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This study aims to determine whether common protein altering single nucleotide polymorphisms (SNPs) in DNA repair or cell cycle checkpoint genes are associated with higher or lower than average neurofibroma numbers. For this allele association study we are collecting DNAs and RNAs from NF1 patients that represent the top and bottom 20% of neurofibroma burden. In the first year of this project we established contacts with 169 interested patients, identified 99 eligible individuals, and enrolled 43. DNAs from 50 anonymous patients that match our eligibility criteria have also been offered by a clinical collaborator. With recruitment ongoing, we focused on developing genotyping assays. Rather than only analyzing the limited number of genes included in an MIT Genome Center SNP discovery screen as originally planned, we used information generated by the human genome project to generate a database of 325 missense SNPs in 185 candidate genes. 57 SNPs are of special interest because their variant allele frequency is in the range for which we can expect statistically significant results. Single base extension fluorescence polarization genotyping assays for these SNPs are being developed.

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Introduction

Neurofibromatosis type 1 (NF1 or von Recklinghausen NF) is a common autosomal dominant genetic disease, affecting approximately1 in 3,500 worldwide. NF1 patients are at increased risk of developing a long list of diverse symptoms, the most common of which include skin pigmentation defects, neurofibromas (benign tumors associated with the peripheral nervous system), and learning disabilities (1). NF1 is paradigmatic for a disease with variable expressivity, which makes many NF1 patients feel as if they are living with a time bomb. Genetic studies have argued that modifier genes play important roles in determining the outcome of NF1, but nothing is known about the identity of such genes. This study focuses on identifying modifier genes that control neurofibroma numbers in NF1 patients for three main reasons. Firstly, neurofibromas contribute significantly to the overall morbidity of NF1, with some patients developing thousands of these tumors. Secondly, there is excellent genetic evidence that modifier genes play an important role in determining neurofibroma numbers (2). Finally, we hypothesize that variability in neurofibroma numbers reflects differences in DNA repair capacity between individuals. Such differences would be reflected in different rates of loss of the wildtype *NF1* allele, which is believed to be a rate-limiting step in neurofibroma formation (3). To test this hypothesis we aim to determine whether protein-altering SNP alleles of DNA repair genes are associated with higher or lower than average neurofibroma numbers in a case-control study of 600 NF1 patients that represent the top and bottom 20% of neurofibroma burden.

Body

Task 1 of our statement of work was to create computerized patient and modifier gene databases. This task has been accomplished. The password-protected Filemaker patient database includes names, sex, dates of birth, clinical information (neurofibroma numbers), contact information, details about consent procedures, summaries of email messages and other contacts, codes used to identify samples in the laboratory, and other information if available.

The modifier gene database includes records for 185 potential neurofibroma modifiers. This list was compiled from recent surveys of mammalian DNA repair genes (4, 5). In our original proposal, we indicated that we would focus on genes that had been included in a cancer-related

gene SNP discovery screen at the MIT genome center. In the end, many genes that we were particularly interested in turned out to be excluded from the MIT screen. Thus, rather than focusing exclusively on those genes analyzed at MIT, we decided to broaden the scope of our project by analyzing a comprehensive list of potential modifier genes, using dbSNP (6) and HGBase public SNP databases to identify common protein altering polymorphisms. This comprehensive approach was made possible by the identification of well over two million human SNPs as part of the human genome project (7, 8). In our own SNP survey, we identified 325 missense SNPs among the 185 potential modifiers. It is important to note that allele frequencies for many of these SNPs remain unknown. However, we identified 47 SNPs with a 1-4% variant allele frequency, and 57 SNPs with a variant allele frequency of 4% or higher. We are particularly interested in this latter category, for which we are most likely to obtain statistically significant results given the intended size of our patient panel.

Task 2 of our statement of work said that during the first 15 months of our project we would collect peripheral blood from 150 high and 150 low neurofibroma number patients, extract DNA and RNA, perform single-base extension, fluorescence resonance energy transfer (SBE-FRET) genotyping assays developed and validated at MIT, and statistically analyze allele associations. Patient numbers were based on estimates provided by Boston NF clinic-associated clinical collaborators. Thus, Dr. Bruce Korf had estimated to contribute between 60 and 100 patients annually and Dr. Mia MacCollin had indicated she would contribute between 40 and 50 patients each year. The remaining patients would be recruited by advertising this study nationally. The programmatic review panel encouraged us to collaborate with Dr. Andreas Kurtz at Georgetown University, who had proposed a complimentary approach to identify mitochondrial modifiers. Our collaboration with Dr. Kurtz was greatly facilitated by his recent move to MGH.

We had allocated 15 months for task 2, but patient recruitment is clearly running behind schedule. Thus, we have at this time established contacts with 169 interested patients, send out consent and blood drawing kits to 99 eligible subjects, and fully enrolled 43 patients. Enrolled means that we have received back signed consent forms and blood samples from which we have extracted DNA and RNA for genotyping. Of the 169 patients, all but ten were recruited with help from NF patient organizations. Many patients expressed delight at being able to participate in

this type of study, and some non-eligible patients were clearly disappointed at not being able to contribute to research on a disease for which there is currently no cure.

There are several reasons for the lower than expected patient recruitment rate. First, we had anticipated recruiting 100-150 patients annually from two local clinics based on projections from collaborators, but only 10 patients have been recruited from these sources. One reason for this severe shortfall is that Dr. Korf no longer directs the Children's Hospital NF clinic. The fact that Army IRB regulations didn't allow us to recruit anyone under 18 years of age also reduced the number of eligible patients from clinics such as the one at Children's Hospital. To make up for the severe reduction in local patients, early in 2001 we contacted NF clinic directors both in this country and in the United Kingdom, Belgium and Spain. Dr. Rosalie Ferner who directs the NF clinic at Guy's Hospital in London has been working for the past six months to obtain IRB approval for this study. When such approval is obtained, she expects to contribute 30 patients annually. Dr Eric Legius who runs an NF clinic in Leuven, Belgium, declined to participate, reflecting his previous experience in failing to get approval to participate in another Army funded study. Dr. Elizabeth Schorry in Cincinnati who sees over 100 NF1 patients annually also declined to participate for the same reason. Both clinicians agreed to contribute patients when NIH grants intended to fund this project beyond the two year duration of this pilot grant are awarded. Finally, Dr. Conxi Lazaro in Barcelona recently offered DNAs from 50 anonymous patients who meet our eligibility criteria. Including these samples would increase the enrolled patients to 93. Just this week we received permission from the MGH IRB to analyze anonymous DNAs, but we will clear their use with the Army IRB before undertaking further action. Dr. Andreas Kurtz, who contributed few patients so far, is in the process of recruiting patients from Germany and we hope to also include these patients if permission to do so is obtained.

With recruitment ongoing, we focused on developing SNP genotyping assays. In our original application we indicated that we would use a single base extension fluorescence resonance energy transfer (SBE-FRET) genotyping protocol like to the one used at the MIT Genome Center. By the time this project started, the SBE-FRET method had been replaced by a lower cost single base extension fluorescence polarization (SBE-FP) protocol. In this latter method a 150-250 bp genomic segment harboring the SNP is PCR amplified from patient DNA.

These and all subsequent steps are performed in 96 well format. The amplification is followed by enzymatic degradation of primers and nucleotides, after which an unlabeled 20-mer primer that abuts the SNP is extended with two fluorescent chain terminators corresponding to the SNP. Incorporation of one or both of the chain terminators is measured as an increase in fluorescence polarization (9).

Our MIT collaborators had committed to providing us with validated genotyping assays for all SNPs discovered in their screen. However, the MIT group has invested much of its recent time in developing multiplex mass spectroscopy-based genotyping methods. These methods are however not yet robust enough to allow reliable genotyping. For this reason, and because we had decided to include SNPs not discovered at MIT, we were left to design and validate our own genotyping assays. Our original plan was also to read genotypes using MIT Genome Center equipment. In reality the heavy use of this equipment during the final stages of the human genome project made this arrangement extremely cumbersome, and made trouble shooting near impossible. We solved this problem by acquiring our own 4x96 well PCR thermal cycler and LJL-Analyst-AD 96/484 well fluorescence polarization plate reader. Part of the $110,000 cost of this equipment was provided by Partners Healthcare/MGH to fund a genotyping core facility.

At this time we have successfully designed SBE-FP genotyping assays for 30 SNPs. To validate the assays we obtained the same panel of 32 Coriell Institute human DNAs that were used in the MIT SNP discovery screen. During assay development, we first assess the robustness of PCR amplification. For SNPs that alter a restriction site, the next step is to genotype the 32 Coriell DNAs by restriction fragment length polymorphism analysis of the PCR products. Next, we perform SBE-FP genotyping and determine whether genotypes obtained by both methods coincide. We consider a SBE-FP assay validated if both genotyping methods provide the same answer in at least 31/32 assays. For SNPs that do not change a restriction site, we validate SBE-FP results by sequencing the 32 PCR products. A minority of SBE-FP assays fail to produce consistent genotypes. For these assays we use an SBE primer corresponding to the other DNA strand. If problems remain, we genotype using acycloterminators and AcycloPol, rather than our standard protocol using ddNTPs and AmpliTaq. If neither procedure works, genotypes may still

be determined by DNA sequence analysis or by mass spectroscopy assays currently being developed at MIT.

The development of genotyping assays has used up 100% of the effort of the technician funded by this project. We currently aim to design assays for all 57 SNPs with variant allele frequencies of 4% or higher, and genotype patient DNAs when they become available.

Key Research Accomplishments

1. Designed and implemented patient information database
2. Designed and implemented candidate SNP database
3. Contacted 169 NF1 patients, identified 99 eligible patients, enrolled 43.
4. Designed successful SBE-FP genotyping assays for 30 SNPs

Reportable Outcomes

- Meeting abstract. NNFF International Consortium for the molecular biology of NF1 and NF2. Aspen, CO. May 20-23, 2001.
- Patient information and DNA repair gene SNP databases.
- Work supported by this award is the basis of an NIH R01 application (Dr. Bruce Korf, Principal Investigator). This collaborative application by Drs. Korf, Gusella, and Bernards aims to identify NF1 modifiers by a combination of clinical and molecular genetic approaches, including SNP genotyping of candidate genes. Work supported by this award was also used as preliminary data in a Harvard Nervous System Tumors Specialized Program of Research Excellence (SPORE) application. One of five projects in this application is a collaborative effort by Drs Korf, Cichowski and Bernards entitled Neurofibromatosis 1: markers of risk for neurofibromas and malignancies.

Conclusions

The goal of this project is to identify genetic risk factors that affect the rate at which neurofibromas develop in NF1 patients. The successful identification of such modifiers may impact patients in two major ways. First, knowing the identify of modifiers would for the first

time allow a prediction of disease severity, thus reducing the anxiety currently experienced by many NF1 patients. Secondly, genes that affect neurofibroma development might present new targets for therapeutic intervention. In a broader sense, we consider it likely that genes that affect neurofibroma development will also affect overall cancer risk. Thus, the successful completion of this project is likely to lead to additional studies to assess overall cancer risk in individuals harboring the identified alleles.

The identification of over 2 million SNPs as part of the human genome project has made available the tools to identify genetic risk factors in many human diseases. This project capitalizes on this development in the hope of eventually improving NF1 diagnosis and therapy.

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Appendices

None