Malignant Peripheral Nerve Sheath Tumors
amplicon search by FISH and protein expression profiling using tissue micro arrays

By

Helge R. Brekke

The recent study was performed during the period March 2003 - December 2004 at

Department of Genetics
Institute for Cancer Research
The Norwegian Radium Hospital

The Cand.Scient degree will be defended at the

Department of Molecular Biosciences
Biological Institute

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Acknowledgements

I would like to take this opportunity and thank the people at the Department of Genetics, Institute for Cancer Research at the Norwegian Radium Hospital. Especially my supervisor Professor Ragnhild A. Lothe has helped me through the time it took to finishing this thesis. Thanks for being so enthusiastic and for believing in me. Your excellent ideas and guidance has made these months both fun and inspiring, and taught me how a successful study should be arranged. I am grateful to post doc Rolf I. Skotheim for guidance in the labwork related to TMA and for patiently answering all my questions There are also some people I would like to mention that I have worked with in Ragnhild's group, Mette Eknæs, Merete Hektoen, Guro Elisabeth Lind, Kristine Kleivi, Chieu Diep, Maja Kraggerud, Marianne Berg, Lin Thorstensen and Terje Ahlquist have all supported me, both professionally and socially, during the master period. The fine work of our collaborator and reference pathologist Bodil Bjerkhagen must also be credited. Finally, I am indebted to Professor Fredrik Mertens at the Department of Clinical Genetics, University Hospital of Lund, Sweden, for the scientific input and social pleasure I experienced during my stay in his laboratory (February 2004). Thank you to all for contributing to my thesis, and I look forward to many coming years of collaboration and new scientific breakthroughs.

Table of Contents

Abbreviations

List of abbreviations mentioned in the text. Genes are written in italic capitals, whereas proteins in non-italic capitals.

ARF	alternative reading frame
ATP	adenine tri-phosphate
Bp	base pair
CCND1	cyclin D1
CCND3	cyclin D3
CCNE1	cyclin E
CDK2, 4, 6	cyclin dependent kinase 2, 4, 6
CDKN1A	cyclin dependent kinase inhibitor 1A ($p21^{CIP1}$)
CDKN1B	cyclin dependent kinase inhibitor 1B ($p27^{KIP1}$)
CDKN1C	cyclin dependent kinase inhibitor 1C ($p57^{KIP2}$)
CDKN2A	cyclin dependent kinase inhibitor 2A ($p16^{INK4A}$)
	or the alternative reading frame ($p14^{ARF}$)
CDKN2C	cyclin dependent kinase inhibitor 2C ($p18^{INK4C}$)
CGH	comparative genomic hybridization
CIN	chromosome instability
DNA	deoxyribonucleic acid
E2F	E2F transcription factor
ERRBB2	erythroblastic leukemia viral oncogene homolog 2 (HER2)
FISH	fluorescence *in situ* hybridization
G1	gap phase 1
G2	gap phase 2
GAP	GTPase activating protein
GDP	deoxyguanosine diphosphate
GEF	guanine exchange factor
GTP	deoxyguanosine trisphosphate
kb	kilo base
kDa	kilo Dalton
LOH	loss of heterozygosity
MDM2	murine double minute-2 protein

M phase	mitotic phase
MPNST	malignant peripheral nerve sheath tumor
mRNA	messenger RNA
NF1	the disorder Neurofibromatosis type 1, or the protein neurofibromin (in order to distinguish between these two the protein will be designated neurofibromin).
RAS	rat sarcoma virus
RB	retinoblastoma
RNA	ribonucleic acid
R point	restriction point
S phase	synthesis phase
TGF-β	transforming growth factor β
TMA	tissue micro array
TSG	tumor suppressor gene
TP53	tumor protein 53
UV	ultraviolet

Summary

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant inherited disorders, and carriers are at greatly increased risk of developing malignant peripheral nerve sheath tumours (MPNST). The NF1 patients are carriers of a germline mutation in the NF1 gene, which encodes the protein neurofibromin. This protein is in normal cells responsible for the deactivation of RAS through GTP-hydrolysis and inactivation of the gene will lead to hyperstimulation of the MAP-kinase pathway. However, inactivations of both NF1 alleles are also found in the benign precursor lesions, neurofibromas, showing that additional genetic events are necessary for malignant transformation.

MPNSTs usually have complex karyotypes showing numerous chromosomal aberrations. We have previously shown recurrent copy number gains and losses, including frequent loss of 9p and 13q, and gain of 17q. The target gene for the 9p losses is the CDKN2A and for the 17q gain there is evidence for *TOP2A* as one target gene. The target gene(s) for the 13q losses remains unknown, although the *RB1* is a possible candidate.

In the present study we have analyzed topoisomerase II α, TOP2A and other chromosome 17 probes in a series of MPNSTs and neurofibromas (n=32) by metaphase- and inter-phase fluorescence in situ hybridization, FISH. Excess of TOP2A signals, relative to the centromere signals of the same chromosome, was found in 12 tumors. This support that gene amplification of TOP2A partly explain the previously observed increased expression of TOP2A in this type of tumors. The fact that TOP2A is the molecular target of several well-established chemotherapeutic agents, and that no consensus for therapy, except surgery, exists, underline the importance of these findings. In search for an additional and more distal amplicon at 17q we first analyzed distal loci by interphase FISH and, indeed we found amplification in four MPNST with normal copy number of the 17 centromere and TOP2A. By a global genomic approach, so called COBRA-FISH, we thereafter identified four MPNST with chromosomal breakpoints involving 17q. This pinpointed the region of interest for further FISH studies using BAC probes in order to identify the gene(s) targets.

The second part of this study focused on in situ expression taking advantage of tissue microarray (TMA) as a tool for analyses of a large clinical series.

Previous studies have suggested that some central cell cycle components, in particular deficient p16, contribute to the development of MPNST. A selected set (n=11) of cell cycle components and proliferation markers (n=2) as well as the neurofibromin protein were analyzed by immunohistochemistry on a TMA with tissue cores from 106 MPNSTs and 3 neurofibromas. The thirteen 5um parallel sections, hybridized with the individual antibodies, were each scanned with high resolution before visual scoring was done from the digital images. The expression data revealed that

topoisomerase IIα (TOP2A) and Ki-67 are indeed expressed in most MPNSTs. Fewer tumors than expected had positive score of tumor protein 53 (TP53). Generally, expression of cyclins and CDKs were common among MPNSTs, leading to phosphorylated retinoblastoma (RB1) and G_1/S transition. The cyclin-CDK-complex inhibitors were on the other side absent from most MPNSTs, confirming the suspicion of lost control of the cell cycle checkpoint at G_1-phase.

MPNST is a highly aggressive cancer disease for which no standard therapy, except surgery, exists. A molecular classification of these tumors in combination with the clinical and pathological evaluations may aid in improved treatment of these patients. The current study has added new knowledge to the understanding of the molecular biology of MPNST.

Preface – a chain of coincidence?

Even today we're not fully aware of our origin. Throughout evolution the last 2-3 billions years, we have evolved via many different species. But how the first cell did come to life is still a mystery. It can be hard to comprehend that life is just a process of random consequence, when life is so complex. Even bacteria, the smallest form of life, have thousands of genes and proteins to control at any time. Take for instance the naturally existing amino acids. Human proteins consist of 20 different amino acids. All of these are left-handed amino acids (L-enantiomers). In fact, almost all life on Earth has come to use left-handed amino acids exclusively, rather than their mirror-image, the right-handed forms (Figure 1). Could this be a coincidence through evolution, or are we created this way? Some new evidence support evolution. Scientists working on a meteorite discovered that it contained an excess of left-handed amino acids. It seems like some process is favoring L-enantiomers, and that this has been going on even before the origin of life on earth, and probably before the formation of our Solar System[1].

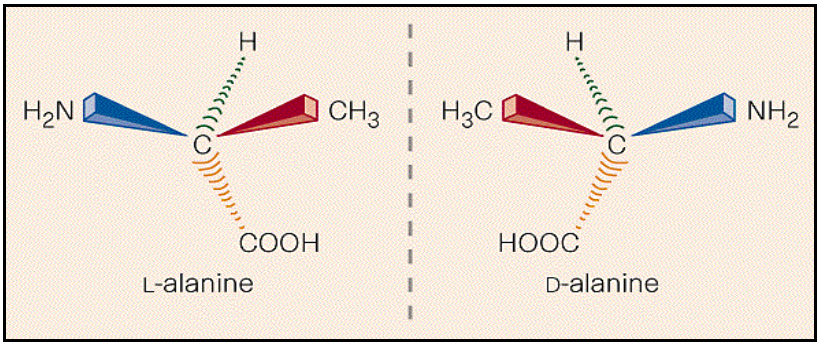

Figure 1. Left-and right-handed versions of the amino acid alanine. A meteoritic excess of L-alanine has been shown not to be from terrestrial contamination, so life's chemical left-handedness may be extraterrestrial.

Introduction

Cancer and evolution

A single celled organism has to "fight" with other organisms in its habitat to survive. Only organisms that are able to feed and propagate well will continue to exist. If the environment is changing, for example to a colder climate, the organism has to be able to comprehend these new surroundings, or to change its habitat. If it also can multiply and reproduce itself into new organisms having the same qualities as it self, it will be a winner through the eyes of evolution. A cancerous cell has these qualities, and becomes a threat to the multicellular individual as it has selective advantages over normal cells. If there is a shortage of nourishment, the cell can adapt new ways of getting more than its neighboring competing cells. This can be done through growth of new blood vessels that cancerous cells actually can guide toward them in a process called angiogenesis. With this single improvement the cell is now able to multiply faster than the normal cells, and is already on its way to generate a tumor (Figure 2).
Key behaviors of a cancer cell were reviewed by Hanahan & Weinberg[5] and are discussed on page 12.

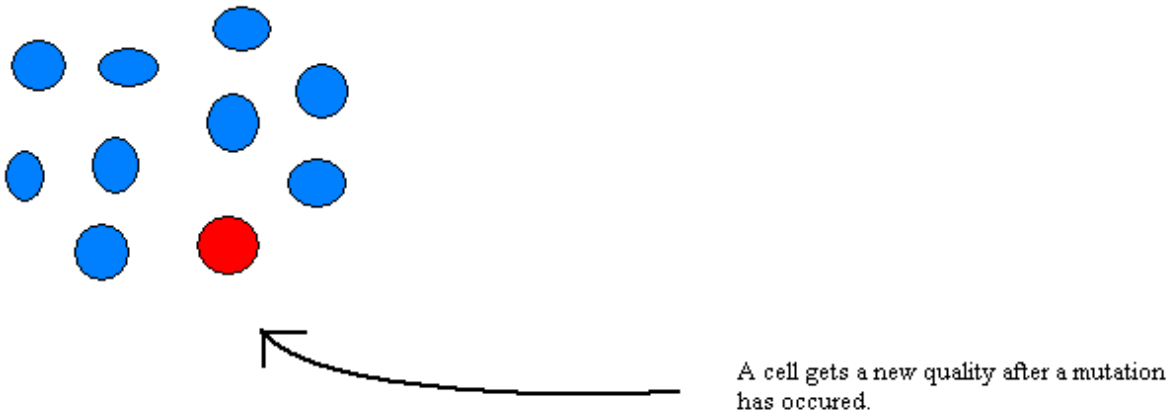

Figure 2. Mutation and evolution. The red cell has by chance evolved a new beneficial quality, and has now a higher probability of dividing and multiply into many new cells, compared with the normal blue cells.

Mutations

The evolutionary record consists of a long series of mutations. It can be hard to imagine that a random chain of coincidence can give a favorable quality. Usually a mutation leads to a disadvantage for the organism, often with a deadly outcome. If an organism gets a mutation that has a drawback, the chances are that it won't produce offspring, and the mutation will die out with the organism. Once in a while a cell will also get a beneficial mutation, which gives it an advantage that no other cell have,

analogous to Darwinian evolution. Through time this has lead a single-cellular organism to evolve to a multi-cellular organism, and has eventually also led to the creation of man. And also, in a stepwise manner, a normal cell can develop into a vicious cancerous cell (Figure 3), that will invade the entire body, with an unfortunate result for the person involved.

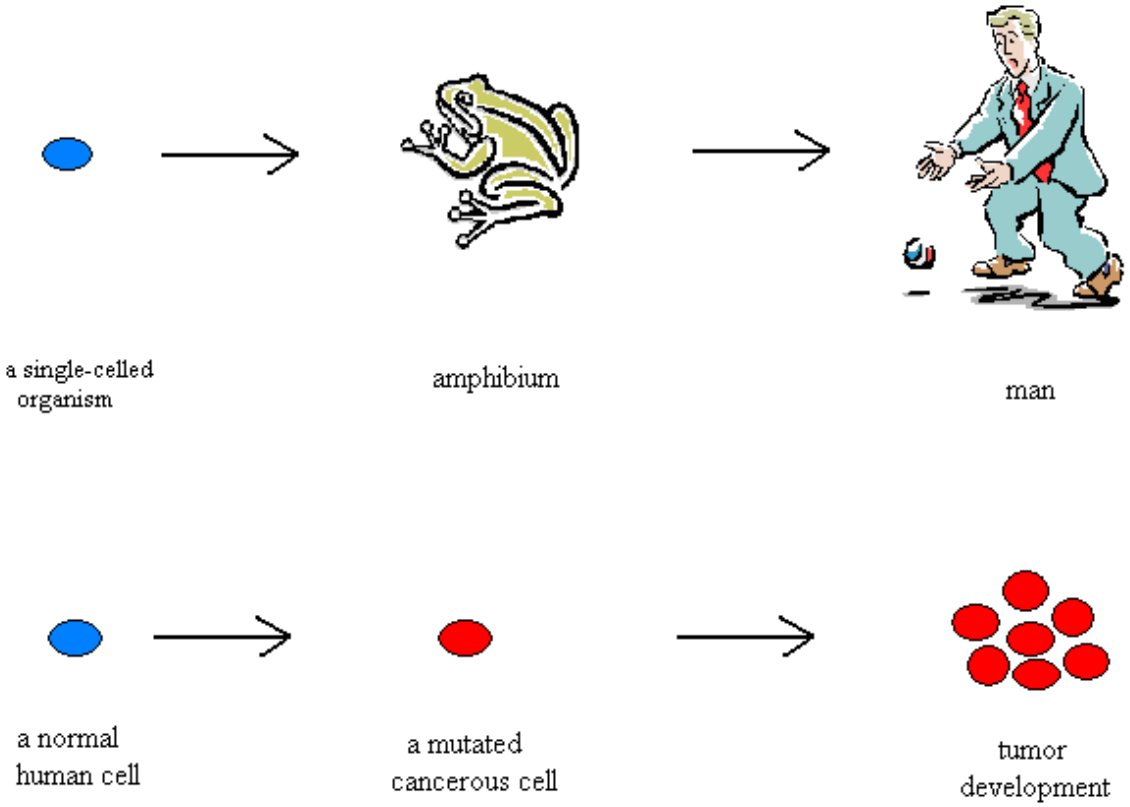

Figure 3. Tumor development. Through a long process man has evolved from a single-celled organism. Tumor development also involves several steps that occur through a period of time, but at another time schedule.

History of cancer

The process of understanding cancer did a leap forward in 1890 when David von Hansemann, a German pathologist discovered that the material he was working on had irregularities compared with normal tissue. He was studying biopsies from several neoplasms, and found that the nuclei often had different shapes, and that the density of them was higher than normal. He also discovered that the mitotic pattern was abnormal in a high number of the cells.

This eventually led to the somatic mutation theory of cancer, which was presented in 1914 by Theodor Boveri in his famous book "*Zur Frage der Entstehung maligner Tumoren*". He meant that cancer is the result of mutations in one cell, and the development of a neoplasm comes from this cell and its daughter cells.

During the next 30-40 years many new approaches were invented, and for example the use of model organisms helped the scientists to great discoveries. For instance has the work on plants (*e.g.*

Arabidopsis thaliana), yeast (*e.g. Saccharomyces cerevisiae*), insects (*e.g. Drosophila melanogaster*), nematodes (*e.g. Caenorhabditis elegans*), and mice (*e.g. Mus musculus*) given us clues on how the genetic material is expressed also during the development of humans. From the 1950s some of the new techniques were used to examine human cells, and a new way of treating cells made it possible to continuously grow new clones, with the exact same genetic material as the original cell (the stem line concept, first defined by Winge (1930)). These include human cancer cells. Soon the benefit of using colchicin, a substance that arrests cells in mitosis by breaking down the spindle apparatus, together with the knowledge that hypotonic salt solution gave good metaphase spreads, resulted in individual chromosomes could be counted and analyzed. All this led to the finding of the first chromosome abnormality in a human cancer. Nowell & Hungerford detected in 1960 a small karyotypic marker in patients with chronic myeloid leukemia. The same error in breakpoint location between two chromosomes was repeatedly seen in cells of many human individuals. Having this mutation the effect always led to the development of leukemia. Nowell & Hungerford named this specific karyotype for the Philadelphia (Ph^1) chromosome. This discovery proved that Boveri's idea from 1914 was correct. It is today a dogma that cancer does start with mutations in one cell (Figure 4). A relationship between the type of cancer, and the often chaotic pattern of human tumor karyotype, was established by Levan and van Steenis in 1966, and then again by Mitelman in 1974. Different chromosomes and chromosome regions are involved in various neoplasms, and specific abnormalities are found to be general for different cancer types and diseases.

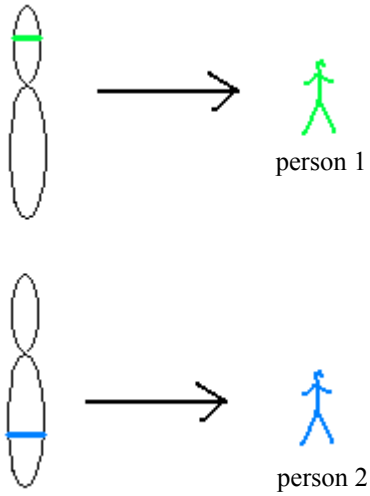

Figure 4. Two specific mutations (green and blue) are pathognomonic for two completely different cancer types.

Cell Cycle

It is through regulation of the processes of cell cycling and apoptosis that a multicellular organism regulates the total number of cells it retains. After the developing epoch is over, the rate of proliferation slows down. But a single cell does have a much shorter life span than its host, so new genetically identical daughter cells are formed to keep up with the right amount of cells required.

A cell is divided into two during the M phase (mitosis). The time between mitosis is called the interphase. During this phase the cell prepares it self to be divided again in the next M phase. It gains weight and copies its DNA. The interphase is divided into G_1 phase (growth), S phase (synthesize) and G_2 phase, where these actions take place.

The progression of the cell cycle is regulated by extracellular signals as well as by internal signals that coordinate the various processes that take place during the different cell cycle phases. Growth factors for instance may act as extracellular signals that tell the cell to continue proliferation, whereas processes such as DNA replication and mitosis are carefully monitored by internal signals. To avoid conflicts of these signals, a series of control points in the cell cycle is secured through evolution. For example, it is critically important that the cell don't begin mitosis until after the genome is replicated. The most important regulatory point is late in G_1 and controls progression from G_1 to S. Before crossing this restriction point (R-point), the cell may be in an arrested state called G_0, where the cell is waiting for external signals for further activity. The cell can remain in G_0 phase for a long period of time without proliferating. Leaving this phase is the start of a new cell division, which is irreversible, even in the absence of further growth factor stimulation.

Other cell cycle checkpoints have more precise tasks. To ensure that incomplete or damaged chromosomes are not replicated and passed on to daughter cells, there are additional control points throughout the cell cycle. In the G_1 checkpoint, DNA damage is allowed to be repaired. Arrest at the G_1 checkpoint is mediated by the action of a key protein known as TP53, which is rapidly induced in response to damaged DNA. *TP53* is the most often mutated gene in human cancers, and loss of function of TP53 may lead to daughter cells with damaged DNA. These cells have an increased frequency of mutations and general instability of the cellular genome, which contributes to cancer development. Germline mutations of *TP53* cause the Li-Fraumeni syndrome and patients with this disease may develop various kinds of cancers. Somatic mutations are known to be present in more than half of all cancers and are often associated to poor disease outcome. If *TP53* or other central tumor suppressor genes (such as cyclin dependent kinase inhibitors) of the cell cycle are non-functioning, the cycle may enter too early into the next phase. Also protooncogenes, that normally

stimulate the cell cycle, may be mutated in cancer and thereby act as oncogenes (such as the cyclins and cyclin dependent kinases, Figure 5). This may lead to a continuous stimulation of the cell cycle and potentially to cancer development.

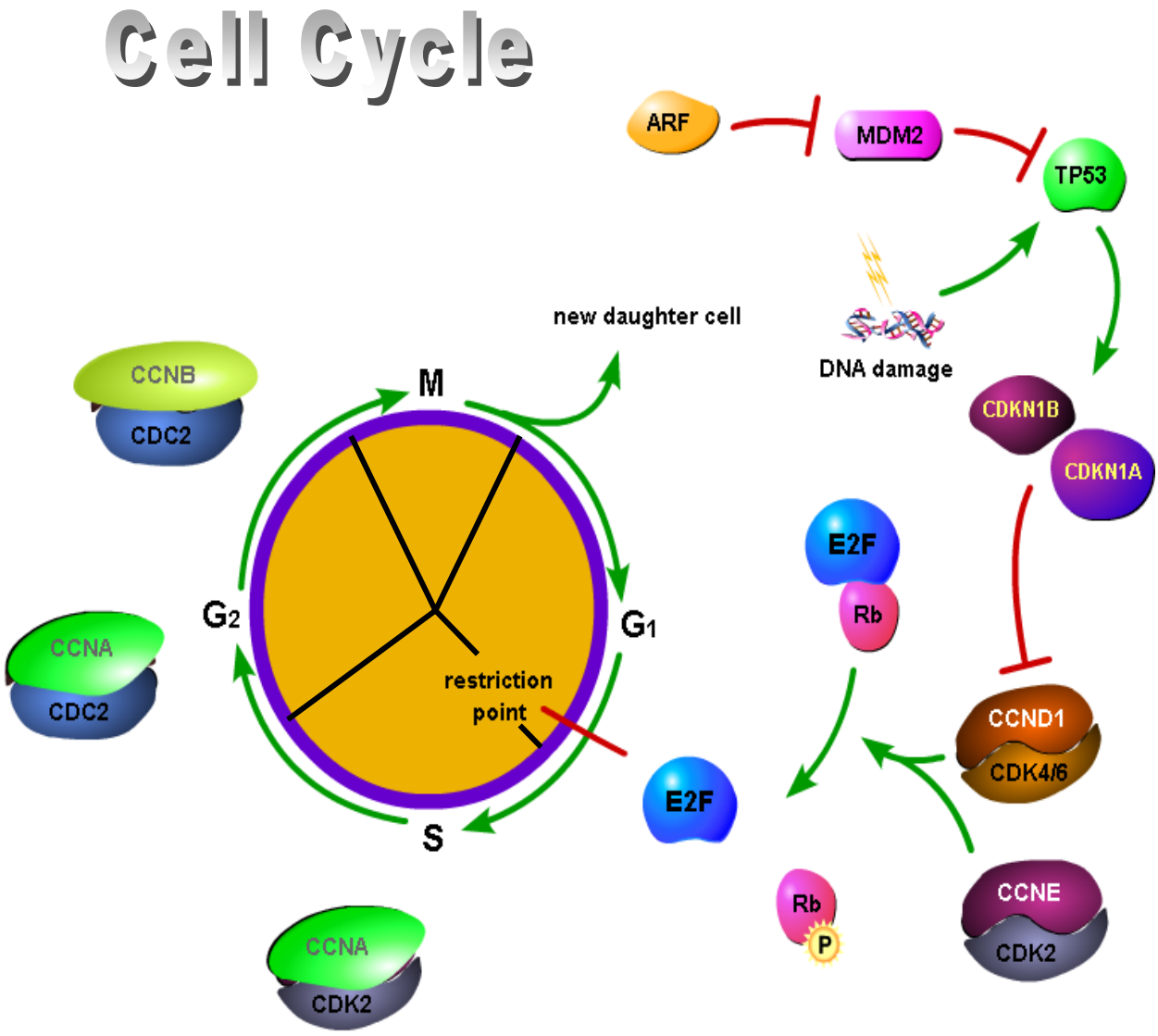

Figure 5. The cell cycle is regulated by intra- as well as extracellular signals that monitor and coordinate the various processes that take place during different cell cycle phases. A major cell cycle regulatory point occurs late in G_1 and controls progression from G_1 to S. Once cells have passed this restriction point, they are committed to entering S phase and go through one cell division cycle. Key regulators to the entrance of the various phases are the CDKs, which only functions in complex with the correct cyclin. A set or phosphorylations and dephosphorylation events, as well as protein degradation processes, further control the action of these complexes. CDKN1A and CDKN1B are some of the CDK-inhibitors that exist. They negatively control the cell cycle by reducing the catalytic activity of CDK-cyclin complexes.

The G_2 checkpoint ensures that the DNA is properly replicated before the cell enters the M phase. In this manner its ensured that incompletely replicated DNA is not distributed to daughter cells. Equally important is making sure that the genome is replicated only once per cell cycle. Therefore control mechanisms exist to prevent initiation of another round of DNA replication after DNA has been replicated once[2]. This way the mother cell is divided into two equally equipped daughter cells.

The key behaviors of cancer cells

What are the steps that will transform a normal cell into a cancerous one? The capability that a single cell has to evolve on a micro-evolutional scale involves dynamic changes in the genome. Over time it has to pass through different obstacles that are necessary to develop malignant cancer. Cytogenetic studies have demonstrated that in many primary tumors all the cells have the same abnormal karyotype, suggesting a unicellular origin, the so called monoclonality theory[3]. This means that when a mutation is beneficial for a cancerous cell, all its offspring will inherit this quality. The regulatory machinery that helps a normal cell to live "peacefully" in harmony with the rest of the human cells is disrupted. The homeostasis within the cell is put out of order, and the cancerous cell is evolving into a more self-centered cell. It no longer lives by the rules that are necessary for a multi cell organism to function. Hanahan and Weinberg[5] pinpointed several physiological features that characterize a cancer cell, shortly described in the following.

1.Growth-inhibitory signals

To become a cancerous cell the normal cell has to ignore antigrowth signals from the other cells nearby. In a healthy multicellular organism, limited growth and proliferation is important to establish a dynamic working "community" of cells. The components involved in the regulation of cell cycle clock normally keep the cell under control, but when mutated, cells will overlook these growth-inhibitory signals and soon outnumber the normal cells in the area. If the retinoblastoma protein that blocks E2F transcription factor is mutated in such a way that it is kept from doing this, E2F will continuously transcribe genes stimulating the cells to premature entry into S phase[4].

2.Growth signals

Not only does a cancerous cell have to ignore inhibitory signals, it also has to make its own growth stimulatory signals. A normal cell is dependent on a continually flow of growth signals from the tissue

around. One type of cell is telling another how to react at a certain time. This is the way cells communicate. When this communication is broken and a cell starts to make its own growth signals, it stimulates itself to proliferate. This is called autocrine stimulation. In about 25% of human tumors, the SOS-Ras-Raf-MAPK pathway is altered and put out of balance, leading to massive growth[5,6].

3.Apoptosis

Even if cells achieve the requirements for continuous growth, they will eventually go into apoptosis. This is a program multicellular organisms have to eliminate cells to maintain a proper number. Apoptosis is also initiated if a cell has unrepairable damaged DNA. It will then be dangerous for the rest of the organism to let the cell continue to proliferate with severe DNA changes. Apoptosis is regulated by signals from the extracellular and intracellular environment. If any abnormalities are discovered the program will kill the cell. This is done by breaking up the cell membrane and degradation of the chromosomes.

TP53 controls apoptosis. In response to upregulated expression of the BAX protein, the mitochondria will release cytochrome C. Within 30-120 minutes the nucleus and the rest of the cell will be fragmented, and the cell will die. All the parts are absorbed by neighboring cells by controlled autodigestion[7].

In more than 50% of all human cancer *TP53* is mutated leading to an inactive TP53 protein[8]. Cells that normally would go into apoptosis will carry on proliferating, even if the genetic material is heavily disrupted. With these conditions a cancerous cell can rapidly evolve.

4.Senescence

Cells will eventually stop dividing. After a specific number of multiplications, a program that is different from apoptosis and independent from extracellular signals will turn the cell off and stop the chance of further proliferation. This is called senescence, and is controlled by the shortening of the telomeres. Under each cell cycle 50-100 bp of DNA are lost from the chromosome ends. Because loss of genetic material in this way would lead to devastating damages over time, an enzyme is set to produce new nucleotides in the ends of the chromosomes. In cancer cells expression of this enzyme, called telomerase, is up-regulated in 85-90%[9] This gives the cancer cell the ability of limitless replication, in contrast to normal human cells that only have the capacity for 60-70 divisions[7].

5.Angiogenesis

Nutrition is crucial for survival. When a tumor is developing rapidly, the cells in the center will be further and further away from blood vessel. Without blood in the proximity, there will be no new supplies of nutrients and oxygen, and expansion is impossible.

A new tissue is formed together with the growth of new blood vessels; a carefully regulated process called angiogenesis. If a tumor is to grow larger than in an early stage, it must acquire angiogenic capabilities.

6.Metastasis

The final stage for a cancer cell is to spread. This is the ability to invade new tissue and to continue to grow there. A benign cell can only keep growing at the same place in the tissue where it originally developed. Treatment for this kind of tumor is done by surgical removal. When a tumor becomes metastatic the chances of survival drop dramatically. The new colonies of cancer cells can in principle settle down anywhere and develop into new tumors. These metastases are responsible for 90% of all human cancer deaths[10]. The molecular mechanisms are not well known, but clearly involve proteins that have to do with cell-cell adhesion, like integrins. The potential for new treatments based on molecular targeting is huge, exemplified with the successful treatment of chronic myelogenic leukemia and gastrointestinal stromal tumors with Gleevec[78].

Cancer, age, inheritance and environment

Mutations accumulate over time, and cancer therefore may develop over a lifetime. Humans continue to extend the lifespan as a consequence of scientific progress. If we are to live to the age of 120-140 years old, cancer will be even more frequent late in our lifetime because there are more time for mutations too occur.

For heritable cancer types the schedules are a different. Approximately 5% of all cancers come from this category. Because these individuals are already born with a mutation that's either passed on from a parent or a new mutation acquired in the germline, cancer may develop much earlier[11]. The retinoblastoma gene (*RB1*) can be found mutated among family members, who have inherited a mutation in one of the two alleles through the germline. If the remaining wild type *RB1* allele is mutated, the cell lose of RB1 function completely. This is known as the "two-hit" mutation theory and was described by Knudson in 1971[13].

Environmental factors can influence the probability of evolving some types of cancers. For instance is tobacco smoke a well-known carcinogen, together with asbestos, radon, outdoor air pollution or exposure to radiation. Today, there is overwhelming evidence that tobacco smoking plays a major role in the epidemiology of lung cancer, cancers at other sites, and a variety of chronic degenerative diseases[14,15]. In a more specific sense, however, environmental factors include only the (natural or man-made) agents encountered by humans in their daily life, upon which they have no or limited personal control. The most important environmental exposures in this strict sense include outdoor and indoor air pollution and soil and drinking water contamination. These incidences are estimated to count for near 2% of all cancer records. Exposure is involuntary but can to a large extent be avoided[16].

Neurofibromatosis type 1

Neurofibromatosis type 1 was first defined by Friedrich von Recklinghausen in 1882. In 1918, Presier and Davenport demonstrated that neurofibromatosis type 1 was an inherited disorder and it is today known as one of the most common autosomal dominant disorders in humans with an incidence of approximately 1 in 4000[72]. Patients with this illness have a germline mutation in the gene *NF1*[17]. In half of the cases the mutation is inherited from one of the parents, but in the remaining half the mutation is new and has evolved through the germline. The patients are heterozygote for the mutation, and any somatic cell will become homozygote with a new mutation in the remaining wild-type allele. The *NF1* gene encodes a protein called neurofibromin, and is expressed in the Schwann cells in the nervous system[18,19]. Schwann cells make the layers that wraps around the peripheral nerves, protecting and isolating the axons. Neurofibromin is also expressed in other tissues, but not as much as in Schwann cells. The expression is particularly high early in the development of the peripheral nerves. *NF1* is located within the chromosome band 17q11.2, and spans 350kb DNA. It contains 60 exons that transcribe an mRNA product of 11-13 kb[20]. Neurofibromin has the properties of a GTPase activating protein (GAP), and therefore plays an important roll in regulating RAS in the MAP-kinase pathway. RAS is an oncogene, and if hyper-stimulated, it will cause the MAP-kinase to produce ectopic amounts of transcription factors downstream. A cell that is under continuous influence of proliferating stimuli will eventually lose control of the cell cycle may build a tumor. Neurofibromin's role in this pathway is to regulate the activity of RAS by converting the active RAS-GTP into the inactive RAS-GDP[21-23] (Figure 6). This activity is reduced in patients with the neurofibromatosis type 1 disease when *NF1* is mutated or if the expression of neurofibromin downregulated. RAS-GTP will stay constitutively activated with the GTPase activity of neurofibromin missing.

NF1 is one of many tumor suppressor genes identified during the last two decades. Many tumor suppressors are key regulators in signaling pathways, but are only relevant in specific tissues. So non-functioning neurofibromin in Schwann cells are extremely critical, and these are the only cell type where the disease develops. However, inactive *NF1* have also been found in other cancer types[73,74,75].

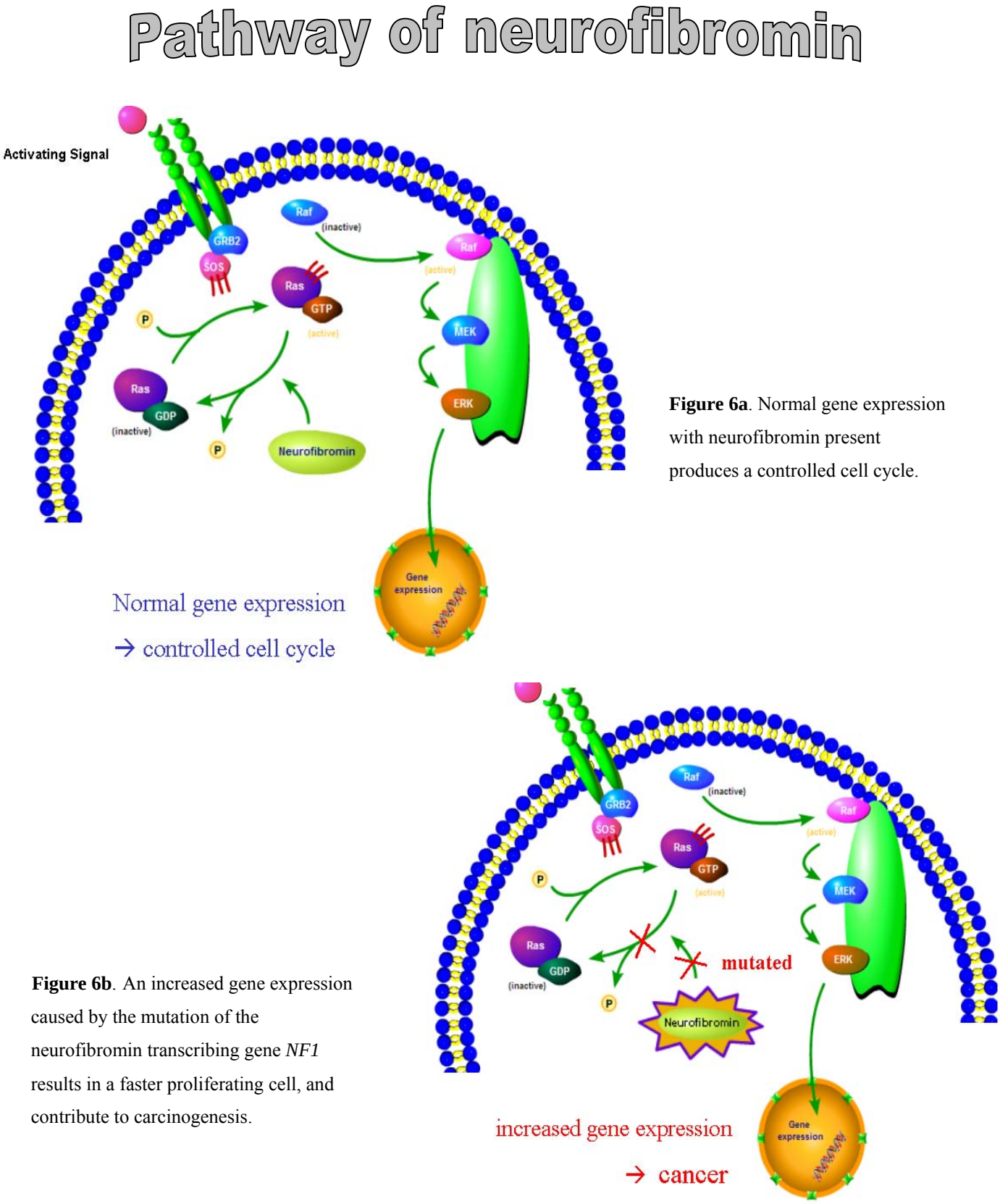

Figure 6a. Normal gene expression with neurofibromin present produces a controlled cell cycle.

Figure 6b. An increased gene expression caused by the mutation of the neurofibromin transcribing gene *NF1* results in a faster proliferating cell, and contribute to carcinogenesis.

The diagnostics of neurofibromatosis type 1 is complicated, but some of the characteristic phenotypic features are plexiform neurofibromas, *café au lait* spots, Lisch nodules, and distinctive bony lesions[24] (Figure 7). Seizures, headache and learning disabilities are also common complications for NF1

patients[25]. The phenotypes can be highly variable even among family members that are carrying the same mutation in the *NF1* gene. Symptoms do in general seem to increase in severity and number over time[26,27].

Figure 7. Some of the characteristic phenotypic features of neurofibromas.

The NF1 patients also have an increased risk for developing other cancer types, but in particular, they have a greatly increased risk compared to the general population in developing malignant peripheral nerve sheath tumor (MPNST).

MPNST – malignant peripheral nerve sheath tumor

About 5% of the NF1 patients develop MPNST[28] (Figure 8). In general it is a rare malignancy, with an incidence of 1 in every 100 000. Therefore, individuals with NF1 account for 50% of all patients with MPNST. The other 50% are sporadic cases. They develop MPNST when they are 20-80 years old, while NF1 patients usually develops MPNST at relatively young ages (15-45 years of age)[29,30].

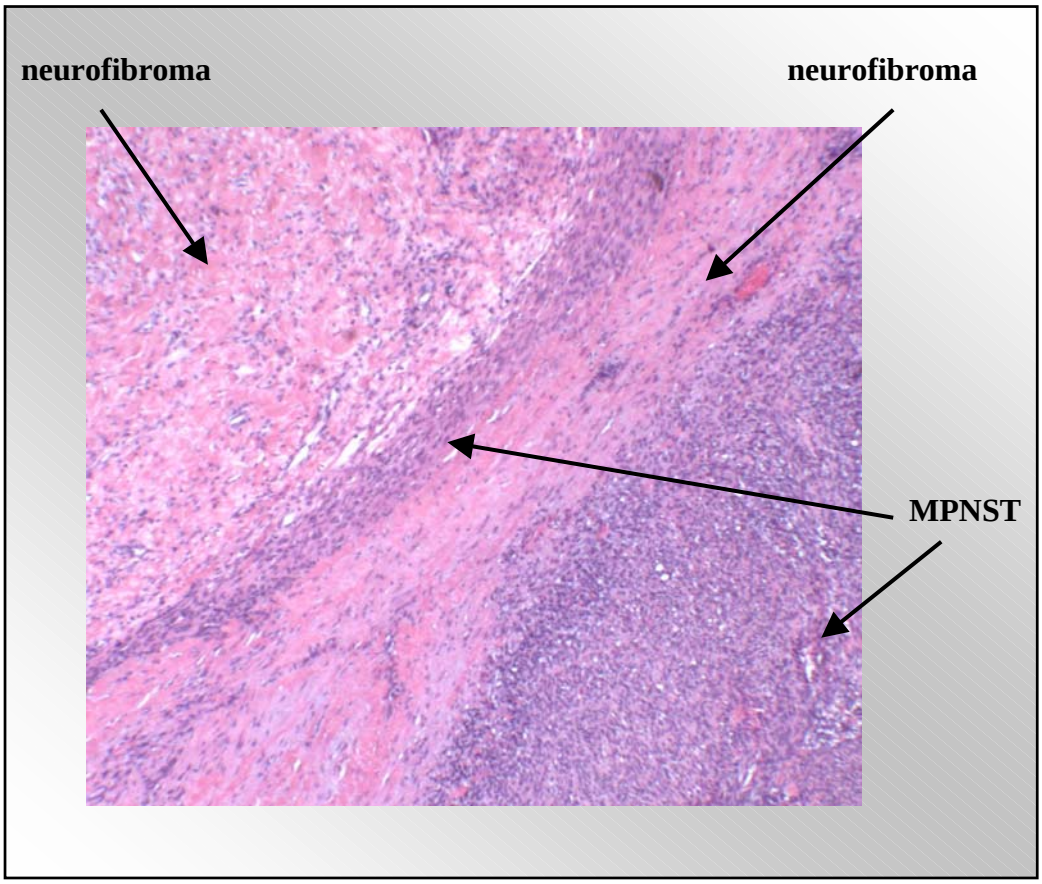

Figure 8. A tumor that consist of a benign part, neurofibroma, and a malignant part, MPNST. A typical pattern in MPNST is the overwhelming amount of nuclei.

At least two thirds of the MPNSTs develop from precursor lesions, the benign neurofibromas, and in particular from the plexiformed, or in rare cases from the dermal subtype of neurofibromas. These lesions contain several cell types, and evidence suggests that the MPNST develop from the Schwann cells. MPNSTs are of neuroectodermal origin (see below) but are classified as under soft tissue sarcomas. This is mainly due to the fact that many of the same complications as seen for soft tissue sarcomas are evident for MPNST patients. Also, many of MPNSTs develop in the extremities. Today, no consensus for treatment exists for this highly aggressive disease

.

Peripheral nerve development

The nervous system develops from the ectoderm. Here, two sets of cells derive - those of the *neural tube,* which forms the central nervous system (the spinal cord and brain, including the retina of the eye), and those of the *neural crest* which gives rise to most of the neurons and supporting cells of the peripheral nervous system[31]. Axons conduct signals from the cell body, while the multiple dendrites receive signals of other neurons and connect cells through synapses. Nerve fibers are usually found grouped together in tight parallel bundles called fascicles invested by a perineurium. Most peripheral nerve neoplasms are assumed to arise from cells of neuroectodermal origin, the Schwann cells.

Schwann cells wrap its plasma membrane around the axon to form a segment of myelin sheath about 1 mm long (Figure 9).

But to cover the possibility that some of the tumors arise from nerve sheath cells other than the Schwann cell, the nonspecific term of MPNST is preferred over malignant schwannoma. Secondly, this also avoids the potential misunderstanding that MPNST arise from benign schwannomas that in reality is extraordinary rare.

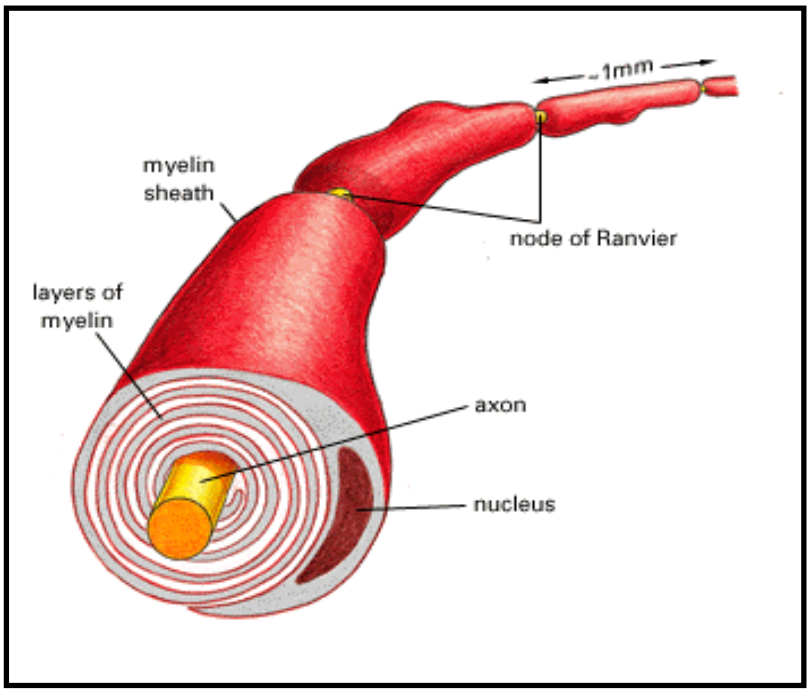

Figure 9. A nerve cell. Schwann cells make up a myelin sheath around the nerve axon by wrapping its plasma membrane (taken from *Ion Channels and the Electrical Properties of Membranes*, the Cell, Fourth Edition 2001).

Indications from clinical behaviors of these two neurofibromas show that plexiform neurofibromas are probably derived from embryonic Schwann-cell lineage, whereas dermal neurofibromas can arise from more mature Schwann-cell lineage [36,37] (figure 10).

Neurofibroma is a heterogeneous tumor that contains every cell type that is present in normal peripheral nerves. These are Schwann cells, fibroblasts, perineural cells, neuronal processes, and mast cells. Schwannoma on the other hand is a homogeneous tumor that is composed of Schwann cells. Both tumors are benign and belong to WHO grade I. MPNSTs on the other hand are grade III-IV [32,38] and have a 5 year survival rate of 34-52%[36].

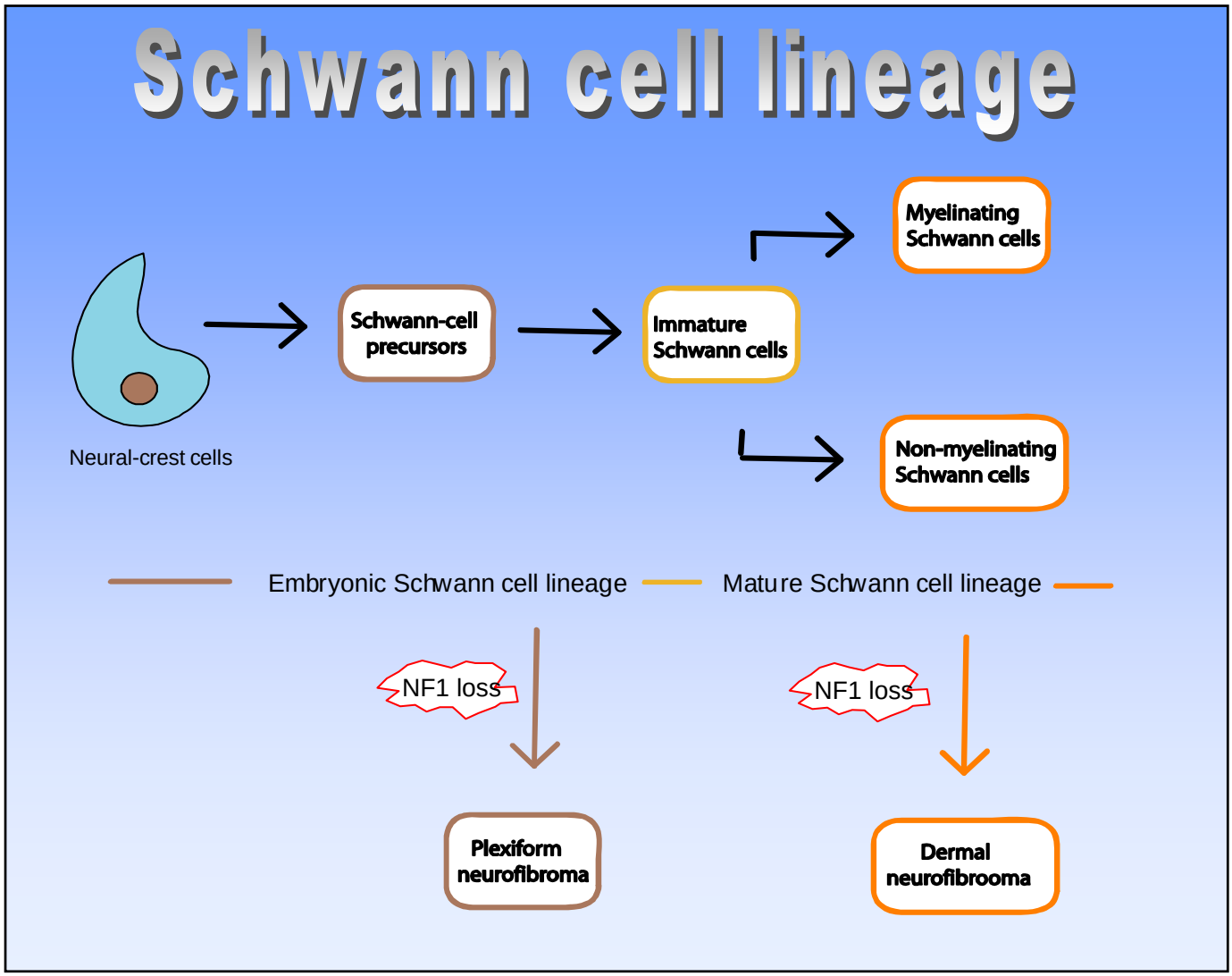

Figure 10. The evolvement of Schwann cells takes place through different stages before becoming mature cells. The type of tumor that forms depends of when the *NF1* mutation occurs. If it is early in the development, the result seems to be more critical for the patient. Modified after Zhu,Y *et al.*[36].

The genetics of MPNST

A loss or a second-hit-mutation in remaining wild type allele of *NF1* can already have happened in the development of benign neurofibromas, before MPNST is fully evolved. Some studies are showing that complete inactivation of *NF1* has been found in benign plexiform neurofibromas[39,40,41,42,43]. This means that inactivation of *NF1* is not sufficient for the development of MPNST.

Some other candidate genes for malignant transformation have been suggested, among these the *TP53*. Previous studies in mice have shown that compound heterozygous $NF1^{+/-}$ $TP53^{+/-}$ mice develop MPNST[44,45]. Also in the human setting, mutations of *TP53* have been found in some MPNST[46,47,48,49], but biallelic inactivation is rare[50]. Large gene rearrangements or deletions of the *CDKN2A* gene, that

encodes INK4A and ARF, are frequent in MPNSTs, but is not present in neurofibromas[51,52,76,77]. ARF stabilizes TP53 by antagonizing the MDM2-mediated degradation[53]. Loss of ARF function therefore supports the idea that TP53-mediated pathways are important for the progression of MPNSTs.
In addition loss of expression for KIP, a component in the RB1-pathway, has been identified in most MPNSTs (91%), compared with only 6% of neurofibromas[54], indicating that the RB1-mediated G_1/S-checkpoint pathway might be important in the tumor progression.

The genomes of MPNST are typically complex as shown by G-banding analyses[56] (see below, and Table 1) and by fluorescence *in situ* hybridization techniques (see below)[58,68,69,70]. No pathognomonic markers have been identified but several recurrent aberrations are detected, possibly pinpointing target genes. In fact, common deletions of 9p21, initially shown by Lothe *et al.*, 1996), strongly suggested *CDKN2A* as a target gene. Berner *et al.*, confirmed this hypothesis in 1999. A CGH study also detected frequent gains of 17q sequences and showed recently that this may reflect an amplification of *TOP2A*, a known target for several chemotherapeutica (Skotheim *et al.*, 2003).

Karyotype nomenclature and terminology

Karyotypes in general are interpreted and described according to an international standard cytogenetic nomenclature[71].

In "Mitelman's Database of Chromosome Aberrations in Cancer"[79] 45000 cytogenetically abnormal neoplasms are gathered. Here, the standard cytogenetic nomenclature is used, which will be described in the following. The autosomal chromosomes are classified according to size where the longest and largest chromosome is given the number 1, the second largest number 2, and so on. The sex chromosomes are called X and Y. Each chromosome has to arms, separated with the location of the centromere. The shortest of them is named p, the longest arm q. These arms are then divided into smaller regions, depending on specific landmarks. They include the ends of chromosome arms (the telomeres), the centromere, and the characteristic bands that appear either dark or light after staining with one or more of the different banding techniques. Each region is given a number, which starts from the centromere and outward along each chromosome arm. This way the two regions next to the centromere are both numbered 1, then the next region numbered 2, and so on. A band used as a landmark is by definition belonging entirely to the region distal to the landmark.

So if a specific band is to be named, four items are required;

- 1. the chromosome number,
- 2. the arm symbol p or q,
- 3. the region number, and
- 4. the band number within that region.

For example, 9q34 means chromosome 9, the long arm, region 3, band 4.

Under prometaphase or early metaphase the chromosomal contractions are not as condensed as later in metaphase. Thus the banding pattern here is more complex, as several of the normal bands in midmetaphase are split into sub-bands. This gives higher resolution or better structure banding, and smaller details can be observed. The name of this sub-band is numbered like midmetaphase bands from the centromere and out, and is placed after a decimal point behind the original band designation. For example, the original band 1q42 may be subdivided into three sub-bands, labeled 1q42.1, 1q42.2 and 1q42.3. If sub-bands are subdivided, additional digits, but no further punctuation, are used. For example: **1q42.12**

When describing a karyotype, the number of the total chromosomes is written first. In a normal cell the number is 46, but in cancerous cells the amount can vary quite a lot. After the number follows a

comma (,) and then the sex chromosome is given. A normal male karyotype is therefore written **46,XY**, or a normal female **46,XX**.

Structurally altered chromosomes are given either a single or a three-lettered abbreviation. The number of the chromosome or chromosomes that are changed is written within parentheses. Following the chromosome is the symbol that describes the rearrangement. If two or more chromosomes are altered, the rule is that the lowest chromosome number is stated first. If one of the sex chromosomes is rearranged, this shall be listed first. The breakpoints are given within their own parentheses, and are specified in the same order as the chromosomes involved. A semicolon (;) is used to separate them. Figure 11 shows some examples of chromosome aberrations.

- **Translocation, t**: this means that a chromosomal segment has moved from one chromosome to another, balanced or unbalanced. The translocation may or may not be reciprocal.
    - t(9;22)(q34;q11) describes two segments that has exchanged places. The distal part from chromosome 9q34 has moved to chromosome 22, where the distal part of 22q11 used to be. This segment is now located at chromosome 9, distal to the breakage at 9q34.

- **Insertion, ins**: a chromosomal segment has moved to a new, interstitial position in the same or another chromosome. The chromosome in which the segment is inserted is always specified first.
    - ins(5;2)(p14;q22q32) describes a breakage at band 5p14 in the short arm of chromosome 5 and that segment from 2q22 to 2q32 in the long arm of chromosome 2 has been inserted between the two parts of the 5p14 breakage.
    - ins(2)(q13p13p23) is an insertion into the same chromosome from the segment between bands 2p13 and 2p23 into the long arm of chromosome 2 at 2q13.

- **Inversion, inv**: This indicates an 180^{o} rotation of a chromosome segment. In the karyotype 46,XY,inv(3)(q26q29), breakage and reunion have occurred at bands 3q26 and 3q29. The segment between these bands is still present but in inverse orientation.

- **Deletion, del**: This means loss of a chromosome segment.
    - del(1)(q23) describes a terminal deletion with the break at band 1q23 and loss of the distal long arm segment. The remaining chromosome consists of the entire short arm of chromosome 1 and the part of the long arm that is between the centromere and band 1q23.
    - del(1)(q21q31) indicates an interstitial deletion with breakage and reunion at bands 1q21 and 1q31.

- **Duplication, dup**: An extra segment has been copied and inserted next to the original segment. The breakpoints describe the duplicated segment, e.g., dup(1)(q21q31).

- **Dicentric, dic**: Is a structurally abnormal chromosome with two centromeres.

- **Double minutes, dmin**: Chromosome fragments that are lacking a centromere are present in multiple copies.

- **Isochromosome, i**: Isochromosomes consist of arms that are mirror images of one another. They are a result of misdivision of the centromere.
    - i(5)(p10) is one example of an isochromosome for the short arm of chromosome 5.

- **Ring chromosome, r**: The name comes from the ring structure that appears when breaks occur in both the short and the long arms, and these two reunite with each other.

- **Marker chromosomes, mar**: Used to symbolize any structurally rearranged unknown chromosome. When the banding pattern is recognizable, however, the marker can be described by the standard nomenclature. The precise definition of a marker is a structurally abnormal chromosome in which no part can be identified.

- When **additional material of unknown origin** is attached to a chromosome region or band, this may be described by the term **add** before the breakpoint designation.
    - add(19)(p13) describes some extra material that has become attached to band 19p13, but the added segment is unknown.

- **Derivative chromosomes, der**: Indicates any structural rearrangements that involve more than one aberration. Can be within a single chromosome, or involve two or more chromosomes.

- **Plus (+) and minus (-)**: These signs are placed before a chromosome number to indicate that it is either an additional chromosome (+), or that the whole chromosome is missing (-). They can also be placed after a symbol to indicate an increase or decrease in the length of a chromosomal arm.
    - 47,XY,+21 is then a male karyotype with 47 chromosome, including an additional chromosome 21,
    - 21q+ indicates an increase in the length of the long arm of one chromosome 21, better described using the add symbol, i.e., add(21)(q?).

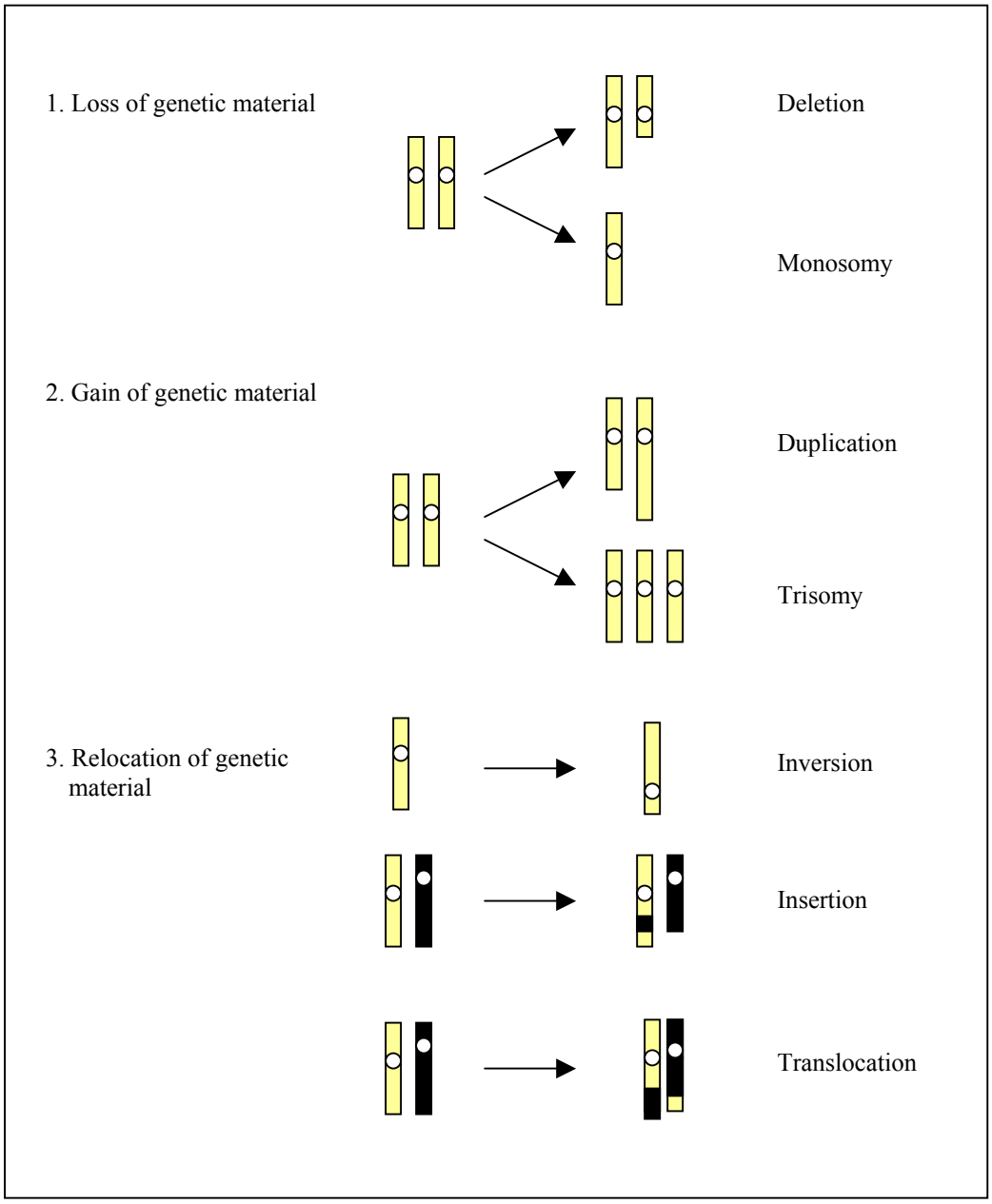

Figure 11. Chromosome alterations.

Aims of the study

The overall aim of the project is to understand the molecular biology behind development of MPNST and to transfer this knowledge into clinical utility.

MPNSTs usually have complex karyotypes showing numerous chromosomal aberrations. We have previously shown recurrent copy number gains and losses, including frequent loss of 9p and 13q, and gain of 17q. The target gene for the 9p losses is the CDKN2A and for the 17q gain there is evidence for *TOP2A* as one target gene. The target gene(s) for the 13q losses remains unknown, although the *RB1* is a possible candidate. Based on this knowledge the present study has two specific aims.

- ✓ One was the localization and identification of additional target genes important in the development of MPNST on chromosome arm 17q.

  [This was searched for by a combination of metaphase and interphase FISH, as well as by G-band karyotyping.]

- ✓ The second aim addressed the potential importance of dysregulated cell cycle components in MPNST development.

  [This was investigated by analyzing *in situ* protein expression of a set of central cell cycle components in a large series of MPNSTs and in benign precursor lesions, neurofibromas, processed into a tissue microarray (TMA). This part of the study required a set of quality control experiments; the growth, harvest, and protein isolation of a series of cell lines to be used as a reference panel for antibody quality control by Western blotting. Secondly, the differential diagnoses of all MPNSTs were re-evaluated by reference pathologists prior to their inclusion in the TMA.]

Brief summary of the results

Report 1. "Identification of breakpoints and copy number changes of DNA sequences at chromosome arm 17q in malignant peripheral nerve sheath tumors."

Probes mapping to a centromere close region and to the distal area of chromosome arm 17q, were used in FISH experiments on MPNST. This was done to map DNA copy number alterations in two areas of the long arm of chromosome 17, knowing that previous genome wide studies have found frequent gains from chromosome arm 17q in MPNST. The locus specific FISH results confirmed amplification of chromosome arm 17q sequences in two thirds of the MPNST series, among which half had chromosome 17 polysomy. The *TOP2A*-probe was amplified in 15 of the 29 tumors, which may partly explain the known overexpression of this protein in MPNST. Four tumors with normal copy number of the centromere and TOP2A showed gain at the distal locus, supporting the assumption of a second amplicon at 17q. Metaphase spreads were used for COBRA –FISH analysis of four MPNST, and breakpoints and marker chromosomes that included chromosome 17 were found in three and one tumors, respectively. The region of interest is currently narrowed down to 2Mb by FISH with selected BAC probes.

Report 2. "*Protein expression of cell cycle components in sporadic and neurofibromatosis type 1 related malignant peripheral nerve sheath tumors*"

Expression data from 14 selected proteins in over a hundred tissue cores taken from MPNST were obtained in this study. A few neurofibroma samples were also included and evaluated against the MPNST results. We confirmed that TOP2A is expressed in the majority of MPNSTs. TP53 was not expressed to the same extent as expected from other studies. Generally the expression of cyclins and CDKs were high, leading to phosphorylated RB1 and G_1/S transition. The cyclin-CDK-complex inhibitors had generally low expression, confirming the lost control of the cell cycle checkpoint at late G_1-phase.

Discussion

The results of the two parts of this study are discussed in the two reports. Therefore I will here focus my discussion on the advantages and limitations by the methods I have used as well as in relation to other alternative methodological approaches. The present study is based on previous results obtained by G-banding and CGH (Table 1), and we have here used COBRA-FISH as well as locus specific FISH. Thus, I found it reasonable to discuss in detail the impact of the various methods.

Table 1. G-banded karyotypes of MPNSTs included in the present study. Performed in the laboratory of Professor Fredrik Mertens at the Department of Clinical Genetics, University Hospital of Lund, Sweden

Tumor	aberration
1224-98	45,XY,**t(10;17)(p10;p10)**,-20
1274-98	47,XY,der(2)t(X;2)(q13;q37),+3,-9,add(15)(q22),**dic(17;19)(p11;13)**,+18,**+mar**/45,X,-Y
1934-98	51-60,XXX,+X,+?X,add(1)(q11),+der(1;3)t(1;3)(p12;q29)ins(1;?) (p12;?),-2,add(2)(q37),-3,add(4)(p11),-5,?add(5)(p11),-6,add(6)(q27), ?der(8)t(1;8)(q25;p21),-9,-10,-10,?del(10)(q11),der(11)add(11)(p15) t(3;11)(p11;q24),der(11)add(11)(q23)de(11)(p13),-13,-13,-13,-14,-14,add(14)(p11),-15,-15,-15,-16,**i(17)(q10),der(?17)t(1;17)(q12;q25)**,-18, -18,-19,add(19)(q13),-20,-20,-21,-21,-22,-22,-22,**+?r**
3201-99	40-43,XY,add(1)(p35),add(2)(p11),del(3)(q?22q?24),-5,I(7)(q10),-9, -9,-10,del(12)(q15),-14,-15,-16,**-17,add(17)(p11)**,-18,+der(?)t(?;5) (?;q1?3),+2-3r,**+mar**
3721-99	78-80,XX,-X,+add(1)(p11),+2,+3,del(3)(q25)x2,+4,add(4)(q35)x2,+5, der(5)t(?1;5)(p32;p15)x2,+6,+7,+8,-9,+11,+12,add(12)(q13)x2,+13, add(14)(q32)x2,-15,-15,+?add(16)(q?)(16)(q?),**-17**,+18,-19,-19,-19,+20,+21, **+der(?)t(?;17)(?;q11)x2**,**+?r**,2dmin
2008-00	46,XX
3687-00	55-59,XY,add(1)(p36),+2,add(2)(p21),-3,+ins(5;?)(q31;?),+add(7)(q31), +8,+9,**-17,-17**,+19,+19,+20,+20,+22,+der(?)t(?;12)(?;14)
3869-01 398-02	45-46,XX,del(6)(p21),add(9)(q34),-13,?del(15)(q12q21,**add(17)(p1?2)**, -18,+der(?)t(?;13)(?;q22),**+mar** 45-46,XX,del(6)(p21),add(9)(q34),-13,?del(15)(q12q21,add(17)(p1?2), -18,+der(?)t(?;13)(?;q22),**+mar** / add(1)(p36)
3043-02	64-69,X,-X,-Y,-1,der(1)t(1;?13)(q11;q12),+2,add(3)(q11),-5,add(5)(p15), -6,+add(7)(22),-8,-8,-9,-10,-11,der(11)t(?1;11)(p21;p14)x2, der(21)t(12;?15)(p12;15)x2,,+13,-14,-15,-15,-15,**-17**,-18,+20,-21,-22, +der(?)t(?;1)(?;q11)x2,+r,**+4-6mar**

Strengths and weaknesses of different cytogenetic screening methods

Banding techniques

Chromosome bands have a great practical significance. They are fundamental units of chromosome organization in which euchromatin alternates with heterochromatin, transcriptionally active DNA with nontranscribed DNA. This gives a resolution of nearly 400 bands in total, and remains today the technique of choice for initial screening for karyotypic abnormalities[56], and are commonly used as a diagnostic tool for both solid tumors, hematological cancers, as well as for many other purposes (Figure 12). The strongest disadvantages with this method is that it requires metaphases, meaning that one need fresh tissue and that one need to grow the cells in short term cultures in order to get them to divide. This in itself usually demands quite large amounts of material, tissue samples. It also requires skills and experience to correctly interpret banded karyotypes.

In the 1970s new banding techniques by Caspersson *et al.*, and Yunis, gave so high resolution that chromosome pattern could now be described much more precisely. The methods first developed for this purpose used quinacrine mustard or quinacrine dihydrochloride to create transverse, fluorescing bands of variable brightness. The procedure is called Q-staining after quinacrine and the resulting bands, Q-bands. Other techniques resulting in a basically identical banding pattern use the Giemsa dye, and are therefore named G-staining methods and G-bands. R-bands give the reverse banding pattern of the G-bands, meaning R-bands are dark where G-bands are light. Other techniques stain only specific chromosomal structures, like the constitutive heterochromatin that gives C-banding and the telomere regions the T-banding.

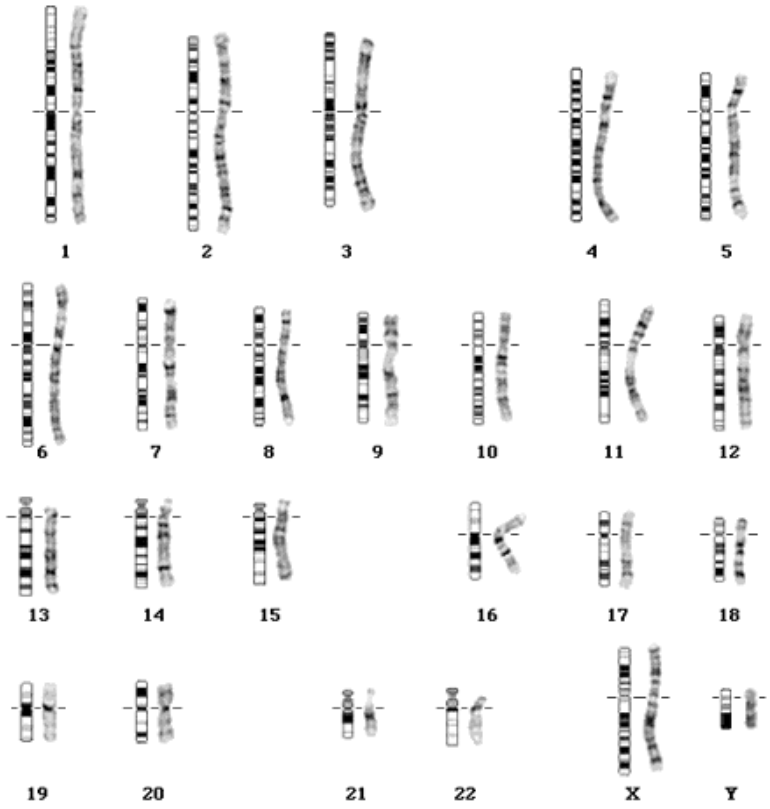

Figure 12. A schematic representation of a human male karyotype. Each chromosome is shown by an ideogram (left), and an actual G-banded metaphase chromosome[57].

FISH-based screening techniques

In the recent years multicolor based techniques have challenged chromosome banding, making pictures representing the entire genome using different colors when marking the chromosomes. This can be used to find possible chromosomal breakpoints and aberrations in, for example, cancer cells. Other techniques with higher precision can then be initiated.

Multicolor-FISH

If all of the human chromosomes are to be colored with painting probes of individual colors (Figure 13), you'll need 24 differentially colored probe sets. The advantage of using multicolor-FISH when detecting and describing a chaotic karyotypic pattern, is that you get the same color on cryptic rearrangements and marker chromosomes (unknown pieces) as the original chromosome (Figure 14). One limitation, though, is that intrachromosomal changes can not be identified, and breakpoints are, at best, vague.

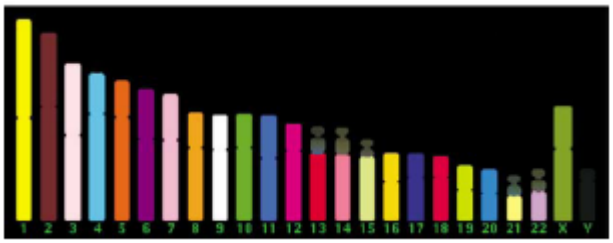

Figure 13. Multicolor FISH. Every human chromosome are colored by different colors.

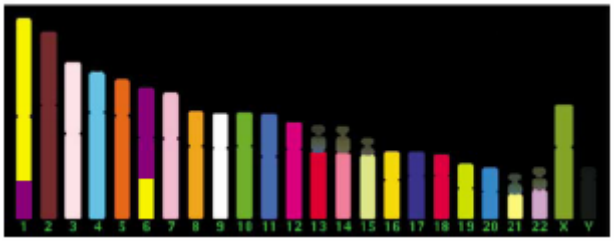

Figure 14. A translocation is easy to see when each chromosome has its own color. In this multicolor FISH example, the distal parts of chromosome arms 1q and 6q have exchanged places.

Spectral karyotyping (SKY)

SKY is a method that uses 24 chromosome specific probes, each labeled with a different combination of five fluorochromes. A single custom designed optical filter is needed to separate the fluorochrome combinations that give distinct colors to all of the 24 different chromosomes[58,59,60,61].

Multiplex-FISH (M-FISH)

M-FISH is very similar to SKY, and also uses five spectrally distinguishable fluorochromes. But with M-FISH the detection of the different combinations needs a separate microscope filter for each of the five fluorochromes. The final result is then given after combining the images in a computer. Compared with SKY, M-FISH has therefore the advantage of permitting the visualization of the signal from each fluorochrome individually[62,63,64].

Combined binary ratio FISH (COBRA-FISH)

COBRA-FISH needs only four fluorochromes to give 24 colors to all the chromosomes. This is achieved by using three fluorochromes pair-wise for ratio labeling a set of 12 chromosome painting probes, and then the last fluorochrome marks a second set of 12 probes[65]. This is used together with the combinatorial labeling described in the two other multicolor-FISH methods.

Cross-species color banding (RxFISH)

Another way of marking human chromosomes is by the use of cross-species color banding. Differentially labeled chromosomes from the gibbon ape are used as DNA probes[66]. Because of the 98% resemblance in DNA sequences of the two species, hybridization will occur throughout the entire genome. But the advantage of using gibbon DNA is the many chromosomal rearrangements that have taken place during evolution, which gives a specific color banding pattern for each chromosome. A chromosome will not have only one color as in the other multicolor-FISH methods. As a result this technique can also be used to detect intra chromosomal rearrangements like deletions and inversions.

Comparative genomic hybridization (CGH)

The technique does not require culturing for tumor metaphases, as in the FISH-methods mentioned above. A limitation with CGH is that balanced translocations and inversions do not change the copy number of the DNA sequences, and will therefore not be detected. But in karyotypes that are complex containing many markers of unknown DNA sequences, CGH is a useful technique.

CGH is a method to DNA copy numbers throughout the genome. For example, DNA from a tumor can be compared against reference DNA from normal cells, an approach first described by Kallioniemi *et al.* in 1992[67]. Labeling the two genomes by different fluorochromes, and hybridizing them together onto normal metaphases gives intensity ratios along the genome, a representation of the DNA copy number ratio (Figure 15). Marking tumor DNA with green and normal DNA with red, changes as losses and gains in the DNA copy number will be shown by the color ratio of red and green. The image is transferred to the computer by a CCD camera and the data is addressed by specialized software. Since first published, several thousand samples have been analyzed by this method.

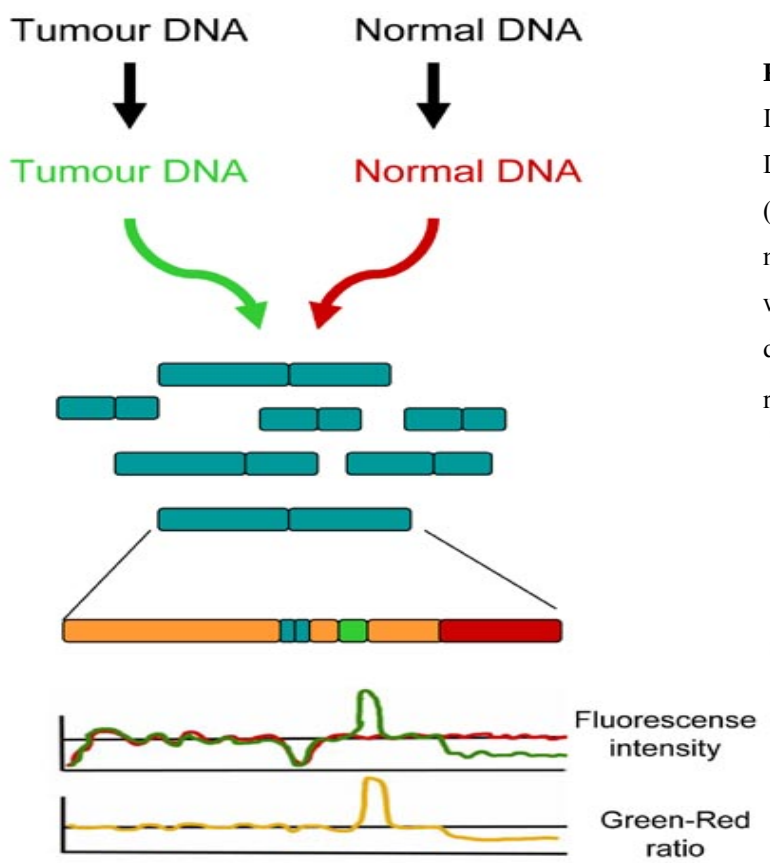

Figure 15. The principles of CGH.
In Comparative Genomic Hybridization, tumor DNA (test) is labeled green, and reference DNA (normal) labeled red, and then cohybridised onto normal human metaphase chromosomes together with human Cot-1 DNA. A color ratio profile describes copy number changes in the test DNA relative to the reference DNA.

Locus specific probes

Since none of the methods written of above can obtain a high enough resolution to describe the whole genome down to single genes, an additional technique is often required. FISH analysis with locus specific probes can detect sequences present in only one copy in the genome. The technique is used in cancer genetics to investigate tumors for deletions, amplifications, inversions and translocations. It is a helpful tool in diagnosis of neoplasm since genomic aberrations often are disease-specific[68,69,70]. The possibility of using these probes in **interphase** cells allows work on archival genetic material and neoplasm with low mitotic activity. Interphase cells, however, give only information of deletions and amplifications. If translocations and inversions are to be detected, locus specific probes have to be used on **metaphase** preparations. In this chromosomal stage, the DNA sequences are assembled closely enough that their order along the chromosome can be seen in the microscope. Locus specific probes can then be used to determine exact breakpoint positions, or to detect the precise nature of specific rearrangements that may be masked in complex chromosomal abnormalities.

Argumentation behind the present locus specific FISH study.

After the identification of an amplified or deleted region by a genome screening technique, one may use FISH probes to further narrow the region of interest. In our previous CGH study (Lothe *et al.*, 1996) we often saw amplifications from the chromosome arm 17q, and the large size of this amplicon implicated that numerous genes were present in too many copies. We therefore used a custom made cDNA microarray, covering all known and many predicted genes from chromosome 17, for detailed gene expression analyses in MPNST, and *TOP2A* was found to have the most highly upregulated expression of all genes on the array. Therefore, we decided to use locus-specific FISH to directly evaluate the DNA copy number of this gene in MPNST samples (Figure 16).

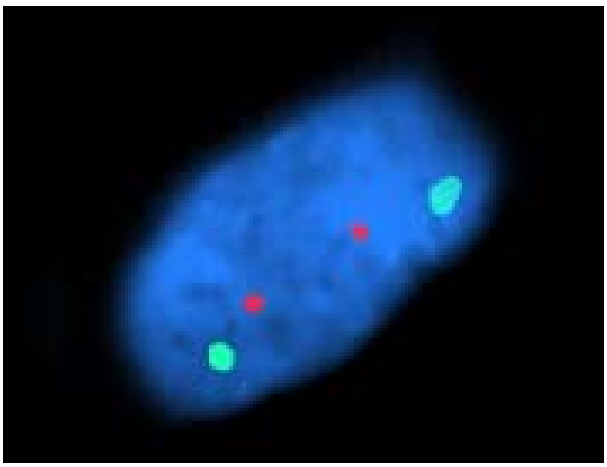

Figure 16. FISH from a cell with normal copy numbers of the two probes tested.

In addition, allelic imbalance studies have suggested a target region on the far distal part of 17q. Thus, we also selected a couple of distal probes for copy number evaluation. Finally, for the few cases we had remaining metaphase preparations from previous G-banding studies, we also performed COBRA-FISH to resolve the karyotypes.

Conclusion regarding choice of genome screening techniques

FISH methods only complement standard chromosome banding analysis, and can so far not replace them. Every technique has its strengths and limitations. The best way to do a chromosome analysis is to start with chromosome banding, and then decide whether any of the FISH methods will be useful to give complementary answers to unresolved parts of the resulting karyotype. If only archival material is available, chromosome banding analyses can not be applied at all, and the CGH method in combination with interphase FISH will provide the best combination for genome analyses. We have here not dealt with the microarray based screening techniques. These offer in principle higher resolution than the chromosome based techniques. However, so far, the BAC clone microarrays have not been available for whole genome coverage, although whole coverage of specific chromosomes has

been published. In addition cDNA microarray or oligo arrays may be used as DNA copy number arrays. A limitation here is that only alterations of coding sequences are obtained. The costs and technical challenges have so far reduce the usefulness of these techniques compared with chromosome based analyses. However, as the technology develops, the applicability will be enhanced, and these new approaches may become the mostly used genome screening tools throughout the scientific community and also in diagnostics.

Comparing protein analyses by Western blotting versus in situ hybridization

Western blot analysis can be used to obtain information about alterations in the protein sizes caused by gene alterations or regulation at the expression level such as phosphorylations and dephosphorylations of proteins. The protein expression levels can also be measured and easily compared with other samples. A limitation with this method is that the subcellular localization of the proteins is lost. Another is that quite large sample material is needed for making the blot.
When constructing a TMA only, small parts of each tissue sample are needed. With immunohisto-chemistry, the protein expression is shown *in situ*, allowing detection of also the subcellular localization of the proteins. Immunohistochemistry has no information of the protein size. To check for intra-sample heterogeneity, replicate tissue cores from the same samples may be included in the TMA. If the sample is heterogeneous, having two phenotypically different cell populations, one can argue that the random sampling of only one tissue core will decrease the impact of the TMA results, not giving necessarily the best answer. Therefore, TMAs are not the platform of choice for diagnostics of individual tumors, but this tool has a tremendous advantage for comparisons of groups within large sample series. The power of simultaneously testing many samples ensures strong statistical distinctions between groups, even in the case of miss-classifications of a few tissues.

Future studies

Chromsome 17 and expression profiling

By a discovery-based research approach, whole genome analyses have identified several recurrent chromosomal aberrations in MPNST. The map positions of these anomalies, pinpoint the localization of potentially important target genes in establishment and progression of MPNST. Amongst the most common changes are gains of 17q sequences. Last year we showed that one of the genes targeted by this amplification is the topoisomerase IIα (Skotheim *et al.*, 2003). The fact that the 17q gains often include large parts of the chromosome and due to previous allelic imbalance studies showing frequent distal 17q gains, we hypothesized that more than one 17q amplicon may contribute to MPNST. The present study has further supported this idea and has shown that upregulated *TOP2A* is indeed partly caused by gene amplification. We are continuing the search for the distal gene targets by FISH analysis using BAC probes covering breakpoints identified by other analysis with locus specific probes or with COBRA-FISH. Currently, an approximate 2Mb region of interest is identified. We have searched for upregulated expression in an existing dataset of 10 MPNST obtained by analysis of a cDNA microarray enriched with chromosome 17 genes. However, only very few genes in the region of interest was present on the array. Therefore, we decided to perform a gene expression analyses using Agilent 22k oligo array covering most genes in the genome. This data set will provide answers to several questions regarding the genetics of MPNST, not only the 17q-specific expression profile. In this new study design we have included precursor lesions of the plexiformed type, the MPNST from which chromosomal translocations involving chromosome 17 were detected, as well a set of MPNSTs from both NF1 patients and sporadic cases. If we detect dysregulation of genes in the specific 17q region that were identified by the BAC-probes, these genes will be considered good candidates for further hypothesis driven mutations analysis.

Cell cycle

The present thesis have provided a standard set of cell lines, representing germ cell tumors, mesenchymal- and epithelial-tumors, to be used, amongst others, for validation of the specificity of antibodies. The staining patterns of the antibodies are examined in large clinical series, of which the MPNST TMA is one. The *in situ* protein expression of central cell cycle components have been investigated in this study, and confirmed the lack of CDKN2A as characteristic to MPNST. It seems like once the CDKN2A is no longer functioning, the remaining proteins analyzed have a normal expression. Among the ones with CDKN2A expression, multivariate statistical analyses will be used further to examine the relationship among the markers as well as the relations to other data. We are now in the process of gathering all clinical information into databases, including type of treatment and

follow-up, for this purpose. In addition, most of the samples are analyzed for genome changes by high-resolution CGH. The complete database of clinical and genomic information will, when finished, be of high value when related to the present protein expression data.

Genome profiling

The chromosome based genome screening methods are of course limited by the resolution of the microscopical evaluation of metaphase chromosomes. We plan to use a DNA microarray approach to further refine the genome picture of these tumors. Currently, the in-house BAC arrays with a 1Mb resolution are available. This resolution is 3-5 times improvement of the resolution from high-resolution chromosomal-CGH for the detection of deletions, whereas the detection limit for amplifications will for the chromosomal-CGH depend on both the size, as well as the degree (number of copies) of the amplicon. Even a 5 Mb deletion in close proximity of a large amplicon may not be detected by chromosomal CGH. Thus, we expect by use of the BAC microarrays to gain novel information about the genomic changes of MPNST and neurofibromas.

Signaling pathways

The *NF1* gene is a tumor suppressor which when inactivated leads to continuous activation of the RAS proto-oncogene. In general, one might expect that inactive *NF1* may be mutually exclusive to mutated RAS or RAF, or *visa versa*. Due to the many exons of the *NF1* gene, mutations have been poorly described in cancer, although the initial report on the cloning of this gene identified somatic mutations in various cancer types[73,74,75]. In addition, few, if any, MPNSTs have been analyzed for point mutations in the coding sequence. For MPNSTs from NF1 patients, a germline mutation of *NF1* is expected, and a possible second hit is usually analyzed by deletion studies in the tumor. Among the sporadic cases of MPNST, even less information is available. However, it has been shown that inactive *NF1* can be present in neurofibromas, indicating that additional changes are necessary for malignant transformation. With regard to this, one may hypothesize that mutated *RAS* or *RAF* may provide the cells with further malignant potential. To test the alternatives of mutual exclusiveness of these three genes or their combined effect in the early neurofibromas and late stages of MPNST, we will perform mutation analysis of all these genes. For the *RAS* and *RAF* genes, direct sequencing will be used, but for the *NF1* gene we will establish the exon by exon screening analyses using the WAVE platform which is based on HPLC. Mutations will affect several down-stream pathways such as the MAPK and WNT-signaling. These pathways are also relevant in many other cancers. We will also test some of the components of the WNT-signaling pathway, for which we have established mutation or methylation assays, as well as examine the potential enrichment of dysregulated WNT components and/or downstream target genes from the expression microarray dataset described above. The latter may provide clues to novel targets for MPNST-development.

Last year, we showed that one of the contributing genes to MPNST is excess of the topoisomerase IIα (Skotheim *et al.*, 2003). This protein is a known target of several drugs commonly used against cancer, and thus this discovery was of high interest. The fact that this cancer disease is rare in the population makes it hard to collect and examine large clinical series, not at least to evaluate the predictive value of biomarkers such as TOP2A since no standard treatment regime exists. Still, in a rather small sample series, *TOP2A* expression was found associated with poor prognosis. We have during the last decade collected fresh/frozen specimens relevant for various molecular studies as well as a series of formalin-fixed archival material. In collaboration with the University of Lund, we have now a total of 106 samples included in the TMA that provide us with a good tool to clinically validate the biomarkers identified in each of the above mentioned discovery-based and hypothesis-driven research approaches. By gaining novel insights into the underlying biology of this disease, and validate sets of relevant biomarkers, the project will aid in the current diagnostic and clinical challenges of this highly aggressive cancer disease of young adults.

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Report I

Identification of breakpoints and copy number changes of DNA sequences at chromosome arm 17q in malignant peripheral nerve sheath tumors.

by

Helge Brekke

Identification of breakpoints and copy number changes of DNA sequences at chromosome arm 17q in malignant peripheral nerve sheath tumors.

This study is part of a collaborative project between our group and the group of Fredrik Mertens, Department of Clinical Genetics, University Hospital of Lund, Sweden. I spent four weeks in the winter 2004 in the Lund laboratory being trained in karyotyping and performing the metaphase FISH analyses. The interphase analyses were performed in our own laboratory.

Summary

Based on previous studies that are implicating chromosome arm 17q amplifications in malignant peripheral-nerve sheath tumor (MPNST; Lothe et al., 1996, Mechtersheimer et al., 1998, Schmidt et al., 1999 and 2000), fluorescence *in situ* hybridization (FISH) was used to establish DNA copy numbers of some selected loci (genes/clones) mapping to a region near the centromere, and to the distal area of chromosome arm 17q. The probes were hybridized onto interphase nuclei or metaphase preparations from MPNSTs from 29 patients. Sixteen individuals had the disorder Neurofibromatosis type 1 (NF1), whereas the remaining 13 were sporadic cases of MPNST. In addition, metaphase spreads were used for COBRA–FISH analysis of the whole genome in order to detect breakpoints and markers that include chromosome 17. The locus specific FISH results confirmed amplification of chromosome arm 17q sequences in 2/3 of the MPNST series, among which half had chromosome 17 polysomy. The last 1/3 of the MPNST had no aberrations in any of the probes tested. TOP2A was found amplified at the gene level in 15 of the 29 tumors, partly explaining the increased expression previously detected at the RNA and protein level. Further, we identified a second amplicon at distal sequences on 17q, where as much as 19 of the 29 tumors were amplified. Five of these had normal copy numbers at the TOP2A/ERBB2 region and at the centromere. COBRA-FISH analyses detected MPNSTs with translocations involving chromosome arm 17q, and two known chromosomes namely chromosomes 1 and 10, and two unknown chromosomes. This study pinpointed a region of interest for further FISH analyses using BAC probes in order to identify gene targets.

Background

Malignant peripheral nerve sheath tumors (MPNSTs) are rare neoplasms classified as mesenchymal tumors, because they often cause the same complications as soft tissue sarcomas and often develop in the extremities. Neurofibromatosis type 1 (NF1) is a common genetic disorder also known as the von Recklinghausen disease, and carriers have a germline mutation in the *NF1* gene. The phenotypic features are several and development of multiple neurofibromas is one of them. These tumors are benign lesions that may become malignant, leading to MPNST. About 8-13% of the NF1 cases develop MPNST [28], which account for 50% of all MPNST cases.

No pathognomonic chromosomal translocation has been found in MPNST. Instead these malignancies often present complex karyotypes, but several recurrent aberrations are identified, including loss of 9p and gain of 17q. Identification of the target genes for these genomic changes will provide new understanding of the disease development and may aid in improving the many diagnostic and clinical challenges associated with this disease.

With the strategy to first obtain a global view of the genomic changes in MPNST, we found that gain of 17q sequences was the most frequent aberration in a series of MPNST as assessed by comparative genomic hybridization (CGH; Lothe et al., 1996). These gains often included large parts of 17q and we therefore went for a large scale expression study in order to detect dysregulated genes along this chromosome arm. We used a custom-made microarray with 636 cDNA clones from chromosome 17, and among the MPNST compared to the benign

BOX 1: The TOP2A gene, located at 17q21-q22 produces topoisomerase II-, a key enzyme in DNA replication, cell cycle progression and chromosome segregation. The nuclear enzyme is involved in processes such as chromosome condensation, chromatid separation, and the relief of stress that occurs during DNA transcription and replication. It catalyzes the breaking and rejoining of two strands of duplex DNA that allows the strands to pass through one another, thus altering the topology of DNA. Two forms of this enzyme exist as likely products of a gene duplication event. The gene encoding this form, alpha, is localized to chromsome 17 and the beta gene is localized to chromosome 3. The gene encoding for this enzyme functions as the target for several anticancer agents.

counterpart, neurofibromas, *TOP2A*, located at 17q21.2, had by far the highest expression level (Skotheim et al., 2003).This gene encodes a protein that is targeted by several drugs in cancer treatment. We did find a statistical association between TOP2A expression and poor patient survival. To further evaluate this target at the gene level we have in the present study evaluated the copy number of *TOP2A* in a Norwegian and Swedish series of MPNSTs. Since the CGH data showed large chromosome gains and since allelic imbalance studies have shown alterations at the distal end of 17q, we also studied distal 17q loci in order to verify the hypothesis of a possible second amplicon. We also analyzed *ERBB2* (alias *HER-2*/*NEU*), located at 17q11.2 – q12, centromeric to *TOP2A*, and encoding a 185 kDa transmembrane cell surface glycoprotein. ERBB2 is a member of the tyrosine kinase family with a high degree of homology to the epidermal growth factor receptor (EGF-R), and amplification of ERBB2 has been reported in several other cancer types. Finally, a multicolor genome screening was done on a subset of the samples to identify possible breakpoints involving chromosome 17.

Materials and methods

Interphase FISH

Tumor samples included in this study were taken from three benign neurofibromas and nineteen MPNST samples from 22 Norwegian and Swedish patients altogether. Ten of the tumors came from NF1 patients. Frozen sections of all samples were examined by a sarcoma reference pathologists. Only samples with confirmed MPNST diagnosis were included. We counted probe signals in nuclei from 100 cells of each tumor.

Methods

Freshly frozen tumor samples were cut in small pieces on a block of dry ice. Tumor fragments were immediately fixed in cold (-20^{o}C) fresh methanol:acetic acid (3:1). The samples were centrifuged and the fixatives were renewed. The tissue fragments were then dissociated in 80% acetic acid and left at room temperature for 2 minutes. One or two drops of the suspension were put on a pre-warmed slide laying on a slide warmer at 47^{o}C, and dried for 5-10 minutes. This gives a nice spread of the cell suspension. The slides were left overnight at room temperature.

We examined the material with dual probes, *TOP2A* or *ERBB2*, together with a probe from the centromere of chromosome 17 (LSI TOP2A, SpectrumOrange ^{TM} / CEP 17,

SpectrumGreen: Vysis Inc, Downers Grove, IL), (ERBB2, Rhodamine / Chromosome 17 Alpha-Satellite-probe, FITC: Qbiogene, Inc. Irvine, CA). A distal 17q-telomere probe was also examined (TEL 17q DNA probe, FITC: Qbiogene, Inc. Irvine, CA). Before hybridizing, the probes and chromosomes were simultaneously denatured. The slides were treated in a 70% formamide solution containing 2 x SSC, and the probe mixture put in a water bath. They were both warmed at 73^{o}C for 5 minutes. The slides were then dehydrated in a series of ethanol washes (70% ethanol for 2 minutes, 85% for 2 minutes, and 100% for 2 minutes) and dried on a 45-50^{o}C slide warmer. A mixture of 1µl probe and 9µl hybridization solution was applied onto the slide, and a glass coverslip was sealed onto the slide. The slides were put in a prewarmed humidified box and placed in a 37^{o}C incubator until the next day. The coverslips was then removed before the slides were washed for 3 x 10 minuets in 50% formamide and 2 x SSC at 47^{o}C. They were then put in 2 x SSC solution for another 10 minutes and then finally 5 minutes in 2 x SSC containing 0,1% NP 40.
The slides were then air-dried away from light, counterstained with 10µl DAPI antifade solution, and sealed with a new coverslip. The nuclei were examined in a Zeiss fluorescence microscope and images were captured by a Cohu camera. Preparations of normal lymphocytes were used as controls for the hybridization conditions.

Control Interphase FISH analyses using triple probe.

To verify that the interphase-FISH results described above was comparable with the metaphase locus specific FISH analyses, we tested the triple probe used in the metaphase analyses on three samples that were already analyzed for the same loci but with dual probes (see above).

Metaphase FISH

Tumor samples included in this part of the study were taken from 10 cultured MPNSTs, all from Swedish patients. Six were tumors from NF1 patients. Three of the same samples were also included in the interphase FISH analyses.

Culturing of tumor cells

Tubes containing MPNST cells, stored in the N_2 tank, were heated in 34°C water. The cells were grown in RPMI-medium at 37 °C for appropriate number of days, and then added Colcemid (GIBCO) for 3-4 hours. Further, they were centrifuged for 10 minutes at 1000 rpm. after added trypsin. The pellet was dissolved in a hypertonic solution (KCl) for 25 minutes, and then washed in methanol:acetic acid solution (3:1). The solution was then spread on glass slides.

Probe preparations

Glass slides were treated in 60°C over night before submerged into 2 x SSC containing 500µl 10% tween20 for 25-30 minutes, also at 60°C. The slides were washed in running water and dehydrated in a series of washes with increasing ethanol contents. 10mg/ml pepsin in 0,01mol/l HCl solution was added onto the slides, which were coversliped and incubated for 10 min on a 37°C slide warmer. The coverslip was removed before washing and dehydrating the slides. We examined the material with a multi color probe detecting both *TOP2A* and *ERBB2* together with the centromere on chromosome 17 (LSI TOP2A/HER-2/CEP 17 Multi-color Probe: Vysis Inc, Downers Grove, IL). We also used a single distal 17q-telomere BAC probe (see below) mapping to 17q25.3 (BAC-probe RP11-388C12: University of Bari, Italy). A total of 10µl probe solution, containing 7µl hybridization solution, 2µl water 1µl and probe, was applied onto the slides laying on a 37°C slide warmer and left there for 5 minutes with a glass coverslip away from light. Then the slides were heated to 74°C for 1.30 minute to denature the probes. The slides were put in a pre-warmed humidified box and placed in a 37°C incubator for 16h to three days. The coverslips were removed, slides washed, dehydrated, and dried on the slide-warmer away from light. Finally, they were counterstained with 10µl DAPI antifade solution, before sealed with a new coverslip, microscopically evaluated.

Metaphase FISH: BAC-probe procedure

The BAC-probe RP11-388C12 was ordered from the University of Bari, Italy, and came in the form of *Escherichia coli* bacteria containing a plasmid with this probe. The *E. coli* was then grown in **LB Medium** (Bacto yeast extract 5 g; Bacto trypton 10 g; NaCl 10 g; H_2O up to 1 Liter, Autoclave) to an appropriate amount, before extracting the DNA probes. The protocol is described here:

The *E. coli* was grown for 16-20 hours together with 30µl chlorampenycol because a resistance gene to this was incorporated in the BAC. 50ml *E. coli* was centrifuged for 20 minutes at 4500 rpm. The pellet was resuspended completely in 3ml of GTE (50mM Glucose, 25mM Tris pH=8, and 10Mm EDTA). Then 6ml of denaturation solution (0.2 N NaOH, 1% SDS, room temp), freshly made, and 5ml of 7.5M Ammonium Acetate were added. The solution was mixed immediately by inverting several times and left on ice for 10 minutes. The solution was again centrifuged for 20 minutes at 4500 rpm. Isopropanol (70% of the total volume) was added before centrifuged for 20 minutes at 4500 rpm. The supernatant was discarded, and the pellet washed with 5ml of 70% ethanol. Solution was centrifuged for 5 minutes at 4500 rpm. The supernatant was discarded, and the pellet resuspended in 1ml of TE, added 500µl 7,5 mol/l Ammonium Acetate. This was put on ice for 1 hour, and then centrifuged for 20 minutes at 4500 rpm. The supernatant was discarded, and resuspended in 3.5ml ethanol and left for 1 hour. Then the solution was centrifuged for 20 minutes at 4500 rpm. The supernatant was discarded, and resuspended in 50-100µl of TE. The solution was left in room temperature over night.
5µl primer was used for every 2000ng of genomic DNA, and put on 95^{o}C water bath for 5 minutes. The primer is universal in that it has a short sequence, and will therefore bind to the BAC at several locations. Then 2µl Florex (green colored dCTP, Amersham), 2µl Klenow polymerase (enzyme) and 17µl ATG-buffer were added and warmed at 37^{o}C for one hour. The next day the reaction was stopped by adding Blue Juice, and centrifuged for 2 minutes at 1500 rpm. The supernatant was discarded, and Cot. mix, ammonium acetate and ethanol were added, and left for one hour away from light. The solution was then centrifuged for 15 minutes at 14000 rpm. The supernatant was discarded, hybridization solution added, and the probe was then ready for use.

Approximately ten additional probes will, along with the use of COBRA-FISH, be performed in the laboratory of Fredrik Mertens on the chromosome arm 17q, in order to further describe the translocation breakpoints.

Results

interphase FISH

❑ **Centromere**

- The number of centromeres is interpreted as the number of chromosomes, either partially or completely intact.
- An increase in copy number was scored if the tumor had more than 1/3 of the nuclei showing three or more probe signals.
- In order to correct for chromosome 17 polysomy, gene amplification was defined as the presence of 4 or more probe signals in at least 5% of the nuclei from a single tumor, plus a gene to centromere ratio of 2 or more.
- 10 of 19 MPNST and 1 of 3 neurofibromas showed gain of centromere 17.

❑ **ERBB2**

- 9 of the 19 malignant tumors had increased copy number of *ERBB2*. All these had polysomy for chromosome 17.
- 8 of these tumors also had cells that were amplified without having polysomy for chromosome 17.

❑ **TOP2A**

- 13 of the 19 malignant tumors showed an increase in copy number of *TOP2A*.
- 10 of these 13 tumors had polysomy for chromosome 17, where 9 also had cells that were amplified without having polysomy for chromosome 17.
- 3 of the 13 tumors had TOP2A gene amplification in at least 5% of the cells, without having the centromere probe amplified.

❑ **Telomere 17q**

- 13 of the 19 malignant tumors had in more than 1/3 of the cells an increased copy number of 3 or more signals of the telomere 17q probe.

Overall evaluation of the neurofibromas

- 3 of the overall 22 samples in this study were benign neurofibromas. Two of these showed no alterations, whereas the last neurofibroma had gain of the centromere signals in 12% of the cells, and at the same time an increased copy number of more than 5% in all of the probes tested. This neurofibroma was therefore considered to be polysomic for the entire chromosome 17 in these nuclei.

Summary of the MPNST interphase FISH results

The interphase results are illustrated in Figure 1, and summarized in Table 1. Only 5 of the 19 MPNST showed a normal copy number for all of the tested chromosome 17 probes.

- 13 of the 19 malignant tumors had the 17q-telomere probe amplified.
- 13 of the 19 malignant tumors had an increase in copy number of *TOP2A*.
- 9 of the 19 malignant tumors had an increase in copy number of *ERBB2*.

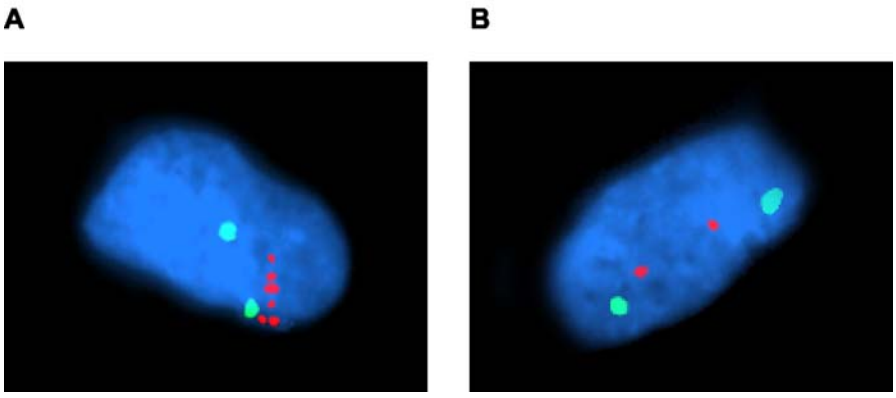

Figure 1A) A nucleus from an MPNST showing an increase in copy number of the *TOP2A* probe signal (six red spots), and a normal number of the centromere probe (two green spots). **B)** A MPNST nucleus having the normal copy number of both the *TOP2A* and centromere probes.

Table 1 (subgroups of MPNST based on interphase FISH probe signals on chromosome arm 17q).

Sample	Diagnose		Centromere 17						ERBB2						TOP2A						17q-tel total					
			0	1	2	3	4	5+	0	1	2	3	4	5+	0	1	2	3	4	5+	0	1	2	3	4	5+
3 (T-rygg)	NF1	MPNST	1	9	51	35	3	1	0	1	26	17	32	24	0	4	36	27	22	11	2	6	46	22	18	6
6	NF1	MPNST	0	4	27	64	4	1	0	8	43	46	3	0	0	0	9	84	5	2	0	1	15	46	28	10
14	NF1	MPNST	4	15	54	25	1	1	0	2	23	30	26	19	0	8	35	29	13	15	1	8	25	29	29	8
753-92	NF1	MPNST	0	7	51	20	19	3	0	5	60	21	9	5	1	1	11	32	18	37	0	2	18	31	26	23
2406-94		MPNST	1	11	53	20	13	2	0	3	54	13	26	4	0	5	47	22	15	11	1	8	54	10	15	12
246-96		MPNST	0	12	74	9	5	0	1	8	37	28	17	9	2	15	51	17	9	6	0	6	32	30	18	14
12	NF1	MPNST.	0	9	87	5	3	0	0	3	41	56	0	0	0	4	57	20	13	6	0	1	62	10	24	3
11		MPNST	0	10	77	12	1	0	0	3	35	60	2	0	0	5	45	37	9	4	0	3	49	45	1	2
650-90		MPNST	2	50	46	1	1	0	0	5	75	17	1	2	0	2	34	14	21	29	0	5	44	47	4	0
763-91	NF1	MPNST	1	9	83	3	4	0	0	5	77	17	1	0	0	2	40	39	14	5	0	7	67	18	4	4
1750-95		MPNST	0	8	91	1	0	0	0	7	92	1	0	0	0	0	25	0	28	47	0	2	37	13	23	25
1615-94		MPNST	0	6	77	15	1	1	0	4	74	20	2	0	0	6	85	8	1	0	0	3	57	35	4	1
5		MPNST	0	10	87	2	1	0	0	6	86	5	2	1	0	7	91	0	2	0	0	3	42	53	1	1
9	NF1	MPNST	0	13	84	2	1	0	0	8	85	6	1	0	0	5	85	4	4	2	1	4	87	7	0	1
8	NF1	MPNST	0	10	90	0	0	0	0	10	90	0	0	0	0	3	97	0	0	0	0	7	89	4	0	0
13		MPNST	0	1	96	1	1	1	2	8	88	1	0	1	1	9	89	0	1	0	0	7	92	1	0	0
2367-90	NF1	MPNST	0	10	89	0	1	0	0	12	87	1	0	0	0	10	88	0	2	0	0	4	88	6	2	0
32-94	NF1	MPNST	0	7	91	1	1	0	0	3	97	0	0	0	0	4	90	5	1	0	0	5	88	7	0	0
3420-95		MPNST	0	16	82	1	1	0	2	15	80	2	1	0	0	9	83	6	1	1	0	6	91	3	0	0
3 (nates)	NF1	Neurofibroma	0	4	82	7	3	4	0	1	87	4	2	6	1	10	76	5	6	2	0	8	79	7	4	2
4	NF1	Neurofibroma	1	14	84	0	1	0	0	15	84	0	1	0	0	8	89	3	0	0	0	7	85	5	2	1
12 (nf3)	NF1	Neurofibroma	0	8	90	1	1	0	0	5	93	1	0	1	0	6	83	8	2	1	1	8	83	6	2	0

Less than 67% of the cells have 2 probe signals.

More than 25% of the cells have 3 or more probe signals.

Polysomy for chromosome 17, meaning that more than 5% of the cells have 3 or more gene signals and 3 or more centromeric signals, in the same cell.

More than 5% of the cells have gene amplification, meaning 4 or more gene signals and only 2 centromeric signals, in the same cell.

Metaphase FISH

FISH on metaphase preparations do not provide the same large amount of interpretable nuclei as interphase preparations, and probe signals from 3-10 cells in each tumor were counted. Since metaphase chromosomes are condensed, the locations of the probes in the genome is seen. This way it is safer to state the actual copy number of the nucleus, if the same pattern is constantly repeated. Translocations can also be certified if the breakpoint is between two of the probe signals used. We also took the opportunity of using one of the multicolor-FISH techniques on a few selected tumor samples, (figure 2). The COBRA-FISH can establish different colors to all the 23 chromosome pairs, and is therefore very useful for detecting translocations and describing other aberrations in a karyotype. There had already been performed G-banding of all these tumors, and some of the results listed under are interpreted with this karyotype in mind. The results of the metaphase FISH are presented in table 2.

Table 2) Numbers of probe signals detected based on metaphase FISH on chromosome 17q.

Patient	Diagnose		Centromere	*ERBB2*	*TOP2A*	17q-telomere	Translocations
1934-98	MPNST	NF1	2-3	4	4	4	
2150-02	MPNST	NF1	2-3	2-3	2-3	5	
3721-99	MPNST	NF1	3	2	2	8-9	t(?;17)
3043-02	MPNST	NF1	3	2	2	4	
3687-00	MPNST	Sporadic	2-3	2	2	4	t(1;?)(p;?)
3869-01	MPNST	Sporadic	2	2	2	3	add(17p)
3201-99	MPNST	NF1	3	2	2	1-2	t(1;17)(p;q) + r17
1224-98	MPNST	Sporadic	2	2	2	2	t(10;17)
1274-98	MPNST	Sporadic	2	2	2	2	t(?;17)(?;TOP2A)
2008-00	MPNST	NF1	2	2	2	2	

Green cells indicate copy number exceeding the set limitations (see above).

- 2 of 10 tumors had increased copy number of all the probes tested.
- 6 of 10 tumors had increased copy number of the 17q-telomere probe (Figure 3).
- 6 of 10 tumors had increased copy number of the centromere probe.
- 3 of 10 tumors had normal copy number of all the probes tested.

- We found four tumors with possible translocations involving chromosome 17. In all these there had been a breakage along the chromosome arm 17q. The distal part of chromosome arm 17q was always translocated away from the centromere.
- In one tumor, the *TOP2A*, but not the *ERBB2*, probe signal was translocated to another chromosome, implying a break point between *ERBB2* and *TOP2A*.

- A translocation of the distal chromosome arm 17q and chromosome arm 1p was seen in one tumor. There was also a ring structure made up of chromosome 17 materials present in the karyotype of this tumor.
- Another tumor had a translocation between chromosome 10 and chromosome arm 17q.

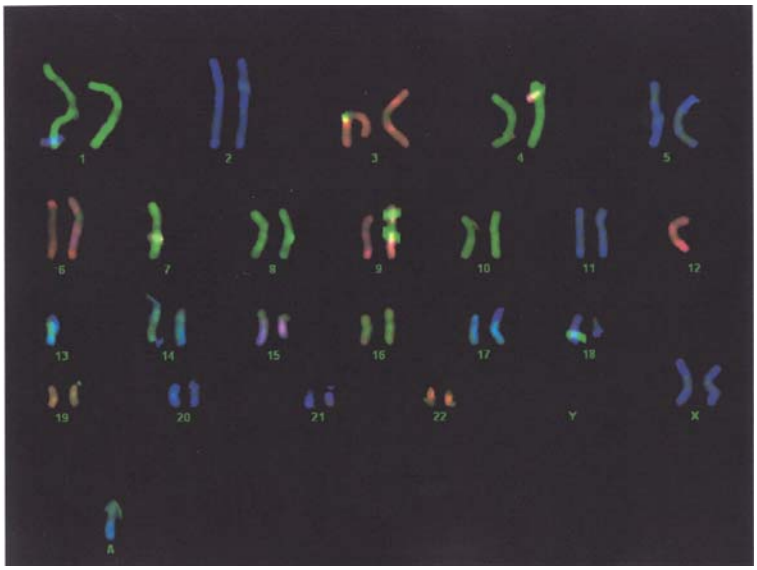

Figure 2. COBRA FISH on MPNST case 3869-01. The karyotype is showing alterations regarding an added sequence to chromosome 17, monosomies of chromosomes 7, 12, and 13, and one unknown marker chromosome on the bottom. The added segment on chromosome 17 is not specified, but looks like a part of chromosome 18.

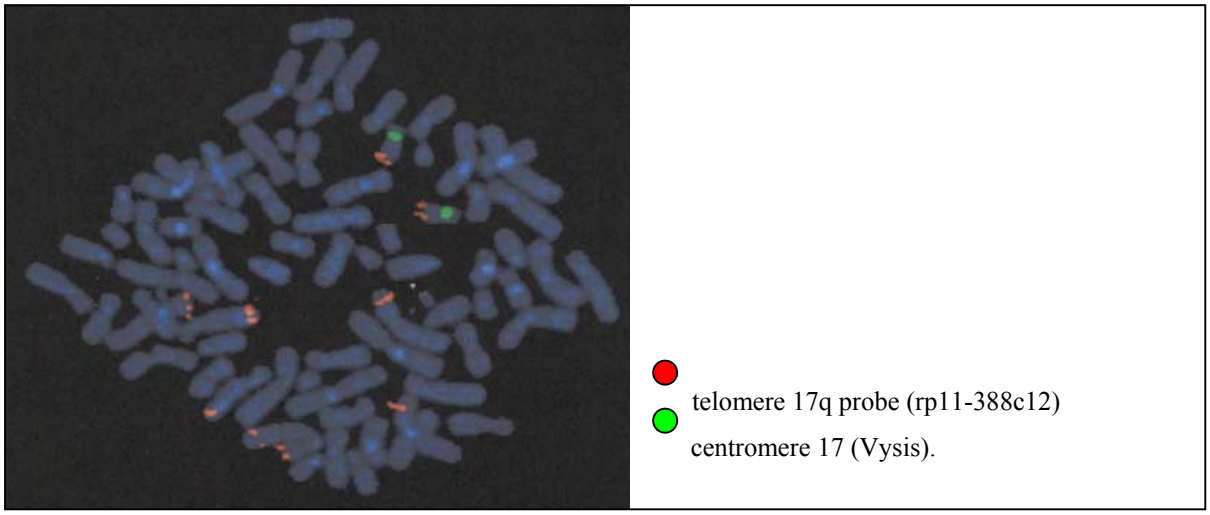

Figure 3. The distal telomere probe on chromosome arm 17q has in this MPNST detected a highly increased copy number. The 17q telomere is here located also to chromosomes other than 17.

Control experiments of different probe combinations used on inter-, and meta-phase preparations

In summary, the results of the tumors analyzed by metaphase FISH differed slightly from the tumors analyzed by interphase FISH. There were fewer samples with increased copy number of *TOP2A* and *ERBB2*, than in the interphase cases. The telomere 17q probe was amplified in a wide extent in both cases, and polysomy of chromosome 17 was seen in about the same number of tumors. Therefore, for some tumors previously analyzed by interphase FISH, we tested the multi color probe on tumor metaphases. We found no differences in the results from the prior work done with the two dual probes. We then used freshly frozen material from some of the MPNSTs which we previously also had cultured and performed metaphase FISH on. This time we counted probe signals from 100 cells in interphase. The results were overall the same as the conclusion from the metaphase cells studied before. No tumor showed a high frequency of increased copy number of *TOP2A* or *ERBB2*, but the centromere probe was amplified in some tumors.

Combined results of interphase- and metaphase FISH of MPNST

All in all, 11 of the 29 MPNSTs on which we performed interphase- and/or metaphase FISH showed polysomy for chromosome 17. Eleven tumors had increased copy number of *ERBB2*, 15 tumors had increased copy number of *TOP2A*, and as many as 19 had an increased copy number of the 17q telomere probe (Figure 4). For 8 of the 29 MPNSTs, we detected no copy number alterations along 17q.

We found no significant difference between MPNSTs from patients with neurofibromatosis type 1 (n=16) and sporadic cases of MPNST (n=13) in relation to chromosome arm 17q copy number.

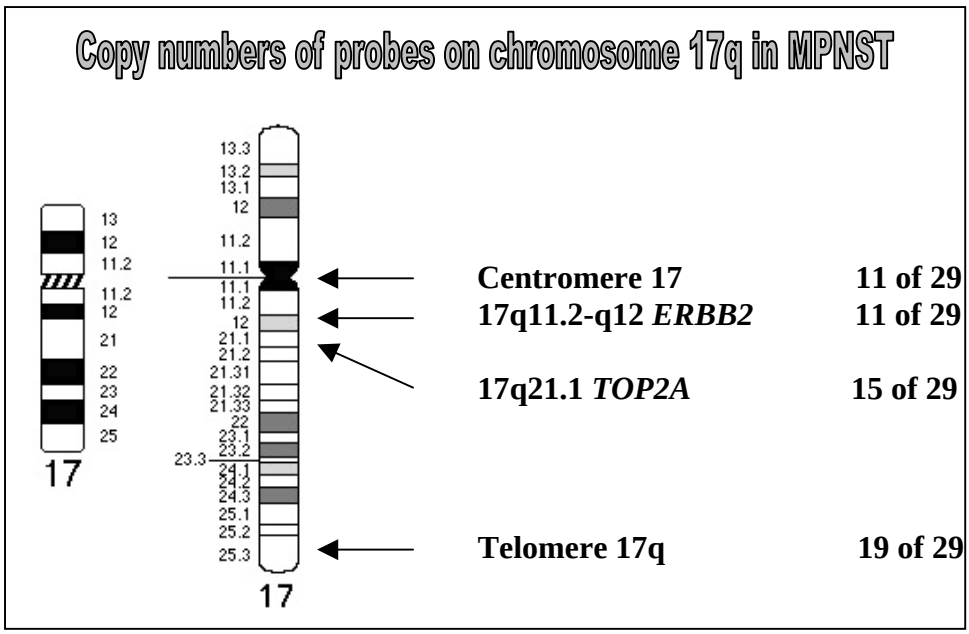

Figure 4) Increased copy numbers along chromosome arm 17q in MPNSTs.

The number of MPNSTs with excess copies of the selected probes on chromosome arm 17q has an increasing trend towards the telomere. One third of the MPNSTs seem to have polysomy for the entire chromosome arm. Another third has parts of the 17q arm in extra copies, with the distal region always included. Often, the chromosome arm 17q is involved in a translocation with another chromosome, but it remains to be seen if this is a general pattern. The last third of the tumors had very few or no aberrations in any of the probes tested.

Discussion

It is generally agreed that cancer development is a multistep process involving natural selections of somatic mutations[11,12]. The karyotype of a cancer cell may look chaotic, but the genetic alterations in a specific tumor are not random. Cancers in different tissues have their own characteristic progression, and exposure to specific classes of carcinogens gives similar genetic profiles[13,14]. Several reports also reveal distinct patterns of genetic alterations in different tumors, thus implying that the carcinogenic process may proceed through alternative genetic pathways[15]. For instance are different genetic pathways to colon cancer closely related to specific segments of the colon[16]. But to differentiate between primary, pathogenetically essential, and secondary evolutionary aberrations is very difficult in solid tumors. At the time of diagnosis most solid tumors have already acquired several aberrations during tumor progression[17]. Telomeres can be directly involved in shaping the karyotypes of tumor cells. This happens if chromosomes connect in telomere fusions, followed by a breakage-fusion-bridge cycle[18], or when essential genes regulating the cell cycle are mutated.

With this in mind, the results of the FISH on MPNSTs reveal two or three possible different pathways in the development of the cancer type. One has chromosome arm 17q sequences amplified during the progression, and is therefore taking advantage of some of the genes along this segment of DNA in the carcinogenic process. Since the other group of MPNST does not have this genetic alteration, it can't be the direct cause of the malignant aggression that evolves in these cells. We know that biallelic mutations in *NF1* are partly responsible for development of MPNST, but have also been found in benign neurofibromas[19]. This implies that *NF1* inactivation is not sufficient for malignant transformation. Alterations within the TP53 pathway[20-22] and the *CDKN2A* gene[3-5], are also known factors in the development of MPNST. In fact, polysomy or partly gain of chromosome arm 17q, are common features in several sarcomas[23,24]. One MPNST had a 1;17 translocation, and this genetic alteration has been seen in several neuroblastomas with gain of 17q and loss of 1p as a result of the translocation[25,26]. Ring chromosome of chromosome 17 material, as found in one MPNST, has also been found in dermatofibrosarcoma protuberans (DP), a tumor of low or intermediate malignant potential[27]. Within the group with 17q gains the two subgroups, one with a centromeric (TOP2A) amplicon the other with a distal amplicon, may also have different clinical impact.

The two groups of MPNST with and without 17q gains as suggested in this study do not pinpoint the initiating event(s) of the carcinogenesis, but reveal important molecular information of diseaseprogression. One group, with gains of 17q sequences, could be associated with a poorer prognosis, as indicated by Schmidt, H. *et al*[9], and Skotheim et al., 2003, and hence have impact on the choice of adjuvant treatments. The potential associations with clinical variables for tumors with a centromeric or distal 17q amplicon remain to be seen.

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Report II

Protein expression of cell cycle components in sporadic and neurofibromatosis type 1 related malignant peripheral nerve sheath tumors.

by

Helge Brekke

Protein expression of cell cycle components in sporadic and neurofibromatosis type 1 related malignant peripheral nerve sheath tumors.

Summary

Previous studies have suggested that some central cell cycle components, in particular deficient p16, contribute to the development of MPNST. A selected set (n=11) of cell cycle components and proliferation markers (n=2) as well as the neurofibromin protein was analyzed by immunohistochemistry on a tissue microarray with tissue cores from 106 MPNSTs and 3 neurofibromas. The tissue microarray sections were scanned with high resolution before visual scoring was done from the digital images. The expression data revealed that topoisomerase IIα (TOP2A) and Ki-67 are indeed expressed in most MPNSTs. Fewer tumors than expected had positive score of tumor protein 53 (TP53). Generally, expression of cyclins and CDKs were common among MPNSTs, leading to phosphorylated retinoblastoma (RB1) and G_1/S transition. The cyclin-CDK-complex inhibitors were on the other side absent from most MPNSTs, confirming the suspicion of lost control of the cell cycle checkpoint at G_1-phase.

Background

The molecular biological understanding of the development of malignant peripheral nerve sheath tumor (MPNST) remains mostly unknown. Patients with the autosomal dominant hereditary disorder Neurofibromatosis type 1 carry a germline mutation in their neurofibromin-producing gene *NF1*, and have a 5% risk of developing MPNST. This patient group accounts for 50% of all the MPNST cases[1]. The *NF1* tumor suppressor gene maps to chromosome band 17q11.2, and the gene product functions, among other things, as a GTPase activating protein (GAP), thereby playing an important roll in the MAP-kinase pathway. The GAP function of neurofibromin regulates the activity of RAS by converting the active GTP bound state of RAS into the inactive GDP bound form[2-4]. RAS is a proto-oncogene and if hyperstimulated it will, through ectopic activation of the MAP-kinase pathway, produce elevated amounts of oncogenic downstream target genes. Inactivation of both *NF1* alleles are also found in the benign precursor lesions, neurofibromas, showing that additional genetic events are necessary for malignant transformation[5-9].

The MPNST karyotype is typically complex, but no pathognomonic structural aberration has been found[10,11]. Previous studies have suggested that mutated *TP53* is important in the development of

MPNST[12-14]. However, the influence of *TP53* itself is debated (15). *CDKN2A* (alias $p16^{INK4A}$) has gene alterations in 50-75% of the MPNSTs[16-18]. The *in situ* protein expressions of these, as well as of a set of other proteins known to control the cell cycle, are examined in the present study.

Materials and Methods

Patients and samples

Tumor samples from 70 patients were included in the study. Data from 44 patients were available. Of these had 22 patients a history of NF1, 13 males and 9 females, the age ranged between 14-71 years with and average of 31. Of the sporadic cases were 11 males and 11 females, and in the age range of 20-85 years with and average of 50 years.

Cell lines

12 tumor types were cultivated in order to test for correct binding of the antibodies used in this experiment. The cell lines included four colon carcinomas (HTC116, Lovo, SW480, HT29) two prostates (PC-3, DU145), three testis (Tera2, NTera2, 2102Ep), one melanoma (129-melanoma), one fibrosarcoma (HT-1080) and one mammary gland (MCF-7). The cell lines were stored in -80^{o}C before use. The cell lines were maintained in Dulbecco's Modified Eagle's Medium (LGC Promochem, Boras, Sweden), supplemented with 10% bovine serum, glutamine and penicillin. The cell line cultures were incubated under standard conditions at 37^{o}C in a humidified environment with 5% CO_2 in the air. When the monolayer was confluent, a split ratio of 1:3 was used for the subcultures. The cells could easily be detached by a trypsin solution. The medium was changed every other day after confluence. On the final day the cell culture were washed in PBS followed by centrifugation for 5 minutes at 1000 rpm. The supernatant was removed, and 1 ml PBS was added to the cell culture. The solution was sonicated (exposure to high frequency sound waves) for 15 seconds, followed by centrifugation at 14000 rcf in 20 minutes at 4^{o}C. Fragments of the cell are collected into the bottom of the tube, while the proteins are now in the supernatant. They were stored in -70^{o}C until use.

Antibodies

The following antibodies (table 1) were used for in situ protein expression and controlled in a western blot procedure, performed on the cell lines described earlier.

Table 1. Primary antibodies used in the present study.

Antibody	Antibody Specification	Manufacturer	Dilution
TP53	Monoclonal mouse IgG2a anti-human (DO-1)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:1000
TOP2α	Monoclonal mouse IgG1 topoisomerase II alpha (3F6)	Novocastra Laboratories Ltd, Newcastle, UK	1:40
CDKN2A	Polyclonal rabbit IgG anti-human p16 (C-20)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:200
CDKN2C	Polyclonal rabbit IgG IgG anti-human p18 (M-168)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:50
CDKN1A	Monoclonal mouse IgG1-kappa anti-human p21WAF1 (EA10)	Oncogene Research Products	1:100
CDKN1B	Monoclonal mouse IgG1 anti-human Kip1/p27 (clone 57)	Transduction Laboratories, Lexington, KY, USA	1:100
CCND1	Monoclonal mouse IgG2a anti-human cyclin D1 (Ab-3)	Oncogene Research Products	1:500
CCND3	Monoclonal mouse IgG1-kappa anti-human cyclin D3 (DCS-22)	DAKO A/S, Glostrup, Denmark	1:50
CCNE1	Polyclonal rabbit IgG anti-human cyclin E (c-19)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:50
CDK2	Polyclonal rabbit IgG anti-human cdk2 (M2)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:200
CDK4	Polyclonal rabbit IgG anti-human cdk4 (C-22)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:50
RB1	Polyclonal rabbit IgG anti-human Retinoblastoma (C-15)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:400
Phospho-Rb	Polyclonal rabbit anti-human phosphorylated Rb (Ser795)	Cell Signaling Technology, Inc., Beverly, MA, USA	1:100
NF1	Polyclonal rabbit IgG anti-human Neurofibromin (D)	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:40
Ki-67	Monoclonal mouse IgG1-kappa anti-human (Ki-S5)	DAKO A/S, Glostrup, Denmark	1:25

Tissue microarray construction and use.

The tissue microarray (TMA) system was initially described in 1998[19]. The present TMA was constructed by transferring 105 cylindrical tissue cores (0.6 mm in diameter) from 67 formalin-fixed and paraffin embedded MPNSTs, and 3 neurofibromas (one plexiformed) (Skotheim et al., 2003, and expanded with more samples; Figure 1). The TMA is stored at room temperature and 4µm sections are cut from the tissue microarray and transferred onto slides using the Instrumedics paraffin tape-transfer system (Instrumedics, Hackensack, NJ, USA). The slide is then UV treated for 30-40 seconds to make covalent bonds between the tumor material and the slide. The tape is removed after using a dissolvent.

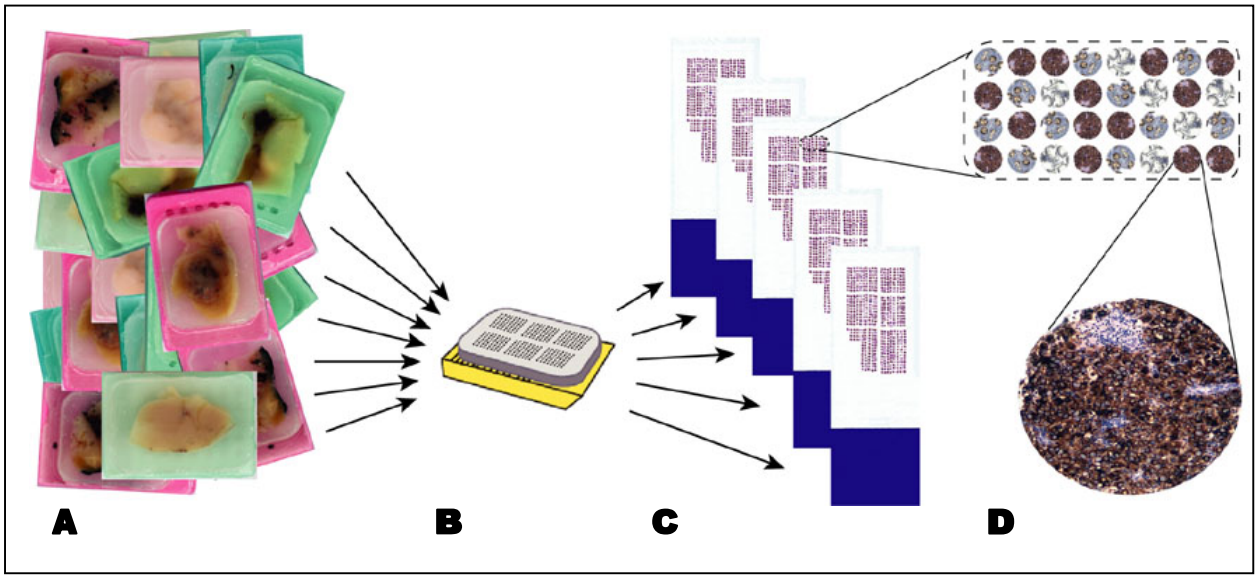

Figure 1. Tissue microarray (TMA) construction. Cylindrical core biopsies from each of the donor blocks (**A**) of formalin-fixed and paraffin embedded tumors are transferred onto a recepient block (**B**) that becomes the TMA. 4µm sections (**C**) are made and each transferred onto slides that will be submitted to immunohistochemistry (**D**). (Figure reproduced Rolf I. Skotheim).

The sections were deparaffinized in xylene and rehydrated via a series of graded ethanol baths (100% ethanol to wash away the xylene, followed by 95% and then 70%). Antigen retrieval was performed by heating in a microwave oven at 900W for 2 x 5 min using different buffer systems, (freshly prepared buffer consisting of either 0.1 m EDTA with 0.1% Tween pH 6.0, or XmM citrate buffer at pH 6.0). The antibodies used in the present study are listed in Table 1. Staining was performed with antibodies using the DAKO Envision kit (peroxidase/DAB). Endogenous peroxidase activity was blocked by applying DAKO's peroxidase block for 5 minutes, before incubation with the primary antibody for 30 minutes at room temperature. Then, the secondary antibody, conjugated with HRP labeled polymer was applied, and incubated for 30 minutes. Staining is completed by a 5 minutes incubation with 3,3'-diaminobenzidine (DAB) + substrate-chromogen which results in a brown-colored precipitate at the antigen site. Excess DAB was removed by distilled water, before the slides

were counterstained with haematoxylin, dehydrated in increasing grades of ethanol, cleared in xylene and mounted in depex.

A schematic layout of the finished TMA construction is shown in Figure 2. The TMA contained in addition to the MPNST and neurofibromas 30 cores from tissues types other than discussed in this report.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	1	3	4	5	5	3 III	40	41	41 II	41 III	41	38	45	46	47
B	3 II	6	7	7	8	9	43	43	44	44	20	20	48	49	50
C	10	10	11	12	13	13	38						51	52	53
D	14	15	15	16	17	17							54	55	56
E	18	18	19	19	20	20	20	2	42	42	25	25	57	58	102
F	21	22	22	23	24	26	28 conn.t.	28 conn.t.	normal kidney C1	normal kidney C1	kidney tumor C6	kidney tumor C6	103	104	105
G	26	27	27	28	29	29	colon carc C12	colon carc C12	Brain BU1-60	Brain BU1-61	Brain BU1-63	ovary C38	106	107	108
H	30	30	31	31	33	33	ovary C39	spinal cord BU1-54	spinal cord BU1-55	testis BU1-68	testis BU1-69	muscle BU1-4	109	110	43
I	32 (1mm)	34	35	35	36	37	liver BU1-40	liver BU1-37	liver BU1-37	Mal. melan.	Mal. melan.	Mal. melan.	41	111 (1)	111 (10)
J	37	39	39 II	39 II	40 II	40	Mal. melan.	Mal. melan.	Mal. melan.	Mal. melan.	Mal. melan.	Mal. melan.			

Figure 2. Schematic layout and contents of the TMA. The numbers refer to the different patients that the tumor samples were taken from. MPNSTs are shown in white, neurofibromas in yellow, and other tissues in grey (not analyzed in this study).

Figure 3 is a picture from a program used after the TMAs were scanned at high resolution. The staining pattern can then be scored on a computer screen, as shown here for antibody TP53. Switching between the cases is done by clicking on the little picture in the upper left corner, showing the TMA layout as in Figure 2. For the cell cycle markers, the immunohistochemistry scoring was done by categorizing the tissue cores according to the frequency of positively staining nuclei. The result was interpreted as negative if less than five percent of the nuclei were stained. Tissue cores with a positive score were classified into three categories; weakly positive (5-10%), positive (10-50%) and highly positive (50-100%).

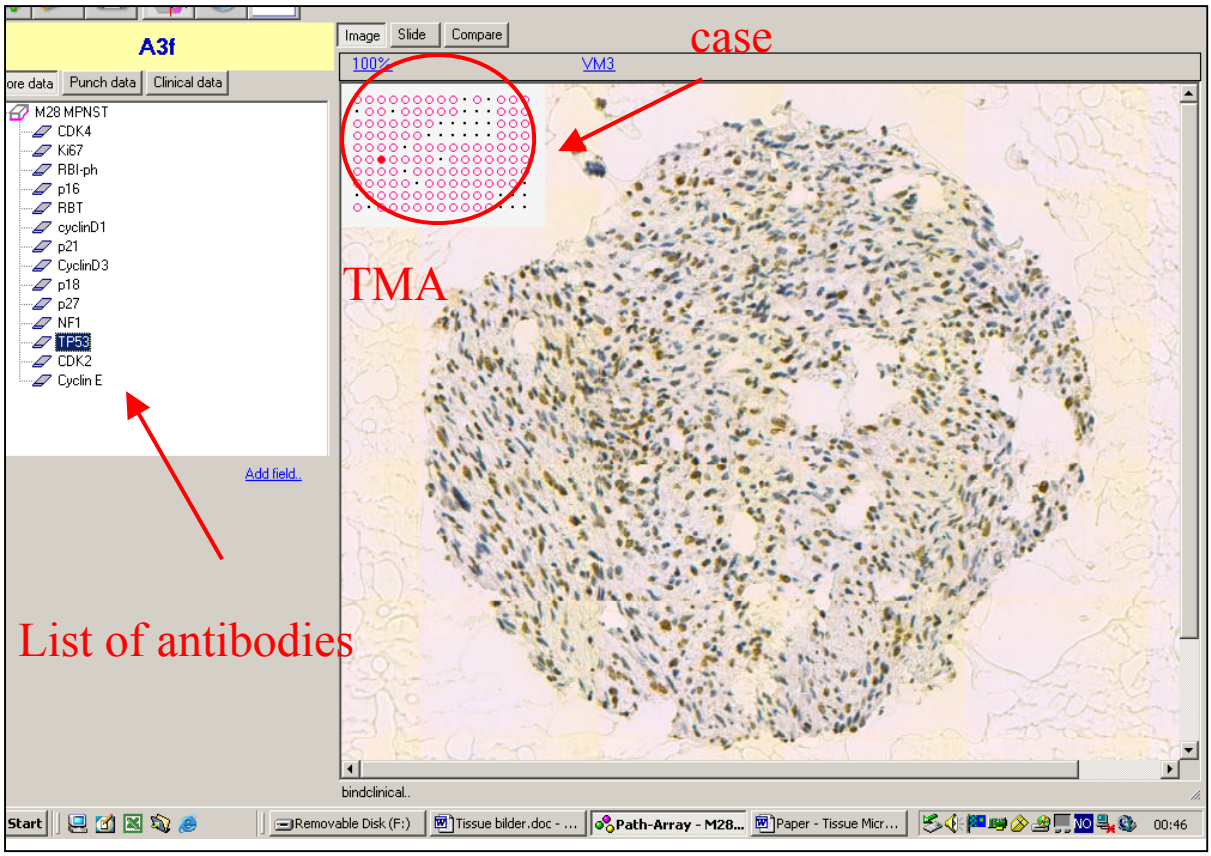

Figure 3. Scoring of the TMA. A screen shot of the software used to score immunohistochemistry on TMAs. The TMA sections were scanned after staining with the respective antibody.

Western blot analyses

In order to verify function and protein binding of the antibodies in use for the in situ expression analyzes, a Western blot procedure was performed. This was done on the 12 cultivated cell lines, assuming that at least some of them would express the protein in question.

Protein measurements

The protein concentration of each sample was measured before Western blot analysis. This was done to ensure that equal amounts of proteins were loaded onto the separation gel before the electrophoresis. The procedure is based on a method first described by Bradford (1976) [20]. The acidic dye, coomassie brilliant blue, binds to proteins with a subsequent change of its color when added to protein solution. The color change is a response to the protein concentration and can be measured with a spectrophotometer because of the switching of absorbance maximum from 465nm to 595nm. Protein concentration can be calculated using a standard curve.

The lysis buffer (M-PER Mammalian Protein Extraction Reagent, Perbio, Pierce) was diluted 1:100 with distilled water. The protein extract was made in the same proportions by adding 495µl distilled water to 5µl extract. 100µl and 200µl of the diluted protein sample were pipetted into two tubes and added 700µl and 600µl lysis buffer. Then 200 µl of Bio-Rad protein assay solution (coomassie brilliant blue G-250, Bio-Rad, Hercules, CA, USA) were added to each tube. To correct for background signals, two tubes with only 800 µl lysis buffer and 200 µl dye reagent were made. A standard curve was prepared by diluting bovine serum albumin (BSA) (Sigma) into six different concentrations, ranging from 0-4.0 µg/ml. Each standard dilution was added 200µl dye reagent and lysis buffer to a total volume of 1 ml. All standard and sample solutions were incubated in room temperature for at least 5 minutes before measured with a spectrophotometer (UV-1601 (Zhimadzu), Bergman, Lillestrøm, Norway) at 595 nm. The measurements were done within 30 minutes. A standard curve was plotted using the absorbance values (y-axis) against the known concentration of the standard samples (x-axis). The mean absorbance values of the samples were calculated and their protein concentrations were determined by using the standard curve.

Western blot procedure

The protein extract from the cell lines was thawed on ice and mixed with ice-cold lysis buffer (M-PER Mammalian Protein Extraction Reagent, Perbio, Pierce). A molecular weight marker (High Range Rainbow Molecular Weight Marker, Amersham) was diluted with lysis buffer. 5µl gel loading buffer (7ml 0,5 M Tris-HCl pH 6,8, 1g SDS (Bio-Rad), 3 ml glycerol, 1,2 mg bromphenol blue (Sigma), and 0.93 g dithiothreitol (DTT)(Sigma)) was added to all samples and the marker. The samples were denatured by boiling for 3 minutes, followed by a short centrifugation and left on ice until loaded on gel.

Western blotting

With the western blot technique a sample of proteins can be separated on a polyacrylamide gel, transferred to a nylon membrane, and then a specific protein can be detected. The process was first described by Towbin *et* al. in 1979, and further developed by Burnette in 1981 [21,22].
Denatured proteins are loaded onto a sodium dodecyl sulphate (SDS) polyacrylamide gel and separated, in accordance to the polypeptide size, by electrophoresis. A marker with a known molecular weight is loaded onto the gel with the rest of the samples, and used to estimate the size of the proteins after separation. The proteins are then transferred to a nylon membrane and treated with a blocking solution to saturate free binding sites and reduce non-specific binding. The antibody for the particular protein of interest is added in a solution so that it is covering all of the membrane. The antibody hybridizes to the antigen on the membrane that it recognizes. Unbound antibodies are washed away. The bound antibodies are detected by labeled secondary immunological reagents coupled to rabbit or mouse horseradish peroxidase (HRP). The detection system used in this study is based on chemiluminescence (ECL detection reagent, Amersham, Oslo, Norway), a substance that produces light during a chemical reaction in room temperature.

<u>SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)</u>
The concentration of the poly-acrylamide depends on the size of the protein that is to be detected. In a solid gel with a high percentage of poly-acrylamide can even small proteins be separated. Two sets of gel types were used, one with 6% poly-acrylamide, and one with 12%. The stock solution was a 40% acrylamide/bis (29:1) solution (Bio-Rad), mixed with 10% SDS, 10% ammonium persulphate (APS, Bio-Rad) 1.5M Tris-HCl pH 8.8, Tetramethylethylenediamine (TEMED), and distilled water. The gel mixture was poured between two glass plates in a vertical position with space left for a 4% stacking gel. A comb for 15

wells was immediately inserted into the stacking gel. One hour later the polymerization was complete, and the comb removed. The wells were then washed with buffer.
The electrophoresis apparatus (Hoefer SE400 Sturdier Vertical Units, Amersham) was filled with electrophoresis buffer (25mM Trisma Base, 50mM glycine, 10g SDS) before mounting the gel and loading of the samples mixed with gel loading buffer and lysis buffer. The electrophoresis was carried out at a constant current at 30 mAmp. for 30-60 minutes.

The proteins were then transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (Immobilon P-transfer, Millipore, Bedford, USA). Together with four pieces of 3MM Whatman filter paper (Merck, Darmstadt, Germany) the membrane was put in methanol, before arranged into a sandwich in a gel holder cassette. This was filled with transfer buffer (48mM Trisma Base, 39 mM glycine, 1.1 mM SDS, 200 ml methanol (Merck), and distilled water). The transfer was done at a constant current at 15 mAmp over night.

If protein visualization was required the membrane was placed in a tray containing amido black staining solution (1g naphtol blue black (Sigma), 450 ml methanol, 100 ml 100% acetic acid (Merck), and distilled water to a total volume of 1l) for 5 minutes. For destaining, the membrane was placed in a destaining solution (800 ml methanol, 20 ml 100% acetic acid and distilled water to a total volume of 1l) until the protein bands became visible and the membrane was not to dark. If the membrane was not used right away, the membrane was air-dried and stored in room temperature. Before hybridization a dried membrane was wet by methanol and washed in buffer.

Antibody staining and scoring

The membrane was blocked in a 10% dried skimmed milk solution (Nestle, Sandvika, Norway) solved in TBST buffer (20 mM Trizma Base, 0.5 M NaCl, distilled water and Tween-20 (Amersham)) for 60 minutes. The primary antibodies were diluted with 5% blocking solution and hybridized with the proteins on the membrane either over night at 4^{o}C, or in room temperature for 120 minutes. The membrane was then washed in TBST buffer before adding HRP-labeled secondary antibody (anti-rabbit IgG HRP / anti-mouse IgG HRP, Invitrogen), diluted in 5% dried skimmed milk blocking solution. After one hour the membrane was again washed in TBST buffer. For visualizing, the HRP-labeled secondary antibody ECL Western Blotting Detection Reagent (Amersham) was used as recommended by the supplier (Figure 4 and Figure 5). The membrane was placed in a film cassette together with an X-ray photo paper (Kodak, Amersham). The exposure time varied according to the staining intensities of the individual antibodies. The film was processed in an automatic developing machine (Curix 60, AGFA, Mortsel, Belgium).

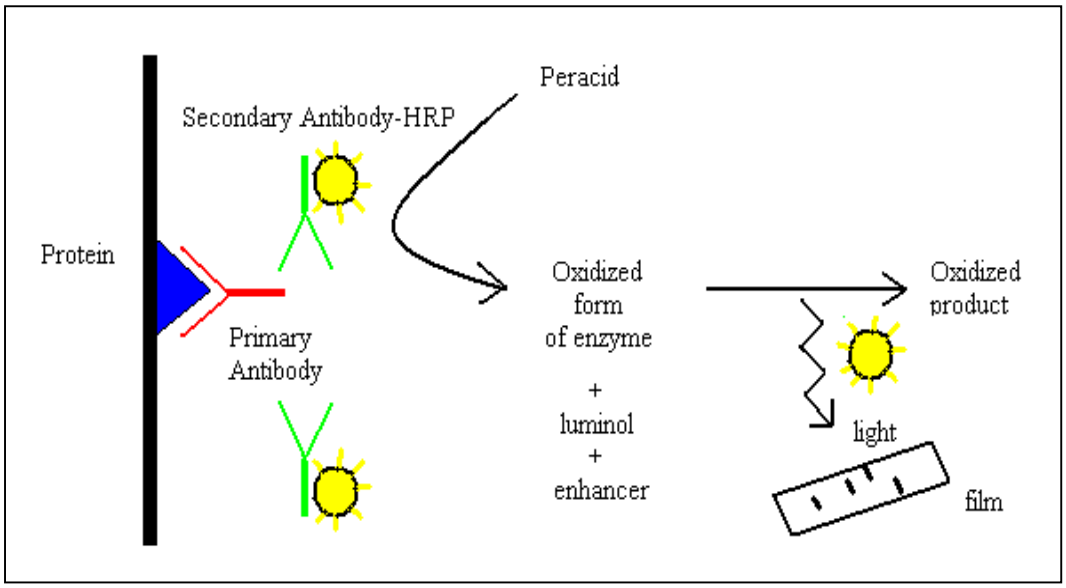

Figure 4) Principles of ECL Western blotting. The primary antibody binds to a specific protein, and is visualized by binding of a horseradish peroxidase (HRP) conjugated secondary antibody. The detection system used in this study is based on chemiluminescence (ECL detection reagent, Amersham, Oslo, Norway); a substance that produces light during a chemical reaction in room temperature (figure modified after Amersham's ECL Western blotting instructions guide).

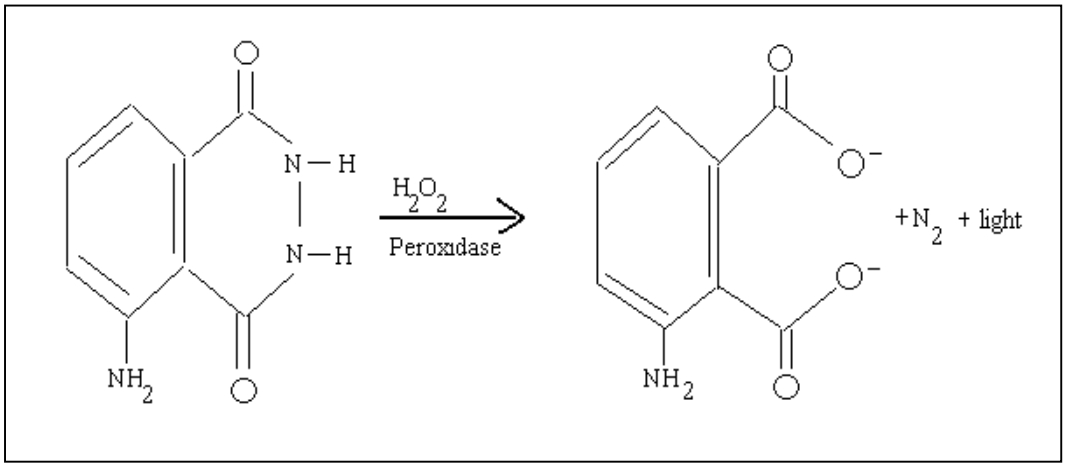

Figure 5) The chemiluminescence reaction. Luminol in the presence of chemical enhancers such as phenols, is in an excited state after oxidation, catalyzed by the HRP/hydrogen peroxide. Luminol then decays to ground state via a light-emitting pathway (figure modified after Amersham's ECL Western blotting instructions guide).

Scoring

Protein sizes were checked against the rainbow colored protein molecular weight marker (Amersham, Figure 6). The expression signals of the proteins on the Western blot films were scored as:

0	-	no expression
+	-	weak expression
++	-	moderate expression
+++	-	strong expression
(+)	-	no wt expression
R	-	rearranged protein
ns	-	not able to score

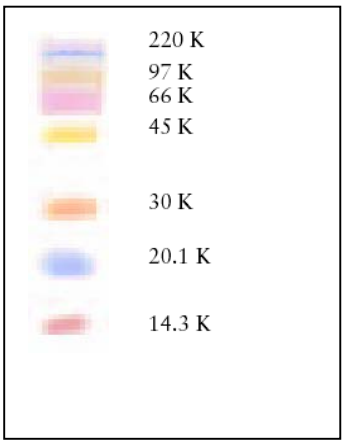

Figure 6. Rainbow marker

Results

In situ protein expression using tissue microarrays

Tumors with nuclear staining in more than 5% of the neoplastic cells were classified as positive, while those with less than 5% were considered negative. Most tumors had replicate tissue cores on the tissue microarray, and a tumor was considered positive when one or more of its tumor tissue cores were positive. The results of the in situ expression analyses are summarized in Table 2.

Table 2. Percentage of the MPNST nuclei with positive staining for a set of antibodies.

	Percentage of stained nuclei					
	0 %	**1-5 %**	**5-10 %**	**10-50 %**	**50-100 %**	number of samples
Antibody	**Percentage of tumors with same level of expression**					
CCND1	9 %	27 %	22 %	28 %	14 %	65
CCND3	59 %	13 %	9 %	12 %	7 %	67
CCNE1	53 %	17 %	15 %	12 %	3 %	59
CDK2	76 %	8 %	11 %	5 %	0 %	64
CDK4	0 %	0 %	0 %	49 %	51 %	43
Ki-67	24 %	15 %	17 %	35 %	9 %	66
TOP2A	4 %	11 %	9 %	45 %	31 %	45
CDKN1A (p21)	80 %	9 %	5 %	5 %	1 %	66
CDKN1B (p27/Kip1)	56 %	24 %	6 %	12 %	2 %	63
CDKN2A ($p16^{INK4A}$)	50 %	11 %	9 %	24 %	6 %	64
CDKN2C ($p18^{INK4C}$)	32 %	42 %	20 %	6 %	0 %	50
RB1	0 %	2 %	11 %	49 %	38 %	63
RB-ph	5 %	5 %	15 %	58 %	17 %	40
TP53	87%	0 %	4 %	5 %	4 %	55

The results of this table are visualized in Figure 7. The three categories of expression are

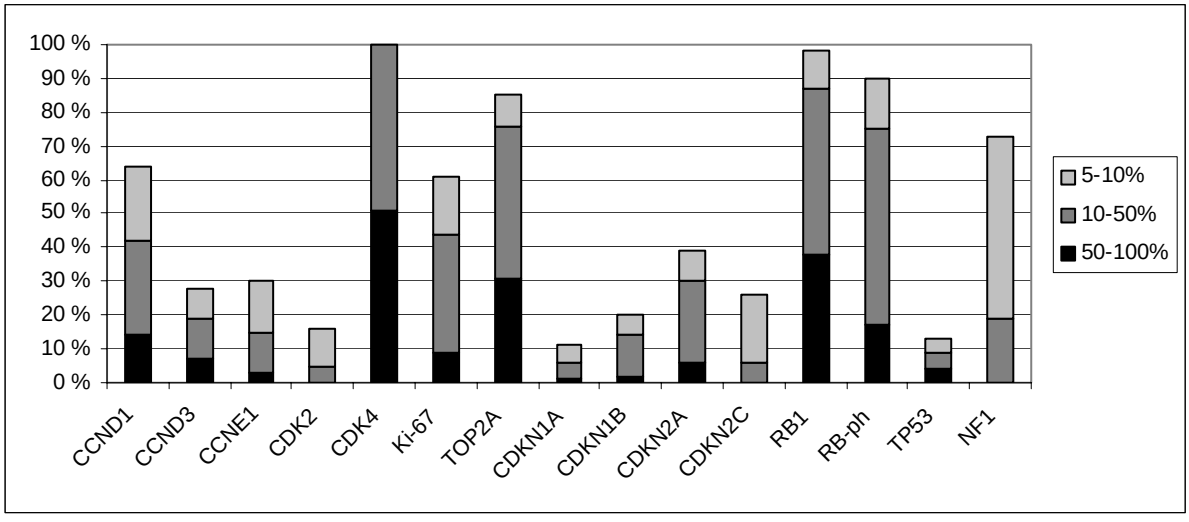

Figure 7. Graphical representation of protein expression in MPNST.

illustrated with different shades in grayscale, the darkest being the highest degree of expression, grey meaning moderate expression, and light grey meaning weak expression. As mentioned earlier the TMA slides with the *in situ* protein expression was scanned, and the

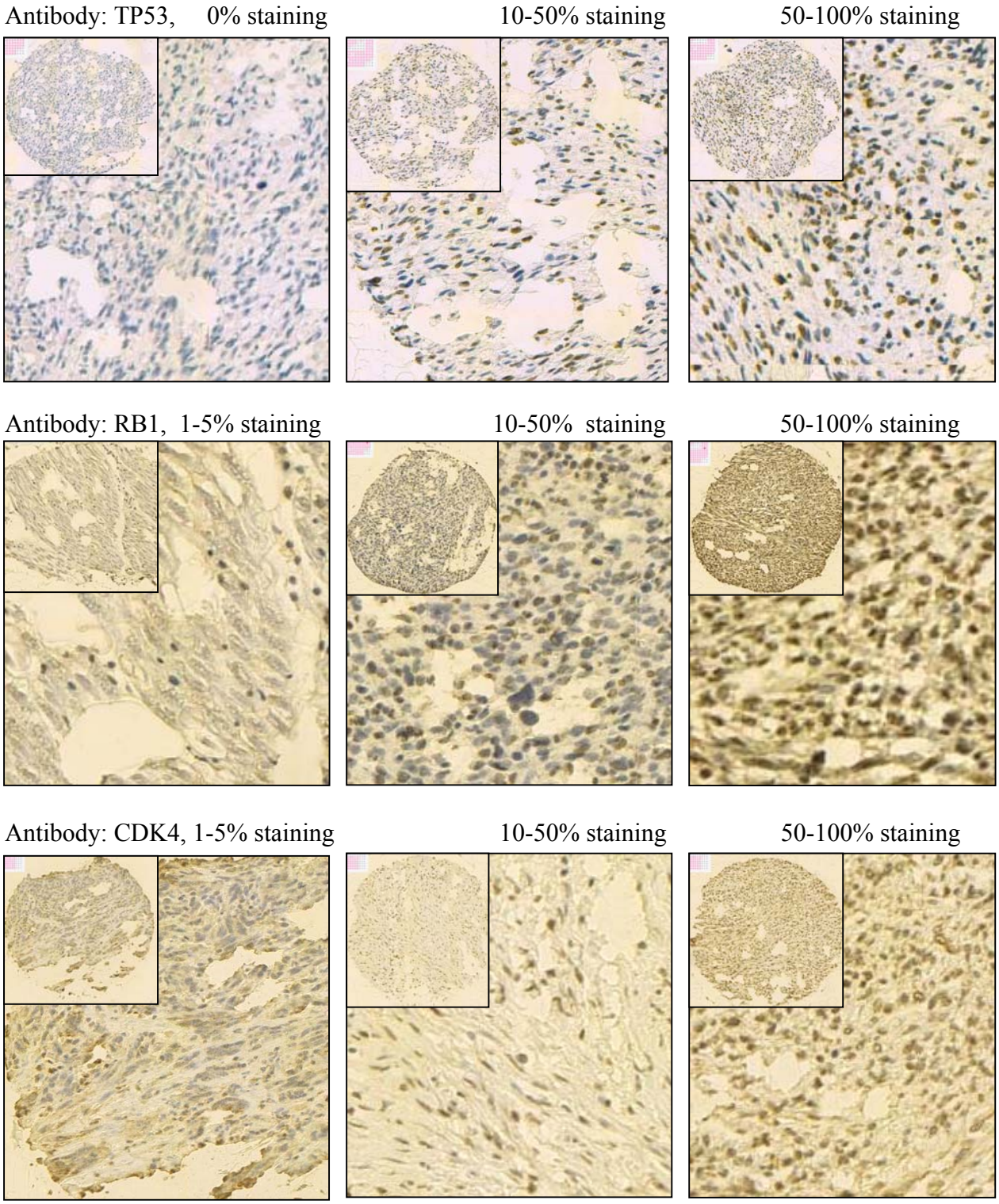

Figure 8. MPNST tissue microarray, immunohistochemical staining. For a collection of the analyzed proteins, representative tissue cores of one negative stained neoplasm, one moderately stained and one highly stained neoplasm are shown. The three antibodies tested here are TP53, RB1 and CDK4.

results were visually scored from digitized images. Figure 8 shows examples from this technique, and has three pictures from each of the antibodies TP53, RB1, and CDK4. The examples in the first column are images scored as negative due to less than 5% staining of the tumor nuclei. The examples in columns two and three have expression of the proteins in 10-50% and more than 50% of the nuclei, respectively.

For cytoplasmic protein expression, we used another set of categories; i.e., no expression, moderate expression and high expression (Table 3).

Table 3. Staining of cytoplasma in MPNST.

Antibody	Nonexistent	Moderate expression	High expression
CCND1	76	24	0
CCND3	99	1	0
CCNE1	84	10	6
CDK2	79	21	0
CDK4	76	14	10
CDKN1A (p21)	100	0	0
CDKN1B (p27/Kip1)	76	24	0
CDKN2A ($p16^{INK4A}$)	98	1	1
CDKN2C ($p18^{INK4C}$)	42	48	10
Ki-67	99	1	0
RB1	99	1	0
RB-ph	99	0	1
TP53	96	4	0
NF1	27	54	19

We also analyzed three neurofibromas, of which one was of the plexiform type. The neurofibromas were analyzed and scored in the same manner as the MPNSTs (Table 4).

Table 4. Degree of nuclear staining with a set of antibodies on neurofibromas.

	Percentage of stained nuclei				
	0	**1-5%**	**5-10%**	**10-50%**	**50-100%**
Antibody	**Percentage of tumors with same level of expression**				
CCND1	100 %				
CCND3	33 %	33 %	33 %		
CCNE1	100 %				
CDK2	67 %		33 %		
CDK4	67 %	33 %			
CDKN1A (p21)	33 %	33 %		33 %	
CDKN1B (p27/Kip1)	100 %				
CDKN2A ($p16^{INK4A}$)	67 %		33 %		
CDKN2C ($p18^{INK4C}$)	33 %			33 %	33 %
Ki-67	67 %	33 %			
RB1			33 %	67 %	
TP53	100 %				
NF1 (cytoplasma)		66 %	33 %		

The most highly expressed gene on chromosome 17 in MPNSTs as compared to neurofibromas, has been found to be Topoisomerase IIα (TOP2A)[46]. The detection of TOP2A expression in 85% of the MPNSTs in the present study was therefore not surprising, and is found highly expressed in other studies too[51].

Published frequencies of CDK4 positive MPNSTs have been ranging 4 % [(48)] to 46% [(51)]. In the current study, 100% of the MPNSTs had high CDK4 expression, whereas only 16% expressed CDK2. In the neurofibromas, there were no CDK4 expression, but one of the three was weakly positive for CDK2.

Expression of RB1 was measured with two antibodies. The "RB1" antibody is binding to both phosphorylated RB1, and none-phosphorylated RB1, while the "RB-ph" antibody is only binding the phosphorylated RB1. 98% of the tumors were positive for the "RB1" antibody, whereas and 90% were positive for the "RB-ph", an eight percent difference. These 8% represent unphosphorylated RB1. The fact that 90% of the tumors had phosphorylated RB1 indicates that the checkpoint between G_1 and S-phase is absent, and hence the restriction of E2F-transcription factors is lost. Overexpression of RB1 in MPNST has also been previously reported[48,49].

TP53 activates CDKN1A (p21) in response to DNA damage and cellular stress[50]. Other studies of sarcomas, including MPNST, have observed TP53 expression in 28%[(49)]-83%[(57)] of the analyzed cases. In this study, however, only 13% of the MPNSTs had TP53 expression. None of the neurofibromas had any expression of TP53, which is in line with previous studies[17,47,48,51,52].

A relatively low frequency (29%) of CCND1 expression in MPNSTs has been reported in previous immunohistochemical analyses[47,49]. In this study, 64% of the MPNSTs expressed CCND1. Only 28% of the MPNSTs expressed CCND3, and 30% CCNE1.
None of the neurofibromas expressed CCND1 or CCNE1, but one of the three expressed CCND3.

The *NF1*-gene product, neurofibromin, is expressed in the cytoplasm. By immunohistochemistry, NF1 expression was detected in 73% of the MPNSTs, but only in one of three neurofibromas. Inactivation of both *NF1* alleles is thought to influence on the malignant progression of the tumor. Therefore is the frequent expression of NF1 in the MPNSTs unexpected. The NF1 antibody (Santa Cruz) is raised against a peptide mapping to the carboxy terminal domain of the human protein. The antibody can detect proteins even if a large part is missing, or specific domains are non-functional, due to mutations in the gene. The expression of proteins with the catalytic GAP-related domain intact or functional can therefore be lower than detected.

In this study both CDKN1A and CDKN1B had expression in 11% and 20% respectively, of the MPNST in the nuclei, whereas CDKN1B had high cytoplasmic staining in 24% of the MPNSTs. This is lower expression for CDKN1A than reported before[49]. CDKN1B has normally weak expression in MPNST[49,51], but a stronger immunostaining in the cytoplasm in MPNST [49,51].
Of the neurofibromas one of three showed positive CDKN1A immunostaining. CDKN1B had no expression.

Thirty-nine percent of the MPNSTs had expression of CDKN2A ($p16^{INK4A}$). In other studies expression has been reported lower (4-9%)[18,48], and higher (46%)[51].
One of the three neurofibromas had some expression of CDKN2A.

CDKN2C ($p18^{INK4C}$) was expressed in 24% of the MPNSTs, and showed high expression in two of the three neurofibromas.

Ki-67 showed protein expression in 59% of the MPNSTs, a result that match another study [49].

Results of the Western blotting

The Western blotting results of the cell lines are shown below for each protein analyzed (Figures 9-18). The known functions of the proteins are indicated in a text box under each Western figure. The weak expression of some proteins in these western blotting, does not mediate with the *in situ* expression done on the TMA. The western blotting serves as a test for whether the antibodies bind to proteins of correct size, and whether the binding is specific.

TOP2A

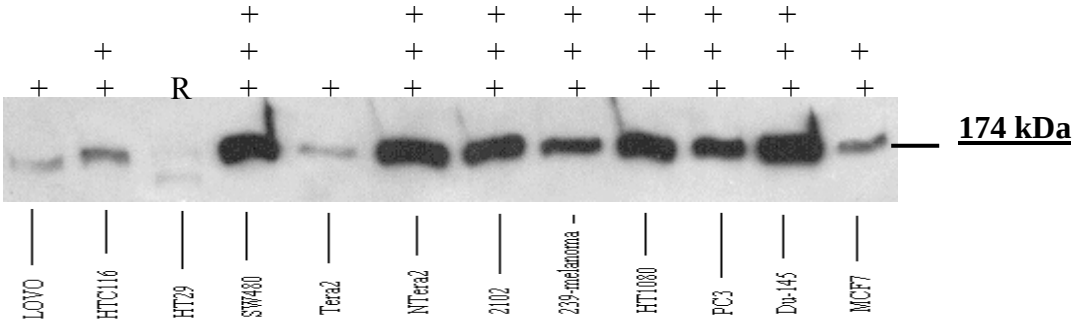

Figure 9. TOP2A. The wild type protein is expected to be of 174 kDa, and was clearly expressed in most cell lines. Seven cell lines had strong expression (SW480, NTERA2, 2102Ep, 129-melanoma, HT-1080, PC-3, Du145) while four had some expression (Lovo, HTC116, TERA2, MCF-7). One cell line, the HT29 showed two weak fragments of colon TOP2A, one wt at 174kDa and one smaller truncated protein at 160 kDa. This suggests a possible rearrangement of the gene due to a mutation.

Topoisomerase IIα is predominantly present in proliferating cells and is modified in M phase by phosphorylation at specific sites. This appears to be critical for mitotic chromosome condensation and segregation[32]. TOP2A is also the molecular target of several cancer chemotherapeutics.

CDK2

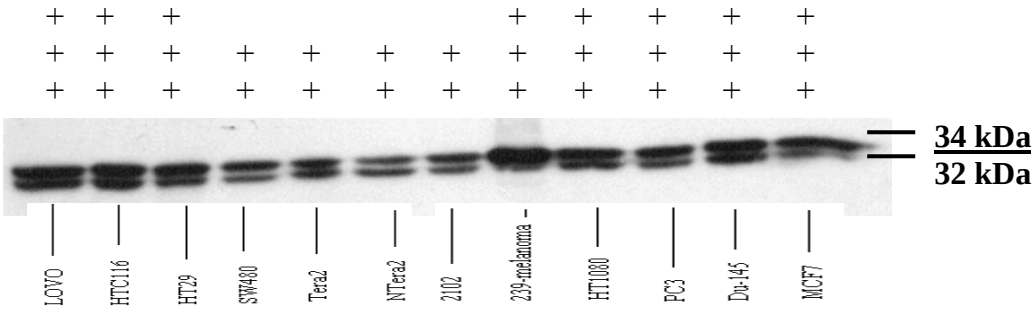

Figure 10. CDK2. The protein is 34 kDa, and was found to be expressed strongly in all of the cell lines. A 32kDa protein did also show up on the western blotting.

CDK2 binds CCNE1 and the complex phosphorylates RB1[37-38]. CDK2 is also essential for the G_2 to M transition in cell cycle control[39].

Cdk4

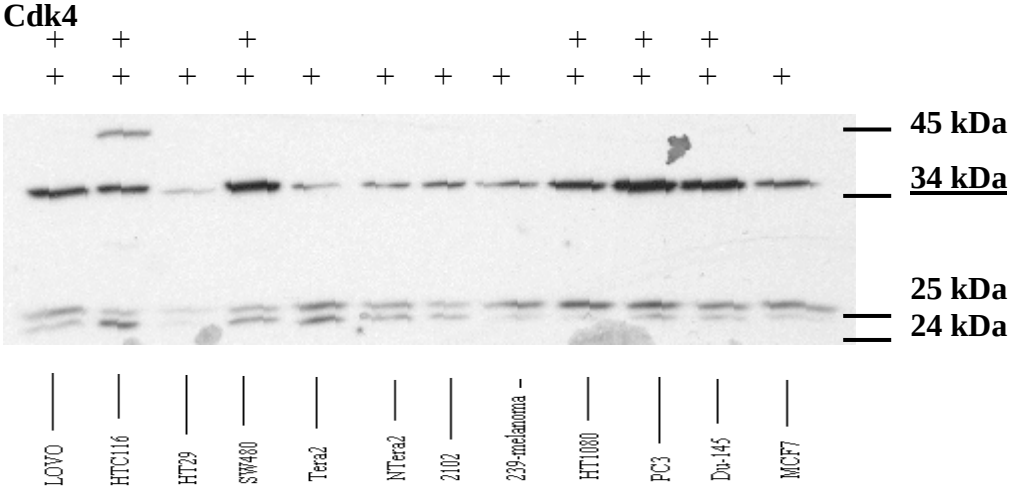

Figure 11. CDK4. The wild type protein is 34kDa, and was found to be expressed in all of the cell lines. Bands at 24kDa and 25kDa proteins also seen for all cell lines.

CDK4 forms holoenzymes with D-type cyclins early in G_1[36], and triggers transitions through late G_1, by phosphorylating RB1.

TP53

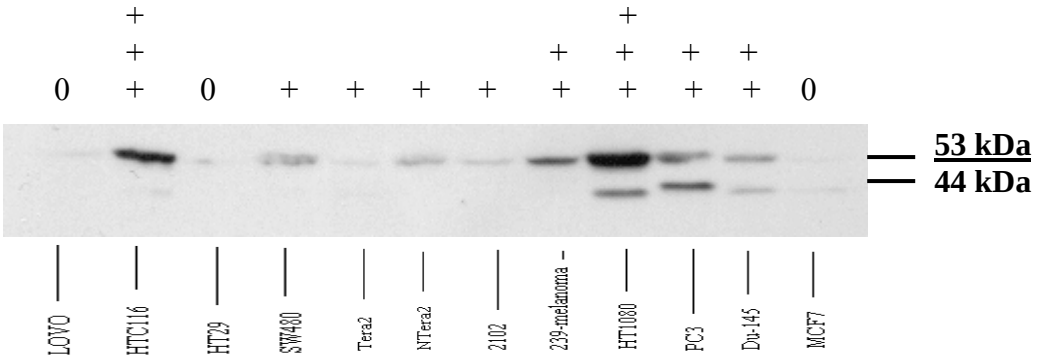

Figure 12. TP53. The wild type protein is 53kDa, and was expressed in 8 of the 12 cell lines. HTC116, 129-melanoma, HT1080, PC-3 and Du145 had moderate to strong expression compared to the four SW480, TERA2, NTERA2, and 2102Ep. Some products of 43-44kDa were found expressed in HT1080, PC3 and Du145. It is possible that these are smaller truncated proteins due to rearrangements of the gene.

> TP53 is a tumor suppressor protein that upregulates growth arrest and apoptosis-related genes in response to stress signals, and therefore controls programmed cell death and cell differentiation[40,41].

CCND1 **(cyclin D1)**

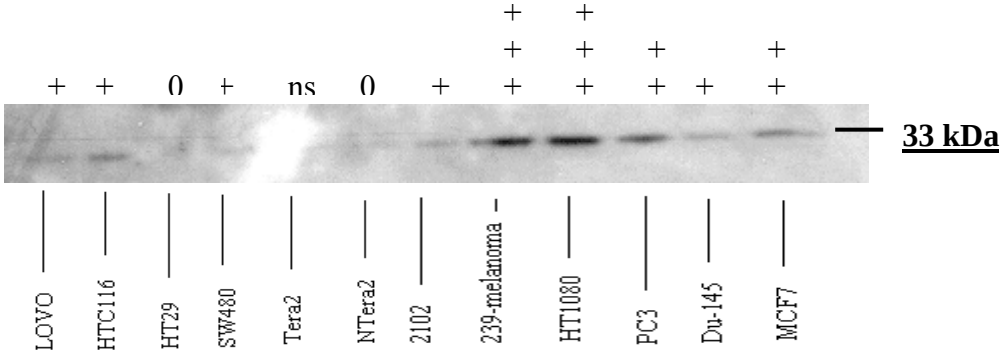

Figure 13. CCND1. The known protein is 33 kDa, and was found to be expressed in most cell lines. HT29 and NTERA2 showed no expression, but the blot is not optimal and has weaker signals than desirable, it is hard to say. It was not possible to score TERA2 due to a bright spot interfering.

> **The D family of cyclins appears in early G_1[33]. They form holoenzymes with CDK4 and CDK6 whose activity triggers transitions through late G_1, by**

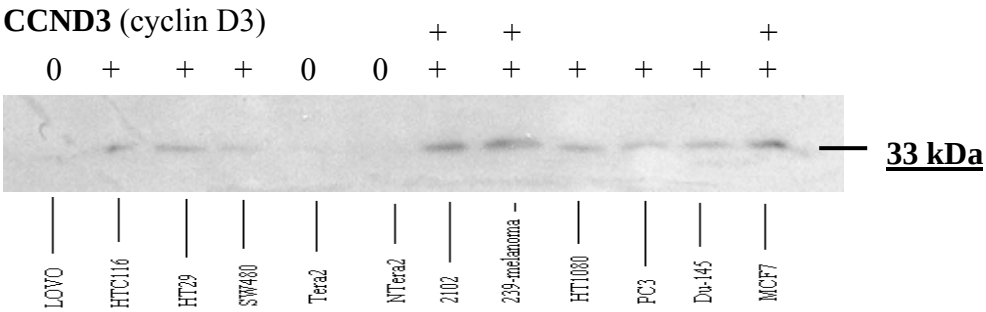

Figure 14. CCND3. The wild type protein is 33 kDa, and had mostly weak expression in most cell lines. Lovo, TERA2 and NTERA2 had no expression. This blot had 18 minutes exposure time with the X-ray photo paper, still the protein expression signals were weak.

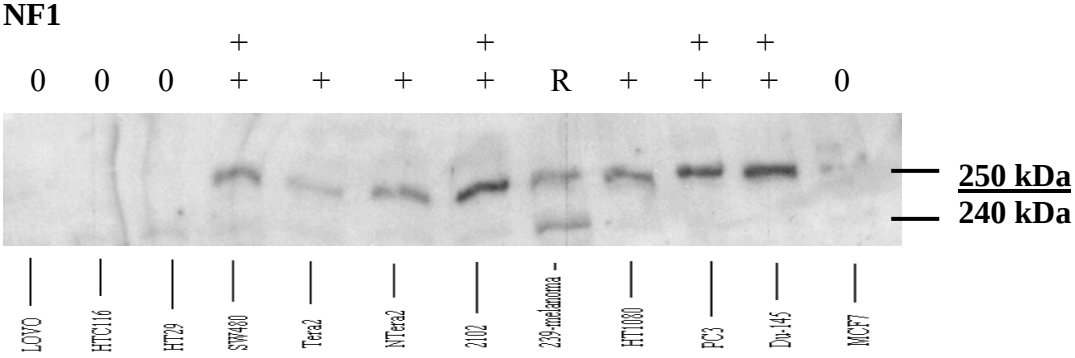

Figure 15. NF1. The wild type protein is 250kDa, and was expressed in 8 of the 12 cell lines. Lovo, HTC116, HT29 and MCF7 did not express any NF1. A 240kDa truncated protein was found expressed in the melanoma cell line (129-melanoma) and can be a mutation due to a rearrangement in the gene.

Although little is known regarding the function of NF1, the homology with the catalytic domain of proteins with GTPase activity suggests that NF1 may also interact *in vivo* with RAS proteins[2-4].

CCNE1 (Cyclin E)

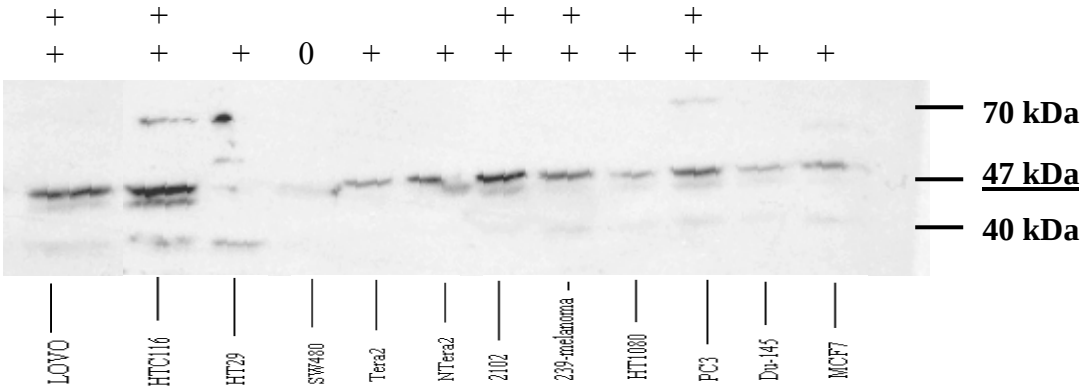

Figure 16. CCNE1. The wild type protein is 47 kDa, and was expressed in all cell lines, except for one colon cell line, SW480. In addition eight cell lines had weak expression of a protein with a protein size at 40 kDa, (Lovo, HTC116, HT29, 129-melanoma, HT1080, Du145, MCF-7). Two cell lines had also expression of a 70 kDa protein, (HTC116 and PC-3).

CCNE1 has its maximal level near the G_1/S boundary, after the cyclin D-CDK4 complex has initiated RB1 phosphorylation. CCNE1 then binds to CDK2, and the complex continues the RB1 phosphorylation, forming a positive feedback loop[37-38].

RB1

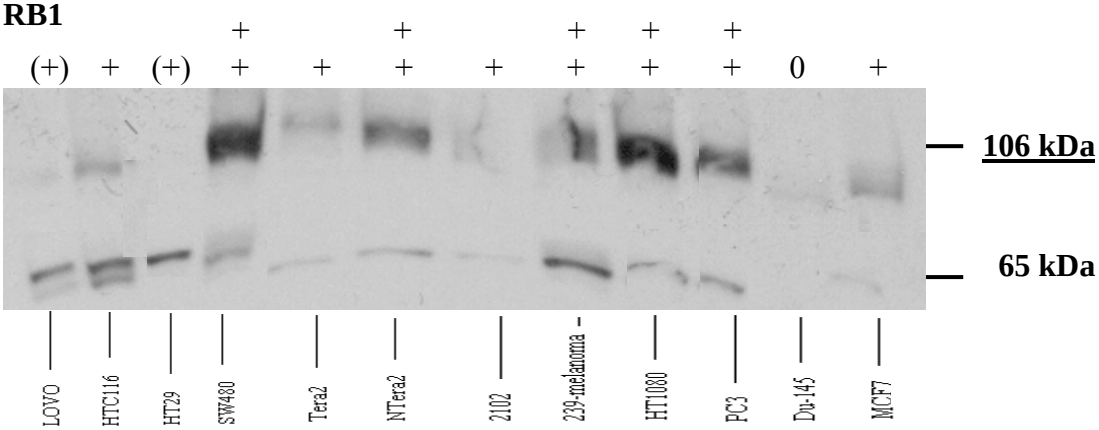

Figure 17. RB1. The wild type protein size is 106kDa and was expressed in nine cell lines. Lovo and HT29 expressed only a second band at 65 kDa, a band that were expressed in all cell lines, except in the prostate cancer cell line Du145, which had no expression at all.

The Retinoblastoma gene encodes a protein that is a potent inhibitor of E2F activation. The protein is phosphorylated from S to M phase of the cell cycle and is dephosphorylated in G1 [45].

Ki-67

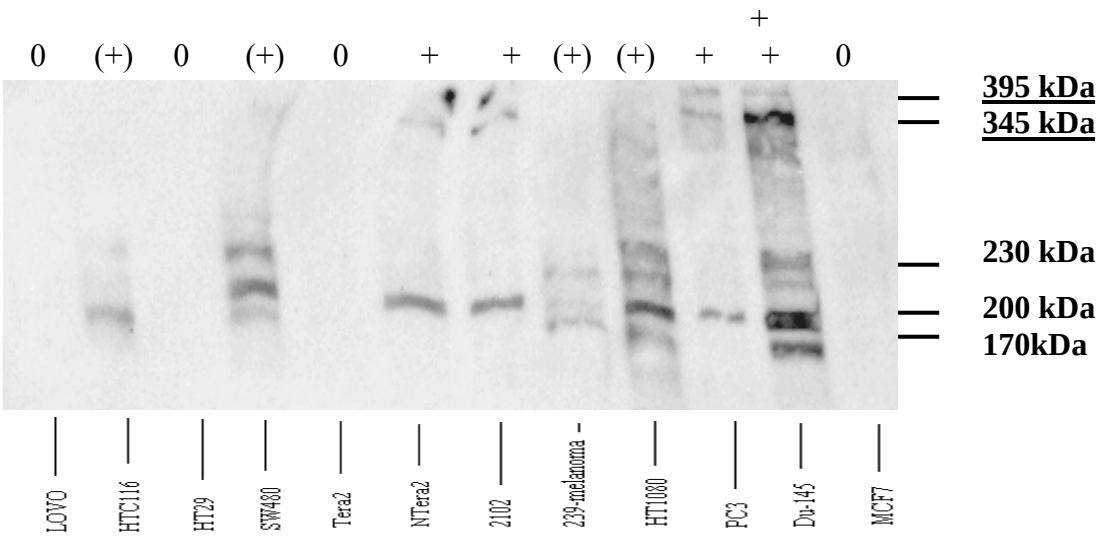

Figure 18. Ki-67. The two wild type protein sizes at 395kDa and 345kDa were detected in four of the cell lines (NTERA2, 2102Ep, PC-3 and Du145). Some other bands were also detected in these cell lines, and were also expressed in HTC116, SW480, 129-melanoma and HT1080. Lovo, HT29, TERA2 and MCF7 had no expression of KI-67.

The proliferation marker Ki-67 (395kDa and 345 kDa) is expressed during all active phases of the cell cycle, G_1, S, G_2 and M-phases, but it is absent in resting cells in G_0 phase[44].

For the inhibitors with a protein size smaller than 20 kDa, i.e. **CDKN2C** ($p18^{INK4C}$) **CDKN2A** ($p16^{ink4}$) **CDKN1A** (p21) and **CDKN1B** (p27/Kip1), were the Western blots not optimal, even after repeated hybridizations. We believe this is a technical problem. We did decide to analyze the antibodies on the TMA, knowing that the antibodies had been successfully used in a previous Western blot study of primary MPNST (Ågesen et al., J Neuropathol Exp Neurology in press).

Discussion

TOP2A is expressed in most MPNSTs. There is an association between high expression of TOP2A and poor patient survival. For patients with TOP2A expressing MPNST, only 38% were still alive five years after diagnosis, but among patients with TOP2A negative MPNST, 83% were still alive[46].

Of the cyclins tested, CCND1 had the highest expression in the MPNSTs (64%), whereas CCND3 and CCNE1 were expressed in about a third of the tumors. The D-type cyclins form complexes with and functions as a regulatory subunit of CDK4 or CDK6. An active CDK4/6-Cyclin D complex is required for cell cycle G_1/S transition. CDK4 was expressed in 100% of the tumors, meaning that the checkpoint of the G_1/S transition is probably out of control, again leading to an ongoing cell proliferation. This is confirmed with the findings of phosphorylated RB1 in 90 % of the tumors. The product of high CDK4 and CCND1 expression is phosphorylated RB1, followed by the activity of E2F transcription factors, and G_1/S transition.

TP53 activity was found in only 13% of the tumors. The gene is expressed after cellular stress or DNA damage, and without TP53's control of cell cycle arrest it is easier for a tumor to grow. The low expression of CDKNA1 and CDKN1B compared with other studies[49,57], can be a consequence of this, which normally would be activated by TP53, and inhibit cyclin-CDK-complexes.

CDKN2A and CDKN2C are also cyclin-CDK-complex inhibitors. CDKN2A was found expressed in 39% of the MPNSTs, which is more frequent than in other reports[18,48,51], whereas CDKN2C was expressed in 26%. It is possible that with low expression of CDKN2C, CDKNA1 and CDKN1B, G_1/S transition control is lost, and that the amount of cyclins and CDKs exceeds the inhibitory effect of the relative high CDKN2A expression alone.

The quality of the expression scorings will be controlled by a pathologist. The total clinical database are under construction. After this is achieved the protein expressions will be evaluated in relation to each other as well as to the clinical variables.

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