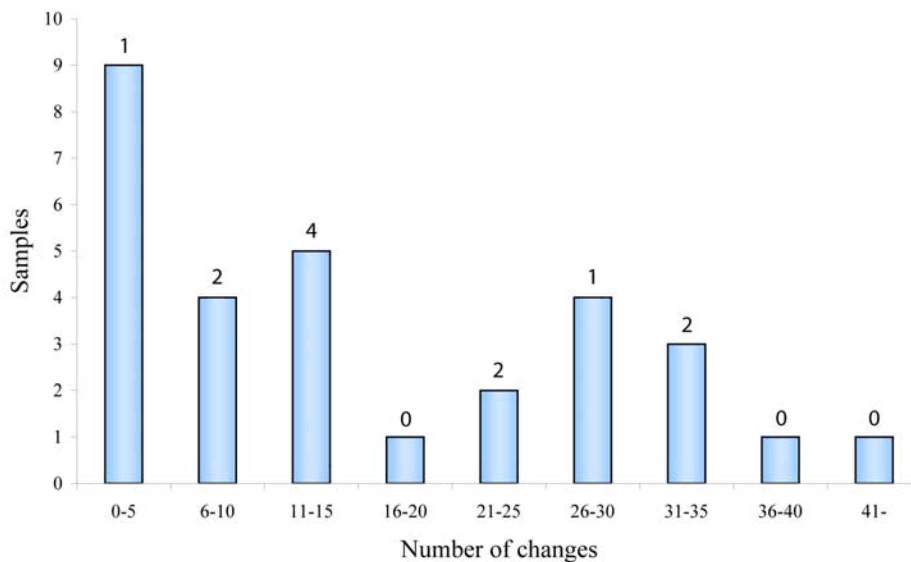


Figure 21: The genetic complexity of the MPNST samples. The changes include gain or loss from the whole or part of a chromosome arm. The numbers on top of the columns show the number of tumors with RASSF1A methylation out of 32 tumors analyzed.

Discussion

Methodological considerations

DNA sequencing is often considered the "gold standard" when it comes to mutational analysis, as the final outcome pinpoints the exact alteration of the DNA sequence. From this information one can identify the respective codon(s) affected and the amino acid alteration(s), and sometimes deduce the biological consequence at the protein level. However, it should be noted that direct sequencing of genomic DNA might not detect large deletions or inversions.

The advantage of using direct sequencing is the requirement of only small amounts of material and the immediate identification of potential mutations. The overall mutation detection level can further be improved by including a highly sensitive prescreening step. A wide variety of such methods exist, including temporal temperature gradient gel electrophoresis (TTGE), single strand conformational polymorphisms (SSCP), and denaturing high performance liquid chromatography (DHPLC), however, most of these prescreening methods are labor-intensive and do not identify the exact alteration (for review [165,166]), and thereby one are in need for DNA sequencing to detect the exact base positions of the aberrations initially detected by the other methods.

Two models of DNA sequencers were utilized to screen *BRAF* for mutations. The 377 DNA sequencer has a mutation detection level of approximately 20%, in contrast to the 3730 DNA sequencer, which has a sensitivity of approximately 10%. Some mutations might therefore have been missed with the first machine, leading to false negatives. However, such mutations

will probably be of little significance for the tumor biology, as the tumor cells still will harbor sufficient amounts of wild type *BRAF*. Since all samples included in the present thesis contain a high percentage of tumor cells, a potentially undetected mutation can only be present in a few clones, and hence not contribute much to the overall phenotype. Heterozygous mutations, which are estimated to be present in approximately 30- 50% of the sample cells, will easily be detected by both sequencers.

Most analyses were performed using the AB 3730 DNA sequencer. This 48 capillary instrument has high capacity and can analyze 96 samples in two hours. However, the most time consuming part is the correct reading of the sequences. A software program from Applied Biosystems is designed to detect the mutations, however this has not yet been tested adequately. The program does not seem to detect the smallest mutations, and therefore all sequences were read manually. For the four genes, a total of 15 fragments x 148 samples were sequenced both ways creating 4440 electropherograms to be manually read. By this approach only small discrepancies were recorded between the two group members reading.

In the present thesis, we have performed sequencing of hotspots and of whole coding regions, chosen according to the existing knowledge about the mutational spectra of the genes in question. Hot-spots for mutation have been reported for selected exons of *KRAS, BRAF,* and *PIK3CA*, whereas no hot-spot mutations have been reported for *PTEN* [53,59,152]. The individual mutation spectra found in this study (figures 17 and 18) are in accordance with the original findings for the respective genes.

Regarding the DNA methylation analysis, bisulphite sequencing is considered to be the "gold standard", as it identifies the methylation status of each individual CpG-site. For monoclonal

cancer cell lines, direct bisulphite sequencing provides results of sufficient quality. The amplified bisulphite PCR product from primary tumors, on the other hand, should be cloned into plasmid vectors, and 10-12 clones from each sample should subsequently be sequenced, as the individual clones might contain different degree of methylation. This is both laborious and time consuming. In contrast, the MSP method is quick and reliable and is now the most widely used technique for studying DNA methylation in large clinical series. The specificity of this assay relies on the match or mismatch of the primer sequence to bisulphite treated DNA and the choice of primers can therefore greatly influence the results. Here, we have used previously published MSP primers. The products obtained, indicating methylated or unmethylated sequences, have shown good associations to lack of/reduced or presence of gene product, respectively [161].

Gene mutations

In colorectal carcinomas, mutations in *APC, KRAS,* and *TP53* are common [46]. Many other genes have also been found to harbor point mutations in colorectal cancer, although not in such high frequencies [46]. Due to development of high throughput methods the last years, mutation analyses of gene families have also been performed, such as the kinome and phosphatome [144,167]. In addition to the actual mutations, the order of events is also important for the development of malignancies. All three cancer types investigated here have identifiable precursor lesions, providing the means to study the tumor progression. Colorectal cancer is a particularly suitable model for this, as pre-invasive lesions at various stages, as well as malignant and metastatic tumors easily can be isolated from the patient. In this developmental cancer model, some of the mutations preferentially arise early, including *APC* and *KRAS*, whereas others appear later, such as *TP53*. However, even though *KRAS* mutations

are frequent early events in colorectal cancer, it is not enough for a malignant transformation, underlined by the high rate of such mutations in benign adenomas. This is also the case for MPNSTs, as *NF1*-mutations are also seen in premalignant stages, as well as in the germline of patients with the hereditary disorder neurofibromatosis.

In sporadic colorectal cancer, the KRAS-BRAF-MAPK and KRAS-PI3K-AKT pathways are frequently altered, and up to 75% of these carcinomas have activating point mutations in either *KRAS, BRAF,* or *PIK3CA* [168], a somewhat higher percentage than we have found (66%). However, the *KRAS* mutations presented here are found within the previously published range of 31-50% in colorectal cancer [53,168-170], showing that our results are in accordance with previous findings. It should further be noted that we selected our series to contain a similar number of MSI and MSS tumors, which do not reflect the distribution among an unselected clinical series. About one third of right-sided colorectal carcinomas show MSI, encompassing most of the tumors with this phenotype.

Davies and co-workers showed that *BRAF* is mutated in a wide range of cancers, including colorectal cancer, with a trend towards cancer types known to harbor *KRAS* mutations [59]. Additionally, it has been shown that *BRAF* mutations are associated with distinct clinical, pathological, and molecular features, such as location in the right side of the colon, poor histological grade, frequent *MLH1* promoter methylation, and MSI [60], which are in accordance with our results. The mutation frequency is usually within the range of 10-15% [59,168,171], however since our series is enriched in tumors showing a microsatellite unstable phenotype, the resulting frequency (22%) is somewhat higher than what would be expected in an unselected series. This is also the case for *PIK3CA* and *PTEN.* Our data are in line with recent studies that have reported *PIK3CA* mutation frequencies spanning from 13.6

to 32.3% not stratified for MSI [71,77]. Mice with a homozygous deficiency of the p110alpha subunit, encoded by *PIK3CA,* were recently shown to be growth-retarded and die during embryogenesis, underlining the critical role for p110 in growth factor and metabolic signaling [172].

PTEN is also a good candidate for involvement in the development and/or progression of sporadic cancer, even though mutations in this gene are relatively infrequent in colorectal carcinomas [173,174] when compared with tumors of the brain and the prostate [80,81]. The functional importance of PTEN is so severe that mutations may in itself provide the tumor cells with a selective advantage, so that only limited numbers of other gene defects are necessary. Indeed, in a study of 8 genes known to affect the WNT signaling, mutation in *PTEN* was the only example of a sole mutation among the investigated genes in single tumors [47]. Only few distinct point mutations have been reported in colorectal cancer, mainly in the two $(A)_6$ repeats in exon 7 and 8 [47,173]. Here, we have identified several additional mutations, located in all but one exon (exon 9;figure 18), which to our knowledge have not been reported for this cancer type, despite of several previous studies of all the exons [174,175]. Hot spots are usually localized in regions that are of functional importance to the gene. The functional domains of *PTEN* are spanning most of the coding region (see figure 18). This is consistent with the fact that the mutations were found evenly distributed throughout the domains instead of appearing in hot spots. This implies that the alterations might have the same functional effect regardless of the affected site. Furthermore, numerous tumors are showing more than one hit, possibly leading to biallelic inactivation, although no consistent pattern can be found. Also deletion of *PTEN* and/or loss of expression have been found in various cancer types, but does not seem to be prevalent in colorectal cancer [79]. Instead it has been hypothesized that other mechanisms, such as promoter methylation, might

be the operative event, and indeed, frequent inactivation of *PTEN* in various tumor types, including colorectal carcinomas [176], have been reported. However, the majority of the primer sets used are mistakenly designed for the frequently methylated pseudogene of *PTEN* showing a 98% homology with the protein encoding gene [177]. Unpublished data from our group show that *PTEN* is not subjected to promoter hypermethylation in colorectal carcinomas nor in colon cancer cell lines (Ahlquist *et al*, unpublished).

In TGCT, the general prevalence of point mutations is low, in contrast to a high frequency of copy number changes. This is exemplified by *TP53*, which is frequently mutated in various cancer types, but not in TGCTs [178,179]. Previous work has documented activating mutations in the *KIT* receptor tyrosine kinase, although at low frequencies [119], with the exception of bilateral tumors. Some years ago it was showed that the protein kinase *STK11* (LKB1) was mutated in a low frequency in TGCT [180]. In a recent large study, the potential role of mutated protein kinases in the development of TGCT were examined by analyzing all the 518 genes of the annotated protein kinase family, however, only a single point mutation in the *STK10* gene was found [181]. This was in contrast to the results from a similar study of colorectal cancer, where 10% of the analyzed genes were changed, affecting 31% of the cases investigated [144]. Mutations leading to activated RAS proteins of both *KRAS* and its homologue *NRAS*, have been detected in TGCT, although at various frequencies. More than a decade ago our group initially showed that KRAS could be mutated in TGCT [54], and in a review of the literature from 1995, the overall frequency of *KRAS* mutations was determined to 11%, with a higher incidence in seminomas than non-seminomas [182]. Our 4% *KRAS* mutations are within this previously published range [54,182]. Expression levels of *KRAS* have also been reported to be slightly increased in wild type TGCT [117], thus, it has been suggested that the activated oncogenic RAS effector pathways may be caused by high

expression of up-stream regulators [183]. Regarding *BRAF* mutations in TGCT, there are only a few cases reported, which was published after the initiation of the present study. McIntyre and co-workers investigated 65 primary TGCTs and 4 TGCT cell lines, and found none [184]. Sommerer and co-workers investigated 62 TGCTs and identified 3 activating mutations in non-seminomas, none in seminomas [185]. In accordance to this, we have only found one *BRAF* mutations, however, it was found in a seminoma rather than a non-seminoma. Summarized, we can now conclude that *BRAF* mutations are rare events in TGCTs. Although of importance to single tumors, these aberrations are not characteristic to the pathogenesis of TGCTs.

The PI3K signaling pathway is suggested to be involved in both neovascularization and cord formation during testis morphogenesis. However, to our knowledge, no reports regarding neither mutations nor expression levels of the PI3K-encoding gene, *PIK3CA*, have so far been presented in human TGCTs. Additionally, since we could not identify any mutations in the gene, it might not be important in the development of this cancer type. On the other hand, inactivation of *PTEN*, which negatively regulates cell growth, migration, and survival via the PI3K-AKT signaling pathway, leads to development of TGCT in heterozygous mice [186,187]. Further it is also is shown that PTEN marks the transition from intratubular germ cell neoplasias (ITGCN) to invasive germ cell tumors in humans by loss-of-expression experiments [188]. ITGCN intensively expressed PTEN, whereas it was virtually absent in over 50 and 80% in seminomas and non-seminomas, respectively. Additionally, the same study showed LOH of the *PTEN* locus, and also revealed two inactivating mutations of *PTEN*. In the present series of TGCT no PTEN mutations were found. Taken together it seems that PTEN is commonly down-regulated in testicular cancer, but this cannot be explained by sequence mutations. Furthermore, regulation of the PI3K-AKT in TGCT may after all also be

disturbed by altered expression of the PIK3CA. The facts that *PIK3CA* has a CpG island in the promoter, and that TGCT commonly include inactivated cancer critical genes through promoter hypermethylation, suggest the *PIK3CA* as an epigenetic target. Methylation studies of *PIK3CA* are currently in progress of investigation.

The molecular basis of MPNSTs is poorly understood, although both loss of the *NF1*-gene and high levels of RAS activity have been described to be common hallmarks. Oncogenic mutations resulting in activated RAS are prevalent in 30% of all human cancers, however, it has not been detected in MPNSTs [55]. It is therefore suggested that the loss of NF1, which product usually accelerate the hydrolysis of active RAS-GTP to inactive RAS-GDP, is the cause of the functional up-regulation of the RAS signaling pathway. Not surprisingly, our results support this theory, as no *KRAS* mutations were identified. The present tumor series were from patients with the hereditary disease neurofibromatosis type 1 and from sporadic cases. The former carry a *NF1* germ line mutation and it has been shown that both alleles are typically inactivated in their tumors. Therefore, it is interesting that the single BRAF mutation found was in a sporadic tumor that potentially does not have both NF1 alleles affected. The *PIK3CA* mutation was found in a tumor from a NF1 patient. The low frequencies may imply that these genes are not important in development or progression of MPNSTs. *PTEN* has been shown to be mutated in several cancer types, but in a previous study of 12 MPNSTs, Mawrin and co-workers failed to demonstrate mutations within the coding region of *PTEN* in MPNSTs [189]. They used the SSCP method, which is known to have somewhat reduced sensitivity, however taken together with the present results, we can conclude that mutations are not commonly found in *PTEN* in MPNSTs.

Gene methylation

In the present study, we analyzed the promoter methylation of *RASSF1A,* which has been shown to be epigenetically inactivated in a variety of tumors, suggesting a major role for this tumor suppressor gene in cancer [68]. *RASSF1A* is shown to be inactivated predominantly by promoter methylation and rarely by somatic mutations.

Several additional genes have been shown to be commonly methylated in colorectal cancer, including *MGMT, MLH1,* and *RASSF2A* [68,190-192]. In accordance with our result, the previously reported methylation frequencies of *RASSF1A* in colon ranges from 12-45% [65,193,194]. The wide frequency range might be due to inter-individual variation in scoring and interpretation of the methylation specific PCR (MSP) results, as well as the small number of samples included in some of the series. It has further been suggested that the *RASSF1A* inactivation in colorectal cancer is a late event, since methylation was rarely detected in benign adenomas (2%), versus more frequent in the carcinomas (16%) [195]. The timeline for *RASSF1A* inactivation in colorectal cancer needs further studies to be conclusive.

About 25 different genes, known to be commonly methylated in various cancer types, have also been analyzed in TGCTs, and only a small number are found frequently methylated [161,164,196,197] (see figure 20 for genes analyzed in our group). Among the most frequently inactivated genes are *MGMT, RASSF1A, APC, HIC1,* and *BRCA1*. It is demonstrated that the nonseminoma group of TGCT show significantly more methylation than do the seminomas [121,164,197], ranging from 29-83% and 0-40%, respectively [161,196-198]. This is in concordance to our findings, resulting in 30% methylation of the nonseminomas and no methylation of the seminoma subgroup. Nonseminomas usually have high sensitivity towards cisplatin-based combination therapy, however, recently it was

demonstrated that promoter methylation of *RASSF1A* is associated with resistance to cisplatin [198], and might serve as a marker for the identification of resistant tumors.

The methylation profile of MPNSTs remains mostly undescribed, as only few studies on promoter methylation have been done in this rare malignancy. The *p16*^{INK4A}, is frequently silenced by promoter methylation or deletions in various cancer types. We initially showed that this gene is commonly deleted or rearranged in MPNST and thereby looses its expression, whereas gene methylation is not seen [142]. We and others have later confirmed the lack of methylation in larger series[142,143,199]. Similar results are reported for *NF1* [200,201]. Regarding *RASSF1A,* a recent report demonstrated a promoter methylation frequency of 18% in MPNST [202], substantially lower than what we found (47%). This might be due to different primer sets utilized for the MSP. However, most likely it reflects the low number (n = 17) of MPNSTs investigated in the Japanese study.

Gene methylation in relation to genomic complexity is different among the three diseases. In colorectal carcinomas there is a general association between gene methylation and near diploidy, whereas in the TGCT model, methylated genes are characteristic to non-seminomas although all TGCT are in the triploid range. Finally, the MPNSTs show a wide range of genetic complexity, which is not related to gene methylation frequency.

Mutually exclusive changes

In nearly all cancer types it has been concluded that *KRAS* mutations are inversely associated with *BRAF* mutations, the exception being pancreatic cancer [203]. The mutual exclusivity is also the case for sporadic colorectal cancer [171,204,205], concordant to our results.

Mutations activates *BRAF* independent of RAS via the RAS-RAF-MEK-ERK signaling pathway, and previous reports provide strong support to the hypothesis that *BRAF* and *KRAS* mutations are equivalent in their tumorigenic effects. Furthermore, in a study published last year, it was suggested that *PIK3CA* mutations in colorectal cancer preferentially occur together with *KRAS* or *BRAF* mutations, suggesting a possible synergistic effect of the signaling pathways controlled by these in the development/progression of the disease [206]. The same trend could also be seen in our data set, although it was far from being statistically significant (see table 4). We found that mutations in *PIK3CA* were equally distributed between tumors with wild type and mutated *KRAS* and *BRAF*.

In 2002, van Engeland and co-workers provided evidence for the interruption of RAS signaling in sporadic colorectal cancer by either genetic activation of *KRAS* or epigenetic silencing of *RASSF1A* [65], although they reported a small proportion (5%) of CRCs that had both *RASSF1A* methylation and *KRAS* mutations. Our findings, showing concomitant hypermethylation and activating mutations of the two genes, are in contrast to the Engeland study but in agreement with, Oliveira and co-workers whom did not find *RASSF1A* hypermethylation versus *KRAS/BRAF* mutations to be mutually exclusive events in MSI tumors [207]. Based on the existing literature, it seems not reasonable to conclude that methylation of *RASSF1A* and mutational activation of *KRAS* are functionally equivalent. To our knowledge, no studies have revealed whether *RASSF1A* inactivation is leading to activation of the RAS signaling pathway and hence providing an alternative pathway for affecting RAS signaling. Whether alterations of *KRAS* and *RASSF1A* are truly mutually exclusive events or not, must await clarification about the biological functions of RASSF1A.

It has been reported that *PTEN* and *KRAS* are mutually exclusive events in endometrial cancer

[208]. To our knowledge, no such connection has been studied in colorectal cancer, however, here we show that there is a strong inversely association between the two genes (*P*=0,002). Furthermore, the opposite result was obtained for *PTEN* and *BRAF*, showing an association for mutations in those genes. This is in accordance with the findings that *KRAS* mutations associate with MSS tumors, whereas mutations of *BRAF* and *PTEN* are more frequently found in MSI tumors. Additionally, somatic mutations in *PTEN* and *TP53* are mutually exclusive in the stroma of breast carcinomas [209]. This seems to be the case for sporadic colorectal cancer as well, as we could also see an inversely association between the two genes. *TP53* activates the transcription of *PTEN* [83], hence negatively regulating the PI3K signaling pathway. Our results indicate that inactivation of the pathway can be achieved by the inactivation of either genes.

No mutually exclusive events were found for *PIK3CA* in colorectal carcinomas, whereas for TGCT and MPNST there were found no mutually exclusive events regarding any of the genes (data not shown).

Master keys in cancer

In this thesis, there were found numerous alterations of *KRAS, BRAF*, *PIK3CA, PTEN,* and *RASSF1A*. We have shown that one or more of these are altered in more than ¾ of all colorectal carcinomas, affecting two signaling pathways, MAP-kinase and PI3K-AKT, which can be referred to as master keys in cancer. Epigenetically targeted master keys do exist, for instance $p16^{INK4A}$ and *MGMT* that are commonly inactivated by promoter methylation. Despite the identification of only few mutations in testis cancer, promoter methylation of the gene analyzed, *RASSF1A*, was found in a subgroup, further supporting this mechanism as

typical to development of TGCTs. The *RASSF1A* gene was indeed altered in a subgroup of all three diseases and summarizing this with similar findings in other cancer types suggest that we may look upon this gene as a key regulator of cancer.

In MPNSTs, a small number of mutations were found in the genes analyzed. In parallel to this study we are currently analyzing *NF1*, which negatively regulates *KRAS,* and is expected to be commonly altered in MPNSTs. In this context, it will be interesting to find out if it turns out to be mutually exclusive in relation to *KRAS, BRAF,* and *RASSF1A* in either disease. Adding the *NF1* data to the current data will further add information to how common one or more of the upstream MAPK- factors are altered across the three diseases.

The two analyzed factors affecting the PI3K-AKT pathway show common mutations only in the colorectal model. However, these components have been shown important in other cancer types, and indeed methylation may again explain the absence of mutation in a subgroup of the samples. Therefore additional analyses must be made before conclusions can be drawn. Aiming to reveal master keys in cancer, there are some additional components that we are considering to investigate. In the PI3K-pathway, the proto-oncogene *AKT1*, which protect the cell from apoptosis and increases proliferation, is recently found to be inactivated by several upstream components. The tumor suppressor gene, *PML*, can inhibit the pathway by inactivating phosphorylated nuclear AKT1 [210]. Loss of *PML* in mice heterozygous of *PTEN,* accelerates tumor development resulting in invasive adenocarcinomas of the colon, whereas *PTEN* $^{+/-}$ mice displayed only pre-cancerous polyps. In addition to this, Krop and co-workers has shown that *HIN-1 (*also known as *SCGB3A1)* is, among other things, also an inhibitor of AKT activation [211]. *HIN-1* expression inhibited *AKT1* phosphorylation induced by EGF. Furthermore, *HIN-1*is found down-regulated in various cancer types due to promoter

methylation, which is also the case for testis cancer (see figure 20) [164]. It seems that the hypermethylation of *HIN-1* to some degree coincide with the hypermethylation of *RASSF1A* in TGCTs, however, *HIN-1* is additionally down-regulated in embryonic carcinomas, which is rarely seen for any other genes. Thus, we consider *PML* and *HIN-1* in the context of upstream signaling key components, to be good candidates for further analyses.

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Appendices

APPENDIX I: Abbreviations and gene symbols

APPENDIX II: Primer sequences

APPENDIX III: Mutation/methylation data

APPENDIX IV: Patient info

Appendix I

According to the nomenclature (Human Gene Nomenclature Committee), human genes should be written in italic capitals, and their gene products in capital without italic. Abbreviations mentioned twice or more in the text are listed here.

AKT1	v-akt murine thymoma viral oncogene homolog 1
APC	Adenomatous polyposis coli
bp	base pairs
BRAF	v-raf murine sarcoma viral oncogene homolog B1
DNA	Deoxyribo nucleic acid
CDK	Cyclin dependent kinase
CDKN2A	Cyclin dependent kinase inhibitor 2A
cDNA	complementary DNA
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CiS	carcinoma *in situ*
CKI	Cyclin dependent kinase inhibitor
CGH	Comparative genomic hybridization
CRC	Colorectal cancer
ddNTPs	dideoxyribonucleotide
dNTP	deoxyribonucleotide (any of the four)
EC	Embryonal carcinomas
EGFR	Epidermal growth factor receptor
FAP	Familial adenomatous polyposis coli
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GTP	Guanine triphosphate
GRB2	Growth factor receptor-bound protein 2
GSK-3β	Glycogen synthase kinase 3 beta
HNPCC	Hereditary non-polyposis colorectal cancer
ITGCN	Intratubular germ-cell neoplasia
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LOH	Loss of heterogozity
MAPK	Mitogen-activated protein kinase
MAPK1	Mitogen-activated protein kinase 1
MAP2K1	Mitogen-activated protein kinase kinase 1
MDM2	Transformed 3T3 cell double minute 2/ prev Mouse double minute 2
MMR	Mismathc repair
MLH1	mutL homolog 1
MPNST	Malignant peripheral nerve sheath tumor
MSH2	mutS homolog 2
MSH6	mutS homolog 6
MSI	Microsatellite instability
MSP	Methylation-Specific PCR
MST1	Macrophage stimulating 1 (hepatocyte growth factor-like)
MTOR	Mammalian target of Rapamycin
NF1	Neurofibromatosis 1
NORE1	Ras association (RalGDS/AF-6) domain family 5 (RASSF5)
OCT3/4	POU domain, class 5, transcription factor 1 (POU5F1)
PCR	Polymerase Chain Reaction
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIP_3	Phosphatidylinositol 3,4,5 triphosphate
PIP_2	Phosphatidylinositol 3,4 diphosphate
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosohatase and tensin homolog
RASSF1A	Ras association (RalGDS/AF-6) domain family 1A
RB1	Retinoblastoma 1
RTK	Receptor tyrosine kinase
SHC1	Src (homology 2 domain containing) transforming protein 1
SMAD	Mothers against DPP homolog
SOS	Son of sevenless homolog 1
TGCT	Testicular germ cell tumor
TGFβ1	Transforming growth factor β
TP53	Tumor protein p53
TSG	Tumor suppressor gene

U	Units
Wnt	Wingless-type

Appendix II

Primer sequences, annealing temperature and elongation time for the various genes analyzed.

Primer		Primer sequence (5' to 3')	Annealing temperature (°C)	Elongation time (sec)
KRAS				
Exon 2	Fwd	ACT GGT GGA GTA TTT GAT AG	50	30
	Rev	GTA TCA AAG AAT GGT CCT		
Exon 3	Fwd	ATAATAGCCAATCCTAA	50	45
	Rev	ATG GCA TTA GCA AAG		
BRAF				
Exon 11	Fwd	TCC CTC TCA GGC ATA AGG TAA	60	45
	Rev	CGA ACA GTG AAT ATT TCC TTT GAT		
Exon 15	Fwd	TCA TAA TGC TTG CTC TGA TAG GA	58	45
	Rev	GGC CAA AAA TTT AAT CAG TGG A		
RASSF1A				
Unmethylated	Fwd	ATG TGT TGT GTA TTG TGT GGG G	62	30
	Rev	CCA CAA CAA CTA CAC TAC CCC		
Methylated	Fwd	ACG CGT TGC GTA TCG CGC G	62	30
	Rev	CCG CGA CGA CTA CGC TAC C		
PIK3CA				
Exon 9	Fwd	GAT TGG TTC TTT CCT GTC TCT G	58	30
	Rev	CCA CAA ATA TCA ATT TAC AAC CAT TG		
Exon 20	Fwd	AAG CCT CTC TAA TTT TGT GAC	54	30
	Rev	AAA CTC CAG TTT ACT TAC ACC		
PTEN				
Exon 1	Fwd	CAG CCG TTC GGA GGA TTA	60/62	90
	Rev	ATA TGA CCT AGC AAC CTG ACC A		
Exon 2	Fwd	GTA CTT TAG TTC TGT GAT GTA TAA ACC GT	60	90
	Rev	CTG AAG TCC ATT AGG TAC GGT AA		
Exon 3	Fwd	ATG TTT GTG AGG GTC GAA TG	60	90
	Rev	GGA CTT CTT GAC TTA ATC GGT TTA G		
Exon 4	Fwd 1	TTG AAA AAG GTG ATC GTT GG	60	90
	Fwd 2	GGT GTG ATA ACA GTA TCT ACT TAA TAG		
	Rev	ATT GTT ATG ACA GTA AGA TAC AGT CTA TCG		
Exon 5	Fwd 1	GAC CTA TGC TAC CAG TCC GTA	60	90
	Fwd 2	ATG CAA CAT TTC TAA AGT TAC CTA C		
	Rev	ATG ATA TGA AAA TGG TAG CGT G		
Exon 6	Fwd	AAT GTA TAT ATG TTC TTA AAT GGC TAC GA	60	90
	Rev	TCA TAA ATA TAA TTT GGC TTC GAC TAC		
Exon 7	Fwd	TTG CTG ATA TTA ATC ATT AAA ATC GTT	60	90
	Rev	AAT AAT CAA GTC TTA AGA AAC GTT AAG G		
Exon 8	Fwd	AGT TGC ACT CAC CGT CCA	60	90
	Rev	ATG CAG CTT TTT TGA CGC T		
Exon 9	Fwd	GGC CTC TTA AAG ATC ATG TTT G	60	90
	Rev	CAC TTT TTA TAA AAC TGG AAT AAA ACG		

Abbreviations: Fwd, forward primer; Rev, reverse primer. For those exons with two forward primes; the first is used for the standard PCR, and the second for the sequence PCR.

Appendix III

COLON									
Tumor sample number	PTEN Ex 1	PTEN Ex 2	PTEN Ex 3	PTEN Ex 4	PTEN Ex 5	PTEN Ex 6	PTEN Ex 7	PTEN Ex 8	PTEN Ex 9
848	wt	wt	wt	wt	wt	wt	wt	wt	wt
854	wt	wt	wt	wt	-	wt	wt	wt	wt
884	wt	T156C (D52D)	wt	wt	-	wt	wt	1 A del (963-968)	wt
887	wt	wt	wt	wt	wt	wt	wt	wt	wt
894I	wt	wt	wt	wt	wt	wt	wt	wt	wt
896	wt	wt	wt	wt	wt	wt	wt	wt	wt
910	wt	wt	wt	wt	wt	wt	wt	wt	wt
912	wt	wt	wt	wt	wt	wt	wt	wt	wt
922	wt	wt	wt	wt	wt	wt	wt	wt	wt
946	wt	wt	wt	wt	wt	wt	wt	wt	wt
948	wt	wt	wt	wt	wt	wt	wt	wt	wt
953	wt	wt	wt	wt	wt	wt	wt	wt	wt
955	wt	wt	wt	wt	-	wt	wt	wt	wt
965	wt	wt	wt	wt	wt	wt	wt	wt	wt
988	wt	wt	wt	wt	wt	wt?	wt	wt	wt
1013	wt	wt	wt	wt	wt	wt	wt	wt	wt
1022	wt	wt	wt	wt	wt	wt	wt	wt	wt
1024	wt	wt	wt	wt	wt	wt	wt	wt	wt
1044	wt	wt	wt	wt	wt	wt	wt	4 bp del (950-953)	wt
1046	wt	wt	wt	wt	wt	wt	wt	wt	wt
1047	wt	wt	wt	-	wt	wt	wt	wt	wt
1066	wt	wt	wt	wt	wt	wt	wt	wt	wt
1103	wt	wt	wt	wt	wt	wt	wt	wt	wt
1117	wt	wt	wt	wt	wt	wt?	wt	wt	wt
1121	wt	wt	wt	wt	wt	wt	wt	wt	wt
1124	wt	wt	wt	wt	wt	wt	wt	wt	wt
1132	wt	wt	wt	wt	wt	wt	wt	wt	wt
1141	2 bp del (GA)	wt	1 T del (166-70)	wt	wt	wt	wt	wt	wt
1166	wt	wt	wt	wt	wt	wt	wt	wt	wt
1167	wt	wt	wt	wt	wt	wt	wt	wt	wt
1190	wt	wt	1 T ins (166-70)	wt	wt	wt	1A ins (795-800)	wt	wt
1193	wt	wt	wt	wt	wt	wt	wt	wt	wt
1194	wt	wt	wt	wt	wt	wt	wt	wt	wt
1197	wt	wt	wt	wt	wt	wt	wt	wt	wt
1268	wt	wt	wt	wt	wt	T542C (L181P)	wt	1 A del (963-968)	wt
1273	wt	wt	wt	wt	-	wt	wt	-	wt
1287	wt	nd	wt	nd	nd	wt	nd	nd	wt
1294	wt	wt	wt	wt	wt	wt	wt	wt	wt
1296	wt	wt	wt	wt	wt	wt	wt	wt	wt
1314	wt	wt	wt	wt	T433A (F145I)	wt	wt	wt	wt
1326	wt	wt	wt	wt	-	wt	wt	wt	wt
1340	wt	wt	wt	wt	wt	wt	wt	wt	wt
1341	wt	wt	wt	wt	wt	wt	1A del (795-800)	wt	wt
1342	wt	wt	wt	wt	-	wt	wt	wt	wt
1349	wt	wt	wt	wt	wt	T542C (L181P)	wt	wt	wt
1363	wt	wt	wt	wt	wt	wt	wt	wt	wt
1364	wt	wt	wt	wt	wt	wt	wt	wt	wt
1369	nd	nd	nd	nd	nd	nd	nd	nd	nd
1388A	wt	wt	wt	wt?	wt	wt	wt	wt	wt
1388C	wt	wt	wt	wt	-	wt	wt	wt	wt
1391	wt	wt	wt	wt	wt	wt	wt	wt	wt
868	wt	wt	wt	wt	wt	wt	wt	wt	wt
886	wt	wt	wt	wt	wt	wt	wt	wt	wt
904	wt	wt	wt	wt	wt	wt	wt	wt	wt
923	wt	wt	wt	wt	wt	wt	wt	wt	wt
927	wt	wt	wt	wt	wt	wt	wt	wt	wt
966	wt	wt	wt	wt	wt	wt	wt	wt	wt
974	wt	wt	wt	wt	wt	wt	wt	wt	wt
976	wt	wt	wt	wt	wt	wt	wt	wt	wt
980	wt	wt	wt	wt	1 T del (267-70)	wt	wt	1 A del (963-968)	wt
984	wt	wt	wt	wt	wt	wt	wt	wt	wt
1027	wt	wt	wt	wt	wt	wt	wt	wt	wt
1029	wt	wt	wt	wt	wt	wt	wt	wt	wt
1060	wt	wt	wt	T242C (F81S)	wt	wt	wt	C895A (E299STOPP)	wt
1069	wt	wt	wt	wt	G389A (R130Q)	wt	wt	T1022G (F1023V)	wt
1111	wt	wt	wt	wt	C388T (R130STOPP)	wt	wt	wt	wt

COLON							
Tumor sample number	**BRAF Ex 15**	**BRAF Ex 11**	**KRAS Ex 2**	**KRAS Ex 3**	**PIK3CA Ex 9**	**PIK3CA Ex 20**	**RASSF1A**
848	wt	wt	wt	wt	A1634G (E545G)	wt	U
854	wt	wt	wt	3 bp del (kod 62)	wt	wt	U
884	T1799A (V600E)	wt	wt	wt	wt	wt	U
887	wt	wt	G34T (G12C)	wt	A1637C (Q546P)	wt	M
894I	wt	wt	3 bp ins (TGG)	wt	wt	wt	U
896	wt	wt	wt	wt	wt	wt	M
910	wt	wt	wt	wt	wt	wt	U
912	wt	wt	G38A (G13D)	wt	G1633A (E545K)	wt	U
922	wt	wt	G35T (G12 V)	wt	G1633A (E545K)	wt	U
946	wt	wt	wt	wt	wt	wt	U
948	wt	wt	G34C (G12R)	wt	wt	wt	U
953	wt	wt	wt	wt	wt	wt	U
955	T1799A (V600E)	wt	wt	wt	wt	wt	U
965	T1799A (V600E)	wt	wt	wt	wt	wt	U
988	wt	wt	wt	wt	wt	wt	NS
1013	A1781G (D594G)	wt	wt	wt	wt	wt	U
1022	wt	wt	wt	wt	wt	wt	U
1024	wt	wt	G34T (G12C)	wt	wt	wt	U
1044	T1799A (V600E)	wt	wt	wt	wt	wt	U
1046	wt	wt	wt	wt	G1633A (E545K)	wt	U
1047	wt	wt	G34C (G12A) / G40A (V14I)	wt	wt	wt	M
1066	wt	wt	wt	wt	wt	wt	M
1103	wt	wt	wt	wt	wt	wt	U
1117	wt	wt	wt	wt	wt	wt	U
1121	wt	wt	G34C (G12A)	wt	G1633A (E545K)	wt	M
1124	wt	wt	G34A (G12D) / G37A (G13D)	wt	wt	wt	NS
1132	wt	wt	G34T (G12V)	wt	G1624A (E542K)	wt	U
1141	wt	wt	wt	wt	wt	wt	U
1166	wt	wt	G37A (G13D)	wt	wt	wt	M
1167	wt	wt	wt	wt	wt	wt	M
1190	T1799A (V600E)	wt	wt	wt	wt	wt	M
1193	T1799A (V600E)	wt	wt	wt	wt	A3140G (H1047R)	U
1194	wt	wt	wt	wt	wt	wt	M
1197	wt	wt	wt	wt	wt	wt	NS
1268	T1799A (V600E)	wt	wt	wt	wt	A3140T (H1047L)	U
1273	T1799A (V600E)	wt	wt	wt	wt	wt	U
1287	wt	wt	wt	wt	nd	nd	ND
1294	wt	G1406C (G469R)	wt	wt	G1633A (E545K)	wt	U
1296	wt	wt	G37A (G13D)	wt	wt	wt	U
1314	wt	wt	wt	wt	wt	wt	U
1326	wt	wt	G37A (G13D)	wt	wt	wt	U
1340	wt	wt	wt	wt	wt	wt	NS
1341	T1799A (V600E)	wt	wt	wt	wt	wt	M
1342	wt	wt	wt	wt	wt	wt	U
1349	wt	wt	wt	wt	wt	wt	M
1363	wt	wt	G37A (G13D)	wt	wt	wt	U
1364	T1790A (L597Q)	wt	wt	wt	wt	wt	M
1369	wt	nd	G34A (G12D)	wt	nd	nd	ND
1388A	wt	wt	wt	wt	wt	wt	M
1388C	wt	wt	G37A (G13D)	wt	G1633A (E545K)	wt	M
1391	wt	wt	GAT G12D	wt	wt	A3140G (H1047R)	U
868	wt	wt	wt	wt	wt	wt	U
886	wt	wt	G34A (G12D)	wt	wt	wt	U
904	wt	wt	wt	wt	wt	wt	U
923	wt	wt	G37A (G13D)	wt	G1624A (542K)	wt	U
927	wt	wt	G34T (G12V)	wt	wt	wt	M
966	wt	wt	wt	A186C (Q61L)	wt	wt	U
974	wt	wt	G34C (G12A)	wt	wt	wt	U
976	wt	wt	G34A (G12D)	wt	wt	wt	M
980	T1799A (V600E)	wt	wt	wt	wt	wt	U
984	T1799A (V600E)	wt	wt	wt	wt	wt	U
1027	wt	wt	G37A (G13D)	wt	wt	wt	U
1029	wt	wt	G34A (G12D)	wt	wt	wt	U
1060	wt	wt	wt	wt	wt	A3073G (T1025A)	M
1069	wt	wt	wt	wt	wt	wt	U
1111	wt	wt	wt	wt	G1633A (E545K)	wt	M

TGCT									
Tumor sample number	**PTEN Ex 1**	**PTEN Ex 2**	**PTEN Ex 3**	**PTEN Ex 4**	**PTEN Ex 5**	**PTEN Ex 6**	**PTEN Ex 7**	**PTEN Ex 8**	**PTEN Ex 9**
30	wt	wt	wt	wt	wt	wt	wt	wt	wt
36	wt	wt	wt	wt	wt	wt	wt	wt	wt
53	wt	wt	wt	wt	wt	wt	wt	wt	wt
59	wt	wt	wt	wt	wt	wt	wt	wt	wt
70	wt	wt	wt	wt	wt	wt	wt	wt	wt
71	wt	wt	wt	wt	wt	wt	wt	wt	wt
75	wt	C132T (G44G)	wt	wt	wt	wt	nd	wt	wt
84	wt	wt	wt	wt	wt	wt	wt	wt	wt
85	wt	wt	wt	wt	wt	wt	wt	wt	wt
86	wt	wt	wt	wt	wt	wt	wt	wt	wt
88	wt	wt	wt	wt	wt	wt	wt	wt	wt
94	wt	wt	wt	wt	wt	wt	wt	wt	wt
95	nd	nd	nd	nd	nd	nd	nd	nd	nd
98	wt	wt	wt	nd	wt	wt	wt	wt	wt
102	wt	wt	wt	wt	wt	wt	wt	wt	wt
109	nd	nd	nd	nd	nd	nd	nd	nd	nd
110	wt	wt	wt	wt	wt	wt	wt	wt	wt
113	wt	wt	wt	wt	wt	wt	wt	wt	wt
118	nd	nd	wt	nd	nd	nd	nd	nd	nd
124	wt	wt	wt	wt	wt	wt	wt	wt	wt
127	wt	wt	wt	wt	wt?	wt	wt	wt	wt
130	wt	wt	wt	wt	wt	wt	wt	wt	wt
132	wt	wt	wt	wt	wt	wt	wt	wt	wt
133	wt	wt	wt	wt	wt	wt	wt	wt	wt
135	wt	wt	wt	wt	wt	wt	wt	wt	wt
136	wt	wt	wt	wt	wt?	wt	wt	wt	wt
137	wt	nd	wt	wt	wt	wt	wt	wt	wt
145	wt	wt	wt	wt	wt	wt	wt	wt	wt
146	wt	wt	wt	wt	wt	wt	wt	wt	wt
216	wt	wt	wt	wt	wt	wt	wt	wt	wt
307	wt	wt	wt	wt	wt	wt	wt	wt	wt
502	wt	wt	wt	wt	wt	wt	wt	wt	wt
564	wt	wt	wt	wt	wt	wt	wt	wt	wt
691	wt	wt	wt	wt	wt	wt	wt	wt	wt
696	wt	wt	wt	wt	wt	wt	wt	wt	wt
737	wt	wt	wt	wt	wt	wt	wt	wt	wt
738	wt	wt	wt	wt	wt	wt	wt	wt	wt
1017	wt	wt	wt	wt	wt	wt	wt	wt	wt
1113	wt	wt	wt	wt	wt	wt	wt	wt	wt
1282	wt	wt	wt	wt	wt	wt	wt	wt	wt
1545	wt	wt	wt	wt	wt	wt	wt	wt	wt
1692	wt	wt	wt	wt	wt	wt	wt	wt	wt
1740	wt	wt	wt	wt	wt	wt	wt	wt	wt
1748	wt	wt	wt	wt	wt	wt	wt	wt	wt
1838	wt	wt	wt	wt	wt	wt	wt	wt	wt
1863	wt	wt	wt	wt	wt	wt	wt	wt	wt
2201	wt	wt	wt	wt	wt	wt	wt	wt	wt
2110	wt	wt	wt	wt	wt	wt	wt	wt	wt
3493	wt	wt	wt	wt	wt	wt	wt	wt	wt
3879	wt	wt	wt	wt	wt	wt	wt	wt	wt

TGCT							
Tumor sample number	**BRAF Ex 15**	**BRAF Ex 11**	**KRAS Ex 2**	**KRAS Ex 3**	**PIK3CA Ex9**	**PIK3CA Ex20**	**RASSF1A**
30	wt	wt	wt	wt	wt	wt	U
36	wt	wt	wt	wt	wt	wt	U
53	wt	wt	wt	wt	wt	wt	U
59	wt	wt	wt	wt	wt	wt	U
70	wt	wt	wt	wt	wt	wt	M
71	wt	wt	G34/35T (G12L)	wt	wt	wt	U
75	wt	wt	wt	wt	wt	nd	U
84	wt	wt	wt	wt	wt	wt	M
85	wt	wt	wt	wt	wt	wt	U
86	wt	wt	wt	wt	wt	wt	U
88	wt	wt	wt	wt	wt	wt	U
94	wt	wt	wt	wt	wt	wt	U
95	nd	nd	nd	nd	nd	nd	nd
98	wt	wt	wt	wt	wt	wt	U
102	wt	wt	wt	wt	wt	wt	U
109	nd	wt	nd	nd	nd	nd	U
110	wt	wt	wt	wt	wt	wt	U
113	wt	wt	wt	wt	wt	wt	U
118	wt	wt	wt	nd	nd	nd	U
124	wt	wt	wt	wt	wt	wt	U
127	wt	wt	wt	wt	wt	wt	U
130	wt	wt	wt	wt	wt	wt	U
132	wt	wt	wt	wt	wt	wt	U
133	G1780A (D594N)	wt	wt	wt	wt	wt	U
135	wt	wt	wt	wt	wt	wt	U
136	wt	wt	wt	wt	wt	wt	M
137	wt	wt	wt	wt	wt	wt	U
145	w	wt	wt	wt	wt	wt	U
146	wt	wt	wt	wt	wt	wt	U
216	wt	wt	wt	wt	wt	wt	M
307	wt	wt	wt	wt	wt	wt	M
502	wt	wt	wt	wt	wt	wt	U
564	wt	wt	wt	wt	wt	wt	U
691	wt	wt	wt	wt	wt	wt	U
696	wt	wt	wt	wt	wt	wt	U
737	wt	wt	wt	wt	wt	wt	U
738	wt	wt	wt	wt	wt	wt	M
1017	wt	wt	wt	wt	wt	wt	U
1113	wt	wt	G34A (G12S)	wt	wt	wt	U
1282	wt	wt	wt	wt	wt	wt	M
1545	wt	wt	wt	wt	wt	wt	U
1692	wt	wt	wt	wt	wt	wt	U
1740	wt	wt	wt	wt	wt	wt	U
1748	wt	wt	wt	wt	wt	wt	U
1838	wt	wt	wt	wt	wt	wt	U
1863	wt	wt	wt	wt	wt	wt	U
2201	wt	wt	wt	wt	wt	wt	U
2110	wt	wt	wt	wt	wt	wt	M
3493	wt	wt	wt	wt	wt	wt	U
3879	wt	wt	wt	wt	wt	wt	U

MPNST									
Tumor sample number	PTEN Ex 1	PTEN Ex 2	PTEN Ex 3	PTEN Ex 4	PTEN Ex 5	PTEN Ex 6	PTEN Ex 7	PTEN Ex 8	PTEN Ex 9
650-90P	wt	wt	wt	wt	wt	wt	wt	wt	wt
2362-90-1	wt	wt	wt	wt	wt	wt	wt	wt	wt
2367-90-1	wt	wt	wt	wt	wt	wt	wt	wt	wt
753-92P-1	wt	wt	wt	wt	wt	wt	wt	wt	wt
32-94R	wt	wt	wt	wt	wt	wt	wt	wt	wt
1615-94P	wt	wt	wt	wt	wt	wt	wt	wt	wt
2406-94P	wt	wt	wt	wt	wt	wt	wt	wt	wt
1046-95P	wt	wt	wt	wt	wt	wt	wt	wt	wt
1944-95P	wt	wt	wt	wt	wt	wt	wt	wt	wt
763-91P	wt	wt	wt	wt	wt	wt	wt	wt	wt
3420-95P	wt	wt	wt	wt	wt	wt	wt	wt	wt
246-96P	wt	wt	wt	wt	wt	wt	wt	wt	wt
11-AEL	wt	wt	wt	wt	wt	wt	wt	wt	wt
12-HK	wt	wt	wt	wt	wt	wt	wt	wt	wt
13-EEO	wt	wt	wt	wt	wt	wt	wt	wt	wt
14-GP	wt	wt	wt	wt	wt	wt	wt	wt	wt
3-HPK	wt	wt	wt	wt	wt	wt	wt	wt	wt
5-GKH	wt	wt	wt	wt	wt	wt	wt	wt	wt
6-OMN	wt	wt	wt	wt	wt	wt	wt	wt	wt
7-GM	wt	wt	wt	wt	wt	wt	wt	wt	wt
8-EMA	wt	wt	wt	wt	wt	wt	wt	wt	wt
9-MO	wt	wt	wt	wt	wt	wt	nd	wt	wt
T85-6544	wt	wt	wt	wt	wt	wt	wt	wt	wt
T84-8124	wt	wt	wt	wt	wt	wt	wt	wt	wt
T89-3925	wt	wt	wt	wt	wt	wt	wt	wt	wt
T91-10391	wt	wt	wt	wt	wt	wt	wt	wt	wt
T92-10261	wt	wt	wt	wt	wt	wt	wt	wt	wt
T95-13713	wt	wt	wt	wt	wt	wt	wt	wt	wt
T95-2487	wt	wt	wt	wt	wt	wt	wt	wt	wt
T97-2719	wt	wt	wt	wt	wt	wt	wt	wt	wt
T97-7036	wt	wt	wt	wt	wt	wt	wt	wt	wt
T98-13182	wt	wt	wt	wt	wt	wt	wt	wt	wt

MPNST							
Tumor sample number	**BRAF Ex 15**	**BRAF Ex 11**	**KRAS Ex 2**	**KRAS Ex 3**	**PIK3CA Ex 9**	**PIK3CA Ex 20**	**RASSF1A**
650-90P	wt	wt	wt	wt	wt	wt	NS
2362-90-1	wt	wt	wt	wt	wt	wt	U
2367-90-1	wt	wt	wt	wt	wt	wt	U
753-92P-1	wt	wt	wt	wt	wt	wt	M
32-94R	wt	wt	wt	wt	wt	wt	U
1615-94P	wt	wt	wt	wt	wt	wt	M
2406-94P	A1742G (N581S)	wt	wt	wt	wt	wt	U
1046-95P	wt	wt	wt	wt	wt	wt	U
1944-95P	wt	wt	wt	wt	wt	wt	M
763-91P	wt	wt	wt	wt	wt	wt	U
3420-95P	wt	wt	wt	wt	wt	wt	U
246-96P	wt	wt	wt	wt	wt	wt	M
11-AEL	wt	wt	wt	wt	wt	wt	M
12-HK	wt	wt	wt	wt	wt	wt	U
13-EEO	wt	wt	wt	wt	wt	wt	U
14-GP	wt	wt	wt	wt	wt	wt	M
3-HPK	wt	wt	wt	wt	wt	wt	M
5-GKH	wt	wt	wt	wt	wt	wt	U
6-OMN	wt	wt	wt	wt	wt	wt	M
7-GM	wt	wt	wt	wt	wt	wt	M
8-EMA	wt	wt	wt	wt	wt	A3140G (H1047R)	U
9-MO	wt	wt	wt	wt	wt	nd	NS
T85-6544	wt	wt	wt	wt	wt	wt	U
T84-8124	wt	wt	wt	wt	wt	wt	M
T89-3925	wt	wt	wt	wt	wt	wt	U
T91-10391	wt	wt	wt	wt	wt	wt	M
T92-10261	wt	wt	wt	wt	wt	wt	U
T95-13713	wt	wt	wt	wt	wt	wt	U
T95-2487	wt	wt	wt	wt	wt	wt	U
T97-2719	wt	wt	wt	wt	wt	wt	M
T97-7036	wt	wt	wt	wt	wt	wt	M
T98-13182	wt	wt	wt	wt	wt	wt	M

Appendix IV

CRC									
Patient	**MSI status**	**Localisation**	**Gender**	**Age**	**Ploidi**	**TP53 status**	**APC status**	**Dukes' stage**	**Differentiation**
848	MSI	Rectum	Male	41	1,9	-	Mut	B	Medium
854	MSI	Right	Female	74	1,0	Wt	Mut	A	Low
884	MSI	Right	Female	90	1,0	-	Mut	B	Medium
887	MSS	Rectum	Female	82	1,1	Wt	Wt	B	High
894I	MSI	Right	Male	80	1,0	-	Mut	B	Medium
896	MSS	Rectum	Female	71	1,1	Mut	Wt	C	Medium
910	MSI	Right	Female	65	1,0	Wt	Mut	B	High
912	MSI	Left	Female	66	1,0	-	Mut	B	Low
922	MSS	Left	Male	71	1,6	Wt	Wt	D	Medium
946	MSS	Left	Male	77	1,0	Wt	Wt	B	Medium
948	MSS	Rectum	Female	61	1,4	Wt	Wt	B	High
953	MSS	Rectum	Male	68	1,5	Mut	Wt	B	Medium
955	MSI	Right	Female	84	1,0	-	Mut	B	Medium
965	MSI	Left	Female	67	1,0	-	Wt	B	Low
988	MSI	Right	Female	66	1,0	Wt	Wt	B	Low
1013	MSS	Rectum	Female	66	1,5	Mut	Wt	B	Medium
1022	MSI	Rectum	Male	33	1,0	Wt	Mut	C	Low
1024	MSS	Left	Female	60	1,0	Wt	Wt	C	Medium
1044	MSI	Rectum	Female	63	1,1	-	Wt	A	Medium
1046	MSS	Left	Male	66	1,5	Mut	Mut	D	Medium
1047	MSI	Rectum	Male	70	1,0	-	Wt	C	Medium
1066	MSI	Left	Female	41	1,0	Wt	Wt	C	Medium
1103	MSS	Left	Female	62	1,0	Wt	Wt	B	Medium
1117	MSI	Right	Male	78	1,0	-	Mut	C	Medium
1121	MSS	Left	Male	71	1,0	Mut	Wt	B	Medium
1124	MSS	Rectum	Male	73	1,4	Wt	Mut	C	Medium
1132	MSI	Right	Female	92	1,0	-	Wt	D	Medium
1141	MSI	Right	Female	76	1,0	-	Mut	D	Medium
1166	MSS	Left	Male	77	1,7	Wt	Wt	B	Medium
1167	MSS	Left	Male	73	3,0	Mut	Mut	C	Medium
1190	MSI	Right	Male	67	1,0	Wt	Mut	C	Medium
1193	MSI	Right	Female	69	1,0	Wt	Wt	C	Low
1194	MSS	Left	Male	44	1,6	Mut	Wt	C	Medium
1197	MSS	Left	Male	71	1,2	Mut	Wt	C	Medium
1268	MSI	Right	Male	71	1,0	-	Mut	B	Low
1273	MSI	Right	Female	68	1,0	-	Mut	B	Medium
1287	MSS	Rectum	Male	77	2,1	Mut	Mut	B	Medium
1294	MSS	Left	Male	73	1,9	Wt	Mut	C	Low
1296	MSS	Left	Male	76	1,7	Mut	Mut	B	Medium
1314	MSI	Right	Female	62	1,0	Wt	Mut	C	Low
1326	MSI	Left	Male	61	1,0	-	Mut	B	Medium
1340	MSS	Rectum	Male	51	1,2	Mut	Mut	C	Medium
1341	MSI	Right	Female	89	1,0	-	Mut	B	Medium
1342	MSI	Right	Male	49	1,0	Mut	Mut	B	Medium
1349	MSI	Right	Female	79	2,0	Wt	Mut	D	Low
1363	MSI	Right	Male	70	1,0	Wt	Wt	A	Medium
1364	MSS	Rectum	Female	60	1,4	Wt	Mut	B	Medium
1369	MSS	left	Female	82	1,5	Wt	Wt	B	Low
1388	MSI	left	Female	61	1,0	-	Wt	D	Low
1388C	MSI	left	Female	61	1,0	-	Wt	D	Low
1391	MSS	Rectum	Female	71	1,9	Mut	Mut	B	Medium
868	MSS	Left	Male	64	1,9	Wt	Mut	B	Medium
886	MSS	Left	Female	61	1,0	Wt	Wt	C	Medium
904	MSS	Left	Male	78	1,6	Mut	Wt	B	Medium
923	MSS	Right	Male	85	1,0	Wt	Wt	C	High
927	MSS	Rectum	Female	73	1,7	Mut	Wt	B	Medium
966	MSS	Rectum	Male	61	1,5	Mut	Wt	A	Medium
974	MSS	Right	Male	73	2,1	Mut	Mut	B	Medium
976	MSS	Rectum	Male	58	1,0	Wt	Wt	B	Medium
980	MSI	Right	Female	75	1,0	Wt	Mut	C	Medium
984	MSI	Right	Female	88	1,0	Wt	Mut	C	Low
1027	MSS	Rectum	Male	79	1,6	Mut	Wt	B	Medium
1029	MSS	Right	Male	83	1,4	Wt	Wt	C	Medium
1060	MSS	Left	Male	70	1,15	Wt	Wt	A	Medium
1069	MSS	Right	Male	74	1,0	Wt	Wt	B	Low
1111	MSS	Left	Female	72	1,0	Wt	Wt	A	Medium

TGCT		
Patient	**Histology**	**Subgroups**
30	Non seminoma	Yolk sac tumor/ITGCN
36	Seminoma	Seminoma
53	Seminoma	Seminoma
59	Seminoma	Seminoma
70	Non seminoma	Mixed
71	Seminoma	Seminoma
75	Seminoma	Seminoma
84	Non seminoma	Immature teratoma
85	Seminoma	Seminoma
86	Non seminoma	Teratoma
88	Non seminoma	Embr.carc/Teratoma
94	Seminoma	Seminoma
95	Seminoma	Seminoma
98	Non seminoma	Teratoma
102	Non seminoma	Immature teratoma
109	Seminoma	Seminoma
110	Non seminoma	Embryonal carcinoma
113	Seminoma	Seminoma
118	Seminoma	Seminoma
124	Non seminoma	Embryonal carcinoma
127	Seminoma	Seminoma
130	Seminoma	Seminoma
132	Seminoma	Seminoma
133	Seminoma	Seminoma
135	Seminoma	Seminoma
136	Non seminoma	Mixed
137	Non seminoma	Embryonal carcinoma
145	Non seminoma	Embryonal carcinoma
146	Non seminoma	Yolk sac tumor
216	Non seminoma	Yolk sac tumor
307	Non seminoma	Yolk sac tumor
502	Non seminoma	Embryonal carcinoma
564	Non seminoma	Embryonal carcinoma
691	Non seminoma	Teratoma
696	Non seminoma	Teratoma
737	Seminoma	Seminoma
738	Non seminoma	Yolk sac tumor
1017	Non seminoma	Embryonal carcinoma
1113	Seminoma	Seminoma
1282	Non seminoma	Teratoma
1545	Non seminoma	Embryonal carcinoma
1692	ITGCN	ITGCN
1740	Non seminoma	Embryonal carcinoma
1748	Seminoma	Seminoma
1838	Non seminoma	Embryonal carcinoma
1863	ITGCN	ITGCN
2201	Non seminoma	Teratoma
2110	Non seminoma	Yolk sac tumor
3493	ITGCN	ITGCN
3879	ITGCN	ITGCN

MPNST		
Patient	**NF1-status**	**Kjønn**
650-90P	Wt	Female
2362-90-1	Mut	Female
2367-90-1	Mut	Male
753-92P-1	Mut	Female
32-94R	Mut	Male
1615-94P	Wt	Female
2406-94P	Wt	Female
1046-95P	Wt	Female
1944-95P	Mut	Female
763-91P	Mut	Female
3420-95P	Wt	Male
246-96P	Wt	Male
11-AEL	Wt	Male
12-HK	Mut	Female
13-EEO	Wt	Male
14-GP	Mut	Male
3-HPK	Mut	Male
5-GKH	Wt	Female
6-OMN	Mut	Male
7-GM	Mut	Female
8-EMA	Mut	Female
9-MO	Mut	Female
T85-6544	Mut	Male
T84-8124	Mut	Male
T89-3925	Wt	Male
T91-10391	Mut	Female
T92-10261	Mut	Male
T95-13713	Wt	Female
T95-2487	Mut	Male
T97-2719	Mut	Male
T97-7036	Mut	Male
T98-13182	Mut	Female