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Advances in Brief

Oncogenic Base Substitution Mutations in Circulating Leukocytes of Normal Individuals[1]

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Abstract

The background frequency of mutations in human tissues is an important issue in cancer susceptibility and genotoxic exposure determinations. Here we report the detection of rare mutant leukocytes containing oncogenic base substitutions of the Harvey-*ras*, N-*ras*, and *p53* genes by the Needle-in-a-Haystack mutation assay with a sensitivity of one cell in a million. Altogether, we detected and identified 17 independent mutations of 66 separate base site analyses of peripheral blood specimens obtained from 19 apparently normal individuals. Two individuals harbored a substantially increased frequency of mutant cells, representing 9 of the 17 independent mutations found. These results suggest that up to 1 in 10 normal individuals may harbor a significant frequency of oncogenic mutations in circulating leukocytes.

Introduction

An increased frequency of mutations in an accessible tissue of an individual may be an indicator of somatic disease, heritable predisposition to somatic disease, and/or exposure to one or more genotoxic agents. The assessment of these mutations may enable the early detection of cancer in individuals or identify individuals who harbor one or more hypermutable clones (1). Because cancer results from the accumulation of mutations essentially in gatekeeper and caretaker genes (2), the determination of the frequency of occurrence of such oncogenic mutations in human tissues is important to the understanding of these disease processes and the risk of cancer development.

The background spontaneous mutation frequency appears to have a strong genetic component as opposed to environmental factors (3). Under nonperturbating conditions, cells lacking any genome-destabilizing gene mutation would probably have a spontaneous DNA mutation frequency at a specific locus of less than one cell in a million, the presently accepted value of null gene mutations (4, 5). Thus, the frequency of DNA mutations is expected to be rare, on the order of less than one in a million cells in individuals who do not harbor an identified genetic disease associated with genomic stability and who have not been exposed to significant levels of genotoxic agents. However, *in vivo* data regarding spontaneous mutation frequencies for DNA base substitution and other point mutations not biased by null gene selection are lacking in the literature.

Using the Needle-in-a-Haystack mutation detection and identification PCR/RE[3]/LCR technique (6), we addressed the question of base substitution mutation frequencies in oncogenic loci in circulating PBLs in apparently normal individuals. Two of 19 individuals had a significantly higher frequency of oncogenic mutations in their PBLs. These data suggest that a substantial proportion of individuals within the human population may harbor a significant frequency of oncogenic mutations in circulating leukocytes.

Materials and Methods

Peripheral Blood DNAs. Peripheral blood specimens were collected from consenting volunteers at The Children's Hospital (Denver, CO). A total of 5–20 ml of venous blood was collected in EDTA vacutainers, and the buffy coat was immediately prepared and harvested after centrifugation. PBL DNA was isolated by standard phenol extraction procedures.

Needle-in-a-Haystack PCR/RE/LCR Analysis. PBL DNAs were assayed for base substitution mutations at sensitivities of one in a million cells or better using the PCR/RE/LCR procedures described by Wilson *et al.* (6). The PCR and LCR primer designs and protocols for the majority of sites analyzed in this work have been described previously by Wilson *et al.* (6). The LCR primers for the second base of codon 12 of the N-*ras* gene and the first base of codon 13 or the N-*ras* gene are listed in Table 1, as are the LCR oligonucleotide mutant template standards for the Ha-*ras* and N-*ras* sites. The LCR primers and template standards for the first base of codon 248 of p53 have been described previously by Wilson *et al.* (6). Human Ha-*ras* genomic sequence plasmids pbC-N1 and pT24-C3 were obtained from American Type Culture Collection and propagated in *Escherichia coli*, as described previously (6). All oligonucleotide primers and templates were obtained as custom synthesis form BioServe Biotechnologies, Ltd. (Laurel, MD).

Briefly, duplicate (and triplicate, where possible) 6 μg aliquots of PBL DNA [or 200 ng of genomic mutant DNA or 6 μg of wild-type control DNA containing 6 pg of mutant (10^{-6} mutant positive control)] were subjected to three cycles of PCR and RE before LCR analyses. The specific protocol for the mutant enrichment PCR/RE procedures was dependent on the locus and nucleotide under study (6). *Msp*I was used for mutant selection in the Ha-*ras* codon 12 and *p53* codon 248 procedures, whereas a *Hph*I site was incorporated into the N-*ras* sequence by PCR for the selection of codon 12 and the first base of codon 13, as described previously by Wilson *et al.* (6). The original sample DNA was either exhaustively digested (overnight) with *Msp*I (New England Biolabs) or amplified by locus-specific PCR primers before digestion with *Hph*I (New England Biolabs). An aliquot of this *Msp*I digest was then amplified for 25 cycles with the outermost set of PCR primers and Taq polymerase (Perkin-Elmer) under standard reaction conditions. The process of *Msp*I (or *Hph*I) digestion (>5 h) and PCR amplification was then repeated using nested primers for 20 cycles and either Taq (Perkin-Elmer) or Pfu (Stratagene) or Pfu Turbo (Stratagene) polymerase. After the third round of 20 cycles of nested PCR amplification, the mutant allele was detected by LCR amplification using ^{32}P-end-labeled primers (labeled invariant oligomers and unlabeled discriminating oligomers), as described previously by Wilson *et al.* (6) and Barany (7). One μl of the above-mentioned PCR-amplified and *Msp*I- or *Hph*I-restricted supernatant was incubated with 1 pmol/μl each of either the wild-type discriminating LCR primer set or all six mutant discriminating LCR primers, 15 units of Taq ligase (a gift from Dr. Francis Barany, Cornell University Medical School, New York, NY, and purchased from New England Biolabs), and 4 μg of sheared salmon sperm DNA in a total volume of 10 μl containing 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 1

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[3] The abbreviations used are: RE, restriction endonuclease digestion; LCR, ligase chain reaction; PBL, peripheral blood leukocyte.

Table 1 *LCR primers and template standards*

LCR primer	5′-3′ sequence
Human N-*ras* codon 12, second base	
HNRasc12s2L	TGATGTTGGGAAAAGCGCCTAT
HNRasc12s2LR	CTGCTCCAACCACCACCA
WtHNRasc12s2L	AAGGTGGTTGGAGCAGG
WtHNRasc12s2LR	AGCTTTTCCCAACATCAC
MtHNRasc12s2TL	AAAAGGTGGTTGGAGCAGT
MtHNRasc12s2TLR	AACGCTTTTCCCAACATCAA
MtHNRasc12s2AL	AAAAAAGGTGGTTGGGAGCAGA
MtHNRasc12s2ALR	AAAACGCTTTTCCCAACATCAT
MtHNRasc12s2CL	ATAAAAAAGGTGGTTGGAGCAGC
MtHNRasc12s2CLR	AAAAAAACGCTTTTCCCAACATCAG
StdHHRasc12s2AL	48-mer template, TAAGCTGGTGGTGGTGGGCGCCGACGGTGTGGGCAAGAGTGCGCTGAC
StdHHRasc12s2CL	48-mer template, TAAGCTGGTGGTGGTGGGCGCCGCCGGTGTGGGCAAGAGTGCGCTGAC
Human N-*ras* codon 12, first base	
StdHNRasc12s1AL	48-mer template, TACAAACTGGTGGTGGTTGGAGCAAGTGATGTTGGGAAAAGCGCCTAT
StdHNRasc12s1CL	48-mer template, TACAAACTGGTGGTGGTTGGAGCACGTGATGTTGGGAAAAGCGCCTAT
Human N-*ras* codon 12, second base	
StdHNRasc12s2TL	47-mer template, AACTGGTGGTGGTTGGAGCAGTTGATGTTGGGAAAAGCGCCTATCCC
StdHNRasc12s2AL	53-mer template, ACAACTGGTGGTGGTTGGAGCAGATGATGTTGGGAAAAGCGCCTATCCCATC
StdHNRasc12s2CL	47-mer template, AACTGGTGGTGGTTGGAGCAGCTGATGTTGGGAAAAGCGCCTATCCC
Human N-*ras* codon 13, first base	
HNRasc13s1L2	ATGTTGGGAAAAGCGCCTATACCGTT
HNRasc13s1LR2	ACCTGCTCCAACCACCACCAGTTT
WtHNRasc13s1L	AAGTGGTTGGAGCAGGTG
WtHNRasc13s1LR	AGCGCTTTTCCCAACATC
MtHNRasc13s1TL	AAAAGTGGTTGGAGCAGGTT
MtHNRasc13s1TLR	AAAGCGCTTTTCCCAACATA
MtHNRasc13s1AL	AAAAAAGTGGTTGGAGCAGGTA
MtHNRasc13s1ALR	AAAAAGCGCTTTTCCCAACATT
MtHNRasc13s1CL	AAAAAAAAGTGGTTGGAGCAGGTC
MtHNRasc13s1CLR	AAAAAAAGCGCTTTTCCCAACATG
StdHNRasc13s1TL	53-mer template, ACAAACTGGTGGTGGTTGGAGCAGGTTATGTTGGGAAAAGCGCCTATCCCATC
StdHNRasc13s1AL	53-mer template, ACAAACTGGTGGTGGTTGGAGCAGGTAATGTTGGGAAAAGCGCCTATCCCATC
StdHNRasc13s1CL	49-mer template, AACTGGTGGTGGTTGGAGCAGGTCATGTTGGGAAAAGCGCCTATCCCGC

mM NAD^+, and 0.1% Triton X-100 (7). The LCR amplification reaction was incubated for 30 cycles at 95°C for 1 min and 65°C for 4 min. The LCR amplification products were separated on a 10% polyacrylamide, 7M urea sequencing gel, dried, and exposed to X-ray film.

Results

After the development of the Needle-in-a-Haystack PCR/RE/LCR assay, the question of the baseline normal mutation frequency in human tissues became an issue (6). If this sensitive mutation detection and identification assay is to be useful in identifying individuals harboring a higher-than-normal frequency of one or more mutations due to either the presence of cancer cells, genetic hypermutability, or a recent mutagen exposure, the background frequency of the mutations under study needs to be known.

To approach this question of background base substitution mutation frequency, human PBL DNAs were obtained from 19 phenotypically normal volunteers. The age of these donors ranged from 2.5–46 years, and both genders were almost equally represented. Four individuals did not provide their age (date of birth), and another 4 of the 19 specimens were obtained anonymously (Table 2).

Five base sites within three separate oncogenic loci were chosen for rare base substitution PCR/RE/LCR mutation analyses. These were the second base of codon 12 of the Ha-*ras* gene, the first base of codon 248 of the *p53* gene, the first and second bases of codon 12 of the N-*ras* gene, and the first base of codon 13 of the N-*ras* gene.

Codon 12 of the Ha-*ras* gene was the first locus to be analyzed. Each duplicate or triplicate sample containing 6 μg of PBL specimen DNA was analyzed by PCR/RE/LCR procedures. A G→T transversion mutation was observed in specimen 93014 (Fig. 1). A standard mix of 10^{-6} mutant (pT24-C3) to wild-type plasmid DNA mix was included in the PCR/RE selection and LCR analyses to ensure the sensitivity of the assay. The shadow observed above the bands in Fig. 1 does not represent additional mutations because no mutations were observed in specimens when the LCR reactions contained only the mutant A primers, whereas LCR bands were observed for the oligonucleotide template standard for mutant A (data not shown). The 1 pmol/μl concentration of oligonucleotide template standard mutant C was saturating because only a single 48-bp band representing the antisense LCR primer set was observed. Lower concentrations of oligonucleotide standards enable the display of both LCR product bands (as noted below).

Only 2 of 17 specimens analyzed at the Ha-*ras* locus were found to contain mutant cells present at a frequency of $\geq 10^{-6}$ (Table 2). All specimens were analyzed in duplicate or triplicate. Although G→A and G→C mutations were assayed simultaneously with the G→T mutation, only G→T mutations were observed in two specimens at this locus (Fig. 1, Table 2).

The number of individual specimens displaying detectable rare base substitution mutations increased slightly at the *p53* codon 248 locus, amounting to 4 of 18 specimens analyzed (Table 2). All of these specimens were assayed for *p53* codon 248 mutations in triplicate. Specimens 890061, 93008, and 93014 provided detectable G→T

Table 2 *Summary of PCR/RE/LCR analysis of human PBL DNAs*

Sample	Age (yrs)	Ha-*ras* codon 12 GGC→GNC	*p53* codon 248 CGG→NGG	N-*ras* codon 12 GGT→NGT	N-*ras* codon 12 GGT→GNT	N-*ras* codon 13 GGT→NGT
890048	—[a]		—	T	T	
890049	—	—	—	—	—	—
890059	—		—			
890061	—	—	T	T	T	A
890062	44	—	T, A			
90093	11	—	—	—	—	—
91116	—	—	—	—	—	
91122	—	—	—	—	—	
92442	8	—				
92443	46	—	—			
92444	—	—	—			
92445	—	—	—			
93008	25	—	T			
93012	20	—	—	—	T	—
93014	2.5	T	T	T	T	
93018	12	—	—	—	T	
93029	41	—	—	—	—	—
93030	44	—	—	—	—	—
93033	4	T	—	—	T	—

[a] Age or date of birth not provided by donor.

transition mutations, whereas specimen 890062 harbored both G→T transition and C→A transversion mutations (data not shown). The triplicate analysis of these samples demonstrated a roughly 10-fold variation in density of the resultant LCR mutant product bands, clearly demonstrating that quantitation of mutant frequency by densitometry would be inaccurate (data not shown). The sensitivity of the PCR/RE/LCR assay is adjustable, so that quantitation of these rare mutants was further attempted with the analysis of both the restricted PCR products from the second and third PCR/RE selection cycles. By reducing the number of PCR/RE selection cycles used, the PCR/RE/LCR assay becomes less sensitive. Specimen 890062 appears to harbor *p53* codon 248 mutant cells at a frequency of less than 1 in 10^6 (data not shown). More accurate quantitation of the proportion of mutant cells within these PBL DNAs by the analysis of smaller and smaller aliquots of DNA (representing smaller and smaller numbers of cells) was not undertaken due to the limited quantities of DNA available for these cases.

Because these mutation analyses were focused on circulating blood cells, and the *ras* gene most commonly associated with leukemias and lymphomas is the N-*ras* gene, the determination of rare cells harboring mutations in the N-*ras* gene would be significant. A G→T transversion mutation in the first base of codon 12 of the N-*ras* gene was also detectable at the frequency of $\geq 10^{-6}$ circulating leukocytes in specimens 890048, 890061, and 93014 (Table 2; see Ref. 6 for representative LCR example).

Transversion mutations were also common at the second base of codon 12 of the N-*ras* gene in these PBL DNA specimens, as shown for specimens 890048 and 93018 (Fig. 2*A*). Half of the 12 specimens assayed at this site provided detectable G→T mutations (Table 2).

Only one of seven specimens was found to harbor a detectable mutation in the first base of codon 13 of the N-*ras* gene (Table 2; Fig. 2*B*). This was a G→A mutation that appears to be a relatively rare non-CpG site transition mutation involving guanosine residue in these oncogenic loci.

Of the 12 individuals assayed for three or more base sites, two stand out with detectable mutations at multiple sites (Table 2). Specimens 890061 and 93014 displayed mutations in four of five sites and four of four sites, respectively, which was significantly different from the other individuals ($\chi^2 = 35$, $P < 0.01$ for 17 mutations from all 19 individuals; $\chi^2 = 22$, $P < 0.02$ for 14 mutations from only the12 individuals tested at three or more base sites).

Discussion

Nineteen PBL DNAs isolated from normal individuals ranging in age from 2.5–46 years were analyzed for rare cells containing oncogenic base substitution mutations in three different genes. At least one rare mutation was detected at frequencies of 1 or more mutant alleles in 10^6 cells in 8 of the 19 PBL DNAs analyzed. Of the 12 individuals tested for three or more oncogenic base sites, 2 individuals were found to have mutations in four of five sites and four of four sites tested, respectively. These two individuals (specimens 890061 and 93014) displayed a significantly increased frequency of base substitution mutations in multiple loci in their circulating leukocytes ($\chi^2 = 22$; $P < 0.02$).

Although isolated and significant genotoxic exposures cannot be entirely ruled out, the data suggest that these two individuals display a hypermutable phenotype. That is, they may harbor one or more hypermutable leukocyte clones or a constitutional predisposition to mutations. Individual AB was reported by Albertini *et al.* (1) to harbor a hypermutable T-lymphocyte clone detectable by culture selection of T-cell receptor gene rearrangements and *HPRT* mutation analyses. If hypermutability is due to a germ-line error, the characteristics of an individual's phenotype vary according to the constitutional error, being dependent on the location, type of mutation, and gene involved (8–11). Alternatively, these two individuals could be displaying a "transient mutator phenotype" in selected stem cells such that these multiple loci PCR/RE/LCR analyses of a PBL specimen obtained at a later date might be negative for hypermutability (12, 13). Thus, the frequency of some form of hypermutable phenotype within the human population may be on the order of 1 in 10.

Discounting the two hypermutable individuals, the overall background base substitution mutation frequency in PBL specimens appears to be less than one in a million circulating cells. Only eight samples were found mutant at one cell or more in a million wild-type cells of 57 separate base site analyses (ignoring the data of the two hypermutable individuals). This is in agreement with the frequency of *HPRT* null gene mutations determined in circulating lymphocytes (4, 5). Limited quantities of DNA restricted the determination of the frequency of mutant cells within individual PBL specimens at each of the base sites studied.

Only missense mutations were assayed in this study. None of the base sites studied were capable of producing a nonsense mutation, and deletions and insertions would not be detected with the LCR designs

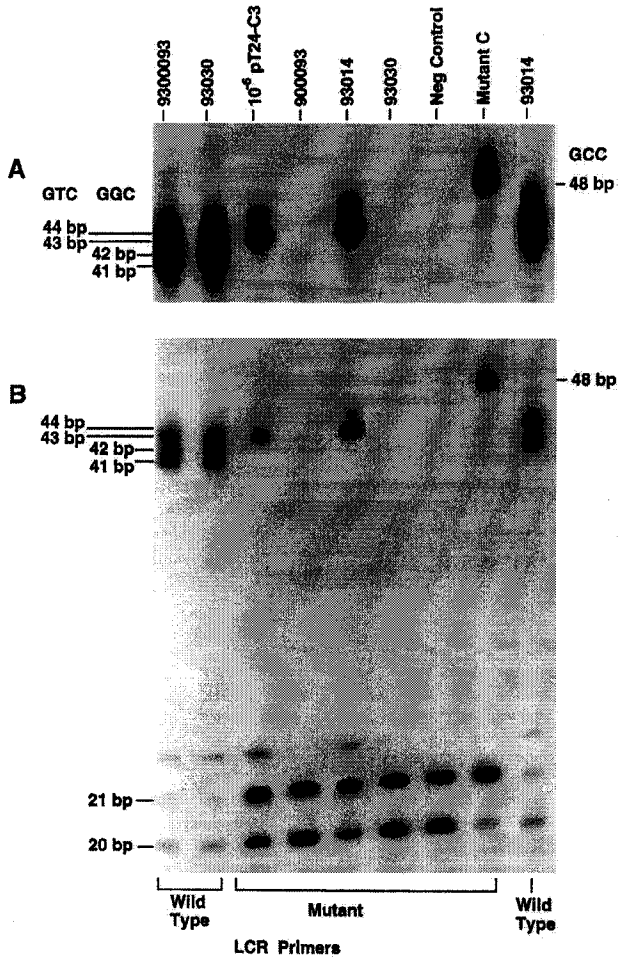

Fig. 1. Detection of Ha-*ras* codon 12 mutations. Autoradiograph of Ha-*ras* codon 12 second base LCR analysis of PCR/RE-selected PBL DNAs. A standard 10^{-6} mixture of mutant plasmid pT24-C3 (GGC→GTC) to wild-type plasmid pbC-N1 DNAs was included in these analyses for detection sensitivity (6). One pmol of the oligonucleotide mutant standards representing the sense strand with a G12D mutation (GGC→GAC) and a G12A mutation (GGC→GCC) were included (see Table 1 for standard template sequences). The LCR primers were chosen to provide 41- and 42-bp product sizes for the sense and the antisense wild-type DNA strands and 43- and 44-bp fragments for the codon 12 G12V mutation (GGC→GTC), 44- and 45-bp fragments for the codon 12 G12D mutation (GGC→GAC), and 46- and 47-bp fragments for the codon 12 G12A mutation (GGC→GCC; Ref. 6). The labeled invariant HHRasc12s2 L and HHRasc12s2LR primers were 21 and 20 bp in length, respectively. LCR reactions contained either both wild-type discriminating primers (WtHHRasc12s2L and WtHHRasc12s2LR) or all six mutant discriminating primers (MtHHRasc12s2TL, MtHHRasc12s2TLR, MtHHRasc12s2AL, MtHHRasc12s2ALR, MtHHRasc12s2CL, and MtHHRasc12s2CLR). *A*, film was exposed for 30 min. *B*, film was exposed for 10 min.

used in this study. Altogether, 17 independent mutations were detected and identified in the 66 base site-individuals assayed. The frequency of individual PBL specimens containing rare mutations varied from loci to loci and by base site within the same loci (N*ras* codons 12 and 13). Only 2 of 17 PBL DNAs (12%) were found to contain rare Ha-*ras* mutant cells, whereas 4 of 18 specimens (22%) contained *p53* codon 248 mutant cells. The higher mutant incidence of the latter probably represents the increased mutability of a known methylated CpG site (14–16). The second base of codon 12 of N*ras* produced the highest mutation incidence within the individual PBL specimens because 50% of the DNAs analyzed contained rare mutant cells. The higher mutation frequency of the N*ras* gene over the Ha-*ras* gene in PBL specimens may be a tissue-specific phenomenon because these data are in accordance with the fact that the N*ras* gene is generally more often associated with leukemias and lymphomas (17). The second base in codon 12 of the N*ras* gene has been reported to be mutated more commonly than the first base (17).

Four of the five mutations identified at the *p53* codon 248 locus were C→T transitions. These data support the literature regarding the incidence of deamination of 5-methyldeoxycytidine residues in DNA (14–16). The first base of codon 248 represents a CpG site that is known to be commonly methylated in most, if not all, tissues (14). The incidence of CpG to TpG mutations is overrepresented in the known genetic disease, causing mutations in the human population and providing more than 30% of all of the disease-associated muta-

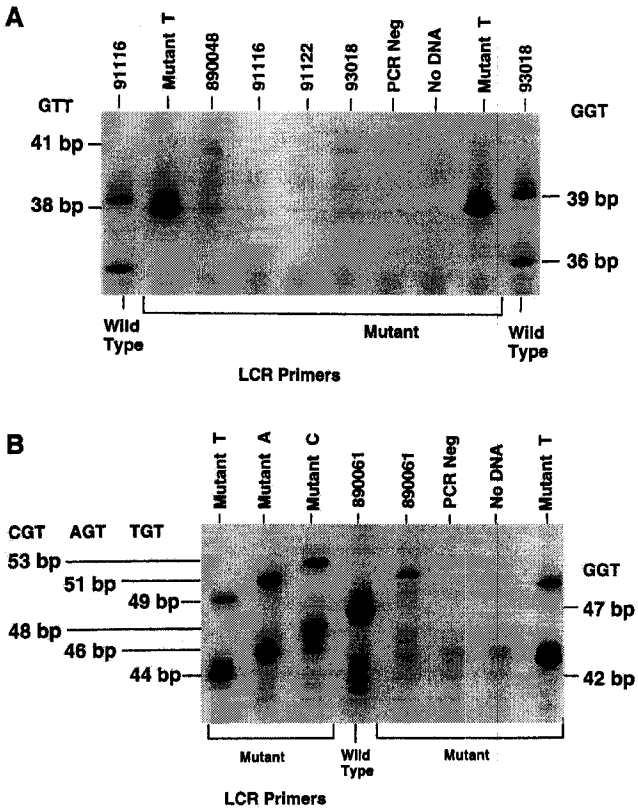

Fig. 2. Detection of mutations in the N-*ras* gene. *A*, autoradiograph of N-*ras* codon 12 second base LCR analysis of PCR/RE-selected PBL DNAs. One pmol of oligomeric standard, mutant T, representing the sense strand with the codon 12 G12V mutation (GGT→GTT), was included. Careful titration of the amount of the sense oligomeric standard added to the LCR reaction enables the appearance of both LCR product bands (see Table 1 for standard template sequences). The LCR primers were chosen to provide 36- and 39-bp fragments sizes for the sense and the antisense wild-type DNA strands and 38- and 41-bp fragments for the codon 12 G12V mutation (GGT→GTT), 40- and 43-bp fragments for the codon 12 G12D mutation (GGT→GAT), and 42- and 45-bp fragments for the codon 12 G12A mutation (GGT→GCT; see Table 1 for primer sequences). LCR reactions contained either both wild-type discriminating primers (WtHNRasc12s2s1L and WtHNRasc12s2LR) or all six mutant discriminating primers (MtHNRasc12s2TL, MtHNRasc12s2TLR, MtHNRasc12s2AL, MtHNRasc12s2ALR, MtHNRasc12s2CL, and MtHNRasc12s2CLR). Film was exposed for 30 min. *B*, autoradiograph of N-*ras* codon 13 first base LCR analysis of PCR/RE-selected PBL DNAs. Oligomeric mutant standards (0.5 pmol) representing the sense strands with codon 13, with either a G13C mutation (GGT→TGT), a G13S mutation (GGT→AGT), or a G13R mutation (GGT→CGT), were included (see Table 1 for standard template sequences). Careful titration of the amount of the sense oligomeric standard added to the LCR reaction enables the appearance of both LCR product bands. The LCR primers were chosen to provide 42- and 47-bp fragments sizes for the sense and the antisense wild-type DNA strands and 44- and 49-bp fragments for the codon 13 G13C mutation (GGT→TGT), 46- and 51-bp fragments for the codon 13 G13S mutation (GGT→AGT), and 48 and 53-bp fragments for the codon 13 G13R mutation (GGT→CGT; see Table 1 for primer sequences). The labeled invariant HNRasc13s1L and HNRasc13s1LR primers were 29 and 24 bp in length, respectively. LCR reactions contained either both wild-type discriminating primers (WtHNRasc13s1s1L and WtHNRasc13s1LR) or all six mutant discriminating primers (MtHNRasc13s1TL, MtHNRasc13s1TLR, MtHNRasc13s1AL, MtHNRasc13s1ALR, MtHNRasc13s1CL, and MtHNRasc13s1CLR). Film was exposed for 15 min.

tions in reported germ-line and somatic abnormalities (14–16). The rate of deamination of 5-methyldeoxycytidine has been reported to be high enough to account for this incidence of CpG→TpG transition mutations, especially if the fidelity of repair of G:T mispairs is less than the repair of G:U mispairs (15).

Aside from the one G→A mutation in the first base of codon 13 of the N-*ras* gene, all of the other independent Ha-*ras* and N-*ras* mutations observed in these PBL DNAs were G→T transversions. The one C→A transversion at the CpG site probably represents a G→T mutation in the complementary strand (18, 19). Although the majority of sites assayed are in the *ras* proto-oncogenes, these data are in agreement with the high nontranscribed strand bias for G→T transversion mutations in *p53* (20). G→T transversions also appear to represent the majority of activation mutations in the human *ras* genes (21).

These mutations are most likely not artifacts of the PCR/RE/LCR assay. The major deoxyguanosine mutation induced by Taq polymerase is a G→A transition, with G→T transversion mutations being the least common error (22, 23). The second and third cycle of PCR and restriction enzyme (PCR/RE) selection use *Pfu* or *Pfu Turbo* polymerases, which have a higher fidelity, but produce G→T transition mutations (24, 25). However, the detection of an artifact-induced mutation during the second or third cycle of PCR/RE selection (after the initial 25 cycles of PCR in the first cycle of PCR/RE selection) is very unlikely in this assay (6). Also, analyses were run in duplicate and in triplicate in selective cases, and no discrepancies were found in the detection and identification of mutations between replicates.

DNA damage such as 8-hydroxydeoxyguanosine could cause mispairing during the initial PCR amplifications because this base adduct also hydrogen bonds with deoxyadenosine, and oxidative damage has been reported to occur during standard DNA isolation procedures (26). However, 8-hydroxydeoxyguanosine would have to occur consistently in the same base site at a high enough frequency to enable the replicate detection of mutations in a given specimen while not occurring significantly at this same site in other specimens. It is more likely that *in vivo* oxidative damage has given rise to these G→T transversion mutations before DNA isolation (26). However, transversions are also associated with exposure to tobacco smoke, aflatoxin, and other environmental carcinogens (5, 11, 27, 28).

In summary, the procedure of PCR/RE/LCR detection and identification of rare mutant cells at multiple loci has the potential to identify individuals who may be hypermutable in response to environmental insults, both endogenous and exogenous. The spectrum of base substitution mutations identified within groups of individuals who may be exposed to specific genotoxic agents will be valuable in the clarification of the agent or agents involved in the etiology of disease (11). Additionally, these techniques are not limited to peripheral blood specimens, but they do require significant quantities of DNA. Additional studies, including a more elaborate and well-designed epidemiological study of a larger number of people, will be required to characterize the potential of this approach. Discerning the *in vivo* dynamics of mutation frequencies per locus per cell division in a chosen tissue will require the study of multiple samples from an individual over time.

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