Division of Cancer Control and Population Sciences
National Cancer Institute
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Mitochondrial DNA and Cancer Epidemiology Workshop

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Draft Summary Report

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Welcome Address

Deborah Winn, Ph.D., Acting Associate Director of the Epidemiology and Genetics Research Program (EGRP), Division of Cancer Control and Population Sciences (DCCPS), National Cancer Institute (NCI), National Institutes of Health (NIH)

Dr. Winn welcomed participants and provided the context for the workshop. DCCPS is the Extramural Division that focuses on public health, clinical practice, and epidemiology areas. She reviewed programs within DCCPS and stated that although they do not focus on the efficacy of screening practice, they do focus on behaviors that influence screening. DCCPS also evaluates the quality of cancer care within the United States. Some of the major initiatives in epidemiology for DCCPS include the creation of consortia of investigators to work on specific epidemiologic problems. For example, DCCPS has initiatives of case-control consortia for head and neck and lymphoma cancers looking at genetic, gene-environment, and epigenetic factors among others.

EGRP is a program within DCCPS that works with investigators and cancer epidemiologists to help move the science forward. EGRP currently is restructuring into four branches: Methods and Technologies, which will bring together the biological sciences and facilitate their application into epidemiological studies; Modifiable Risk Factors; Host Susceptibility Factors; and Clinical Epidemiology.

Dr. Wynn commented that the study of mitochondrial DNA (mtDNA) is a new area of study for EGRP. This workshop is an opportunity to bring together epidemiologists and mitochondrial DNA experts to help formulate the next steps for hypotheses and technologies into large population studies.

Charges to the Participants
Mukesh Verma, Ph.D., Acting Branch Chief, Analytic Epidemiology Research Branch and Program Director, EGRP, DCCPS, NCI, NIH

Dr. Verma presented the objectives of the meeting, which are (1) to review state-of-the-science in the mtDNA field and its utilization in cancer epidemiology, and (2) to develop a concept on mtDNA and cancer epidemiology. Dr. Verma summarized findings from published studies on mtDNA in basic and clinical research. They are summarized below.

- Mitochondrial dysfunction is a hallmark of cancer cells.
- Somatic mitochondrial mutations are common in human cancers, and can be used as a tool for the early detection of cancer.
- MtDNA is 16.5 kb, which makes it easy to sequence.
- Only 37 genes are coded by the mitochondrial genome.
- Evidence exists for the presence of mutations in high-risk populations.
- Examination of human bladder, head and neck, and lung primary tumors reveal a high frequency of mtDNA mutations.
- The majority of these somatic mutations were homoplasmic in nature, indicating that the mutant mtDNA became dominant in tumor cells.
- The mutated mtDNA was readily detectable in paired bodily fluids from each type of cancer and was 19 to 220 times as abundant as mutated nuclear p53 DNA.
- By virtue of their clonal nature and high copy number, mitochondrial mutations may provide a powerful molecular marker for the noninvasive detection of cancer.
- The control region (sometimes called the D-loop) of mtDNA accounts for 6.4 percent of the mitochondrial genome. This region appears to have high rate of variation.
- Changes in mtDNA levels do not correlate with tumor grade and metastasis, suggesting that these alterations may occur in the early stages of tumorigenesis.
- Two strands of mitochondrial DNA differ in their G+T content, and heavy and light strands can be separated on a cesium chloride gradient.
- MtDNA is transcribed as a single polycistronic message.
- Mitochondria play an important role in apoptosis and reactive oxygen species formation.

These findings are suggestive of a significant role for mtDNA in cancer development and risk. The use of technologies, such as the Affymetrix GeneChip® Mitochondrial Resequencing Array (MitoChip), are helping to provide a better understanding of the effect of mtDNA mutations in cancer. Studies have shown that mtDNA mutations are found in numerous cancer types. In addition, mtDNA is associated with various diseases and conditions other than cancer, including diabetes, neurodegenerative diseases, cardiac diseases, rheumatoid arthritis, deafness, blindness, and strokes, as well as antiviral therapies.

Dr. Verma provided a list of charges to participants that represented questions to be addressed during the workshop, and areas of research that need to be assessed to implement progress for increasing the understanding of the role of mtDNA mutations in cancer etiology and progression. The charges include:

- Identify research opportunities in mitochondrial DNA and cancer epidemiology.

- Identify challenges in the field and how to address those challenges.
- Publish the Proceedings of the Workshop.
- Are we ready to utilize mitochondrial DNA information in studying cancer epidemiology? If not, what are the issues?
- How will mitochondrial proteomic knowledge contribute to our understanding of cancer etiology in population sciences?
- What is the value of MitoChip (high-throughput) technology?
- How can we increase the sensitivity, specificity, and predictive values of the assays to detect mtDNA mutation?
- Are there issues of mtDNA sample preparation, such as standardized procedures?
- How much sample is needed?
- What is the origin of mitochondrial mutation: stem cells?
- Do somatic mtDNA mutations actually provoke pathological states or should they be considered epiphenomena? (Somatic mtDNA mutations: the chicken or the egg?)
- When can we use this in the clinic?
- Identifying a cohort of patients with suspected mitochondrial disorders is a challenge.
- Are there founder effects?
- How useful is mitochondrial haplotype analysis in identifying a high-risk population?

Overview

Keshav Singh, Ph.D., Workshop Chair, Associate Professor, Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY

Dr. Singh provided a detailed overview of mitochondria-to-nucleus intergenomic cross-talk and tumorigenesis. An expansive amount of research has been conducted on the structure of mitochondria and its function in the energy production pathway, but the focus only recently has shifted to the role of mtDNA and non-inherited mitochondrial diseases. The number of mitochondria, their shape, and mtDNA copy numbers vary from tissue to tissue. MtDNA is a 16.6 Kb molecule that encodes 37 genes, is maternally inherited, and is homoplasmic in normal mitochondria. The D-loop region, which is the control region, is where most of the mutations occur in mtDNA. Because approximately 1,500 genes encoded by the nuclear DNA (nDNA) that comprise the mitochondrial proteome, cross-talk is a major factor that contributes to the functioning of the mtDNA.

Mutations in mtDNA can affect any organ in the body. Mitochondria also are the major site of free radical production; it has been reported that each mitochondria may produce as many as 10 million reactive oxygen species (ROS) per mitochondria per day. The ROS production may be a major factor in causing mutations in mtDNA. The mutations may be point mutations and small or large deletions that lead to various mitochondrial pathologies.

Dr. Singh presented information on mtDNA mutations and specific identified mitochondrial diseases. He commented that there is a lot not known about the specific mutations and specific diseases, other than in some heritable mitochondrial diseases. Mutations in mtDNA also are used in haplotyping studies. For example, the ongoing International HAPMAP Project has reported that North Americans of European descent may belong to one of nine haplotype groups according to mtDNA mutations.

MtDNA polymorphisms are associated with numerous chronic diseases, including diabetes, Alzheimer's disease, Parkinson disease, bipolar disorders, and cancer. Dr. Singh presented findings from his research on mtDNA and breast and ovarian tumors to study previous findings that depletion of mtDNA occurs in these tumors. Results indicated that copy number changes in mtDNA leads to nDNA instability. Further studies in Rho^0 phenotype cell lines, characterized by a lack of the mitochondria genome, indicated that they could evade apoptosis through over-expression of magnesium superoxide (MnSOD) production. A model was developed, comparing *Saccharomyces cerevisiae* with the wild type and Rho^0 phenotypes, indicated that mitochondria-to-nucleus communication play a role in mutagenesis, and may be implicated in carcinogenesis.

Dr. Singh suggested research questions regarding mtDNA copy number polymorphisms. These included the following:

- Identify D-loop region polymorphisms resulting in changes in mtDNA copy number;
- Identify nuclear genes controlling mtDNA copy number;
- Identify functional changes associated with mtDNA copy number;
- Identify genetic alteration in the nDNA associated with mtDNA copy number changes;
- Identify epigenetic alteration in the nDNA associated with mtDNA copy number changes; and
- Identify genetic and epigenetic changes in the nucleus associated with mtDNA polymorphisms.

Breast Cancer Population Studies
Jeffrey Canter, M.D., M.P.H., Investigator, Center for Human Genetics Research, Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN

Dr. Canter presented results from his recently published epidemiologic study on breast cancer population studies indicating that mitochondrial-generated ROS production damages mtDNA and nDNA; the resulting mutations are associated with increased susceptibility to breast cancer, especially in African-American women. He provided an overview of procedures used to develop these findings, and said that this was his first foray into cancer epidemiology.

Initial interest in mitochondrial polymorphisms and cancer, which became the basis for Dr. Canter's study, began with a patient who was employed at a nuclear facility and was diagnosed with a mitochondrial disease associated with a mutation in 8344—this mutation is associated with a mitochondrial disease known as MERRF (myoclonic epilepsy and ragged red fibre myopathy) and generally is a catastrophic disease seen in children. A pedigree of his family showed that this was endemic within his family across many generations. The disease, however, was seen in various levels within the family, which means that the mutations existed in different proportions in different members of the family. Heteroplasmy was determined; the higher the levels of the mutation were discovered, the more devastating the disease was.

Dr. Canter described three realms for study in mitochondrial epidemiologic research: (1) classic germline mutations, (2) somatically acquired mutations, and (3) germline polymorphic variation. In each realm, variations in the mitochondrial genome may have variable role in common complex phenotypes.

In the study of African-American women and breast cancer, Dr. Canter focused on the 10398 mutation (single nucleotide polymorphism [SNP]) associated with Parkinson disease, Alzheimer's disease, Friedrich's Ataxia, and possibly Amyotrophic Lateral Schlerosis (ALS) and aging. A pilot study and larger population resulted in associations of the 10398 mutation and increased risk of breast cancer. Other reported studies showed that the mutation also was associated with an increased risk of prostate cancer among Caucasian men. Although more studies need to be performed to determine the strength of the associations, these results suggest that mtDNA mutations may be important in determining cancer risk in these populations.

Discussion

Dr. Petros asked whether it was counterintuitive that the SNP is found to be important in African-American women, but is more common in Caucasian women. Dr. Canter responded that this is true but may not be able to be discerned at this time. It may be that the 10398 SNP in African-American women may be just a biomarker of something else. Dr. Wong added that she will be discussing 10398 in her presentation tomorrow. Dr. Canter observed that Caucasian mtDNA is easy to study and that other population groups are more interesting because they tend to be more complex.

Dr. Naviaux asked about any concordance between somatic and germline mtDNA mutations. Dr. Canter commented that it would be an interesting question to pursue. Dr. Winn wanted to know whether mtDNA mutations have environmental causes other than those associated with ROS. Dr. Canter responded that he is not sure from his research, but epidemiologic studies may not have enough information in areas outside ROS. Dr. Singh added that there are multiple pathways, including reactive nitrogen species pathways that are known to cause mtDNA mutations.

Dr. Franceschi wondered about plans to examine nDNA polymorphisms for an interaction, and asked whether the morphology of breast cancer differed if associated with mtDNA mutations. Dr. Canter said that it is critical to look at interactions among mtDNA and nDNA; differences have been seen in some haplotypes. As for morphology, this has not been investigated.

Dr. Kolonel asked if mtDNA in oocytes is homoplastic or heteroplastic. Dr. Canter explained that in the families he studied, the oocytes were heteroplastic in the original female with the mutation. Dr. Ness expressed her amazement that anything was found in the study because of the complexity of the issues. In addition, she said that she is skeptical at the lack of specificity with the association of mtDNA with effects found in the study. Dr. Canter responded that certain polymorphisms appear often in associations, which in an epidemiologic study would be interesting if there was not a biochemical basis for the findings. He posited that the totality of the evidence is an indication that some association exists. Dr. Ness added that the challenge is the computation of such high-level interactions.

Dr. Kolonel asked if there are genes turned on in certain tissues but turned off in others with the same polymorphism. Dr. Canter responded that he is not sure, but this would be an area that could be investigated.

Additional comments addressed some of the challenges in understanding the associations and validating them. There are evolving studies that are attempting to distinquish between mtDNA mutations and differentiated disease states. It seems reasonably certain that heteroplasty results in different symptoms across many diseases, but this needs to be clarified and quantified. There also are other mtDNA polymorphisms that are being studied in population studies (e.g., Fox Chase Cancer Center and in Europe).

Mitochondrial Haplogroups and Cancer and Metabolic Diseases
Masashi Tanaka, M.D., Ph.D., Research Director, Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, Japan

Dr. Tanaka presented information on mitochondrial haplogroups, cancer, and metabolic diseases. His mitochondrial studies in Japanese centenarians and super-centenarians (those 105 years or older) showed specific mtDNA mutations that were associated with haplotypes in these age groups. Centenarians appear to be resistant against lifestyle-related diseases such as type 2 diabetes, myocardial infarction, and cerebrovascular infarction, as well as geriatric diseases such as Alzheimer's disease and Parkinson disease. Super-centenarians have mitochondria and mtDNA that are associated with less impact from ROS than those of controls, and progenitor cells of super-centenarians may be maintained by protection against apoptosis. In addition, the studies identified the historic origins of Japanese haplogroup populations.

The GIFU International Institute of Biotechnology in Japan has the world's most expansive database of mitochondrial genome polymorphisms, and includes entire mtDNA genome sequences of centenarians, marathon runners, individuals with diabetes, obese and non-obese males, and individuals with diabetes and angiopathy. This database offers a rich base of information for understanding mtDNA associations with aging and disease. Luminex100^{TM} technology is used to investigate haplotypes. Haplogroups N and M have been identified for study among Japanese subjects.

Dr. Tanaka described the origins of population groups associated with mtDNA haplotypes and suggestions about the functional character of mtDNA mutations that confer disease protection and longevity. Results indicated that longevity is associated with the D4a haplogroup, whereas mitochondrial haplogroups F and A are genetic risk factors for diabetes. Haplogroup N9a was found to be a protective genetic factor against diabetes, especially in women. He suggested that large-scale association studies will have a major impact on the functional studies of mitochondrial haplogroups and on the elucidation of their contributions to longevity or pathogenesis of lifestyle-related diseases. For cancer, Dr. Tanaka described a study of 149 mtDNA polymorphisms and 30 haplogroups. Results indicated that haplogroup M7b2 is a risk factor for leukemia.

There is a need for further studies of mtDNA SNPs and disease, and this could be accomplished at present in Japan because of the existing database and what is known about Asian haplotypes. The United States will need to construct a system for analyzing haplotypes and SNPs that are characteristic of Caucasian populations. Research into the functional differences among SNPs and haplogroups is ongoing and would benefit from more frequent collaborations.

Cytochrome C Oxidase Subunit I Mutations and Haplotype Predisposition in Prostate and Renal Cancer

John Petros, M.D., Associate Professor, Department of Urology, Pathology and Laboratory Medicine and the Winship Cancer Institute, Emory University and the Atlanta VA Medical Center, Atlanta, GA

Dr. Petros reviewed the associations between mtDNA mutations and cancers of the colon, head and neck, bladder, ovary, and breast, and the diagram of the mitochondria and known genes. He described an investigation that began with the identification of an mtDNA mutation in one renal cell carcinoma (RCC) patient. This became the impetus to look for mtDNA deletions in other cancers. It was known that large deletions occurred in various cancers, but the focus was on prostate cancer.

Dr. Petros described a haplotyping project that identified haplotype U as over-represented in RCC and prostate cancers; haplotype T was under-represented in RCC. A point mutation study in 10 colon cancer samples found that 70 percent had specific pathogenic point mutations. Study of the surrounding tissue of the 10 samples resulted in the finding that the tumors were homoplasmic mutant, and the surrounding tissue was homoplastic wild-type. This changed thinking in this area, and has led to the theory that transformation has occurred and that the mutants had a growth advantage.

The frequency of a CO1 missense mutation was high in a number of prostate cancer patients and low in patients without prostate cancer. The prostate cancer patients were segregated into four groups characterized by specific mutations. Further studies in animals of conserved mutations allowed the creation of cybrids with a prostate cancer nucleus and either the patient's 8993 wide-type or mutants. This point mutation appeared to allow protection for the mutant against apoptosis. Functional studies showed that increases in ROS may account for the increased risk of prostate cancer.

Mitochondrial Correlation Microscopy in Single Cell Cancer Diagnosis

Paul Gourley, Ph.D., Distinguished Member of Science Staff, Biomolecular Materials and Systems, Sandia National Laboratories, Albuquerque, NM

Dr. Gourley presented information on phenotypes of cells and mitochondria morphology, and new technologies used to study mitochondria. New types of light sources and semiconductor materials that have unique spectral properties can be used to probe tissues, cells, and organisms. For example, the Vertical Cavity Surface-Emitting Laser fits into this category. It can penetrate tissue and are not absorbed. This led to development of Biocavity Laser Spectroscopy, which combines semiconductor with light-emitting properties that can be sandwiched together with glass dielectrics to produce a small flow cytometry on a chip. Dr. Gourley presented information on this technology and its uses, for example in the detection of cancer cells. He showed examples to illustrate that this method can identify organelles as well as cells in cancer. The mitochondria have been viewed using these technologies, and it was found that mitochondria are responsible for a majority of the light scattering in healthy cells. In normal mouse liver cells, there is a clustering of mitochondria around the nucleus; in cancer cells, the mitochondria are dispersed randomly throughout the cell. A digital-imaging process technique

(i.e., an autocorrelation image) was applied to normal and cancer cells, and it was found that normal cells have a high probability that mitochondria are clustered, which is not the case in cancer cells.

Dr. Gourley provided a summary of findings from optical studies of mitochondria. They are:

- Mitochondria are dynamic, involved in many diseases, and useful as markers to study spatial correlation;
- Mitochondria can dominate light scatter from cells;
- Mitochondria are more spatially correlated in normal cells and exhibit stronger scatter;
- Mitochondria are larger, less optically dense, and unorganized in diseased cells;
- Experimental data presented here show that it is feasible to detect cancer in a single cell rapidly using biocavity laser spectroscopy; and
- The biocavity laser represents a unique and powerful new tool for the rapid analysis of cells and mitochondria.

Biocavity Laser Spectroscopy of Cancer Cells and Genetic Forms of Mitochondrial Disease
Robert Naviaux, M.D., Ph.D., Workshop Co-Chair, Associate Professor, Department of Medicine, University of California, San Diego

Dr. Naviaux related his discussion to the previous presentation by Dr. Gourley. Frustration with the lack of clinical fact and with the complexity of the heritable mitochondrial phenotypes led to the search for a better understanding of diseases associated with these phenotypes. The hope was to find optical characteristics of haplotypes and germline mutations that could assist with the diagnosis of these conditions. At the time, it required more than 20 techniques to reveal abnormalities in mitochondria associated with disease. The goal was to find one technology through which to study the biophysical determinants of mitochondrial function.

The integrated biolaser chip, developed by Dr. Gourley, allowed the identification of mitochondrial dysfunction by nanolaser spectroscopy. It has been shown that normal mitochondria have a single red-shifted spectral band; mitochondria associated with disease have a wide range of characteristic spectral bands. Dr. Naviaux showed examples of the technique using primate stem cells. Diffuse distribution of mitochondria, which is characteristic of mitochondria from differentiated stem cells, is associated with high lactate production, high oxygen consumption (i.e., aerobic glycolysis), and increased mitotic potential.

In studies of human patients, unique spectral distributions are associated with specific mitochondrial diseases. There is greater diagnostic resolution using this method than in biochemical assays for the same diseases.

In summary, Biocavity Laser Spectroscopy is sensitive to biophysical changes in mitochondria associated with inherited forms of oxidative phosphorylation disease in patients, yeast mutants, and cancer; the diagnostic resolution of the biocavity laser surpasses that of gold standard biochemical assays of mitochondrial function; and a shift from Gaussian to skewed or Poisson-like spectral distributions is associated with many mitochondrial disease states and cancer.

Skin Cancer mtDNA Mutation
James Sligh, M.D., Ph.D., Assistant Professor, Division of Dermatology, Vanderbilt University Medical Center, Nashville, TN

Dr. Sligh presented information on non-melanoma skin cancer (NMSC) and mtDNA mutations. NMSC is the most common human malignancy, is easily diagnosed, and generally easily treated. Because many cases of NMSC arise from well-defined precursor lesions, it represents an opportunity to determine the role of mtDNA mutations in the etiology of the disease. MtDNA point mutations have been identified in photoaged skin and are hypothesized for other heritable and nonheritable, cutaneous conditions.

Dr. Sligh described an investigation of mtDNA mutations in skin tumors and aging skin. NMSC tumor and surrounding cell samples were acquired by micrographic surgery in the clinic, amplified the genome in a single PCR reaction, and resolved it with field inversion gel electrophoresis (FIGE). Results indicate that older patients have more mtDNA deletions—often the only mutation present; in addition, long deletions were more common and appear to arise from damage in margin skin. Common deletions among these samples were found to be at the 3715 and 6278 break points; these deletions previously had not been reported in the literature. These deletions may represent possible biomarkers in aged skin to predict future NMSC.

Dr. Sligh presented information on mtDNA changes in aged skin to identify other common mutations. Early results suggest that multiple mutations can exist in multiple cell lines and in most areas of the mtDNA genome. This information still is being analyzed. In studies of primary tumors and margin tissue, there appear to be changes in margin skin and tumor, which may indicate that a third source of tissue, possibly blood from these patients, needs to be studied to better understand this phenomenon.

Screening assays that have been used in this research are DNA-High Performance Liquid Chromatography (dHPLC) and temperature-gradient capillary electrophoresis (TGCE), known as the REVEAL system and developed by Spectrumedix. Dr. Sligh provided detailed descriptions of procedures used with each screening assay. Future directions for NMSC research includes validation of mouse TGCE screening technique, determination of the utility of Affymetrix technology for screening human mtDNA , and evaluation of phenotypic changes caused by somatic mtDNA mutations in cybrids.

Discussion

Dr. Naviaux asked if there are patches of epidermis that occur naturally in humans that confer higher risk of ultraviolet (UV)-induced carcinogenesis. Dr. Sligh commented that there may be a natural mosaicism in the epidermis that may give rise to hyperkeratotic disease. Dr. Franceschi noted that people develop multiple skin cancers. He asked if these are the same. Dr. Sligh responded that aside from those with genetic predisposition, at age 50 years a person who is diagnosed with NMSC has approximately a 50 percent chance of being diagnosed with a second NMSC within 5 years. Generally, a person's UV exposure makes it likely that this will happen.

Dr. Wong asked whether the blood of older patients was assessed for multiple deletions. Dr. Sligh responded that he did not have blood from the original patients in the study. This will be done in the future, if is possible. Dr. Wong added that, in her studies of oral cancer, deletions are found only in the surrounding tissue, not in the tumor. Dr. Sligh agreed that this is true for deletions as it may be for point mutations.

The Role of mtDNA Alterations in Body Fluids in Diagnosing Cancer
Edward Sauter, M.D., Ph.D., Professor, Department of Surgery, University of Missouri Columbia

Dr. Sauter described the role of mtDNA alterations in body fluids regarding the diagnosis of cancer. Alterations include mutations; an increase in mtDNA copy number and their association with transfer of mtDNA to the nucleus as often seen in gliomas; and alterations in the expression of mtDNA-encoded protein components as seen in RCC.

DNA mutations are seen in almost all cancers, but vary in frequency even among cancers from the same site. One cause for these disparities may be in the different methods of detecting mutations (e.g., the method of sequencing or the percentage of the mtDNA genome sequenced). This does not include differences in the stage or grade of disease, or the prior treatment before samples are taken. This variability also exists in the identification of mtDNA mutations in precursor lesions. Moreover, for noninvasive techniques, such as needle biopsy in breast cancer, mutations are found at approximately the same rate as found in solid tumors.

In a comparison of matched body fluids with tumor tissue, variability exists. For example, in bladder cancer, there is a very high detection of the same mtDNA mutations in urine and tissue. One problem with the data presented by Dr. Sauter is that it is difficult to know whether the specimens (i.e., body fluid or tissue) containing these mutations were from the same patient. This is an area that needs to be elucidation by further research.

Dr. Sauter presented information on screening techniques and how the method influences the detection rate. The gold standard for detection is direct sequencing of all bases evaluated. Other methods, including restriction fragment length polymorphism (RFLP), single-stranded conformation polymorphism (SSCP), and temporal temperature gel electrophoresis (TTGE) are used to sequence only the abnormal areas and may over- or under-represent the mutations. The MitoChip is a newer technique that needs 300 nanogram (ng) genomic DNA, covers the entire coding sequence, but does not cover the D loop; has few failed signals; has > 99.99 percent reproducibility of base calls within and between chips; and in one report, miscalled 5 percent of mutations compared to direct sequencing.

Dr. Sauter concluded by listing the current state of screening for alterations in body fluids. They are:

- The prevalence of mtDNA mutations in tissue varies based on disease origin.
- MtDNA alterations occur early in disease development.
- Mutation prevalence in body fluids varies, likely dependent on tumor/normal cell ratio, as well as cell number.

- No current method of detection combines high throughput, optimal accuracy, and screens the entire mitochondrial genome.

Discussion

Dr. Franceschi clarified that the newer MitoChip array does contain the D-loop region. He asked if anyone has investigated mtDNA alterations after radiotherapy. Dr. Sauter responded that it has been investigated and does cause mtDNA mutations. There is, however, little information on the type of alterations that occur, and reports rarely mention the type of radiotherapy administered. Dr. Verma commented that often patient information is collected with the sample, so one can know what other factors should be considered. For example, it is important to know that the patient who donated the sample also had alcoholism, *k-ras* mutations, or other factors.

Dr. Wong commented that the most important application of measuring mutations in body fluids is to follow the patient to know the progression of the disease and whether it re-occurs.

hMitoChip3 and Mitochondrial Transcriptome in Melanoma
Yan Su, M.D., Ph.D., Associate Professor of Biochemistry and Molecular Biology, Associate Director of Catherine Birch McCormick Genomics Center, The George Washington University Medical Center, Washington, DC

Dr. Su described the use of hMitoChip3 technology in ascertaining expression profiles and molecular pathways related to the mitochondrial transciptome in melanoma. The hMitoChip3 is a new technology that can provide information about the 37 genes found in mtDNA, as well as about the 1,098 nDNA-encoded and mitochondrial-related functional genes and 225 control elements. Dr. Su described the inter-gene consistency and reproducibility of the hMitoChip3 array. The bioinformatic support software for the array allows a high level of data analyses and database interpretation.

Dr. Su described preliminary investigations of detection of gene expression in melanoma cell lines and melanocytes using hMitoChip3, and the types of data available. He showed the relational data and gene information files provided from the chip array using the bioinformatic tools. What was learned from these preliminary investigations is that a wealth of information is available using these methods.

Discussion

Dr. Wong asked if the chip and software programs are able to pick up mild effects. Dr. Su responded that the investigator can set the parameters. In response to Dr. Franceschi's question of whether the specific cell line used would effect or bias the results, Dr. Su affirmed that this was possible. Dr. Wong asked if there is a possible application of hMitoChip3 to the diagnosis of mitochondrial disorders. Dr. Su replied that this is possible, but a validation study must be completed on a larger tissue sample. Dr. Wong added that she would like to see this occur with more samples from different types of tissue.

Dr. Singh added that he has a similar chip that is being validated at the National Institute of Standards and Technology (NIST). Dr. Sauter commented that the use of this technology for high-throughput is exciting, although validation with real tissue and standard sequencing would be convincing. Dr. Singh responded that this is what NIST will be validating.

Depletion of mtDNA in Prostate Cancer
Masahiro Higuchi, Ph.D., Assistant Professor, Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR

Dr. Higuchi presented recent results from studies on the depletion of mtDNA in prostate cancer. Androgen ablation has been the most common treatment for prostate cancer, and there may be a role for mtDNA in etiology or in treatment and recurrence. Dr. Higuchi focused his research on prostate cancer cell lines that contained non-androgen dependent cells, which do not respond to androgen ablation, to determine what factors separate these cells from the more common androgen-dependent cells.

Dr. Higuchi described investigations of the C4-2 androgen-dependent cell line, inoculated with the androgen-dependent LNCaP cell line into castrated mice, and found that normal mtDNA was reduced and there was an accumulation of large-depletion DNA. This led to the loss of androgen dependence in the resulting cells. This finding suggests that mtDNA determines the androgen status of prostate cells; this may be key in determining the progression of disease during and after treatment for prostate cancer.

The mechanism that may be responsible for the loss of androgen dependence includes superoxide and ROS production resulting from androgen ablation therapy. This damages the mtDNA and results in reduced respiratory function, and eventual induction of cancer-related signals such as NFκB, Akt, CREB, and AP-1.

Discussion

Dr. Naviaux asked if Dr. Higuchi has prepared a slide of a traditional model of androgen signaling pathway and how it has to be modified in the face of these results. Dr. Higuchi responded that he has, and one of the issues in this investigation is the androgen receptor, which is lost in androgen-independent C4-2 cells. Signaling pathways also are changed in the absence of androgen.

Mitochondrial DNA Mutations and Reactive Oxygen Species Stress in Cancer Cells: Molecular Players and Clinical Implications
Peng Huang, M.D., Ph.D., Associate Professor, Department of Molecular Pathology, The University of Texas M.D. Anderson Cancer Center, Houston

Dr. Huang recounted that the two most prominent metabolic abnormalities in cancer cells are the Warburg Effect, characterized by increased glycolysis for ATP generation even in the presence of oxygen, and redox imbalance, which causes increased ROS generation and oxidative stress. Dr. Huang presented information on primary chronic lymphocytic leukemia (CLL) and the detection of mtDNA mutations. CLL cells from patients with prior chemotherapy exhibit a

higher frequency of heteroplasmic mtDNA mutations. Assay of these cells indicate that a G to C mutation of 7762 is associated with a significant increase in oxygen radical generation leading to increased oxidative stress and production of ROS. Another finding is that CLL cells have more mitochondria than normal lymphocytes, which may make those cells more resistant to chemotherapy.

Dr. Huang described investigations to determine the mechanisms of mutations in mtDNA and resulting increases in oxidative stress that influence the lack of response to chemotherapy. It is possible that the loss of *p53* function plays a role, possibly by protecting the mtDNA. Mitochondrial respiration malfunction also causes redox imbalance, leading to Akt activation and drug resistance. Changes in glycolysis for ATP generation, attributable in part to mitochondrial malfunction, are possible, which suggests that inhibition of glycolysis represents a potential novel strategy to overcome drug resistance.

Discussion

Dr. Higuchi commented that, in the utilization of glucose, there would be a significant increase in the amount of lactate production and a change in pH to acidic. He asked how this affects chemotherapy. Dr. Huang responded that this is an important question and the characterization of increased acidity is correct. This situation does not help chemotherapy. Dr. Singh asked about the *p53* localization and whether a mitochondrial signal is associated with this. Dr. Huang said that p53 translocation is not in the mitochondria but is influenced by increased ROS generation caused by mutated mtDNA.

Dr. Franceschi added that *p53* is capable of influencing cell senescence; in addition to its effect on DNA repair, which stops cancer growth. He asked if Dr. Huang could explain what is happening in chemotherapy on apoptosis induced by mitrochondrial-dependent apoptosis and cell senescence. Dr. Huang responded that this has been studied this and the response to chemotherapy is much better with *p53*.

Discussion Panel Leaders
Dr. Naviaux, Panel Moderator

Konstantin Khrapko, Ph.D., Assistant Professor, Department of Medicine, Beth Israel Medical Center, Harvard Medical School, Boston, MA

Laurence Kolonel, M.D., Ph.D., Director, Epidemiology Program, Cancer Research Center, University of Hawaii, Honolulu, HI

Roberta Ness, M.D., Ph.D., Professor and Chair, Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, PA

Alexander Parker, Ph.D., M.S., Assistant Professor of Epidemiology, Department of Urology, Mayo Clinic College of Medicine, Jacksonville, FL

Mukesh Verma, Ph.D., Acting Branch Chief, Analytic Epidemiology Research Branch and Program Director, EGRP, DCCPS, NCI, NIH, Bethesda, MD

Dr. Naviaux, Panel Moderator, explained that the panel discussion will occur in two phases. The epidemiologists will attempt to organize the use of specific biomarkers, both for their predictive value for risk of cancer and their value for determining treatment, and in their prognostic capability in predicting mortality. The second phase will be a discussion of questions developed by Dr. Verma to guide the discussion.

Dr. Ness began the discussion from an epidemiologic view by establishing a context of design issues to understand etiologic risk factors for use as early detection markers, as a factor used to design appropriate treatments, and for determining prognosis. Whether somatic or germline mutations are etiologic, they can be used for risk stratification and as biomarkers. Knowing the mechanisms of a mutation that has been identified by epidemiology is not as important as knowing that it is associated with higher risk; it is ideal if it is causal.

Panel members discussed whether heteroplasmy is important if the mtDNA SNP is not found in all mitochondria. In homoplasty, cells are clonal and those SNPs will occur in all cells. There is mtDNA mosaicism in the oocytes, but the vast majority of oocytes have a homogenous haplotype. Siblings have exactly the same mitochondrial haplotype, but unrelated individuals can vary between 30 to 50 mutations (SNPs).

Dr. Ness summarized a few principles that epidemiologists may follow regarding studies of mtDNA mutations:

- Cancer epidemiologists and researchers don't talk about "cancer" *per se*, but about specific kinds of cancer. It is nonsensical to talk about mtDNA mutations and cancer, but it is logical to talk about mtDNA mutations and specific cancers.
- It is important for epidemiologists to report when specific mtDNA mutations for specific cancers are found in specific populations.

A panel member addressed the function of adenosine triphosphate (ATP), which involves a six-step process that involves a specific enzyme found on the inner membrane of mitochondria. This means all cellular pyrimidine processes depend on the integrity of normal electron transport because of a redox reaction that must occur to produce what is required for normal growth and biosynthesis. This function of mitochondria is more important for cancer cells than ATP synthesis because of the ATP in cancer cells is made by glycolysis. In addition, in an evolutionary sense, cells prefer their pyrimidine to be delivered intact; it is only in rogue (i.e., cancer) cell membranes that upregulated pyrimidine synthesis is seen.

Dr. Ness added that it is important to be convinced that predisposition toward an association exists. It is unlikely, however, that there will be a strong association from a particular haplotype because of factors such as age, somatic mutations, and post-translational modifications. Therefore, a future topic for research will be to look at gene-gene and gene-environment interactions. The most effective approach to this problem is to develop consortia to involve all known haplotypes.

Dr. Tanaka mentioned that it would be difficult to separate risk factors from haplotypes, especially in older populations. Age is a confounder in any study, but it is notable in a field such as this where there is increased risk just with aging.

Dr. Franceschi discussed the importance of creating a consortium in this field. It would need to include a large number of patients and controls. Even with such a large study, one would have to study all the interactions among mtDNA and genes, nuclear genes, and scores of mutations. Dr. Wong added that at this point, it is not clear whether any mtDNA mutation actually causes cancer. Dr. Singh reminded the panelists that mtDNA needs communication and direction from nDNA, and this cross-talk plays a role in mtDNA mutations. This is an enormous problem to unravel.

After a discussion of what is needed to design an mtDNA genome study, the following table was developed to represent a comprehensive approach. Each of the paired tissue samples would undergo the same battery of tests. Blood would be an appropriate control for the study.

TABLE: Possible Design for mtDNA Epidemiologic Study

	Blood	**Tumor**	**Tumor-Adjacent Sample (normal)**
Nuclear DNA	x	x	x
Mitochondrial DNA	x	x	x
SNP Analysis	x	x	x
Gene Analysis	x	x	x

In addition to the samples listed in the table, risk-factor data on individuals participating in the study would allow determination of gene-environment interactions. Suggestions for such a study included tapping into NCI tumor repositories of tissue and developing consortia to provide enough samples to arm the epidemiological study with appropriate statistical power.

Dr. Kolonol offered that he has been associated with various NCI population-based cohort studies that have large numbers of samples from many population groups, although blood samples were not taken from everyone in the studies. Dr. Ness interjected that cohorts do not produce many cases. It may be better to begin with case-control studies to be able to see enough cases. In addition, NCI programs, such as the Early Detection Research Network (EDRN), may be able to provide samples. It may be best to begin with a very clear population at the beginning to determine which factors are clues that then could be assessed in a larger sample.

Other suggestions were to begin the study in a population that does not receive chemotherapy (e.g., renal cell carcinoma) because those mutations would not be present to subvert the application of results to the entire population. Another suggestion was to use buccal cells for DNA capture, although there would be differences in smokers and nonsmokers that would have to be considered. This leads to the issue of stratification of mtDNA mutations being present and linking this back to known risk factors, such as smoking. Dr. Verma informed the panel that a recent paper did look at mtDNA mutations in smokers, former smokers, and never smokers.

This data showed that there are changes that occur in smokers who do not have overt disease at the time.

Dr. Franceschi suggested that it may be interesting to study the individuals who smoke heavily, are older, and have never been diagnosed with cancer to see if they have mtDNA mutations that are protective. This would be approaching the problem in a different manner than traditional studies. He also suggested that it may be beneficial to use the candidate-gene approach. Dr. Ness found this suggestion provocative and asked whether other "natural" experiments could be suggested that are based on this same concept. Dr. Franceschi noted that this type of study has been proposed in Italy among centenarians at high risk of cancer who do not get it even after having high-risk behavior over many years. Other participants suggested using the same concept to study siblings and twins of cancer patients who are cancer-free, as well as other populations that could be identified as fitting these criteria.

Dr. Naviaux described the basic approaches for designing a study. One could begin with epidemiology and find associations (i.e., use a top down approach), or begin with mechanistic findings and approach the study from the bottom up. This does not mean that a functional basis for an epidemiologic finding would not strengthen the direction of the study.

Dr. Kolonel suggested that it might be better to look at tissue with more mtDNA turnover than other tissues and whether there are more mutations in the mtDNA with large numbers of mutations compared to tissue with less mtDNA. In discussions, the point was made that it is the accumulation of mutations rather than the mitochondrial number that is important.

Dr. Naviaux noted that among families with hereditary mitochondrial diseases, an effect of heteroplasty and homoplasty could be studied—namely, there is a prediction that the mother who passes on these mutations may have a higher risk of cancer, although studies have not been conducted. One of the problems is that the study of mitochondrial genetics has been in existence only since 1988, so long-term data currently are not available. Dr. Winn added that patients with mtDNA genetic diseases can be followed over time, just as they are in other genetic epidemiologic studies.

Dr. Ness addressed the issue of biologic plausibility. Epidemiologists are cognizant that clinical trials often reveal that many issues with biologic plausibility do not turn out to be causative.

Dr. Tanaka addressed the confusion with labeling haplogroups and their subgroups with names that do not describe their functional significance. Using cybrids, it may be possible to develop a risk scale that would better identify functional differences between sub-haplogroups. Dr. Naviaux agreed this would be an interesting method for classification, but observed that different cell lines would function differently.

Discussion of Questions

The remainder of the session addressed questions developed by Dr. Verma. After each question, the points raised during the discussion are listed.

1. ***Are we ready to utilize mtDNA polymorphisms in studies of epidemiology in cancer?***
   Yes. There are a few models for the design of archived paired-sample databases with sufficient epidemiologic data.

2. ***Are mutations causative or merely reflective of nuclear genome instability?***
   Most researchers would agree that individual mutations are not causative, but that mtDNA mutations occur and may precede nuclear genome instability.

3. ***Do somatic mtDNA mutations actually provoke pathological states or should they be considered epi-phenomenon (e.g., chicken or egg)?***
   This may be a reason to conduct case studies before conducting cohort studies. The question is not answerable at this time, but an approach for answering it may be possible.

4. ***Are nuclear and mitochondrial processes independent events?***
   Most people posit that they are interdependent or complementary. There is no case, however, where large mtDNA changes occur without nDNA instability occurring at the same time.

5. ***What is the origin of mtDNA mutations?***
   There were various opinions on this question. Some thought they originated in the stem cells, but the general opinion was that they originated in post-stem cell compartments, but before final differentiation. It was pointed out that when differentiation begins in stem cells, methylation also begins.

6. ***What are the mitochondrial types and the risk of cancer?***
   This has been discussed previously and in sufficient detail.

7. ***What are the founder effects in mtDNA?***
   This relates to haplotypes but is more relevant for nDNA mutations than mtDNA mutation effects. In populations where people are less dispersed compared to the United States, this can be a problem.

8. ***Identifying a cohort of patients with mitochondrial disorders is a challenge?***
   This has been a challenge for developing clinical trials for mitochondrial because it is difficult to accrue a homogenous population of inherited mitochondrial disease. The problem is to find patients with the same disease who have the same expression of the disorder, such as a group that all have been diagnosed with Mitochondrial myopathy, encephalopathy, lactacidosis, stroke (MELAS) syndrome and diabetes or cardiomyopathy. Treatment studies are funded primarily by pharmaceutical companies that are assessing new agents for the disease. Even in experimental mouse models, very few concrete findings have helped in this area. In addition, it appears that mice are homogenous for mtDNA by continent.

9. ***Do patients with mitochondrial disease get cancer?***
   They do, but it is unknown whether it is at a higher rate than people without mitochondrial disease. The current rate among people with mitochondrial disease is less than 10 percent

in those over age 50. One participant added that mutation 10398 A to G is associated with the protection of breast cancer among Caucasian women, but appears to be associated with a higher rate of prostate cancer in Caucasian men. Another mutation, 9055, was found to be protective against Parkinson disease but is a risk factor for breast cancer. Because the estrogen receptor exists in the mitochondria, there should be research in this area to help understand these conflicting findings.

10. ***Will mitochondrial proteomics knowledge contribute to the understanding of cancer etiology in the general population?***
Dr. Naviaux commented that researchers in genomics, proteomics, and metabolomics have an interest in results from mitochondrial research, but each area works within a different time period. For example, genetics can explain what happens over long time periods; proteomics may determine what is occurring in days; and metabolomics can tell what is happening over minutes. Each "-omic" can be beneficial. At this time, proteomics and metabolomics do not have sufficient techniques and methodologies to fully participate in mitochondrial research.

11. ***What can be offered by MitoChip and high-throughput technologies?***
This is a challenge for epidemiologists because of the multiple variables that can be attached to demographic data. There may be a need to develop data-compression methods to allow the use of all the data that can be collected using these methods. All data methods will need to be validated. A significant challenge will be handling the two types of data collected. There will be straightforward data from the investigation of the candidate gene, and then a flood of additional categorical variables to account for in the data. There must be an incubator to identify candidate genes so that research groups can use the information to determine disease associations.

12. ***What do we know about the sensitivity and specificity of the assays to detect mutations?***
This depends on the technology used. Most sequencing methods are poor at detecting heteroplasty. The MitoChip can detect only approximately 25 percent, but version two appears to yield better results. PRC-FLP is approximately 15 percent and dHPLC is approximately 2 percent for detecting heteroplasty. It was noted that this is only important for research on somatic factors rather than for the study of homoplasmic factors.

13. ***What are the issues regarding mitochondrial sample preparation?***
It was noted that alkali or standard plasmid kits should not be used for mitochondrial investigations because they interact with the mtDNA strands. Additionally, not all mtDNA is 2-stranded. Another issue is that it is difficult to secure enough mtDNA during sample preparation for use in multiple investigations. Approximately 200 ng is needed for the MitoChip and this is not always possible to obtain. This makes the use of mtDNA for screening impractical at this point, although future research may be able to overcome this challenge. One participant also raised the point that it is difficult to collect enough mtDNA to use for long deletions; this is not the case for study of short deletions.

FRIDAY, SEPTEMBER 8, 2006

The Origins of mtDNA Mutations
William Copeland, Ph.D., Senior Investigator, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC

Dr. Copeland presented background on the origins of mtDNA mutations, which include DNA damage from environmental factors and oxidative stress, as well as from spontaneous errors in DNA replication, translesion synthesis, and repair re-synthesis. He described the DNA replication errors involving DNA polymerase γ (pol γ), which is the only human polymerase found in the mitochondria that have been the focus of his research. Two other roles in disease processes have been described for pol γ: it is the only replicative DNA polymerase that is sensitive to a wide variety of nucleoside reverse transcriptase analogs, and the gene of pol γ was identified for several mitochondrial diseases because it contained point and stop-code mutations.

Dr. Copeland described repair genes and the characteristics of mtDNA that facilitate the identification of specific mutations and their causes, and pol γ has a rather high fidelity rate in mtDNA. There, however, is a higher mutation rate in mtDNA than in other DNA models studied, such as the DNA of *E. coli*. Using this information, a pol γ exonuclease-deficient knockout mouse has been developed that exhibits premature aging and is able to accumulate numerous mutations and generates apoptosis without an increase in ROS generation.

A review of spectrographic data has been conducted to determine what type of spectra are found with pol γ mutations. These indicate that the number of errors in mutations increase linearly. The conclusion is that endogenous errors mediated by DNA pol γ constitutes the primary source of mitochondrial point mutations in human tissues, supported by the fact that 83 percent of mutations detected *in vivo* were reproduced *in vitro* with recombinant DNA pol γ.

To answer the question of the contribution of nucleotide pools to mtDNA mutagenesis, Dr. Copeland described experimental and animal studies to determine that:

- The concentrations of the four deoxynucleotide triphosphates (dNTPs) are not equal in mitochondria isolated from several tissues of both young and old rats,
- Mitochondrial 2'-deoxyguanosine 5'-triphosphate (dGTP) concentrations are high relative to the other dNTPs, and
- The fidelity of DNA synthesis *in vitro* by normally highly accurate mitochondrial DNA pol γ is reduced because of the increased formation of template T-dGTP mismatches that are inefficiently corrected by proofreading.

Further studies to determine the role of ROS in mtDNA mutations looked at 8-oxoguanine (8oxoG). It appears that most of the ROS is ameliorated by pol γ, which explains why ROS is not considered to be a major factor in mtDNA mutations.

Discussion

Dr. Petros asked if pol γ varies in different tissues. Dr. Copeland responded that it is the same and can have an effect on fidelity, although it is not known if this occurs in humans. Dr. Petros followed up with a question on the model system used. There were different rates of mutation depending on the organ investigated. Dr. Copeland said that they were within 2-fold of each other, but they were being studied under different conditions based on the tissue nucleotides.

Dr. Franchschi commented that the pol γ knockout mice model of aging has been criticized by many researchers. Dr. Copeland said that a new study shows that, in the heterozygous mouse model, there is a 500-fold increase in accumulated mutations and there is no aging phenotype. Dr. Singh asked if they had looked at adaptive responses in these mice. Dr. Copeland said he was unsure, but other studies have shown that it does not involve an increase in ROS, and it proceeds directly to apoptosis.

Dr. Sauter asked for a clarification on why mismatch repair is not present in mtDNA errors, as they appear to be high in colon cancer. Dr. Copeland responded that mismatch repair is important in nDNA but not in mtDNA. There is unpublished data on this. Dr. Verma added that, in colon cancer, hypermethylation is dominating mismatch repair, meaning that epigenetic events are more important than genetic events.

mtDNA Involvement in Aging/Longevity and Age-Related Diseases
Claudio Franceschi, M.D., Ph.D., Professor, Department of Experimental Pathology, University of Bologna, Italy

Dr. Franceschi addressed mtDNA involvement in aging and age-related diseases. He provided background information showing that coevolution of nDNA and mtDNA has been shaped by natural selection, and that these changes have not been neutral regarding human aging and disease. Dr. Franceschi presented evidence from his investigation of centenarians showing that in the area of Sardinia in Italy, there are higher numbers of centenarians than in other areas of Italy. In addition, in Italy there is a North to South gradient in the female:male ratio in centenarians, and male centenarians are more frequent in the South. One theory suggests that increased longevity is associated with cross-talk between the nuclear and mitochondrial genomes.

Dr. Franceschi reviewed studies showing that the mtDNA C150T mutations in the D-loop is associated with homoplastic transition in leukocytes in an Italian population, and is more frequent in centenarians. The increased frequency of C150T in Italian centenarians also has been found in centenarians from Finland and Japan. Further studies in cybrids from different haplogroups has produced results that indicates that the C150T mutations are associated with reduced inflammation associated with aging. This finding may contradict the accepted aging theory based on free radicals.

Studies to understand the effect of mtDNA mutations on inflammation associated with aging indicate that cytocine and cytokine receptors are modulated by such mutations. In specific, mitochondrial mutations that modulate Interleukin-6 (IL-6) production are associated with

longevity in Italian men. A study of haplotypes in Alzheimer patients with variants of apolipoprotein-e (apoE) showed that there are differences in risk according to haplotype—particularly between haplotype U, which is neutral, and haplotype J, which is not.

A *p53* polymorphism of Arg/Pro at codon 72 has a higher propensity to interact with *p21* and lead to apoptosis. This phenomenon is called "allele timing," which means that an allele that is neutral when individuals are at a young age can have an impact in older individuals. The mechanism for this is not known. This phenomenon also is seen in older breast cancer patients. Preliminary data from a population study in central and northern Italy on 26 repair genes with 70 SNPs have associated three polymorphisms with longevity. These data are still being collected and analyzed.

Discussion

Dr. Ness wanted to know the concordance for siblings living to be centenarians. Dr. Franceschi responded there is no genetic advantage to age 60 years in twins, but after after age 60, one twin would have a concordance of 0.2 for every year lived by the co-twin. This is a very strong impact of genetics. Offspring, parents, and non-twin siblings of centenarians also live longer than their respective cohort, and have a better health status related to cardiovascular disease and cancer. This does not extend to spouses.

Dr. Naviaux asked whether there is evidence that chronic inflammation alters intracellular nucleotide pools. Dr. Franceschi commented that his research group is trying to determine if there is an impact in this area. Dr. Naviaux also asked if there is a possibility that the large databases could be analyzed to see if any two factors play a role (e.g., synergy). Dr. Franceschi said he is looking at this very issue to ascertain whether the data can be looked at in this way, but a manuscript on this topic has been rejected by a journal with a request to repeat the analysis.

Dr. Singh recalled that a recent paper in *Cancer Research* discussed the role of CD4 in the surveillance of mtDNA mutations.

Dr. Petros requested a comment on the J-type haplotype and longevity and whether there is a strong association to aging. Dr. Franceschi responded that he continues to seek stronger associations to the haplotype. A study is underway with 3,000 people. The hypothesis is that an interaction between haplotype J and C150 allows longevity. Some uncoupling occurs, but enough stability remains to allow the mitochondria to function at a lower level than normal. Although this may be counter-intuitive, it is strong theoretically. Dr. Petros commented that he finds this unusual because there is the uncoupling phenomenon, but there does not appear to be as strong a consideration of reactive oxygen. Dr. Franceschi said that, most importantly, the data suggest that mtDNA is not neutral. Most centenarians do not get cancer, which suggests that they should be studied to determine why they can live to 100 years or more without getting a disease that afflicts so many younger people. He said that he is looking for genes that can account for this.

Somatic mtDNA Mutations and Germline mtDNA Polymorphisms in Cancer
Lee-Jun Wong, Ph.D., Professor, Department of Molecular and Human Genetics, and Director, Mitochondrial Diagnostic Laboratory, Baylor College of Medicine, Houston, TX

Dr. Wong shared her experience in studying mtDNA mutations and germline mtDNA polymorphisms in cancer. She discussed breast cancer and mtDNA mutations, including point mutations, large deletions, mtDNA depletion or amplification, and microsatellite instability in the D-loop hypervariable and coding regions. Using TTGE, investigations are able to show heteroplasmic point mutations and are capable of identifying homoplasmic-homoplasmic, homoplasmic-heteroplasmic, and heteroplasmic-heteroplasmic changes. She described characteristic point mutations in each situation.

In breast cancer, the same changes are seen, even though most of the changes are in the D-loop with several in the coding region that involve single base mutations. In liver cancer, most changes are in the D-loop but a few have been found in the coding region, which may be more significant; there also are missense changes. Dr. Wong presented the types of changes also in papilloma, medullablastoma, and esophageal cancers, each of which have characteristic changes, including which region the mutations are found. These are all somatic mutations; a summary of mutations indicate that most are in the D-loop region.

Dr. Wong presented information on mutations in the D-loop regions, where most changes may not be significant, although a few have been identified with functional impacts in cancer. It may be that many D-loop mutations increase copy number, causing faster growth of the tumor, and they may affect DNA or RNA synthesis. Although the majority of mutations may be passenger mutations, some may have functional effects (e.g., missense and nonsense mutations affect RNA processing or stability of ribosomal or transfer RNA).

Dr. Wong described germline mutations that influence cancer or other disease risk. She presented summary data from blood samples in non-Jewish Caucasians indicating that some mutations (e.g., G9055A and A10398G) may be protective against Alzeihmer disease. T3197C and G13708A may be associated with increased breast cancer risk. Haplotype studies in this group also indicated that haplotype K is associated with increased breast cancer risk, whereas haplotype U is associated with decreased breast cancer risk.

In summary, the following results and suggestions were presented by Dr. Wong regarding somatic and germline mutations:

- Somatic mtDNA mutations occur in all types of cancers although whether they are a cause or consequence is largely unknown. There is a need to know the effect of mtDNA content and the impact of large deletions and their functional effects, with single molecule analysis being a method to consider.
- Germline mtDNA SNPs or haplogroup influence an individual's cancer risk either protectively or detrimentally. Gender, ethnicity, disease, and gene-gene/gene-environmental interactions are important factors. Increasing the sample size and more funding for studies can help accelerate progress in this area.

Discussion

Dr. Huang asked about the single molecule technique used and what the chances are of identifying a mutant versus the wild type. Dr. Wong responded that either can be identified and sequenced. When mutant and wild type are mixed together, sequencing is done only if heteroplasmy is detected.

New Approaches to the Whole Mitochondrial Genome Sequencing

Konstantin Khrapko, Ph.D., Assistant Professor, Department of Medicine, Beth Israel Medical Center, Harvard Medical School, Boston, MA

Dr. Khrapko presented information on newer methodological approaches to whole mitochondrial genome sequencing by first indicating that the price for sequencing the entire mtDNA genome is becoming less expensive as new techniques are introduced. Cost remains a significant factor in the conduct of large studies. At the present time, whole genome sequencing costs approximately $800 per sample for regular sequencing; the use of MitoChip has reduced the price to approximately $150 per sample. He described the process of whole mitochondrial sequencing and advantages of various techniques.

Dr. Khrapko said that the methods are not entirely satisfactory at this point, and there is a need to develop alternative approaches. The "single-template, random sampling" approaches, such as 454 and Solexa, were described. Each of these, although still under development, use algorithms to reduce errors. A challenge for this technique is that mtDNA is too small for analysis unless a barcode is attached to a 16 Kb strand (i.e., tagging), mixed with additional tagged strands, and the resulting 100 Kb strands are analyzed. This results are mtDNA strands large enough for the "single-template, random sampling" approaches.

The key points made by Dr. Khrapko include the following:

- Random sampling, single-molecule sequencing approaches may be highly competitive for whole mtDNA sequencing.
- This method is equivalent to the very popular cloning-sequencing approach in mutational analysis.
- These approaches provide the sequence and the "digitized" read-out of the mutant fraction.
- These approaches, if they will be used for mtDNA, need additional testing and development.

Discussion

Dr. Franceschi indicated that there are studies being conducted that should provide more information about the utility of the 454 technique. It is experimental, but it is being used to check a large number of mtDNA samples and is a feasible technique.

Dr. Canter commented that he has used the Mitochip version 2, and problems exist with some of the chips. He said his research group is working with Affymetrix to correct some of these issues.

One of the problems is that the D-loop is over-represented. It is important to consider a head-to-head test of the various chips to find the ones that have the greatest research utility.

RECOMMENDATIONS

Presented to the Group by the Chair and Co-Chair

Dr. Naviaux introduced the session by asking whether any circumstances have arisen where the point mutation frequency in mtDNA is seen without deletion(s)—in other words, are they produced by different factors or similar factors. Dr. Sligh commented that there are different genotoxic stresses that are being subjected to cells and at times one stressor may dominate another. Long-term exposure to ROS associated with aging is a traditional example where the common deletion is the key factor. There are, however, environmental situations where there could be different types of stressors at the same time promoting different base changes; tobacco smoke and the oral cavity is an example. Dr. Wong added that point mutations and deletions are not always seen together, but this may be a function of the detection limitation. Dr. Petros proffered that it may be a function of what one looks for in the investigation. Dr. Khrapko suggested that the two types of mutation are different, and Dr. Franceschi commented that this indicates a need to conduct full genome studies.

Dr. Naviaux presented a summary slide of recommendations made during the previous presentations. As the recommendations were presented, participants commented and suggested revisions. The following represents the results of this discussion, including a list of remaining questions.

Overall Recommendations

A. **Approach**—NCI should consider an RFA for developing a consortium to conduct studies controlled for ethnicity and mtDNA haplogroup. Suggestions for the studies include:

- Three specific tissue types for any mtDNA study: blood, tumor, and normal tissue adjacent to the tumor.
- Case control studies of common cancers (e.g., breast and prostate) and rare cancers; (e.g., thyroid).
- Mitochondrial patient family studies (e.g., nuclear gene mutations; ANS POLG, TP-MNGIE, mtDNA; MERRF, MELAS, NARP). These studies could be in partnership with other NIH Institutes, such as NIDDK and NIA.
- Cancer free high-risk subjects (e.g., elderly smokers, etc.).
- Nested case control studies within existing cohorts.

B. **Data Collection**—(tumor, adjacent normal, blood, with linked demographic data).

- Mitochondrial DNA—assessing SNPs, deletions, and copy number polymorphisms
- Nuclear genes—genes encoding the mitochondrial proteome (including retrograde response genes). Possible target genes include:
    1. oxphos—82 genes

2. non-oxphos mitochondrial proteins—approximately 1500 genes
3. DNA replication and damage repair—approximately 50 genes
4. mtDNA damage copy number control genes—approximately 50 genes (based on yeast studies)

C. Functional/Mechanistic Studies/Resource Facilities

- Centralized cybrid production core facility
- Quantitation of mtDNA mutator phenotype and nDNA instability (e.g., microsatellite changes)

D. Tools We Have

- Mitochip2 and Luminex for mtDNA SNP analysis and haplotyping
- Real-time PCR for mtDNA deletion analysis and copy number polymorphisms (not currently a high-throughput technology)

E. Tools We Need

- Head-to-head test of MitoChip, 454, and Solexa sequencing technologies
- Specialized nDNA chip for genes (and promoters) encoding the mitochondrial proteome
- mtDNA SNP database (such as the one at NCBI)
- Centralized mtDNA mutation database (mitomap.org, NIH, other centralized resources)

Basic Research Questions Needing Possible Attention

1. To what extent do mtDNA mutations cause cancer, or influence cancer risk?
2. What is the impact of cross-talk between nDNA and mtDNA for influencing changes that may increase or decrease the risk of cancer?
3. Is there a benefit for having an mtDNA genome study; will sequencing the mtDNA genome lead to a beneficial understanding of the impact of mtDNA mutations on cancer risk?
4. Is there a need to define what "normal" is regarding mtDNA?
5. How can gene-environmental interactions be determined regarding mtDNA and cancer risk?
6. What is the effect of copy number changes?
7. Have the key mtDNA polymorphisms been identified; do we know enough about their influence on cancer risk?
8. Are there mtDNA mutations that confer reduced cancer risk, even in individuals with a number of risk factors for cancer?
9. Is there a benefit in researching individuals who have reached an advanced age but have not been diagnosed with cancer (i.e., centenarians)?
10. Do we know enough about the genetic mitochondrial diseases?

NCI FUNDING OPPORTUNITIES AND NEXT STEPS

After the discussion was completed and recommendations developed, Dr. Verma informed participants of NCI information pertinent to advancing the study of mtDNA. The following NCI Program Announcements (PAs) and Requests for Applications (RFAs) were presented:

PAR-06-294 Small Grants Program for Cancer Epidemiology (R03)
PA-06-314 Pilot Studies in Pancreatic Cancer (R03)
PA-06-303 Pilot Studies in Pancreatic Cancer (R21)
PA-06-338 Research on Malignancies in AIDS and Acquired Immune Suppression (R21)

CA-07-024 Innovations in Cancer Sample Preparation (R21/R33)
CA-07-023 Innovations in Cancer Sample Preparation (R33)
CA-07-022 Innovations in Cancer Sample Preparation (R21)

Information on these funding opportunities may be found by visiting the NCI Web Site at http://www.cancer.gov/researchandfunding#fundingopportunities.

Dr. Verma thanked participants and the chair and co-chair for a lively meeting. He said that, in the next week, the recommendations will be presented to the NCI Council, which will make a decision on new PAs or RFAs that will be developed by NCI. In addition, the summary proceeding from the workshop will be published.