CELLULAR LOCALIZATION ANALYSIS OF

ELLIS-VAN CREVELD SYNDROME PROTEINS

A Major Qualifying Project Report

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ABSTRACT

Ellis-van Creveld (EVC) Syndrome is a recessively inherited genetic disorder characterized by dwarfism, craniofacial abnormalities, skeletal deformities and dysplasia, and an approximate 60% incidence of heart defects. EVC syndrome is associated with mutations in two genes, EVC and EVC2 (limbin), located on chromosome 4p16, whose functions remain unknown. The purpose of this MQP was to obtain clues to the functions of EVC and EVC2 proteins through the study of their cellular localization. Synthetic peptides from parts of the EVC and EVC2 proteins were used to make antibodies for Western blot and immunocytochemistry analyses. Immunocytochemical localization of the EVC2 protein in normal and patient cultured fibroblasts suggests that the EVC2 protein is normally found in the nucleus, but is absent in some EVC patients. In contrast, the EVC protein normally appears in a peri-nuclear location. This research should provide information that helps identify the function of these proteins.

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BACKGROUND

Introduction to Ellis-van Creveld Syndrome

History

EVC syndrome was first described by Richard W.B. Ellis of Edinburgh and
Simon van Creveld of Amsterdam. The pair first wrote of EVC in 1940, after meeting en
route to a medical conference. While talking they came to realize that each had a patient
with the characteristics of a syndrome that would later bear their names. At the time they
called the disease chondroectodermal dysplasia. Throughout the 1950s various case
studies were published, but little else became known about the disease.

Description

EVC is a form of chondrodysplastic dwarfism. It is an autosomal recessive
disorder, characterized by short limbs, short ribs, polydactyly, and dysplastic teeth and
nails. A large percentage of patients also present with congenital cardiac defects. The
most common is a defect in the atrial septation that produces a common atrium.
(Galdzicka et al, 2002; Laufer-Cahana, 2002).

Prevelance

EVC appears to affect families worldwide, with patients in Europe, the Americas,
and even the Middle East. In the general population, the disorder occurs about once in
every 60,000 live births, but it is particularly prevalent among the Amish population

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occurring in as many as 5 of 1,000 live births in the Amish communities of Pennsylvania (Laufer-Cahana, 2002).

Life Consequences

A diagnosis of EVC syndrome usually occurs in utero or immediately following birth. Once the diagnosis is made, a heart exam is usually performed. Surgery can be performed for craniofacial abnormalities, polydactyly, or orthopedic abnormalities.   A child with Ellis-van Creveld syndrome may be prone to respiratory infections such as pneumonia, and may have more difficulty recovering from illnesses because of a small chest or hypoplastic lungs (Laufer-Cahana, 2002). EVC does not cause adverse mental or developmental abnormalities. Despite some severe cases, the average EVC child can grow to 43-61 inches.

Etiology of Ellis-van Creveld Syndrome

Genetics

Presently the etiology of Ellis-van Creveld syndrome is unknown.   Metrakos and Fraser (1954) first described a possible hereditary factor in chondroectodermal dysplasia. It was not until 42 years later, in 1996, that Ellis-van Creveld syndrome was mapped to chromosome 4p16 (Polymeropoulos, et al 1996).   It is believed that EVC follows a typical autosomal recessive inheritance. However, there is evidence that in some families the inheritance does not follow traditional Mendelian rules. In these families, more than fifty percent of the children are affected.

Gene EVC-1

Gene EVC was the first gene determined to play a role in Ellis-van Creveld syndrome, hence its designation (McKusick, 2000). Translation of the coding sequence gives a 990 amino acid long protein also known as EVC or DWF-1. It has a molecular weight of 111.8 kDa. Motif searches found a leucine zipper, three putative nuclear localization signals and a possible transmembrane domain (McKusick, 2000).

Gene EVC-2

EVC2 was the second gene found to play a role in Ellis-van Creveld syndrome. EVC2 encodes a 1228 amino acid long protein, spans 166.4kb, and is divergent to and shares a promoter with EVC (Galdzicka et al, 2002). Figure 1 shows the chromosomal location of EVC-2 relative to EVC.
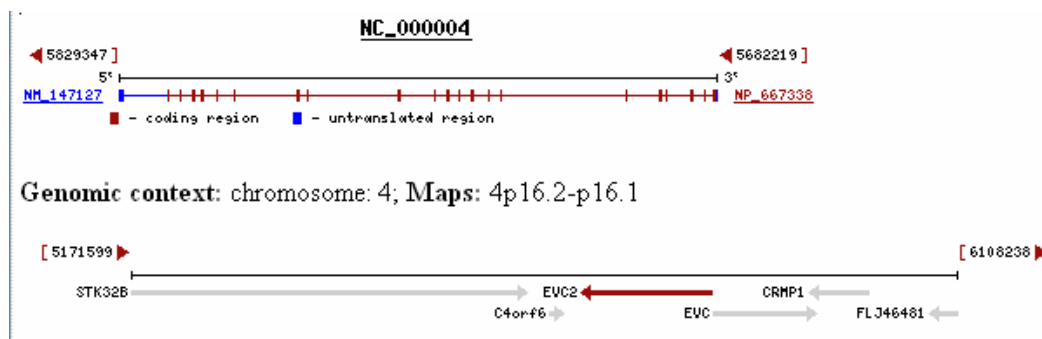

Figure 1: Gene Map Showing The Location of EVC2 Relative to EVC on Chromosome 4.   Source: http://www.ncbi.nlm.nih.gov/ entrez/ query.fcgi?db=gene&cmd=Retrieve&dopt= Graphics&list_uids=132884)

The transcriptional start sites of EVC and EVC2 are only 1643 bp apart. Protein EVC2 is predicted to have one transmembrane segment, three coil coiled regions and one RhoGEF domain. (Galdzicka et al, 2002)

Exclusion of the MSX1 Homeobox Gene

As with the EVC-1 and -2 genes, the MSX1 homeobox gene also maps to chromosome region 4p16.1. The msx genes are known to be involved in pattern formation of the developing limb bud and craniofacial bones in the mouse embryo (Ide et al, 1996). The gene family shares a 180-kb sequence of DNA encoding the highly conserved homeodomain. Members of the family have been found in various classes of organisms from Drosophila to chickens, frogs, and humans.

To determine whether MSX1 may be related to EVC syndrome, Ide et al 1996, created Msx1-defient mice which exhibited abnormalities in mandible and tooth development, as well as craniofacial development. There were, however, no abnormalities in the limbs or heart as one would expect in EVC. The authors suggested that despite the lack of limb abnormalities the "[msx1] expression pattern in embryos in the forming limb bud and neural crest implicate this gene in skeletal dysmorphologies…" Later sequence analysis of msx-1 in EVC patients, obligate carriers, and normal individuals, revealed no mutations in the coding region of the gene. Consequently, mutations in the msx homeobox domain were ruled out as a possible cause for Ellis-van Creveld syndrome.

Immunochemistry

The term immunochemistry was first used and coined by the scientist Arrhenius in 1907 (the Antibody Resource Page). This phrase summarized the combination of biology and chemistry in the study of antibodies. It is now used in the study of additional cellular products, but uses antibodies as the main method of identifying the target.

Western Blots

A western blot, also known as an immunoblot, is a combination of gel electrophoresis, protein transfer and an antibody assay used for detecting proteins in a supernatant or other solution. There are other similar types of blots that are used to identify RNA, DNA and other types of cellular products. A western blot uses antibodies with peptide sequences that have been created to bind with a specific peptide sequence in a protein. These peptide sequences are injected into animals to induce an immune response to the foreign protein, and the antibody is collected from the serum of the animal. These antibodies are known as the primary antibodies, as they make direct contact with the desired protein. In order to detect the bonded antibodies and protein, a secondary antibody is created and a type of label is attached. The secondary antibody is a product induced in a different animal than the first one, used to create the primary. The primary antibody is injected into the second animal, and the immune response creates an antigen which will then attach to the primary antibody.

The secondary is used to detect the protein that the primary has already discovered and makes its presence known through the use of a label. One of several different types of label is attached; radioactive molecules, fluorescein, horseradish peroxidase, etc., to act as a detection tool when the membrane containing the protein is

placed next to X-ray film. For this MQP, the label of choice was an enzyme conjugate that chemically converts the chemilluminescent substrate to emit light detected by the film. The combination of primary and secondary antibodies allows the visual ability to locate and identify the presence of specific proteins and their sizes.

Immunocytochemistry

The technique immunocytochemistry is very similar to that of western blots. The use of primary and secondary antibodies, the methods of antibody induction, the procedure for carrying out the technique are all the same. However, this practice is carried out on whole cells or tissues. The purpose of immunocytochemistry is to enable a scientist the ability to locate the target they are studying in the cell.

PROJECT PURPOSE

The purpose of this MQP was to obtain clues to the functions of EVC and EVC2 proteins through the study of their cellular localization. Synthetic peptides from parts of the EVC and EVC2 proteins were used to make antibodies for Western blot and immunocytochemistry analyses. Once identified, the proteins were used in immunofluorescence localization experiments to help assign a cellular function for EVC and EVC2.

METHODOLOGY

Western Blots for the different antibodies were performed using EVC patient samples X193, B136, 1352, 895, 891, 877, 874, 873, and 850. The original form of the samples was a cell pellet taken from patient blood samples, and a protein extraction step was required to make the desired EVC protein accessible in the supernatant. The EVC antibodies were created using chickens by Aves Labs, Inc® from peptide sequences that Dr. Ginns and Dr. Galdzicka had requested, and the Limbin EVC2 antibody was commercially available through AbCam®.

Western Blots

Preparation of Whole Cell Lysates

Cell lines of patient samples collected from blood, were maintained and subsequent cell cultures were frozen and stored at -80°C for future use. After thawing on ice, each of the sample pellets were resuspended in approximately 750 µL of Extraction Buffer, 60mM Potassium Phosphate with 0.1% Triton X-100 at pH 5.9. After the resuspension, the samples were flash frozen on dry ice to lyse the cells. Once the samples were frozen they were returned to ice to allow them to slowly thaw. Gentle rubbing was used to aid the thawing process.

Once the samples had thawed they were brought to the UltraSonic Sonicator™ at 50 Watts for 10 seconds three times. In between each sonication, the sample was kept on ice. The samples were then centrifuged at 14,000rpm for 5 minutes at 4°C to pellet cell

debris. The supernatant, which contained soluble proteins, was removed and placed into clean 1.5 mL tubes that were labeled with the sample numbers, and stored at –20˚C.

The remaining cell debris pellet was also processed for analysis by resuspension in an additional 750 µl or a five times approximate volume of the Extraction Buffer. The suspension was homogenized for 30 seconds and stored at -20˚C.

BCA Protein Assay

The Pierce BCA Assay Kit™ was used to determine the protein concentrations present in the cell lysate and pellet samples. Using the 2.0 mg/ml BSA standard, 8 standard curve dilutions were set up, as in Table 1.

Table 1. BSA Assay Standard Concentrations.

Volume BSA	Volume dH$_2$O	Final BSA Concentration
300 ul Stock 2.0 mg/ml BSA	0 ul	2000 µg/ml
375 ul Stock BSA	125 ul	1500 ug/ml (A)
325 ul Stock BSA	325 ul	1000 ug/ml (B)
175 ul of A	175 ul	750 ug/ml (C)
325 ul of B	325 ul	500 ug/ml (D)
325 ul of D	325 ul	250 ug/ml (E)
325 ul of E	325 ul	125 ug/ml (F)
100 ul of F	400 ul	25 ug/ml (G)

To prepare Reagent Mix, Reagent A from the kit was diluted 1:50 with Reagent B. (24.5 mL of Reagent B + 500 µL of Reagent A). The tube containing the mix was vortexed to ensure the reagents were homogeneously distributed. EVC sample tubes contained 100 µL of a 1:10 dilution of the sample. 2 mL of the Reagent Mix was then

added to each tube and the sample was mixed using the micropipette and placed back in the rack. One test tube containing only 100 μL of water and the 2 mL of reagent was made and used as a "blank." All of the test tubes were covered with parafilm and placed in a 37˚C water bath for 30 minutes. After the incubation, the test tubes were immediately placed on ice to stop the reaction. Each sample was read in the spectrophotometer relative to the blank. Sample concentrations were determined by comparison to the BSA standard curve.

Sample Preparation for Electrophoresis

A volume of sample containing 15 μg of protein was mixed with dH_20 to make 5 μl, then 5 μL of loading buffer plus reducing agent was added. Sample tubes were placed into boiling water for two minutes then briefly microfuged.

Gel Electrophoresis

For electrophoresis pre-made gels were purchased. To prepare the gels, they were removed from their protective packaging and the comb was removed to open the wells and the tape removed from the hard shell. Using a clean disposable pipette, 1x running buffer was used to clean the wells and remove any excess storage fluid and to eliminate any bubbles. Two gels were loaded into the gel box and then one half of the cover was forcefully inserted to create a seal between the gels and the probe. To test the seal, the external portion of the box was filled half way to ensure that no running buffer was leaking into the center between the two gels. Once that had been confirmed, the rest of the box could be filled with Electrode buffer, including the center to make sure that the wells were completely covered in buffer.

Once the box was set up, the samples were loaded into a well plus two markers: XP BenchMark Antibody Ladder and the Pre-Stained Ladder. Elecrophoresis was at 40 mA (one gel) or 80 mA (two gels) for about 90 min until the blue dye nearly reached the bottom of the gel.

Protein Transfer

Once the gel has completed its run, the next step was to transfer the protein from the gel to a membrane for antibody staining. Approximately 2 L of transfer buffer was used for each run. In a graduated cylinder transfer buffer was made with 400 mL of Methanol, 28.82 g of Glycine, and 12.5 mL of Tris HCl pH 8.3. The Tris HCl was made using 6.25 mL of Tris HCl pH 9.0 and 6.25 mL of Tris HCl pH 7.4. The total volume was then brought up to 2 L of ultra pure filtered water.

While the buffer was mixing 3MM filter paper and Immunoblot-P PVDF blot membranes were cut into the same size and shape of the electrophoresis gel. Two pieces of filter paper and membrane were needed for each gel. A western blot tray, which contained 100% methanol, was used to soak the membranes, while another tray containing the transfer buffer soaked the large and small sponges and filter paper. After the membrane had soaked for a few minutes in the methanol, it was moved to the tray containing the transfer buffer to rinse off. Once the gel was ready, a plastic cage was used to keep the sponges, gel and membrane together. The cage was laid flat; one or two sponges were placed on either side, followed by a thick white sponge on top of the sponges, then a piece filter paper, and then the membrane, followed by the gel. The cage was closed together and was tightly packed. The gel should be in the middle of the

"sandwich" with identical items flanking it on both sides. The membranes and gel must be kept wet at all times using the transfer buffer.

The sandwich was then taken to the transfer chamber which was filled with the transfer buffer. The cover was placed on the chamber and the transfer program of 25 mAmps for 20 minutes, 70 mAmps for 4 hours, and 100mAmps for 12-24 hours was set. This process was run overnight.

Our results had large amounts of non-specific binding which lead to intense background, and in many cases it over-powered the banding signal. Through Aves it was discovered that chicken antibodies are infamous for their non-specific binding, and the effect is increased by using PVDF membranes. To try to counteract the non-specific binding the methodology was altered by using BlokHen II as a different blocking agent over the dry milk, and switched the PVDF membranes to nitrocellulose as recommended by Aves. To then determine the best combination of membranes and blocking agents, a "Dot Test" was performed using antibody 3693 to optimize the methodology.

Membrane Incubations

Before removing the cages from the transfer box the washing buffer of 1x PBS plus 0.05% Tween 20 was made. Usually 500 mL of the solution was made at a time, with 250 μL of Tween 20 added to 500 mL of 1x PBS. The blocking solution of dry milk and PBS wash solution was made by adding 5 g of dry milk to 100 mL of wash. It was set on a plate and using a stir bar, was mixed for 15 minutes.

The membrane that had taken up the Prestained Ladder was also the membrane that all of the protein from the gel had transferred to; the other membrane was empty and was discarded. Depending on whether the gel had been set up for one Western Blot or

two, if the gel had two identical sample sets then the membrane was cut down the XP

Marker lane. Each membrane was placed into a dish labeled with the antibody that it

would be washed with. To each dish containing a membrane, approximately 20 mL of the

milk blocking solution was added. The dishes containing membrane and solution were

placed on a shaker in an incubator at 37˚C for 1 hour. While the membranes were

incubating the primary antibody solution was made. The solution was made with 1:500

dilution of the primary antibody that was to be applied to the membrane. It was made

with 20 mL of the blocking milk solution and 40 μL of the specified antibody. The 20 mL

of the solution was added to the dish with the membrane that was set up to receive the

antibody and was returned to the 37˚C incubator and shaken for 1 hour.

       After the hour the primary antibody solution was drained off the membranes and

then approximately 15 mL of PBS Tween washing solution was added. The dishes were

then placed on a shaker at room temperature for 5 minutes. After the 5 minutes, the

washing solution was decanted and fresh washing solution was added and then the dishes

were returned to the shaker for an additional 5 minutes. This was repeated one more time.

       During the final wash, the goat anti-chicken alkaline phostphatase secondary

antibody solution was prepared following the directions from the WesternBreeze

Chemilluminescent Kit. A 1:5000 dilution in 20mL of wash was used with the secondary

antibody. Approximately 20 mL of the secondary antibody solution was added to the

dishes and placed on the shaker in the 37˚C incubator for 30 minutes. The PBS Tween

washes were then repeated three times only this time each wash was done for 10 minutes.

Immno-Dot Test

To optimize the banding signal and minimize the non-specific binding and background of the Westerns a dot test was performed for eight different sets of conditions (see Table 2), performed on both PVDF and nitrocellulose membranes.

Table 2. Conditions for the Immuno-Dot Test.

Sample	Blocker	Primary	Wash
1	Milk	3693	PBS Tween
2	Milk	3693	PBS
3	BlokHen II	3693	PBS Tween
4	BlokHen II	3693	PBS
5	BlokHen II	None	PBS Tween
6	BlokHen II	None	PBS
7	Milk	None	PBS Tween
8	Milk	None	PBS

The PVDF membranes were treated as usual, but the nitrocellulose membranes followed a slightly different methodology. The PVDF membrane was activated in methanol, and the nitrocellulose was activated in purified water before being rinsed in transfer buffer. Both membranes were placed in a 50 mL conical tube and rotated at 37ºC in a rotator. The blocking step was preformed for an hour using 10 ml of the listed blocking solution. The washes were also performed in the incubator using 15 ml of the wash solutions. Each wash was performed three times at 10 minutes each. The primary incubation was also done in the incubator at 37°C for an hour. Blocker/Diluent A and B solutions from the WesternBreeze kit were used to make the solution, as instructed by the kit, to dissolve the

antibody. The primary antibody 3693 was diluted to 1:500, while the membranes that did

not receive the primary antibody were incubated in the blocker/diluent solution alone.

The incubation followed another series of washing as before. The secondary incubation

was done in the incubator for 30 minutes. The goat anti-chicken alkaline-phosphatase

secondary antibody was also dissolved into the blocker/diluent with a dilution of 1:5000.

A final series of washes was performed on the membranes, prior to chemiluminescence.

X-Ray Photographs

Once the washes were completed the chemilluminescent substrate, blotting paper,

pipette with 5 mL pipette tips, and transparency paper were taken to the lab's darkroom.

The developing tank was filled with clean water and the developer and fixer solutions

were made. Each bottle of Kodak GBX Fixer and Replenisher™ or Kodak GBX

Developer and Replenisher™ was dissolved in water and brought up to 3.8 liters of total

volume, or 1 gallon. Once the tank was ready, the membrane was taken out of the wash

solution and placed on a plastic transparency sheet where 2.5 mL of the

chemiluminescent substrate was applied and sat for 5 minutes. After the 5 minutes, the

excess substrate was blotted off with blot paper and the membrane was covered with the

other transparency. The lights were turned off and only the red safe light was on in the

room. A piece of X-ray film was taken out of its protective cover and placed on top of the

membrane and then covered by a black phosphor screen. Each X-ray film was exposed

for differing amounts of time depending on how previous exposures appeared, but the

average exposure was about five minutes. Once the exposure was done, the X-ray film

was attached to a hanger and placed in the developer for 3 minutes. After the 3 minutes

the hanger and film were transferred to the water for 2 minutes to wash the developer off.

The film was then moved to the fixer for another 3 minutes before being moved to the

water to wash for a final 2 minutes. Once the film was in the water after the fixer, the

lights could be turned back on to see the film image. The films were placed on another

hanger where they could hang and dry.

Immunocytochemistry

Fibroblast cell lines from EVC patients were maintained in DMEM with 10%

FBS in 6 well plates until they reached confluency. The media was siphoned off and the

wells were washed with 1x PBS for 5 minutes. The PBS was then siphoned off and the

10% buffered formalin was added and allowed to incubate at room temperature for 1 hour

to fix the cells. After the hour, the formalin was removed and a PBS wash was performed

three times at 5 minutes each.

To block the cells, the PBS was removed and then approximately 750 µL of 0.1%

BSA was added and incubated on a shaker at 37°C for 1 hour. The BSA was removed

before adding 750 µL of the 1.0% BSA + PBS solution containing the Limbin primary

antibody in a 1:1000 dilution. The primary was incubated at 37°C for 1 hour on a shaker.

This primary antibody solution was removed and placed in a container where it was

stored at 4°C and used for multiple incubations. The cells were then washed with 2-3 mL

of PBS + 0.05% Tween 20 five times at five minutes each. Once the washes were

complete the PBS wash was removed and the cells were incubated with 750 µL of the

1:5000 diluted secondary antibody using rabbit anti-goat IgG (H+L) Alexa Fluor 488

from Molecular Probes™ for one hour at 37°C. Once the secondary antibody had been

added, the plate was wrapped in aluminum foil to minimize light exposure, which can

bleach the fluor. The secondary antibody solution was also removed and stored at 4°C for

multiple incubations. The final five washes were performed with 2-3 mL of PBS + 0.05%

Tween 20 for five minutes each prior to immunofluorescent detection performed on a

PixCell II Microscope™.

Immunocytochemistry Fixation Test

An optimization of the immunocytochemistry procedure was performed to

maximize the fluorescence of the limbin primary when attached in the cell and to

minimize the background of secondary non-specific binding. The optimization was

performed on a 12 well cell culture plate containing ML1 normal EVC patient fibroblast

cells using six different sets of conditions (see Table 3). Each condition was performed

twice, one with primary solution and the other without primary to act as a negative to test

for non-specific binding.

Table 3 Fixation Optimization Conditions

Condition	Fixer	Fixer Time (minutes)	Detergent	Detergent Time (minutes)
1	Methanol	15	4mM Sodium Deoxycholate	15
2	Methanol	30	4mM Sodium Deoxycholate	15
3	Methanol	15	4mM Sodium Deoxycholate	30
4	Methanol	30	4mM Sodium Deoxycholate	30
5	Ethanol	15	4mM Sodium Deoxycholate	15
6	Ethanol	30	4mM Sodium Deoxycholate	30

The optimization immunoassay was carried out similar to the immunocyto-chemistry protocol. Wells were washed with PBS quickly to remove media before the fixation with the listed solution and time was performed. The wells were then washed three times at 5 minutes each using only PBS. Each condition was blocked for one hour using 1.0% BSA + PBS at 37°C on a shaker. The BSA was removed and 500 μL the 1:1000 dilution of limbin primary antibody solution was applied for an overnight incubation at 4°C. The primary solution was then pooled and stored at 4°C for future use. Each well was washed five times for 5 minutes each using approximately 1.5 mL of PBS + 0.05% Tween 20. The rabbit anti-goat secondary solution with a 1:1000 dilution was added to the wells and incubated for one hour at 37°C. The plate was wrapped in aluminum foil for the remainder of the experiment to minimize light exposure. The secondary solution was collected and saved at 4°C before being washed five times at 5 minutes each using PBS + 0.05% Tween 20.

The cells were kept in PBS after the final PBS + 0.05% Tween 20 wash to be viewed under the PixCell II™ microscope. Each well was viewed and compared to one another. An assessment on strength of signal was rated using a number system 1 thru 10 with the strongest possible signal receiving a 10 and the lowest a 1. This information was used to select the most optimal fixation and permeability times.

Immunoassay Secondary Antibody Dilution Test

The next step was to determine the optimal dilution of secondary to have the maximum amount of signal, while attempting to minimize the amount of background. A

6 well plate containing ML1 normal EVC cell lines using the fixation method selected

from the previous test, 15 minute fixation with Methanol followed by a 15 minute

permeability, were tested for three different secondary solution dilutions (see Table 4).

Table 4 Secondary Antibody Optimization of Dilutions

Series	Dilution of Secondary Antibody
1	1:1000
2	1:5,000
3	1:10,000

Each condition was performed twice, once with 1:1000 primary dilution and the

other without primary antibody to act as a negative control. The same protocol for the

immunoassays was repeated except for a few changes requested by Dr. Ginns. The plate

was fixed with 3 mL methanol for 15 minutes, and then permeabilized with 4 mM

Sodium Deoxycholate for 15 minutes. The plate was washed three times at 5 minutes

each using PBS before 3 mL of the blocking solution of 1.0% BSA + PBS was added. At

the request of Dr. Ginns, the blocking was performed for two hours over the usual one

hour, at 37°C. The theory is that for the longer amount of time the cells are blocked,

lower amounts of the secondary antibody will bind non-specifically to proteins in the cell.

The primary dilution of 1:1000 was then added in amounts of 750 μL to each well and

incubated overnight at 4°C. The primary was then collected and saved at 4°C for future

use and washes were performed five times at 5 minutes each using PBS + 0.05% Tween

20. The next change requested was that the series of secondary dilutions being tested

were performed for an hour at room temperature. The plate was loaded with 750 μL of

the listed dilution of secondary, covered with aluminum foil and incubated on a shaker at

room temperature for one hour. The secondary was removed and saved at 4°C for future use and the wells washed with 3 mL of PBS + 0.05% Tween 20 five times at 5 minutes each and then stored in fresh PBS at 4°C.

The cells on the plate were observed using the PixCell II™ and rated on the strength of their signal. An assessment on strength of signal was rated using a number system 1 thru 10 with the strongest possible signal receiving a 10 and the lowest a 1. This information was used to select the most optimal dilution of the rabbit anti-goat secondary to minimize the amount of secondary needed to obtain the strongest signal and minimal amounts of background.

RESULTS AND ANALYSIS

Prior to performing any bench research, a bioinformatic approach was used to identify potential glycosylation sites on the two EVC proteins to predict the extent of glycosylation and their expected sizes on immunoblots.

Computer Analysis of EVC Protein Sequences

The NetOglyc program, from the Center for Biological Sequence Analysis (CBS), was used to carry out an analysis of the EVC sequence data, obtained from Dr. Galdzicka. The NetOglyc server produces neural network predictions of mucin type GalNAc O-glycosylation sites in mammalian proteins. Analysis gave possible O-glycosylation sites at 478 and 545. Position 478 carried a 0.236 G-score, and an I-score of 0.704, while position 545 had scores of 0.153 and 0.563, respectively, using the parameters previously established for analyzing mammalian O-glycosylation sites (Julenius et al, 2005).

Another program, NetNGlyc, available from CBS, was used to determine whether there were potential N-glycosylation sites. The NetNglyc server predicts N-Glycosylation sites in human proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons. Analysis gave three possible N-glycosylation sites -109 (0.7858 potential); 136 (0.6538 potential); and 831 (0.4996 potential). Jury agreement for the three positions varied greatly. Position 109 was declared a high probability (+++) N-glycosylation site with a 9/9 jury; 831 was ruled out with a 3/9 jury result, and 136 was declared a possible N-glycosylation site, but it prompted a "pro-x1" warning, meaning a

proline occurs just after the asparagine residue. Due to conformational restraints the asparagine is prevented from being glycosylated (Gupta, 2004).

Analysis of the EVC2 sequence with the NetNGlyc 1.0 server identified possible N-glycosylation sites at seven positions, all with jury values between 7/9 and 9/9. Positions 110 (potential 0.7583), 140 (0.6123), 208 (0.7287), 333 (0.7142), 485 (0.7482) each received jury result of 9/9, and are predicted N-glycosylation sites. Positions 119 and 193 were just as successful with jury results of 8/9 and 7/9, respectively (Gupta et al, 2005). Analysis of EVC2 with the NetOGlyc 3.1 server identified just one possible O-glycosylation site at position 1069. With a g-score of 0.240 and an I-score of 0.619 this position could be an O-glycosylation site, but it is unlikely (Julenius et al, 2005).

On the basis of the bioinformatic identification of potential glycosylation sites, we determined that because several such sites are present, the two EVC proteins likely exist as glycoproteins within the cell, so their sizes on immunoblots may be larger than predicted solely on the basis of their amino acid compositions.

Antibody Sequencing

Antibodies 3695 and 3693, induced against synthetic peptides EDH EER KLQ HLK TLQ and SRR RKR EVQ MSK DKE, respectively, were analysed using the Sequencher™ 4.2 program to determine where on the EVC protein the antibody recognition domain resided (see Figure 2).
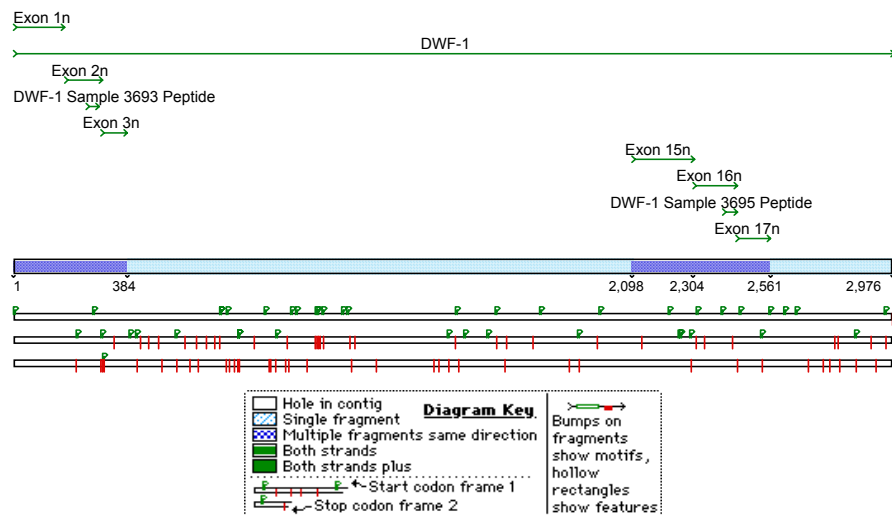
Figure 2: Locations of Antibody 3695 and 3693 Epitopes on EVC.

The peptides that had been selected for injection were then analyzed using the Sequencher 4.2™ program to identify on which exon in the gene they resided. For 3695's recognition sequence resides in exon 16 of the EVC1 gene. Due to the fact that one of the prominent EVC mutations being studied causes a stop codon on exon 13, the exon 16 amino acid sequence would likely not be present in patient samples, so this ruled out the usage of antibody 3695 in future experiments (including the proposed immunohistochemistry experiment).

Antibody 3693 recognizes an epitope located in exon 2 of EVC. This antibody was used in subsequent immunoblot and immunofluorescence experiments, since exon 2 should be present in most patient samples.

Using the NCBI website (http://www.ncbi.nlm.nih.gov) it was determined that antibody 3660 recognizes an epitope present in the EVC2 gene starting at the 565th peptide of the 1228 peptide long protein. This places the antibody almost exactly in the center of the protein, before the known stop codon, so this antibody was also a viable option for subsequent experiments.

Western Blot Analysis

Prior to the start of this project, chicken and rabbit antibodies were induced against specific regions of the EVC peptides. Those antibodies, and a goat antibody (termed Limbin) directed against EVC2 (obtained from Abcam, Inc.) were assayed to see which had the highest affinity for EVC or EVC2 proteins. Western blots using antibodies 3693, 3695, 3626 and 3627 for EVC, and Limbin and 3660 for EVC2 were used in immunoblot experiments to test immunoreactivity using cell lystates prepared from EVC

patient samples (see Table 5).

Table 5. EVC Patient Samples.

Sample Number	Sample Type	Identity	Mutation	Gene	Procedure Utilized For
B100	ASHKENAZI	PATIENT	homoz nonsense	EVC2	Western Blot
B132	American	PATIENT	heteroz missense	EVC2	Immunocytochemistry
B136	American	PATIENT	homoz del_> frameshift->Stop	EVC	Western Blot; Immunocytochemistry
B145	American	PATIENT	heteroz del-> frameshift->Stop/ heteroz missense	EVC	Immunocytochemistry
B162	Taiwan	PATIENT	homoz nonsense	EVC2	Immunocytochemistry
EVC850	AMISH	PATIENT	homoz splice-> Stop	EVC	Western Blot
EVC869	AMISH	PARENT (OF 889)	heteroz splice-> Stop	EVC	Western Blot
EVC870	AMISH	PARENT (OF 889)	heteroz splice-> Stop	EVC	Western Blot
EVC873	AMISH	PARENT (OF 891)	heteroz splice-> Stop	EVC	Western Blot
EVC874	AMISH	PARENT (OF 891)	heteroz splice-> Stop	EVC	Western Blot
EVC877	AMISH	PARENT (OF 895)	heteroz splice-> Stop	EVC	Western Blot
EVC887	AMISH	PATIENT	homoz splice site mut -> Stop	EVC	Immunocytochemistry
EVC889	AMISH	PATIENT	homoz splice-> Stop	EVC	Western Blot, Immunocytochemistry
EVC891	AMISH	PATIENT	homoz splice-> Stop	EVC	Western Blot
EVC895	AMISH	PATIENT	homoz splice-> Stop	EVC	Western Blot
NSB1012	NORMAL	NORMAL	normal	none	Western Blot
NSB1048	NORMAL	NORMAL	normal	none	Western Blot
NSB1342	NORMAL	NORMAL	normal	none	Western Blot
NSB1353	NORMAL	NORMAL	normal	none	Western Blot
X193	Canadian (French)	PATIENT	heteroz del-> frameshift->Stop/ heteroz splice site mut	EVC	Western Blot Immunocytochemistry
T0313	American	PATIENT	heteroz missense	EVC	Immunocytochemistry
RD	Netherland	PATIENT	unknown		Immunocytochemistry
ML1	NORMAL	NORMAL	normal	none	Immunocytochemistry

In the beginning, both soluble proteins and debris pellets were analyzed from lysates prepared from stored patient tissue pellets to determine whether the EVC protein resided in the soluble fraction or debris pellet (see Figure 3). The lysate samples, seen in lanes 1 through 4, were remarkably different from the pellet samples seen in lanes 7-10 (lanes 5 and 6 were a prestained ladder and XP ladder, respectively). Using limbin antibody, bands were seen in the lystate lanes around 100 kDa in all patients tested on this blot (889, 1012, 1048, and 1353).
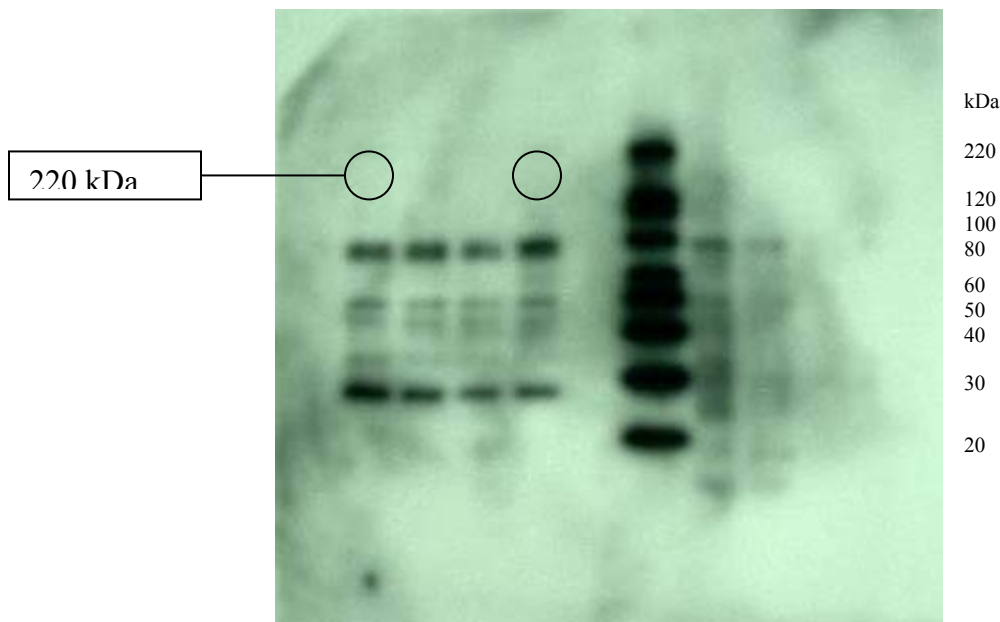
Figure 3: Analysis of Soluble Lysate Versus Pellet Samples for the Commercially Available Limbin Antibody. Lane 1, extract 889; Lane 2, 1012; Lane 3, 1048; Lane 4, 1353; Lane 5, XP Marker.

Eventually, the analyses concluded that only the soluble portion of the lysate samples should be used based on the observation that bands were found around 220 kDa in lysates of patient sample 889, but not in the pellet samples associated with this patient.

Unfortunately, our results had large amounts of non-specific binding which lead to intense background, and in many cases it overpowered the band signals. It was learned

through Aves Labs, Inc. (Tigard, Oregon) that chicken antibodies are infamous for their non-specific binding, and the non-specific effect is increased by using nylon-based membranes. To try to counteract the non-specific binding, the methodology was altered by using BlokHen II as a different blocking agent over the dry milk, and by switching from the PVDF membranes to nitrocellulose as recommended by Aves.

To determine the best combination of membranes and blocking agents, a "Dot Test" was performed using antibody 3693 (discussed below) to optimize the methodology.

Antibody Limbin (Against EVC-2) Immunoblot

Patient lysates were analyzed using the commercially available Limbin antibody against EVC-2 (Figure 4). The 5 minute exposure (data not shown) showed the ladder as a smear so an attempt was made to try to resolve the ladder.
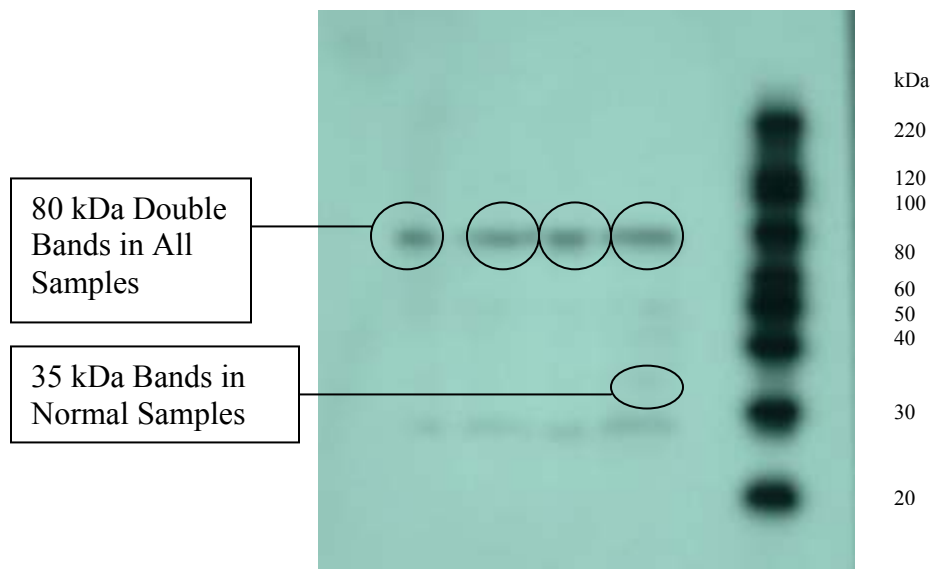

Figure 4: Analysis of Patient Samples Using Limbin Antibody Against EVC-2. Lanes 1,2 Patient Sample 891; Lanes 3,4 Normal Sample 1012. 30 second exposure.

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The 30 second exposure (Figure 4) showed several bands. Nothing was observed at the 220 kDa mark in any sample (220 kDa was deemed worthy of consideration due to the fact that it was a non-expected band which may indicate different mutations not yet known in the EVC2 gene). However, a band around 35 kDa was present in normal samples but not present in the patient samples, as expected if the patient samples had a mutated EVC protein. Also, there appears to be double bands around 80 kDa in all samples (marked in the figure with circles).

On the basis of these findings, antibody Limbin against EVC2 was chosen for further analysis (see below) because two different anomalies of interest between patient and normal samples were found.

Antibody 3660 Against EVC2

Portions of this blot were not useable because of a bubble. This bubble was the result of a bad transfer. Portions unaffected by this bubble were still valuable.
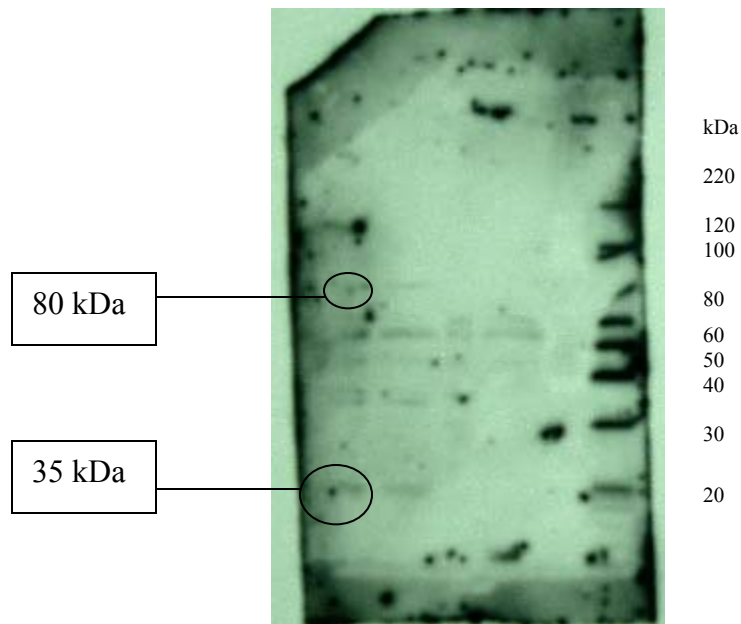
Figure 5: Test of Antibody 3660 Against EVC-2. Lanes 1, 2 Patient Sample 891; Lanes 3, 4 Normal Sample 1012. Five minute exposure.

Bands were observed around 35 kDa and 80 kDa in patient 891 but not in normal (1012) samples, which is because the location of 3660 is different from the location of Limbin. 3660 is located in the center of the EVC2 gene while limbin is located at the very end. This would result in different banding between the different blots.

Antibodies 3626, 3627 (EVC2)

These antibodies recognize the same EVC2 epitope as 3660 but come from different hens. The experiment shown in Figures 6 and 7, failed to yield any results because the gel lost its consistency, although the pre-stained ladder transferred correctly.
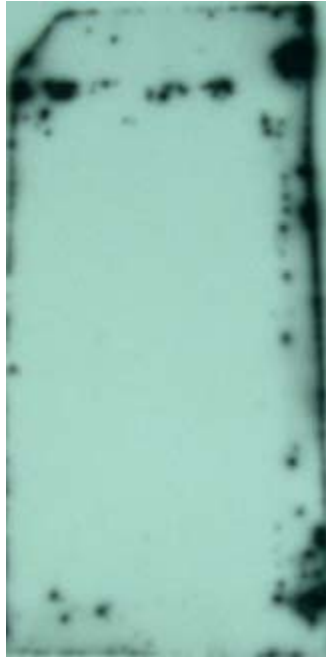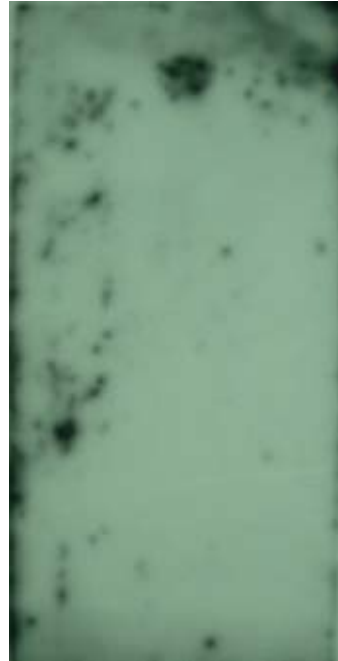
34

Figure 6: 3626 One minute exposure

Figure 7: 3627 One minute exposure

Antibody 3693Against EVC, Exon 2

Bands were observed around 100-85 kDa and 35 kDa in patients, but not in normal samples.
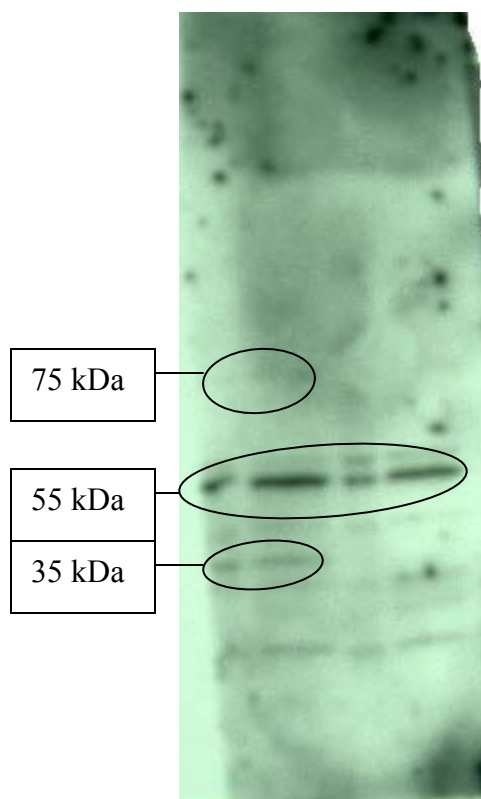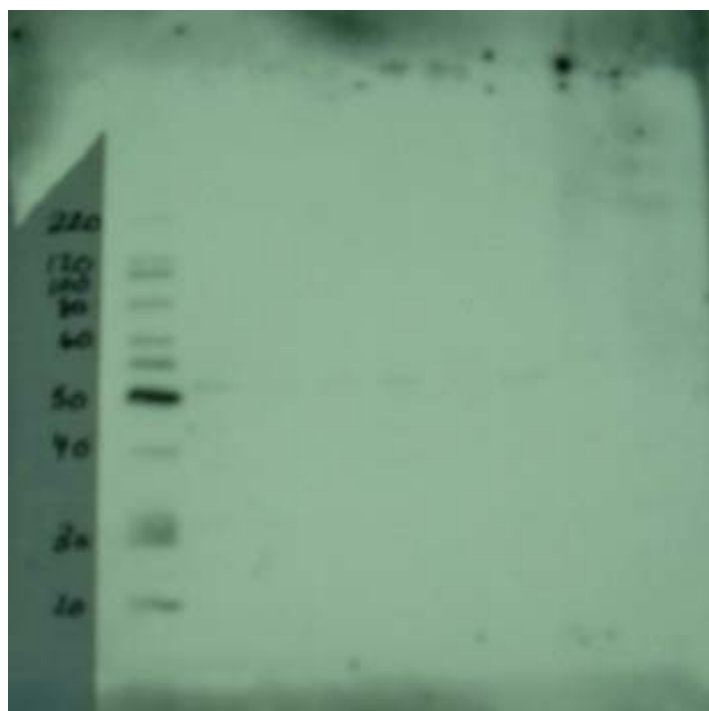
Figure 8: 3693 **Figure 9: 3693**
Lanes 1, 2 Patient Sample 891; Lanes 3, 4 Normal Sample 1012 for both

A second blot confirmed the presence of the 100-85 kDa and 35 kDa bands in patient samples and but in the controls. There was also a very strong band in all samples around 55 kDa.

Another attempt was made but this experiment failed due to an unexplained error. The ladder became disorganized and had extra bands (data not shown).

Antibody 3694 Against EVC1

A 30 second exposure was underdeveloped, however the 1 minute exposure produced bands (Figure 10).

Figure 10: 3694 Overnight exposure   **Figure 11: 3694 One minute exposure**
Lanes 1, 2 Patient Sample 891; Lanes 2, 3 Normal Sample 1012
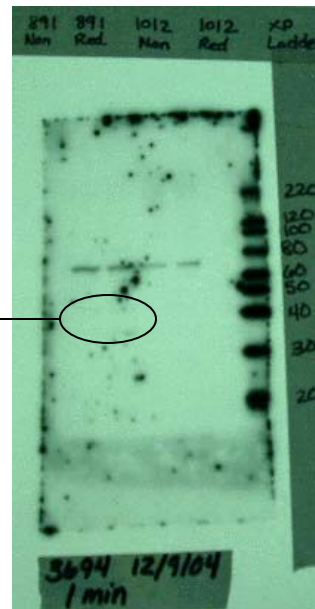
The five minute exposure showed strong signals at 75 kDa in all samples. The overnight

exposure showed what might be weak bands in the patient samples at 35 kDa.

Antibody 3695 (EVC-1, exon 16)

This antibody was not used for analysis because its recognition epitope resides on

exon 16, which comes after the stop mutation within exon 13 in a frequent EVC

mutation. At no point was the affinity for 3695 established by sequencing. Despite

attempts at optimization the antibody continued to demonstrate large amounts of non-

specific binding (data not shown).

Optimization of 3693

One of the problems encountered while testing the chicken antibodies was a high background. Contact with Aves indicated that PVDF and other nylon based membranes cause higher than normal non-specific binding, due to the interaction of nylon and chicken IgGs (Aves Labs, Inc., 2005). Thus nitrocellulose was tested for antibody 3693. The nitrocellulose membrane was incubated with 3693 as the primary antibody for two different lengths of time (one hour and overnight). A 10-minute exposure of the first blot yielded faint bands below the 20 kDa marker (data not shown). An overnight exposure, of the one hour incubation (Figure 12) yielded slightly darker bands at approximately 33 kDa in patient (B100, 895) pellet samples. Also notable is banding seen below the 20 kDa mark in B100 and 895 pellets. The results of the overnight incubation were not as expected. The only observable signals after a thirty-minute exposure were a very faint XP ladder, a possible band in the 895 pellet around 45-50 kDA, and a smear in the B100 patient lystate.

Figure 12: Test of EVC Antibody 3693 with Nitrocellulose Membrane. Overnight Primary. 30 minute exposure.

Dot Test

After the failure of the optimization procedure several other western blots were performed on nitrocellulose membranes using a procedure from Aves. These blots used antibodies 3693 and 3695 but most of these blots appeared not to have any bands on them. In an effort to determine the best way to blot, a dot test was performed.

Sixteen different dot tests (refer to Methodology page 18 for more information) were performed to optimize the Western Blot protocol (Figure 13). Some of the factors tested were nitrocellulose versus PVDF, washing in PBS Tween versus PBS, use of primary antibody versus no primary antibody, and blocking with a milk solution versus BlokHen II.


Figure 13: Nitrocellulose Dot Test.
For "Dot Conditions" See Table 2

The results for the nitrocellulose membranes (shown in figure 13) were quite interesting. The 5-minute exposure showed a limited response from the nitrocellulose membranes, regardless of the conditions, however blot N8, carried out with milk block and washed with PBS Tween, had a very faint signal. When using a longer overnight exposure, only the samples that were blocked using milk (N1, N2, N7, and N8) had a

visible signal. All of the membranes that had been blocked with BlokHen II had no signal. N1 and its slightly stronger companion N2 had no background and detectable signal. N7 and its companion with slightly stronger signal N8 were blocked using milk and did not receive 3693 during the primary incubation yet their signals were equal to those receiving primary antibody. This raised concerns about the validity of the signal obtained using a nitrocellulose membrane. This also raised concerns about the validity of any signal obtained using the goat anti-chicken alkaline-phosphatase secondary because obtaining a signal meant that the secondary was binding any number of unrelated proteins. The lack of response on the nitrocellulose membrane tests raised the possibility that our EVC proteins were not binding to the nitrocellulose for some reason.

The PVDF dot test results (Figure 14) were more promising. The 5-minute exposure showed several signals. The conditions for the PVDF dot test were the same as those for nitrocellulose but were performed on PVDF membranes instead. (See Table 2 for exact conditions.) P1 had a strong signal with a mostly clean background. P2 had a strong signal, but had the strongest non-specific binding and darkest background. P3 which used BlokHen II, 3693 as the primary incubation and was washed using PBS Tween, had a clear signal, and one of the cleanest backgrounds. P4 had a very weak signal mainly because the background was nearly as strong as the signal itself. Blots P5 and P6 had barely visible signals and were both carried out with no primary antibody added to the Blocker/Diluent solution. P7 had a clear signal with minimal background. P8 had a clear signal, with the second most background. Overall it was determined that the blots blocked in milk had too strong of a background, while those blocked with BlokHen II had an acceptable balance of signal and background. Based on the information

obtained from the exposures the P3 procedure was determined to be the optimal

procedure.
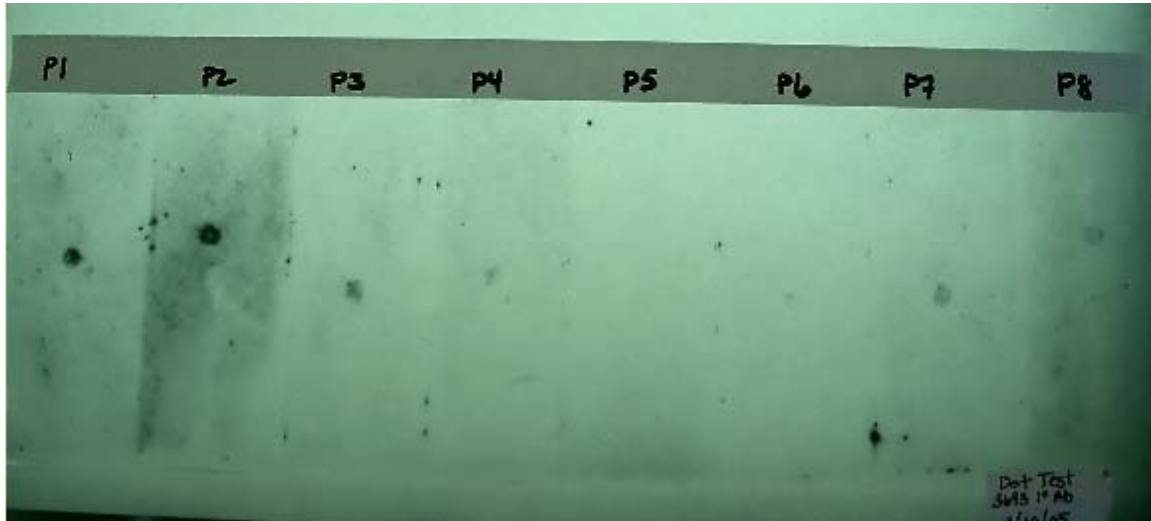
Figure 14: PVDF Dot Test. Five-minute Exposure
For "Dot Conditions" See Table 2.

Immunocytochemistry

Several immunocytochemistry experiments were performed on human fibroblast

tissue culture samples of both normal and EVC patients. The EVC2 (limbin), chicken and

rabbit antibodies were used to localize the EVC proteins in the cells.

Immunoassay Fixation Test

Initially, the variation in fluorescence between positive and negative EVC2

(limbin) tests in fibroblast tissue culture was minimal, so optimizations to enhance the

differences were carried out. An optimization was performed to create an ideal fixation

process, which combined the fixing of the cells to the plate and making the cells

permeable to uptake the limbin primary antibody and fluorescent rabbit anti-chicken

secondary. The entire procedure for this process and the conditions tested can be found in the Methods section, on page 21.

The system to compare the variations between the different tests was a rating method assigning values on a scale of 1 to 10. Values were assigned based on the brightness of signal in the cells in the different conditions compared to one another. The strongest signals received larger numbers than the cells with weaker signals. The condition with the largest positive difference between the limbin positive and negative samples was chosen as the fixation process to proceed with (see Table 6).

Table 6: Results of Fixation Optimization

CONDITION	Limbin (1:1000 dilution)	NEGATIVE	DIFFERENCE
1	7	1	+6
2	1	2	-1
3	3	3	0
4	6	3	+3
5	8	3	+5
6	3	4	-1

Condition 1 had the largest difference between the positive and negative test, a difference of +6, and was selected as the fixation process to continue experimentation. This condition utilized a 15 minute fixation step with Methanol and 15 minute 4 mM Sodium Deoxycholate permeability step.

Another aide in the selection process for the optimal protocol included the appearance of the cells under the microscope. Cells that had been exposed to Methanol for 30 minutes and 4 mM Sodium Deoxycholate for 15 minutes had a shriveled appearance. Cells that were exposed to both solutions for 30 minutes had a bright fluor,

but the cells appeared to dissolve and were no longer intact, which would not aide in identification of a location in the cell. Ethanol had a similar shriveling and dissolving effect to the cells as Methanol, except that the cells were destroyed within 15 minutes. With the optimal times for the fixation process decided upon, the next step was to determine the optimal dilution for the secondary antibody.

Secondary Dilution Series Test

The purpose of the dilution test (data not shown) for the secondary antibody was to determine the least amount of secondary antibody needed to give a fluorescence signal while minimizing the background. Because each well had a fluor, with a noticeable difference between the negative and the Limbin positive, it was concluded that the largest dilution, 1:10,000 was acceptable and was to be used in the immunocytochemistry methodology.

Immunocytochemistry of Patient Samples with EVC2 (limbin) Antibody

ML1 were the normal samples used as a comparison to the patient samples. When the EVC2 antibody (limbin) was used as the primary, and the ML1s were observed under a PixCell II Microscope, a signal was prominent (Figure 15). Most notable was the presence of bright dots located in the nucleus. This is indicates that the normal and healthy EVC2 protein resides in the nucleus.
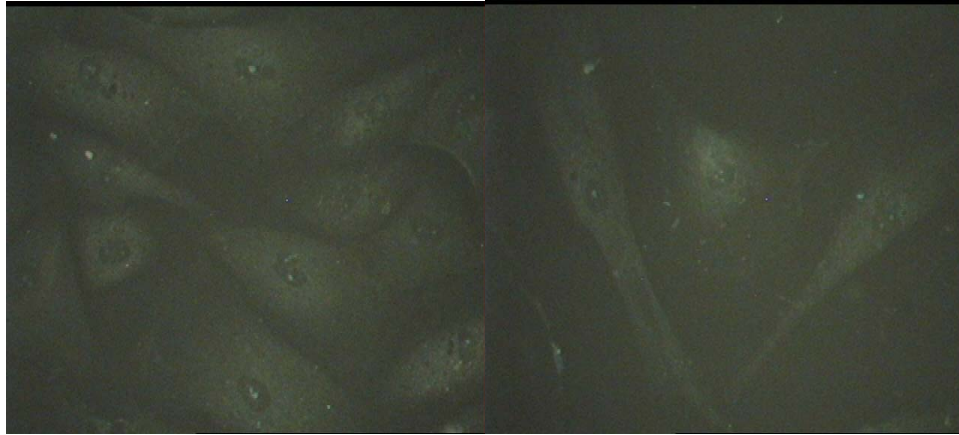
Figure 15: ML1 Limbin Antibody Primary (1:1000)

There are many different forms and severity of the disease, and numerous mutations have been identified. Patient sample 887 has a distinct morphological difference than the normal ML1 fibroblast structure and the dots in the nucleus remain present (see Figure 16).
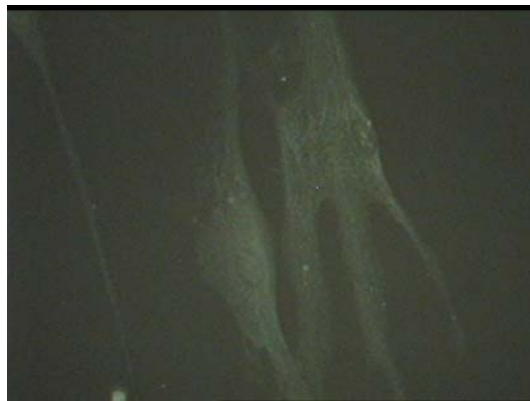

Figure 16: Immunomicroscopy of Patient 887 With Limbin Primary.

The patients B136, B132, B142, and B145, were all children when they died from the disease. According to Dr. Ginns, who maintained the cell lines, these cells were the most difficult to grow and were the last to adhere to the wells. They also have the most severe morphological changes from the healthy cells. The dots in the nucleus are absent

in B142 and it is clear that the cells lack the normal ML1 fibroblast appearance (see
Figure 17).

Figure 17: Immunomicroscopy of Patient B142 With Limbin Antibody.

Immunocytochemistry of Patient Samples with EVC Chicken Antibodies

ML1 were the normal samples used as a comparison to the patient samples. When
the EVC antibody 3693 was used as the primary, and the cells observed under a PixCell
II Microscope, a signal was prominent (Figure 18). Most notable was the presence of a
halo around the nucleus. This is indicates that the normal and healthy EVC protein
resides in a peri-nuclear location. This may also indicate an association between the
EVC2 protein (located within the nucleus) and the EVC protein (located peri-nuclear).

Figure 18: Immunomicroscopy of ML1 With 3693 Primary

This peri-nuclear localization was also seen when the EVC antibodies 3694 and 3695 were used as the primary, and the cells observed under a PixCell II Microscope (Figures 19 and 20).

Figure 19: Immunomicroscopy of ML1 With 3694 Antibody.

Figure 20: Immunomicroscopy of ML1 With 3695 Antibody.

Immunocytochemistry of Patient Samples with Rabbit Antibodies

ML1 human fibroblasts were the normal samples used as a comparison to the patient samples. Rabbit antibodies that had been previously prepared by Dr. Galdzicka were tested against EVC patient cell lines. When the 809 antibody was used as the primary, and the cells observed under a PixCell II™ Microscope, a prominent signal was detected (Figure 21). The cells tested for the EVC protein with antibody 809 were clearer than the other rabbit antibodies, the least grainy, and showed a striking presence of a halo around the nucleus.
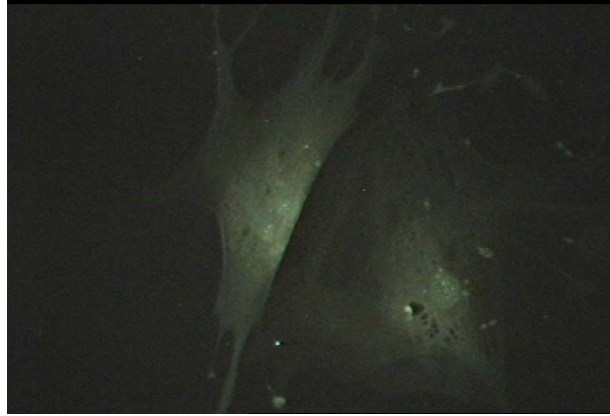
Figure 21:Immunomicroscopy of ML1 With 809 Primary Antibody

808K had the second best signal. It demonstrated a peri-nuclear localization, but there was not significant difference between this signal and background signal. Antibody 802 had the worst affinity, and no picture was readily found to exhibit this.

Figure 22: Immunomicroscopy of ML1 With 808K Primary.

DISCUSSION

Dot tests, fixation experiments and other assays assisted in the determination of optimum conditions. The nitrocellulose versus PVDF dot test confirmed that PVDF membranes, blocked in BlokHen II, and washed in PBS-tween was the optimum immunoblot procedure (see Figure 14). A 15-minute soak in methanol followed by 15-minute soak in 4 mM Sodium Deoxycholate was determined to be the best possible fixation procedure based on the results of the fixation optimization (see Table 6). Throughout the various experiments performed, the commercially available Limbin antibody exhibited positive affinity for EVC2 proteins. This affinity varied between normals and some patients, such as normal 1012 having an additional 35 kDa band which was clearly absent in patient 891 (See Figure 4). This discrepancy indicates the 35 kDa band may play an important part in the expression of the disease in patient 891 and potentially other patients. In culture, the limbin antibody produced a punctuate pattern in the nucleus of normal and some patients (see Figures 15 and 16).

A total of eighteen different patient, and five normal samples were tested between the immunoblot and immunocytochemistry experiments. Samples 887, B145, B162, RD, and TO313 were used for immunocytochemistry experiments only. The remaining samples were used in immunoblots. Patient samples 889, B136 and X193 were used in both immunoblot and immunocytochemistry experiments (see Table 5). Sample 889 demonstrated double banding around 80 kDa, and banding around 35 kDa in immunoblots incubated with limbin. B136, and X193 did not stand out against other samples in immunoblots. The X193 cell line did not grow well in culture and immunocytochemistry results were not conclusive. Both samples 889 and B136

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demonstrated fluor in the nucleus under EVC2 staining.

Patient samples B145 and B136, which were negative for EVC2 protein, were the only patients from the study that possessed frameshift mutations in the EVC gene. A third patient line, X193, contained the mutation but did not grow adequately in culture. This frameshift potentially results in downstream errors in EVC2 translation. During the course of the experiment it was noted that B145 samples had a minority of cells in each well demonstrating a positive fluor for the EVC2 protein. B145 contains an additional mutation, a missense in EVC, which is unlikely to affect the EVC2 gene. This produces a potential explanation for the lack of fluor uniformity of B145 cells, despite the large portion of cells, which tested negative.

These results also demonstrate a relationship between EVC and EVC2 genes. This supports previous research, performed by Galdzicka et al, which suggested that the EVC and EVC2 genes are arranged in a "head to head" configuration and may influence one another during transcription. The failure of EVC to properly function may inhibit the transcription or packaging of EVC2 as evidenced by the lack of fluor in samples B145 and B136, which contained the frameshift mutations.

EVC banding was expected at 112 kDa, while EVC2 banding was expected at 140 kDa. At this point in time there is uncertainty whether any of the bands observed in our immunoblots had the expected sizes for either of the glycosylated proteins. Double bands were consistently present around 80 kDa in both patient and normal samples when tested with the EVC2 antibody. This was unexpected.

Regarding the immunofluorescence experiments, Kamei et al (1995) have identified dot-like structures in the nucleus as well, these structures were known to contain mostly

coilin, an 80 kDa protein. While the research performed by Kamei et al was not done to gain information on Ellis-van Creveld Syndrome, or used any of the same antibodies, the similarities to its presence and structure in the nucleus is why the information could point to a direction in the function of the "dots" in EVC and should be pursued. These coiled bodies are thought to be involved in RNA processing (Kamei, 1995). Further research into these findings may reveal a co-localization of colin and EVC2 protein with these nuclear structures.

The optimization of cell line X193 (see Table 5) would allow tests to be conducted in an effort to identify any similarity to B145 and B136. If similarities exist then it can be suggested that an EVC mutation causes a later mistake in EVC2 translation or transcription, thus demonstrating a relationship between EVC and EVC2. Similarly, An organ blot should be performed to determine whether EVC proteins are tissue-specific. The analysis could be extended to include tissues at various stages of development to help determine EVC's role those processes.

Rabbit antibodies against EVC peptides were induced in an attempt to avoid the high background associated with chicken antibodies. Preliminary investigations with rabbit antibodies showed a promising beginning in cell culture experiments for affinity to EVC. These results have been consistent with those obtained with the anti-EVC chicken antibodies. Both demonstrate EVC protein localized in a peri-nuclear fashion. Further research should be conducted into these antibodies. However, confirmation should be made through Western blots or other assays to determine if these are in fact identifying EVC.

The results of these findings and future recommendations could help in the identification of the functions of these otherwise unknown proteins and potentially uncover a treatment or cure for this debilitating and rather obscure disease.

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