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AWARD NUMBER DAMD17-97-1-7286

TITLE: Detection of Mutations Using a Novel Endonuclease

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The detection of mutations in the genes of breast cancer patients and their families is an important part of the effort to counter breast cancer. This laboratory has discovered a family of nucleases that has the ability to cut DNA at the sites of mismatches, such as those that are formed when a DNA containing a mutation is paired with a copy of the normal DNA. The enzyme we use is CEL I from celery. The CEL I mismatch detection (CMD) assay has shown high efficacy. In the work supported by this grant, we have tested the detection of mutations in the *BRCA1* gene of many research participants. CMD detected mutations using either the PCR products amplified from DNA, or the RT-PCR products amplified from their mRNA. The results showed that CMD will be useful in the genetic screening against breast cancer. Through this work, we obtained insights on approaches that will further enhance the sensitivity of CMD. These methods will be tested in the following period of research support by this grant.

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FOREWORD

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(X) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 32 CFR 219 and 45 CFR 46.

(   ) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Insitutes of Health.

Anthony Leung
Principal Investigator's Signature

5/27/98
Date

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Anthony T. Yeung, Ph.D.
[redacted] communication

Detection of Mutations using a Novel Endonuclease

INTRODUCTION

The focus of this research is to develop an effective method to screen for mutations in genes important to the development of breast cancer. The method developed in this laboratory is called CEL I mismatch detection (CMD). CEL I, from celery, is representative of a family of mismatch-specific endonucleases found in plants. The neutral pH optimum of these nucleases allows mismatch incision without excessive DNA helix unwinding seen at acidic pH ranges when S1 nuclease is used. In CMD, PCR primers of two fluorescent colors are used to PCR to amplify an exon of a chosen gene of a research participant, or a piece of the mRNA by using RT-PCR. When the PCR product is denatured by heat and then renatured, heteroduplexes will be formed in a fraction of the DNA. CEL I cuts these heteroduplexes at the 3' side of the mismatch, in one DNA strand per duplex. Because two different mismatches can form from the PCR products, CEL I can cut the mismatch in either the top strand or the bottom strand at a mutation, and produces one truncated band for each color. The sizes of the bands are measured by GeneScan software, with the help of internal standards in each lane, in the ABI automated DNA sequencer. The sum of the products of the two colors should be one nucleotide longer than the full-length PCR product.

Our institution is favorably situated for testing CMD because the Dyson Foundation/Family Risk Assessment Program directed by Dr. Mary Daly of our institution. This Foundation has over 2000 research participants who has agreed to allow their DNA be used to further the causes of mutation detection. Many of these research participants are from families with an elevated risk for breast and ovarian cancers. Our co-investigator, Dr. Andrew Godwin, is in charge of this DNA collection and performs genetic analysis of the mutations. He provides samples of PCR products to us to conduct blind tests of CMD as well as other samples for methods development. We provide him with CEL I to compare CMD with other traditional methods in use in his laboratory. All results are confirmed by direct DNA sequencing of both DNA strands. We do not know the identities of the donors. In our initial test of this method, CMD had detected the mutations and polymorphisms of the *BRCA1* gene of 30 research participants with no false positive or false negative predications. An application for patent protection of this method has been allowed. In the current proposal, I intend to use the DNA samples of 120 research participants to test our ideas on how to improve and demonstrate the efficacy of CMD. In the first year of this grant, I have organized my laboratory and established collaborations for this effort, including training an additional person in my laboratory to assist the leading technician in this work. We will showed below that CMD can detect mutations in a mixture of normal alleles. We also tested that it can detect mutations in templates spanning multiple exons, made by RT-PCR of the mRNA. The latter study included 82 research participants. Some plans we have for improving the signal to noise ratio of CMD will be presented.

BODY

The CMD project required a two-technician team, Kate Oleykowski assisted by Colleen Bronson, prior to the start of the grant. Colleen returned to graduate school prior to the start of the grant, therefore we eventually hired David Besack to replace Colleen to

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assist Kate. Funds from another source other than this grant was used to hire David, about six months after this grant started. The first three months of Kate's effort was spent on improving the purification of the CEL I enzyme. The availability of a good preparation of CEL I enzyme is essential to the success of this project.

We found that CEL I aggregates with other similar proteins at the later stages of its purification, especially during storage at 4 °C. We improved the purification of CEL I by the addition of Triton X-100 to the buffers in the later steps of enzyme purification. The reverse of the aggregation allowed further ion-exchange chromatography, and more importantly, the use of size resolution on gel filtration columns from which we obtained a CEL I fraction containing only proteins of about 30,000 to 40,000 molecular weight. Several protein bands are still present in this fraction. However, CEL I is about 20 times more active in the presence of Triton X-100 than the preparations we previously used.

We also developed our patent application of the CEL I mutation detection method during this period of time to the stage of being "allowed" by the U.S. patent office. Having patent protection is essential to the widespread distribution of a new method because the patent allows the commercial sector to invest in research support and promotion of the method.

The following report describes the progress with respect to the tasks for "year one" described in my original proposal:

Task 1: Months 1-6 Screening of first 30 research participants using current mutation screening procedure while new methods are being developed.

Kate has used this task to train our new recruit David Besack, on PCR, gel electrophoresis, and CMD. Various exons containing known mutations and polymorphisms were used to practice CMD and to improve on the understanding of the GeneScan software that assists us in data interpretation. During this time, we learned that the CEL I purified in the presence of Triton X-100 is almost 20 times higher in mismatch endonuclease activity than the previous preparations that has formed aggregations during storage. We also felt that the original CMD procedure was too cumbersome because of the need to use Magic Prep PCR cleanup kit for each sample, and gel filtration to desalt each CMD product prior to gel loading. As shown in Task 2, CMD can work with only one fifth as much DNA sample as before, and without a cleanup step after PCR.

Task 2: Months 7-12 Screening second 30 research participants using the newer protocols developed in months 1-6.

In collaboration with the laboratory of Andrew Godwin, our co-investigator in our institution, we have practiced the mutation detection of specific exons of the *BRCA1* gene of 180 research participants, using CMD. PCR was performed from DNA templates. CMD was performed with no cleanup of the PCR products, and with only 1/5 of the amount of PCR products as we used in previous studies. The goal was to streamline the CMD operation. Most of the mutation identification was done with visual inspection of the CMD gel fluorescence image reconstructed by the Genescan software at the end of the automated DNA sequencing reaction. Not all exons were tested for each research participant, only exons where the mutations were most prevalent in this set of research participants were tested. The process took eight weeks.

Of the 57 mutations that are known to exist in these patients, CMD identified 54 of the mutations. The three mutations that were missed are mutations that CEL I is known to be able to identify. We are requesting more samples of those false negatives to further our understanding of why false negatives occurred. There were no false positives.

Task 5: Months 1-3 Protocols to cover the whole *BRCA1* gene as six fragments of about 1 kilobase will be developed. RT-PCR will be tested. Bridging PCR will be tested.

The work initiated in this task is the screening by RT-PCR of a region of 450 bp of the mRNA of 82 research participants, with RT-PCR coupled to CMD. The test was conducted blind. The operator, Kate, was asked to make all the mutation calls without consulting other scientists. The experimental conditions were very challenging because our collaborator could only provide us with RT-PCR products of a single color at this time, the same color for both PCR primers. The use of a single color makes the noise in the assay twice as high as would be in a two-color assay. Further, the ability to add up peaks of two colors to the full length, as a means to rule out some of the false positive mutation calls, was not available when a single color was used. In spite of the high noise level in this sample set, we made 83% correct calls of the positives and 95% correct calls of the negatives in this sample set.

This process of 82 samples took 6 weeks to complete because of the need to clean up the PCR products by Wizard Prep. and agarose gel evaluation prior to CMD. These extra steps were taken as a precaution because the RT-PCR products were of lower quality than our usual PCR products. The CMD was analyzed on both the ABI 373 and the ABI 377 automated DNA sequencers with essentially the same results obtained in both cases. Peaks were sharper, however, in the Model 377 automated DNA sequencer.

From this study, we learned that CMD makes false positive calls when the operator reads too much into the data and attempted to identify a putative mutation even when the signal is close to the level of the noise. This accounted for five of six false positive calls made in this study. The last false positive produced CEL I cut peaks that are too strong to be a false positive in our opinion. We are asking our collaborator to repeat this sample to confirm that there has not been an error of contamination or mis-identification. Of the five false negatives, two were insertions and deletions of over 300 bp that we saw on the agarose gel analysis as extra bands of PCR products, but unable to identify in the CMD assay. Because the RT-PCR had failed to give PCR products in 14 other samples that we have not included in this analysis, we propose that the other three false negatives may be due to unbalanced PCR that produced mainly one of the two alleles, thereby led to little heteroduplexes being formed. We have requested our collaborator to provide the RT-PCR products again to be certain that there is not some features about these alleles, with respect to CMD, that we should study further.

With two-color PCR CMD, we expect all false positives to be eliminated. The methods we proposed at the end of this report, to improve the signal to noise ratio in CMD, may allow us to eliminate the false negatives encountered in this trial.

Task 6: Months 4-5 Double-nested overlap-primer PCR approach to minimize the possibility of allelic loss in PCR will be tested.

We have not yet initiated this task because it is less urgent relative to other tasks. Allelic loss has not been a frequent encounter so far. We intend to complete this task in

"year two" because it should add reliability to CMD. We believe this task will be predictably successful because it is essentially nested PCR and nested PCR is more robust than standard PCR.

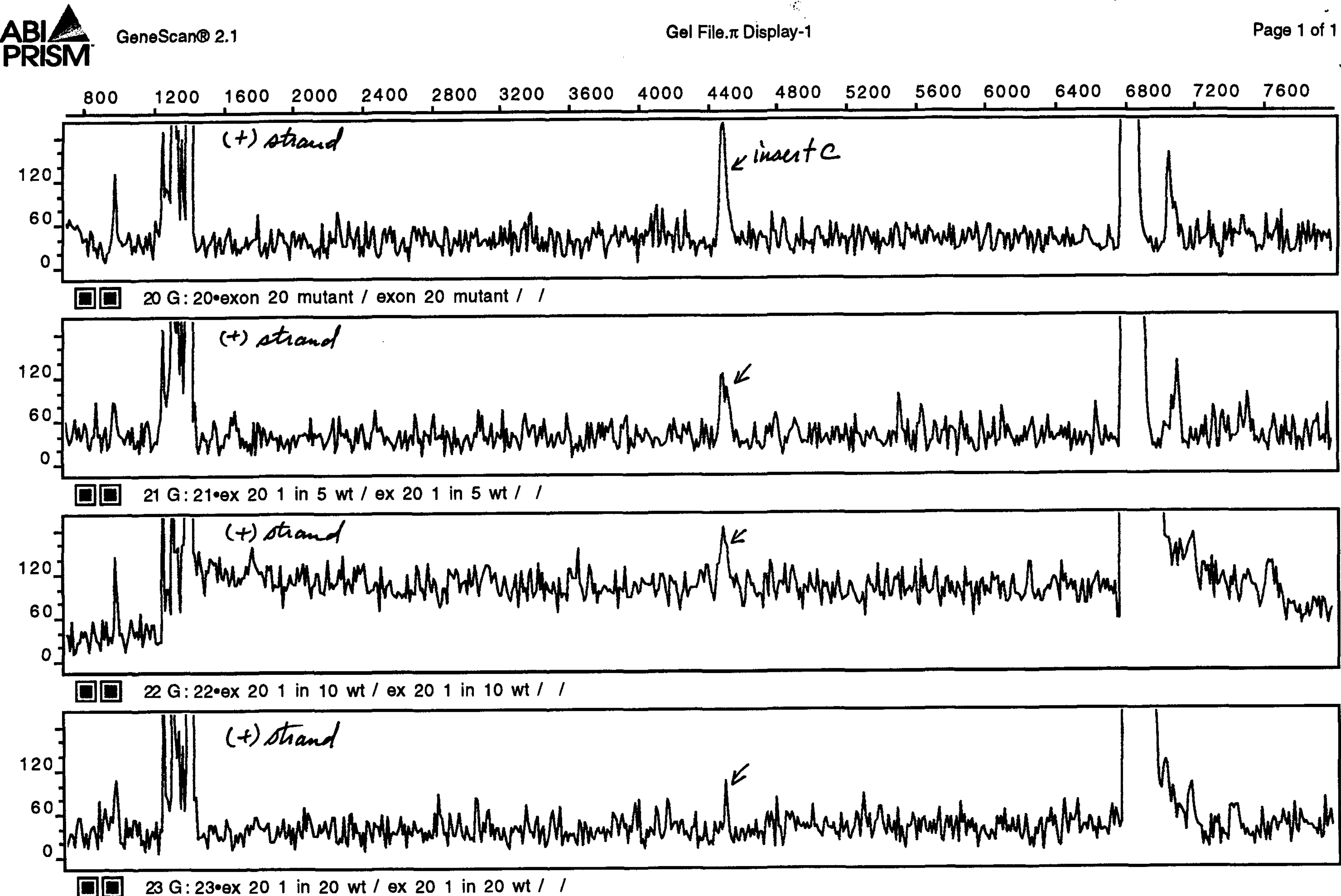
Task 7: Months 6-8 Multiplex and grid analysis of 300 basepairs long PCR products will be tested.

This task involves the detection of a mutant allele among many normal alleles. Using an xyz coordinate pooling scheme, if CMD can detect one allele in 10 people, we will be able to screen for mutations in 1000 people in just 30 reactions. In order to use CEL I to detect single nucleotide polymorphisms efficiently, we began a systematic evaluation of detecting a mutation among many normal alleles. Figure 1 shows the detection of a C insertion mutation in a 401 basepair PCR products of exon 20 of the *BRCA1* gene. Top panel is the heterozygous patient. Second, third and fourth panels illustrate the signal strengths from mixing the heterozygous PCR product with the PCR products from 4, 9, or 19 other normal patients, respectively. This mixing gives the mutant alleles as 1/10, 1/20, and 1/40 of the normal alleles in the assay. Insert C is a medium strength CEL I signal. The high background in the lanes are believed to be from having too much salt in this sample. The salt problem is being addressed.

Figure 2 tests the same procedure for a T to G substitution, a 235 bp PCR product of exon 5 of the *BRCA1* gene. This mutation produces a G/A and a T/C mismatch. It is a weaker CEL I signal, but not the weakest. Incision in both the (+) and (-) strands are shown in alternate panels in Figure 2 as sizes 80 and 155, respectively. Panels top and second are the heterozygous patient sample. Panels 3 and 4 are one allele among five people. Panels 5 and 6 are one allele in 10 people. Panels 7 and 8 are one allele in 20 people, where mismatch-specific signals were no longer observed. We hope that further improvements in CMD by mixing CEL I homologs from various plants will enhance CMD sensitivity on all different mismatches. This study also suggests that CMD can be easily adapted for the purpose of screening for single-nucleotide polymorphisms that are believed to occur at a frequency of one in three people. This later application is a current priority of the human genome project.

Figure 1

Insert C, 401 bp exon 20



T→G substitution, 235 bp exon 5

Figure 2

GeneScan® 2.1 | Gel File.π.1 Display-2 | Page 1 of 1

Wed, May 20, 1998 | - 1 - | Not For Use In Diagnostic Procedures

Plans to improve the signal to noise ratio of CMD.

1. Test various heteroduplex formation conditions to see whether there is room for improvement.
2. To develop a DNA-CEL I buffered system in the CMD assay to make the assay easier to perform. Excess calf-thymus DNA, oligonucleotides, and CEL I will be present and balanced such that variations in the amount of PCR products input will not substantially alter the kinetics of the assay.
3. Evaluate the use of volatile buffers and salts in the assay systems so as to lower background.
4. Evaluate the use of nick-translation to remove non-specific backgrounds.
5. To develop methods to separate CEL I cut DNA from non-cut DNA to lower background fluorescence.
6. To broaden the mismatch specificity of CMD by mixing CEL I-like nucleases from various plants.
7. To test other DNA targets for the sensitivity of CEL I in multiplexed detection of single nucleotide polymorphisms.

CONCLUSIONS

Progress on the CMD project is satisfactory to date. We have trained adequate manpower so that we can now test ideas that may improve the CMD procedures. Much has been learned on how to coordinate the CMD studies at our institution. The ease of doing the RT-PCR CMD of over 80 persons in six weeks is reassuring. With further improved procedures that can make the mutations easier to identify, we expect to accomplish most of the stated goals of this proposal on time.

REFERENCES

none

APPENDICES

none

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