Screening of patient sera against a cDNA library produced from melanocytes in an attempt to find autoantibodies in vitiligo patients

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Abstract
Several studies have made the observation that vitiligo is caused by an autoimmune reaction against the pigment cells, melanocytes. Although autoantibodies and auto reactive T-lymphocytes it remains unclear what role they play. Autoantibodies have also been observed in malignant melanoma patient with a vitiligo like depigmented patch. The depigmented patch can be seen as a halo around melanoma and is called halo nevus. An increased survival has been observed in patients with vitiligo-like depigmentation. In this project we will try to find out if autoantibodies are expressed in vitiligo patients by screening sera from a boy with a giant nevus and vitiligo against a cDNA library prepared from cultured melanocytes from a healthy person.
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1 Introduction

Our immune system is a very complex system that manages to protect us from different pathogens and infections. The most characteristic feature of the immune system is its ability to distinguish self (tissue) from nonself (antigen). When the immune system fails to distinguish self-tissue from nonself (antigen) it will start an immune response, where the T- and B-lymphocytes will generate a cell-mediated or humoral response directed against self-antigens. The consequence of this is the development of autoimmune diseases. The aetiology of autoimmune diseases is yet not completely understood and is estimated to affect 5-7% of the population around the world. There are several types of autoimmune diseases such as, diabetes mellitus type-1, rheumatoid arthritis and vitiligo.

Vitiligo is an idiopathic acquired skin disorder that is believed to arise as a result of autoimmune reaction since it usually occurs in conjunction with other autoimmune disorders. It is characterised by white symmetrical patches on the skin due to the loss of pigment cells. Autoantibodies and auto reactive T-lymphocytes targeting the pigment cells, melanocytes, have been detected in many vitiligo patients. Although this observation has been made, it remains unclear what role autoantibodies play in the destruction of melanocytes. Autoantibodies have also been observed in malignant melanoma patient with a depigmented patch around the tumour. The depigmentation usually occurs spontaneously or during melanoma treatment and is very similar to the one seen in vitiligo patients. Depigmentation can be seen as a circular clearing around the melanoma, which is called halo nevus. It has been observed that there is an increased survival among melanoma patients with vitiligo-like depigmentation. This increased survival is believed due to the autoimmune reaction against the pigment cells also combats tumour cell by destroying the melanocytes, which are shared by both normal healthy skin cells and cancer cells (melanoma).

The objective of this project is to try to find out if autoantibodies are expressed in vitiligo patients? This is done by screening sera from a boy with a giant nevus and vitiligo against a cDNA library prepared from cultured melanocytes from a healthy person.

The cDNA library that I have used in this thesis is made from melanocytes taken from a healthy person and cultivated until enough cells have been produced. From these cells, mRNA is then extracted and used as a template for synthesis of cDNA strands that are subsequently incorporated into bacterial phage, each of which will express a protein when they infect bacteria. This library can be used to screen for potential disease-related antigens/ protein in vitiligo or malignant melanoma patients with halo.
2 The Skin

The human body is covered by the bodies’ largest organ, the skin. This organ has several functions of which the most important is to form a physical barrier allowing and limiting inward and outward passing of water and electrolytes. The skin also provides protection from microorganisms, ultraviolet radiation, toxic agents and mechanical insult. Epidermis, dermis and subcutis are the three structural layers of the skin. Hair nails, eccrine and apocrine sweat glands are all considered to be part of the skin. Epidermis is the outer layer of the skin and serves as the chemical and physical barrier between the interior of the body and the exterior environment; the dermis provides structural support of the skin and is the middle layer of the skin and subcutis which is the last layer is an important depot of fat. The skin is has a rich innervation of nerve cells and different receptors especially in hands, face and genitals to enhance the senses of these areas.

2.1 Epidermis

Epidermis is the outer visible part of the skin composed of stratified squamous epithelial and the main cells are keratinocytes which synthesizes the protein keratin. Keratinocytes are connected by protein bridges called desmosomes and they are constantly in transition from the deeper layers to the superficial. Keratinocytes are shed once they reach the surface of the skin and they are replaced by new cells. Epidermis varies in thickness from and it contains four layers; stratum basale, stratum spinosum, stratum granulosum and stratum corneum. In stratum basale a cell type called melanocyte is found scattered throughout the entire basal layer. Epidermis also contains Langerhans’ cells that are part of the bodies’ immune system and protect us from infections. 

2.2 Melanocytes

Melanocytes are derived from the neural crest cells and are very similar to nerve cells in appearance. Melanocytes produce melanin the protein that is responsible for giving the skin and hair its Colour. Melanin has also an important function in protecting us from UV radiation by absorbing the radiation and therefore protects our DNA from damage and development of cancer. UV-radiation causes the keratinocytes to proliferate leading to thickening of epidermis. Melanocyte granules form a protective shield over the nuclei of keratinocytes in the inner layers of epidermis and in the outer layer melanin granules are more even distributed. Production of melanin occurs in special organelles called melanosomes which are localized in the cytoplasm of melanocytes. Melanosomes contains the enzyme tyrosinas, which oxidizes the amino acid tyrosine to 3, 4 dihydroxyphenylalanin (dopa), which, is than further transformed and polymerized to melanin. The melanin is packed in melanosomes and then transported along the dendritic processes of melanocytes to keratinocytes where it’s transferred through phagocytosis. Keratinocytes functions as a reservoir for melanin and contains more melanin than the cell that produced it.
3 Vitiligo

Vitiligo is the most common skin disorder that affects about 0.1-2% of the world population and is defined as an acquired progressive disorder that results after selective disappearance of some or all melanocytes in interfollicular epidermis and sometimes in the hair follicular as well. [10-11] The precise etiology of vitiligo is not yet clear but it has been discussed by many scientists that autoimmunity may be the cause to loss of melanocytes. Autoimmunity is when the body’s immune system produces an inappropriate immune response against its own tissues (in this case the melanocytes). It has been observed that some patients manifests autoantibodies directed against melanocytes or melanocytes proteins. [3, 9]

The clinical picture of vitiligo is characterized of white macules on the skin that are often symmetrical and affects areas of the skin that are under pressure or tensile forces. During the active state of vitiligo the disorder may be exacerbated by injury or inflammation. [10-11]

There are two major clinical subtypes of vitiligo, segmental and non-segmental vitiligo (generalized). Non-segmental vitiligo is bilateral and characterized by symmetrical depigmented patches. Segmental vitiligo is unilateral and affects one segment of the skin. It does not cross the midline or have the classical dermatome distribution. [3, 11]

Vitiligo lesions are especially prominent on the face, in the axillae or umbilicus, back of hands or wrists. The disorder is more striking around the eyes, nostrils, mouth, nipples and genitalia. The depigmentation is more noticeable on dark-skinned patients or tanned patients since that the depigmented macules don’t tan. [10-11]

4 Malignant melanoma

Malignant melanoma is a fatal skin tumour which is the leading cause of death due to skin disease. Malignant melanoma can develop at any age but it has its peak of incident between the ages of 20-45. It is the second most common cancer form in women between the ages of 30-34, breast cancer being the most common. If identified at an early stage melanoma is largely curable. Development of skin cancer is associated to the exposure of UV-radiation. People who have an outdoor occupation, have fair skin or lives in a region receiving higher per capita sunlight are at greater risk for developing skin cancer. Melanoma cells are derived from melanocytes that have lost control over their own growth and start to grow uncontrollably. [12-13]
5 Halo Nevus

Halo nevus is a symmetrical circular depigmented area around a metastatic melanoma lesion and is commonly found on the backs and trunks of children and young adults. These phenomena of halo nevus have been observed several times in patients with a personal or familiar history of melanoma. Halo nevus has been reported to be coexistence with vitiligo in about 20% of the cases, although it is not yet understood how the relationship between these two acquired leucoderma works.

Halo nevus are defined as banning although it has been reported that malignant melanoma associated with a halo around the lesion are most likely to regress or stop metastasize leading to an increased survival rate. This suggests that halo nevus may have a protective function and can be seen as a tumour-inhibitor.

5.1 Giant congenital nevomelanocytic nevus (CNMN)

Congenital nevomelanocytic nevus affects 0.2-2% of all the new-borns. It is characterised by pigmented skin lesions that are light to dark brown and round or oval. The skin lesions can be any size, from small to very large and they can also vary in colour from light brown to dark brown or even black. Although all CNMN are benign they can still be precursors of malignant melanoma regardless of size. The prevalence of CNMN is equal in both females and males and affects all races. CNMN is the result of a developmental defect in neural crest–derived melanoblasts. The trunk is the most common location of the skin lesions in CNMN followed by the extremities and the head and neck. These larger lesions may sometimes be associated with multiple smaller satellite lesions. Lesions may over time become less pigmented and sometimes even more.

6 Homo sapiens E1A binding protein p300 (EP300), mRNA

Homo sapiens E1A binding protein p300 is a transcriptional regulator and is involved in essential processes such as cell proliferation, cell differentiation and tumour progression. E1A binding protein is also known as KAT3B/EP300, and function as a transcriptional co-activator of various transcriptional factors. This protein is involved in many cellular processes such as DNA repair, cell growth, differentiation, cell death and migration. EP300 is an oncogenic protein and it has been observed in lung, colorectal, breast and prostate cancer but its overexpression is a poor prognosis indication. EP300 gene encodes the adenovirus E1A binding protein which is as mentioned above a transcriptional co-activator protein. This protein functions as a histone acetyltransferase, transcription regulation is done by chromatin remodelling, an important process in cell proliferation and remodelling. The EP300 gene is located on the long arm of chromosome 22; chromosomal translocation and rearrangement on chromosome 22 have been observed to be associated with certain types of cancer. These genetic changes occurred in certain cells and during a person’s life time (somatic). In cancer cells the region with chromosome 22 and expression of EP300 is disrupted. A
mutation prevents EP300 gene from producing any functional protein leading to uncontrollable cell growth and eventually development of tumours. [21]

7 Methods and Materials

7.1 Expression cloning

Make phage-competent bacteria:

A strain of E-Coli bacteria (XLI-Blue) were streaked on LB-agar with tetracycline (12, 5µg/ml) and then incubated over night at 37° C. The bacteria can be stored for a week in a refrigerator.

One bacterium colony was cultured in 100ml NZY medium or LB-medium with 1ml 1M MgSO₄ and 1ml 0, 2% (w/v) maltose in a flask. The flask was then put in a shake incubator overnight in 30°C.

The following day, bacteria stock solution was checked upon to see if they had grown. OD₆₀₀ was measured with the help of a spectrophotometer at the wavelength 600 nm. LB-broth was used to reset the spectrophotometer to zero before measuring the OD₆₀₀ of the bacteria which should be 0, 5. The bacteria stock solution was transferred into a 50 ml falcon tube and centrifuged at 500xg for 10 minutes in 4°C, the supernatant was poured out and the pellet was left on the bottom and resuspended in 10mM MgSO₄ to OD₆₀₀ 0,5 The bacteria pellet was resolved by inverting the tube 4-6 times and was then stored in the refrigerator.

Preparation of bacterial culture plates:

NZY-agar (1, 5%) was first melted in the microwave for approximately 20 minutes the flask cork was loosen before heating the flask. Five 15 cm petri-dishes were placed on a flat surface (special horizontal plate). After 5-10 minutes of cooling NZY-agar was poured into the five petri-dishes on the horizontal plate. The agar plates were left to dry on the horizontal plat without their lids for a couple of hours.

Preparation of filter:

10 mM IPTG was poured in one petri dish. Hybond-C extra filters (Amersham, Buckinghamshire, UK) were dipped in the IPTG (137mm for 15cm and 87mm for 10 cm petri dish) by using a tweezers. The filters were left to dry on whatman paper for 1h. This procedure was repeated once again. The filters were than marked from 1-5.

Preparation of buffers for expression cloning:

1L 10xTBS: 100mM Tris-HCL, 1g/L, 1,5M NaCl (87,75g/L) were mixed in a bottle and mixed with milliQ water to 1L. The PH was adjusted by adding HCl until it reached PH 8.

3L TBS-GT: 0,3 L 10xTBS+ 0, 1% gelatin(3g/3L) and 0,05% Tween 20 (1,5ml/5L). Gelatine and tween 20 were mixed with a small amount deionised water in a goblet and then dissolved by warming
in the microwave. The solutions were poured into a flask together with deionised water to 3L and mixed by inverting the flask a few times.

1L Colour reaction buffer:

12.1g Tris and 0.12g MgCl\(_2\) 6H\(_2\)O were mixed in a bottle and filled with milliQ water to 1L. The pH was adjusted to 9, 5 by adding HCl.

Infecting bacteria with the phage library and plating of them:

The newly made NZY agar-plates were preheated in 42°C in an incubator before use. Meanwhile two water-baths were preheated to 48°C and 37°C respectively. Top-agar(0.7% NZY-agar) was melted in a microwave oven and then left in the 48°C water-bath for 20 minutes to be tempered. The concentration of the melanocyte cDNA library was 10\(^8\) pfu (plaque forming unit)/µl and needed to be diluted to 50 000pfu/µl before use.

600µl phage competent bacteria per 15 cm petri-dish (300µl per 10 cm) and 1µl of the phage library were added to sterile falcon tubes. The bacteria-phage mix was thereafter incubated for 15 minutes in 37°C. Thereafter 7ml (15 cm plates, 4ml for 10 cm) top-agar was pipetted into each tube at a time and immediately poured on the preheated the NZY-agar plates and spread evenly. This was done on a horizontal plate and the NZY-agar with bacteria-phage mix was left to dry before incubating the plates for 3.5-4h in 42°C. The plates were marked 1-5.

After 4h the Hybond C-extra nitrocellulose filters were placed on top of each agar-plate then incubated overnight in 37°C. Filters were marked by making five holes with the help of a cannula. The following morning the filters were removed carefully with the help of a flat tweezers one by one and were put in separate 15cm petri-dishes with the down-side facing up. The filters were washed 3x5 minutes in 15ml (10 ml for 10 cm plates) TBS-GT and placed on a shaking table during each wash.

The filters were blocked for unspecific antibody binding for one hour with 15ml TBS and 1% gelatine and then washed as above. Meanwhile Block medium with normal goat serum (NGS) was diluted 1:1000 and multiplied with the amount of petri-dishes in 15ml TBS-GT. Block-medium was pipetted to each filter and then left for 1h.

Primary antibody (serum from a boy with a giant nevus) was diluted 1:300 in TBS-GT and the filters were washed before incubation with the primary antibody overnight on the shaker with a slow speed.

After washing, the filters were incubated for 1.5h in alkaline phosphates conjugated goat anti human antibody (BIO-RAD) diluted 1:3000 in TBS-GT.

The filters were washed with 15 ml (10ml for 10 cm petri-dish) TBS+0.1% gelatine 3x<5 minutes (the total time should not exceed 15 minutes). For 100 ml colour reaction buffer 1ml of BCIP and NBT respectively was added. For development 15 ml of the colour reaction buffer was transferred to each filter. The colour reaction was stopped by washing with water. Filters were left to dry for
approximately two hours before positive colonies could be distinguished and then plucked and then put in 400µl (200 µl for 10cm plates) SM buffer and 20 µl (10 µl for 10cm plates) chloroform and stored in the refrigerator.

Secondary screening:

Each plucked positive clone from primary screening was rescreened on 10 cm plates. Approximately 500 plaques were plated on each plate. Two individual plaques were thereafter picked, one positive and one negative as a control. These plugs were also put in 200 µl SM-buffer and 20 µl chloroform before storage in the refrigerator.

Tertiary screening:

Each picked clone from secondary screening was screened once more on 10 cm plates to make sure that an individual (Pure) clone could be picked. The agar plugs were also put in SM buffer and Chloroform just as above. PCR was done on the pure clones that had been obtained to verify their purity.

7.2 PCR Amplification

Expand HIFI PCR system kit (Rotche diagnostics GmbH, Penzaberg, Germany) was used to amplify clones from tertiary screening. Two master mixes were prepared M1 and M2 (the amount multiplied with number of samples).

Master Mix 1 multiplied with number of samples

- H₂O 16,6µl
- dNTP 0,4 µl
- Primer M13 20 M 1,5µl
- Primer BK reverse 1,5µl

Master Mix 2

- H₂O 19,25µl
- 10xPCR buffert 5,0 µl
- Enzym 0,75 µl

DNA 5,0 µl

All material was placed on an ice block. M1 and M2 are mixed in the RNA-hood in two micro centrifuge tubes. A stripe of PCR tubes is taken out and filled with 20 µl of M1 and 25 µl of M2. The PCR tubes are then moved to the DNA-hood were only DNA is handled. 5 µl of DNA from tertiary screening is added to each PCR tube. PCR tubes are than placed in a PCR machine and the reaction can get started.

Program: Hifi 52 Cycles

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<td>15’’</td>
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<tr>
<td>94°</td>
<td>15’’</td>
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</tbody>
</table>
7.3 PCR Analysis

PCR products were analysed with electrophoresis on 1% agarose gel (Invitrogen, Carlsbad, CA, USA) which was mixed in 100 ml TAE buffer and heated in the microwave until resolved. EDTA was added to the mixture and the solution was poured on a casting tray with a comb generating 16 wells and left to solidify.

PCR products were then taken out from the PCR heating block and 5 µl of the products from each tube were mixed with 10 µl loading buffer and were thereafter loaded in the wells of the agarose gel. 3 µl of DNA ladder (Bio-Rad) was thereafter loaded on each side of the samples.

Single clone Excision:

XLI-BLUE and XLOLR bacteria were grown and incubated overnight in 37°C with shaking. XLI-BLUE and XLOLR bacteria were grown on separate e-flask overnight in 50 ml LB-broth mixed with 0.5 ml 20% maltose and 0.5ml 1M MgSO₄. The bacteria were transferred to two separate 50 ml falcon tubes.

The bacteria concentrations, OD₆₀₀ was measured (OD₆₀₀=1.0) and the bacteria were centrifuged (500xg). The supernatant was poured out and each of the cell pellets were resuspended in 10mM MgSO₄ (Calculate the volume by using V₁xC₁=V₂xC₂).

200 µl of XLI-Blue, 150 µl of phage stock and 1 µl of the ExAssist helper Phage were mixed in 6 Falcon 2059 tubes and incubated in 37 °C water bath for 15 minutes. After 15 minutes 3ml LB-broth, 30 µl MgSO₄, and 30 µl 20% maltose were added and then incubated for 3h at 37°C with shaking.

Then the tubes were heated in 67°C for 20 min and spun down for 15 minutes (500xg). The supernatant containing excised pBK-CMVphagemid packaged as filamentous phage particles was poured into 6 clean Falcon tubes and stored at 4°C.

200 µl of freshly grown XLOLR cells were transferred to twelve micro centrifuge tubes. 100 µl of the phage supernatant from the step above were added to six of the micro centrifuge tubes and 10 µl of the phage supernatant were added to the remaining six tubes. Each tube was marked with phage number and volume and incubated at 37°C for 15 minutes. Thereafter 300 µl NZY-medium was added and incubated at 37°C for 45 min. 150 µl of the cell mixture from each micro centrifuge tube were plated on LB-kanamycin (50 µg/ml) agar-plates and incubated overnight at 37°C. The following morning plaque had formed on all plates.

7.4 Plasmid DNA purification (Mini preps)

A single colony from each LB-kanamycin plate was picked and cultured in 3ml LB-broth and 1.5 µl kanamycin (stock 100mg/ml) overnight at 37°C. The bacteria were then centrifuged for one minute at +4°C (20 000xg). The supernatant was removed with a vacuum suction and a Pasteur pipette.
The Quiagen mini prep kit was used for purification of the plasmid. The bacterial pellet was resuspended in 250 µl P1 buffer and then 250 µl P2 buffer was added and mixed by inverting the tube. 350 µl N3 buffer was transferred and was mixed by inverting the tubes 3-6 times until the blue colour have disappeared. The tubes were centrifuged for 10 minutes in 13000xg.

The supernatant were decanted on to QIA prep spin column and spun down for 30-60s. 0,5 ml PB-buffer was added and the tubes were centrifuged for 30-60s, the liquid was discarded.0,75 ml PE-buffer was added to the spin column and centrifuged for 30-60s. It is very important to discard the liquid and centrifuge the QIA prep spin column for one minute, for the dispose of the remaining washing buffer (PE-buffer). QIA prep spin column are placed in micro centrifuge tubes, 50 µl EB-buffer is added to each tube which are left to filter through for one minute, and then centrifuged for one minute. The amount DNA was measured in each tube using nano drop.

Sequencing:

DNA template (the purified plasmid), primer (T7 and T3) and dH2O the mini prep were mixed in 0,2 ml PCR-strips( total volume per sample = 18 µl) and sent for sequencing at Uppsala genome centre. The sequence result were analysed with the help of a program called sequence scanner. NCBI BLAST was thereafter used to translate the sequences into a known protein.

7.6 Invitro translation and transcription (ITT)

12,9 µl nucleus free water, 2 µl TnT reaction buffer, 1 µl RNAsinhobitor, 1 µl Aa mix-met, 2 µl 35S-met 10mCi/ml, 5,1 µl DNA template 1µg/50µl, 1 µl TnT RNA polymerase T7 and 12,5 µl TnT reticulocytes were mixed in micro centrifuge tubes in a fume cupboard and incubated in 30°C for 1,5h. One tube contained one positive control with DNA and one negative control without DNA, the last tube contained our purified plasmid.

To be able to analyse the ITT-product, 100µl 10M NaOH, 67 µl 30% H2O2 and 833 µl H2O was mixed in a micro centrifuge tube and then put on ice. 2 µl of the ITT product was taken and added together with 100 µl of 10M NaOH, H2O2 and H2O- mixture in new micro centrifuge tubes. The tubes were than incubated for 10 minutes at 37 °C. After the incubation, 900 µl cold 25% TCA was added to each micro centrifuge tube, which was then left 30 min. Vacuum suction was taken and small round whatman filters were placed on top of each well. The filters were than washed with 5% cold TCA buffer.

The ITT-product in the micro centrifuge tubes was then transferred to each filter on top of the vacuum suction. The filters were washed five times with 1 ml 5% TCA and then twice with 1ml acetone using a dispenser pipette. 2 µl from the positive control was transferred to a new whatman filter; the filters were than left to dry under a lamp so that the protein can be attached to the filter. The filters were than each put in a scint can containing 2, 5 ml scint solution. Radioactivity was then measured in a beta counter.
7.7 Immune precipitation

Preparation of microtiter plates:

200 μl 1xDUNK was pipetted to the plates with filter (Millipore, Billerica, MA, USA) and were left in room temperature for 1h. 1xDUNK was then discarded and replaced with 200 μl 1xDUNK with 1% Bovine serum albumin for 2h. The wells were thereafter washed twice with 200 μl 1xDUNK with 0.05% Tween 20 and then with 200 μl ITT-wash buffer. The plates were than stored in the fridge overnight.

Immune reaction

25 μl of ITT-wash buffer was added to each well of the microtiter plates with conical bottom (Nunc, Roskilde Denmark). This was followed by pipetting 2,5 μl in double or triple samples of serum from 59 different vitiligo patients including our patient serum, 34 blood donors and BSA as a negative control.

To achieve >20000cpm in each volume of 25 μl, the ITT product was diluted in appropriate amount of ITT-wash buffer. 25 μl of ITT-wash buffer plus ITT product was then added to each well with conical bottom and was left on shaking with 300 rpm at 4°C overnight.

Washing of protein A sepharose:

A falcon tube was taken and filled up with an appropriate amount of 75 % protein A sepharose and ITT-was buffer. The falcon tube was than centrifuged at 500xG for 5min, this procedure was repeated one more time. ITT-wash buffer was then used to dilute the sepharose to get 50% slurry.

50 μl of this slurry was then taken and pipetted to each well of the microtiter plate with filter. The filter plates were then put on shake at 4°C for 45 min.

Washing
The liquid was removed from the wells with the help of a vacuum suction. Thereafter by a washing procedure according to this scheme:
A) 200μl ITT-wash buffer 3X
B) 150μl ITT-wash buffer, 300rpm shake for 5min at 4°C
Repeat A)

Repeat B)
200μl ITT-wash buffers 2X
After these steps, the bottom cover of the microtiter plates were removed and placed under a lamp for 1h until the sepharose had dried and cracked. The plates were then put on a holder whose bottom was covered with a plastic wrapper. Scint solution was added to each well and then the whole plate was covered with a plastic wrapper. The plates were thereafter placed in a beta counter.

8 Results

The melanocyte cDNA library was screened with a serum from a young boy with giant congenital nevomelanocytic nevus. From the five plates used at primary screening 24 positive plaques were plucked. The positive clones seen below went through secondary and tertiary screening (purification) to get as pure clones as possible. When all the clones were purified, there were only six plaques of positive clones. The six positive clones were sent to Uppsala genome centre to be sequenced.

This picture is of nitrocellulosa filters with positive and negative plaques. The picture to the left shoes plaques from primary, secondary and tertiary screening. The arrows in the pictures on the right hand side shows how the positive clones looked like.

After Sequencing we found that the sequences of the six positive clones we had plucked encoded for the following proteins.

1. Homo sapiens E1A binding protein p300 (EP300), mRNA
2. Homo sapiens procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3), mRNA
3. Homo sapiens structural maintenance of chromosomes 6 (SMC6), transcript variant 1, mRNA
4. Homo sapiens prune homolog 2 (Drosophila) (PRUNE2), mRNA
5. Homo sapiens RAB GTPase activating protein 1 (RABGAP1), mRNA

Of these five proteins, we chose to pursue with homo sapiens E1A binding protein p300 (EP300) to make the ITT-analysis and went forward with screening the ITT-product with serum from vitiligo patient and blood donors. The purpose of the ITT-analyses is to see if other vitiligo patients express antibodies against the protein EP300 that we found during screening with the serum from the young boy with giant congenital nevomelanocytic nevus.
This staple diagram represents the results från ITT-analysis. The Blue staples shows vitiligo patients and the pink staples shows blood donors. The Y-axis show CPM and the x-axis show vitiligo patients (v) and blood donors (BG).

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This diagram shows the index values from the ITT-analysis, were the positive control (patient serum V1) is set to 100. By plotting the vitiligo patients and blood donors and then calculating the index, we can see which of the patients are negative in comparison with blood donors. The staples that go above the red line and has an index value over 400 are considered to be positive values. As we can see only one vitiligo patient (V19) expresses antibodies against the protein EP300, which means that V19 is positive.
9 Discussions

The screening of the cDNA library made from cultured melanocytes with a serum from a young boy with a giant nevus made it possible to find five different proteins (potential antigens). Homo sapiens E1A binding protein p300 (EP300), mRNA, homo sapiens procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3), mRNA, homo sapiens structural maintenance of chromosomes 6 (SMC6), transcript variant 1, mRNA, homo sapiens prune homolog 2 (Drosophila) (PRUNE2), mRNA and homo sapiens RAB GTPase activating protein 1 (RABGAP1), mRNA.

After doing some research on the internet, the protein homo sapiens E1A binding protein was found to be the most interesting candidate to proceed with in doing ITT-analysis. The EP300, also known as KAT3B was found to be involved in essential processes such as cell proliferation, differentiation and tumor progression. The transcriptional regulator had been observed in several types of cancer, for example, lung, breast and prostate cancer. This protein is considered to be an oncogene and that is why we chose to continue working with it and performed ITT-analysis.

The ITT-analysis was performed so that we could see if other vitiligo patient also expressed antibodies against the protein EP300. Unfortunately our results were not as good as we had hoped for them to be. The results showed that only one patient was positive and due to the grate variation among the blood donors’ values, it is hard to conclude anything from the results. The reason behind these poor results is due to an accident that we had while preparing for the ITT-analysis. During one time all our samples accidently leaked out from the microtiter plate to the bench paper and left some of the wells empty, leading to us having uncertain results.

To be able to be sure if the EP300 protein is an important antigen target a new ITT-analysis would have been needed to be done, but since that I only had limited amount of time this was not possible for me to do.

Because the protein EP300 have been observed in other cancer types, it might not have a big significance in the development of malignant melanoma. It might be one of the proteins that are involved in the development of skin cancer but maybe not the key protein. I assume this because EP300 is a transcriptional regulation factor that is involved in many different cells development (proliferation and regulation) and it seems to be a common cause of tumour expression in many cancer cases. I think that the protein involved in the development of malignant melanoma is probably located and has its function within the skin cells.

The ITT-analysis that I did was initially not included in the plan but I was lucky to be able to do it and learn more about how it is done and also see what kind of information one can obtain from the analysis. My supervisor will have to continue with the analysis of the EP300 and the remaining proteins that I did not have time to analyse.
10 Acknowledgement

I would like to give special thanks to Eva Hagforsen, Håkan Hedstrand and to all the people who provided me with assistance and advice during my weeks at the institution.

11 References

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Images:

All the images were given to me from my supervisor.