EXAMINING THE DNA OBTAINED FROM SALIVA SAMPLES IN RELATION TO THE PHARMACOGENETICS OF KIDNEY TRANSPLANTATION

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ABSTRACT

The recent application of pharmacogenetics to the kidney transplantation process has provided a new method for creating drug dosage regimes that are specific to each patient. These regimes are based on a patient's genotype, which is usually obtained from a blood sample. The purpose of this study was to determine whether saliva samples could be used as a less invasive alternative to blood samples for examining genes of interest in transplant patients. To test this hypothesis, the amount and purity of DNA was measured from four saliva samples. Following this, the purified DNA was run with the human primer for the SNP RS7767396 to determine if it had potential for use in further testing. The results from this work suggest that saliva samples produce an amount of DNA useful for further pharmacogenetic testing, though the resulting product contains more impurities than the DNA obtained from blood. These results imply that saliva samples could possibly be used as an alternative to blood samples in the future, making it more likely that patients would agree to participate in trials.

INTRODUCTION

Kidney transplantation is one of the most common transplant procedures that occur each year in the United States. It is one of the few solutions to end-stage renal disease, which is mainly caused by diabetes and high blood pressure (NIH 2006). The transplantation procedure involves many drugs; some of the most important of these are involved in immunosuppressant regimes to avoid organ rejection. These drugs offer a possibility for the introduction of personalized medicine, or treatment based on a patient's genetic code, to the transplantation process.

The need for immunosuppressive drugs in kidney transplantation is based in the body's recognition of foreign cells as a threat to the immune system. This is largely due to human leukocyte antigens (HLAs) within the immune system that are specific to each person and help to eliminate pathogens that enter the body. Transplantation across HLA barriers without immunosuppressive regimes can also lead to rejection of a foreign organ. Suppression of the immune system is therefore important for overall graft survival in kidney transplantation.

The drugs involved in this suppression of the immune system offer a possible application of pharmacogenetics to the kidney transplantation process. Through knowledge of a patient's genetic code, doctors are able to create a specific dosage regimen for each person. Two drugs that are of special interest in this area are Tacrolimus and Cyclosporine A, which are the most commonly prescribed immunosuppressant drugs currently available. Both are calcineurin inhibitors: they inhibit the protein phosphatase that activates T cells, lymphocytes that play a role in cell-mediated immunity, in the immune system (Coto 2011). Tacrolimus, or Tac, suppresses the immune system by preventing the transcription of several cytokine genes involved in immune responses, thereby repressing the activation, proliferation and differentiation of T cells (Zhao 2009). Cyclosporine A, or CsA, is structurally distinct from Tac and binds to a different cytoplasmic receptor, cyclophilin A (Webster 2009). While Tac is associated with a greater risk of developing post-transplant diabetes, it is also associated with a decrease in graft loss when compared to CsA. Tac is also related to a more favorable cardiovascular risk profile, a reduced lipid profile and lower arterial blood pressure, and is therefore the more commonly prescribed immunosuppressant drug between the two (Webster 2009).

Studies have shown that Tacrolimus is associated with a number of genes involved in drug metabolism. In terms of pharmacogenetics, however, it appears that currently the most

important of these is the CYP3A5 enzyme. This is the major enzyme involved in the metabolism of Tac and therefore has a large impact on the bioavailability of the drug (Coto 2011). The wild-type allele for this gene (CYP3A5*1) leads to a functioning protein product, meaning that Tac will be quickly metabolized. However, there is a single nucleotide polymorphism (SNP) that has been found to greatly impact the overall metabolism of Tac. This SNP is CYP3A5*3, which affects splicing of the pre-mRNA allele, leading to poor metabolizing phenotype and absence of protein activity (Coto 2011). Both the CYP3A4 and the ABCB1 genes were also involved in the metabolism of Tac, but neither showed a large amount of pharmacogenetic variability and therefore is not as likely of a candidate for future research. Further studies are necessary to better implement pharmacogenetics in kidney transplantation (Elens 2012).

The purpose of this research was to find a possible method to increase the number of participants in such a study. In order to find genes of interest and therefore specify dosage for immunosuppressant drugs, DNA must first be obtained from a patient. This is usually done through a blood sample, which requires a more invasive procedure and a trained professional for analysis. Both of these elements decrease the likelihood that a patient would want to participate in a trial, as well as complicate the process of applying personalized medicine to his treatment plan. Saliva samples are a possible alternative to blood samples, if sufficient DNA can be obtained for testing. The overall goal of this research was to begin looking into the possibility of less invasive methods to obtain DNA from kidney transplant recipients for the eventual application of pharmacogenetics in their immunosuppression regimens. In this study, it was hypothesized that saliva samples could produce an adequate amount and quality of DNA to be used in pharmacogenetic trials.

METHODS

Prior to the determination of quantity and quality of DNA obtained from saliva samples, the DNA had to be obtained and purified. Samples of saliva (2 mL) were taken from four subjects using a DNA Genotek Kit. They were mixed with stabilizing liquid and shaken, then stored at room temperature for one day. The samples were then incubated in an air incubator at 50° C for 2.5 hours to ensure that the DNA was released and that the nucleases were permanently inactivated. The DNA was then purified using two methods, a Genotek and a QIAgen kit, to determine the efficacy of both.

Genotek Purification

A portion of each sample (500 µL) was transferred to a microcentrifuge tube. PT-L2P (20 µL), a reagent used for extraction of DNA, was added and the samples were vortexed. The samples were incubated on ice, and then centrifuged at 12,800 rpm. The supernatant was collected from the samples and transferred to a new microcentrifuge tube. To this, 95% ethanol (600 µL) was added at room temperature, and then the solutions were mixed gently by inversion and allowed to stand until the DNA fully precipitated. They were then centrifuged at room temperature.

To the DNA pellet, 70% ethanol (250 µL) was added and allowed to stand. The ethanol was then removed without disturbing the pellet; complete removal was necessary so as not to disrupt the downstream assay. TE solution (100 µL) was added to the samples to dissolve the pellet, and each sample was vortexed.

QIAgen Purification

Saliva from each sample (50 µL) was mixed with Buffer ATL and proteinase K, then pulse-vortexed and incubated at 56° C. The samples were briefly centrifuged before ethanol (50

µL) was added, and then the solution was vortexed and incubated at room temperature. The lysate was then transferred to a QIAamp MinElute column and centrifuged again. Buffer AW1 was added (500 µL) and the solution was centrifuged again. The same process was repeated with Buffer AW2. Ethanol (700 µL) was then added and the solution was centrifuged, and then centrifuged again at full speed to dry the membrane of the column completely. The column was then incubated at room temperature before distilled water was applied to the center of the membrane, following which it was again incubated at room temperature and centrifuged at full speed.

Gel Electrophoresis

To quantify the amount of DNA, samples were then run using agarose gel electrophoresis: 250 mL of 0.8% TAE gel was used. A portion of each previously prepared DNA sample (1.2 µL) was mixed with bromophenol blue dye (4 µL) and loading buffer (5 µL) and injected into the wells along the negative end. A 1 kb ladder was injected into the wells on either end of the gel for sizing and quantification purposes. Once injected, the DNA was allowed to run for 45 minutes. The DNA was visualized through staining of the gel with ethidium bromide. After it was stained, the DNA was viewed under an ultraviolet transilluminator.

Nanodrop Analysis

The concentrations of DNA were quantified using a Nanodrop spectrophotometer. The spectrophotometer was zeroed with water, and then a small amount of sample (2 µL) was placed on the pedestal. Data was obtained for each sample, and the pedestal was wiped clean between each trial.

Picogreen Analysis

A Picogreen assay was also preformed to determine more exact values for the concentrations of DNA. This was done using a Picogreen spectrophotometer, with a small amount of sample (2 µL) mixed with a fluorescent nucleic acid stain. The values were compared with standard values for DNA concentrations to determine concentration.

Polymerase Chain Reaction (PCR) and Primer

The DNA from both methods of purification was run with a human primer for the SNP RS7767396. The PCR was set up by adding to each sample HotMaster Buffer (1 µL), 2 mM dNTPs (1 µL), 10 µM forward primer (0.25 µL), 10 µM reverse primer (0.25 µL), HotMaster Taq DNA polymerase (0.25 µL) and water (6.75 µL). The thermal cycling conditions for the reaction were a start of 94° C (2 minutes), then 35 cycles of 94°C (20 seconds), 62° C(10 seconds), and 65° C (30 seconds). The resulting product was run in a 1.5% TAE gel for electrophoresis.

RESULTS

Electrophoresis

Gel electrophoresis was used as a primary method to determine if the purified samples contained DNA, as well as to determine if there was variation between the amounts obtained from the different purification methods. The results from the gel electrophoresis (Figure 1) showed that DNA was obtained from each of the saliva samples, though the amount obtained from each sample varied. Comparison with the 1 kb ladder showed that there were many different sizes of DNA fragments, and the different bands visualized in the UV light showed that

the samples purified with Genotek contained a larger amount of DNA than those purified through QIAgen.

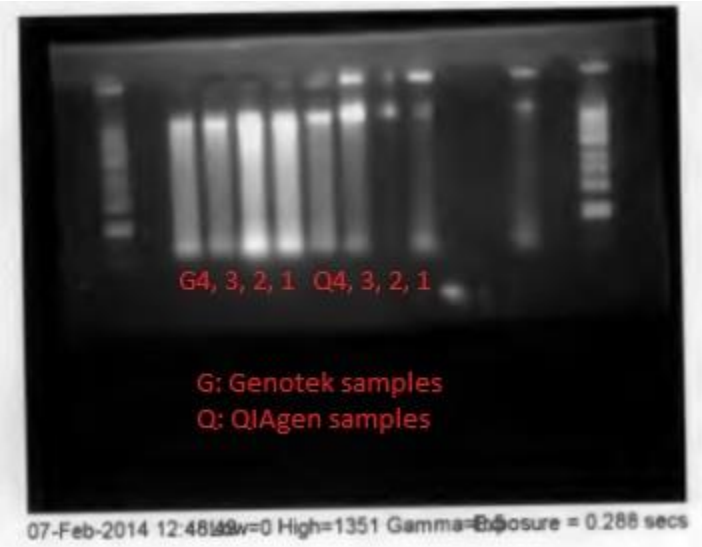

Figure 1. Image generated from the agarose gel electrophoresis, done in 0.8% TAE gel. The exposure was 0.288 seconds with gamma = 0 x 5, a low of 0 and a high of 1351. G denotes Genotek samples, and Q denotes QIAgen samples. The Genotek samples show a greater amount of DNA than those purified by QIAgen., with the most DNA in G3 and G4.

Nanodrop Analysis

A nanodrop spectrophotometer was used to obtain both the concentration of DNA and the purity of each sample. The results from the Nanodrop showed variation in the amount of DNA obtained from each sample. It was found that Genotek samples had a higher average concentration of DNA than QIAgen, with values of 85.375 ng/µL and 43.375 ng/µL respectively (Tables 1 and 2). The A260/280 ratio from the Nanodrop for each sample is indicative of protein contamination, as aromatic proteins have a strong UV absorbance at 280 nm. The majority of QIAgen samples had values over 1.8, which indicates a high level of purity (Table 1). The majority of values obtained for Genotek-purified samples were lower than 1.8, indicating that the samples were protein-contaminated (Table 2). The A260/230 ratio was indicative of organic

impurities, and again the values were below 1.8 indicating a large amount of impurities (Tables 1 and 2).

	Amount of DNA (ng/µL)	A260/280 Ratio	A260/230 Ratio
Sample 1 (QIAgen)	49	1.92	0.31
Sample 2 (QIAgen)	72.1	2.01	0.46
Sample 3 (QIAgen)	23.4	2.03	0.17
Sample 4 (QIAgen)	29	1.19	0.2
Average	43.375	1.7875	0.285
Average Without Outlier		1.99	

Table 1. Values for concentration and purity of QIAgen purified samples, determined through Nanodrop analysis. Averages calculated for the A260/280 both with the outlier (Sample 4) values and without the outlier. As a value of 1.8 indicated high purity, QIAgen samples 1 – 3 are above this with an average of 1.99.

	Amount of DNA (ng/µL)	A260/280 Ratio	A260/230 Ratio
Sample 1 (Genotek)	72.5	1.71	0.75
Sample 2 (Genotek)	140	1.81	1.59
Sample 3 (Genotek)	85.2	1.73	0.98
Sample 4 (Genotek)	43.8	1.7	0.87
Average (Genotek)	85.375	1.7375	1.0475

Table 2. Values for concentration and purity of Genotek purified samples, determined through Nanodrop analysis. Purity is lower than that of QIAgen, with an average A260/280 of 1.7375 which is lower than the desired minimum of 1.8 and indicated high protein contamination. The average amount obtained, however, is higher than QIAgen, with an average of 85.375 ng/µL compared to 43.375 ng/µL (Table 1).

Picogreen Assay

Another method used to obtain the concentration of DNA in each purified sample was a Picogreen assay, which involves fluorescence enhancement of the DNA and comparison with standard concentrations. The calibration of the standards is shown in Figure 2. The data from the Picogreen assay also shows that Genotek-purified samples produced a larger amount of DNA

than QIAgen, with average amounts of 35.62 ng/µL and 10.37 ng/µL respectively. These values for DNA concentration are considered more accurate than those of the Nanodrop.

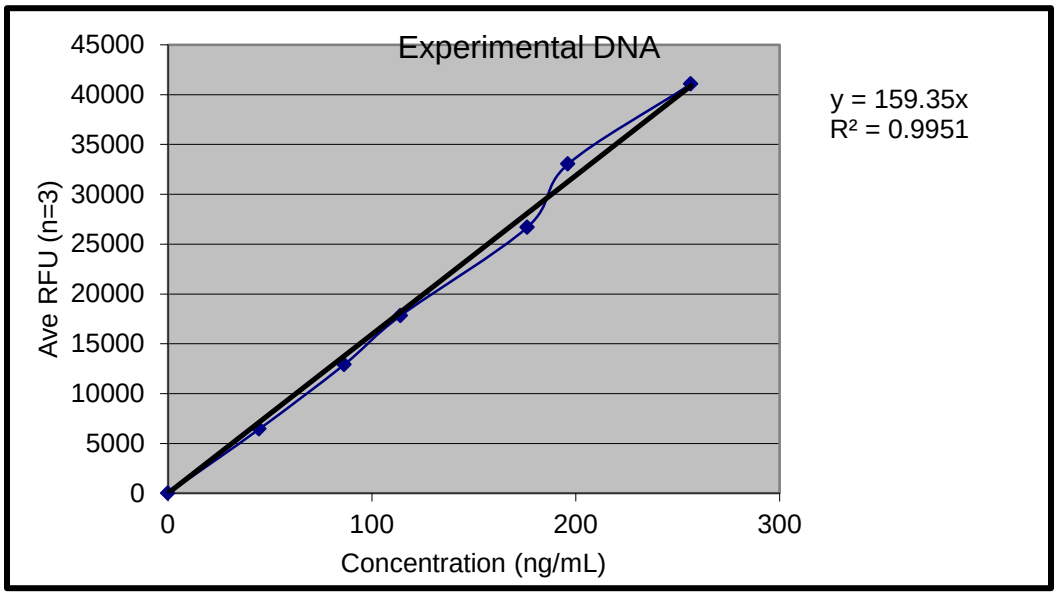

Figure 2. Analysis of the standard DNA concentrations used in the Picogreen assay, with an R^2 value of 0.99507. This indicates that the standards were very close to desired concentrations, which validates the concentrations of DNA from the samples.

	Concentration of Experimental DNA (ng/µL)
Sample 1 (QIAgen)	11.19
Sample 2 (QIAgen)	17.73
Sample 3 (QIAgen)	5.84
Sample 4 (QIAgen)	6.71
Average	10.37

Table 3. Concentrations of DNA determined from the Picogreen assay for the QIAgen purified samples. The average is lower than that determined by the Nanodrop for QIAgen samples (Table 1), as well as lower than the average determined by the Picogreen for Genotek samples (Table 4).

	Concentration of Experimental DNA (ng/µL)
Sample 1 (Genotek)	28.53
Sample 2 (Genotek)	94.79
Sample 3 (Genotek)	10.42
Sample 4 (Genotek)	8.74
Average (Genotek)	35.62

Table 4. Concentrations of DNA determined from the Picogreen assay for the Genotek purified samples. The average is lower than that determined by the Nanodrop for Genotek samples (Table 2), but still higher than the concentration determined by the Picogreen for QIAgen samples.

	Concentration of DNA (ng/µL) for Picogreen	Concentration of DNA (ng/µL) Nanodrop	A260/280 Ratio
Average (QIAgen)	10.37	43.38	1.99
Average (Genotek)	35.62	85.38	1.74

Table 5. Averages from both Picogreen and Nanodrop analyses of DNA concentrations. Average for QIAgen A260/280 ratio excludes outlier. Both values for the Genotek-purified concentrations are higher than those for the QIAgen purified samples. The A260/280 ratio for QIAgen is higher, indicating a higher level of purity.

Primers

Each of the purified samples was run with a primer for the SNP RS7767396. This was to examine the quality of the purified DNA and whether or not a specific gene could be pulled from it using PCR. Following this, gel electrophoresis was used to visualize the results (Figure 3). Each of the samples produced a large amount of the desired gene.

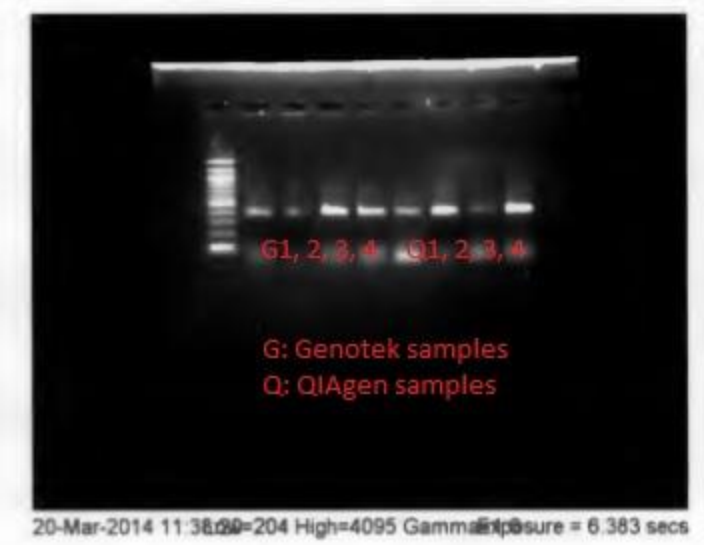

Figure 3. Image generated from the agarose gel electrophoresis, done in 1.5% TAE gel. The exposure was 6.383 seconds with a low of 204 and a high of 4095. The desired product was 350 base pairs long; the bright bands in the image are product, the lighter smears are primers. G denotes Genotek samples, and Q denotes QIAgen samples. While there is some variation in the amount from each sample, both methods were effective in purifying the DNA to an extent that it could be used to obtain a certain sequence of DNA, indicated viability in further pharmacogenetic testing.

DISCUSSION

These findings suggest that an adequate amount of DNA for further pharmacogenetic testing can be obtained and purified from saliva samples. While the Genotek kit produced a larger amount of DNA, the QIAgen kit produced an adequate amount with greater purity. It would be necessary to consider this high amount of impurities when choosing between uses of saliva or blood samples, as DNA taken from blood would likely be less contaminated.

The results from running the samples with primers (Figure 3) show that the DNA from saliva samples is viable for use with primers and PCR. Given this information, future experimentation will have to be done, such as running the DNA in a large-scale experiment with a set of 13 primers for genes commonly tested for in pharmacogenetics. Success of DNA from saliva in such circumstances would further support using saliva samples instead of blood samples.

These results are especially applicable to kidney transplantation, as participants in studies would be more likely to participate if given the option of a saliva sample instead of having blood drawn. Saliva samples also add another benefit to possible future studies in that they could be analyzed in the lab rather than being sent out to special facilities. This could greatly expedite the process of determining a patient's DNA and therefore lead to a faster application of a specific dosing regimen for that patient. It could also possibly lower the overall cost of studies conducted in this area, making it a favorable choice in comparison to blood samples.

Further studies are necessary to determine the extent to which saliva samples can be implemented in terms of pharmacogenetic usage. It will be necessary to test the purified DNA with a set of primers specifically designed for pharmacogenetic use, such as for the genes involved in the metabolism of Tacrolimus and Cyclosporin A. If the DNA can be successfully run on a large scale with primers for the CYP3A5 gene, it is likely that they could be an effective tool in the personalized medicine of kidney transplantations.

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