NOVEL MARKERS OF THE PROGRESSION OF HUMAN MELANOMA WITH SPECIAL CONSIDERATION OF THE ALTERNATIVE SPLICE VARIANTS OF CD44

PhD thesis

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1. List of abbreviations

12-LOXarachidonate 12-lipoxygenase

17AA17 amino acid

ADP Adenosine diphosphate

AMLAcute myeloid leukaemia

APAdult primary (tumour)

AON antisense oligonucleotid

ASP alternative splice pattern

ATPadenozin-trifoszfát

BBP branch point binding protein

bFGFbasic fibroblast growth factor

CD20cluster of differentiation 20

CD33cluster of differentiation 33

CD34cluster of differentiation 34

CD44cluster of differentiation 44

CD61cluster of differentiation 61

CD117cluster of differentiation 117

CSF-1colony stimulating factor 1

DEPCdiethylpyrocarbonate

ECD extracellular domain

ECM extracellular matrix

EDTAethylenediaminetetraacetic acid

EMTepithelial-mesenchymal transition

ERestrogen receptor

ESEexonic splicing enchancer

ESS exonic splicing silencer

FGFRfibroblast growth factor receptor

GISTgastrointestinal stromal tumour

HER2human epidermal growth factor receptor 2

HEV high endothelial venule

HBMEhuman brain microvascular endothelial (cell)

hn-RNPheterogenous nuclear ribonucleoprotein

HUVEChuman umbilical vein entothelial cell

HPETE5-hydroperoxyeicosatetraenoic acid

ICD intracellular domain

ISE intronic splicing enchancer

ISS intronic splicing silencer

IVLCintravenously implanted lung colony

kDakilo Dalton

KITLGKIT ligand

KSKaposi sarcoma

KTSlysine threonine serine

LMD laser microdissection

LTA4leukotriene A4

LXA4lipoxin A4

LXB4lipoxin B4

MAPK 1MAP kinase 1

MAPK3MAP kinase 1

MEKmitogen activated protein kinase

MPNMmetastasis from the primary tumour created by implanting cells from a metastatic tumour of a newborn animal

mRNAmessenger ribonucleic acid

NCTCnewborn circulating tumour cells

NLMnewborn lung metastasis

NMnewborn metastasis

NSCLCnon-small cell lung cancer

NPnewborn primary tumour

PAR-1protease-activated receptor

PCRpolymerase chain reaction

PNMprimary tumour created by implanting cells from a metastatic tumour of a newborn animal

RAF1rapidly accelerated fibrosarcoma 1 gene

RAR-αretinoic acid receptor alpha

RPMIRoswell Park Memorial Institute

sCD44soluble CD44

SCFstem cell factor

SCFRstem cell factor receptor

snRNP small nuclear ribonucleoprotein

snRNSsmall nuclear RNA

SR protein serine and arginine–rich protein

SSMsuperficially spreading melanoma

TGF-ßtransforming growth factor beta

TPARE12-O-tetra-decanoyl-phorbol-13-acetate TPA response element

VEvariable exon

VE-cadherinvascular-endothelial cadherin

VEGFvascular endothelial growth factor

VEGFR-2vascular endothelial growth factor receptor-2

WHOWorld Health Organistaion

WT1 Wilms' tumour 1

2. Introduction

With the advent of modern molecular biological techniques in the late 1980's and early 1990's our knowledge on cancer pathogenesis and progression has expanded leading to a revolution in clinical practice. Numerous new drugs are being developed to target new markers, and they are in various stages of trials and clinical practice. Targeted therapy has now become the goal.

As the available techniques get more sophisticated, our understanding of the molecular biology of cancer becomes more complex. In the bottleneck of the process of translating basic research into therapy, is diagnostics. The tools of histo-, cyto- and molecular pathology must be created with the understanding of underlying molecular events as well as their manifestation through applying the available diagnostic methods. One must be prepared to integrate new data into ones daily practice and alter it accordingly. Deviation from the anticipated immunohistochemical panel should not be put down anymore as a result of poor technique and thought should be given to other underlying causes, such as molecular events, that would change the result of such a reaction. The implications are hugely important, as diagnoses and prognoses could be altered this way.

Better understanding of the complexity of molecular events might also lead to uncovering causes leading to 'resistance' to these newly developed drugs.

However, the technological boom in molecular biology did not happen on its own. It was accompanied by the rapid development of information technology. Today, we have huge databases, hundreds of articles published every day, new techniques being developed, used and then altered to fit the latest research findings. It is worth taking a step back, to look at our tools and re-examine what examination results really mean.

Our research group has developed and worked with models of tumour progression for decades. As the available techniques evolved so have our understanding of molecular processes. The current work aims to examine novel markers of tumour progression at molecular level, possible candidates for targeted therapy.

2.1. Tumour progression

The death of cancer patients is, for the most part, caused by disseminated, metastatic disease. Metastasis formation is a multi-step process, the so-called metastatic cascade, all steps of which need to be completed for successful tumour progression (Figure 1). If

the cell fails to achieve all steps, or if the process is interrupted by human intervention at any point, the formation of distant metastasis is unlikely.

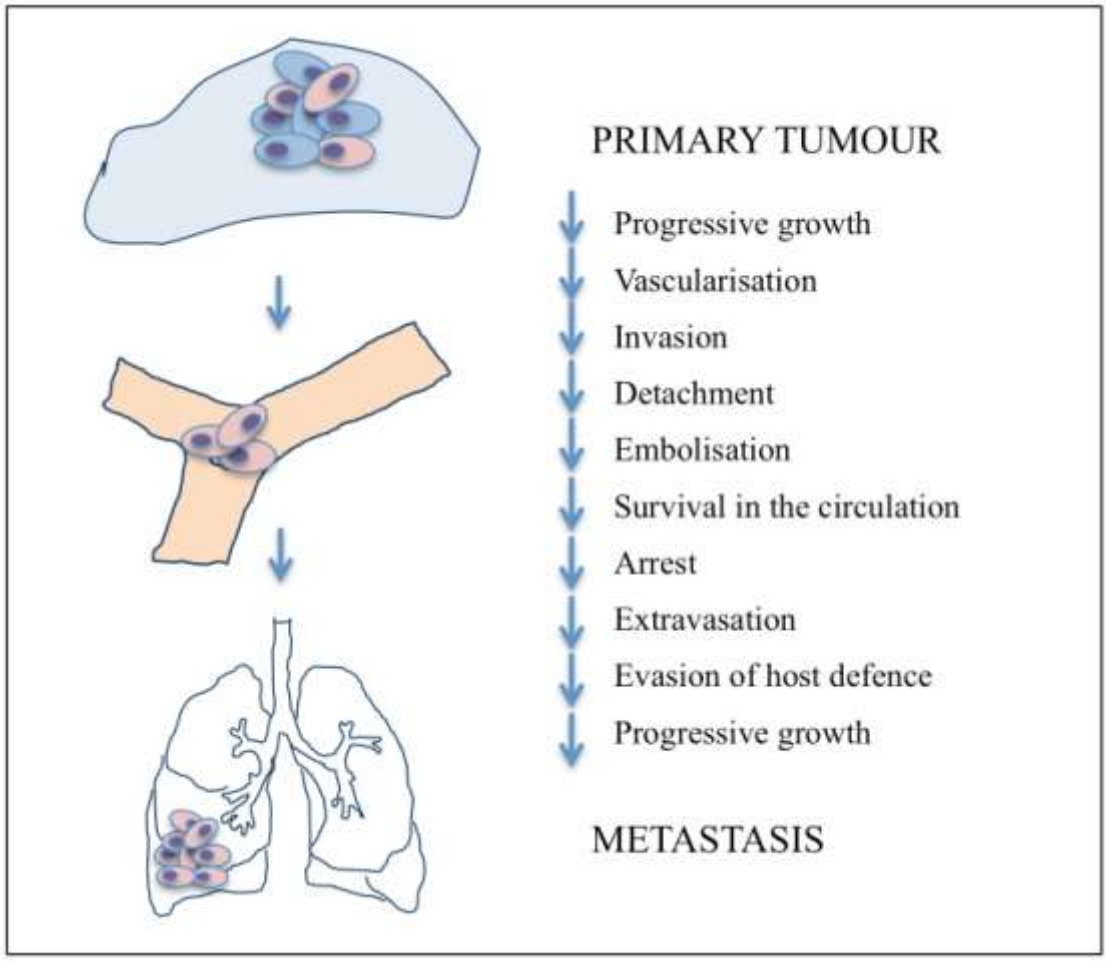


Figure 1. The steps of metastasis formation (1)

Better understanding of this process and identifying the individual molecules and their interactions can provide more than just data in the literature. It might uncover markers that can be used in the diagnosis and differential diagnosis of malignancies as well as provide possible clinical markers of progression and targets for anti-tumour therapy.

For example, much focus has been on bevacizumab (Avastin), a monoclonal antibody, that interferes with one of the firsts steps of tumour progression by inhibiting angiogenesis through binding to vascular endothelial growth factor A (VEGF A). This drug is now part of the therapeutic panel in the treatment of metastatic colorectal carcinoma, non-squamous non-small cell lung carcinoma, metastatic renal cell carcinoma and recurrent glioblastoma multiforme. (2)

However, unfortunately, the available drugs are currently being utilized (Figure 2) during the treatment of disseminating / disseminated disease and much emphasis has been put onto identifying targets during the earlier stages of carcinogenesis (2).

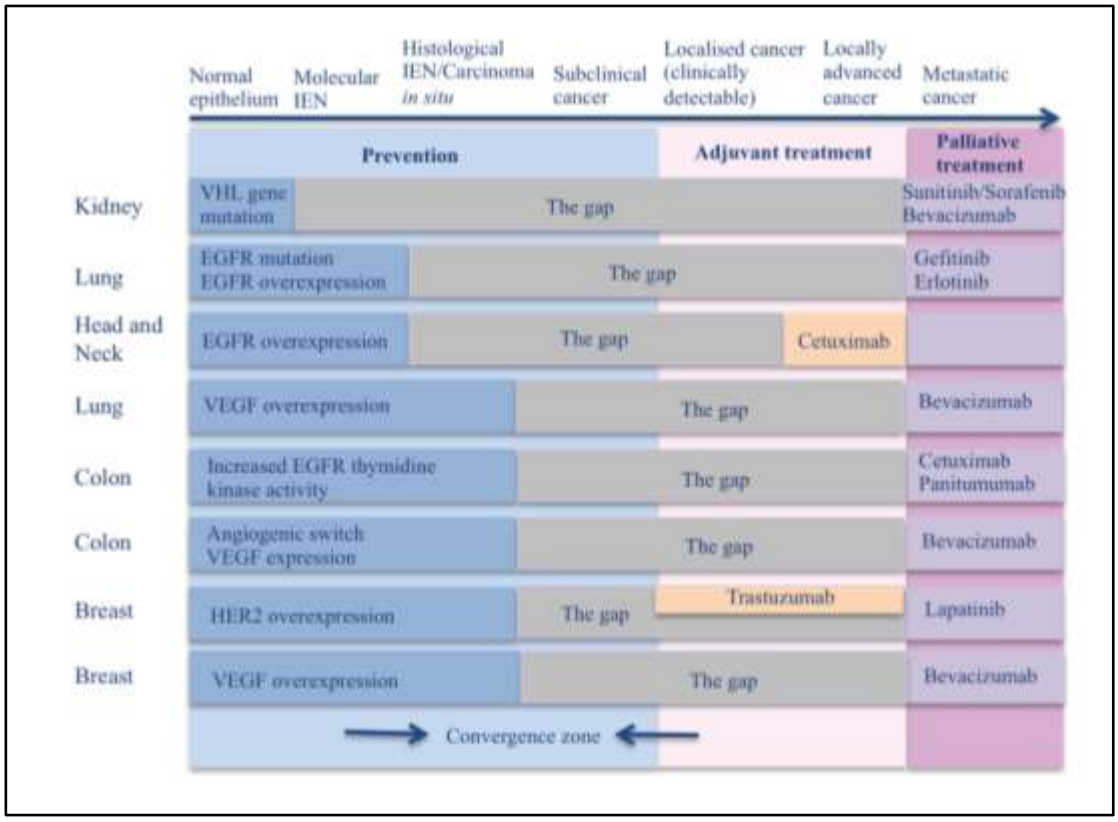


Figure 2. Available targeted therapies are mainly being applied in the later, progressive phase, rather than the early, carcinogenic stage. (2)

Therefore, there is much need to characterize the earliest stage of carcinogenesis and stages of tumour progression, to provide points of intervention in an earlier, more manageable stage.

2.1.1.Our previous studies on tumour progression

As it has been described above, much hope has been put into characterizing the process of angiogenesis to provide early access to the process of tumour progression. To study this, one must characterize the expression pattern of angiogenesis related molecules during tumour progression.

There are a number of other tumour derived molecular markers, which might support an increased angiogenic phenotype. The above-mentioned angiogenic cytokines are

heparin binding for the most part and their function depends on cell surface heparin sulphate proteoglycans. In another series of experiments, we examined the expression of the expression of the v3 variable exon of the CD44 proteoglycan, which enables the protein to bind heparin-binding proteins, *in vitro* human endothelial cells (HBME, HUVEC, KS) and in tumour vessels *in vivo*. PCR measurements using v3 specific primers proved the constitutive expression of CD44v3 in KS and HUVEC, while no such expression was observed in HBME. CD44v3 expression was further confirmed at protein level within the above cell lines as well as in the microvasculature of human melanoma and glottis carcinoma *in vivo*, which can be further proof of tumour induced angiogenesis. (3)

Detection of the Wilms tumour 1 gene (WT1) one can also serve as a novel endothelial marker. Endothelial cells are derived from angioblasts that differentiate from bone marrow stem cells, both of which show characteristic constitutive WT1 expression. We described the expression pattern of the different WT1 splice variants in HBME and KS cell lines using PCR. This expression was further confirmed at protein level in the above cell lines as well as in tissue derived from he biopsy samples of haemangioma, Kaposi sarcoma and angiosarcoma. This shows, that WT1 expression is conserved during angiogenesis and malignant transformation therefore WT1 can also be regarded as an endothelial marker. (4)

WT1 expression was then further examined in metastatic and non-metastatic animal models of Kaposi sarcoma, developed by our group. In this model, the primary and metastatic tumours as well as the circulating metastasizing tumour cells were all found to be WT1 positive. WT1 therefore could be used as a marker of progression of the endothelial-derived Kaposi sarcoma. (5)

Angiogenesis was thought to be the result of ingrowing, host-derived endothel. However, in one of our studies, we detected the presence of bone marrow derived endothelial progenitor cells (BMEP) in the peripheral blood of patients suffering from non-small cell lung carcinoma (NSCLC), which correlated with said patients' prognosis. Quantitative PCR measurements showed decreased expression of CD33, VE-cadherin and CD34 stem cell markers, while the expression of VEGFR-2 increased in the peripheral blood of NSCLC patients. Also, this increased VEGFR-2 level remained increased after therapy of therapy resistant cases. (6)

The most detailed, and one could argue most accurate, picture could of course be obtained from analyzing the entire genome of the tumour. Our group also performed full genome analysis of non-metastatic and metastatic human melanoma. (7)

2.1.2. Malignant melanoma and its progression

Malignant melanoma, the malignant tumour of melanocytes, and as such it mainly occurs in the skin, but a smaller, yet still noteworthy percentage arises in other organs, such as bowel or the retina (uveal melanoma).

The incidence is on the rise, with 160 000 newly diagnosed cases and 48 000 deaths worldwide, according to the most recent WHO reports (8). The effects of this disease are even more devastating as the last decades showed a marked decrease of the average patient age from 46-50, to 15-34. The death of the patients is for the most part caused by the metastases of the disseminated disease, with a median survival (MS) of 6-9 months and a 3-year survival of 10-15% historically.

Although early detection of malignant melanoma is improving, thanks to patient education and visibility of the primary tumour, making surgical treatment of the earlier stages more and more effective there are limited treatment available for progressing and advanced disease. This tumour type is highly resistant to chemo and radiation therapy and there has been a remarkable lack of progress in the development of new treatment modalities.

The most recent clinical trials are centered on targeted therapies, such as non-selective (Sorafenib, failed in phase III) and selective (Vemurafenib, promising phase III results despite increased risk of cutaneous squamous cell carcinoma) BRAF inhibitors, MEK (AZD6244 and GSK1120212, phase II) and c-kit (Imatinib, phase II) inhibitors. Collecting data on possible markers of progression and targets for therapy is ever pressing, with the likes of the Targeted Therapy Database of the Melanoma Molecular Map Project now being available online.

2.1.3. Markers, commonly associated with an aggressive phenotype, examined by us

2.1.3.1. α2bβ3(GpIIbIIIa)integrin

The GPIIb/GPIIIa is a platelet membrane glycoprotein integrin complex, made up by glycoproteins IIb and IIIa forming the alpha and beta subunits respectively. It mediates platelet aggregation and thrombus formation by acting as a receptor for fibrinogen. The

receptor activation is under strict control: ADP binding results in a calcium dependent association of the subunits. The complex also acts as a receptor for von Willebrand factor and fibronectin. Certain mutations of the GPIIb or GPIIIa genes result in Glanzmann thrombastenia, an autosomal recessive bleeding disorder caused by failure of platelet aggregation and decreased / absent clot retraction.

The inhibition of this integrin's functions is of importance in cardiovascular medicine. GpIIbIIIa inhibitors, such as abciximab, eptifibatide and tirofiban are used to prevent platelet aggregation and thrombus formation during percutaneous coronary intervention and to treat acute coronary syndromes.

Although its expression is considered highly megakaryocyte-specific [integrin β3 is a cluster of differentiation on thrombocytes (CD61)] and most of its importance is in haemostasis and haematological disorders, there are studies describing ectopic GpIIbIIIa expression in human colorectal (10) and prostatic adenocarcinoma (11, 12). This ectopic expression does not only enable tumour cells to exhibit platelet-like characteristics and interaction with platelets, but also provide a possibility of targeted therapy using anti-platelet agents.

2.1.3.2. bFGF (basic fibroblast growth factor, FGF-2)

As one of the 22 members of the fibroblast growth factor family (FGF), bFGF has a wide range of functions, including mitogenic, angiogenic, and neurotrophic effects. This 18kDa non glycosylated polypepdtide consists of 146 amino acids in its mature form, which derives from a 155 amino acid precursor. (13) Members of the FGF family bind to the four transmembrane tyrosin kinase FGF receptors (FGFR 1-4) to a varying degree. bFGF binds all four of them with high affinity as well as binds to heparin sulfate proteoglycanswith lower affinity.

It is expressed by almost all tissue types of mesodermal and neurectodermal origin and cancer types derived from these tissues. Its expression shows a strict chronological and topological pattern and many of the cells store the protein in a biologically inactive form. Inactive forms can be activated during inflammatory processes and proliferation of malignancies.

bFGF stimulates the growth of many cell types, including endothelial cells, fibroblasts, myoblasts, neuronal cells, chondrocytes and myoblast.

bFGF is also a potent angiogenic factor (14) by controlling the proliferation and migration of vascular endothelial cells, enchancing their plasminogen activator and collagenase activity. (15)

It is also one of the genes involved in tumour angiogenesis, facilitating the invasive growth and metastasis formation. Strong bFGF expression has been described in gliomas (16), in melanoma (17) and prostate tumours, where its been linked to tumour progression (15).

bFGF also plays an important physiological role in tissue regeneration and wound healing.

Monoclonal antibodies directed against bFGF inhibit the growth of tumour cells. (18)

2.1.3.3. Arachidonate 12-lipoxygenase (12-LOX)

12-LOX is anarachidonic acid metabolizing enzyme of the lipoxygenase pathway. It is a non-heme iron-containing dioxygenase that catalyses the stereo-specific peroxidation of free and esterified polyunsaturated fatty acids generating a spectrum of bioactive lipid mediators. Its main substrate is arachidonic acid, that is converted to (12S)-hydroperoxyeicosatetraenoic acid/(12S)-HPETE but can also metabolize linoleic acid. It also converts leukotriene A4 (LTA4) into lipoxin A4 (LXA4) and lipoxin B4 (LXB4). Through the production of specific bioactive lipids like (12S)-HPETE it regulates different biological processes including platelet activation. (19)

It enhances the expression and secretion of the angiogenic factor, vascular endothelial growth factor (VEGF) thus providing a direct link between this enzyme and angiogenic properties. Such angiogenic properties have been demonstrated in prostate cancer. (20-22)

2.1.3.4. c-kit (Mast/stem cell growth factor receptor (SCFR), tyrosine-protein kinase Kit or CD117)

C-kit, coded by the KIT gene, is a receptor tyrosine kinase. When activated by its ligand, stem cell factor (SCF/KITLG) it activates various intracellular signal transduction pathways resulting in survival and proliferation of the cell. It also plays an important role in the regulation of haematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration and function, and in melanogenesis. In response to KITLG/SCF binding, KIT can activate several signalling pathways, including AKT1, RAS, RAF1 and MAPK1 (ERK2) and/or MAPK3 (ERK 3).

It is important cell surface marker of hematopoietic stem cells (HSC), multipotent progenitors (MPP), and common myeloid progenitors (CMP) of the bone marrow. It is also expressed by mast cells, melanocytes and interstitial cells of Cajal in the digestive tract.

Activating mutations of c-kit are associated with gastrointestinal stromal tumours (GIST) (23), classical seminoma of the testis (24), acute myeloid leukaemia (AML) (25) and malignant melanoma. Detection of the mutational status of these tumours is important, as only mutations in exon 11 (GIST) and not exon 17 (testicular seminoma, most AML) will result in therapeutic response to treatment with tyrosine kinase inhibitor Imatinib.

2.2. The problem studying the expression and behavior of single molecules

Working in a research group heavily involved in the basic research of tumour progression, one is presented with the opportunity to be involved at studying the different aspects of the different steps of the metastatic process. There are many different techniques involved and new discoveries might changeour view on past results.

There is a phenomenon, which we constantly came across and started to deal with from early on. A molecular process which changes how we interpret our results at every single level. This phenomenon is alternative splicing.

2.3. Alternative splicing

The discovery of alternative splicing brought revolutionary changes into the world of molecular biology disproving the ’single gene - single protein’ theory. However, at the same time it has helped to resolve the inequality between the proteom and genome described by the Human Genome Project, i.e. that there are a lot less functioning genes than it was expected on the basis of the protein functions. It is now obvious, that even according to the most careful of estimations, at least 74% of all genes posses at least one, but rather more splice variants. This might result in the synthesis of structurally and functionally different protein isoforms from the same gene (26).

Contrary to constitutive splicing, many different variants are synthetised, which together form the alternative splic pattern (ASP) of a given gene. This pattern frequently changes depending on the type, the environment, the level of differentiation of the cell, therefore trying to characterise the role and function of the original gene might be

challenging without knowing the attributes and functions of its splice variants. The ASP is very finely regulated, so that the gene can fulfill its role in the physiological function of the cell, therefore changes of this pattern can lead to pathophysiological processes, such as malignancies. (27)

2.3.1. Constitutive splicing

Alternative splicing is in fact a 'modified version' of constitutive splicing, therefore one must understand constitutive splicing before starting to learn about alternative splicing.

The process through which the introns are removed from the pre-mRNA is called constitutive splicing – this happens within the spliceosome. The classical spliceosome is formed by the dinamically changing system of six (U1, U2, U3, U4, U6 and U6) snRNPs (small nuclear ribonucleoprotein) of different functions. The splice site is marked by characteristic sequences on the introns: the 5' (donor) site at the beginnig of the intron starting with a GU dinucleotid and the 3' (acceptor) site at the end of the intron, a sequence ending with AG dinucleotid (GU-AG rule). 20-50 base pairs away from the 3' (acceptor) splice acceptor site in 5' direction one finds the branch point, which is coded by a single adenin nucleotid, conserved across all the existing genes, within the middle a consensus sequence [CU(A/G)**A**(C/U)]. A pirimidin-rich region is also needed, the enrichment of which in purin nucleotides can weaken the binding site (Figure 3). These are the sequences the snRNAs (small nuclear) bind to with their complementary sequences and from the different snRNPs with the surrounding proteins.

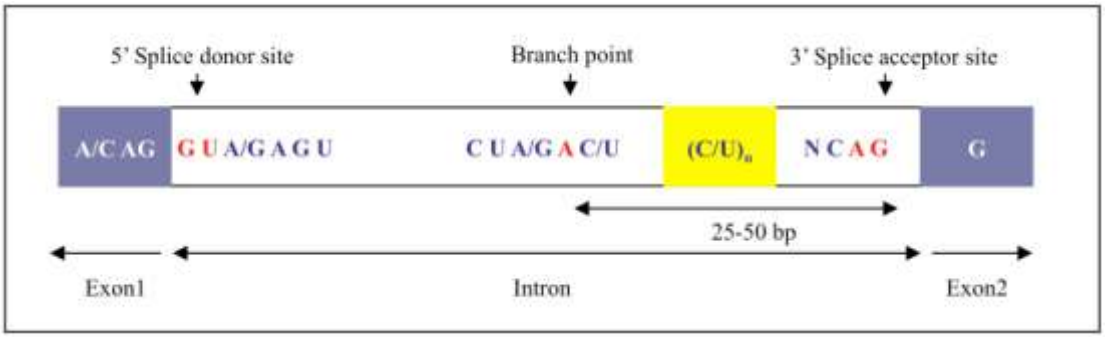


Figure 3. Position of the sequences needed for splicing.

First the BBP (branch point binding protein) and U2AF bind the branch point (Figure 4A), later giving place to the U2 snRNP (Figure 4B), which binds to the branch point with its consensus sequence while the U1 snRNP binds the 5' splice donor site. At this point the U4/U6•U5 'triple' snRNP (Figure 4C) enters the spliceosome, in which the U4

and U6 snRNAs bind to each other strongly with their comlementory sequences, while U5 has a weaker interference with the two others. This is followed by the re-arrangement of multiple RNA-RNA interactions, including U4 being rejected from the spliceosome before the end of the splicing, which allows the now cathalytically active U6 to bind to the 5' splice site, taking over from U1 (Figure 4D). Further re-arrangements foms the active binding site of the spliceosome and bring the pre-mRNA substrate into appropriate position for splicing (Figure 4E) (28). The energy needed for these RNA-RNA interactions is provided by ATP hydrolysing proteins, also parts of the snRNP (29).

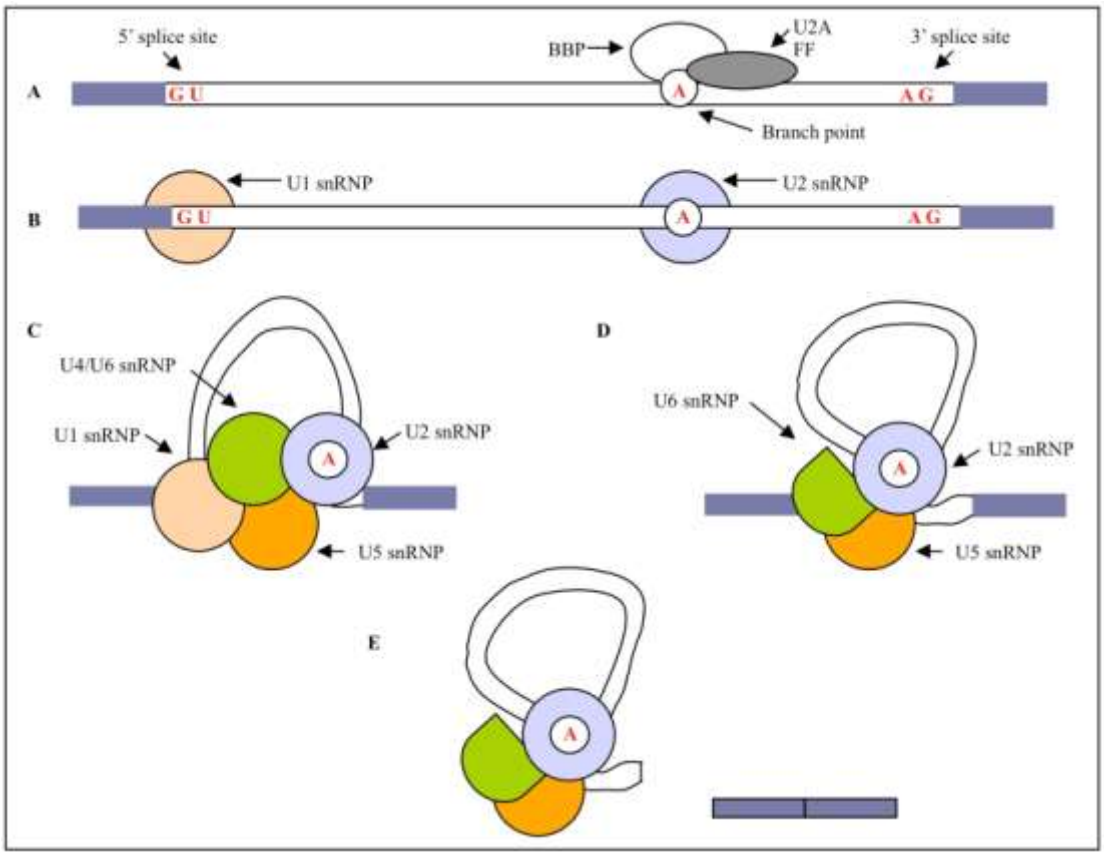


Figure 4. The mechanism of splicing. Binding of the BBP and U2AF proteins (A) after the detachment of which the U2 and U1 snRNPs bind (B). The U46U6•U5 triple snRNP enters the spliceosome (C), the splice loop forms followed by the separation of the 5' and 3' ends (D). The process end with the fusion of the exons (E).

There is another splicing mechanism, spliceosome, using the AU-AC rule, in which the sequence at the 5' donor site starts with AU and the sequence at the 3' acceptor site ends with AC dinucleotids (30). However, it is important to emphasize that while the

middle exons are bordered by 5' donor and 3' acceptor regions, the 5' end of the first exon is formed by the cap region and the 3' end of the last exon is forlem by the polyadenilation region (poly-A tail), which might be of importance for alternative splicing.

2.3.2. Alternative splicing

The donor and acceptor consensus sequences of the constitutive splicing are strong binding sites for the snRNPs. However, they can become weak binding sites by changing a bases: their affinity to the binding proteins / snRNPs weakens according to the number of the altered bases. This observation might explain the process of alternative splicing, namely there are such cis acting short, ~10 bp long sequences, which facilitate or arrest the recognition of these weak binding sites with the help of regulatin SR proteins (serin/arginin rich proteins) and hn-RNPs (heterogenous nuclear ribonucleoprotein), or by forming such secondary structure of the pre-mRNA, that prevents SR proteins from binding to the neighbouring regulatory sequences (31). These sequences are called ESE (exonic splicing enchancer), ESS (exonic splicing silencer), ISE (intronic splicing enchancer) and ISS (intronic splicing silencer), based on their position within the exon / intron and whether the given exon / intron will be part of the mature mRNA due to their mutation (Figure 5).

This is how certain point mutations can affect splice sites by creating silencer/enchencer sequences and giving rise to the synthesis of new mRNA isoforms (32). Strong acceptor/donor sites can become weak as well, activating alternative splice sites. The possibility of forming alternative splice site sequences also arises, which also results in the synthesis of different mRNA/protein isoforms. The new protein isoforms generated via alternative splicing are frequently structurally as well as functionally different from the wild type protein (33).

There are many different forms of alternative splicing depending on the type and the localisation of the splice sites.

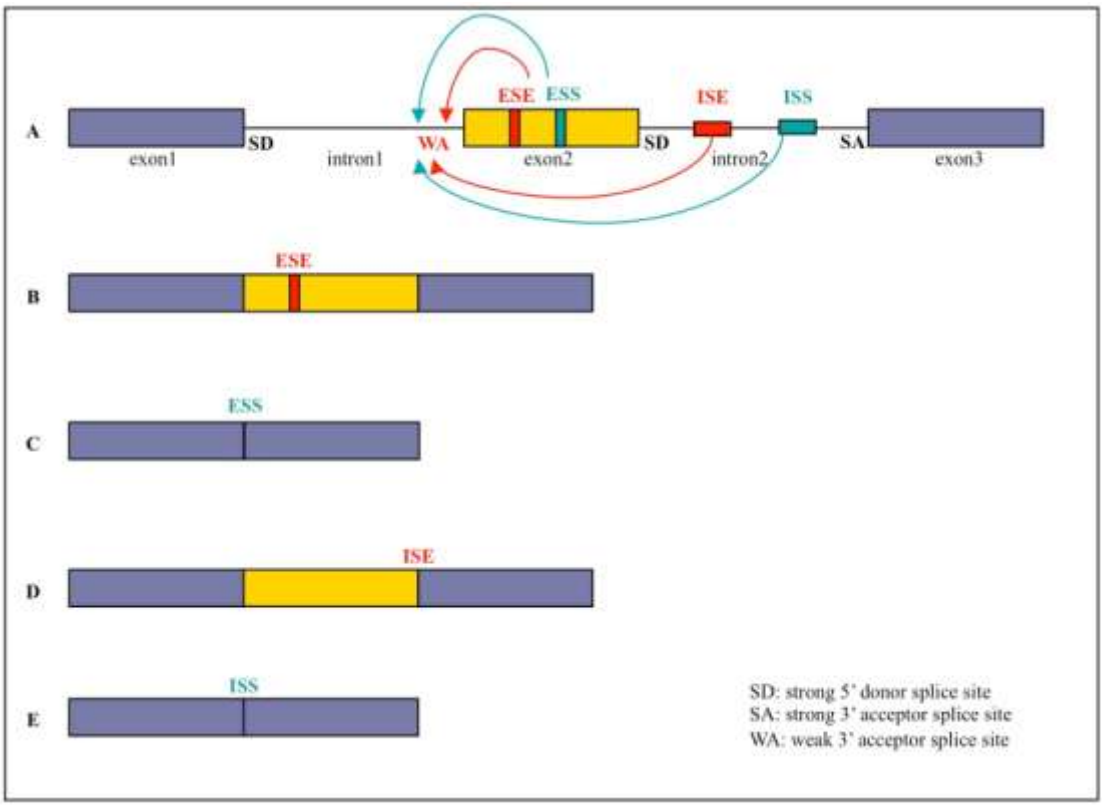


Figure 5. Use of the weak splice site. The presence of a splice enhancer (ESE) sequence on a given exon results in a functioning cis position weak binding site (A), therefore the exon will be part of the mature mRNA molecule, while the presence of a splice silences sequence (ESS) arrests the function of the same binding site (B), therefore the exon will not be part of the mature mRNA molecule. Equally, the exon can be 'retained' due to the presence of a intronic splice enchancer (ISE) sequence affecting the weak splice acceptor site at its 5' side (C) or 'drop out' due to the presence of a intronic splice silencer (ISS) sequence affecting the weak splice acceptor site at its 5' side (D).

2.3.2.1. Exon skipping

In the presence of overlapping enchancer and silencer sequences on the exon, the inhibitory protein binding the ESI prevents U2 binding to the weak 3' splice acceptor site at the 5' end of the exon and if the ESI forms such secondary structure of the mRNA that prevents SR protein binding to the ESE region, the exon will be spliced out via alternative splicing (Figure 6C) (31).

In case of an overlapping enchancer and silencer sequences on the exon, the ESI region binding inhibitory protein inhibits U2 binding to the weak 3' splice acceptor site at the 5' end of the exon resulting in such secondary structure of the ESI and mRNA molecule, which inhibits SR protein binding to the ESE region and results in splicing of

the exon itself (Figure 6C). However, succesful SR protein binding stops the inhibitory protein from working and the exon will be expressed in the mature mRNA molecule. (Figure 6B).

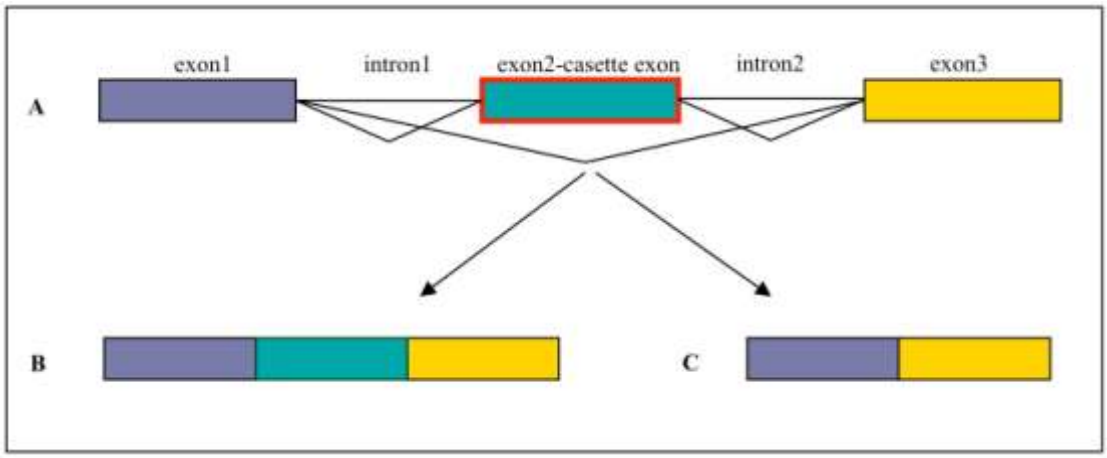


Figure 6. During the process of exon skipping from the pre-mRNA molecule (A) both a wild type (B) and a casette exon free isoform (C) could be syntesised

The function of the isoform, which does not contain the exon could differ from the function of the wild type isoform. It might even result in constitutive activation, like it is the case after exon11 skipping of the Ron molecule. (34) The most recent studies already examine the possible therapeutical utilisation of exxon skipping. There are clinical studies underway to examine the role of certain antisense oligonucleotids (AON), which could restore the reading frame and resulting in a functionally intact protein in Duchenne muscle dystrophy via targeted exon skipping. (35)

2.3.2.2. Alternative 5’ splice site

In addition to the proximal, 5’ donor site, there might be an alternative 5’ splice site (5SS)on the exon positioned at the 5’ end of the intron,competing for the 3’ acceptor site of the intron. (Figure 7) The U1 snRNP might bind to both 5SSs, initiating the use of the alternative 5’ splice site with the rest of the splicing machinery. (36)

SF2/ASF, one of the most important SR protein splicing factor increases the U1 binding of all 5SS sites and its increased expression will push the balance towards using the proximal splice site on the intron. On the other hand, the presence of hnRNP A1, which competes for mRNA binding with SF2/ASF, will decrease the mRNA binding capability of U1, resulting in the use of the 5’ alternative splice site on the exon. (37)

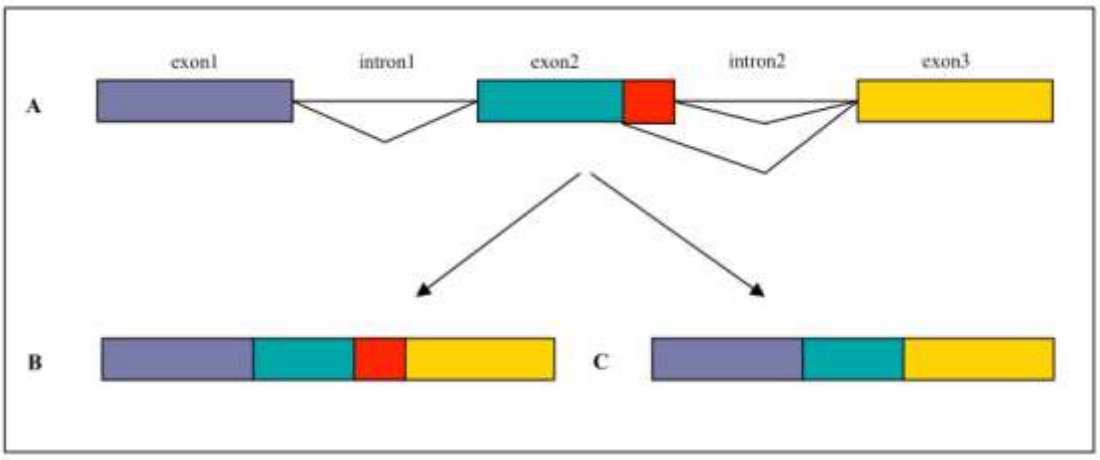


Figure 7. The presence of an alternative 5’ splice site within the exon will result in the splicing of the 3’ end of the exon with the adjacent intron (C) resulting in a new isoform that can be synthetised from the same pre-mRNA molecule (A) in addition to the wild type isoform (B)

There are other factors, which also influences the use of the alternative 5’ splice site, such as the splicing regulatory protein TIA1 (38), or the Hsp47 heat shock protein (39). The use of alternative 5’ splice site might result in an isoform with a different function – it might even have an opposite function, like the anti-apoptotic function of such alternative isoform of the PKC protein (40).

2.3.2.3. Alternative 3’ splice site

EST based bioinformatics studies showed, that approximately 25% of all splice events are happening by using an alternative 5’/3’ splice site. Half of these events happen by using the alternative 3’ splice site of a tandem NAGNAG motif, 3 nucleotids away from the 3’ end of the intron. (41) The regulation of using the 3’ splice site is just as complex as that of the alternative 5’ splice site. (Figure 8)

The deletion of coding and non-coding regions containing the authentic 3’ splice site of the KIT oncoprotein results in an in frame deletion of 27 nucleotids and the formation of a new alternative 3’ splice site. This will then lead to the constitutive activation of the molecule and will be the driving force behind the formation of gastrointestinal stromal tumours. (42)

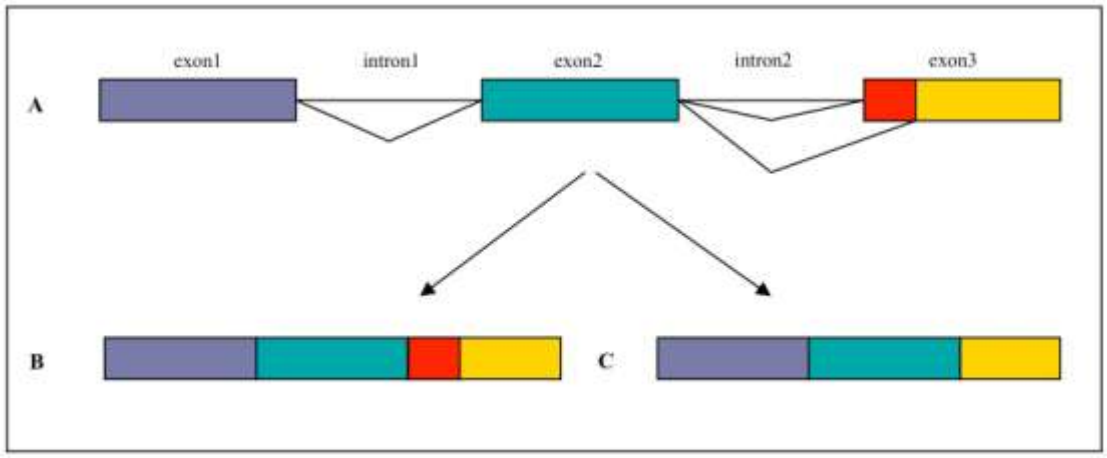


Figure 8. The presence of an alternative 3' splice site within the exon will result the splicing of the 5' end of the exon with the adjacent intron (C) resulting in a new isoform that can be synthetised from the same pre-mRNA molecule (A) in addition to the wild type isoform (B)

2.3.2.4. Intron retention

Intron retention is the rarest form of alternative splicing. (Figure 9).

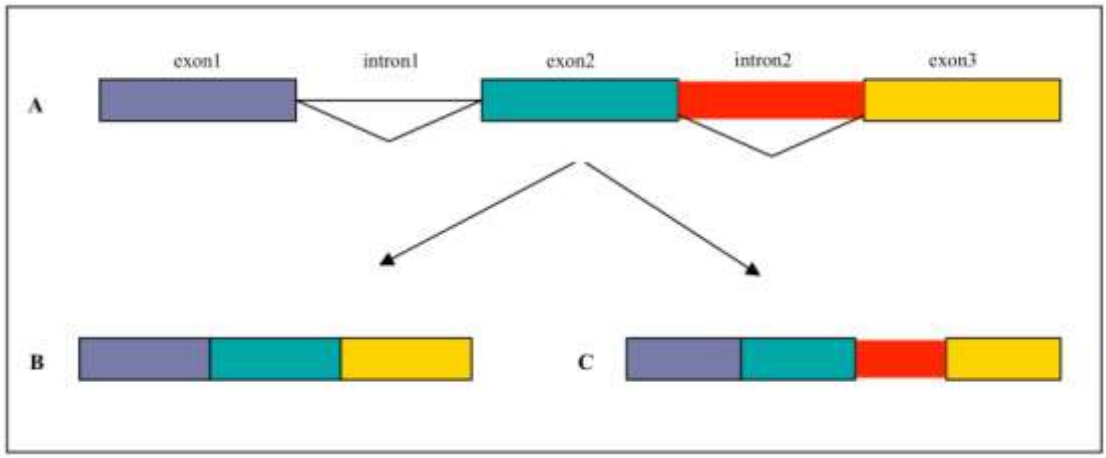


Figure 9. Certain introns can remain within the mRNA (C) during the process of intron retention resulting in an isoform, which differs from the wild type (B) during the course of pre-mRNA editing (A)

It is most likely to occure in the combined presence of weak splice sites, short intron length and a high ESS and ISE expression level. (43)

Retention of introns 9 and 18 of the CD44 has been described in colorectal carcinoma. (44)

2.3.2.5. Variable exons

Variable exons might also form part of the mature mRNA as a result of a summative effect of the regulatory factors, much like in the case of exon skipping. (Figure 10)

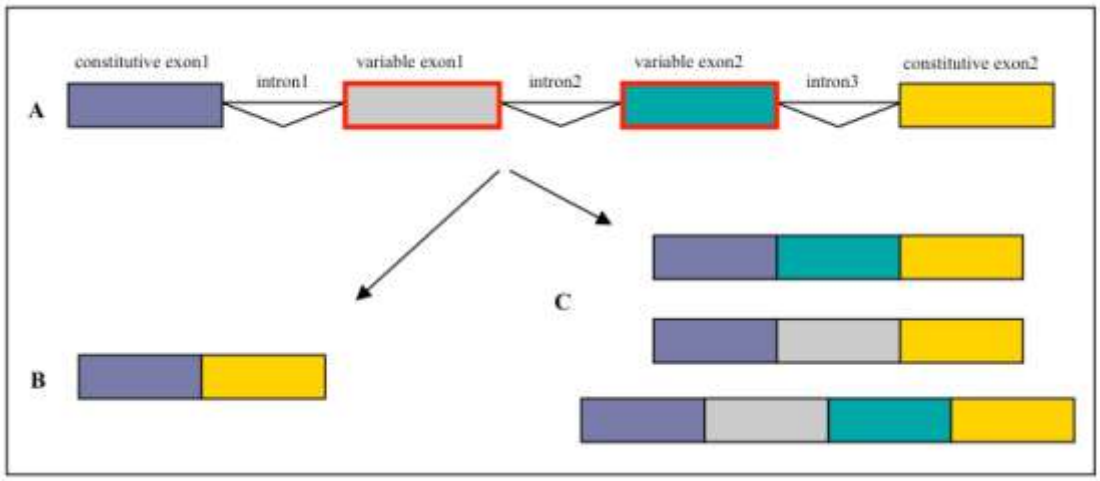


Figure 10. During the editiing of the pre-mRNA molecule (A), in addition to the wild type isoform (B) the variable exons can appear in any combination (C)

One of the best known examples of this phenomenon happens during the expression of the CD44 gene, which might give rise to thousands of different isoforms using its 10 (v1-10) variable exons.

2.3.3. The role of alternative splicing in tumour biology

The discovery of alternative splicing shed a new light onto and re-assessed the role of gene alterations, that were thought to be of importance in carcinogenesis and tumour progression. Point mutations, deletions and other types of alterations might weaken already existing splice sites, create new alternative splic sites or alter the use of the pre-existing splice sites by forming silencer or enchancer sequences. The expressed alternative isoforms are often present within the same cell, which makes studying the function of a ’single gene’ difficult, as the overall ’gene function’ might be a summation of the functions of the structurally and frequently functionally different alternative isoforms. This phenomenon has forced the study of tumour biology to descend to the level of studying the mechanisms promting and regulating alternative splicing. The number of publications on the effect of alternative splicing on tumours is ever rising,

expanding our knowledge about the alternative splice variants of a single gene and their role in tumorigenesis and tumour progression. (Table 1) (45)

Tumour type	Gene	Function	Reference
Leukaemia	Fyn	Tyrosin kinase	46
Leukaemia	Caspase-8	Apoptosis	47
Leukaemia	PASG	Chromatin modelling	48
Thyroid	MUC1	Adhesion, metastasis	49
Thyroid, Colon	Insulin receptor	Tyrosin kinase	50, 51
Colorectal	Rac1	Signal transductional GTPase	52, 53
Stomach	KAI1/CD82	Metastasis	54
Stomach	WISP1	Invasion	55
Pancreas	Secretin receptor	Growth inhibition	56
Pancreas	Gastrin receptor	Proliferation	57
Liver	DNMT3b4	Chromatin modelling	58
Liver	SVH	Novel	59
Lung	NRSF	Transcription factor	60
Lung	C-CAM	Adhesion	61
Lung	VEGF	Angiogenesis	62
Lung	Actinin-4	Adhesion, metastasis	63
Endometrium	SHBG	Hormon signal transduction	64
Emdometrium	Integrin β1c	Adhesion	65
Breast	AIB1	Hormon jelátvitel	66
Breast	Androgen receptor	Transcription factor	67
Breast	Estrogen receptor	Transcription factor	68
Breast	Syk	Metastasis	69
Breast	uPAR	Adhesion, proteolysis	70
Breast, brain	FGFR1	Growth signalling	71
Brain	Crk	Migration, invasion	72
Brain	NF1	Signal transductional GTPase	73
Numerous	TCG101	Proteolysis	74, 75, 76
Numerous	MDM2	Proteolysis	77,78,79
Numerous	CD44	Proliferation, angiogenesis	80, 81, 82
Numerous	Tenascin-C	Adhesion inhibitor	83
Numerous	Fibronectin	Angiogenesis	84

Table 1. Functions of certain genes, which have at least one alternative splice variant present in the given tumour type with reference

It is therefore really important to detect alternative splicing, so the appropriate function can be attached to the different isoforms. There are more and more ways to study alternative splicing, from commercially available alternative splicing detecting microarrays (e.g. SpliceArray™, Agilent), through online databases (85-93) to individually tailored experiments.

2.4. The effects of alternative splicing on medical practice

Its takes time to incorporate new methods into the diagnostic toolkit and for it to be adequately used and understood.

2.4.1. Histopathology / Immunohistochemistry

Histopathology is at the top of the clinical diagnostic pyramid. It not only provides the diagnosis, but also information on the prognosis and more recently on the behavior and response of the tumour to certain treatment type. Its toolkit has recently expanded and after decades of stagnation, there are new techniques at our disposal. These techniques are still new, and we are leaning their use and their affect on our daily practice.

Classical techniques, such as macroscopic and microscopic examination of the tissues with a range of tissue stains is still very much the basis of the diagnostic work, we heavily rely on the use of immunohistochemistry for the diagnosis, differential diagnosis and sometimes even to determine prognosis of most tumour types.

The antibodies only detect the presence of the epitope, but they are not capable of detecting its position, i.e. the entire structure of the protein. For example, in a hypothetical gene, which contains to variable exons and an alternative 3' splice site (Figure 11A), there is a possibility of 8 different mature mRNA (Figure 11B), hence 8 different protein isoforms to be synthesized. These protein isoforms will be structurally different, and quite likely to be of different function.

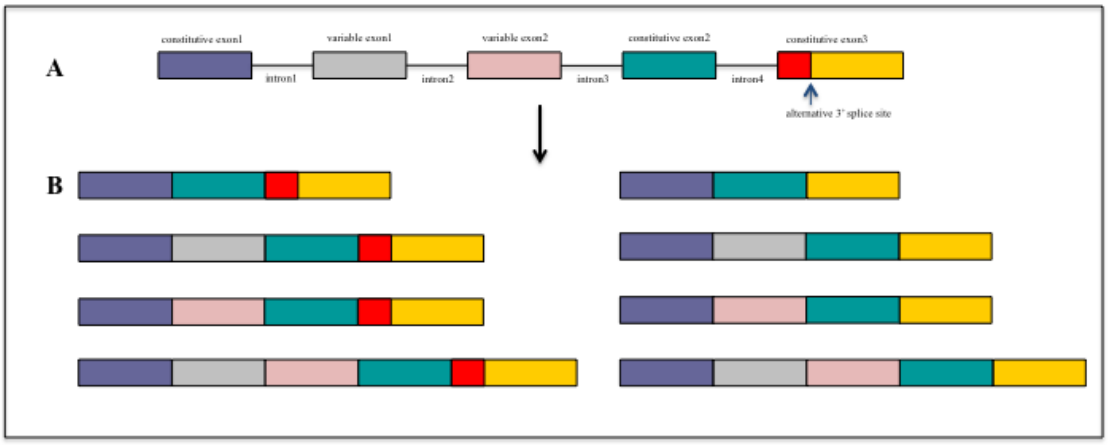
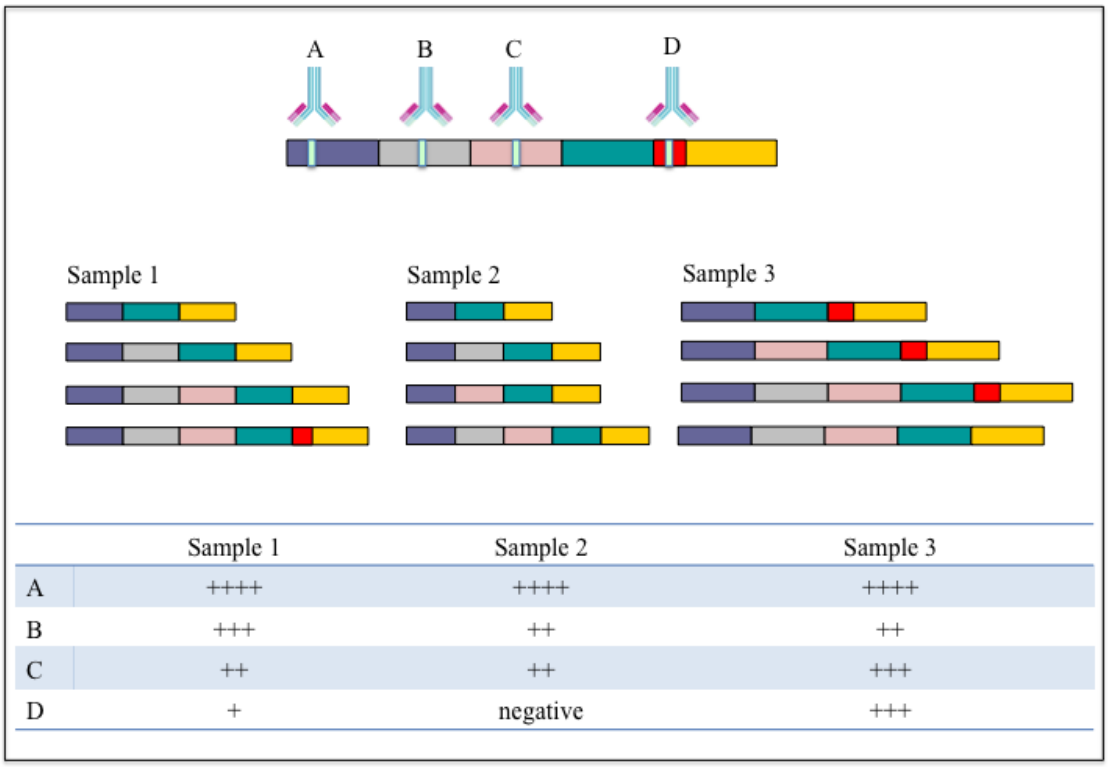


Figure 11. Possible mRNA isoforms of a hypothetical gene with two variable exons and an alternative 3' splice site

Furthermore, as the cells are not limited to 'choose' a single isoform of any given protein, there is a possibility for a set of different isoforms of the same protein to be present within the cell. Different cells might have a different set of isoforms. In the case of our hypothetical gene, samples of different tissue/cell types have different sets of protein isoforms (Figure 12 – Samples 1-3). When examining them using antibodies

against the constitutively expressed region (Fig. 15A), the two variable exons (Figure 12B and C) and a region that could be cut out via using the alternative 3' splice site (Figure 12D) a range of immunohistochemical appearances could be detected (Figure 12 Table).



	Sample 1	Sample 2	Sample 3
A	++++	++++	++++
B	+++	++	++
C	++	++	+++
D	+	negative	+++

Figure 12. Immunohistochemical detection of the different isoforms of a single protein (synthesized from the mRNA in Figure 11) in three different samples, using antibodies directed against four different regions (A: constitutively expressed region, B and C: variable exons, D: a region that could be spliced out using an alternative 3' splice site).

It is very rare, that more than one antibody against a protein in routine diagnostics. The drawback of this is clearly seen, as sample 2 would be deemed negative on immune using antibody D and sample 1 might be considered weakly positive, although both samples could contain the same amount of the protein as the strongly positive sample 3, albeit different isoforms. In the current diagnostic climate, where negativity for certain immune markers would mean withdrawal of therapy (e.g. CD20 positivity as a pre-requisite for Rituximab therapy of various lymphoma types) and where the strength of the immunostain influences therapeutic means (e.g. ER and HER2 positivity in breast carcinoma), we must make sure, that we now for certain that we have dealt with every

single isoform. This is, of course, a huge practical challenge and needs a lot of basic research.

There are currently no models, or approaches to deal with the above described phenomenon, even in the case of genes with the most simple splice pattern.

2.5. Markers, which undergo alternative splicing, that were included in our studies

2.5.1. WT1

WT1 has recently gained a whole new lease of life, as it has been identified as the 'top cancer antigen, for targeted therapy', based on its therapeutic function, immunogenicity, specificity, oncogenicity, expression level within the tumour, stem cell expression, number of patients with antigen positive cancers, number of epitopes, cellular location and expression. (94)

The WT1 gene, which was originally described to play a significant role in embryonic stem cells was later also identified in the case of numerous other human tumours (e.g. breast cancer, lung cancer, colon carcinoma) (95). Though it cannot be accepted as an unambiguous fact, the expression of the WT1 seems to be detected in tumours type, where it plays a significant functional role in the stem cells of the originating organ during embryonic growth, or, in rare cases, in the fully developed organism. The expression of the WT1 gene controls the differentiation of the mesenchymal blastema cells into epithelial structures in the embryonic kidney (96). Wilms' tumour, which expresses the WT1 gene in the majority of the cases, evolves from these so-called residue blastema cells in the kidney. WT1 also plays a definitive role during the early differentiation of the haematogenous medullary stem cells. This is supported by the fact that it is able to bind to the promoter region of genes – e.g. CSF-1, TGF-β, RAR-α, c-myc and bcl-2 – expressed in numerous haemopoietic stem-cells (97). Furthermore, in most cases of leukaemia independently of their type, its expression recurs in the immature CD34+ tumour cells in the peripheral blood. WT1 shows considerable expression in the mesotheliomas, and in the light of this fact it is not surprising that WT1 controls the differentiation of the mesothel during embryogenesis. The WT1-null mutant mouse "dies a multiple death" already at embryonic stage. It has no kidneys developed, and the correct differentiation of the mesenchyma into epithelium does not take place, the consequence of which is the development of heart dysplasia, pericardial bleeding and massive oedema (98). If despite of all the above mentioned problems the

mouse would born it would have reproductive difficulties because WT1 also plays an important role in determining the sexes. This fact has been approved by clinical examples because in the case of numerous post-WT1 mutation symptoms (e.g. Frasier syndrome, Denys-Drash syndrome) a so-called inter-sex aberration can be observed. Moreover, certain organs of the urinary and genital system are those rare exceptional sites where the expression of WT1 can be detected even in the adult age. For example: in the pre-Sertoli and Sertoli cells of the testicles, in the granulose and epithelial cells of the ovary, and in the urothel epidermis of the kidney (99). In this way, appearance of WT1 expression is not surprising in ovary and testicular tumours, so it can be dealt with as a "differentiation" marker. Relationship with the melanomas has been supposed owing to the fact that numerous cell groups express the WT1 gene during differentiation of the nerve system. WT1 is registered as a tumour suppressor gene. It was detected first in Wilms' tumours (nephroblastoma) and for a long time, its overexpression was brought into relation with this single tumour type. WT1 can be characterised with strict local and temporal expression pattern, it is not expressed by the normal body cells after the embryonic growth has been completed. Exceptions are certain cells of the kidney, testicle and ovary, detailed above. The gene is evolutionarily very conservative, the homology of the human and rat WT1 protein exceeds 98%. Its localisation in the human genome: 11p13. The gene consists of ten exons: the exons from 7 to 10 are constituted by four Krüppel-like Cys-Hys zinc fingers (100). In the majority of the tumours it is present in non-mutated form (though the mutated form also occurs). Its expression (may) cause(s) a problem from two points of view. Namely the intact, non-mutated protein is also functionally competent, i.e. if it "had an occasion" it fills in its role. This means that it induces or suspends the function of genes essential for the cell cycle such as EGFR, bcl-2, TGF-β, syndecan, etc. which perhaps should operate in an entirely different manner in a given tissue environment. The other not less interesting fact is that in accordance with the discoveries of the last few years we cannot speak simply about WT1 gene product or WT1 protein. In fact, the single gene encodes 24 different proteins that (may) also behave differently functionally. Change in the proportion of these isoforms can often be brought into relation with abnormal modifications at the level of the cell or even the organism.

Depending on the presence or absence of the sequence (Figures 13) (101) inserted between the third and fourth zinc fingers and encoding three amino acids (KTS), the protein can operate as transcription a factor (WT1KTS-) or post-transcription modifier

(WT1KTS+). Its reason is simple. The presence of the three amino acids conformationally hinders the use of all four zinc fingers of the protein, i.e. binding to the promoter region of the correspondent genes, being able to control their transcription. Today it is known that the change of the optimal rate of the WT1KTS- and WT1KTS+ isoforms may have a dramatic consequence equal even to a "rude" mutation. At the fifth exon, there is a possibility for the insertion of a new sequence (Figure 13) encoding 17 amino acids (17AA). Its biological importance is less clarified than that of KTS. (In fact, this is a brand new evolutional acquisition, its appearance is known from the vertebrata, albeit WT1 itself has been present in insects.) Merely by using these two splice sites, four different proteins may be synthesized from the WT1 mRNAs (17AA-KTS-, 17AA- KTS+, 17AA+ KTS+, 17AA+ KTS-). Furthermore, to date, 9 splice variants have been published altogether. (Figure 14) Further twenty proteins can be synthesized through frame shift (frame shifts at 60-62 kD, 52-54 kD, 36-38 kD), the products of which may be proteins with fundamentally different size and function, making the interpretation of the role of WT1 gene in a given tissue environment difficult. This can explain that rather discrepant results were reported in the literature during the 1980's, following the examination of WT1 transfected cell lines. The limited tissue and temporal patterns of appearance of the WT1 gene products makes possible to use those regions in the diagnostics and perhaps in therapy, which are common in some isoforms (consensus regions). This means that expression exceeding the normal blood level (which is actually very low), for example, with orders of magnitude, indicates the presence of tumour cells (e.g. leukaemia) in high likelihood. The expression pattern of the isoforms may also carry information. The most hopeful observation of the last few years was that in the peripheral blood of some 25% of the patients suffering from leukaemia there were antibodies against the NH-terminal part of the WT1 protein (its COOH-terminal part is less immunogenic) (102). Several experiments support that such a CD8+ cytotoxic T-lymphocyte clone can be produced against the synthetic WT1 protein, which is toxic for the leukaemia cells but does not affect the colony formation of the normal marrow stem cells, that is, it offers an immuno-therapeutic possibility in the medication of the leukaemia.

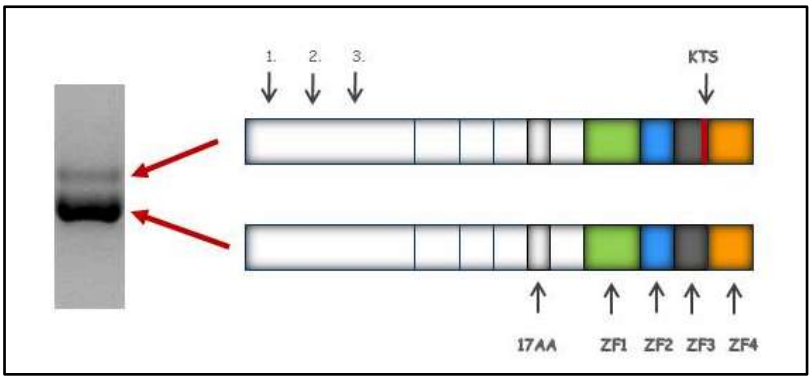
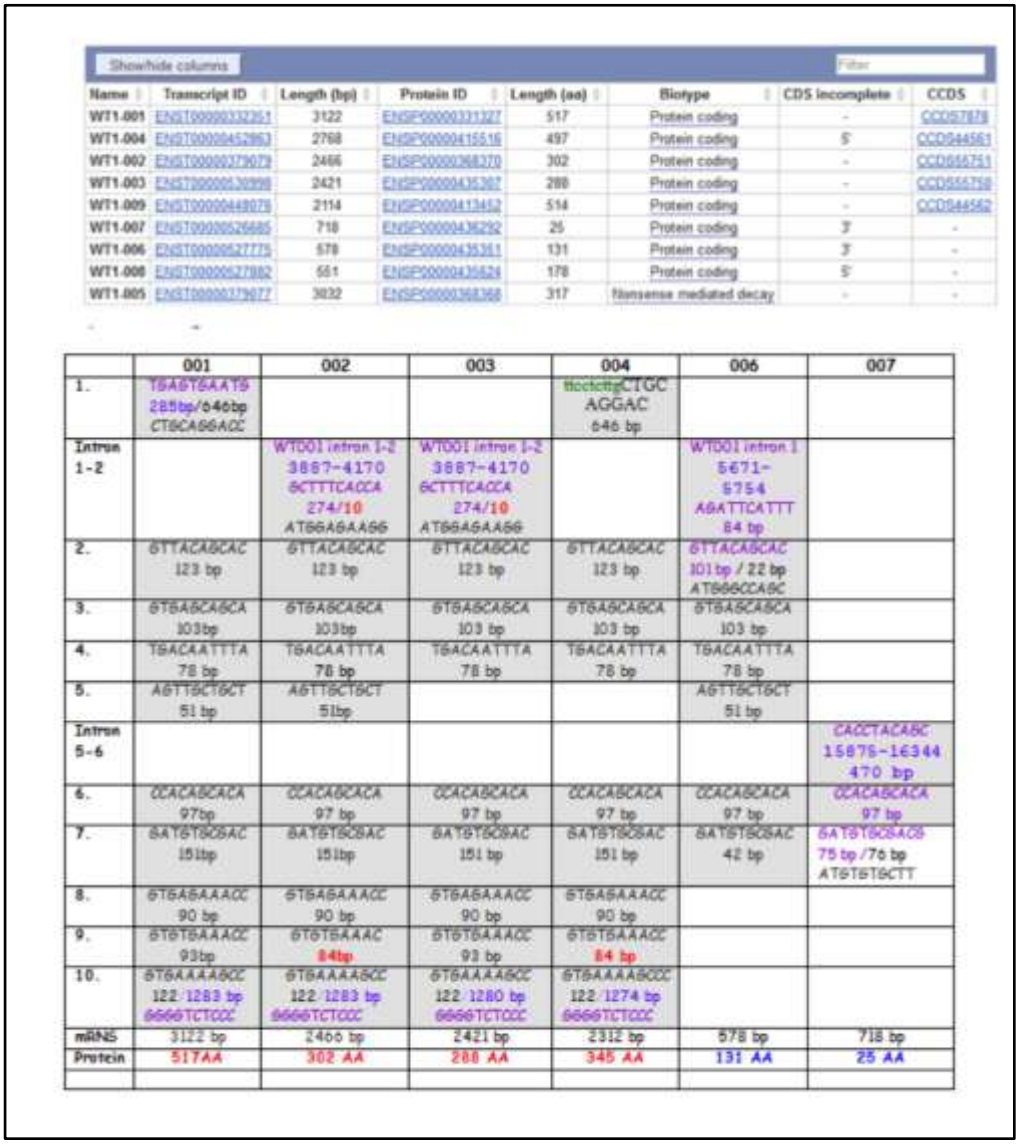


Figure 13. Structure of the WT1 protein, and the formation of its isoforms through alternative splicing (17AA and KTS) and frame shifts (1. 60-62kD, 2.52-54kD,3. 36-38kD).



Show/hide columns

Name	Transcript ID	Length (bp)	Protein ID	Length (aa)	Biotype	CDS incomplete	CCDS
WT1-001	ENST00000332351	3122	ENSP00000331327	517	Protein coding	-	CCDS7878
WT1-004	ENST00000452863	2768	ENSP00000415516	497	Protein coding	5'	CCDS44561
WT1-002	ENST00000379079	2466	ENSP00000368370	302	Protein coding	-	CCDS55751
WT1-003	ENST00000430998	2421	ENSP00000413367	288	Protein coding	-	CCDS55758
WT1-009	ENST00000449078	2114	ENSP00000413452	514	Protein coding	-	CCDS44562
WT1-007	ENST00000526685	718	ENSP00000436292	25	Protein coding	3'	-
WT1-006	ENST00000527775	578	ENSP00000435361	131	Protein coding	3'	-
WT1-008	ENST00000527882	551	ENSP00000435624	178	Protein coding	5'	-
WT1-005	ENST00000379077	3032	ENSP00000368368	317	Nonsense mediated decay	-	-

	001	002	003	004	006	007
1.	TGAGTGAATG 285bp/646bp CTGCAGGACC			tcctcttgCTGC AGGAC 646 bp		
Intron 1-2		WT001 intron 1-2 3887-4170 GCTTTCACCA 274/10 ATGGAGAAGG	WT001 intron 1-2 3887-4170 GCTTTCACCA 274/10 ATGGAGAAGG		WT001 intron 1 5671-5754 AGATTCATTT 84 bp	
2.	GTTACAGCAC 123 bp	GTTACAGCAC 123 bp	GTTACAGCAC 123 bp	GTTACAGCAC 123 bp	GTTACAGCAC 101 bp / 22 bp ATGGCCAGC	
3.	GTGAGCAGCA 103bp	GTGAGCAGCA 103bp	GTGAGCAGCA 103 bp	GTGAGCAGCA 103 bp	GTGAGCAGCA 103 bp	
4.	TGACAATTTA 78 bp	TGACAATTTA 78 bp	TGACAATTTA 78 bp	TGACAATTTA 78 bp	TGACAATTTA 78 bp	
5.	AGTTGCTGCT 51 bp	AGTTGCTGCT 51bp			AGTTGCTGCT 51 bp	
Intron 5-6						CACCTACAGC 15875-16344 470 bp
6.	CCACAGCACA 97bp	CCACAGCACA 97 bp	CCACAGCACA 97 bp	CCACAGCACA 97 bp	CCACAGCACA 97 bp	CCACAGCACA 97 bp
7.	GATGTGCGAC 151bp	GATGTGCGAC 151bp	GATGTGCGAC 151 bp	GATGTGCGAC 151 bp	GATGTGCGAC 42 bp	GATGTGCGACG 75 bp /76 bp ATGTGTGCTT
8.	GTGAGAAACC 90 bp	GTGAGAAACC 90 bp	GTGAGAAACC 90 bp	GTGAGAAACC 90 bp		
9.	GTGTGAAACC 93bp	GTGTGAAAC 84bp	GTGTGAAACC 93 bp	GTGTGAAACC 84 bp		
10.	GTGAAAAGCC 122/1283 bp GGGGTCTCCC	GTGAAAAGCC 122/1283 bp GGGGTCTCCC	GTGAAAAGCC 122/1280 bp GGGGTCTCCC	GTGAAAAGCCC 122/1274 bp GGGGTCTCCC		
mRNS	3122 bp	2466 bp	2421 bp	2312 bp	578 bp	718 bp
Protein	517AA	302 AA	288 AA	345 AA	131 AA	25 AA

Figure 14. Alternative splice variants of WT1 (Protein level: red known protein coding, blue putative protein coding)

2.5.2. CD44

2.5.2.1. Functions of CD44

CD44 is the main hyaluronic acid binding protein of the cell surface (103), which enables the cells to partake in cell-cell and cell-ECM interactions. CD44 expression on the surface of mature lymphocytes play a key role in lymphocyte homing through its interaction with HEVs (high endothelial venules). (103, 104)

Polymorphism of the gene results in the formation of the indian blood group antigenes [In(a) and In(b)] (105), which are resposible for the majority of the transfusion, and some of the foeto-maternal transfusion reaction. (106)

Although most of its functions, as an adhesion molecule are performed through its extracellular domain (ECD), under proteolytic effect, its intracellular domain can get translocated into the nucleus, where it can act as a transcription factor through the 12-O-tetradecanolforbol-13-acetate responsive element (TPARE), which regulates the expression of genes of diverse function, including CD44 itself. (107)

There is also a soluble form of CD44 (sCD44), which is capable of binding to the cell surface CD44 protein altering its function in an agonistic/antagonistic manner. (108)

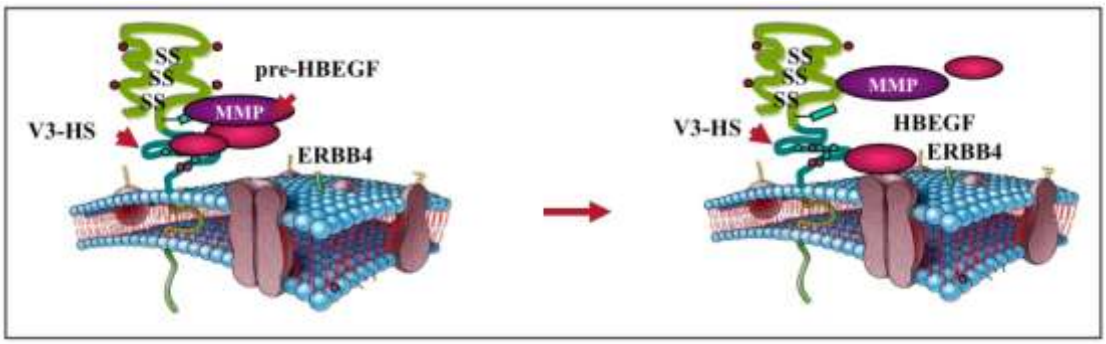


Figure 15. The role of v3 within the CD44 protein

Many of the variable exons' individual functions have been examined individually demonstrating significant functional changes in signaling pathways.. CD44 is the principal cell surface receptor for hyaluronate. When v3 is expressed (109) hyaluronate binding is weaker. Permitting further chondroitin and heparan sulphate glycosylation, results in the presentation of heparin-binding growth factors such as HB-EGF, b-FGF and amphiregulin. (110) (Figure 15)

For activating c-met by its ligand, HGF/SF, v6 is needed and important for the intracellular signal pathway via MEK and Erk. (111) (Figure 16A-B)

In Ras pathway-regulated CD44 alternative splicing, those isoforms containing v6 together with HGF form a positive feedback loop on Ras, causing the downregulation of non-v6 containing isoforms (112). (Figure 16C)

However, there is discrepancy around the data on the role of CD44 and its isoforms in tumour biology.

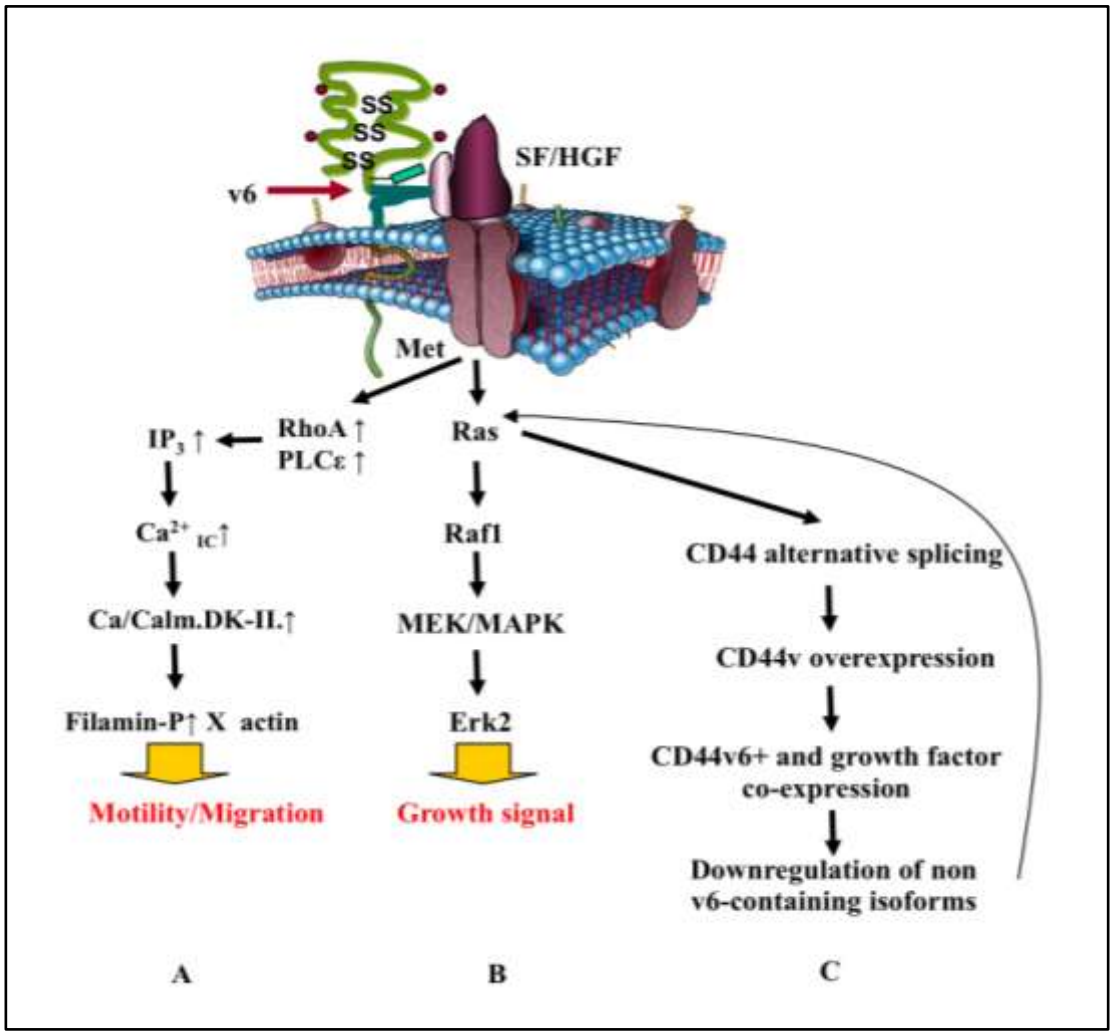


Figure 16. The expression of exon v6 exon activates signal pathways of motility/ migration (A) and growth (B) as well as forms part of a positive feedback loop activated by growth factors and their receptors (C).

2.5.2.2. CD44 in tumour biology

Despite the fact that it has been known for a number of years now that healthy tissues have specific CD44 Alternative Splice Patterns (ASPs) (113), and a PCR based method has already been proposed to analyse the different CD44 splice variants (114), there has not been any follow up to this study. Conversely, most data on CD44 alternative

splicing in neoplasia and tumour progression have focused on the associations of the expression of single CD44 variable exons. There are problematic discrepancies in the role of CD44v in tumour biology.

For example, in colorectal carcinoma some studies indicate that v2 has a predictive role in progression (115), v3 and v6 play a role in cancer free survival (116) and v6 again in distant metastases (117, 118). Other studies have failed to demonstrate any such role (119, 120, 121).

Similar discrepancies emerge in the changing patterns of CD44v expression in human melanoma. Dome et al. demonstrated the up-regulation of v3 results in a higher visceral metastatic potential (122) while Pacifico et al. showed that CD44v3 expression is associated significantly with a better outcome(123). Again v10 (124) and v6 (125) may be involved in metastasis formation, or not. One study suggests that human melanomas do not express CD44v isoforms at all and only express the CD44S form at a higher level, and does not correlate with the prognosis of this tumour type (126). These varied findings may reflect the focus on the expression of solitary exons, rather than the profile of all ASPs.

Other studies do analyse the co-expression of two or more variable exons (127, 128), although not as a part of the ASP.In an alternative splice pattern, many different isoforms are present. The functional importance of any single variable exon may be dependent on the full expression pattern. Detecting the presence of a single, or multiple variable exons across all of these isoforms does not provide any information as to where these variable exons are expressed, and crucially what other variable exons are present alongside. On the other hand, the presence of additional variable exons on a particular isoform may actually change or not permit the function of the variable exon in question, and thus without knowing the entire alternative splice pattern, this restricts what one can say about detecting the presence of a single variable exon in these studies. (Figure 17)

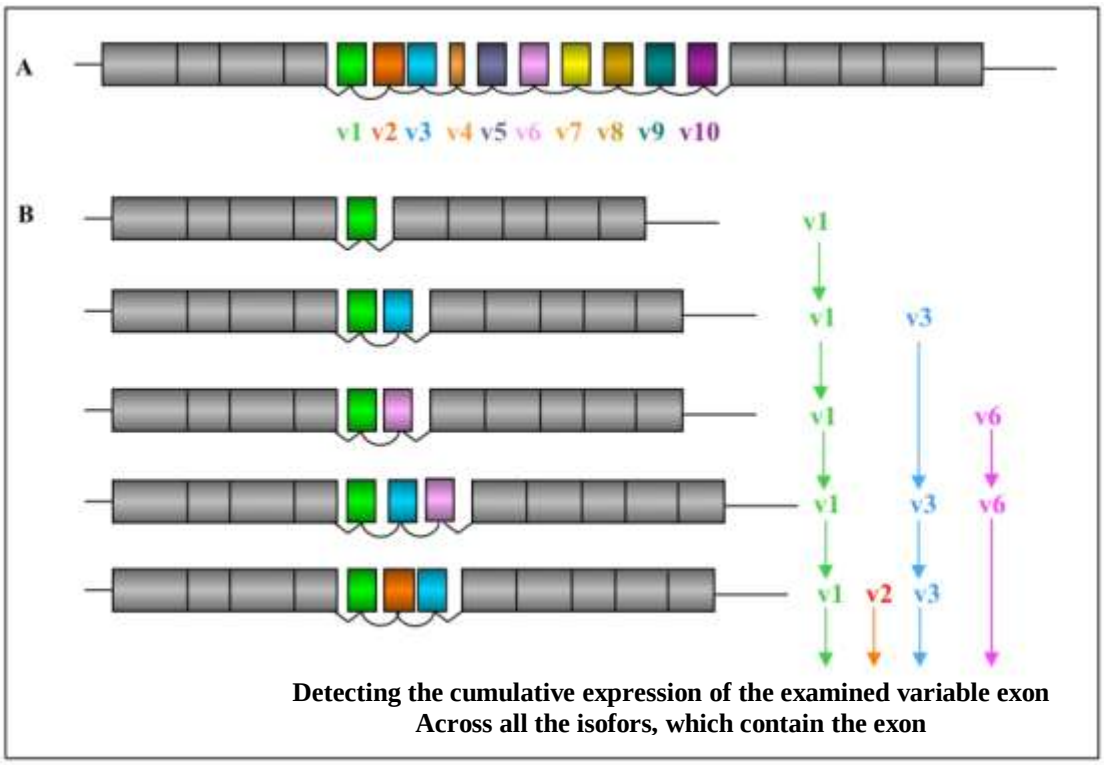


Figure 17. In case of multiple, expressed variable exons (A) a range of differenr mRNA isoforms can be expressed (B) the variable exon content of which cannot be separated, therefore the expression of a single variable exon is a summation of the expressed exon across all the isoforms, which contain it

For the same reason even the 'co-expression' of two exons proven by immunohistochemistry (129) does not mean that they are on the same molecule as the presence of two or more different CD44 isoforms in the same cell at the same time is also possible.Although the expression level changes of one variable exon might still show a correlation with the progression in one tumour type, there is no such obvious example in the literature as there are lots of contradictions even during the examination of the same tumour type.

Some more recent studies have analyzed the role of CD44v isoforms rather than single exons in tumour progression (130, 131), but not as a part of a complex, finely regulated pattern.

A more holistic view of the alternative splice event is needed to examine the role of CD44 variants.

3. Materials and methods

3.1. Cell lines and culture conditions

Melanoma cell lines

The A2058 melanoma cell line was provided by LA Liotta (NCI, Bethesda, MD). HT168 and HT168M1 lines are derivatives of A2058. HT199 was developed in the 1st Department of Pathology and Experimental Cancer Research (Semmelweis University, Budapest, Hungary – A Ádám, A Ladányi). WM983A, WM983B and WM35 were gifts from M. Herlyn (Wistar Institute, Philadelphia, PA). The α2b and β3 were transfected into WM983B cells in the Wistar Institute (19H and 19L, ESH and ESL), a mock-transfected line was also created (3.1P). Sub-clones of 19H (8F3- and 6E5+)and 19L (9B4+, 9E3+) were created by culturing one cell suspensions of the original line(National Institue of Oncology, Budapest - A. Ladányi, J. Dobos).The murine B16a cell line was obtained from the Animal andTumor Bank, Division of Cancer Treatment, NIH (Bethesda, MD)

Other cell lines

The HT25 colorectal carcinoma cell line arrived from M. Hendricks laboratory(Iowa), and HCR31was developed in the 1st Department of Pathology and Experimental Cancer Research (Semmelweis University, Budapest, Hungary – E Rásó).The HT29 (ECACC 91072201) and HCT116 (ICLC HTL95025) human colorectal carcinoma cell lines, MDA-MB-231 human breast adenocarcinoma (ECACC 92020424), PE/CA PJ15 (ECACC 96121230) and PE/CA PJ41 (ECACC 98020207) human oral squamous cell carcinomas, K562 human chronic myelogenous leukaemia (ECACC 89121407) and A431 human vulva squamous carcinoma (ECACC 85090402) cell lines were purchased from the sources specified.The melanocyte (C-12403), keratinocyte (C-12003) and fibroblast (C-12360) cells were derived from Promo Cell.

Culture conditions

All tumour cell lines were maintained in RPMI 1640 medium (Lonza) supplemented with 10% fetal bovine serum (*Sigma*-Aldrich) and1% Penicillin/Streptomycin (*Sigma*-Aldrich). The melanocytes were maintained in Melanocyte Growth Medium M2 (PromoCell), the keratinocytes in Keratinocyte Media 2 (PromoCell) and the fibroblasts in Fibroblast Media (PromoCell). All cell lines were maintained in a *37*°C, 5% *CO2* incubator.

3.2. Total RNA isolation, reverse transcription

Total RNA was isolated from the frozen homogenized tumour samples and cell cultures from the in vivo experiments using TRI Reagent™ (Sigma®)according to the manufacturer instructions**.** Possible DNA contamination was eliminated using TURBO DNA-free™ kit (Ambion®). For reverse transcription 1 µl of 10mM dNTP mix (Finnzymes,Espoo, Finland) and 1µl of random primer-oligo dT were mixed for a final concentration of 2.5 µM and used with 2 µg of purified total RNA. After incubating at 70 °C for 10 min, 1µl of M-MLV reverse transcriptase (200 units/µl), 2 µl of 10x M-MLV RT Buffer (both from Sigma), 0.5 µl RNase Inhibitor (40 units/µl, Promega, Madison WI) and 6.5 µl DEPC treated water was added for 20 µl final volume and incubated at 37 °C for 50 min and then at 85 °C for 10 min. The occurrence of reverse transcription was checked by polymerase chain reaction withhuman and mouse (in the case of B16a-experiments) β-actin primers (Figure 18).as a housekeeping gene. RNA of the same sample was used as negative control for detection of DNA contamination and DEPC treated water as non-template control.

Human WT1, transcript variant D Ref.seq: NM_024426.4		
	5'end primer	3'end primer
WT1KTSo	GGCATCTGAGACCAGTGAGAA (1333-1353)	GAGAGTCAGACTTGAAAGCAG T (1815-1794)
WT1KTSi	GCTGTCCCACTTACAGATGCA (1402-1422)	TCAAAGCGCCAGCTGGAGTTT (1744-1724)
WT117AAo	GCTGCTGAGGACGCCCTACAGCAG (1039-1062)	CAAGAGTCGGGGCTACTCCAGG (1325-1304)
WT117AAi	CAGATGAACTTAGGAGCCACC (1112-1132)	CACATCCTGAATGCCTCTGAA (1294-1274)
WT1k	GTTACAGCACGGTCACCTTC (837-856)	GTGGCTCCTAAGTTCATCTGATT (1131-1109)
Mus musculus Wilms tumor 1 homolog (Wt1) Ref.seq: NM_144783.2		
MWT1o	GCCTACCATCCGCAACCA (895-912)	TCCAGATACACGCCGCAC (1387-1370)
MWT1i	TGACCTGGAATCAGATGAACCT (1179-1200)	TCCAGATACACGCCGCAC (1387-1370)
Homo sapiens actin, beta (ACTB) NM_001101.3; Mus musculus actin, beta (Actb) NM_007393.3		
Human β-actin (ACTB)	GTGGGGCGCCCCAGGCACCCA (187-205)	CTCCTTAATGTCACGCACGATTTC (726-703)
Mouse β-actin (Actb)	TCTGGCACCACACCTTCTAC (333-352)	GTCCTTAATGTCACGCACGATTTC (714-698)

Figure 18. Sequence and localisation of human and mouse WT1 and β-actin primers

3.3. PCR detection of WT1 and CD44 variant expression

The PCR reaction mixture contained12,5 µl AmpliTaq Gold® 360 Master Mix (Applied Biosystems, called Life Technologies today), 2.5-2.5µl of the appropriate primer pair designed with Array Designer (Premier Biosoft International). 2µl of the cDNA and 5.5µl DEPC treated water for the final volume of 25 µl.

	Cycling conditions
WT1KTSo	94 °C 1 min; 64 °C 1 min; 72 °C 2 min – 30 cycles
WT1KTSi	94 °C 1 min; 64 °C 1 min; 72 °C 2 min – 35 cycles
WT117AAo	96 °C, 95 °C, 94 °C, 1 min; 65 °C 1 min; 72 °C 1 min – 2+2+36 cycles, touch down
WT117AAi	94 °C 1 min; 57 °C 1 min; 72 °C 2 min – 35 cycles
WTk	94 °C 1 min; 55°C 1 min; 72 °C 2 min – 35 cycles
MWT1o	94 °C 1 min; 55 °C 1 min; 72 °C 1 min – 30 cycles, final extension: 72 °C, 7 min
MWT1i	94 °C 1 min; 55 °C 1 min; 72 °C 1 min – 30 cycles, final extension: 72 °C, 7 min
Human β-actin (ACTB)	95°C 1 min, 55°C 1min, 72°C 2 min - 35 cycles, final extension 72 °C for 10 min
Mouse β-actin (Actb)	94 °C, 1 min; 60 °C 1 min; 72 °C 2 min – 21 cycles

Figure 19. Cycling conditions for human and mouse WT1 and β-actin

The cycling conditions for all of the CD44 primers were: 97°C for 10min once, then 95°C for 1 min, 55°C for 1min, 72°C for 2 min for 35 cycles, 72 °C for 10 min. The sequence and localisation of human and mouse WT1 primers and cycling conditions are shown in figures 18 and 19 respectively. The sequence and localisation of the CD44 primers are shown in figure 20.

RefSeq: NM_000610.3

	5' end primer	3' end primer
Standard region (S)	AGTCACAGACCTGCCCAATGCCTTT (827-852)	TTTGCTCCACCTTCTTGACTCCCATG (2316-2342)
Variable exon 2 (v2)	GACAGCAACCAAGAGGCAAG (1133-1153)	TTTGTGTTGTTGTGTGAAGATGATT (1200-1225)
Variable exon 3 (v3)	CGTCTTCAAATACCATCTCAGCA (1234-1257)	ATCTTCATCATCAATGCCTGA (1317-1341)
Variable exon 4 (v4)	AACCACACCACGGGCTTT (1361-1379)	CATCCTTGTGGTTGTCTGAAGTA (1444-1467)
Variable exon 5 (v5)	ATGTAGACAGAAATGGCACCAC (1471-1493)	GTGCTTGTAGAATGTGGGGTC (1565-1585)
Variable exon 6 (v6)	GGCAACTCCTAGTAGTACAACG (1592-1514)	GTCTTCTCTGGGTGTTTGGC (1672-1692)
Variable exon 7 (v7)	CTCATACCAGCCATCCAATGC (1726-1747)	CTTCTTCCTGCTTGATGACCTC (1822-1843)
Variable exon 8 (v8)	CTCCAGTCATAGTATAACGCTTCA (1856-1880)	GTTGTCATTGAAAGAGGTCCTGT (1926-1948)
Variable exon 9 (v9)	AGCAGAGTAATTCTCAGAGCTTC (1951-1974)	CAGAGTAGAAGTTGTTGGATGGT (2008-2031)
Variable exon 10 (v10)	ATCATTCTGAAGGCTCAACTACTT (2077-2101)	TAAGGAACGATTGACATTAGAGTTG (2216-2240)

Figure 20. Sequence and localization of the exon specific CD44 primers

PCR products were separated on a 2 %, occasionally 3% agarose gel and captured with the MULTI GENIUS Bio Imaging System (Syngene, Frederick, MD) after ethidium bromide staining. Transcribed fragments could be exactly identified according to the estimated length of the separated products. This band was excised and DNA purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).

3.4.Sequencing analysis

All PCR products of this thesis were verified via direct sequencing according to the following protocol.

The purified PCR fragments of PCR-products were analysed by direct sequencing in both sense and antisense directions. The sequencing reaction was done with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems™ – by Life Technologies™) according to the manufacturer protocol (same primers as the ones used for PCR amplification reactions). Before analysis, purification of the sequencing reaction products was done by the BigDye® XTerminatorTM Purification Kit (Applied Biosystems ™ – by Life Technologies™).

PCR products were analyzed by a 4-capillary automated sequencer (Applied Biosystems 3130 Genetic Analyzer, Applied Biosystems).

3.5. Flow cytometry

One cell suspensions were made containing 2×10^6 cells, 4 test tubes each from both HT168-M1 human melanoma and positive control K562 (CML blastic crisis) human leukaemia cell lines. They were both labelled for cell surface WT1 and its isotype control, as well as intracellular WT1 and its isotype control. The cells were fixed with 1% paraformaldehyde, and then for the intracellular labelling the cell membrane was permeabilised with 0.2% saponin (MERCK). For the cell surface examinations the membrane permeabilisation step was omitted. In both cases, after blocking with 1% BSA (bovine serum albumin) for 10 minutes, the cells were washed with PBS. Then incubated with anti-human WT1 (PharMingen, Becton Dickinson) monoclonal mouse (IgG) primary antibody, or, in the case of the negative control, with isotype-matched IgG for 45 minutes. After PBS wash, the cells were incubated for 45 minutes with FITC conjugated anti-mouse IgG (Amersham) secondary antibody. After PBS wash, the cells with PBS to a 500 μl final volume, and the intensity of the fluorescence of 10,000 cells (Becton Dickinson Immunocytometry Systems, FACSscan, 488 nm laser) was measured.

3.6. PCR detection and sequencing of CD44 fingerprint

The PCR conditions were as described above. The primer pairs were the following: S5'-S3', S5'- v33', v35'- S3', S5'- v63', v35'- v63' using the exon specific primers (Figures

20, 21 and 22). PCR products were separated on a 3% agarose gel and detected with MULTI GENIUS Bio Imaging System (Syngene, Frederick, MD)after ethidium bromide staining.

PCR products were re-isolated from the agarose gel (High Pure PCR Product Purification Kit, Roche, Mannheim) in the case of all bands. The DNA sequences were determined by BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems™ – by Life Technologies™).

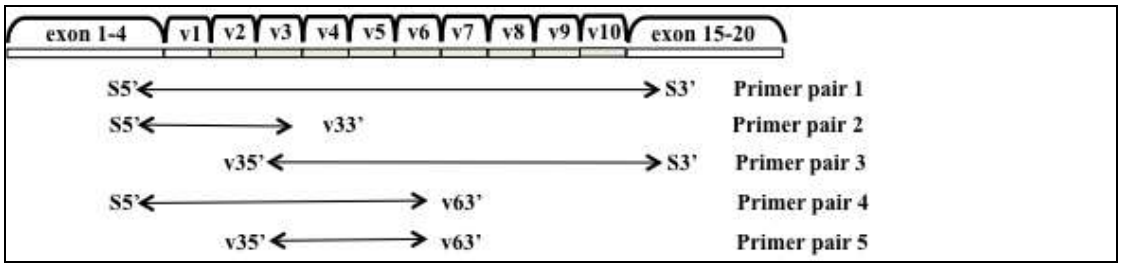


Figure 21.Localization of the primer pairs used to create the fingerprint.

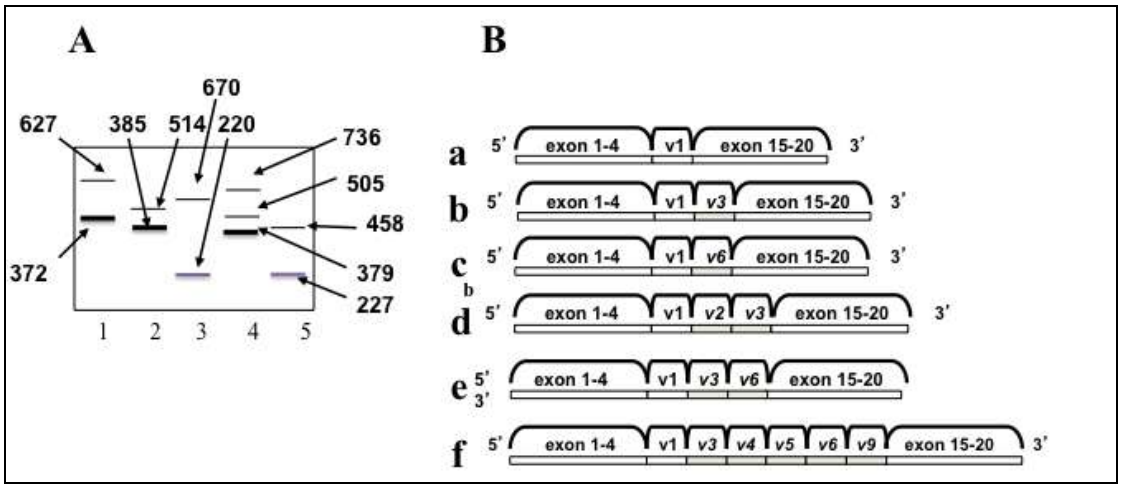


Figure 22.The melanoma fingerprint.**A**. Fingerprint with the product sizes. **B.**Predicted isoforms based on the qualitative fingerprint. From the qualitative picture, the following isoforms can be identified in melanomas: CD44S which does not contain variable exons and appears as 372bp product in lane 1 of the fingerprints (a); CD44v3 containing only v3 exon appearing as 385 bp product on lane 2 and 220 bp product in lane 3 of the fingerprint (b); CD44v6 with also one variable exon (v6) as the 379 bp product of lane 4(c); CD44v2v3 with two expressed variable exons, v2 and v3 represented by the 514 bp product of lane 2 (d); CD44v3v6 is also two variable exon containing isoform which can be identified from the 627 bp product of lane 1, the 505 bp product of lane 4 and the 227 bp product of lane 5 (e) and CD44v3v4v5v6v9 as the biggest isoform with five

expressed variable exons detected as the 670 bp product of lane 3, the 736 bp product of lane 4 and the 458 bp product of lane 5 (f). As the variable exons are very similar in size with sometimes only a few base pair difference other isoforms might be present as well and the presence of v7, v8, v9 and v10 is also possible. For instance the 204bp long v10 and the 207 bp long co-expressed v5 and v9 are very hard to distinguish as they would appear as 'one' band on lane three and only v5v9 co-expression can be proved by the appropriate sized products of lanes 4 and 5. This is further confirmed by cloning and next generation sequencing (Figures 35, 36 and 37).

3.7. Cloning the CD44 PCR products from the fingerprint

The PCR products of the fingerprint were re-isolated from the 2% agarose gel (as above) inserted into vectors in the pGEM®-T Vector Systems (Promega ®) according to the manufacturer's instructions. The plasmid DNA was re-isolated using GeneJET $^{(TM)}$ Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's instructions and the products were sequenced with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems™ – by Life Technologies™).

3.8. Next-generation sequencing

During a library preparation the ligated amplicon generation was used. The PCR products from HT199, A2058 and WM983B melanoma fingerprints were amplified, purified and their ends were polished and ligated with Roche 454 multiplex identifier (MID) adaptors, to generate universal primer binding sites for emulsion PCR in every sample reactions. The amplicons were than separated with magnetic Ampure beads (Agencourt) to eliminate the non-binded adaptors. Emulsification of the ligated emPCR was done according to the manufacturer's protocol (Roche 454). The library was than sequenced with clonal pyrosequencing technic (454 GS Junior - Roche) with 200 cycles, in 400 bases read length mode. After sequencing, image processing and signal processing (amplicon pipeline) the amplicon variant analyzer software (Roche Diagnostics) was used to demultiplex the samples after MIDs, to trim the primers and to align the reads to the reference cDNA sequence. In the case of splice variants the difference between variants and the complete reference cDNA sequence can be too high. Therefore the reads were aligned to every exon separately to identify the real exon combination. The alignment was successful if there was higher identity than 90% in

more than 20 bases length in every exons. The exon combinations which have more than 50 reads were reported.

3.9. Using extracellular matrix-components for cell cultures

Fibronectin (FN) (Sigma-Aldrich), laminin (Sigma-Aldrich), Collagen Type IV from (BD) Matrigel, Hyaluronic acid (HA) (Sigma-Aldrich) (each 50 μg/ml) and 0,9% NaCl solution (as control) were administered into different wells of a 6-well plate. After 3 hours of incubation on RT, supernatants were removed. 1-1 ml of $5x10^4$ cell/ml suspensions of HT168M1 was administered on the prepared matrix-films. After 72 hours of incubation, we removed supernatants, and started the process of total RNA isolation by pipetting TRI Reagent™ (*Sigma*-Aldrich ®) straight onto the cell cultures.

3.10. Metastasis models using *scid* mice

This study was carried out in strict accordance with the recommendations and was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (TUKEB permit number: 83/2009). All surgery was performed under Nembutal anaesthesia, and all efforts were made to minimize suffering.

Cultured HT199 and HT168M1 human tumour cells were injectedsubcutaneously ($5x10^5$/50μl volume) at the same lower back localisation into 10 newborn and 10 adult *scid* mice as well as intravenously into 5 adult *scid* mice for both cell line. On the 30th day, the animals were sacrificed by bleeding under anaesthesia. Primary *in vitro* cell cultures were formed from the primary tumour, circulating tumour cells and the lung metastases of the same animal implanted as a newborn. Also, the primary tumour, circulating tumour cells and the i.v. transplanted lung colonies from the adult animals were used to create cell cultures the same way (Figure 23).

For comparative measurements the different tumours, i.e. primary tumour, circulating tumour cells, lung metastasis, always derived from the same animal to allow standardisation of the host.

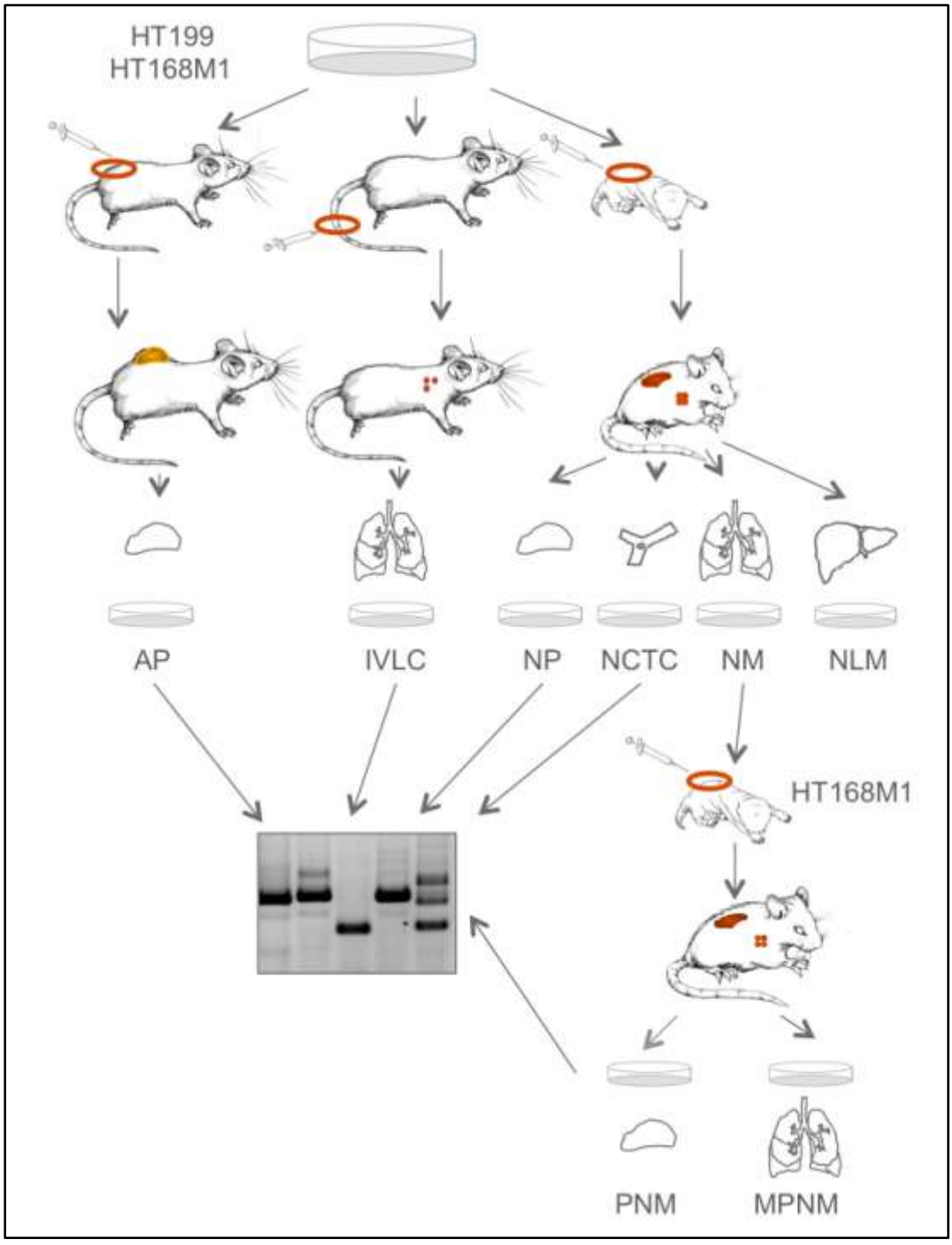


Figure 23.Schematic structure of the *in vivo* human melanoma (HT199 and HT168M1) metastasis animal model. The same melanoma cell suspension was implanted subcutaneously into adult and newborn *scid*mice as well as intravenously into adult *scid* mice. The primary adult [(subcutaneously (AP) and i.v. implanted (IVLC)] and newborn tumours (NP) were removed along with the liver (NM) and lung (NLM) metastases, that were only formed in newborn mice, on the 26th post-implantation day. Cell cultures were created from all the above tumours and the circulating tumours cells

(NCTC) of newborn mice. A cell culture created from a single HT168M1 lung metastasis of a newborn mouse was then re-injected subcutaneously into newborn *scid* mice and the primary tumour (PNM) and its lung metastasis (MPNM) were also removed and cultured on the 26th post implantation day.

3.11.Quantitative PCR analysis

For semiquantitative measurement of the expressed WT1 andCD44 variable exons q-PCR reactions were used (iQ SYBR® Green Supermix,Bio-Rad), by cycling conditions 3 min at 95°C, then 40 cycles at 95°C 30 sec, 55°C 30 sec, 72°C 1 min. Starting quantities were defined on the basis of standard fivefold dilution series (1x-625X) carried out with control cDNA (K562 in the case of WT1 and A431 in the case of CD44) and by normalizing the starting quantities to the housekeeping β-actin starting quantities from the same cDNS sample. Three parallel measurements were carried out on each sample in every case.

3.12. Statistical analysis

Real-time PCR results, relative expression rates (normalized on beta-actin expression rates) were compared with two-sample T-tests (data were showing normal distribution). Statistical significance was assumed at $p< 0.05$.

4. Goals

Our goal was to identify novel markers and study their behavior during the course of the melanoma progression.

1. Study the expression and effect of a number of genes, which would endow melanoma cells with more metastatically potent phenotype.

Namely:

- α2bβ3,
- 12-LOX
- c-kit
- WT1

2. The results of any gene expression study can be significantly skewed by not considering the changes of all expressed alternative splice variants of said gene during tumour progression. When studying CD44, a known ‘metastasis associated gene’, and WT1 to a lesser extent, we aimed to

- Detect all expressed isoforms of the gene in the given experimental setting
- Examine the qualitative and quantitative changes of the expressed isoform pattern during the course of tumour progression
- Examine, if any detected change is tumor-specific
- Consider the effect of our findings on the evaluation of experimental results and on everyday diagnostic work

5. Results

5.1.Novel markers of melanoma progression

The author has contributed to the work described in this chapter as a co-author by performing experiments at mRNA level and real-time PCR measurements. Therefore, these results will be focusing on the author's work.

5.1.1. Effect of ectopic expression of α2bβ3 besides avβ3 on angiogenic phenotype of melanoma

As it has been described above, much hope has been put into characterizing the process of angiogenesis to provide early access to the process of tumour progression. To study this, one must characterize the expression pattern of angiogenesis related molecules during tumour progression. One of the best model systems metastasis formation is that of human melanoma, considering that metastatic involvement of the regional lymph nodes and distant organs happens in the earliest stages of tumour growth. Our research group has described the ectopic expression of α2bβ3 (GpIIbIIIa) in 2005. This integrin is the main adhesion receptor of platelets, which plays a key role in platelet aggregation through its fibrinogen binding. Its role in melanomas was not yet clear at the time, although it was though to influence the same step of progression that α5β3 integrin does. To characterize its role, we studied the change of the *in vivo* behavior and gene expression pattern of a human melanoma cell line (WM983B), which does not originally express α2bβ3, but does so α5β3. The α2b and β3 were transfected into the cells in the Wistar Institute (19H and 19L, ESH and ESL), and a mock-transfected line (3.1P) was used as control in all cases.

5.1.1.1. Parallel expression of alphaIIbbeta3 and alphavbeta3 integrins promotes ortho- and heterotropic growth of human melanoma cell lines

When implanted heterotopically, into the spleen of *scid* mice, all cell lines were tumorigenic, but the α2bβ3 transfected ones showed a significantly more rapid growth when compared to the mock transfected one (Figure 24). Orthotopic, intracutaneous implantation of 19H and 19L into *scid* mice, again, showed significantly faster growth in the early phases of tumour development, than the mock-transfected 3.1P (Figure 25). However, α2bβ3 expression did not seem to alter metastatic potential, as the rate of metastasis formation of the transfected lines was not significantly higher than the non-

transfected one in our three different *in vivo*experimental setups, i.e. after intravenous, intrasplenic and intracardiac injection of the cells into *scid* mice.

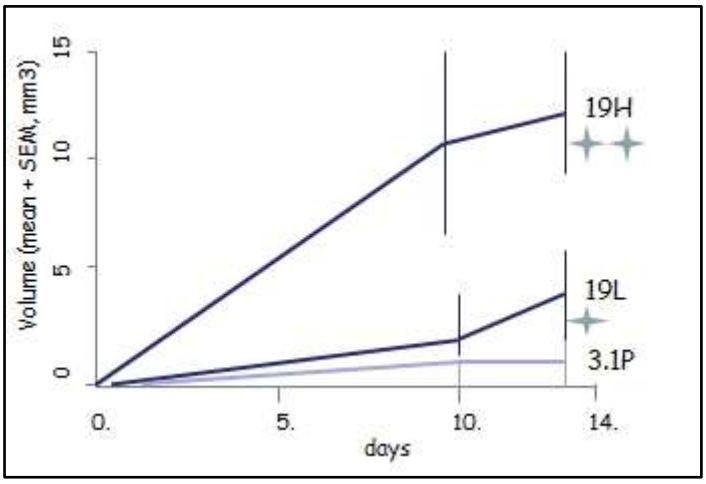


Figure 24. Growth of melanoma clones in SCID mice in the spleen-liver (i.s.) model.10^6 tumor cells were injected into the spleen of SCID mice and the size ofthe primary was determined on the 30th post-injection day

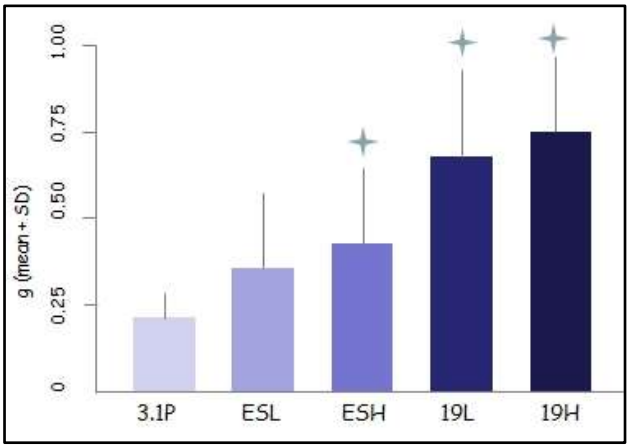


Figure 25. Growth of melanoma clones in SCID mice in the orthotopic model.One hundred thousand tumor cells were injected intradermally into the animals, tumor diameters were measured and the tumor volume was calculated on the 10th and 13th post-injectiondays

5.1.1.2. Parallel expression of alphaIIbbeta3 and alphavbeta3 integrins promotes vascularisation and the angiogenic phenotype of human melanoma cells

Immunohistochamical examination (using CD31) of intratumoral blood vessels in orthotopically implanted primary tumours of 19H, 19L and 3.1P, revealed a higher density of vessels in the transfected lines, which was significant in the case of 19H.

Flow cytometric measurements of VEGF and bFGF, two of the most significant angiogenic factors in melanoma, revealed constitutive expression of VEGF across all the cell lines (tranfected and mock tansfected alike); while bFGF was only detected in the transfected lines. This was further confirmed on *in* growing cell lines using bFGF antibody and their *in vivo* growing primary tumour counterparts using confocal microscopy, where 19H showed strong cytoplasmic bFGF positivity in the majority of tumour cells.

5.1.1.3. Parallel expression of alphaIIbbeta3 and alphavbeta3 integrins correlates with an altered gene expression profile of human melanoma cells

The quantity of the α2bβ3 protein in the examined individual melanoma cell lines (19H, 19L, ESH, ESL, 8F3, 3.1P) showed a straight correlation with the relative expression of bFGF (Figure 26).

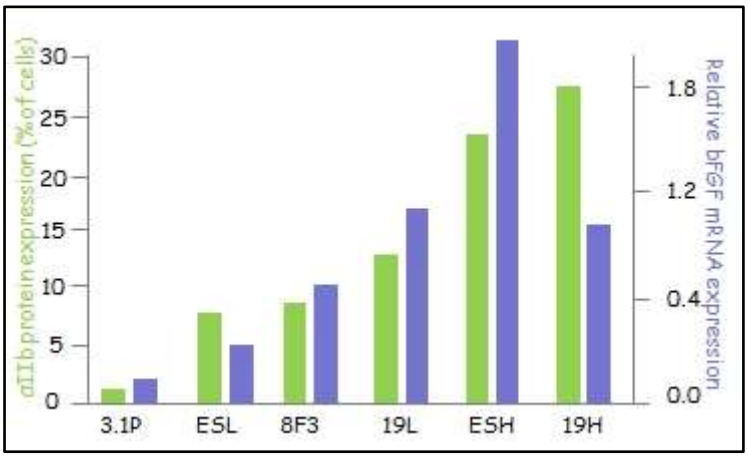


Figure 26.bFGF mRNA levels in transfected melanoma clones determined by quantitative PCR analysis with parallel deminstration of αIIb-proteinexpression

Microarray (3600 genes) studies showed the overexpression of 36 genes (including three vasculogenic mimicry genes: CD34, endothelin receptor B, Prostaglandin I-2 synthase) in 1919H compared to 3.1P, which could potentially be related to its

progressive propensity. The upregulation of the β3 endonexin in the α2bβ3 transfected cell lines, and the fact that the bFGF mRNA level was downregulated by tyrosine kinase inhibitors seemed to have support the theory that the change in angiogenic phenotype caused the increased progressive phenotype of α2bβ3 transfected human melanoma cell lines.

5.1.2. Platelet-mimicry of cancer cells: epiphenomenon with clinical significance

It is a well-documented phenomenon, that certain tumour types might have a so-called 'stem cell mimicry' resulting in ectopic gene expression. These stem cell-like properties might play a role in the epithelial-mesenchymal-transformation (EMT) of certain tumour types, or in the vascular mimicry of other tumour types. In another one of our studies, we identified the thrombocyte mimicry of certain tumour types (α2bβ3, 12-LOX, thrombin-receptor), in the background of which we detected c-kit and WT1 expression of human melanomas. Both of these markers are characteristically expressed in bone marrow progenitor cells, and their ectopic expression was thought to be possibly resulting in the appearance of thrombocyte cell surface markers. This might also promote haematogenic dissemination of the tumour and provide a target for anti-tumoural therapy.

5.2.The role of isoforms generated through alternative splicing in the diagnosis and progression of human melanoma

The results included in this chapter were from experiments designed and performed by the author.

5.2.1. WT1

5.2.1.1. WT1 ASP of human tumor cell lines

To characterize the WT1 expression of human melanoma, we examined the known splice sites, i.e. the 17AA+/- and KTS+/- alternative splice pattern of 12 human melanoma cell lines. We included WM983B human melanoma cell line as well as its α2bβ3 tranfected clones, described in chapter 5.1.1. These cell lines allowed us to study the correlation between the different proliferation activity and alternative splicing. We detected WT1 expression at mRNA level in 10 cell lines. (Figure 27A). Remarkably, WT1 expression disappeared in two of the transfected cell lines. One of them is the

mock transfected cell line (3.1 pool), which makes us question the assumption that the transfection of the empty vector is an authentic control to study the properties and behaviour of the cell lines transfected with the target gene.

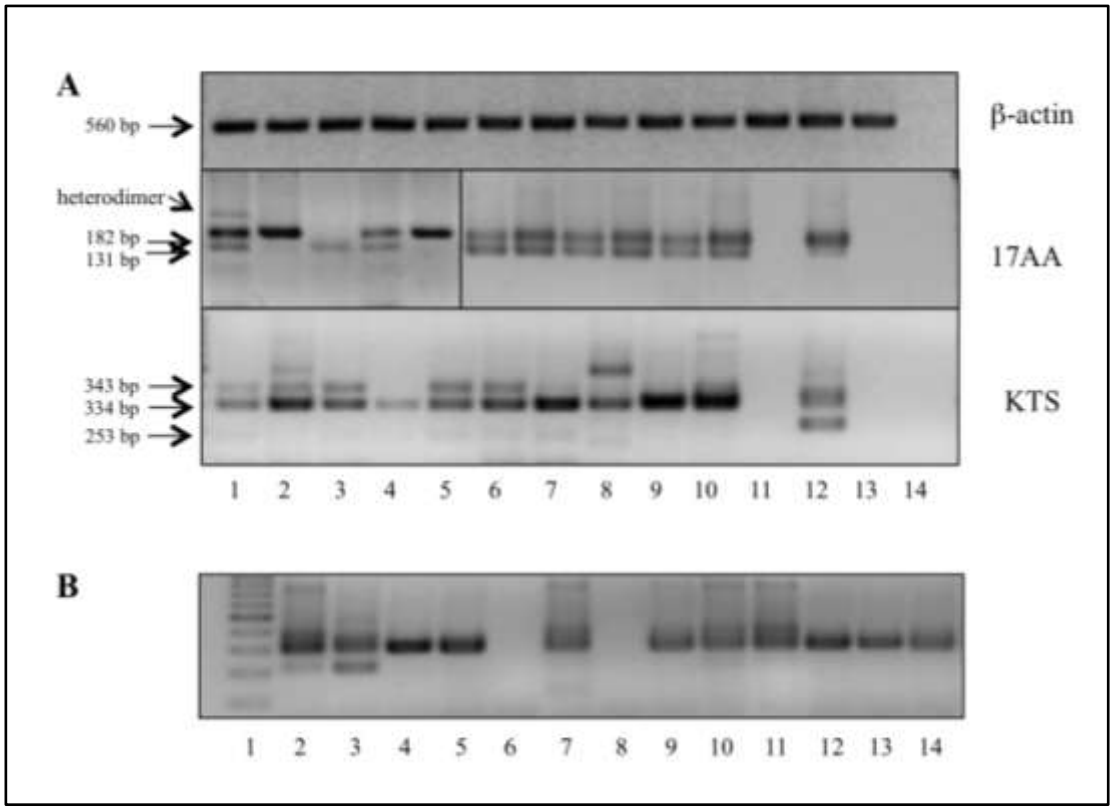


Figure 27.WT1 alternative splice pattern of different tumour types.**A**. WT1 expression in human melanomas at the two alternative splicing sites of the gene. 1. 8F3-, 2. 9E3+, 3.6E5+, 4.HT168M1, 5.HT199, 6.WM983A, 7.HT168, 8.A2058, 9.WM983B, 10.A431, 11.9B4+, 12.WM983B, 13. 3.1 pool, 14. H2O; **B**. The expression pattern of the WT1 zinc-finger region of different tumour type. 1. Marker, 2. HT29 colorectal adenocarcinoma, 3.HT25 colorectal adenocarcinoma, 4.PE/CA PJ 15 head and neck squamous cell carcinoma, 5.CCRF /CEM acute lymphoblastic leukaemia, 6.RAJI Burkitt's lymphoma, 7.PC3 prostate adenocarcinoma, 8.K562A chronic myelogenous leukaemia, 9.PANC1 pancreatic carcinoma, 10. MOLT acute T-lymphoblastic leukaemia, 11. HCR 25 colorectal adenocarcinoma, 12. LNCaP 13. OVCAR ovarian adenocarcinoma, 14 HL60 promyelocytic leukaemia

In the expression pattern of the splice variants there was no essential difference between the cell lines. In the case of all WT1+ cell lines, expression ratio of the KTS- and KTS+ isoforms was variable despite out expectations and the already published data from the literature, however, it showed no consistent pattern. Interestingly, the KTS- isoform was consistently absent from A2058, as well as from its *in vivo* selected, metastatically less potent version (HT168) and the cell line gained from its liver metastasis (HT168M1). In the pattern of the 17AA+ and 17AA- isoforms there was a difference too, which was independent of the metastatic propersity established on the basis of the previous examinations of the laboratory (Table II).

We also examined the WT1 splice pattern of the zinc finger region in 14 non-melanoma cell lines (Figure 27B).We were not able to identify a consistent, melanoma-specific splice pattern. This might partially be due to the fact, that PCR examination of the W1 zinc finger region is technically challenging. Our research group later developed a completely new technique to examine this region.

Cell line	Lung colony formation*	RNA (PCR)		Protein-FCM (intracellular, %)		
		KTS	17AA	WT1	MelanA*	Gp100*
HT168-M1	++	- +	++	71	24	24
HT168	++	- +	++	41	32	15
HT199	++	++	+ -	ND	29	42
A2058	++	- +	++	ND	44	52
WM983A	++	++	++	ND	12	7
WM983B	+	++	++	ND	50	55
3.1pool	+	- -	- -	ND	ND	ND
9E3+	+/-	++	+ -	ND	ND	ND
8F3-	+/-	++	+ -	ND	ND	ND
9B4+	+/-	-	+ +	ND	ND	ND
6E5+	+/-	++	- +	ND	ND	ND
WM35	-	++	++	ND	23	14

Table II Metastasis formation of human melanoma cell lines in SCID mice and their connection with WT1 splice variants expression, and melanoma antigenes (FCM: flow cytometry; previous results of the laboratory are marked with *; ND: not determined)

As an unexpected finding, we also identified a new splice variant with the in frame skipping of zinc-finger 3. (Figure 28)

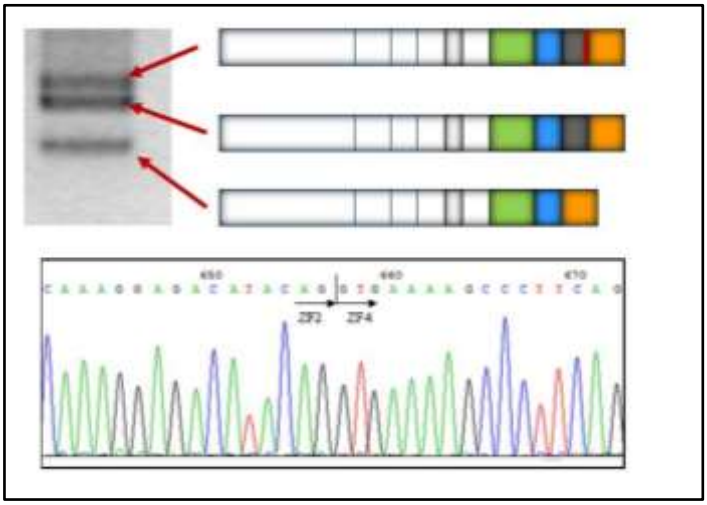


Figure 28. Electropherogram of KTS+, KTS- and ZF3- WT1 isoforms, the latter is also shown on a sequenogram of direct sequencing.

5.2.1.2. Quantitative change of WT1 expression during tumour progression

We compered the WT1 expression of tumours from hosts permissive and non-permissive to metastasis formation, focusing on two regions of alternative splicing. These included the zinc finger region (WT1KTS) and a potentially consensus region, that does not contain known alternative splice site (WTk). Our results showed that a number of the examined melanoma cell lines (WM983B for example) were truncated in this region, therefore these tumours should be treated separately when studying the behaviour of the gene.

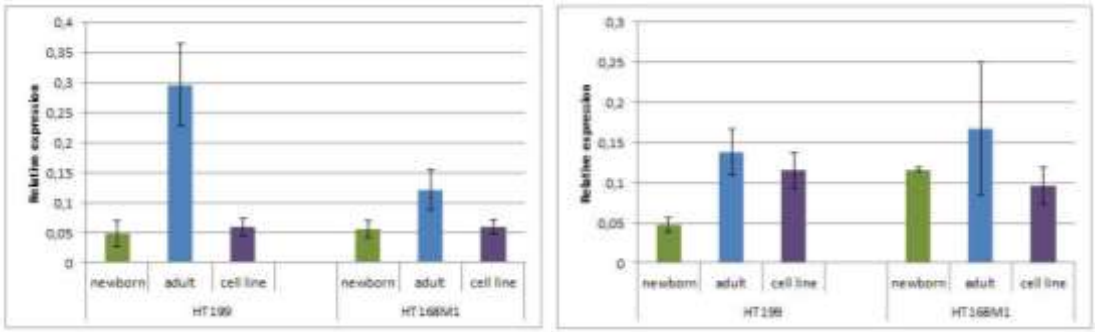


Figure 29. Real-Time PCR measurement of WT1 relative expression (normalised to β-actin) of two human melanoma (HT199 and HT168M1) growing in hosts permissive (newborn) and non-permissive (adult) to metastasis formation and *in vitro* (cell line)

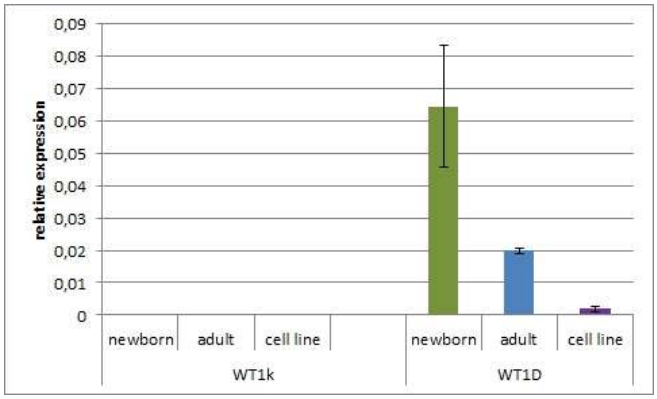


Figure 30. Real-Time PCR measurement of WT1 relative expression (normalised to β-actin) of WM983B growing in hosts permissive (newborn) and non-permissive (adult) to metastasis formation and *in vitro* (cell line) using primers for the consensus (WT1k) and zinc finger (WT1D) regions. The same cDNA sample was used with both primers, samples had uniformly high housekeeping gene (β-actin) expression.

The results of this experiment showed that higher WT1 expression correlates with better prognosis from the point of view of metastasis formation (Figure 29). However, we could not identify such correlation with the expression change of any given splice variant. For the better detection of the splice variants, our group later developed a probe based Real Time PCR assay, which was validated on human leukaemia samples. The technique will soon be applied to study the behaviour of the WT1 splice variants during the progression of human melanoma as well.

The behaviour of 5' end truncated WT1 isoforms (WM983B), most likely differs from that of the non-truncated isoforms during metastasis formation, although, again, the rest of the expressed isoforms should also be identified (Figure 30). This might be of diagnostic / prognostic importance.

5.2.1.3. Flow cytometric detection of the WT1 protein

As WT1 is considered as the 'number one' tumour antigene by the National Cancer Institute, therefore confirmation of WT1 protein expression might be of clinical importance.

Flow cytometry confirmed the expression of the WT1 protein, which has mostly intracellular localisation according to the expectations (i.e. according to the function of the WT1 protein) **(**Figure 31).

Compared to out earlier results(Table II), the WT1 protein shows an intracellular localisation in the examined HT168 and HT168-M1 cell lines, and it is present in a higher percentage of the cells than the known melanoma antigenes MelanA and gp 100. The cell surface labelling for WT1 is significantly less intense in these cells (Figure 32).

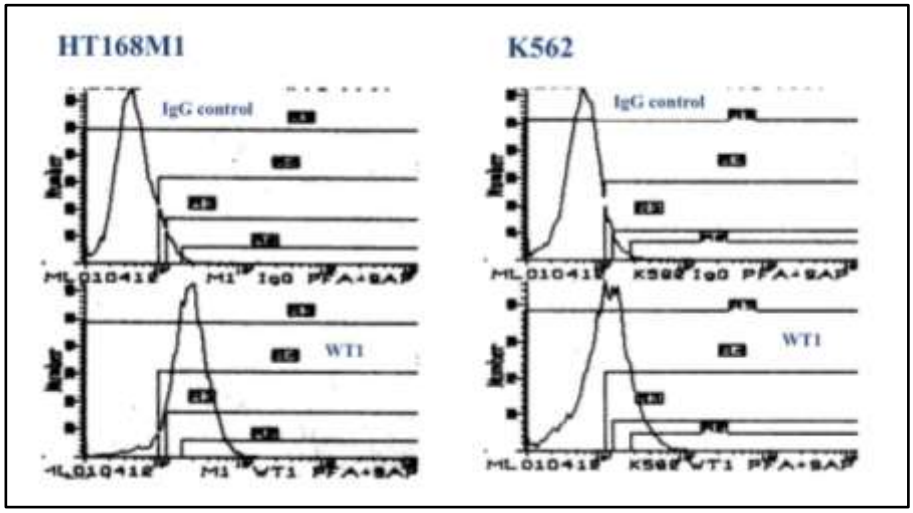


Figure 31.Detection of the WT1 protein by flow cytometry. Intracellular labelling. (Fluorescence intensity above control: HT168-M1: 17.75; K562: 5.82)

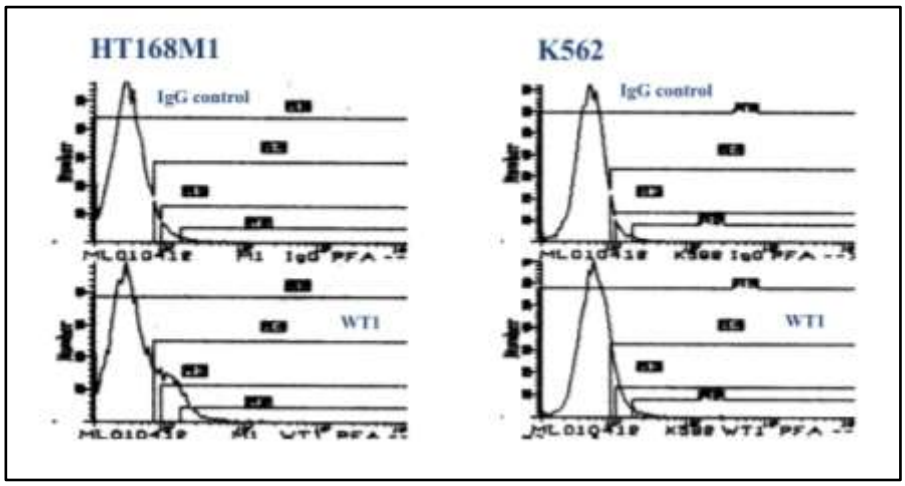


Figure 32.Detection of the WT1 protein by flow cytometry. Cell surface labelling. (Fluorescence intensity above control: HT168-M1: 1.71; K562: 0.75)

5.2.1.4. Expression of WT1 in mouse melanoma

Much like its human counterpart, the B16a mouse melanoma cell line, expresses the corresponding species specific (mouse) WT1 gene. This fact may suggest that (like in the case of kidney blastema cells or lymphoblasts) it plays an important role in the differentiation of melanoblasts (Figure 33).

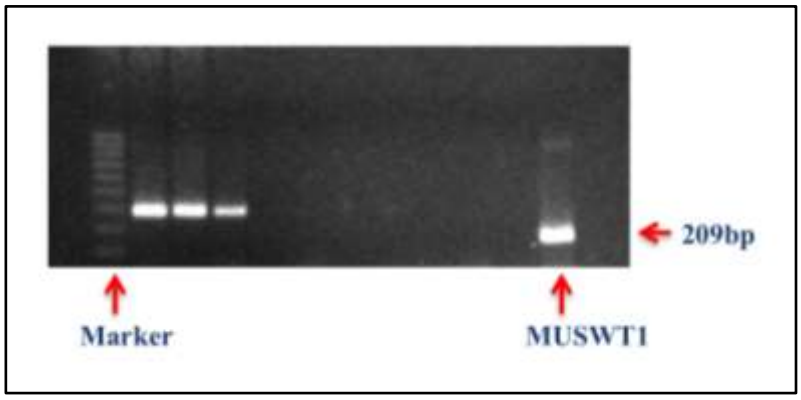


Figure 33.Expression of the mouse Wilms' tumour 1 (MUSWT1) gene in B16a mouse melanoma cells

5.2.1.5. Detection of WT1 positive circulating tumour cells

The serial dilution **(**Figure 34).made with human blood from the HT168-M1 tumour cell line confirms that the PCR technique used by us is able to detect the presence of 10^2 tumour cells in 10^6 normal lymphocytes. Consequently, it may be appropriate to detect the presence of circulating tumour cells in the peripheral blood of cancer patients.

Figure 34.Serial dilution made from the HT168-M1 tumour cell line with human blood containing 10^6 leukocytes

5.2.2. The CD44 alternative splice pattern of human melanoma

5.2.2.1. CD44 variable exons and possible isoforms at mRNA level

We visualized the expression of CD44 variable exons in HT168 human melanoma by performing PCR reactions pairing the sense (5') primers of variable exons with the common antisense (3') primer localized on exon 16 and variable exon's antisense (3') primers with the common sense (5') on the standard exon 4. Our results showed, that all the variable exons, which are considered variable in databases (v2-v10) were present. Also, this method with the overlapping sequences allowed us to construct some of the isoforms (Figure 35 and Figure 36), although, this still seems rather inaccurate as some of the exons seemed to have been of slightly different size.

This size difference can possibly be explained by the fact that by next generation sequencing on the same tumour, we identified a daunting number of small deletions across the CD44 isoforms (data not shown).

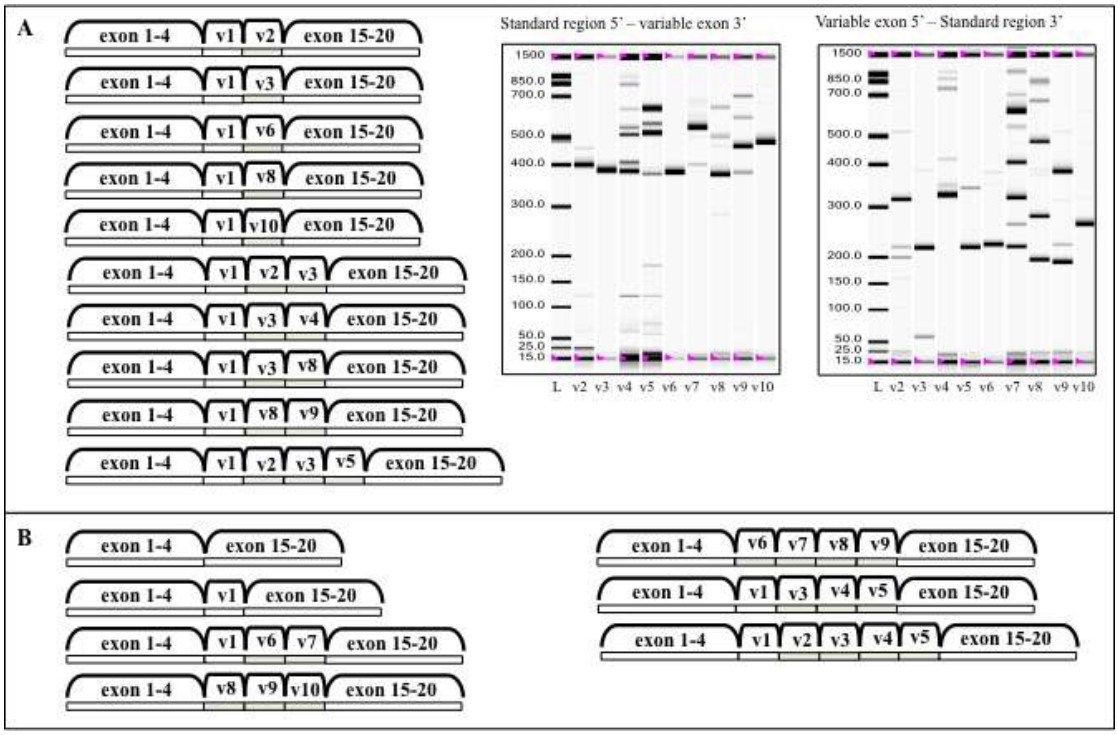


Figure 35. CD44 isoforms validated by next generation sequencing **A.** CD44 isoforms from the qualitative picture of pairing the variable exon specific primers with the standard region specific ones both 5' and 3' directions in HT168 human melanoma cell line. These isoforms were validated by next generation sequencing. **B.** Further validated isoforms from next generation sequencing with the primer pairs of the fingerprint**.**

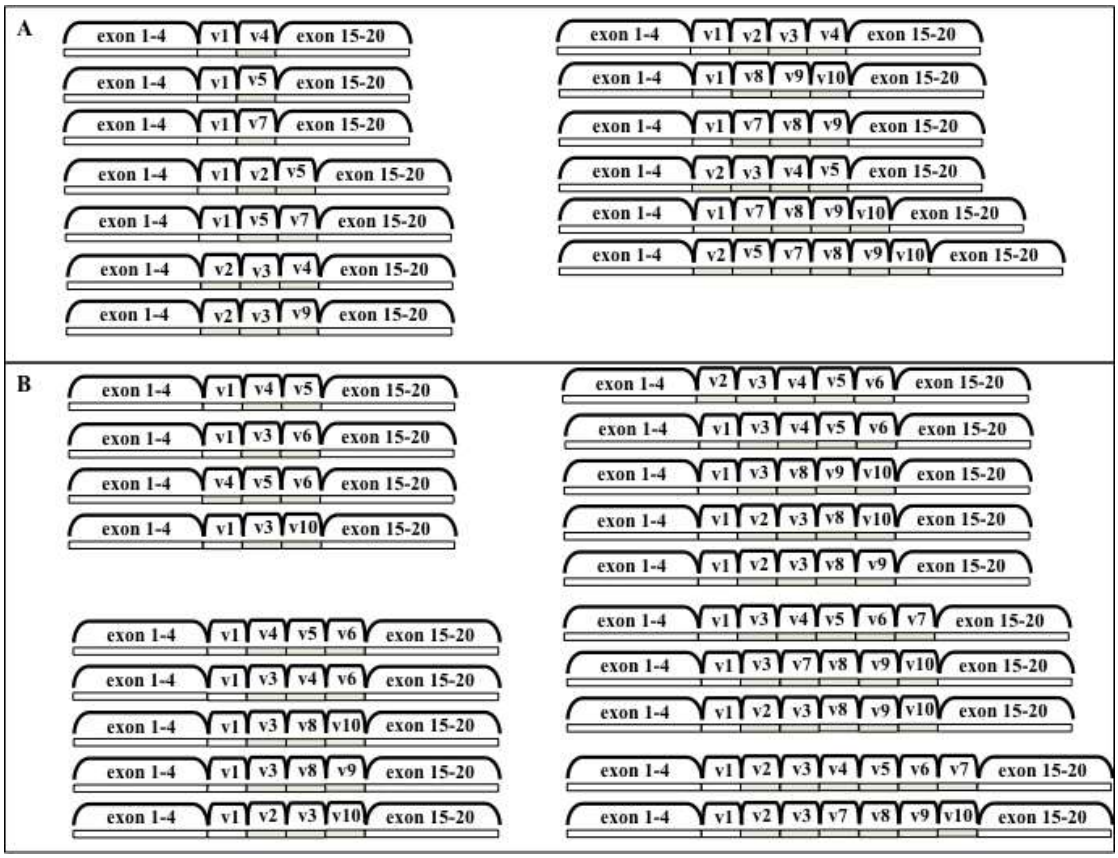


Figure 36. Further Hypothesized CD44 isoforms **A.** Hypothesized CD44 isoforms from the qualitative picture of pairing the variable exon specific primers with the standard region specific ones both 5' and 3' directions in HT168 human melanoma cell line. **B.** Hypothesized isoforms using next generation sequencing with the primer pairs of the fingerprint

We made further attempts and cloned our PCR products from A2058 and HT168 M1 human melanoma cell lines, which resulted in certain isoforms being more dominant and inserting at a higher rate, but yet again, the full set of the expected/calculated isoforms could not be identified. However, direct sequencing of some of the cloned sequences confirmed that v1, is in fact missing in some of the isoforms, which tied in nicely, with our above mentioned PCR-based results (Figure 37A). Furthermore, some isoforms contained a truncated version of v1 (Figure 37B).

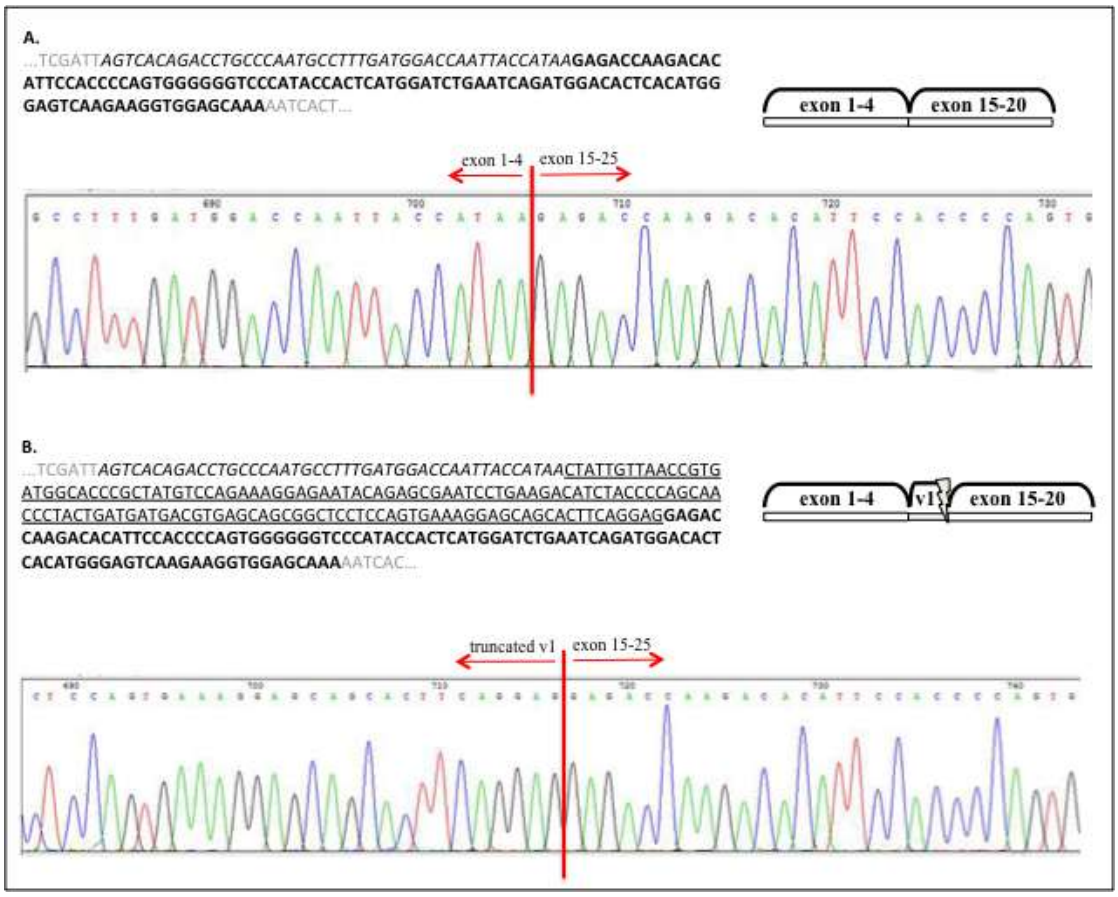


Figure 37.Cloned PCR products from the 5' (exon 4, italic) and 3' (exon 16, bold) primer combination of CD44 in A2058 human melanoma cell line. Direct sequencing shows a CD44 isoform with no v1 or any other variable exons (A) as well as one with truncated v1 (underlined)

5.2.2.2. The CD44 melanoma fingerprint

In light of the complexity of CD44 isoform expression simple method to represent this pattern was developed which included v3 and v6 – the exons considered to be of importance for melanoma progression.

For this purpose, we designed a five primer pair containing PCR-reaction series using a certain combination of the exon specific primers, which covered the whole variable region with five overlapping sequences. The first primer pair was located on the two standard regions allowing detection of all variable exons (S5'-S3', primer pair 1). The second was on exon 4 of the standard region and v3, which makes possible to detect v2-v3 co-expression (S5'-v3', primer pair 2). The third straddles the v3-v10 region by binding to v3 and exon 16 of the standard region (v35'-S3', primer pair 3). The fourth

pair was designed to exon 4 and v6 detecting the co-expression of v2-v5 with v6 (S5'-v63', primer pair 4). The fifth one was detecting v4-v5 expression of v3-v6 co-expressing isoforms by binding to v3 and v6 (v35'-v63', primer pair 5). The five PCR products of the same sample were run always in this same order in every case, so the pattern of the bands were comparable across all of our samples in the different experimental models. This bar code-like pattern is what we define as the 'fingerprint', a simplified representation of the CD44 ASP.

With this method we examined the CD44 ASP of human melanoma cell lines (HT168M1, WM35, WM983B, A2058 and HT199) in culture to establish whether there is a pattern that is conserved across these genetically different tumours. As it is shown in Figure 39, we found a consistent pattern throughout, which we refer to as the melanoma fingerprint. Some of the isoforms, which were predicted from the melanoma fingerprint, based on the size of the bands, were confirmed by next generation sequencing. Although the reading frame of 454 GS Junior is significantly wider than that of similar techniques, its higher limit is still 400-700bp. Therefore, even though next generation sequencing is rather promising, we are still relying on estimations in the case of larger products. 10 isoforms were confirmed (Figure 35A) and a further 26 predicted (Figure 36) as part of the melanoma CD44 fingerprint.

We then compared this pattern to that of other human tumour cell lines grown in culture. These included cell lines derived from human colorectal adenocarcinoma (HT29, HT25, HCT116), human oral squamous cell carcinoma (PE/CA PJ15 and PE/CA PJ41), vulval squamous cell carcinoma (A431) and K562 human erythromyeloblastoid leukemia cell lines. (Figure 38 and 39) Comparison was also made with primary cultured human melanocytes, skin keratinocytes and skin fibroblasts (Figure 39). In each case the fingerprint differed unambiguously from the melanoma fingerprint, raising the possibility of a melanoma specific isoform expression pattern.

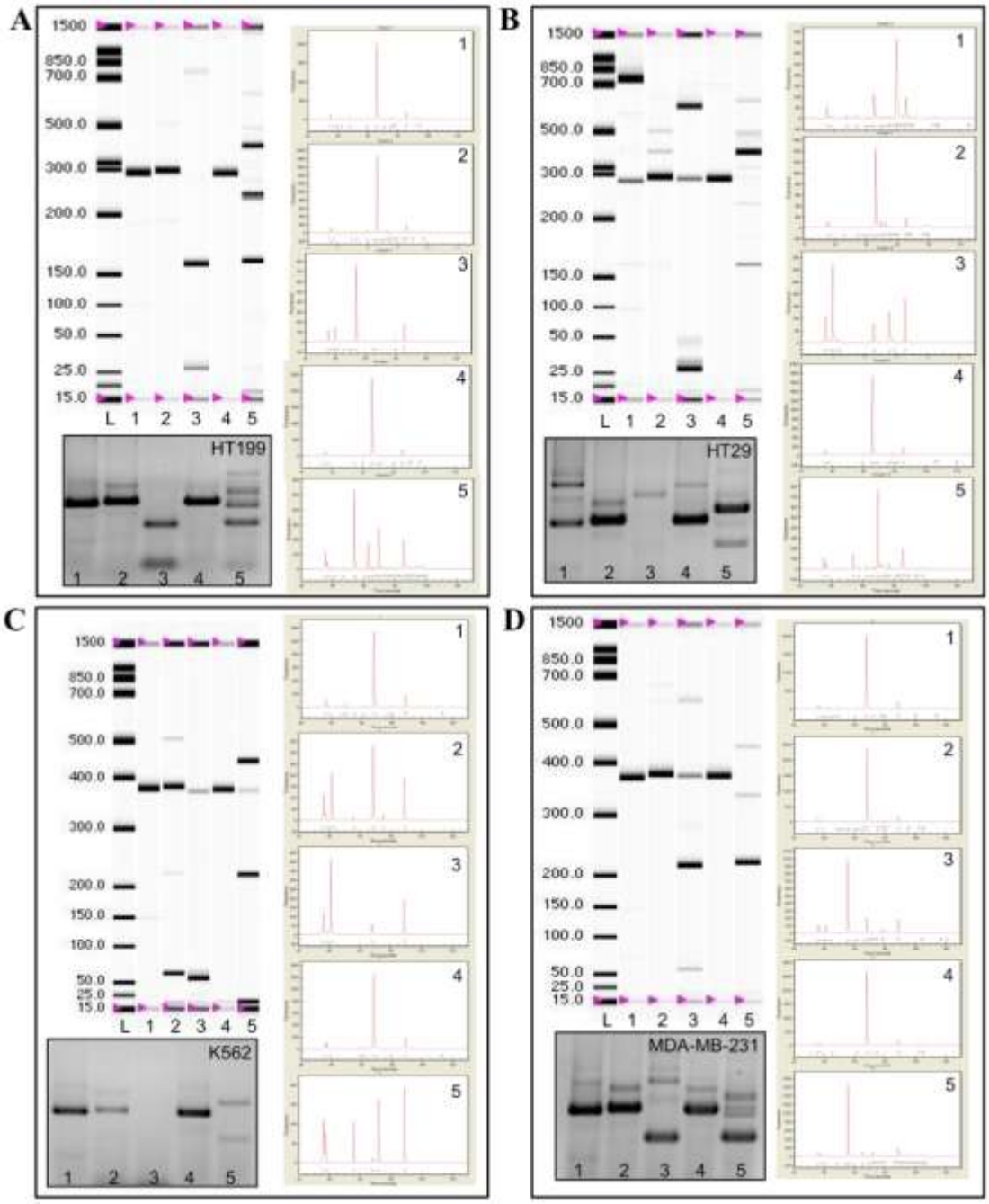


Figure 38. The CD44 alternative splice pattern of different human tumour cell lines demonstrated by virtual gels and electropherograms generated by Experion DNA Capillary Electrophoresis System and corresponding agarose gel picture.**A.** HT199 human melanoma cell line **B.** HT29 human colorectal adenocarcinomacell line**C.** K562 human erythromyeloblastoid leukemia cell line **D.** MDA-MB-231 human breast carcinoma cell line

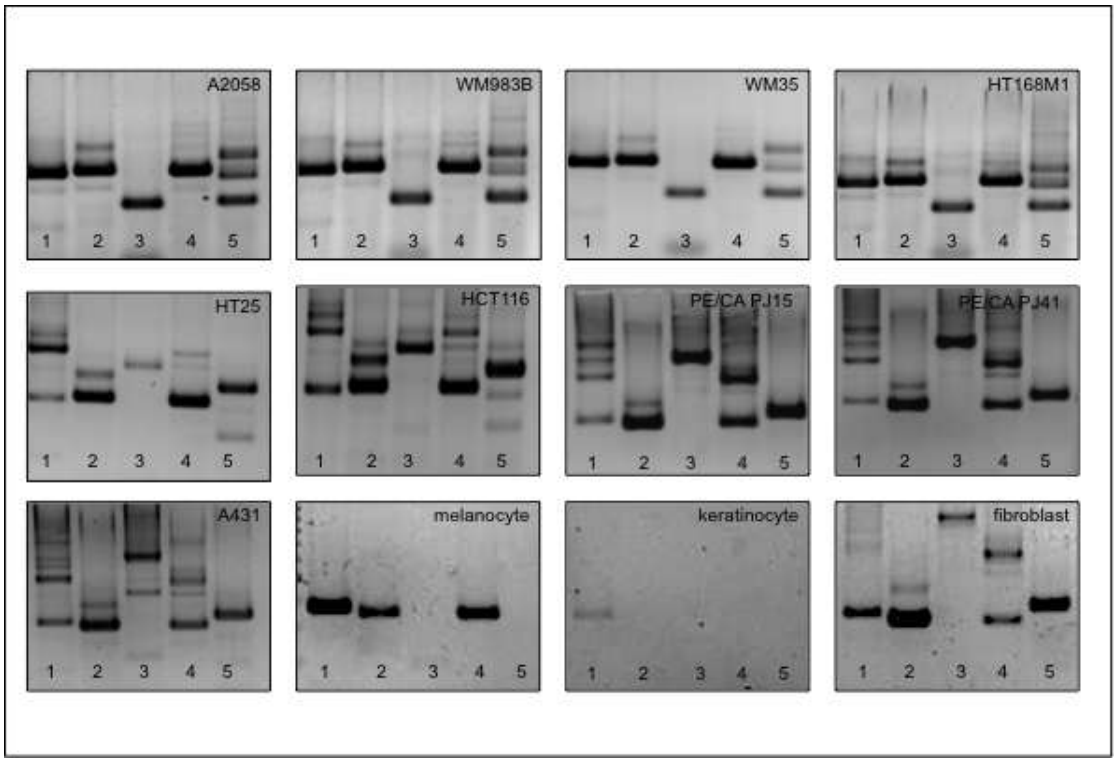


Figure 39.The CD44 alternative splice pattern of different human tumours is different, but preserved throughout samples from the same the tumour type as it is demonstrated by the agarose gel electropherograms of human melanoma (A 2058, WM983B, WM35 and HT168M), colorectal adenocarcinoma (HT25 and HCT116), oral squamous cell carcinoma (PE/CA PJ15 and PE/CA PJ41) and vulval squamous cell carcinoma (A431) cell lines. The melnanoma CD44 fingerprint also differs from that of non neoplastic melanocyte, keratinocyte and fibroblast cell lines as constituents of the microenvironment.

5.2.2.3. Modeling the effects of the microenvironment *in vitro*

To decide whether the *in vitro* melanoma CD44 fingerprint is maintained *in vivo* despite the influence of the microenvironment, we compared the CD44 splicing pattern of several, genetically different human melanoma cell lines (A2058, HT199, WM35, WM983A, M35) growing on plastic or different matrices. We also investigated HT168, a cell line cultured from the *in vivo* xenograft variant of A2058; HT168M1, a cell line which is the *in vivo* selected metastatic version of HT168; WM983B, cultured from a lymph node metastasis from the patient whose primary tumour gave rise to WM983A. Since CD44, as a cell surface glycoprotein, plays an important role in cell-matrix interaction, it was important to examine whether different matrix components change the alternative splicing pattern, or whether the ASP is stable and possibly inherent to

melanoma-specific behavior. Therefore as a first step we determined the CD44 fingerprint of HT168M1 human melanoma cell line growing *in vitro* on different matrices, namely fibronectin, laminin, collagen and matrigel. After 48 hours incubation time the CD44 fingerprint was found to be unchanged in the case of every matrix type (Figure 40). This fingerprint was found to be consistent through all examined cell lines growing on different matrices (only HT168M1 shown). It is interesting, that the fingerprint is retained in the cell lines derived from the primary tumours and their metastases alike (HT168 versus HT168M1 and WM983A versus WM983B).

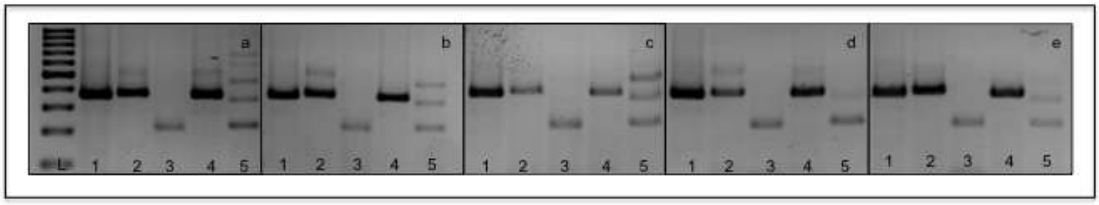


Figure 40. CD44 'fingerprint' of HT168M1 human melanoma cell line growing on different matrices namely plastic (a), fibronectin (b), laminin (c), collagen (d) and matrigel (e). L stands for molecular weight marker.

5.2.2.4. CD44 fingerprint *in vivo*, in surgical samples

However, the *in vivo* microenvironment is far more complex, than the isolated affects of individual molecules of the extracellular matrix. We must consider other host related factors, such as cytokins, growth factors and hormones. The way to prove the melanoma specificity of the fingerprint, would be to detect the same fingerprint in surgical melanoma samples. However, considering that stromal components might have their own CD44 pattern, the total RNA isolated from the macroscopically cut tumour fragments contains both stromal and tumour derived CD44 isoforms, resulting in a mixed 'fingerprint' (Figure 41).

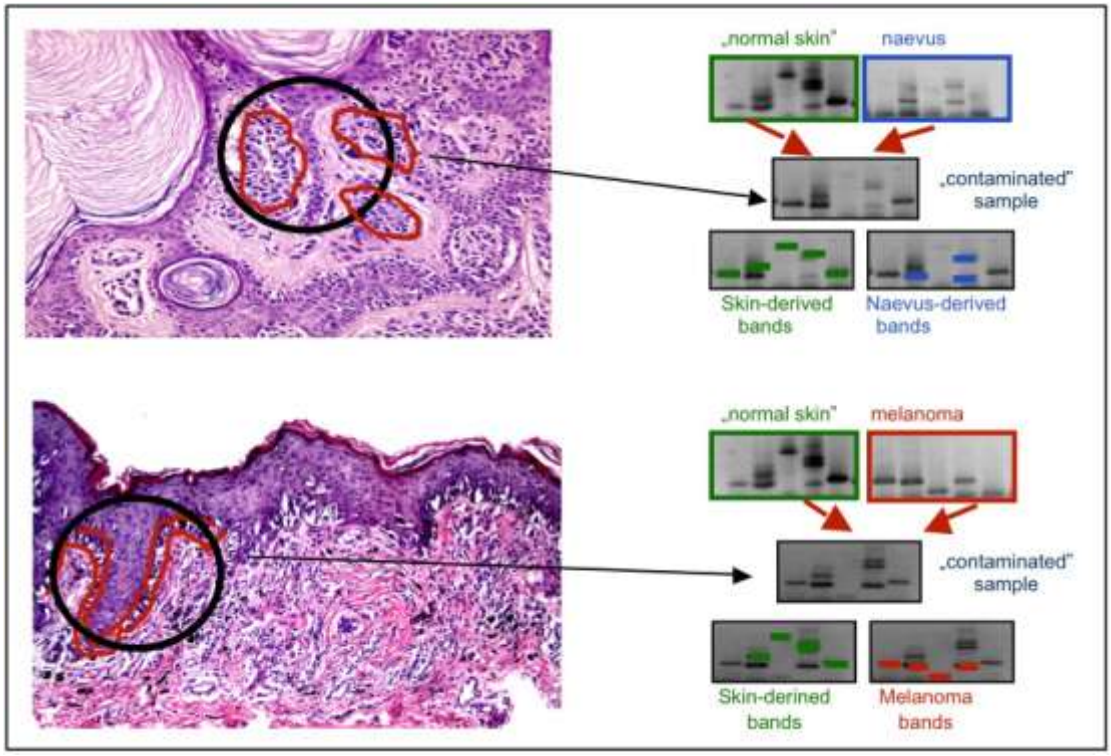


Figure 41.Macroscopically cut tumour samples might result a mixed pattern with ’contaminating’ isoforms from the surrounding tissue(s)

To examine separately the tumour without stromal components we used laser microdissection (LMD) on surgical human melanoma samples (Figure 42). With this method we could determine the ‘fingerprint’ of surgical melanoma samples and compare it to the surrounding normal epidermis and naevus of the same patient removed and stored at the same time. After doing this, we found that the ‘fingerprint’ of melanoma was still the one known from the *in vitro* investigations and mouse model, and completely different from the fingerprint of epidermis and naevus (Figure 42).

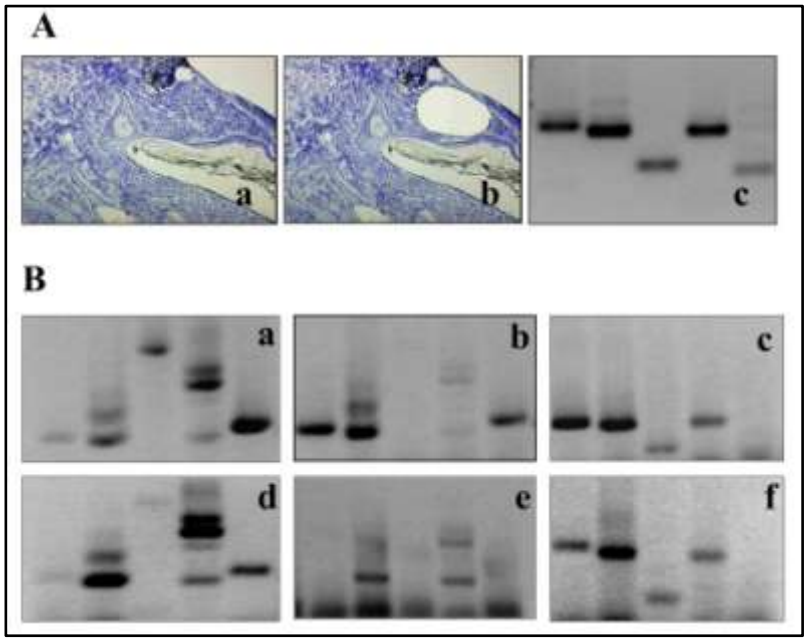


Figure 42. PCR-based detection of CD44 splice variant pattern *in vivo* human melanomas **A.** Superficially spreading human melanoma (SSM) surgical samplebefore (a) and after (b) laser captured microdissection (LCM) and its CD44 isoform pattern (c). **B.** The CD44 expression pattern of two surgical melanoma samples (c and f) is the same known from *in vitro* experiments and basically differs from the pattern of the normal epidermis (a and d) and naevus (b and e) of the same patient shown in the same lane.

5.2.2.5. The CD44 melanoma fingerprint *in vivo* in our animal model

As the *in vivo* microenvironment is far more complex than the influences of the extracellular matrix, we used an animal model to evaluate the CD44 melanoma fingerprint *in vivo*. This model has been developed by our group, following the observation that semi-orthotopically (subcutaneously) implanted human melanomas always formed metastases in newborn *scid* mice (permissive host), yet never did so in adult ones (nonpermissive host). This model made it possible to examine the melanoma 'fingerprint' during the metastatic processes.

In vivo expression patterns were evaluated on two human melanoma cell lines HT199 and HT168M1.

We performed our PCR reaction series on theprimary subcutaneous tumour, circulating tumour cells obtained from blood and lung metastases from transplanted newborn *scid* mice, as well as the primary subcutaneous tumours from transplanted adult mice. In

addition lung tumours were generated in adult animals by intravenous injection (Figure 23).

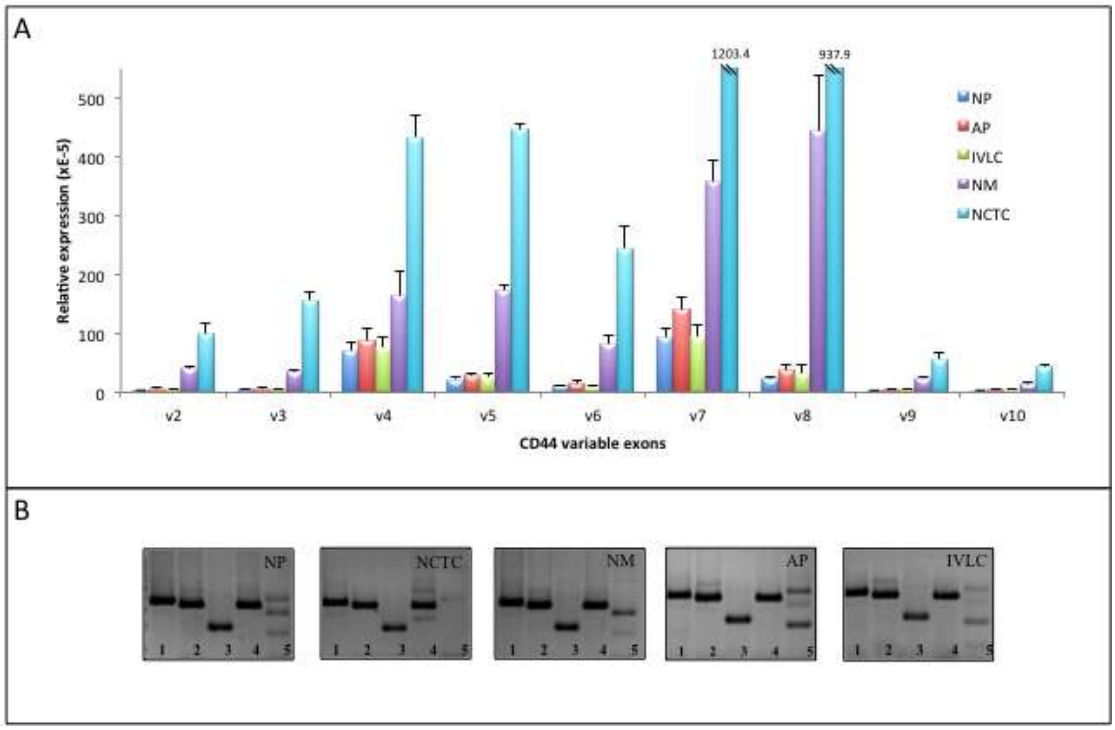


Figure 43.Relative quantitative expression of CD44 variable exons in cell cultures from metastatic (newborn) and non-metastatic human xenograft model (Real-Time PCR measurement) of HT199, a human melanoma cell line of originally low variable exon expression level.. **A.** The relative expression level of all variable exons is raised in circulating metastatic cells (NCTC) and metastatic cells (NM) compared to their levels in primary tumours [newborn primary (NP) and adult primary (AP)] and lung colony (IVLC) **B.** The qualitative fingerprint (bottom line) remains unchanged

For HT199 we found that the CD44 fingerprint demonstrated *in vitro* was unchanged throughout the sampled sites (Figure 36B). These findings do not explain published observations that the expression of certain CD44 exons correlates with metastatic potential. Our results suggest that the CD44 ASP behind the 'fingerprint' is the same in all these cases, meaning that the same isoforms are present. The cited quantitative expression changes of single variable exons should therefore be explained differently.

We made a further quantitative PCR analysis with our variable exon specific primers on the same samples. We examined the quantitative changes of the individual variable exons (VE) during tumour progression in the *in vivo* animal models of two genetically

different human melanoma cell lines (HT199, HT168M1). It has become clear after the first measurements, that the two cell lines have 3 orders of magnitude difference in their CD44 VE expression relative to beta-actin housekeeping gene, but despite this, their malignant potential was practically identical. CD44 in HT199, the cell line with a low base VE expression, behaved as a 'classical' metastasis gene. The non-metastatic adult primary (AP), the metastatic newborn primary (NP) and the lung colony formed after intravenous injection into adult animals (IVLC), which is also a form of primary tumour, all expressed the VEs within the same order of magnitude (Figure 43A). The circulating tumour cells (NCTC) and lung metastases (NM) from the animal implanted as a newborn showed 21 times and 9 times increased expression respectively.

In the case of HT168M1, which expresses the CD44 VEs in 3 times larger order of magnitude compared to HT199, the role of CD44 is more complex. In this case, we created cell cultures from different localisations of the primary tumour of individual animals. The individual lung metastases (newborn animals) and lung colonies (adult animals) of the individual animals were also cultured separately. The non-metastatic adult primary tumour (AP) showed a higher expression level of all CD44 VEs than the metastatic newborn primary tumour (Figure 44A). The reason behind the rather large error bars seen on the measurements from the newborn lung metastases (NM) is that the individual lung metastases showed huge VE expression level differences. Cells from the cell line created from the lung metastasis showing the highest CD44 VE expression level (NM=S1T2, Figure 44B) were then re-implanted subcutaneously into newborn animals. Cell cultures were then created from three different localisations of the primary tumour (PNM) and three random lung metastases (MPNM) of the chosen animal (Figure 23). They showed no difference in CD44 VE expression level compared to each other, however they showed on average 24 times lower expression than the cell culture (newborn lung metastasis, NM=S1T2) of origin. We also consistently detected lower CD44 VE expression in liver metastases (LMIVLC) from lung colonies (IVLC), which are practically secondary metastases (Figure44C).

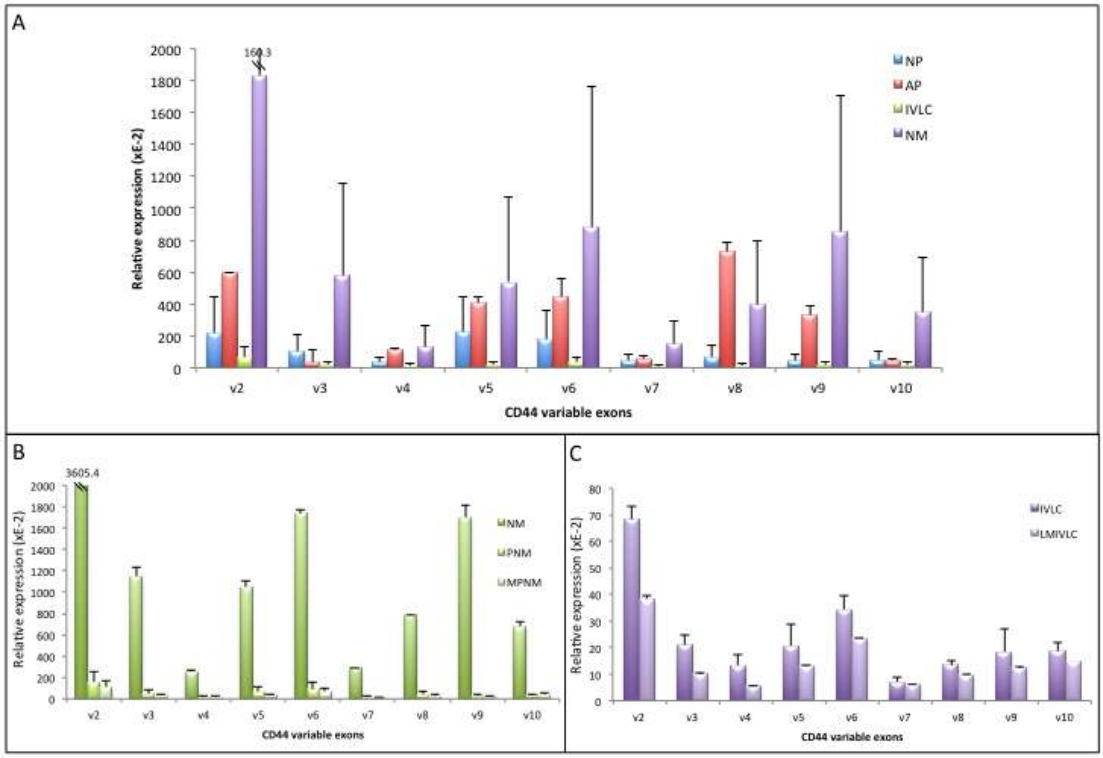


Figure 44. Relative quantitative expression of CD44 variable exons in cell cultures from metastatic (newborn) and non-metastatic [adult (AP)] human xenograft model (Real-Time PCR measurement) of HT168M1, a human melanoma cell line of originally high variable exon expression level. **A.** The relative expression level of all variable exons is raised in certain lung metastasis (NM), when remaines low or even decreases in other lung metastases resulting in large error bars. It should be noted that the expression level of the primaries from different localisations [newborn primary (NP), adult primary (AP) and intravenously implanted lung colony (IVLC)] are all comparable, although the slightly higher expression observed in the adult primary is an unexpected finding. **B.** We used the lung metastasis from the newborn animal with the highest CD44 variable exon expression level (NM=S1T2) for subcutaneous re-implantation into another newborn animal. The expression level in the primary tumour (PNM) and its metastases (MPNM) was 24 times lower on average. **C.** The liver metastases (LMIVLC) from the intravenously implanted lung colonies (IVLC) showed a decrease in expression, when compared to the lung colonies (IVLC).

5.2.3. CD44 colorectal adenocarcinoma

The ultimate proof of the CD44 alternative pattern being a tumour-specific phenomenon and that progression, and metastatic potential related changes CD44 changes are quantitative, rather than qualitative, we examined the CD44 ASP of human colorectal adenocarcinoma.

5.2.3.1. CD44 variable exons and possible isoforms at mRNA level

Using the same set of five primers [although in different order: S5' – S3' (A-B), S5'-v3 3'(A-D), S5'-v6 3'(A-E), v3 5'- S3' (C-B), v3 5' – v6 3' (C-E)], (Fig. 2A) as we did in the case of human melanoma, we identified 26 expressed mRNA sequences behind the fingerprint, some of which were proven with next generation sequencing, others by estimation based on product sizes. Fourteen of these were full-length isoforms from the AB primer combination (Figure 45), the rest were segments using the rest of the primer pairs (data not shown).

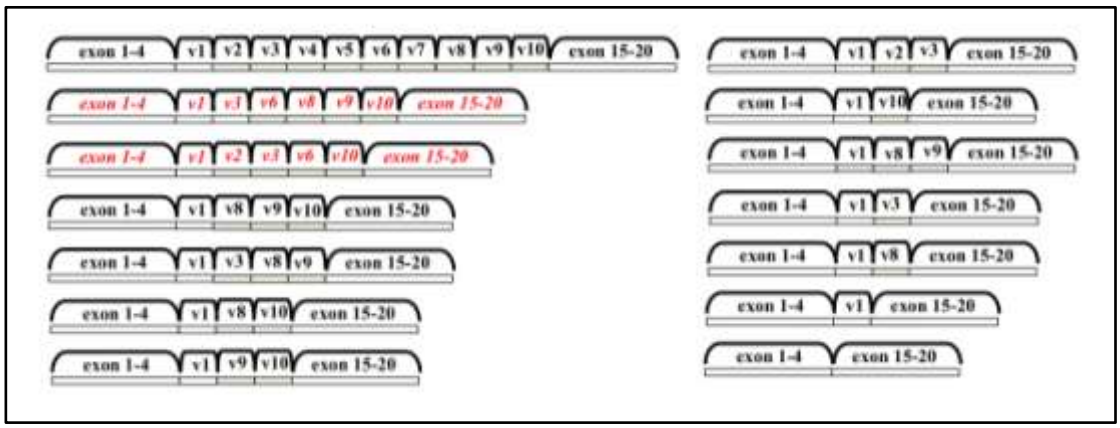


Figure 45. Expressed full CD44 isoforms in HT29 human colorectal cancer cell line. Agarose gel electrophoretograms of the PCRs performed with A-B primer combination.A total of 14 different, expressed mRNA isoforms were identified and either confirmed by next generation sequencing, or predicted (red italic) based on product size.

5.2.3.2. The CD44 fingerprint of human colorectal adenocarcinoma

With the five primer pair PCRseries method[using the following combination: S5' – S3' (A-B), S5'-v3 3'(A-D), S5'-v6 3'(A-E), v3 5'- S3' (C-B), v3 5' – v6 3' (C-E)], , we

examined the CD44 ASP of human colorectal carcinoma cell lines (HT29, HCT116 and HT25)in culture.The pattern was found to be conserved across these genetically different tumours. (Figure 46)

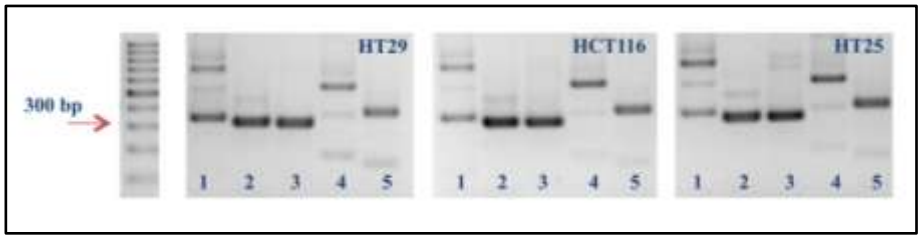


Figure 46. CD44 fingerprint of human colorectal carcinoma Agarose gel electrophoretograms demonstrating CD44 isoform expression pattern of human CRC (colorectal specific „CD44 fingerprint"). CD44 fingerprint of HT29, HCT116 and HT25 colorectal carcinoma cell lines our five primer reaction series (1. A-B; 2.A-D; 3.A-E; 4.C-B; 5. C-E).

5.2.3.3. The CD44 colorectal fingerprint *in vivo* in our animal models

To examine the specificity of the *in vitro* identified qualitative CD44 colorectal carcinoma fingerprint, we performed the same five primer pair based PCR reaction series on samples from our animal models.

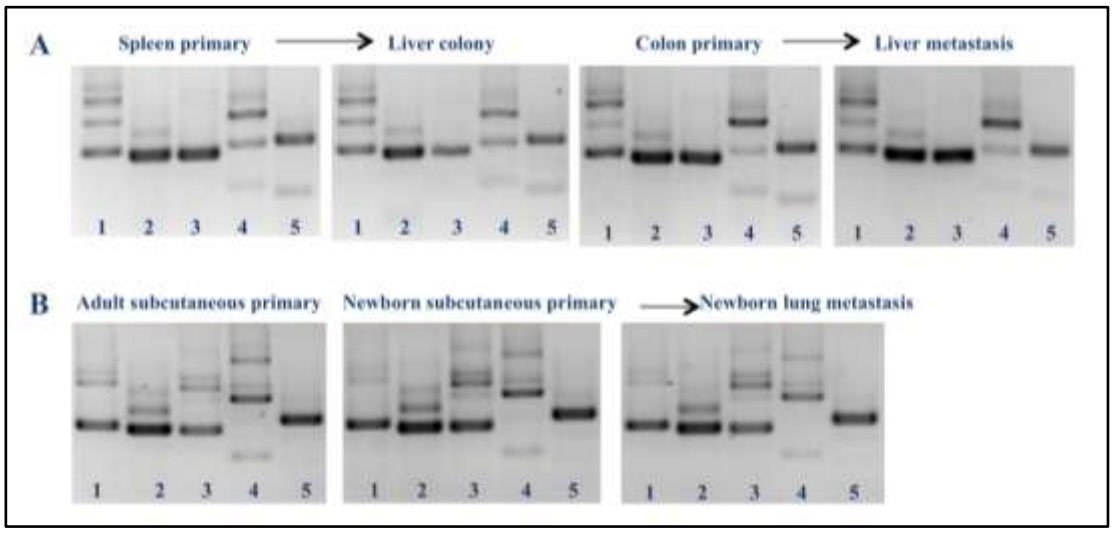


Figure 47. CD44 fingerprint *in vivo*A. CD44 pattern of HT29 colorectal carcinoma xenografts in orthotopic implantation and liver colonization models B. CD44 fingerprint in metastatic and non-metastatic version of subcutaneously implanted HCT116 human colorectal carcinoma cell line in spontaneous pulmonary metastasis model.

The fingerprint of HT25 and HCT116 (data not shown) *in vivo* was identical to the one detected in the injected suspension (*in vitro* sample) of these cell lines, and it was conserved throughout all samples from the respective spleen primaries and their liver colonies of our liver colonisation model, and the colon wall primaries and liver metastases of our implantation model.

Non-metastatic and metastatic versions of primary HT29 tumours subcutaneously implanted into adult and newborn *scid* mice respectively, and lung metastases showed the same CD44 pattern detected in the HT29 cell culture (*in vitro*) used for implantation. (Figure 47B)

Much like the melanoma fingerprint, the CD44 fingerprint of human colorectal carcinoma was found to be tumour specific and showed no qualitative change during tumour progression in our different animal models in vivo.

5.2.3.4. Quantitative changes of v3 and v6*in vivo* in our animal models

5.2.3.4.1. Mouse C26 isograft system

The relative expression of the examined variable exons (VE) was found to be one magnitude larger in the liver metastasis when compared to its orthotopically implanted colon wall primary, while no such difference was detected between the spleen primary and its liver colony. However, interestingly the relative expression level of v3 and v6 in both the colonic wall and splenic primaries was comparable. (Figure 48)

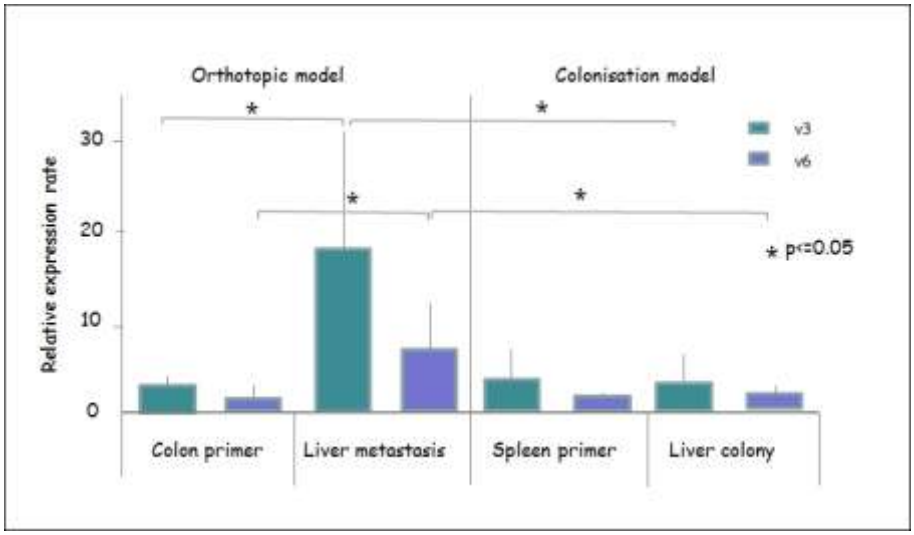


Figure 48. Relative expression of CD44v3 and CD44v6 in orthotopic implantation and liver colonization models of C26 mouse isograft colorectal carcinoma. There is a

significantly higher expression of these variable exons in true liver metastases, when compared to their colonic wall primaries (orthotopic implantation), while there is no such difference between the liver colonies formed after intrasplenic transplantation (liver colonization model)

5.2.3.4.2. Human colon cancer xenografts

Although all of the examined HT29, HT25, HCT116 human colorectal carcinoma cell lines possessed a similar metastatic rate of approximately 50-65%, they expression level of the examined VEs showed rather different patterns in our orthotopic and liver colonization models. (Figure 49)

CD44 v3 and v6 expression in HT29 showed identical pattern to that observed in our C26 mouse isograft model with elevated expression levels in true liver metastases, no increased expression in liver colonies when compared to their respective primaries, and comparable levels in both primaries.

HT25 showed quite the opposite behavior with comparable levels of expression in colonic primary and its true liver metastasis, while expression level was elevated in the liver colonies, when compared to its splenic primary.

In the case of HCT116, where the primary tumours showed extremely high CD44v3/v6 expression levels compared to the other two cell lines, liver metastases/colonies from both primaries showed a magnitude lower expression level.

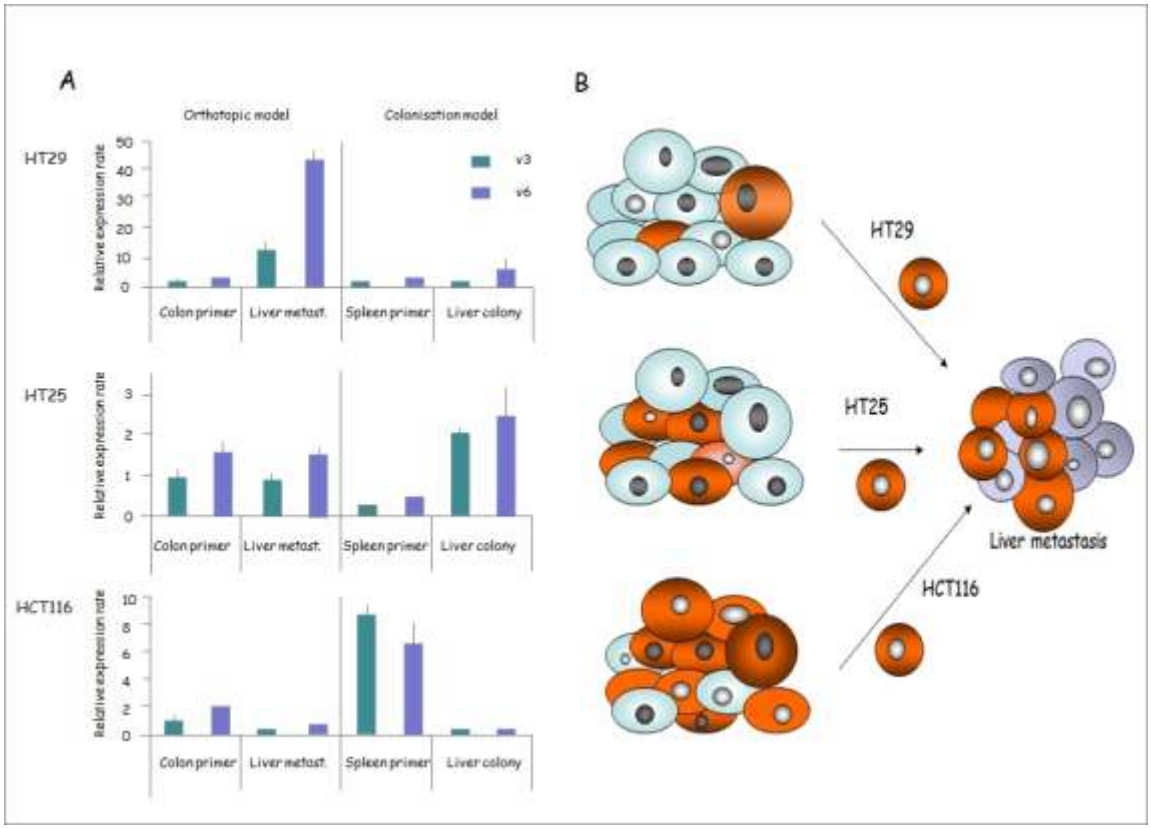


Figure 49. Relative expression of CD44v3 and CD44v6 in human metastatic and liver colonization colorectal carcinoma xenograft models CRC metastatic and liver colonization system. A. The changes in the expression levels between primaries and their metastases / colonies follow three different patterns in the three examined cell lines (HT29, HT25 and HCT116). B. Model of the metastatic clone selection in each of the three cell lines

5.2.3.4.3. Spontaneous lung metastasis xenograft model from subcutaneous implantation

We have found that, in all three of the examined cell lines (HT25, HT29, HCT116)CD44v3 and CD44v6 expression levels were significantly, 2-3 fold higher in the metastatic primary, growing in *scid* mice implanted as newborns, compared the non metastatic primary version of the same tumour, growing in mice implanted as adults. (Figure 50).

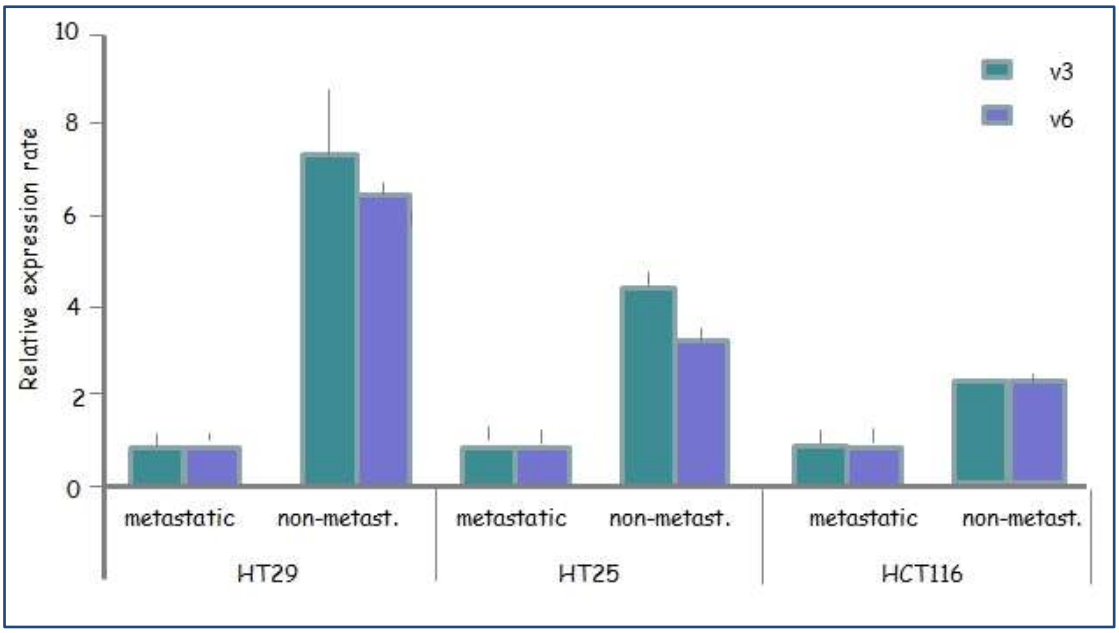


Figure 50. Relative expression level of CD44v3 and CD44v6 in our spontaneous lung metastases *scid* mouse xenograft model of three genetically different human colorectal carcinoma cell lines (HT29, HCT116 and HT25). The relative expression level was significantly higher in the metastatic (newborn) primary subcutaneous tumour compared to the non-metastatic (adult) one

6. Discussion

The death of melanoma patients (similarly to most other tumour types) is caused by disseminated disease. The average survival ratio is less than 4% after the development of distant metastases. Therefore it is rather important to find new markers not only to better understand the progression of the disease, but also to serve as diagnostic markers of progression, or even targets for therapy.

6.1. Novel markers of a more metastatically potent melanoma phenotype

The first steps in the dissemination of the primary tumour is the formation of and extravasation through blood vessels, and it seems that a tumour that possesses molecules that are involved in these processes will be more successful at forming metastases. This was, proven to be the case when we studied the behaviour of our α2bβ3 integrin transfected human melanoma cell line (WM983B). The presence of

α2bβ3, which is a main adhesion receptor of platelets playing a key role in their aggregation, with the parallel upregulation of bFGF expression resulted in an angiogenic, more progressive phenotype.

In addition to α2bβ3 expression, we also identified 12-LOX, c-kit and WT1 expression of human melanoma. The expression of these thrombocyte (platelet-mimicry) and stem cell markers would not only enable more progressive properties, but also would provide a target for anti-tumoural therapy.

6.2. WT1 expression of human melanoma

It is a non-negligible fact that once a gene undergoes alternative splicing any meaningful conclusion about its function and behaviour can only be drawn after careful examination of all expressed alternative isoforms. Ten of our examined human melanoma cell lines were found to be positive for WT1 at mRNA level with inconsistent KTS+ and KTS- isoform pattern throughout. Although the 17AA isoform ratio was inconsistent (7 containing both 17AA- and 17AA+ isoforms, 2 containing 17AA-, and 1 containing 17AA+ isoform only), there was no association of any particular phenotype with metastatic potential. Also, the splice pattern of the WT1 zinc-finger region of human melanoma was not found to be melanoma specific, when compared to other tumour types.

We also identified a new splice variant with the in frame skipping of zinc-finger 3.

Although no association was found between the WT1 ASP, or its qualitative change and the metastatic potential, our quantitative Real Time PCR measurements showed that higher WT1 expression correlates with better prognosis in our experimental animal model. However, cell lines with truncated zinc finger region showed different behaviour, which underlines the importance of identifying all isoforms of alternatively spliced genes, if one wants to examine the role of the given gene during tumour progression.

WT1 expression was also confirmed at protein level in HT168 and HT168M1 human melanoma cell lines, with predominant intracellular localisation, where it was present oh higher percentage, that the classical melanoma markers gp100 and Melan-A.

This raises the possibility of WT1 to be used as a marker for melanoma, as well as a target for anti WT1 immunotherapy (antibody-, T-cell mediated and anti-sense, respectively) in the therapy of the melanoma. Also, our PCR measurements showed high sensitivity in detecting WT1 mRNA expression of circulating tumour cells. This

would make WT1 a good candidate for a new marker for the clinical diagnosis of progression.

6.3. CD44 expression of human melanoma

While WT1 has quite a few alternative isoforms, the task of studying the gene in its entirety with all alternative isoforms is a rather daunting task in the case of CD44 with its thousands of different possible isoforms. A reliable and reproducible method is needed to be able to study these large number of molecules at the same time.

Our first step was to determine which variable exons, other than the ones most studied in the literature, are expressed at mRNA level in human melanoma. We showed that all the variable exons are expressed in human melanomas and predicted a number of paralelly expressed CD44 isoforms. We also found that v1 was missing from some of the isoforms, although it is not considered as a variable exon, also that some of the isoforms contained a truncated v1 exon. This was confirmed by direct sequencing of our cloned molecules. However, next generation sequencing studies have surfaced a whole other level of 'complications' by identifying a number of deletions across the variable exons.

As a surrogate for representation of all of the expressed alternative splice variants of CD44, we developed a method to determine a 'fingerprint' of expression in human melanoma. This appeared to be stable in cell culture and mouse xenograft models and differed substantively from that found in colorectal adenocarcinoma, squamous cell carcinoma and primary cultures of human melanocytes, kerationcytes and fibroblasts. This technique bypasses the attempts to link the expression or co-expression of individual variable exons to metastasis formation, a strategy that loses crucial contextual information about the complex ASP underlying the CD44 protein set. This oversimplification may also account for some of the contradictory evidence on the associations of CD44 expression with the generation of a metastatic phenotype.

Previous work in our laboratory has shown that under the effect of host derived selection factors, xenografts of tumour cells growing in new born *scid* mice differ in gene expression pattern than those growing in adult mice. It is not yet clear whether this pattern is related to formation of metastasis or reflects a summation of changes resulting

from local effects of graft:host interaction. Adaptation to a new microenvironment is a crucial factor in the formation of metastasis: This may require events in changing patterns of gene expression prior to implantation or may reflect post hoc modification of expression in response to the metastatic niche. To be able to study the pattern of expression during tumour progression we have established an experimental mouse model in which the expression pattern of pure cultured cells from a primary implanted tumour, circulating cells in the peripheral blood stream and cells within established metastases in newborn *scid* mice from the same, individual animal could be studied. In addition expression patterns could be compared with those generated in adult *scid* mice either as primary tumours as lung colonies since spontaneous metastases are not formed in this animal population. We followed the CD44 VE expression changes during tumour progression of two human melanomas, that express CD44 VEs at different orders of magnitude, in this experimental animal model. We found that CD44 VE expression and metastasis formation showed inverse correlation. The adult primary tumour, newborn primary tumour and lung colony of HT199, the human melanoma cell line with low base CD44 VE expression level, all expressed the variable exons in the same order of magnitude and within the error bar. However, as only a limited number of cells within the primary tumour are capable of forming metastasis, the above finding is not surprising. The dramatic increase in CD44 VE expression level seen in the metastatic tumours means, that the variants most likely play a role in metastasis formation. The extreme high level of CD44 VE expression in the circulating tumour cells of the newborn animals seems to indicate, that their role is mainly in getting into and / or surviving in the circulation. It seems, that for the new population of metastatic cells in the target organ, CD44 VE expression level is not as much or even not at all important.
The CD44 VE expression level in HT168M1, which has a high base CD44 VE expression, varies within the metastases, ranging from barely detectable to approaching the level detected in circulating tumour cells. When a population with extreme high CD44 VE level is then re-implanted, the expression level dramatically decreases in the metastases while the qualitative picture (fingerprint) remains unchanged.

From these results, we suggest that predicting the role of CD44 variable exon expression in tumour progression is more complex than previously anticipated. Our qualitative studies identified a melanoma-specific CD44 ASP, or fingerprint, which is different to the pattern of other examined tumour types. While this certainly raises the

possibility to use this fingerprint to identify the unknown primary of metastases, the stability of this fingerprint during tumour progression also shows that no isoforms changes occur that could be correlated to metastatic phenotype or prognosis. However, our real-time PCR measurements suggest that quantitatively, melanoma is not a uniform group and CD44 variable exon expression levels can follow different patterns. Primary tumours with low overall CD44 VE expression level harbor metastatic clones with high expression level, which appears to be needed for entering the circulation and forming metastases. On the other hand, primary tumours with high base CD44 VE expression also contain metastatic clones, which either have sufficient CD44 expression or 'utilize' other molecules to facilitate metastasis formation. This, however, does not explain the extreme low CD44 VE expression levels detected in lung metastases, and further research in this area is needed. In any case, in both scenarios the CD44 VE expression level of the true metastatic clone is not immediately obvious from the overall expression of the primary tumour, which partially explains the contradicting results described in the literature.

We hypothesise that the metastatic clone is not the result of ratio changes or even an 'appearing/disappearing variable exon', which might be one of the several factors to give metastatic property to that clone. Ultimately, these results show that even in one of the 'most simple' CD44 fingerprints, melanoma, we cannot talk about 'CD44' as a single molecule anymore.

6.4. CD44 expression of human colorectal adenocarcinoma

Similarly to melanoma, we found that human colorectal adenocarcinoma also has a specific CD44 fingerprint, which is conserved across *in vitro* growing genetically different colorectal cancer cell lines and their subcutaneous, intrasplenic and orthotopic implanted primary tumors and their lung/liver metastases/colonies.

We identified a number of definitely expressed isoforms and further predicted some more using next generation sequencing and product size based estimation based on the appearances of the fingerprint.

Our studies into the qualitative changes of the expressed v3 and v6 variable exons seemed to tie in nicely with out findings in melanoma. Similarly to what was observed in HT199, a higher v3 and v6 expression level was detected in true liver metastases from orthotopically transplanted primaries of both our isograft (C26 in Balb/C mice) and one of our xenograft (HT29 in *scid* mice) tumours, while liver colonies from splenic

transplantation showed no increase in these variable exons. This confirms again, that the expression level of v3 and v6 is not the effect of the liver microenvironment, but it is a pre-existing feature of the metastatic clone cells within the primary tumour.

HT25 showed comparable v3 and v6 expression levels in orthotopically transplanted colon primaries and their true liver metastases, and showed increased levels of expression in liver colonies, when compared to their splenic primaries. This, along with the fact that HCT116, a cell line with extremely high CD44v3/v6 expression level, showed a third pattern of behaviour, wherein liver metastases/colonies from both primaries showed a magnitude lower expression level, further confirms our conclusions based on results from HT168M1, the cell line with high base VE expression level. We, again suggest, that primary tumours of different base CD44 VE expression levels harbor different numbers of highly expression, metastatically more potent clones, which is not immediately evident from the overall expression level of the primary tumour. From a practical point of view, this means thatthe ‘lack’ of overexpression would not exclude metastatic potential or even a worse prognosis.

The role of the microenvironment was proven in out subcutaneously implanted xenograft model of HT25, HT29, HCT116, where CD44v3/v6 expression levels were significantly higher in the metastatic version (animals transplanted as newborn) of the same tumour, when compered to the non-metastatic version(animals transplanted as adult), as these genetically identical host were only physiologically different.

7. Conclusion

To sum up our studies into identifying novel markers of melanoma progression, our research resulted in a number of findings, with potential clinical importance:

1. We described a number of new melanoma markers (α2bβ3, 12-LOX, c-kit and WT1), which characterize the angiogenic phenotype and platelet mimicry of human melanoma, both of which endows the cells with a more progressive phenotype. These markers can potentially be used to better characterise the progression and predict the prognosis of this malignant tumour, as well as provide possible diagnostic, and/or therapeutic targets.
2. When examining the expression WT1 alternative isoforms in human melanoma, we found that:
    a. Although ten of the twelve examined human *melanoma cell lines expressed WT1 at mRNA level*, there was no consistent, melanoma specific WT1 ASP, when compared to other non-melanoma cell lines.

b. There is *no correlation between WT1 alternative splice pattern and metastatic propensity*, and no qualitative splice pattern change can be observed during tumour progression.
c. Higher *WT1 expression level correlates with better prognosis* in our animal model.
d. Detection of the metastatic, circulating melanoma cell via PCR detection of WT1 mRNA can be a *useful marker for the detection of dissemination*.
e. The expressed WT1 protein can serve as a target, for *anti-WT1 therapy*.

3. As the main body of our research, we described the CD44 alternative splice pattern of human melanoma and showed that it can not be looked at as a single molecule and that all isoforms must be considered to gain any meaningful data on its function and role during tumour progression. We showed, that:
    a. Although many of the actual expressed mRNA isoform can be confirmed via a combination of techniques, such as next generation sequencing and cloning, and some more can be predicted based on product sizes of PCR reaction with varying primer combination, most of them will still remain undetectable using the currently available technologies. We *proved the presence of certain CD44 alternative splice isoforms and predicted the presence of other ones* in human melanoma.
    b. There is a *characteristic melanoma-specific CD44 alternative splice pattern* (ASP), that is conserved across genetically different samples of this tumour type *in vitro* as well as *in vivo*
    c. The *CD44 melanoma ASP is different to the ASP of other human tumour types* examined by us (i.e. colorectal adenocarcinoma, oral squamous cell carcinoma and squamous cell carcinoma of the vulva), which my a suggest a role in the differential diagnostics of difficult cases
    d. When tested in our experimental animal model, the melanoma CD44 ASP showed *no qualitative change during the progression of the tumour*, i.e. was conserved across samples from the non-metastatic (transplanted into adult *scid* mice), metastatic (transplanted into newborn *scid* mice) and spontaneous lung metastasis of the examined tumour type. This showed, that there is no isoform shift, that would correlate with progression or metastatic potential, and that the previously described

variable exon expression level changes in the literature had to be explained differently.

e. There are *definite quantitative changes* of the stable CD44 isoform sets during the progression of human melanoma in our experimental animal model, which is characterised by increased CD44 expression level of the metastatic clone detectable in the circulating tumour cells and spontaneous lung metastases, but not necessarily in the primary tumour, meaning thatthe 'lack' of overexpression would not exclude metastatic potential or even a worse prognosis

4. We suggest, that the above described *behaviour of the CD44 ASP*, i.e. tumour specificity with stability across *in vivo* and *in vitro* samples, and characteristic quantitative, but not qualitative changes during tumour progression, *might be consistent* as we proved it to be so in the case of human colorectal adenocarcinoma.
5. The qualitative stability of the CD44 ASP raises the possibility of CD44 being used in the diagnosis of occult primaries, although extensive further work is needed to test the stability of the CD44 ASP of other tumour types during their progression.
6. Ultimately, our results show that we *cannot talk about 'CD44' or other genes, which undergo alternative splicing, as single molecules* anymore and must keep it in mind, when targeting certain parts of the isoform/isoforms during diagnostic (i.e. immunohistochemical) or therapeutic (i.e. targeted therapy) processes.

8. Summary

The aim of the studies, described in the thesis, was to identify and examine novel markers, that might play a significant role during the progression of human melanoma.

We have proven the expression of a number of markers (α2bβ3, 12-LOX, c-kit and WT1), which we suggest results in a more metastatically potent phenotype of human melanoma.

However, the majority of our experiments was centered on examining the pattern of splice variants, isoforms with potentially different functions, and characterizes their behavior during the progression of human melanoma.

WT1 showed no melanoma specific alternative splice pattern, when compared to other human tumour types and no change, which would correlate with the metastatic potential. However, detection of WT1 mRNA in the circulating tumour cells might be a useful clinical marker of disease progression / dissemination. The expressed WT1 protein can serve as a target of anti-WT1 therapy of human melanoma.

The role of CD44 in the progression of human melanoma has mostly been characterised by qualitative changes in expression of its individual variable exons. These exons

however, may be expressed to form a number of molecules, the alternative splice variants of CD44, which may be structurally and functionally different. Using real-time PCR measurements with variable exon specific primers we have determined that all are expressed in human melanoma. To permit comparison between different tumours we identified a stable CD44 variable exon (CD44v) expression pattern, or CD44 ‘fingerprint’. This wasfound to remain unchanged in melanoma cell lines cultured in different matrix environments. To evaluate evolution of this fingerprint during tumour progression we established a *scid* mouse model, in which the pure expression pattern of metastatic primary tumours, circulating cells and metastases, non-metastatic primary tumours and lung colonies could be studied. Our analyses demonstrated, that although the melanoma CD44 fingerprint is qualitatively stable, quantitative changes are observed suggesting a possible role in tumour progression. Such behaviour of the CD44 alternative splicing pattern was proven to be consistentphenomenon, as the characteristic CD44 ASP of human colorectal adenocarcinoma also showed tumour specificity with stability across *in vivo* and *in vitro* samples, and characteristic quantitative, but not qualitative changes during tumour progression. This raises the possibility to use CD44 in the daily diagnostic work not only for differential diagnosis, but also for the diagnosis of occult primaries.

Összefoglalás

A dolgozat alapjául szolgáló munka célja a humán melanómák progressziójában szerepet játszó új markerek azonosítása és azok kísérletes vizsgálata volt.

Igazoltuk néhány gén (α2bβ3, 12-LOX, c-kit és WT1)expressziójának szerepét a humán melanómák metasztatikus fenotípusának kialakításában.

A kísérletek döntő hányadát azonban az alternatív splice variánsok, mint potenciálisan új funkciót ellátó izoformák mintázatának vizsgálata képezte a humán melanómák progressziója során.

Más humán tumorok splice mintázatával összevetve, a WT1 nem mutatott melanóma-specifikus mintázatot sem metasztázis-asszociált változásokat.Azonban a keringő tumorsejtek által expresszált WT1 mRNS a progresszió/disszemináció klinikai követésére használhatónak bizonyult. Az expresszált WT1 fehérje új célzott terápiás targetként szolgálhat melanómák esetén.

A CD44 humán melanóma progressziójában betöltött szerepét az irodalmi adatok többnyire az egy-egy variábilis exon expressziójának kvalitatív/kvantitatív változásával hozták összefüggésbe. Ezek az exonok azonban több expresszált CD44 molekula, azaz splice variáns részét is képezhetik, amelyek nemcsak szerkezetileg, de akár

funkcionálisan is különbözhetnek egymástól. CD44 alternatív splice mintázatának (ASM) elemzése során azt találtuk, hogy a humán melanóma rendelkezik egy, csak rá jellemő, más vizsgált tumortípustól jól megkülönböztethető CD44 variábilis exon (CD44v) mintázattal – CD44 'ujjlenyomattal', amely nem mutat kvalitatív változást a tumor mikrokörnyezetének *invitro* megváltoztatása során. A CD44 ujjlenyomat tumorprogresszió során bekövetkező változásának *in vivo* vizsgálatára létrehoztunk egy olyan kísérletes rágcsáló modellt, amelyben az áttétképzés lépcsőfokai külön-külön is vizsgálhatók. A CD44 ujjlenyomat ugyanazon állatból származó primer, keringő- és áttéti tumorban valamint a kolóniákban is megtartotta kvalitatív stabilitását, ugyanakkor szignifikáns kvantitatív változások voltak mérhetők a tumorprogresszió során jelezve annak a lehetőségét, hogy szerepet játszik a folyamat kialakulásában. A CD44 ujjlenyomat hasonló viselkedését találtuk humán kolorektális daganatokban: a tumorspecifikus mintázat jellegzetes kvantitatív, és nem kvalitatív változást mutatott a tumorprogresszió során. A splice mintázat kvalitatív stabilitása felveti a daganat-specifikus CD44 'ujjlenyomat' használatának lehetőségét a differenciáldiagnosztikában és okkult primer tumorok azonosításában.

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10. List of the author's publications

Publications closely related to the dissertation:

1. **Raso-Barnett L** - Banky B, Barbai T, Becsagh P, Timar J, Raso E. (2013) Demonstration of a Melanoma-Specific CD44 Alternative Splicing Pattern That Remains Qualitatively Stable, but Shows Quantitative Changes during Tumour Progression.*PLoS One.* 8(1):e53883. Epub 2013 Jan 14.,
2. Bánky B -**Rásó-Barnett L**, Barbai T, Tímár J, Becságh P, Rásó E. (2012) Characteristics of CD44 alternative splice pattern in the course of human colorectal adenocarcinoma progression.*Mol Cancer.* 2012 Nov 14;11:83.
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6. Rásó E, **Mészáros L**, Albini A, Tímár J. (2004) A WT1 expressing metastatic human Kaposi sarcoma xenograft model. *Pathol Oncol Res* 10:22-25
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