

Meeting Report

Third International Workshop on Collaborative Interdisciplinary Studies of *p53* and Other Predisposing Genes in Li-Fraumeni Syndrome

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A workshop on LFS² and inherited *p53* mutations was held under the sponsorship of the National Cancer Institute in Bethesda, MD, June 25-26, 1996. Clinical and laboratory investigators summarized the current state of knowledge, presented new data, and extended prior discussions of collaborative clinical and basic research on the syndrome (1, 2).

Joseph F. Fraumeni, Jr. (National Cancer Institute) highlighted unanswered questions regarding LFS. Germline *p53* mutations have been found in approximately one-half of LFS families and in a smaller fraction of LFS-like families who have some features of the syndrome. The mutated gene(s) in other LFS and LFS-like kindreds remain unknown. Explanations are also needed for the wide range of neoplastic phenotypes among family members who have the same germline *p53* mutation and for the seeming differences in penetrance among families. The risk of second primary tumors should be quantified, and the carcinogenic effects of radiotherapy and chemotherapy studied. The challenges of offering genetic counseling and testing and the development of effective interventions are daunting. Laboratory studies using LFS biospecimens should shed additional light on the role of *p53* as "guardian of the genome" through regulation of cell proliferation, repair, and apoptosis. Progress will be speeded through international, multi-institutional collaborations that share patient resources and research findings for this rare disorder.

Descriptive and Molecular Epidemiology Studies

Jillian M. Birch (Royal Manchester Children's Hospital, Manchester, United Kingdom) and Jenny M. Varley (Paterson Institute for Cancer Research, Manchester, United Kingdom) analyzed a series of 30 LFS and LFS-like families. Their LFS families fulfilled the following criteria: proband with sarcoma prior to the age of 45; a first-degree relative with any cancer diagnosed by age 45; and another first- or second-degree relative with any cancer before age 45 or a sarcoma at any age. In

LFS-like families, the proband had a childhood cancer or a sarcoma, bone tumor, or adrenocortical cancer prior to the age of 45; a first- or second-degree relative with a typical LFS component tumor (breast, sarcoma, brain, adrenocortical carcinoma, and leukemia) diagnosed at any age; and another first- or second-degree relative with any cancer diagnosed prior to the age of 60. Most other investigators have used the same criteria for identifying LFS families, whereas LFS-like families differ among institutions.

DNA sequence analyses were performed for *p53* exons 1-11, including 200 nucleotides upstream of the transcription start site in 34 LFS and LFS-like families. Twelve of 19 (63%) LFS and 4 of 15 LFS-like (27%) families had germline *p53* mutations, with predominance of previously described mutations at codons 175, 248, and 273. One LFS family had a 167-bp deletion that removes exon 1 and part of intron 1. Another had a novel germline missense mutation within the tetramerization domain (L344P, Ref. 3). In Saos cells, a mutation in this domain resulted in loss of both G₁ growth arrest and tetramer formation (4). The finding of 2 of the 16 mutations outside the "hot-spot" domains shows the importance of screening the entire gene.

No genotype-phenotype correlations were observed when families with truncating mutations were compared to families with missense mutations. The 16 families with *p53* mutations appeared to have higher frequencies of brain tumors and adrenocortical carcinomas and a decreased frequency of lymphoreticular neoplasms, when compared to families with wild-type *p53*. The two groups of families showed no differences in frequency of sarcomas and breast tumors. However, members of families with *p53* mutations seemed to develop soft tissue sarcomas and breast cancers at earlier ages and as second or subsequent cancers. On the basis of small numbers, codon 248 mutations might be associated with increased breast cancer frequency and R175H with acute leukemia.

LOH studies were performed by restriction enzyme digestion and sequence analysis. Among 35 tumors from 14 families with germline *p53* mutations, 16 (46%) had LOH at chromosome 17p that resulted in loss of the wild-type *p53* allele. However, two tumors showed loss of the mutant *p53* allele as well as other regions of 17p. The combined data from Manchester and the published literature show that six of seven breast carcinomas from family members had 17p LOH. Germline mutation at codon 248 seemed to be associated with 17p LOH in breast cancers but not other cancers in the family.

Laurence Brugières and Jean Feunteun (Institut Gustave-Roussy, Villejuif, France) are conducting a hospital-based family study that is part of a French collaborative registry of germline *p53* mutations. At the Institut Gustave-Roussy, they identified 33 children with multiple primary cancers and 341 children who have solid tumors and a first or second degree relative with a cancer diagnosed under 46 years of age. Blood samples from 132 selected probands were examined for germline *p53* mutations. To date, mutations have been detected in 14

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²The abbreviations used are: LFS, Li-Fraumeni Syndrome; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; TDGS, two-dimensional gene scanning; NER, nucleotide excision repair; NMC, nitrosomethylurea; OAT, ornithine aminotransferase.

patients: 6 of 13 (46%) probands in LFS families, 5 of 58 (9%) in LFS-like families, and 1 of 16 (6%) patients with multiple primary cancers. Studies of other family members revealed germline *p53* mutations among multiple relatives in 10 families, and *de novo* mutations in the probands of 4 families. These four families had some features of LFS: one proband with a medulloblastoma had a cousin who had a neuroblastoma; another, who had a choroid plexus tumor, had a maternal aunt with ovarian cancer; the third, who had osteosarcoma, had a parent with Wilms' tumor; and the fourth had both adrenocortical carcinoma and rhabdomyosarcoma.

Rosalind A. Eeles (Institute of Cancer Research, Royal Marsden Hospital, Sutton, United Kingdom) examined germline *p53* mutations in 57 LFS-like families. Each family had at least two first- or second-degree relatives with two different LFS component tumors diagnosed at any age. Exons 1-11 of the *p53* gene were scanned by SSCP under conditions that reportedly yield $\geq 90\%$ sensitivity (5). Directed DNA sequencing revealed that four (7%) had germline mutations (exon 5 in two probands and in exons 7 and 8 one each). Three of these were missense mutations, and one was a deletion of codon 151. The *p53* mutation frequencies in this series and the Brugières and Feunteun series (see above) are virtually identical (9 percent), but lower than the 25% frequency reported by the Birch and Varley (see above), who used more stringent criteria for LFS-like families.

A review of the published literature suggests a trend toward a slightly lower frequency of *p53* insertion/deletion mutations and transversions in LFS-like families when compared with LFS families. Because few LFS-like families are due to *p53* mutations, other susceptibility genes might be involved. Germline *BRCA2* mutations have been identified in one affected member in each of two LFS-like families who do not have inherited *p53* mutations.³ Whether *BRCA2* mutations cosegregate with cancer in these families is being determined.

David Malkin (Hospital for Sick Children, Toronto, Ontario, Canada) has also studied several LFS and LFS-like families. For LFS families without germline *p53* mutations, any gene encoding proteins involved in the *p53* pathway, including *p21* and *mdm2*, might be candidates to account for the familial clusters of cancer. Preliminary mutation analysis of such genes and appropriate functional assays in more than 50 LFS and LFS-like families from the Hospital for Sick Children Registry are currently in progress.

A study of the psychological and social impact of DNA-based predictive testing for familial cancers is also in progress in Toronto. This prospective study is evaluating parents of childhood cancer patients for their understanding of genetics and genetic testing, as well as the potential impact of predictive genetic testing results. Thus far, 103 sets of parents who have children with cancer, including four LFS/LFS-like families, have been interviewed. Comparisons within groups and between groups are being made to characterize common and distinctive features that may be relevant to the development of guidelines for predictive testing in these kindreds.

Catherine Bonaïti-Pellié (Institut Gustave-Roussy) discussed using Analysis of Risk Corrected for Ascertainment and Age at Diagnosis (ARCAD) to estimate cancer risk among germline *p53* mutation carriers from family data (6). ARCAD allows for mutation carriers to be ascertained through affected relatives and for information on the carrier status of untested

individuals to be calculated through their relatives' genotypes. Ten families in the French Childhood Cancer Study had a proband with both childhood cancer and a germline *p53* mutation, and at least one first- or second-degree relative with cancer diagnosed before the age of 45 years. Estimates of penetrance suggest that 40% of *p53* mutation carriers develop cancer before the age of 16. A higher cancer incidence was found in female family members that was due mainly to breast cancer development between ages 16 and 45 years.

Louise C. Strong (M.D. Anderson Cancer Center, Houston, TX) summarized her hospital-based cohort study of 159 sarcoma patients diagnosed at ages 0-19 years. A segregation analysis of their family history data used an autosomal dominant model and a mixed model. Twelve kindreds showed evidence of familiarity and most of them met the criteria of LFS. Interestingly, the evidence for familiarity was strongest when cancer susceptibility was transmitted from an affected father to the proband; additional studies of patterns of transmission are in progress.

Frederick P. Li (Dana-Farber Cancer Institute) has completed a follow-up study on second cancer risk in 24 LFS families ascertained between 1968 and 1986. Among 200 family members who were followed after development of cancer, 30 developed a second primary cancer, 8 developed a third, and 4 developed a fourth cancer. The cumulative incidence of subsequent cancers is 57% at 30 years after first cancer diagnosis. Breast cancer and sarcomas were the commonest neoplasms and were the first cancer in 104 (52%) of the 200 patients.

Members of families with germline *p53* mutations can develop cancers from causes other than the inherited disorder. Among affected families, a wide spectrum of cancers have developed that might represent rare phenotypes of germline *p53* mutations or chance associations. To identify the rare cancer phenotypes, review was made of the literature and unpublished data from several centers. Among *p53* mutation carriers and their first-degree relatives with 50% likelihood of the mutation, the tumor spectrum included multiple cases of early-onset melanoma; lymphoma; and carcinomas of the stomach, colon, and lung that may represent rare features of LFS.

Laboratory Investigations and Animal Models

Charis Eng (Dana-Farber Cancer Institute) described a novel DNA-based mutation analysis technology that is sensitive and cost-effective and has a high throughput. Loss of function mutations affecting tumor suppressor genes, such as *p53*, are characteristically scattered throughout the length of the gene and have a wide spectrum. Common mutation analysis techniques, which involve exon-by-exon examination, are labor intensive, have varied sensitivity and specificity, and are often expensive. These issues make large-scale mutation analysis in the clinical diagnostic or molecular epidemiological setting impractical.

RBI, the susceptibility gene for hereditary retinoblastoma, comprises 27 exons spanning approximately 180 kb, and mutations are numerous and scattered. TDGS was developed as a single-reaction multiplex PCR-based mutation detection system that discriminates by size (first dimension) and melting characteristics (second dimension) and displays all exons on a single gel. Target amplicons were prepared by an initial 6-plex long-distance PCR, followed by a 25-plex PCR encompassing 26 of the 27 exons, and finally, by a round of heteroduplexing. The final mixture of amplicons was then subjected to size fractionation followed by denaturing gradient gel electrophore-

³ R. Eeles *et al.*, unpublished data.

sis. TDGS was able to detect mutations (and polymorphisms) in a set of DNA from 33 bilateral retinoblastoma patients (7). Preliminary TDGS studies on 90 previously unanalyzed samples from hereditary retinoblastoma patients revealed that more than 83% had mutations.⁴ Thus far, 21 samples that showed TDGS variants have been confirmed to have mutations by sequence analysis. Direct cost per sample is about \$100 for TDGS, technician salary, equipment depreciation, overhead charged at 100%, and directed sequencing of a single exon. In addition to *RB1*, TDGS can be designed for other susceptibility genes, such as *p53*, *BRCA1*, *hMSH2*, *hMLH1*, and *NF1*.

Jean Feunteun (Institut Gustave-Roussy) reported on a functional assay for *p53* that is applicable to analysis of *p53* mutations (8). The template cDNA is placed into an appropriate expression vector by homologous recombination and transfected into yeast cells carrying the *ADE2* gene downstream of a *p53*-response element. Wild-type *p53* results in transcription of *ADE2*, and these yeast colonies are white. Mutant *p53*, which does not interact with the response element, yields the *ADE2*-phenotype colonies, which are red. Advantages of this functional assay include the ability to search for functionally meaningful mutations and to evaluate the level of expression of the transfected *p53* gene. However, validity of this assay is dependent on a stable transcript of the mutant allele, and the mutant protein must be defective in transactivation. The assay will not detect a mutation that only results in aberrant tetramerization, as in a mutation at codon 337, or a nonsense mutation at codon 214 that results in defective *p53* transport from nucleus to cytoplasm and absence of mutant RNA in the cytoplasm.

Curt C. Harris (National Cancer Institute) discussed the functions and interactions of *p53* protein (9). *p53* participates in many cellular functions, including transcription-dependent apoptosis, transcription-independent apoptosis, cell cycle arrest, and NER. NER and transcription have only recently been shown to be linked. Two genes mutated in xeroderma pigmentosum, *XPB* and *XPD*, encode DNA helicases that are involved in NER. *XPB* and *XPD* are two of the six components of TFIIH, a general transcription factor. TFIIH can be recruited to the site of damaged DNA by the XPA protein encoded by a gene that can also be mutated in xeroderma pigmentosum and acts as a repair factor (10). *p53* has been shown to bind to *XPB* and *XPD* *in vitro*. Consistent with these observations, LFS fibroblasts have decreased NER.

To test the hypothesis that *p53*-dependent apoptosis can be modulated by TFIIH, expression vectors containing various *p53* mutant constructs were transfected into primary human fibroblasts. These cells were examined in apoptosis assays and gene expression studies of proteins downstream of *p53*. Missense mutation in codon 517, which is within the *XPB* and *XPD* binding domain, resulted in fewer apoptotic cells when compared with the wild-type construct. *p21* expression was detected in 30% of cells with the wild-type construct. However, *p21* expression (*p53* acts as a transcription factor for *p21*) was found in 80% of cells with the codon 517 mutant construct, reflecting markedly increased transactivation of *p21* but loss of apoptotic potential. Also, injection of wild-type *p53* resulted in abnormal apoptotic responses in *XPB* and *XPD* cells, but not in *XPA* and *XPC* cells. *p21* expression could still be induced. Thus, *p53*-mediated apoptosis appears to be defective in the *XPB* and *XPD* pathways. This defect can be corrected by microinjection

of wild-type *XPB* and *XPD* genes. These and other studies demonstrate that *p53* functions as a guardian of the genome (11). Knowledge of the protean role of *p53* might lead to novel prevention and therapeutic strategies.

James M. Phang (National Cancer Institute, Frederick, MD) described transgenic *p53*-knockout mice as models for studying carcinogenesis and chemoprevention. *p53*-knockout mice (C57BL/6) have increased cancer incidence and mortality. Cancers occur more frequently and at earlier ages in homozygous null mice than in hemizygotes (12). *p53*-null mice showed an increased survival when subjected to 60% calorie reduction, when compared to similar mice with *ad libitum* oral intake ($P \leq 0.001$; Ref. 13). In chemoprevention studies, *p53*-null mice were given one of the following agents: α -limonene (an inhibitor of farnesyl transferase and modulator of G proteins), all-trans retinoic acid, quercetin (a flavanoid), dehydroepiandrosterone, a sex steroid hormone precursor, or placebo. Only dehydroepiandrosterone prolonged survival, with reduced deaths from lymphomas but not sarcomas (14). Hemizygous *p53* knockout mice with low rates of spontaneous tumorigenesis were examined for susceptibility to NMU, a directly acting carcinogen. NMU induced more tumors in hemizygous null mice as compared with wild-type mice. However, *p53* mutation rates as assessed by SSCP analysis were similar in NMU-SSCP treated hemizygotes and NMU-treated wild-type mice (15).

Additional studies showed that *p53* may be involved in the regulation of "housekeeping enzymes," such as OAT. OAT plays a central role in routing carbon transfer between the urea cycle and the tricarboxylic acid cycle and is modulated by dietary protein and glucose. Caloric (carbohydrate) restriction is associated with increased OAT expression in wild-type mice, but this response is not observed in *p53*-knockout mice (16), suggesting that the dietary regulation of hepatic OAT may involve *p53*.

Genetic Counseling and Testing

Judy E. Garber (Dana-Farber Cancer Institute) updated the *p53* Predisposition Testing Program for at-risk members of LFS families with known mutations. To date, 83 individuals have been invited to participate, and 27 (33%) have entered this research program by completing the initial in-person counseling program visit. Fifteen of them have received results during a subsequent visit. There were no differences between participants and nonparticipants in demographic factors, and number of first-degree relatives with cancer. At study enrollment, 62% of participants had health insurance, 37% had life insurance, and 19% had disability insurance. Fifty-two % made one or more visits to their doctors in the preceding year. Overall, 35% visited doctors at least once a year, whereas 65% visited their doctors every 2-5 years. Among individuals at 50% risk of inheriting their family-specific mutation, only 18% were found to be mutation positive. The likely explanation is that participation was first offered to older unaffected relatives, after excluding family members who had already developed cancer. Prior to receiving results, 19% believed that they were carrying an altered gene, 15% felt they were not, 23% had no opinion, and 43% were unsure. Few would share test results with their physicians.

Katherine A. Schneider (Dana-Farber Cancer Institute) presented preliminary data from the genetic counseling component of the *p53* Predictive Testing Program. The average age of participants was 38 (range, 20-75). One-third have a high school diploma only, and the remainder have at least some college education. On a baseline knowledge questionnaire, the

⁴ C. Eng *et al.*, unpublished data.

average score was 68%, with the fewest correct responses in the cancer genetics subscale. Ninety-three % of participants knew which of their first-degree relatives had been diagnosed with cancer and correctly identified the site of cancer. There was decreased knowledge of more distant relatives, particularly regarding cancer site. All but one participant had a 50% *a priori* risk of carrying a *p53* mutation, and almost all could correctly state their cancer risk. However, perceptions of risk ranged from "low" to "very high." None reported any prior insurance problems because of their family history, but one-third expressed "high concern" about possible discrimination based on test results. The majority of participants with children would like their offspring tested. Although the Dana-Farber program is limited to adults, one-third of the participants have children under 18.

Karen H. Rothenberg (University of Maryland School of Law, Baltimore, MD) discussed the legal and ethical aspects of genetic testing. Some of these issues are global, whereas others are specific to the United States. Issues of concern include informed consent, discrimination, privacy, confidentiality, family rights and responsibilities, and liability. There are benefits and risks determined by the biological characteristics of the gene and resultant cancers and by the clinical utility of the genetic findings. Potential benefits of genetic testing may include relief of anxiety, early cancer detection through effective surveillance, and the possibility of sharing genetic information with family members. Potential risks include increased anxiety, change in self image, lack of privacy or confidentiality, altered familial relations, social stigma, and various aspects of legal and employment discrimination. However, the value of predictive testing for many inherited cancer syndromes, including LFS, is unknown. There are limited clinical data on the effectiveness of interventions, and the impact of predictive testing on health behavior is unknown. Finally, there might be unforeseeable risks and benefits.

Laws in at least 13 states prohibit, to varying degrees, health insurers from using genetic information in underwriting policies. However, most of these laws focus rather narrowly on the genetic test itself. Most do not include genetic information obtained by other means, such as family history. In addition, self-funded insurance proposals are exempt from these state laws. A number of federal insurance plans have been introduced that address, to varying degrees, genetic discrimination and privacy issues.

For genetics caregivers, the standard of care is still evolving. Uncertainties exist regarding the extent to which there is a duty to disclose both the benefits and the risks of testing, to decide when to disclose or not to disclose test results to minors, the duty to warn patients of genetic transferability, the duty to keep genetic information confidential, and the duty to provide appropriate follow-up and recontact when necessary.

Caryn Lerman (Georgetown University, Washington, DC) is conducting prospective observational studies of uptake and impact of *BRCA1* testing in at-risk male and female members of families with inherited *BRCA1* mutations; her results may be relevant to testing for *p53* and other inherited susceptibility genes. Using families from the Creighton Registry maintained by Dr. Henry Lynch, *BRCA1* testing has been offered to 279 at-risk members of families with *BRCA1* mutations and one *BRCA1*-linked family (multipoint logarithm of odds score 6.2; Ref. 17). After the initial letter of introduction, telephone interview, and family information session, approximately 60% requested results and 40% declined. The rate of uptake for *BRCA1* results was significantly higher among females than males (66% versus 48%), those with education beyond high school, those with health

insurance, and those with higher numbers of first degree relatives with breast cancer.

At 1 month postdisclosure, individuals with negative results (no *BRCA1* mutation) showed significant reductions in both distress and functional impairment when compared with those who declined testing and individuals with a *BRCA1* mutation. Overall, however, those with a *BRCA1* mutation did not show further increases in distress or impairment of functioning at 1 month follow-up. Subgroup analyses have suggested that some who were *BRCA1* mutation positive, especially those who were unmarried, may be at higher risk of adverse psychological consequences. These preliminary results suggest that uptake of offers of genetic results is lower than expected, and that learning genetic results may have short-term psychological benefits for members of high-risk families who test negative. However, these study subjects were a relatively homogeneous group that is well educated about breast cancer genetics. Future studies should examine the long-term impact of testing and identify determinants of responses to genetic information.

Alfred G. Knudson, Jr. (Fox Chase Cancer Center, Philadelphia, PA), the summary speaker, noted, "[T]his is a fascinating time in the history of LFS and cancer genetics in general. Basic questions remain, such as the operational definitions of LFS and LFS-like syndrome. The relationship between LFS and inherited *p53* mutations can be visualized as two partially overlapping circles. The overlap is approximately 50% in LFS families and as reported by meeting participants, 5–25% in the more heterogeneous LFS-like families. The possibility exists that subtle *p53* mutations have been missed in standard DNA assays. Several speakers have also suggested other candidate genes, such as *BRCA2*, might account for the remaining familial cancer clusters. A fruitful strategy might be to focus on genes involved in functional *p53* signaling pathways. In this regard, validated functional assays have the advantage of simultaneously examining the products of multiple genes."

Knudson noted the frustrations of investigators and clinicians in providing care to LFS families. No standard protocol appears to exist regarding medical surveillance of at-risk relatives, a problem that also exists in varying degrees for other disease susceptibility genes. No effective interventions exist for Huntington disease. On the other hand, hereditary retinoblastoma can be diagnosed through surveillance of neonates with a family history of the disease, leading to early treatments that help preserve eyesight. Genetic testing for *RB1* mutations in siblings can also provide additional information that spares noncarrier infants from multiple examinations under anesthesia. Although such interventions are not available presently to families with *p53* mutations, the situation could improve rapidly. An example is the active program of screening and early detection that became available after cloning of the *VHL* gene for Von Hippel-Lindau disease.

Summary and Future Studies

Dr. Louise Strong led the discussion to plan future studies. Meeting participants agreed on the need to pool resources because of the rarity of LFS families. A multidisciplinary group that pursues both molecular and clinical studies can hasten accrual of new knowledge. Workshop participants identified a series of projects that could answer some of the questions raised in this meeting. Investigators who have not participated in the workshops will be sought as future collaborators.

Eight research issues were identified for collaborative studies:

- (a) No universal criteria exist presently for LFS and LFS-like families, and proposed criteria should be developed and presented at the next meeting in November 1997 (Manchester, United Kingdom).
- (b) Data are needed on penetrance and genotype-phenotype correlations of various *p53* mutations. Comparisons should be made of the characteristics (age at cancer diagnosis, sex, and tumor site) of families with *p53* mutations, and those with no known mutations.
- (c) Risk of second tumors by age, sex, therapy for the first cancer, and mutation status should be determined.
- (d) Linkage analyses are needed for families without germline *p53* mutations.
- (e) Comparisons should be made of sensitivity and specificity of various mutation detection techniques (SSCP, two-dimensional gene scanning, functional assays, apoptosis assays, Saos assay, and DNA sequencing), using a common set of specimens.
- (f) Development of a common database is needed, along with a computerized registry.
- (g) Collaborative studies should examine the frequency of *de novo* mutations and the association between tumor-specific mutations and LOH.
- (h) Investigators should develop a common genetic predisposition counseling and testing protocol and recommendations for screening and surveillance.

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