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Survey of the Class I VNTR Allele 814 and Insulin Dependent Diabetes Mellitus in Alaskan Native Tribes

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Survey of the Class I VNTR Allele 814 and Insulin Dependent Diabetes Mellitus in Alaskan Native Tribes.

Submitted in partial requirement for graduation with honors from the Department of Natural Sciences at Carroll College. Helena, MT

Jason Tyler Davis
April 9, 2001
This thesis for recognition by the Department of Natural Sciences has been approved by:

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April 9, 2001
# Table of Contents

Acknowledgements...........................................................................................................iii
List of Tables......................................................................................................................ii
List of Illustrations..............................................................................................................ii
Abstract..............................................................................................................................iv
Introduction.........................................................................................................................1
Materials and Methods........................................................................................................4
Results.................................................................................................................................8
Discussion............................................................................................................................11
References...........................................................................................................................14
List of Tables
Table 1....................................................................................................................9
Table 2....................................................................................................................9
Table 3..................................................................................................................10
Table 4..................................................................................................................12

List of Illustrations
Figure 1.................................................................................................................11
Acknowledgements

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Abstract

A VNTR (variable number tandem repeat) region upstream of the human insulin gene may affect susceptibility to type I diabetes mellitus (also called insulin dependent diabetes mellitus or IDDM) in humans (Lucassen et al., 1993 Bennett et al., 1995). I used the polymerase chain reaction and digestion by the restriction enzyme Hph I to screen for the 814 allele of the class I VNTR that is associated with susceptibility to insulin dependent diabetes mellitus in nine Siberian Yupiks and one Tlingit. The Qiagen and Gentra methods were used to isolate the DNA. Because northern native tribes (Eskimo/Aleut and NaDene) are more closely related to each other than either is to Amerindians (Shields et al., 1993), I hypothesized that northern groups may differ from others in their susceptibility to IDDM. While none of the samples analyzed possessed allele 814, my data are limited due to a small sample size and difficulty in determining the correct buffer concentration in the PCR amplification. More data are needed to properly determine the frequency and distribution of the 814 allele in these groups.
Introduction

Diabetes is a disease that affects millions of people. Type 1 insulin dependent diabetes mellitus is a form of the disease in which the immune system targets the insulin producing cells in the pancreas. When these cells are destroyed the body can no longer produce insulin in order to regulate the level of sugar in the blood. This forces the afflicted individual to receive insulin injections and to monitor their diet closely. Even with these treatments, however, the disease has serious effects such as blindness, organ failure, and death.

Diabetes is not a disease caused by a single factor. Instead diet, lack of exercise, and genetic makeup often work in concert to lead to the onset of diabetes (Weiss and Lonnquist, 2000). While diet and lifestyle often contribute to the disease’s onset, genetics can influence how susceptible a person is to the disease. It has recently been discovered that the length of a variable number of tandem repeat (VNTR) near the insulin gene (INS) greatly affects susceptibility to the disease. There are three classes of this VNTR based on size with I being the shortest and III the longest. The class I allele leads to increased susceptibility (Lucassen et al., 1993; Bennett et al., 1995).

The mechanism by which this locus affects susceptibility to diabetes is best seen by examining how class III alleles function. Pugliese et al. (1997) showed that the class III allele led to decreased production of mRNA for insulin in the pancreas. This phenomenon was not enough, however, to cause protection from diabetes (Bennett et al., 1996). Pugliese et al. (1997) then studied the effects of the two alleles on proinsulin production within the thymus, the organ in which the killer cells of the immune system develop. In childhood proinsulin is made in the thymus in order to prevent an
autoimmune response by T-cells. It was found that the class III allele led to increased production of the proinsulin protein within the thymus. This increase in proinsulin within the thymus leads to increased tolerance toward insulin by allowing the body, through negative selection, to eliminate the cells capable of an immune response to insulin. Because these T-cells are destroyed, they cannot attack the pancreas and the individual gains protection against diabetes. Individuals with class I alleles do not produce large amounts of proinsulin within the thymus, and therefore do not receive the protection from their T-cells described above. This greatly increases their risk of diabetes (Pugliese et al., 1997).

Few studies have been done concerning type I diabetes in Alaskan natives due to the disease’s low prevalence in these groups. Type I affects two percent of the native population compared to seven percent afflicted with type II diabetes (NIDDK, 1999). Also, most cases of type I diabetes in native tribes are seen in individuals who have both American Indian and Caucasian heritage. These numbers, however, are from the combined populations of Amerindians, NaDene, and Eskimo-Aleut. Since Shields et al. (1993) showed that NaDene and Eskimo-Aleut are genetically different from Amerindian groups, it is important to gain some understanding of susceptibility to IDDM among these groups. Also, Bennett et al. (1996) has questioned how the inheritance of the gene affects its activity, stating that the level of susceptibility may be dependent from which parent the allele is inherited. Due to these factors it is important to study the prevalence of allele 814 in these tribes.

Based on the majority of data, there should be a correlation between the appearance of the class I VNTR and the onset of insulin dependent diabetes mellitus.
However, such a correlation is not certain. Therefore, I propose that the genomes of some of the members of these tribes be examined using PCR and the restriction enzyme Hph I to determine if they contain allele 814, the most common of the class I allele. I hypothesize that there will be a correlation between the presence of allele 814 and insulin independent diabetes mellitus.

This research is important because it allows the medical community to know how prevalent diabetes may become in northern native populations. With this information possible treatment and prevention plans could be developed for high-risk individuals. It could also allow the medical community to prepare for possible cases of type I diabetes. This is especially important as more fatty foods are being introduced into the diets of native tribes in the north. Finally, the discovery of the linkage of this gene with diabetes is still fairly new and more data would be useful in finding the exact mechanism for the disease. This could eventually lead to better medical treatment of type I diabetes.
Materials and Methods

Dr. Gerald Shields provided blood samples of Siberian Yupik (Eskimo/Aleut) and Tlingit (NaDene). I used the Qiagen method (1999) and Gentra protocols (1997) to isolate DNA. The Qiagen method consisted of the following steps. First, I pipetted 20μl of Qiagen protease into a 1.5ml microcentrifuge tube. Next, 200μl of blood was added followed by 200μl of Qiagen buffer AL. The solution was pulse-vortexed for 15 seconds and then placed in a 56°C water bath for 10 minutes. Next, the tube was briefly centrifuged to remove fluid from the lid of the tube. I then added 200μl of 100% ethanol to the sample, pulse-vortexed for 15 seconds, and briefly centrifuged to remove drops from the lid. The mixture was transferred to a Qiamp spin column in a 2ml collection tube and centrifuged at 6000xg for one minute. Then the Qiamp column was transferred to a new collection tube and the old tube along with the filtrate was discarded. Next, 500μl of Qiagen buffer AW1 was added to the column. The column was then centrifuged once again at 6000xg for one minute. Following centrifugation, the column was once again placed in a new collection tube and the previous tube discarded. I next added 500μl of Qiagen buffer AW2 and centrifuged the column at 20,000xg for three minutes. The collection tube was then discarded and the column was placed in a new microcentrifuge tube and 200μl of Qiagen buffer AE or distilled water was added to the column. Next, the column was centrifuged at 6000xg for one minute. The filtrate was then stored at 4°C for immediate use or -20°C for long-term storage.

I used the Gentra Puregene Isolation Kit and the following protocol. 100μl of whole blood was placed in a 1.5ml microcentrifuge tube containing 300μl of Gentra RBC Lysis Solution. The mixture was incubated for one minute at room temperature and
inverted gently three times during the incubation. The mixture was centrifuged for one minute at 13000-16000xg. The supernatant was removed except for 5-10µl. The tube was vortexed for 10 seconds to resuspend the white cell pellet in the remaining fluid. Next, 100µl of cell lysis solution was added to the suspended cells. The mixture was then pipetted up and down to facilitate cell lysis.

I then added 33µl of protein precipitation solution to the lysed cells. The solution was then vortexed for 20 seconds to mix the contents of the microcentrifuge tube, followed by centrifugation of the tube at 13000-16000xg for one minute. The supernatant was placed in a clean microcentrifuge tube containing 100µl of isopropanol. The tubes were inverted gently 50 times and centrifuged at 13000-16000xg for one minute. The supernatant was discarded and the tube drained briefly on absorbent paper. Next, I added 100µl of 70% ethanol and centrifuged the tube at 13000-16000xg for one minute. The ethanol was then poured off and the tube drained on absorbent paper for five seconds.

The DNA pellet was rehydrated with 33µl of Gentra DNA hydration solution. The mixture was vortexed at a medium speed for five seconds and incubated at 65°C for five minutes. Following incubation the tube was again vortexed at medium speed for five seconds. The DNA solution was then stored at -20°C until it was used.

The presence of DNA in the isolate was verified by running the isolate through a 0.8% agarose gel. Once the DNA was isolated the protocol followed that of Rutledge (pers. comm.) as follows. The concentration of DNA was first determined by spectrophotometry. A dilution of the DNA was done at 1:25 by adding 40µl of DNA solution to 960µl of TE buffer. The solution was then analyzed using a UV
spectrophotometer at wavelengths of 260 and 280nm. The absorbance was then used in the equation: $[\text{DNA}] (\mu\text{g/\mu l}) = (A_{260})(50\mu\text{g/ml})(25)(1\text{ml/1000\mu l})$.

The PCR reaction was then used to amplify the amount of DNA to a level that could be effectively analyzed. The PCR cocktail and amplification cycle are seen in Tables 1 and 2.

**Table 1. Reagents used in PCR Cocktail.**

<table>
<thead>
<tr>
<th>Component</th>
<th>X1</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR Buffer</td>
<td>2\mu l</td>
</tr>
<tr>
<td>dNTPs 2.5mM each</td>
<td>2\mu l</td>
</tr>
<tr>
<td>Primers 5uM each</td>
<td>2\mu l</td>
</tr>
<tr>
<td>Taq</td>
<td>0.2\mu l</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>12.4\mu l</td>
</tr>
<tr>
<td>DNA sample</td>
<td>1\mu l</td>
</tr>
<tr>
<td>Total</td>
<td>20\mu l</td>
</tr>
</tbody>
</table>

**Table 2. PCR Amplification Cycle.**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>6 min</td>
</tr>
<tr>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>66°C</td>
<td>2 min</td>
</tr>
<tr>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Steps 2-4 were repeated fifty times. The primers for the PCR reaction were INS15 5’-CATCAAGCAGGTCTGTTCCA-3’ and INS16 5’-AAAAAGTGACCTGACCCC-3’.

Next, the restriction enzyme Hph I, which is specific for a GGTGA section of allele 814, was used to check for the presence of the allele. The digestion cocktail is as given in Table 3.
Table 3. Digestion Cocktail Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>X1</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NEB buffer 4</td>
<td>2µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>7µl</td>
</tr>
<tr>
<td>Hph I</td>
<td>1µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>10µl</td>
</tr>
<tr>
<td>Total</td>
<td>20µl</td>
</tr>
</tbody>
</table>

Amplified DNA was allowed to be digested with Hph I overnight at 37°C. Finally, the digested DNA solution was run through 2% agarose gels to visualize digestion products. Presence of the 814 allele would correspond to a band at about 200-175 base pairs. Absence of the allele would correspond to a band at 250 base pairs.
Results

After isolating and restricting the DNA samples, nine Siberian Yupik samples and one Tlingit sample underwent successful isolation, amplification, and restriction. Figure 1 shows the results for four Siberian Yupiks. The main band is present at about 250 base pairs, along with a doublet at about 100-150 base pairs. This is the position expected for samples that do not contain the allele (Rutledge, pers. comm.). None of the samples in the figure possess allele 814. If allele 814 were present, there would be a smaller band at 200-175bp rather than the band at 250 bp seen here.

Figure 1. Gel of digested Siberian Yupik DNA samples.

Attaining these results required the changing of several of the methods used at the beginning of this project. Throughout much of this project, the results determined the materials and methods used. Preliminary DNA isolation attempts using RSB and SLS proved ineffective. 260/280 ratios were taken using a UV-Vis spectrophotometer to quantify the DNA. These ratios were between 1.0 and 1.1. None of these samples when used in PCR gave any amplified product. In an attempt to obtain better results the Qiagen
procedure was used. This procedure produced isolates with average 280/260 ratios of 1.40. The isolate was then run through a 2% gel and the presence of DNA was confirmed by visualizing a band of DNA in the gel. A preliminary PCR reaction with 1mM MgCl₂ gave no amplified product. Further attempts at amplification with a variety of MgCl₂ concentrations gave some DNA product from PCR but the formation of product was not consistent and the product never formed a distinct band that could be used in the restriction reaction.

The study was continued at the University of Washington in order to determine problems with my protocol. A PCR of five samples with the university's reagents gave a product of just over 500 base pairs in all but one lane, while a PCR of the same samples with our reagents gave only one faint band. To determine the problem with my PCR protocol, DNA isolates were amplified using a mixture of the University of Washington's and my own reagents. In each reaction one of my reagents from Table 1 was used with the rest being supplied by the university. I also did a reaction with only my reagents as well as one with only the university's reagents. A single sample of Alaskan native DNA was used for all the reactions. The results from this assay are seen in Table 4.

Table 4. Results of PCR assay.

<table>
<thead>
<tr>
<th>Reagent of mine used</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR Buffer and Taq</td>
<td>Smear</td>
</tr>
<tr>
<td>dNTPs 2.5mM each</td>
<td>Band</td>
</tr>
<tr>
<td>Primers 5uM each</td>
<td>Smear</td>
</tr>
<tr>
<td>All my reagents used</td>
<td>Band</td>
</tr>
<tr>
<td>All UW reagents used</td>
<td>Band</td>
</tr>
</tbody>
</table>

The smears were unusable in any restriction enzyme reaction while the bands signified a successful PCR attempt.
Further PCR attempts at the University of Washington were successful. Preliminary calculations using the 260/280 ratio of the DNA isolate indicated no DNA was present; however, tests run of isolate through 0.8% agarose gel showed the presence of DNA. Twenty-four isolates consisting of different Alaskan native tribes were then used in PCR with eleven samples showing a strong amplification and four samples showing a weak one. The samples were then restricted and scored on an agarose gel. The nine Siberian Yupik and one Tlingit sample all gave identical results, with no one possessing the 814 allele.
Discussion

The frequency of class I and class III alleles in the native tribes studied seems similar to other groups that have been studied, such as populations in Finland and the United Kingdom (Bennett et al. 1996, Rutledge pers comm). While the small sample size does make it difficult to make population conclusions, preliminary data fit with the original hypothesis that the allele is not present in Alaskan native tribes.

While the sample size is small, the data obtained can be compared to previous results obtained by earlier workers. While Julier et al (1991) obtained results showing a nearly even prevalence of class I and class III genes in a general non-diabetic population and a ratio of 3:1 of class I to class III alleles in a diabetic population, this is not widely seen in other literature. Studies at the University of Washington however, indicate that the class III allele is the most prevalent allele in the general population (Rutledge, pers comm). This high number of class III alleles is somewhat surprising though, as recent data show that the class I allele is preferentially passed on to offspring from heterozygous parents (Bennett et al., 1996). However, the rate of its preferential transference does vary among different ethnic groups that are physically separated in some way (oceans, mountains etc). I cannot say whether or not that is the case in these tribes since there were no comparisons involving parents and their offspring.

The identical results for members of the two tribes is not surprising since Siberian Yupiks and Tlingits are closely related populations (Shields et al., 1993). Yet, the data in this study are insufficient to make a conclusion about similarities regarding this locus. This is because differences in allele frequencies have only been seen in populations separated by severe physical barriers such as water or mountain ranges, while the United
States and Canada have similar frequencies for the 814 allele (Bennett et al., 1995). Therefore, native Alaskan tribes may not be very different from one another in regard to this allele.

The original lack of success in DNA isolation procedures may have been due to the age of the blood samples. The samples had been frozen for several years and many of the samples showed severe clotting. Because of this stress many of the white blood cells present within the sample may have already been lysed and some of the DNA may have been degraded. This could explain why the Qiagen kit was the most successful protocol used. Since the kit contains a membrane that binds DNA until an elution buffer is added, the membrane would trap any DNA from cells that had been lysed due to freezing. This DNA would have been lost in other forms of DNA isolation. This may not be true, however, as DNA was unable to be amplified from fresh blood using the Qiagen technique.

The reasons for the lack of success for PCR at the Carroll College lab have not yet been fully determined. The PCR 10x buffer seems to be the most likely reason, based on preliminary results from the University of Washington. Smears obtained by running PCR product through 2% agarose gels at the University of Washington resemble smears seen in the Carroll College lab. Also, the micropipettes used to mix the buffer at Carroll with MgCl₂ can be error prone when used to measure the small volumes needed to create the concentrations used. This problem was eliminated in the Seattle lab as premixed buffer was used there.
The 260/280 ratio that indicated absence of DNA in samples isolated at the University of Washington was based on the type of equation used to determine the concentration from the ratio. The equation:

\[
\text{Absorption Ratio} = \frac{\text{Abs 260nm-Abs 320nm}}{\text{Abs 280nm-Abs 320nm}}
\]

can give misleading values at small concentrations due to the correction absorbance at 320 nm. This is the most likely reason that DNA seemed absent based on the 260/280 ratio but was found to be present when run through an agarose gel.

Overall the data obtained support the original hypothesis. More samples must be studied however, to determine the frequency and distribution of the 814 allele in Alaskan natives.
References


