Genetic Analysis of Children of Atomic Bomb Survivors

Chiyoko Satoh,1 Norio Takahashi,1 Jun-ichi Asakawa,1 Mieko Kodaira,1 Rork Kuick,2 Samir M. Hanash,2 and James V. Neel3

1Department of Genetics, Radiation Effects Research Foundation, Hiroshima, Japan; 2Department of Pediatrics; and 3Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan

Studies are under way for the detection of potential genetic effects of atomic bomb radiation at the DNA level in the children of survivors. In a pilot study, we have examined six minisatellites and five microsatellites in DNA derived from 124 children. We detected a total of 28 mutations in three minisatellite loci. The mean mutation rates per locus per gamete in the six minisatellite loci were 1.5% for 65 exposed gametes for which mean parental gonadal dose was 1.9 Sv and 2.0% for 183 unexposed gametes. We detected four mutations in two tetranucleotide repeat sequences but no mutations in three trinucleotide repeat sequences. The mean mutation rate per locus per gamete was 0% for the exposed gametes and 0.5% for the unexposed gametes in the five microsatellite loci. No significant differences in the mutation rates between the exposed and the unexposed gametes were detected in these repetitive sequences. Additional loci are being analyzed to increase the power of our study to observe a significant difference in the mutation rates at the 0.05 level of significance. — Environ Health Perspect 104(Suppl 3):511–519 (1996)

Key words: children of atomic bomb survivors, germ cell mutation, ionizing radiation, mutation rate, minisatellites, microsatellites, 2-dimensional gel electrophoresis (2-DE), DNA, single copy sequence, human doubling dose

Introduction

Extensive studies of the children of survivors of the atomic bombings in Hiroshima and Nagasaki have thus far yielded no statistically significant increases in genetic effects compared to a control population (1–8). Recently, the feasibility of detecting radiation-induced mutations at the DNA level has been explored (9–11). As samples for further screening, we have established cell lines from the peripheral B lymphocytes of over 800 families composed of father–mother–child trios. In half of the families, one or both parents were exposed to A-bomb radiation of more than 0.01 Sv (gonadal dose), whereas the other half is composed of control families in which parents were not exposed or exposed to less than 0.01 Sv.

DNA sequence analysis can detect both nucleotide substitutions and insertion/deletion/rearrangement (I/D/R) mutations. However, its efficiency is too low considering that 1.2 × 1010 nucleotides must be examined not only for the children of the exposed parents but also for the control children in order to detect a significant difference in the mutation rates between the two groups of children (11). On the other hand, a single scanning technique capable of detecting a sequence difference in a fragment of hundreds of nucleotides is able to detect only one of the two types of mutations. Therefore, we have introduced and modified two types of scanning techniques for our studies, one for nucleotide-substitution mutations and one for I/D/R mutations. We have improved efficiencies of techniques such as the RNase A method (12) and the modified denaturing gradient gel electrophoresis (DGGE) method (13,14). Although we confirmed that these approaches were effective for detecting nucleotide substitutions, small deletions, and insertions, the efficiencies of these techniques were still too low to study the huge number of nucleotides estimated necessary to detect significant differences between the two groups of children.

For the detection of the I/D/R mutations believed to predominate among radiation-induced mutations, we chose two types of DNA as targets: repetitive sequences, such as minisatellites and microsatellites, and single copy sequences. Minisatellites or VNTRs (variable number of tandem repeats) (15,16) are dispersed throughout the human genome and show a strong tendency to cluster in telomeric regions (17). Minisatellites consist of short-sequence units iterated in tandem to form arrays and show substantial allelic variation in the number of repeat units. At several minisatellite loci, extreme variabilities were observed that were associated with high mutation rates for new length alleles in the germline (18). The mutation rates at these minisatellites were measurable by pedigree analysis using the standard Southern blotting technique.

Microsatellites consist of around 10 to 50 copies of motifs from 2 to 6 nucleotides long that can occur in perfect tandem repetition, as imperfect repeats, or together with another repeat type. They are highly polymorphic in copy number and are randomly distributed in human DNAs, and they occur frequently. The aberrant expansion of exonic trinucleotide repeats has recently been found to result in several genetic diseases such as fragile X syndrome (FRAXA) (19–21), myotonic dystrophy (DM) (22,23) and spinobulbar muscular atrophy (SBMA) (24). Among normals, triplet repeats such as CGG, CAG, and CAG in fragile X mental retardation-1...
(FMR-1), DM, and androgen receptor (AR) genes, respectively, which are causative for these diseases, are highly polymorphic in number. In patients, the triplet repeats are expanded well above the normal range. The numbers of repeats may differ among affected members of a given family or even among the cells of one individual (25,26). This diversity in the repeat number is thought to be caused by the instability of the triplet repeats in meiosis and mitosis, but the mechanism of this aberrant expansion has yet to be discerned. High spontaneous mutation rates in some of the tetranucleotide repeats are also reported (27,28). We have examined minisatellites and microsatellites to determine whether atomic bomb radiation affected the instability of these repetitive sequences.

For the detection of the ID/R mutations in single copy sequences, we have introduced a new two-dimensional gel electrophoresis (2-DE) approach termed restriction landmark genome scanning, reported by Hatada et al. (29). Without using probes, this method provides over 2,000 DNA fragments (spots) from a genomic DNA digest on a single gel. We use NotI as one of three restriction enzymes to digest the DNA, and the resulting NotI sites, which are frequent in the unmethylated CpG islands are labeled with 32P. This strategy is thought to assure that a high proportion of visualized fragments originate from active genes (30). Because a fresh mutation would usually be detected in a heterozygote with one normal and one mutated allele and only the normal allele would be at the usual position, a quantitative analysis searching for a 50% decrease in spot intensity is required.

In this report we describe results obtained in the pilot studies on minisatellites and microsatellites. In addition, we also describe the efficiency observed in a preliminary study on the 2-DE technique as a screen for mutations because the 2-DE technique seems promising for the screening of a large number of nucleotides, which is our stated goal.

Materials and Methods

Families and DNA Samples

We studied 50 exposed families and 50 control families. These 100 families are a subsample of approximately 800 families from Hiroshima and Nagasaki consisting of father, mother, and all available children from whom permanent cell lines have been established by using Epstein-Barr virus transformation of peripheral B lymphocytes. In these 800 families, all parents were younger than 25 years of age at the time of the bombings. In each of the families, one or both parents belong to the Adult Health Study cohort that is monitored biennially by our institution (31) and at least one of the children has been examined for protein mutations in a previous study on the children of the atomic bomb survivors (4). In 400 families, termed the exposed group, one or both parents received A-bomb radiation of more than 0.01 Sv (gonadal dose). For the integration of two types of radiation released by the atomic bombs (predominantly gamma and a small neutron component) into a single figure expressed in Sv, we employed a value of 20 as the relative biological effectiveness (5–8). The other 400 are control families in which one or both parents were exposed to less than 0.01 Sv or were not in Hiroshima or Nagasaki at the time of the bombing.

Table 1 summarizes the numbers of children of the 100 families belonging to the exposed and the control groups and the numbers of gametes derived from the exposed parents and the unexposed parents. Among 65 exposed gametes, 33 were maternally exposed and 32 were paternally exposed, with mean doses for gametes being 1.7 Sv and 2.1 Sv, respectively. Among 63 unexposed gametes in the exposed group, 31 and 32 gametes were derived from mother and father, respectively. Because most of the children from the exposed families in our study were born more than 10 years after the bombings, these children are assumed to be derived from gametes irradiated at the spermatogonial stage or the oocyte stage.

DNA samples were extracted from cell nuclei of the cell lines or peripheral lymphocytes as previously described (32). For screening purposes, DNA samples extracted from the cell lines were used. A portion of each of the samples was stored separately for checking abnormal results. For the confirmation of mutations, DNAs extracted from granulocytes or lymphocytes that had not been treated with Epstein-Barr virus were used.

Minisatellite Probes

We used five human minisatellite probes (λTM-18, ChdT-C15, pAg3, λMS-1, and CEB-1) and one mouse minisatellite probe (Pc-1) for this study. Each probe detects a single hypervariable minisatellite locus with multiple-length alleles. The Pc-1 (33), λTM-18 (34), and ChdT-C15 (35) probes were kindly provided by Ryo Kominami of Niigata University. Gilles Vergnaud of Centre d’Etudes du Bouchet provided the CEB-1 (36) probe, and Alec J. Jeffreys of the University of Leicester provided the λMS-1 (18). For detecting alleles at the pAg3 locus, we used a DNA fragment amplified by the method of Jeffreys et al. (37).

Southern Blotting

Samples of DNA (5 μg) were digested with HindIII (New England Biolabs, Beverly, MA) or AluI (Takara, Kyoto, Japan) and electrophoresed on a 25-cm-long 1.0% agarose gel (Agarose type I; Sigma Chemical, St. Louis, MO). Separated DNA fragments were transferred to BAS85 nitrocellulose filters (Schleicher & Schnell, Dassel, Germany). The filters to be hybridized were used sequentially with all probes; before reuse of the filters, the preceding probe was removed by submerging the filters in 20 mM NaOH for 15 min at room temperature.

Each probe was labeled with α-32P-dCTP (110 TdQ/mml, 370 MBq/ml) by using the multiprime labeling system (Amersham International, Amersham

| Table 1. Summary of the 100 families examined in the pilot study. |
|------------------------|------------------------|------------------------|------------------------|
| Exposure group        | Number of families     | Number of children     | Parental exposure status (≥0.01 Sv; RBE = 20) |
|                       |                        |                        | Exposed parents     | Unexposed parents |
| Exposed               | 50                      | 64                     | Both parents exposed (n = 1) | 2                  | 0                   |
|                       |                         |                        | One parent exposed (n = 63) | 63                 | 63                  |
| Control               | 50                      | 60                     | Neither parent exposed | 0                  | 120                 |
| Combined              | 100                     | 124                    |                         | 65                 | 183                 |

RBE, relative biological effectiveness. *In families with only one exposed parent, the unexposed parent (exposed to less than 0.01 Sv or was not in Hiroshima or Nagasaki at the time of the bombings) was included among unexposed parents in this study. **Parents in the control group were exposed to less than 0.01 Sv or were not in Hiroshima or Nagasaki at the time of the bombings. ***62 females and 62 males.
Place, U.K.). Hybridization was performed as previously reported (38). Filters were washed three times at 65°C in 0.5× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate) and exposed on Fuji X-ray film (Fuji, Kanagawa, Japan) with a DuPont Gronex H1-Plut intensifying screen (Wilmington, DE).

**Microsatellite Analysis**

Sequences including the repeats were amplified by polymerase chain reaction (PCR) and electrophoresis of the products was carried out on a polyacrylamide sequence gel. The repeat number in the products was deduced from the lengths of bands by comparing with the M13 sequence ladder and confirmed by sequence analysis.

For amplification of the sequences with CTG repeats in the DM genes, we used primers 453 (sense) and 454 (antisense) (primers are designated by numbers assigned within our laboratory) whose sequences are identical with those used by Fu et al. (22). By using these primers, the PCR product having a sequence with 5 CTG-repeats was 78-base pairs (bp) long. The PCR reaction mixture, final volume 12.5 μl, contained 50 to 150 ng genomic DNA; 0.3 unit Ampli-Taq DNA polymerase (Perkin-Elmer Cetus, Foster City, CA); 200 μM each deoxyribonucleoside triphosphates (dNTP; dATP, dCTP, dTTP, and dGTP); 50 mM KCl; 1.5 mM MgCl2; 10 mM Tris–HCl (pH 8.3); 0.5 μM each primer, 0.01% gelatin; and 7.4×10−6 M of α-32P-dCTP. After denaturation of genomic DNA by heating at 95°C for 10 min, 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 90 sec, and elongation of DNA at 72°C for 60 sec were performed in a programmable thermal controller, PTC 100 (MJ Research, Watertown, MA). The final elongation step was prolonged for an additional 7 min. After the addition of an equal volume of stop solution (86% formamide, 17 mM ethylenediaminetetraacetate (EDTA), 0.04% bromphenol blue, 0.04% xylene cyanol FF), the mixture was heated for 5 min in boiling water, cooled in ice water, and immediately applied to a 6% polyacrylamide gel containing 7 M urea. Electrophoresis was carried out for 18 hr and autoradiography was performed in the same manner as that for the Southern filters, except a Fuji Green Emitting Intensifying Screen (Fuji Photofilm, Tokyo, Japan) was used instead of a DuPont Screen.

We used primer 443, 5'-TCCA-GAATCTGTTCCAGAGCGTGTC-3' (sense), which is identical to one of the primers used by La Spada et al. (24), and primer 446, 5'-CAGGACCGGTAC-CTGTGGG-3' (antisense) for amplification of the sequences with CAG repeats in the AR genes. By using this primer set, an amplified sequence with 16 CAG triplets was 245-bp long. Except for the primers, the reaction mixture and the conditions for the PCR amplification were identical with those used for the DM gene.

For amplification of the sequences with CGG repeats in the FMR-1 genes, we used 449 and 452 primers whose sequences are 5'-GGCGTCAGCTCCGTGCC-3' (sense) and 5'-TCCTCATCTTCTCTCAGGC-3' (antisense), respectively, which are slightly different from those of Kremer et al. (39). By using this primer set, an amplified sequence with 16 CGG repeats was 248-bp long. In the reaction mixture, 7-deaza-2'-deoxyguanosine 5'-triphosphate (7-deaza-2'-dGTP) was used instead of dGTP and 10% dimethyl sulfoxide (DMSO) was added. In the PCR, the temperature cycle number was increased to 27 and the annealing temperature was decreased to 55°C.

For amplification of the sequences composed of different numbers of CTTT repeats and CTTT repeats, which we describe as CCTT/CTTT repeats, in the macrophage colony-stimulating factor 1 receptor (CSF1R) genes, hemi-nested PCR was performed following the method of Hästbacka et al. (27). The three primers 453, 464, and 465 have identical sequences with primers used by these investigators. A first PCR was performed with primers 463 and 464 using a reaction mixture identical to that used for the DM gene, but without α-32P-labeled dCTP, for 27 cycles in the Iwaki thermal sequencer TSR 300 (Iwaki Glass, Tokyo, Japan). The cycling conditions were: 95°C for 75 sec, 66°C for 160 sec, and 72°C for 100 sec followed by a final 300 sec extension step at 72°C. Using 1 μl of the amplification product from the first PCR reaction mixture as template, a second PCR was performed in a thermal controller (MJ Research) in a final reaction mixture of 10 μl using primers 463 and 465. The concentration of each material in the reaction mixture was identical with that used for the DM gene amplification. We found that some individuals had a 107 bp sequence with 4 CTTT repeats but no CCA repeats or a 111 bp sequence with 5 CTTT repeats and no CCA repeats. These short sequences could not be effectively amplified under the conditions optimal for amplification of the sequences longer than 194 bp containing CCA/ CTTT repeats. Thus, we made two 10-μl reaction mixtures using two 1-μl aliquots from a single first PCR reaction mixture, and these were employed in the second PCR under different conditions. For longer sequences, the cycling conditions after 5 min at 95°C were 26 cycles: 95°C for 35 sec and 71°C for 150 sec followed by a final 7-min extension step at 72°C. Electrophoresis was carried out for 18 to 20 hr. For short sequences, after 10 min at 95°C, the cycling conditions for 28 cycles were 95°C for 35 sec, 68°C for 120 sec, and 72°C for 70 sec, followed by a final 7-min extension step at 72°C. Electrophoresis was carried out until the xylene cyanol-band moved to the position of 39 cm from the origin.

Primers 468 and 469, identical to those used by Hästbacka et al. (27), were used for amplification of sequences including TAGA repeats in the CSF1R gene. By using this primer set, an amplified sequence with 16 TAGA repeats was 192-bp long. After 10 min at 95°C, the cycling conditions (26 cycles) were 95°C for 35 sec, 67°C for 60 sec, and 72°C for 30 sec, followed by a final 7-min extension at 72°C in the thermal controller (MJ Research).

**Sequencing of Trinucleotide and Tetrinucleotide Repeats**

Fragments to be sequenced were amplified following the methods used for the amplification of the sequences in the screening for mutations, but the final volume was scaled up to 100 μl and α-32P-dCTP was excluded. The second PCR was carried out using 10 μl of the amplification product from the first PCR reaction mixture as template in a final reaction mixture of 100 μl for each of several reaction mixtures. After the second PCR, reaction mixtures from several tubes were combined and electrophoresis was carried out on 3% agarose (2% NuSieve GTG agarose and 1% SeaKem LE agarose; FMC Bio Products, Rockland, ME) gel using Tris-borate-EDTA (TBE) buffer. After staining DNA fragments with ethidium bromide, bands to be sequenced were cut from the agarose gel, and the fragments were extracted using a QIAEX gel extraction kit according to the QIAEX DNA gel extraction protocol (DIAGEN, GmbH; Hilden, Germany/QIAGEN, Chatsworth, CA). Purified DNA fragments were sequenced using the dsDNA cycle sequencing system obtained from...
GIBCO BRL (Gaithersburg, MD) following their protocol.

2-Dimensional Gel Electrophoresis

DNA samples were extracted from cell lines derived from three father–mother–child trios. None of the parents had been exposed to the A-bomb radiation. Experimental conditions for the sample preparation and 2-DE, data collection and analysis, and construction of a genomic DNA library were carried out following the methods described in our previous reports (30,40,41). In short, genomic DNA was digested with NotI and EcoRV and the NorI-derived 5' protruding ends were α-32P-labeled. These fragments were electrophoretically separated in an agarose disc gel, which was subsequently treated with HindIII in situ. The resulting fragments were separated in a 5.25% polyacrylamide gel (33 cm × 46 cm × 0.08 cm). Autoradiograms were obtained and digitized with a Kodak charge-coupled device camera. Software to detect and quantify DNA fragments and software for the camera were obtained from BioImage (Ann Arbor, MI).

Results

Mutations at Minisatellite Loci

We examined HindIII digests of DNA from members of 50 exposed families and 50 control families (Table 1) for mutations by probing with the five human minisatellite probes and one mouse minisatellite probe, Pc-1. The Pc-1 probe can detect a mouse locus identical to the Msh6-hm (42) and cross-hybridize with a human minisatellite locus. The loci that can be detected with these probes are denoted as λTM-18, ChdTC-15, pAg3, λMS-1, CEB-1, and Pc-1. Because each probe detects a single locus with multiple-length alleles and because heterozygosities for these six loci range between 70% (for Pc-1) and 97% (for λMS-1 and CEB-1) (Table 2), each allele in children can be traced back to one parent. We compared the bands of the children with those of their parents and identified mutant bands when bands with identical lengths were absent in both parents. Whenever we detected the mutant bands in children, we confirmed these assignments by retesting these families using DNA digested with AluI, which cuts at positions outside the repeat-unit block that are different from the HindIII digestion sites. To exclude the possibility that mutations could have been generated during establishment and proliferation of the cell lines, we examined HindIII digests of DNA from granulocytes or lymphocytes from members of these families. For all mutations, we observed mutant bands identical to those detected in the cell-line DNA of the children, confirming that the mutations almost certainly occurred in the germ cells of one parent.

The results of these studies are summarized in Table 2. In screening 124 children for mutations, we detected 1, 12, and 15 mutations at the pAg3, λMS-1, and CEB-1 loci, respectively. The mean mutation rate (mutations/locus/gamete) in these minisatellite loci was 1.5% [6+ (65 × 6)] in the exposed gametes and 2.0% [22 + (183 × 6)] in the unexposed gametes. We observed no significant difference in the mean mutation rates for six minisatellite loci between the exposed gametes and the unexposed gametes (p = 0.37, Fisher’s exact probability test). In our analysis, detectability of mutations depends on the length of bands because gains or losses of small numbers of repeat units in large alleles is difficult to detect. There was no significant difference between the mean values of the lengths of bands at each of the six loci derived from the two groups of parents (data not shown); however, small deletions may have escaped detection in these analyses.

Mutations at Microsatellite Loci

In 124 children of the 100 families, we determined the number of trinucleotide repeats in the DM, FMR-1, and AR genes that, when expanded, are known to be responsible for DM, FRAXA, and SBMA, respectively. The DM gene is on chromosome 19 and the FMR-1 and the AR genes are on the X-chromosome. We also examined two types of tetranucleotide repeats in the CSFRI gene that is identical with c-fms on chromosome 5, which encodes the macrophage colony stimulating factor 1 receptor. We compared bands of PCR-amplified sequences that include trinucleotide or tetranucleotide repeats from children with those from their parents and identified mutant bands when bands with identical lengths were absent in both parents. When we detected apparent mutant bands in the children, we confirmed the assignments by retesting the families using the second aliquot of the samples separately stored and examining samples of DNA prepared from granulocytes or lymphocytes that had not been immobilized.

In the examinations of trinucleotide repeats, we detected several children who showed abnormal bands that were not identical with any of their parental bands, when DNA samples extracted from the cell lines were examined. However, when DNA samples from untransformed lymphocytes or granulocytes were examined, the abnormal bands were absent. Therefore, the abnormal bands must have resulted from mutations that occurred in the process of transformation or in succeeding cultures, or parental lymphocytes had been mosaic and cells with one type of alleles were selectively amplified in the cell culture process. Thus, we detected no mutations in the trinucleotide repeats in the three genes (Table 3).

The tetranucleotide repeat in intron 2 of the CSFIR gene was composed of CCTT repeat and CTTT repeat. A sequence including CCTT/CTTT repeat was amplified by PCR and products of single families were compared. Two mutations that resulted in decreased band length were detected. The first, detected in a female child from Nagasaki, seemed to be derived from her mother who had received a gonadal dose of less than 0.01 Sv and had been classified as an unexposed parent. The second mutation, also detected in a female Nagasaki child, originated from her mother whose gonadal dose was again less than 0.01 Sv. Thus, the mutation rate was 1.1% per gamete in the unexposed parents. We detected two additional mutations that resulted in an increased number of TAGA repeats in intron 6 in two children from

| Locus        | Mutation rates  | Heterozygosity (%) |
|--------------|-----------------|--------------------|
| Exposed gametes | Unexposed gametes |                   |
| λTM-18       | 0/65            | 0/183              | 77                     |
| ChdTC-15     | 0/65            | 0/183              | 95                     |
| pAg3         | 1/65 (1.5%)     | 0/183 (0%)         | 92                     |
| λMS-1        | 1/65 (1.5%)     | 11/183 (6%)        | 97                     |
| CEB-1        | 4/65 (6.2%)     | 11/183 (6%)        | 97                     |
| Pc-1         | 0/65            | 0/183              | 70                     |

*Heterozygosity for each locus was determined by counting the number of heterozygotes among 200 unrelated parents in the 100 families.
Table 3. Mutation rates at five microsatellite loci.

| Locus | Chromosome | Repeating unit | Exposed gametes | Unexposed gametes |
|-------|------------|----------------|-----------------|------------------|
| DM    | 19         | CTG            | 0/65            | 0/183            |
| FMR1  | X          | C3G            | 0/56            | 0/130            |
| AR    | X          | CAG            | 0/56            | 0/130            |
| CSF1R | 5          | CCT/CTTT       | 0/65            | 2/183 (1.1%)     |
| CSF1R | 5          | TAGA           | 0/65            | 2/183 (1.1%)     |

Hiroshima families. We could not define parental origins of these mutations, but both were from unexposed families (Table 4). Thus, these were spontaneous mutations, and the mutation rate at the TAGA repeat locus was also 1.1% per gamete in the unexposed parents. These four mutants were also detected in DNA from untransformed lymphocytes or granulocytes.

As shown in Table 3, we detected no mutations in a total of 307 alleles derived from the exposed gametes and four mutations in 809 alleles derived from the unexposed gametes. The mean mutation rates in these five microsatellite loci were 0% for the exposed gametes and 0.5% for the unexposed gametes. Thus there was no significant difference in the mean mutation rate for the five microsatellite loci between the children of the exposed and the unexposed parents.

Preliminary Results Obtained by 2-Dimensional Gel Electrophoresis

By choosing three restriction enzymes, NotI, EcoRV and HinfI, approximately 2,000 DNA fragments (0.3–2.0 kb) from a single DNA sample were separated and visualized as spots by autoradiography on a polyacrylamide sheet gel (Figure 1). After optimization of experimental conditions, gel patterns were of such quality that duplicate electrophoretic patterns of spots derived from a single DNA sample were superimposable. In principle, this system will detect two types of genetic variations: variations due to gain or loss of a cut site for the three restriction enzymes used to digest DNA, and variations due to I/D/R events.

The intensity of any spot on the gel is, in the usual case, expected to be determined by two homologous DNA fragments; however, in a heterozygote with a normal allele and a hereditary variant allele or a mutant allele whose length is different from that of the normal allele, only one DNA fragment would be at the usual position and the intensity of this spot should be decreased by about one-half. The variant or the mutant fragment may migrate to an altered position on the gel as a new spot, not enter the gel, or migrate off the gel. New spots may appear on the gel as a result of change in a fragment that does not normally appear on the gel. Some I/D/R events could eliminate a second fragment.

We have analyzed DNA samples from three mother–father–child trios. For each sample, gels were prepared in duplicate and autoradiograms were analyzed at the University of Michigan Medical School. Of approximately 2,000 spots on the autoradiogram, 774 spots were selected as potential candidates because they were distinct, they were not near the margins of the gel, and they were not one of the very large spots on the gel. As a measure of the variation in the integrated density of each spot (i.e., in spot intensity), we employed a

![Figure 1. Digital image of the autoradiogram of a 2-DE gel. The fragment sizes in each dimension are indicated. On the basis of published DNA sequences, the position of many of the very intense spots matches the predicted position of ribosomal DNA fragments or Epstein-Barr virus fragments.](image-url)
which goes with normal study coefficient of variation (CV), obtained by dividing the square root of the unbiased estimator of the variance by the mean spot intensity for each set of nine gels; we used the average of the two intraset CVs as a single measure of spot reproducibility. Among the 774 spots, 482 spots were selected because the average CV for their intensities for the 2 sets of 9 gels was less than 0.12. In a system in which the CV intensities of the spots is less than 0.12, a spot whose intensity is 50% of the normal value should be detectable. We have detected heterozygotes with normal and deleted alleles using this criterion in our previous screening for enzyme deficiency variants (43) and in a study to detect heterozygous carriers of a deletion in the families of Duchenne muscular dystrophy patients and of a hemophilia B patient (32). Thus, the 482 spots, which include 43 spots showing genetic polymorphisms, are suitable to detect mutations; however, we detected no mutations among the spots of three children of the three trios. When we rely on these spots, the number of base pairs that can be screened on a gel from one individual will be $2.5 \times 10^6$ bp ($= 500 \times 2,500$ bp $\times 2$), where the average size of each spot is estimated to be 2,500 bp.

Two polymorphic systems whose spots are included in the 43 spots are shown in Figure 2; each of the six digital images was taken from a whole 2-DE gel image of three members of different families. In the digital image of Child 2 from Family 2, two sets of polymorphic spots are observed. In the first set, Child 2 has two spots termed 1A and 1B; Father 2 and Mother 2 from Family 2, have spots corresponding to spot 1B and spot 1A, respectively. The intensity of spot 1B of Father 2 and that of spot 1A of Mother 2 is almost twice as great as that of spot 1B or spot 1A of Child 2. Thus, Child 2 is heterozygous for spot 1B and spot 1A. Father 2 is homozygous for spot 1B, and Mother 2 is homozygous for spot 1A. In the second set, both Child 2 and Father 2 have two spots termed 2A and 2B, whereas Mother 2 has a single spot corresponding to spot 2B. The intensity of spot 2B of Mother 2 is almost twice as great as that of spot 2B of Child 2 or of Father 2. Therefore, both Child 2 and Father 2 are heterozygous for spot 2A and spot 2B, whereas Mother 2 is homozygous for spot 2B. Concerning the first polymorphic system in Family 1, Father 1, Child 1, and Mother 1 are homozygous for spot 1B. For the second system, both Father 1 and Mother 1 are heterozygous for spot 2A and spot 2B, whereas Child 1 is homozygous for spot 2B.

**Discussion**

We examined frequencies of germline mutations at six minisatellite loci in the exposed and the control families. The mean mutation rate in these loci was 1.5% in the exposed gametes and 2.0% in the unexposed gametes. The observed mean mutation rates are similar to the spontaneous mutation rate of 1.2% (48/4099 observed for six minisatellite loci, $\lambda_{MS-1}$, $\lambda_{MS-31}$, $\rho_{lg} 3$, $\lambda_{MS-43}$, $\lambda_{MS-8}$, and $\lambda_{MS-32}$, in 40 families from the Centre d’Etude du Polymorphism Humain panel comprising 344 offspring (18,44) and the mean mutation rate of 1.2% (55/4611 observed for five minisatellite loci, $\lambda_{MS-1}$, $\lambda_{MS-31}$, $\rho_{lg} 3$, $\lambda_{MS-43}$, and YNH24, in Caucasian families (45,46).

We found that all 15 mutations in the $CEB$-1 locus were of paternal origin. Vergnaud et al. (36) reported an identical result. In the examinations of the $rho_{lg} 3$ locus, only one mutation that occurred in an exposed father was detected in a son who also showed a mutation in the $CEB$-1. The bias toward the paternal origin of the mutation at the $CEB$-1 locus may reflect the large number of cell divisions during spermatogenesis compared with oocyte formation, which makes the frequency of accumulated replication errors in male germ lines higher than that in female germ lines and suggests the involvement of mitotic events in the generation of mutations.

Among 12 mutants detected at the $\lambda_{MS-1}$ locus, 3 were of maternal origin and 9 were of paternal origin. However, no significant differences were seen between the mutation rates at the $\lambda_{MS-1}$ locus of male and female germ cells ($p = 0.07$, Fisher’s exact probability test). Jeffreys et al. (18) reported that paternal and maternal mutation rates were similar (5.5% and 4.9% per gamete, respectively) at the $\lambda_{MS-1}$ locus, and suggested that the germ-line mutations at the $\lambda_{MS-1}$ locus might arise during meiosis. Origins of mutations at the $CEB$-1 and $\lambda_{MS-1}$ loci may be different. Mutations originating from replication errors, such as those at the $CEB$-1 locus, may be less sensitive to radiation exposure compared with those occurring at the $\lambda_{MS-1}$ locus, which are not related to replication errors. A distinct minisatellite locus might respond differently to radiation.

In this pilot study, we examined the children of families in which primarily one parent was exposed or in which neither parent was exposed (control families). However, for loci with high heterozygosity where parental origins of mutated alleles in the

**Figure 2.** Digital images from six individuals who are members of two families. Each image is part of a whole autoradiogram of the 2-DE gel for each of the six individuals: Father 1, Child 1, and Mother 1 are members of Family 1; Father 2, Child 2, and Mother 2 are members of Family 2. Spots 1A and 1B are components of one polymorphic system and Spots 2A and 2B are components of the other polymorphic system. Black arrows indicate spots that exist on gels. White arrows show where spots would appear if they were present. Spot 3 is not described in this report.
CHIL IDREN OF ATOMIC BOMB SURVIVORS

children can be easily deduced, examining the children of the control group is unnec-
esary. Using this strategy, the numbers of gametes derived from the exposed mothers and the exposed fathers must be the same in order to have an equal number of unex-
posed controls for both of them, a criterion that is fulfilled in our exposed families. By ex-
cluding the control group, efficiency of the screening increases. In addition, in a child carrying one exposed allele and one unexposed allele, the background (genetic and environmental) effects are identical on both alleles, which is preferable.

We estimated the numerical require-
ment to demonstrate a significant differ-
ence between the mutation rates at the
minisatellite loci of the exposed and the unexposed germ cells employing standard power function statistics (a type I error of 0.05 and a type II error of 0.2). Because the most probable human gametic dou-
bling dose for acute radiation exposure has been estimated to be between 1.7 and 2.2 Sv (5) and the mean gonadal dose for the 65 exposed gametes was 1.9 Sv, we assume that they had received the estimated dou-
bling dose. For a locus with a spontaneous mutation rate of 0.02 per gamete, the mean mutation rate of the six loci exam-
ined in this study, we calculated that we would need to survey two samples (exposed and unexposed) of 1,188 germ cells each to observe a significant difference at the 0.05 level of significance. By examining the 64 children from the exposed families at six loci, we have already examined a total of 390 exposed alleles and 378 unexposed alleles in this study. Thus, in these children we have to examine an additional 13 minisatellite loci having a mutation rate of 0.02/gamete/locus.

There are a few published reports con-
cerning radiosensitivity of mouse minisatel-
litie loci. Sadamoto et al. (47) reported that an increase in the mouse paternal germline mutation rate at the Pe-1 locus was statistically significant for irradiation with 2-3 Gy at the spermatogonial stage but not at the spermatogonial stage. They confirmed the results with a larger number of offspring (48). On the other hand, Dubrova et al. (49) reported that an increase in the mouse paternal germline mutation rate in DNA fingerprints induced by 0.5 Gy y radiation to which the increase in the mutation rate at the Pe-1 locus is believed to contribute was statistically significant at the spermatogonial stage but that induced by 1.0 Gy irradiation was not significant. Results based on mouse data are not consistent with regard to the doubling dose and radiation-sensitive stage, and further study is necessary to provide suitable data for humans.

In the examination of the microsatel-
rites, we compared the lengths of the
PCR-amplified fragments including trinu-
cleotide or tetrancleotide repeats in single families. When we sequenced fragments with CTTT/CTTT repeats from more than 30 individuals, we discovered that repeat numbers of CTTT and CTTT var-
ed among individuals who nevertheless had identical total lengths of their frag-
ments. Our present screening method to examine the total number of repeats cannot detect differences in numbers of these two types of repeats. We have established a method to determine each of these two types of repeats independently, and their repeat numbers are being examined.

Recently, hereditary nonpolyposis col-
orectal cancer (HNPCC) and some other
colon cancer cells were observed to have an abnormality called microsatellite insta-
bility in which mutations occur at several microsatellite loci on different chromo-
somes (50,51). HNPCC can be caused by germline mutations of the mismatch repair genes (52–54). In each of the mutations

we detected, however, the mutation had occurred at only one microsatellite locus. Thus, these mutations do not seem to be caused by an abnormality in the mismatch repair genes.

For characterization of mutant frag-
ments that will be detected by 2-DE in the future, we have constructed a genomic
DNA library prepared from DNA frag-
ments digested with NotI and EcoRV. We have also developed a method that permits
target cloning of DNA spots obtained from
the 2-DE gels. By using the cloned DNA spots as probes in the screening of
the DNA library and Southern blot
analysis of the 2-DE gels, we have isolated
and characterized DNA fragments that
represent new hereditary polymorphic
variants detected in the 2-DE gels. Asakawa et al. (40) reported an example in
which there was a HinfI site sequence
(AGGAGTCGGG–) in the smaller frag-
ment, but the larger fragment that did not
have this site was characterized by the
sequence (AGGAGTTGGG–).

The mutational yield in the 2-DE
study can be calculated only approxi-
ately. If we assume that the spontaneous
mutation rate is 1 × 10–5/fragment/genera-
tion (30), we would then expect one muta-
tion per 100 gels from control children
(500 diploid fragments scored per gel).
This calculation is based on having 500 good spots from the 2,000 derived from the
NotI/EcoRV fragments of 1 to 5 kb
separated in the first dimension elec-
trophoresis. Considering this assumed
mutation rate, it is not surprising that we
detected no mutations in the spots from
the three children examined in this preli-
nary study using the 2-DE technique. We
are now making an effort to develop a
2-DE pattern from the 5- to 20-kb NotI/
EcoRV fragments, which will visualize a
new 2,000 spots.

REFERENCES

1. Neel JV, Schull WJ. The effect of exposure to the atomic
bombs on pregnancy and termination in Hiroshima and
Nagasaki. In: The Children of Atomic Bomb Survivors.
A Genetic Study. Washington: National Academy Press,
1991:13–269.
2. Kato H, Schull WJ, Neel JV. A cohort-type study of survival in
the children of parents exposed to atomic bombings. Am J
Hum Genet 16:214–230 (1966).
3. Awa AA, Honda T, Neriishi S, Sofuni T, Shimba H, Ohkaki K,
Nakano M, Kodama Y, Itoh M, Hamilton HB. Cytogenetic
studies of the offspring of atomic bomb survivors. In:
Cytogenetics: Basic and Applied Aspects (Obe G, Basler A,
ed). Berlin: Springer, 1987:166–183.
4. Neel JV, Satoh C, Goriki K, Asakawa J, Fujita M, Takahashi
N, Kageoka T, Hazama R. Search for mutations altering pro-
tein charge and/or function in children of atomic bomb sur-
vivors: Final report. Am J Hum Genet 42:663–676 (1988).
5. Neel JV, Schull WJ, Awa AA, Satoh C, Kato H, Otake M,
Yoshimoto Y. The children of parents exposed to atomic
bombs: estimates of the genetic doubling dose of radiation for
humans. Am J Hum Genet 46:1053–1072 (1990).
6. Otake M, Schull WJ, Neel JV. The effects of parental expo-
sure to the atomic bombings of Hiroshima and Nagasaki on
genital malformations, stillbirths and early mortality
among their children: a reanalysis. Radiat Res 122:1–11
(1990).
7. Yoshimoto Y, Neel JV, Schull WJ, Kato H, Soda M, Eto R, Mabuchi K. Malignant tumors during the first 2 decades of life in the offspring of atomic bomb survivors. Am J Hum Genet 46:1041–1052 (1990).

8. Yoshimoto Y, Schull WJ, Kato H, Neel JV. Mortality among the offspring (F2) of atomic bomb survivors, 1946–85. J Radiat Res 32:327–351 (1991).

9. Satoh C, Hiyama K, Takahashi N, Kodaira M, Neel JV. Approaches to DNA methods for the detection of heritable mutations in humans. In: Mutation and the Environment, Part C (Mendelsohn ML, Albertini RJ, eds). New York:Wiley-Liss, 1990:197–206.

10. Satoh C. A review of forty-five years study of Hiroshima and Nagasaki atomic bomb survivors. Biochemical genetics study. J Radiat Res (Tokyo) 32 Suppl:378–384 (1991).

11. Neel JV, Satoh C, Myers R. Report of a workshop on the application of molecular genetics to the study of mutation in the children of atomic bomb survivors. Mutat Res 291:1–20 (1993).

12. Hiyama K, Kodaira M, Satoh C. Detection of deletions, insertions and single nucleotide substitutions in cloned β-globin genes and new polymorphic nucleotide substitutions in β-globin genes in a Japanese population using ribonuclease cleavage at mismatches in RNA: DNA duplexes. Mutat Res 231:219–231 (1990).

13. Takahashi N, Hiyama K, Kodaira M, Satoh C. An improved method for the detection of genetic variations in DNA with denaturing gradient gel electrophoresis. Mutat Res 234:61–70 (1990).

14. Satoh C, Takahashi N, Asakawa J, Hiyama K, Kodaira M. Variations among Japanese of the factor IX gene (F9) detected by PCR-denaturing gradient gel electrophoresis. Am J Hum Genet 52:167–175 (1993).

15. Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E, White R. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235:1616–1622 (1987).

16. Wong Z, Wilson V, Patel P, Povey S, Jeffreys AJ. Characterization of a panel of highly variable minisatellites cloned from human DNA. Ann Hum Genet 51:269–288 (1987).

17. Royle JN, Clarkson RE, Wong Z, Jeffreys AJ. Clustering of hypervariable minisatellites in the proroginal regions of human autosomes. Genomics 3:352–360 (1988).

18. Jeffreys AJ, Royle NJ, Wilson V, Wong Z. Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature 322:278–281 (1988).

19. Oberlé I, Rousseau F, Heitz D, Kreitz C, Deves D, Hanauer A, Bouret B, Boissin MF, Mandel J-L. Instability of a 550-bases pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097–1102 (1991).

20. Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu Y-H, Kuhl DPA, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, van Ommen GJB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT. Cloning of the human DNA probe (EMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X Syndrome. Cell 65:905–914 (1991).

21. Yu S, Pritchard M, Kremer E, Lynch M, Nancarrow J, Baker E, Holman K, Mulley JC, Warren ST, Schlessinger D, Sutherland GR, Richards RI. Fragile X phenotype characterized by an unstable region of DNA. Science 252:1179–1181 (1991).

22. Fu Y-H, Pizzuti A, Fenwick RG JR, King J, Rajnarayan S, Dunne PW, Dubel J, Nasser GA, Ashizawa T, Jing PD, Wieringa B, Korneluk R, Perryman MB, Epstein HF, Caskey CT. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science 255:1256–1258 (1992).

23. Mahadevan M, Talbeldi C, Sabourin L, Shuter G, Amemiya C, Jansen G, Neville C, Narang M, Barceló J, O'Hoy K, Leblond S, Earle-Macdonald J, Jong PJD, Wieringa B, Korneluk RG. Myotonic dystrophy mutation: an unstable CTG repeat in the 3'-untranslated region of the gene. Science 255:1253–1255 (1992).

24. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77–79 (1991).

25. Milet M, Mulley J, Loeber D, Turner G, Donnelly A, Gideon A, Hillen D, Kremer E, Lynch M, Pritchard M, Sutherland GR, Richards RI. Fragile-X syndrome: unique genetics of the heritable unstable element. Am J Hum Genet 50:968–980 (1992).

26. Fu Y-H, Kuhl DPA, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJMH, Holden JJA, Fenwick RG Jr, Warren ST, Oostra BA, Nelson DL, Caskey CT. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the sherman paradox. Cell 67:1047–1058 (1991).

27. Hästbacka J, de la Chapelle A, Kaitila I, Sistonen P, Weaver A, Lander E. Linkage disequilibrium mapping in isolated founder populations: diastrastic dysplasia in Finland. Nature Genet 1:204–211 (1992).

28. Mahtani MM, Willard HF. A polymorphic X-linked tetranucleotide repeat locus displaying a high rate of new mutation: implications for mechanisms of mutation at short tandem repeat loci. Hum Mol Genet 2:431–437 (1993).

29. Hatada I, Hayashizaki Y, Hirotsume S, Komatsubara H, Mukai T. A genomic scanning method for higher organisms using restriction sites as landmarks. Proc Natl Acad Sci USA 88:9523–9527 (1991).

30. Asakawa J, Kuick R, Neel JV, Kodaira M, Satoh C, Hanash SM. Quantitative and qualitative genetic variation in two-dimensional DNA gels of human lymphocytoid cell lines. Electrophoresis 16:241–252 (1995).

31. Wong FL, Yamada M, Sasaki H, Kodama K, Akiba S, Shimaoka K, Hosoda Y. Noncancer disease incidence in the atomic bomb survivors: 1958–1986. Radiat Res 135:418–430 (1993).

32. Asakawa J, Satoh C, Yamaakai Y, Chen S-H, Accurate and rapid detection of heterozygous carryers of a deletion by combined polymerase chain reaction and high-performance liquid chromatography. Proc Natl Acad Sci USA 89:9126–9130 (1992).

33. Mitani K, Takahashi Y, Kominami R. A GGGAGG motif in minisatellites affecting their germline instability. J Biol Chem 265:15203–15210 (1990).

34. Honma M, Mitani K, Mizusawa H, Sofuni T, Muramatsu M, Kominami R. A new VNTR-type RFLP probe (ATM-18) on chromosome 1 (D15157). Hum Mol Genet 1:554 (1992).

35. Honma M, Mitani K, Mizusawa H, Sofuni T, Muramatsu M, Kominami R. A new VNTR-type RFLP probe (ChdTC-15) on chromosome 12 (D12655). Hum Mol Genet 1:559 (1992).

36. Corugnud G, Muyten D, Apisova F, Muyten J, Lauthier V. The use of synthetic tandem repeats to isolate new VNTR loci: cloning of a human hypermutable sequence. Genomics 11:135–144 (1991).

37. Jeffreys AJ, Wilson V, Neumann R, Keyte J. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nuclear Acids Res 16:10953–10971 (1988).

38. Kodaira M, Hiyama K, Karakawa T, Kameo H, Satoh C. Duplication detection in Japanese Duchenne muscular dystrophy patients and identification of carriers with partial deletions using pulsed-field gel electrophoresis. Hum Genet 92:237–243 (1993).

39. Kominami E, Pritchard M, Lynch M, Yu S, Holman K, Baker E, Warren ST, Schlessinger D, Sutherland GR, Richards RI. Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence (CCG). Science 252:1711–1714 (1991).

40. Asakawa J, Kuick R, Neel JV, Kodaira M, Satoh C, Hanash SM. Genetic variation detected by quantitative analysis of end-labeled genomic DNA fragments. Proc Natl Acad Sci USA 91:9052–9056 (1994).

41. Kuick R, Asakawa J, Neel JV, Satoh C, Hanash SM. High yield of restriction fragment length polymorphisms in two-dimensional separations of human genomic DNA. Genomics 25:345–355 (1995).
42. Kelly R, Bulfield G, Collick A, Gibbs M, Jeffreys AJ. Characterization of a highly unstable mouse minisatellite locus: evidence for somatic mutation during early development. Genomics 5:844–856 (1989).

43. Satoh C, Neel J, Yamashita A, Goriki K, Fujita M, Hamilton HB. The frequency among Japanese of heterozygotes for deficiency variants of 11 enzymes. Am J Hum Genet 35:656–674 (1983).

44. Armour JAL, Patel I, Thein SL, Fey MF, Jeffreys AJ. Analysis of somatic mutations at human minisatellite loci in tumors and cell lines. Genomics 4:328–334 (1989).

45. Henke L, Cleef S, Zakrzewska M, Henke J. Population genetic data determined for five different single locus minisatellite probes. In: DNA Fingerprinting: Approaches and Applications (Bulke T, Dolf G, Jeffreys AJ, Wolff R, eds). Basel:Birkhauser Verlag. 1991:144–153.

46. Henke J, Fimmers R, Baur MP, Henke L. DNA minisatellite mutations: recent investigations concerning distribution and impact on parentage testing. Int J Leg Med 105:217–222 (1993).

47. Sadamoto S, Suzuki S, Kamiya K, Kominami R, Dohi K, Niwa O. Radiation induction of germline mutation at a hypervariable mouse minisatellite locus. Int J Radiat Biol 65:549–557 (1994).

48. Fan Y-J, Wang Z, Sadamoto S, Kamiya K, Kominami R, Niwa O. Germline mutation of a hypervariable mouse minisatellite locus induced by ionizing radiation [Abstract]. J Radiat Res 35:285 (1994).

49. Dubrova YE, Jeffreys AJ, Malashenko AM. Mouse minisatellite mutations induced by ionizing radiation. Nature Genet 5:92–94 (1993).

50. Aaltonen LA, Peltonäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin J-P, Järvinen H, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B, de la Chapelle A. Clues to the pathogenesis of familial colorectal cancer. Science 260:812–816 (1993).

51. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science 260:816–819 (1993).

52. Leach FS, Nicolaides NC, Papadopoulos N, Liu B, Jen J, Parsons R, Peltonäki P, Sistonen P, Aaltonen LA, Nyström-Lahti M, Guan X-Y, Zhang J, Meltzer PS, Yu J-W, Kao F-T, Chen DJ, Ceresaletti KM, Fournier REK, Todd S, Lewis T, Leach RJ, Naylor SL, Weissbach J, Mecklin J-P, Järvinen H, Petersen GM, Hamilton SR, Green J, Jass J, Watson P, Lynch HT, Trent JM, de la Chapelle A, Kinzler KW, Vogelstein B. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 75:1215–1225 (1993).

53. Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Earabino C, Lipford J, Lindblom A, Tannergaard P, Bollag RJ, Godwin AR, Ward DC, Nordenskjold M, Fishel R, Kolodner R, Liskay RM. Mutation in the DNA mismatch repair gene homologous hMLH1 is associated with hereditary non-polyposis colon cancer. Nature 368:258–261 (1994).

54. Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseultine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Hamilton SR, Petersen GM, Watson P, Lynch HT, Peltonäki P, Mecklin J-P, de la Chapelle A, Kiznzer KW, Vogelstein B. Mutation of a mutS homolog in hereditary colon cancer. Science 263:1625–1629 (1994).