PhD. Theses

CHARACTERIZATION OF THE EFFECTS AND THE MECHANISM INDUCED BY THE ACTIVATION OF NOTCH-1 SIGNALING ON HUMAN PRIMERY MELANOMA PROGRESSION IN IN VITRO AND SCID MOUSE MODELS

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1. Introduction, Aims

Malignant tumors are the second most common cause of death after cardiovascular dieseases. The incidence of melanoma malignum – a tumor derived from the pigment producing cells of the skin- is increasing in the world, and the rate of the increase doubles every 15 years. The patients can be treated successfully if the melanoma malignum is recognized early, and if the tumor is removed on time, however if metastasis occurs the disease can rapidly lead to death. Melanoma cells are resistant to radio- and chemotherapy, as well as to programmed cell death.

Cancer is a complex, multistep process (induced by genetic and enviromental factors), which requires several molecular abnormalities to occur. Several research laboratories have been studying the biology of melanoma malignum via different in vitro and *in vivo* models, however the process hasn't been fully understood.

The Notch-receptor family which was discovered in the last few decades hasn't been well studied. Notch is a transmembrane receptor that regulates embryonic development, and Notch has been recently demonstrated to play a role in tumorigenesis. In human T-cell leukemia some of the cancer cells possess a specific chromosomal translocation, which leads to permanent activation of Notch and to uncontrolled cell growth. The first publications discussing the role of Notch-1 in the development of solid tumors (lung, cervical and renal cancer) appeared in print around year 2000. The role of Notch-1 receptor in the development, progression and metastasis formation of melanoma malignum is not known. The aim of our study was to determine the importance of the Notch-1 receptor function in tumor growth and lung metastatis formation of melanoma malignum using in vitro cell culture models and *in vivo* SCID mouse models.

We wished to contribute to the deeper understanding of the pathogenesis of human melanoma with our work. For this very reason we designed the following experimental strategy:

1. Analyze the expression of functionally active Notch-1 receptor on human melanocyte and melanoma cell lines, as well as on human naevus pigmentosus and melanoma malignum tissue specimens.

2. Study the effects of the inhibition of Notch-1 receptor on melanoma cell growth in vitro and *in vivo*.

3. Construct a recombinant lentiviral mammalina expression vector expressing the active form of Notch-1 receptor. Overexpression of active form Notch-1 in human melanoma cell lines.

4. Characterize the effect of Notch-1 overexpression on cell and tumor growth (in vitro & *in vivo* SCID mouse model).

5. Investigate the effect of Notch-1 overexpression on melanoma cell adhesion, and on the expression of adhesion molecules in melanoma cells.

6. Test the effect of Notch-1 overexpression on the expression and function of β-catenin molecule (as possible downstream target).

7. Study the effects of the inhibition of β-catenin expression (using siRNA) on cell proliferation, apoptosis and tumor growth in melanoma cell lines overexpression active form Notch-1.

2. Irodalom

2.1.*Melanoma malignum*

The incidence of melanoma malignum in Hungary is 7.6/100,000, and the mortality caused by the disease is 1.6/100.000. (WHO, 2005) The number of new cases presented in the Department of Dermatology and Venereology of the Medical and Health Science Center of the University of Debrecen varied between 80 és 118 per year during 1998-2006. The melanoma malignum cases derived from the skin can be devided into 4 groups. The superficially spreading melanomas give 2/3rd of the melanoma cases. The nodular melanomas, tumors from vertical growth phase count for 10-15% of the cases. The lentigo maligna melanoma (LMM), -its in situ form is called lentigo maligna- usually develops on sunexposed areas such as the face. Acral

lentiginosus melanoma (ALM) localizes to the palm, sole and subungual area. The most useful prognistic indicator of melanoma malignum are the Breslow thickness, and the presence or absence of ulceration. Melanoma malignum is a highly metastatic tumor, 2/3rd of the metastases occure in the first 3 years after the diagnosis.

The development of melanoma is a multistep process. Based on the Clark model: melanoma most frequently develops on the basis of naevus pigmentosus, then after radial and vertical growth phases it leads to metastasis formation. Malignant cells alter from the normal cells in the following features: unlimited cell proliferation, resistence to apoptosis, ability for tissue invasion and metastasis formation, insensitivity to anti-growth signals, sustained angiogenesis, and self-sufficiency in self-growth signals.

Based on the recent knowledge the most important molecular changes contributing to melanoma development and progression effect the following genes: Mitogen-aktivated protein kinase (MAPK) pathway, RAS, BRAF, retinoblastoma, p53, cell cycle, PI3kinase pathway, MITF, and c-KIT.

2.2 *Notch-1 receptor*

The Notch receptors are part of a signaling pathway, and are type I. transmembrane receptors with a highly conserved structure. To date, four mammalian receptors have been identified: Notch 1-2-3-4. The receptor consists of a ligand binding (extracellular) domain, a transmembrane domain, and an intracellular domain which directly participates in the signal trunsduction. Notch receptors are produced as single precursor molecules, which undergo a 3-step activation process. After cleavage by metalloprotases such as TACE (ADAM17) and γ-secretase (presenilin) the Notch intracellular domain (NIC) is released and translocates to the nucleus, where it binds to the transcription factor CSL and activates target gene transcription. The genes known to be regulated by Notch are the following: a basic helix-loop-helix (bHLH) hairy/enhancer od split (Hes) gene family, p21, cyclin-D1, myc, PTEN, EphrinB2; Notch related genes: Nrarp, a Deltex-1, as well as a pre-T cell receptor-α, and smooth muscle actin. To date, 5 mammalian Notch ligands

have been identified: Delta-1, Delta-3, Delta-4, Jagged-1 and Jagged-2, which also belong to the type I. transmembrane receptors. The regulation of the Notch pathway happens mostly post-translationally, through the glycolization or proteolysis of the extracellular domain of the ligand and the receptor.

Notch signaling is critical for developing and maintaining tissue homeostasis in multicellular organisms, and its function effects all three germ layers (endo-, meso-, ectoderm). It has been demonstrated that Notch signaling functions as oncogene in breast, colon, pancreatic, early stage cervical cancer and in certain brain tumors. In a few other cancers such as the basal cell carcinoma (skin cancer from from keratinocyte origin), certain B-cell leukemia, late stage cervical cancer, hepatocellular carcinoma and thyroid cancer the Notch receptors act as a tumorsuppressor.

The Notch-1 gene collaborates with other cellular proto-oncogenes to accelerate tumor growth in different tissue types. The consequences of the Notch signaling depend on signal strength, timing, and especially cellular contex.

2.3 *The role of adhesion molecules*

The metastatic cascade involves a series of steps: dissociatation of the tumor cells from the primery tumor, invasion of the surrounding stroma, intravasation, circulation through blood and lymphoid vascular system, extravasation, homing/attachment and adhesion at distant sites, and growth as a new lesion. Whether these cells die, survive in silence or form a clinically diagnosed metastasis, depends on the influence of the immune system and the microenvironment. The adhesion molecules (cadherines, integrines, selectines, and members of immunoglobulin family) which mediate the cell-cell and the cell-extracellular matrix connections play an important role in every steps mentioned above, including the development of metastasis.

When melanocytes transform into malignant melanoma cells, and when the melanoma cells move from the non invasive, radial to the invasive vertical growth phase, the repertoire of the adhesion molecules on the cell surface radically changes. It has been shown that the most important changes effect

the following molecules: E and N-cadherin (Ca2+ dependent cell adhesion molecule), Mel-CAM, and αvβ3 integrin (vitronectin receptor). The Mel-CAM protein has been found to be present in 80% of the melanoma metastases, and to be in direct proportion with the Breslow thickness. The expression of Mel-CAM on melanoma cells increases their agressiveness and metastatic potential. When the expression of Mel-CAM was induced on early, radial phase melanoma cells, and when the cells were injected into mice, metastases were detected in 70% of the cases.

Several publication suggest, that tumor cells are able to regulate their own adhesion through inside-out signaling via the integrin molecules. Mechanical effects (pressure, sheer pressure, deformation) cause changes in the function of the mechanical sensors of the cytoskeleton, such as Scr, PI3 kinases, focal adhesion kinases (FAK), and Akt-1.

2.4 *Beta-catenin*

A previous publication (Nicolas et al) suggested the possible involvement of β-catenin as a downstream element in the Notch signaling pathway, as they found that this molecule transmits the anti-tumor effect of Notch-1 in basal cell carcinoma. β-catenin is a 92-97kDa protein, which /together with the α-, γ- es δ-catenin molecules (plakoglobins)/ binds to the intracellular region of the E-cadherin receptor, and is part of the zonule adherens. β-catenin can be found in multiple intracellular compartments. The membrane associated β-catenins participate in the adhesion, while the cytoplasmatic β-catenin participate in the process of signal transduction. β-catenin is part of the wingless (WNT) signaling pathway, where it regulates segmentation during embryonic development as atranscription factor. The WNT pathway is one of the three major signaling pathways (Wingless/WNT, Sonic-Hedgehog es Notch) which regulate the embryonic development.

3. Methods

Cell culture: Human melanoma cell lines [WM35 (RGP), WM115, WM278, WM3248 (VGP), WM239A, and 1205Lu (Metastatic)] derived from different

progression stages were isolated from human melanoma specimens; normal human primary melanocytes FOM104, FOM105 and FOM117 were isolated from human epidermis.

Cell adhesion: Melanoma cells were plated into 24-well plates, and incubated for 30 minutes (37℃), then the cells attached to the surface were counted. To study the homotypic adhesion of the melanoma cells we plated melanoma cells onto 96 well plates pre-coated with 1% agar followed by incubation for 48 hrs, then the spheroids were photographed. Due to the agar coating of the wells the cells stayed in a non adherent state.

Three-dimensional spheroid growth in agar: Spheroids were prepared with the „liquid overlay" method: melanoma cells were seeded onto 96-well plates pre-coated with agar. The plates were incubated for 48 hrs, after which the spheroids were collected and implanted into bovine collagen type I. After 72-96 hours of incubation the spheroids were stained with LIVE/DEAD viability kit, and then were photographed.

Detection of Notch-1 receptor and adhesion molecules (proteins): The protein molecules were detected with Western blot (immunoblot) and immunohistochemistry. The human tissue specimens (15 cases of nevus pigmentosus, and 15 cases of melanoma malignum) were prepared in the Department of Dermatology and Venereology of the Medical and Health Science Center of the University of Debrecen. The paraffin- embedded sections were stained with peroxidase method. The enzyme activity was detected with AEC chromogen substrate.

Real-time PCR and semi quantitative PCR: Total RNA was isolated from melanoma cells and melanocytes with the Trizol method, and then cDNA was synthesized, which was used for real-time PCR reaction. The results shown are average of three measurements. For the evaluation of the results a standard curve was created using the threshold cycle (Ct) values. The samples were normalized with β-actin control.

Cell growth and apoptosis: The cell proliferation was measured with MTT method or with the ^{3}[H]timidin incorporation method. Apoptosis was measured through the detection of single stranded DNA.

Soft agar colony formation assay: Melanoma cells in single cell suspension were embedded into agar gel containing FBS in 6-well plates. The wells were precoated with agar to avoid the attachment of the cells to the plastic surface. The experiment was carried out in triplicates. Colonies (defined as minimum of 4 cells) were counted after 10 days of incubation.

Recombinant retro- és lentiviral vectors: For gene transfer, we constructed viral vectors. GFP/lentivirus and a NIC-GFP/lentivirus was constructed; to created the last one: a gene fragment NIC was inserted into pIRES2-EGFP plasmid. After removal of the small fragment containing the GFP gene, the remaining plasmid was cloned into H1UG, which vector derives from the FG12 lentiviral vector. All generated plasmids were confirmed by restriction enzyme digestion and DNA sequencing. Production of pseudotyped lentivirus was achieved by co-transfecting 293T cells with the 3 lentiviral plasmids. Green cells were counted under fluorescence microscope. Retroviral vectors MAML1/pBabe, DN MAML1/pBabe and empty bBabe were described previously (Jeffries, Mol.Cell.Biol. 2002). Recombinant retroviruses were generated by transfection of vector into Phoenix (AMPHO) helper free retrovirus prodecer cell line using the calcium phosphate method. Construction of small interfering RNA lentiviral vector: details to be found under “Results”.

Viral infection of targeting cells: To infect target cells by lentiviruses or retroviruses, we exposed cells overnight to viruses with an MOI ranging from 2 to 10 in the presence of polibrene. Cells were washed, cultured with regular complete medium for 2 additional days, and analyzed for protein expression by immunoblot or pooled for subsequent analysis as indicated in the individual experiments.

Animal experiments: Six-week–old SCID CB-17 mice were purchased either from the Charles River Laboratories or derived from the SCID Mouse

Animal Facility of the Department of Dermatology of the University of Debrecen. For subcutaneous injection, $3x10^6$ cells per mouse were injected with 8 mice in each group. Subcutaneous tumor growth was measured once per week, and tumors were harvested after 7 weeks, weighed, and stored in -70℃ embedded in OCT. For the lung colony formation assay, $4x10^5$ cells were injected into 12 SCID mice in each group via the tail vein. The experiment was terminated at week 7; the lungs harvested, tumor colonies were macroscopically counted, and samples were subjected to histological evaluation.

Luciferase assay: Cells grown in 24-well plates were transiantly infected using Lipofectamin 2000 and either the TOPflash or FOPflash reporter plasmids. Cells were incubated for 24 hours at 37℃ in serum free culture medium. Cell lysates were prepared and luciferase activity was measured following the manufacturer`s protocol.

4. Results

4.1 Comparison of the expression of Notch-1 receptor protein in naevus pigmentosus and melanoma malignum tissue specimens

We first carried out immunohistochemistry to compare the expression of Notch-1 protein in 15 human naevus pigmentosus and 15 human melanoma malignum tissue specimens deriving from different individual donors. The average age of patients with naevus pigmentosus was 28.26 years (n=15), SD=17.33; the average age of patients with melanoma malignum was 55.53 years (n=15), SD=15.12. (Both genders were equally represented). Notch-1 was present in 10 out of 15 melanoma lesions but was only detectable in only 1 out of 15 naevus samples. Notch-1 appeared to be expressed in the cytosol and nucleus, which implies that the active form Notch-1 (NIC) was translocated to the nucleus, meaning the Notch-1 was in activated status.

4.2 Expression of Notch-1 receptor and its target genes in human melanocytes and human melanoma cell lines of different growth phases

Six melanoma cell lines derived from different growth stages of progression and 3 normal primary human melanocytes were tested with immunoblot using an antibody specifically recognizing activated Notch-1. Compared with the level of activated Notch-1 in melanocytes, higher levels were of Notch-1 was observed in all 6 melanoma cel lines. SUP-T1 (T-cell leukemia cell line) and N^{IC}/lenti transfected mel888 (mel-N^{IC}) were used as positive controls. Almost all cell lines expressed the Jagged-1 gene (Notch ligand), but we saw no significant change in levels between melanocytes and melanoma cells.

In addition, we analyzed expression of Notch target genes HEY1, HEY2, HES1 and HES2 using quantitative real-time RT-PCR. We found at least 1 Notch target gene was upregulated in a given melanoma cell line compared with melanocytes, although the type of Notch-target gene varied among differenht melanoma cells.

4.3 Suppression of Notch pathway in human melanoma (RGP, VGP, Met) and human melanocyte cell lines

To inhibit the Notch pathway, we used a γ-secretase inhibitor, a N-[N-(3, 5-difluorophenacetyl-l-alanil)]-S-phenylglicine t-butyl ester (DAPT) in low (0.2μM) and high (1μM) doses. Treating melanoma cells with a high dose of DAPT significantly reduced their growth rate. However, at a low dose DAPT selectively inhibited primary but not metastatic tumor cells. Similarly, colony formation of primary melanoma cells was selectively inhibited by a low dose of DAPT. These results suggest a difference in sensitivity to DAPT treatment of primary versus metastatic tumor cells at a low dosage.

4.4 Suppression of Notch induced transcription via blocking MAML-1 transcriptional regulator

To specifically and selectively block the Notch pathway, we utilized a second method, as well. Mastermind like-1 (MAML-1) is a transcriptional regulator downstream of Notch-1. A dominant negative (DN, functionally inactive, truncated) mutant of MAML1 was overexpressed in WM35, WM3248, WM239a and 1205Lu melanoma cell lines using a retroviral vector (pBabe). Expression of DN-MAML1 protein significantly reduced the rate of cell growth

and colony formation in soft agar in early phase melanoma cell lines but did not in metastatic cells compared with those in pBabe-transfected cells. Cell growth was measured via MTT assay. After this we injected WM3248 melanoma transfectants subcutanelously into SCID mice, where tumor growth was decreased when MAML-1 was inhibited. Taken together, our data suggest that Notch-1 signaling plays an important role in stimulating primary melanoma proliferation and implicate the Notch pathway could be a potential therapeutic target.

4.5 Constitutive activation of Notch-1 in human melanoma cells

To investigate the role of aberrant Notch-1 signaling on cellular properties of human melanoma, we tested the effect of activated Notch-1 on melanoma cell growth in vitro by introducing the NIC into the cells. For this approach we utilized a modified lentiviral vector system because of its universal infectivity rate, low toxicity rate and high flexibility of the system. We have successfully created a lentiviral vector coding activ form Notch-1-GFP and a control vector coding only GFP. Six melanoma cell lines (2 RGP, 2 VGP and 2 MET) were transfected with NIC-GFP-lenti and used for in vitro and *in vivo* experiments. Expression of the transgene was tested under fluorescent microscope based on the level of green fluorescence (expression of N^{IC} was double checked with immunoblottal as well).

4.6 The effects of constitutive Notch-1 activation on cell growth and colony formation in soft agar

The ^{3}[H]timidin incorporation experiments showed that the growth rate of active Notch-1 infected primary melanoma cells (RGP &VGP) significantly increased compared with that of GFP-control cells or NIC transfected metastatic melanoma cells. Similarly, substantially increased colony formation was observed in early phase (WM35 és SbCl2) primary melanoma cells, but not in metastatic melanoma cells.

These results show that constitutively active Notch-1 signaling promotes primery melanoma cell growth and anchorage independent growth in vitro. The later one is characteristic feature of malignant cells.

4.7. The effects of constitutive Notch-1 activation on apoptosis of melanoma cells in three-dimensional spheroid model

In this experiment our goal was to study the effect of N^{IC} on survival and apoptosis of WM278 melanoma cells in the three-dimensional spheroid model. The spheroids created from WM278-NIC-GFP and WM278-GFP cells were implanted into type I. bovine collagen, incubated for 96 hours in serum and growth factor free media followed by a DEAD/LIFE fluorescent staining. In the spheroids containing the GFP transfected cell lines we found countless dead cells, but in the spheroids of WM278-N^{IC}-GFP cells we detected only a few apoptotic cells. The increased survival capacity of the cells of the last case manifested itself in larger size of the spheroids and increased invasion into the collagene. These data show that the activation of Notch-1 increases the survival, cell growth and the ability of the melanoma cells to invade into collagene in three-dimensional spheroid model.

4.8. The effect of constitutive Notch-1 activation on cell adhesion and the expression of cell adhesion molecules

Even number of WM278-N^{IC}-GFP and WM278-GFP melanoma cells were plated and incubated in a 24-well plate for 30 minutes, then the cells found to be attached to the surface were counted. We have found that the N^{IC}-GFP cells adheared stronger as compared with the control ones, which means that Notch-1 increased the adhesion of melanoma cells. We have tested the homotypic (cell-cell) adhesion in the melanoma cells, as well. In order to inhibit the adhesion to the plastic surface the wells of a 96-well plate was coated with agar. After culturing the cells for 48 hrs in those wells, we found that the WM278-N^{IC}-A NIC-GFP built larger and more organized spheroids as compared to WM278-GFP cells. This demonstrates that the cell-cell adhesion was increased after Notch-1 activation. When detecting the level of activation of the focal adhesion kinase (FAK) we found that it was increased in WM-278-N^{IC}-GFP cells consistently with the increased adhesion capacity. We used immunoblot to investigate the expression level of the cell surface molecules E-cadherin, N-cadherin and Mel-CAM (known to be related to tumorigenicity of melanoma). The level of N-cadherin and Mel-CAM was

found to be increased in N^{IC}-GFP transfected cells as oppose to the control (GFP) ones. Our data demonstrates that the activation of Notch-1 receptor increases the adhesion of melanoma cells, through the manipulation of the FAK, N-cadherin and Mel-CAM proteins.

4.9. Characterization of the effect of constitutiv Notch activation in *in vivotumor growth,* SCID mouse model

SCID CB-17, is a mouse strain with spontaneous „scid" mutation. This is a unique strain which because of a mutation effecting the (V(D)J recombination is able to accept foreign cell and tissue transplants such as human tumor cells. We have used 8 week old, male and female mice negatively tested for immunglobulin production.

We have transplanted two early phase melanoma cell lines (WM35 and WM3248) constitutively expressing N^{IC}-GFP a WM 35 and WM3248 into SCID mice. After 7 weeks of observation the experiment was terminated, and the tumor size measured. The results showed that the N^{IC}-GFP tumors were seven times larger compared with the control (GFP) ones. We concluded, that the Notch-1 receptor functions as an oncogene in melanoma.

In a different animal experiment we tested the effect of Notch-1 receptors on the progression, the metastatic ability of the early phase melanoma cells. WM3248N^{IC}-GFP and WM3248-GFP cells were injected into the tail vein of the SCID mice, and after 7 weeks the mice were sacrificed. We found only a few metastatic nodules in the lungs in the case of the WM3248-GFP cells, but fond countless metastatic nodules covering the whole surface of the mouse lungs in the mice injected with the WM3248-N^{IC} cells. The constitutive activation of Notch-1 induced a more agressive, malignant phenotype of the melanoma cells. One of the explanation could be a change in the level of adhesion molecules (such as N-cadherin, Mel-CAM) on the surface of melanoma cells, which molecules are known to play a role in the process of extravasation, homing, and adhesion of melanoma cells to the new organ.

To conclude our results, we can say that the Notch-1 signaling pathway plays an oncogenic role in the development and progression of primary melanomas.

4.10. Effect of Notch-1 activation on the stabilization of β-catenin

In the next step we investigated the mechanism of the Notch-1 signaling in details. In immunoblot experiments we have shown that the overexpression of Notch-1 receptor increased the expression of β-catenin protein in N^{IC}-GFP-lentivirus transfected early phase melanoma cell lines (Sbcl2, WM35, WM115, WM3248 and WM278) as compared with the GFP cells. We have not detected this change in the metastatic melanoma cells, which, again suggests a stage-specific role for the Notch-1 receptor. Our results were supported by the fact that the inhibition of the maturation of the Notch-1 receptor (DAPT treatment) reduces the production of β-catenin.

We have tested whether the Notch-1 induced β-katenin proteins are functionally active measuring the level of transcription of human T-cell factor gene (downstream gene regulated by β-catenin). The TCF-luciferase test demonstrated that in the WM3248-N^{IC}-GFP and WM115-N^{IC}-GFP cells the amount of actively functioning TCF is significantly higher that in the control cells, meaning that the β-catenin molecules induced by Notch-1 receptor are functionally active.

As the next step we decided to construct a lentiviral vector coding specific small interfering RNA (siRNA, gene silencing technique) to permanently knock out β-catenin of the cells. Based on a previous publication we inserted a gene sequence coding inhibitory sequence for β-catenin into H1UG, a mammalian expression vector. We constructed three different lentiviral vectors coding different siRNA. Melanoma cells were transfected with different lentiviral vectors, and the most efficient was chosen for experiments. WM35-N^{IC}-GFP and WM3248-N^{IC}-GFP cells were transfected with siRNA coding lentivirus to establish a stable phenotype. This way we have created two new cell lines, which were named as β-cat-siRNA/WM35-N^{IC} and β-cat-siRNA/WM3248-N^{IC}. The decreased protein level of β-catenin was tested via immunoblot, which showed that the siRNA transfected cells exhibited 78% (WM35) and 90% (WM3248) less amount of β-catenin. The supression of β-catenin significantly decreased the rate of cell proliferation in melanoma cells in vitro. It can be explained by the fact that the inhibition of β-catenin caused

the induction of programmed cell death, the apoptosis. After the measurement of apoptosis (ssDNA) we found that the number of dead cells increases after the inhibition of β-catenin.

To further adress the above mentioned issue we injected the β-kat-siRNS/WM3248-N^{IC} cells into SCID mice intravenously, we found that the inhibition of β-catenin reduced the number of lung metastases as compared with the control mice. These results indicate that β-catenin mediates, at least partly as a functional downstream target of Notch-1 signaling in primary melanoma cells.

5. Discussion

In this study, we investigated the role and importance of Notch-1 receptor in primary melanoma progression in different experimental settings. Our goal was to discover biological changes induced by Notch-1 receptor activation in melanoma cell lines or in *in vivo* SCID mouse tumor model. We hope that through our work we could pinpoint a new molecule which can be used as therapeutic target for drug development also against melanoma.

In this work, we demonstrated that Notch-1 signaling is activated in human melanoma. We were able to detect Notch-1 protein expression in humna melanoma cell lines, and melanoma tissue speciemens, but it wasn`t detectable in melanocytes or naevus pigmentosus tissue. The involvement of Notch-1 receptor in melanoma biology was suggested by a few scientific publications, but none of them studied it in details. We demonstrated that the component of the Notch-1 pathway: downstream elements such as HEY1, HEY2, HES1, HES2 are expressed in melanoma cell lines of all three grwoth phase. Similar results were published by Hoek et al where they used cDNA based microarray data to detect Notch-1 target genes.

Tumor progression is a complex process, which involves several changes in multiple levels of the cellular functions such as increased cell proliferation, survival, migration, invasion etc. We chose to study the level of cell proliferation and anchorage independent growth (soft agar assay). We

found that if we block the function of Notch-1 receptors (by both methods) it reduces the proliferation and anchorage independent growth of melanoma cells. Suwanjunee at al has demonstrated as well, if Notch signaling is blocked via γ-secretase inhibitors, it inhibits the cell proliferation in lymphoma and hepatoma cell lines.

Most mammalian cells prefer to be adheared to a surface. Cells are able to regulate their proliferation via anchorage dependence, but this control mechanism is lost in transformed, malignant cells. The anchorage independent cell growth indicates tumorigenicity and invasivity in severel cell types. Colony formation in soft agar („soft agar assay") is a commonly used experimental method, which examines the anchorage independent cell growth. Our results showed that the inhibition of Notch-1 (via DAPT or at transcriptional level) decreases the growth of RGP and VGP melanoma cells in soft agar, but has no effect on metastatic melanoma cells. It seems that the signaling pathways which may collaborate with the Notch pathway differ in primery and metastatic melanoma cells.

The Notch receptor family may play different role in the pathomechanism of different cancer types. This kind of a functional diversity can be seen during the regulation of tissue homeostasis and development by Notch receptors. We reported the oncogenic effect of Notch-1 in melanoma, but it has been published that Notch-1 functions as tumor supressor in basal and squamous cell carcinomas (in animal models). It has been shown that Notch signaling can play different roles (positive and negative) in the regulation of cell differentiation, cell proliferation, survival and tumorigenesis. This is why it is not surprising, that in malignant tumors of the same organ but of different histological types – the Notch receptors induce opposite effects. For example in B-cell development, Notch-1 induces growth arrest and apoptosis whereas in T-cells, it induces cell proliferation. Notch signaling elicits oncogenic activities in acute lymphoblastic T-leukemias, mouse mammary tumors, and transformed kidney cells. On the other hand, it act as growth antagonist or tumor supressor not only in murine basal cell carcinoma but also in small cell lung cancer, hepatocellular carcinoma and prostate cancer.

We are the first to report experimental results exploring the effects of constitutive activation of Notch-1 in human melanoma cell lines. Activation of Notch-1 increased the proliferation and anchorage independent cell growth in vitro and *in vivo* (in SCID mouse model). This effect showed a stage-dependent characteristics. Activation of Notch-1 receptor promoted the progression of primary melanoma cells but had little effect on metastatic cells. The stage-dependent function of Notch-1 receptor has been demonstrated also in cervical cancer. Notch-1 receptor is known to act as oncogene in the early stage, and acts as a tumorsupressor in the late stage of cervical cancer. One of the possible reasons behind the proliferation induced by Notch could be, that Notch-1 induces the expression of cell-cycle proteins (sych as cyclin D1). Probably multiple genetic changes are required in the same time to start the malignant transformation by Notch-1. The fact that the effect of Notch-1 activation depends on the stage of the tumor, is similar to the phenomenon seen during embryonal development: consequences of Notch signaling depend on signal strength, timing and cellular context. The precise mechanism of this dual action remains to be explored.

We demonstrated that the adhesion of melanoma cells is increased after Notch-1 activation, as well the expression of N-cadherin and Mel-CAM molecules. We showed that the activation of Notch-1 increases the invasion and survival of melanoma cells in three-dimensional spheroid model. We proved that Notch-1 induces the activation of focal adhesion kinase (FAK). Based on our results, we can conclude that constitutive activation of Notch-1 increases the number of lung metastasis in SCID mouse model. One of the explanation for increased proliferation, migration, adhesion and metastasis formation is increase in the level of N-cadherin and Mel-CAM protein expression after Notch-activation in the melanoma cells.

It is also possible that the function of the FAK kinases are regulated by Notch-1 via the cadherins. We examined the direct effect of Notch-1 on FAK kinases, and we found that the Notch-1 activation increased the amount of active, phosphorylated kinases in the melanoma cells.

A previous publication (Nicolas et al) suggested the possible involvement of β-catenin as a downstream element in the Notch signaling pathway, as they found that this molecule transmits the anti-tumor effect of Notch-1 in basal cell carcinoma. We found that the increased function of the same β-catenin molecule mediates an oncogenic and not an anti-tumor effect in human melanoma malignum. Whether the effect via β-catenin will be oncogenic or anti-tumoral, probably depends on the level of Notch-1 activation (type and amount of ligand), and the original role of Notch-1 in that tissue type during embryonal development. It has been published that the aberrant activation of β-catenin cause by mutation, and the aberrant intra-nuclear accumulation of a wild type β-catenin or the stabilization of a β-catenin is linked to the progression of melanomas. β-catenin plays an important role in the regulation of E-and N-cadherin (Ca^{++}cell surface molecules) mediated cellular adhesion. β-catenin is an important member of the WNT signaling pathway, where it induces the transcription of the T-cell factor (TCF) gene.

Our data show that the increased production (or stabilization) of β-catenin is only specific to early phase (RGP and VGP) melanoma cells, it is not the case in metastatic melanoma such as 1205Lu cells. One of the reasons for this could be that Notch-1 receptor and its target genes are already activated in the metastatic melanoma cells, even before the overexpression of the receptor. The permanent, external activation of Notch-1 is not able to increase the level of β-catenin any more, and is unable to further induce the cell proliferation. It is possible that in melanoma cells several oncogenic pathways are active, and the downstream elements (β-catenin or cell cycle machinery) works on maximum efficiency, which can not be further increased anymore. It is also probable that there are other unknown feedback mechanisms in the melanoma cells which are antagonizing the effects of Nocth-receptors. This link between β-catenin and Notch pathway has an impotant implication: it connects two major intracellular signaling pathways, the Notch and the Wnt pathways. Alves-Guerra et al has suggested that there is a relationship between the Notch and the Wnt pathways, as they demonstrated that the transcriptional regulator MAML-1 is a co-activator for not only the Notch-1 receptor but also for the β-catenin pathway.

The phenomenon of RNA interference (RNAi) was discovered a few years ago. It is a universal intracellular mechanism based on the fact that small double stranded RNA sequences (siRNA) become single-stranded after attachment to the protein, Slicer, and then form an RNAi silencing complex (RISC), which is able to recognize genes complementary to the small RNA sequence and catalyze their degradation. During our work we constructed a retroviral vector coding a siRNA sequence specifically for the inhibition of β-catenin. Then we demonstrated that the inhibition of a β-catenin via siRNA was able to inhibit cell growth in vitro and *in vivo* in melanoma cells, furthermore that it induces apoptosis. We conclude, that a β-catenin is required for the r egulation of melanoma cell growth. Our hypothesis was supported by Delmas et al, who showed that a β-catenin causes the immortalization of melanocytes through the supression of16/Ink4a, and through cooperating with the Ras pathway.

The treatment of human malignant tumors are usually frustrating, because very often drug-resistent clones arise in the patients during or after the therapy of the tumor. This step leads to the re-occurrence of the tumor and the appearance of untreatable metastases. One of the new ideas in oncological research is the use of therapeutic agents targeting genetic alterations specific to the individual. Useful targets of this aproach could be the members of three major signaling pathway regulating differentiation, cell proliferation and apoptosis: WNT, Sonic-Hedgehog and Notch pathway.

It has been suggested that maybe cancer stem-cells, which are drug-resistent and have unlimited growth potetial could be eliminated via therapeutics developed against Notch-receptors. We hope that our work contributed enough evidence to consider the possibility of anti-Notch-1 treatment in melanoma malignum.

6. New findings:

1. Notch-1 receptor and its target genes are expressed ini human melanoma.

2. Inhibition of Notch-1 receptor blocks the proliferation and anchorage independent growth in agar of RGP and VGP melanoma cell lines.

3. Activ form of Notch-1 receptor can be successfully overeexpressed in melanoma cells with lentiviral vector.

4. Constitutive activation of Notch-1 increases proliferation, and anchorage independent growth of RGP and VGP melanoma cells in vitro, and increases tumor growth *in vivo (*subcutaneously) and the development of lung metastases in SCID mose tumor model.

5. Constitutive activation of Notch-1 increases the adhesion and inhibits apoptosis of melanoma cells in three-dimensional spheroid models, as well as induces the expression of N-cadherin, Mel-CAM adhesion molecules and the phosphorylation of focal adhesion kinase.

6. Activation of Notch-1 increases the amount of free β-catenin in early phase (RGP and VGP) melanoma cells, and has no effect in the metastatic cells.

7. Inhibition of β-catenin in melanoma cells with permanently activ Notch-1 receptor decreases the proliferation and induces apoptosis in vitro, as well as is able to reduce metastasis formation in lung in SCID mouse tumor model *in vivo*.

7. Summary

Notch-1 receptor is a highly conserved transmembrane protein that determines differentiation, proliferation and survival during embryogenesis. Upon ligand activation, the receptor is cleaved by metalloproteases, and then its intracellular domain translocates to the nucleus, and induces subsequent activation of target gene transcription. Involvement of Notch signaling in several cancers is well known, but its role in melanoma remains poorly characterized.

In our work, we studied the role of the Notch-1 receptor and the Notch signaling in the progression of melanoma. Here we show that the Notch-1 receptor is expressed in human melanoma malignum tissue specimens, and that the Notch1 pathway is activated in human melanoma. We have showed that blocking Notch signaling suppressed the proliferation of primary melanoma cell lines. We have demonstrated that constitutive activation of the Notch1 intracellular domain enhanced the primary melanoma cell growth both in vitro and in vivo, as well as enhanced their anchorage independent growth, yet had little effect on metastatic melanoma cells. We have found that activation of Notch1 signaling enabled primary melanoma cells to gain metastatic capability. Notch1 activation increases tumor cell adhesion, up-regulates N-cadherin and Mel-CAM expression, as well as FAK phosphorylation. Notch1 activation also enhances tumor cell survival when cultured as three-dimensional spheroids. Furthermore, the oncogenic effect of Notch1 on primary melanoma cells was mediated by β-catenin, which was up regulated following Notch1 activation. Inhibiting β-catenin expression reversed Notch1-enhanced tumor growth and metastasis.

We conclude that the Notch-1 signaling is an active mechanism in the pathogenesis of melanoma. Our data therefore suggest a β-catenin–dependent, stage-specific role for Notch1 signaling in promoting the progression of primary melanoma. This is the first study to show the oncogenic role of Notch-1 in human melanoma malignum.

8.Key words:

Melanoma malignum, Notch-1 receptor, tumor progression, beta-catenin, SCID mouse model, γ-secretase, lentiviral vectors

9. Publications:

Publications on the subject of the thesis:

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