Weak Yet Distinct Mutagenicity of Acrylamide in Mammalian Cells

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Background: Despite concern raised with the announcement that common heating processes such as frying introduce acrylamide, a known rodent carcinogen, into food, the mutagenicity of acrylamide in mammalian DNA is controversial. Methods: Big Blue mouse embryonic fibroblasts, which carry a λ phage cII transgene, were treated with acrylamide. Formation of DNA adducts was determined by terminal transferase-dependent polymerase chain reaction. Mutational events were detected with a λ phage-based mutagenesis assay and expressed as the frequency of the number of mutant cII plaques per total number of plaques screened. Mutations were confirmed by DNA sequence analysis. All statistical tests were two-sided. Results: In vitro treatment of the cells with acrylamide at millimolar concentrations induced DNA adducts along the cII gene. Treatment with acrylamide at micromolar concentrations increased the frequency of mutations in the cII gene up to twofold relative to control treatment (13.8 × 10⁻⁵, 95% confidence interval [CI] = 12.3 to 15.3 × 10⁻⁵ versus 6.9 × 10⁻⁵, 95% CI = 6.5 to 7.3 × 10⁻⁵, df = 2, 21; P<.001; ANOVA). The specificity of acrylamide in inducing cII gene mutations was shown by a statistically significantly different mutational spectrum from that in control-treated cells, with an excess of G → C transitions and A → G transitions (P = .024; Adams and Skopek test). Although some of the frequently mutated sites in the cII gene co-localized with sites of preferential DNA adduct formation, there was no direct relationship. Conclusion: Acrylamide had distinct mutagenicity in transgenic mouse embryonic fibroblast cells, which might potentially be ascribed to its DNA adduct-inducing property. Whether acrylamide has the same effects on human cells is yet to be determined. [J Natl Cancer Inst 2003;95:889–96]

The recent discovery of acrylamide, a probable human carcinogen (1), in a variety of fried and starch-based food products has raised concern worldwide (2–4). To address this public health issue, the World Health Organization and the Food and Agriculture Organization of the United Nations held an urgent expert consultation in Geneva, Switzerland (5). Because of a lack of adequate epidemiologic and experimental data, the consultation could not establish the full extent of human health risks associated with dietary exposure to acrylamide; however, it encouraged the scientific community to engage in expeditious research on acrylamide to explore the chemical mechanisms governing its formation in food, its fate and bioavailability, and its toxicological effects, especially genotoxicity, carcinogenicity, and neurotoxicity (5).

Recently, two independent groups showed that acrylamide can be generated from food components during heating processes as a result of the Maillard reaction between amino acids...
and reducing sugars (6,7). The implication of this finding for human cancer epidemiology has prompted a resurgence of investigations into the genotoxicity of acrylamide and its mode of action in mammalian cells. To date, acrylamide has consistently had no effect in bacterial gene mutation assays with various strains of Salmonella, Escherichia coli (E. coli), and Klebsiella pneumoniae, in either the presence or the absence of an exogenous activation system. By contrast, glycidamide, the epoxy derivative of acrylamide, induced mutations in Salmonella strains TA100 and TA1535 (8,9). However, in vitro mammalian gene mutation assays, acrylamide has inconsistently induced mutations in mouse lymphoma cells at the thymidine kinase and hypoxanthine–guanine phosphoribosyl transferase loci (8–11). Likewise, in vivo in MutaMouse, acrylamide weakly induced mutations in the lacZ gene (12), whereas in the morphological specific-locus test and mouse spot test, acrylamide increased meiotic mutations in male germ cells (13) and induced spots of genetic relevance indicative of point mutations/chromosomal loss/somatic recombination in the (T × HT)F1 embryos, respectively (14). In carcinogenicity experiments, acrylamide administered via different routes increased the incidence of lung adenomas and initiated skin tumorigenesis in mice and induced scrotal mesotheliomas, thyroid adenomas, mammary gland tumors, uterine adenocarcinomas, clitoral gland adenomas, and oral papillomas in rats (8,9).

Given the non-conclusiveness of the data on acrylamide genotoxicity and the ambiguity of its mechanism(s) of action, an appealing hypothesis is that acrylamide triggers mutagenesis by damaging DNA. To test this hypothesis, we sought a methodology to determine whether acrylamide–DNA adduct formation could induce mutations at the gene level. A transgenic mouse system was chosen to allow us to examine mutation induction and DNA adduct formation in a single-test system. The in vitro system consists of Big Blue mouse embryonic fibroblasts with recoverable λ shuttle vectors that carry the mutational target, the cII transgene (15). This system has several major advantages over similar systems, including the ability to control the experimental conditions, easy recovery of the target transgene, and the cost-effectiveness of DNA sequence analysis and DNA adduct mapping of the entire cII gene (294 base pairs [bp]) (15).

#### MATERIALS AND METHODS

### Cell Culture and Chemical Treatment

Acrylamide (acrylamide for electrophoresis, purity: 99.9%; Boehringer Mannheim, Indianapolis, IN) was dissolved in double-distilled water and sterilized by passage through a 0.2-μm filter. Early-passage embryonic fibroblasts of Big Blue mice (prepared from 13.5-day-old embryos) were grown to monolayer confluence in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. After the cells reached confluence, the medium was removed and replaced with serum-free DMEM for several hours before the experimental treatments were begun. The cells were treated with various doses of acrylamide or control solvent (i.e., double-distilled water) in serum-free DMEM for 4 hours. For some experiments, the cells were harvested with trypsin immediately or 24 hours after the treatments to be used for the analysis of cell viability and DNA adduct formation. Cell viability was determined with the trypan blue exclusion assay. After the treatments, the medium was removed and replaced with complete growth medium and the cells were grown for an additional 8 days for use in mutation analyses. Each experimental condition was assessed in triplicate, and each experiment was done two or three times. In a separate experiment, cells were treated with increasing concentrations (0.01 μM, 0.1 μM, 1 μM) of the known carcinogen benzo[a]-pyrene diol epoxide (BPDE; Midwest Research Institute, Kansas City, MO) or control solvent (dimethyl sulfoxide) for 45 minutes in the dark. The cells were subsequently handled using the same protocol as indicated above for mutation analyses.

### Genomic DNA Isolation

Genomic DNA was isolated using a standard phenol and chloroform extraction and ethanol precipitation protocol (16). The DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and kept at −80°C until further analysis.

### Terminal Transferase-Dependent Polymerase Chain Reaction for Mapping of DNA Adducts

The entire length of the cII gene was subjected to terminal transferase-dependent polymerase chain reaction (TD-PCR) as described (17), with some modifications. Briefly, genomic DNA (~1 μg) was used as a template, and single-stranded products were made by repeated primer extensions. The extension protocol consisted of primer U1: 5'-AATCGAGAGTGCGTTGCTT-3' (Tm = 49.9 °C) in a mixture of Vent (exo-) DNA polymerase (New England Biolabs, Beverly, MA); and a thermocycler setting of 2 minutes at 95°C, 2 minutes at 53°C, 3 minutes at 72°C, nine cycles (in which one cycle consisted of 45 seconds at 95°C, 2 minutes at 53°C, and 3 minutes at 72°C), 45 seconds at 95°C, 2 minutes at 53°C, and 10 minutes at 72°C. The resulting product was precipitated with ethanol and a salt solution containing 10 M ammonium acetate, 0.5 M EDTA (pH 8.0), and glycogen at 20 mg/mL, and then subjected to the homopolymeric ribotailing and adapter ligation. The ligated fragments were PCR-amplified using primer U2: 5'-GCCTTGCCTTAA CAAAATCGCAATGCT-3' (Tm = 63.1 °C). The thermocycler was set for 2 minutes at 95°C, 2 minutes at 62°C, 3 minutes at 72°C, 18 cycles (in which one cycle consisted of 45 seconds at 95°C, 2 minutes at 62°C, and 3 minutes at 72°C), 45 seconds at 95°C, 2 minutes at 62°C, and 10 minutes at 72°C. Final primer extension of the PCR products was done using a fluorescence infrared dye-labeled primer (IRD-700; LI-COR, Lincoln, NE) U3: 5'-GCAATGCTTGAACCTGAGACAGC-3' (Tm = 61.4 °C). The thermocycler setting was 2 minutes at 95°C, 2 minutes at 65°C, 3 minutes at 72°C, six cycles (in which one cycle consisted of 45 seconds at 95°C, 2 minutes at 65°C, and 3 minutes at 72°C), 45 seconds at 95°C, 2 minutes at 65°C, and 10 minutes at 72°C. The labeled reaction products were subjected to gel electrophoresis (5% acrylamide/urea) in an IR2 Long Ranger 4200 system with simultaneous detection (LI-COR). The sites of DNA adduct formation were identified as the locations in which the presence of the lesions stopped the DNA polymerase from progressing, resulting in an intense dark band (dependent on the lesion frequency) in the sequencing gel.

### cII Mutation Frequency Analysis

The cII mutation frequency was quantified by using the λ Select-cII Mutation Detection System for Big Blue Rodents (Stratagene, La Jolla, CA). The assay system is based on the ability of the λ phage to multiply either lytically or lysogenically...
in *E. coli* host cells (15). The commitment of the λ phage to lysis or lysogeny upon infection of the host is regulated by a chain of events, of which cII transcription is a determiner (18). The cII protein activates the transcription of cl repressor and λ integrase, both of which obligate the phage to undergo lysogenization (18). Only λ LIZ shuttle vectors carrying mutant cII can enter the lytic pathway and, as a result, form visible plaques on an *E. coli* lawn (15). The λ LIZ vector, however, harbors a cI857 temperature sensitive (ts) mutation that makes the cI(ts) protein labile at temperatures exceeding 32 °C (19). Hence, under nonselective conditions, e.g., incubation at 37 °C, all λ LIZ phages, regardless of their cII mutant/nonmutant status, multiply lytically in the host *E. coli* (15).

Briefly, the λ LIZ shuttle vectors were recovered from Big Blue mouse embryonic fibroblast genomic DNA (=5 µg) and packaged into viable phage particles by Transpack packaging extract, according to the manufacturer’s instructions (Stratagene). The phages were then pre-adsorbed to G1250 *E. coli*, and the bacteria were plated on TB1 agar plates. The plates were incubated for 48 hours at 24 °C or overnight at 37 °C (regarded as selective and nonselective conditions, respectively). The cII mutation frequency was expressed as the ratio of the number of plaques formed on the selective plates to the number formed on the nonselective plates. As recommended by the manufacturer (Stratagene), a minimum of 3 × 10⁵ rescued phages was screened for each experimental condition. For quality assurance, control phage solutions containing a mixture of λ cII+ and λ cII− with known mutant frequencies (Stratagene) were assayed in all runs.

**cII Mutational Spectrum Analysis**

The cII plaques containing putative mutations were all verified after being replated under the selective conditions on a second TB1 agar plate. The verified plaques were subsequently amplified in a PCR by using a λ Select-cII sequencing primer (Stratagene), according to the manufacturer’s recommended protocol. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced by a Big Dye terminator cycle sequencing kit on an ABI Prism 377 DNA Sequencer (Applied BioSystems, Foster City, CA).

**Statistical Analysis**

Results are expressed as means and 95% confidence intervals (CIs). Mutation frequency data were analyzed by one-way analyses of variance (ANOVA). Mutational spectra were analyzed with the hypergeometric test of Adams and Skopek (20) and the chi-square test, where appropriate. All statistical tests were two-sided. Values of P≤.05 were considered statistically significant. The statistical software packages included Statview SM™ Graphics (Abacus Concepts, Amsterdam, The Netherlands) and DNA Mutation Analysis Application (21) available at: ftp://anonymous@sunsite.unc.edu/pub/academic/biology/.

**RESULTS**

**Acrylamide Cytotoxicity**

We first determined whether acrylamide was cytotoxic to Big Blue mouse embryonic fibroblasts. The cells were exposed to increasing concentrations of acrylamide (32 nM, 320 nM, 3.2 µM, 32 µM, 320 µM, 3.2 mM, 16 mM, 160 mM, and 320 mM) for 4 hours, and cell viability was determined by trypan blue dye exclusion over a period of 24 hours (Fig. 1). The cytotoxic effects appeared only when the cells were treated with concentrations of acrylamide in the millimolar range and were time- and dose-dependent. The cytotoxicity and reduction in the number of viable cells at doses of 3.2 mM or greater were accompanied by almost negligible cell division in the surviving cells (data not shown).

**Acrylamide-DNA Adduct Formation**

To determine whether acrylamide induced the formation of DNA adducts, cells were exposed to 3.2 mM and 16 mM acrylamide or control solvent (double-distilled water) for 4 hours. Genomic DNA was subsequently isolated and subjected to TD-PCR, a method that allows the mapping of polymerase-blocking lesions at nucleotide resolution (16). Our preliminary work showed that the TD-PCR results were more quantitative for samples treated with millimolar rather than micromolar doses of acrylamide (data not shown). Preferential adduct formation was observed at specific nucleotide positions along the cII gene in DNA isolated from acrylamide-treated cells when compared with DNA isolated from the control-treated cells (Fig. 2). The formation of DNA adducts at most nucleotide positions was dose-dependent.

**Acrylamide cII Mutation Induction**

We next determined whether acrylamide induced specific mutations in the cII gene. The cells were treated with incremental concentrations of acrylamide (32 nM, 320 nM, 3.2 µM, 32 µM, 320 µM, 3.2 mM, and 16 mM) for 4 hours and then allowed to grow for an additional 8 days. This time frame was chosen to permit multiple population doublings and fix any potential mutations into the genome. After 8 days, the cells were amplified in a PCR by using a λ Select-cII sequencing primer (Stratagene), according to the manufacturer’s recommended protocol. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced by a Big Dye terminator cycle sequencing kit on an ABI Prism 377 DNA Sequencer (Applied BioSystems, Foster City, CA).
analyzed to determine the mutation frequency and the mutational spectrum of the cII gene.

Induction of mutations in the cII gene by acrylamide was initiated at a concentration of $3.2 \times 10^{-5}$ M ($11.2 \times 10^{-5}$ to $16.2 \times 10^{-5}$) and increased dose dependently thereafter. The induced cII mutant frequency was maximal at an acrylamide dose of $320 \mu M$, showing a twofold increase in the number of mutations relative to control ($13.8 \times 10^{-5}$ to $15.3 \times 10^{-5}$, 95% CI = 6.5 to 7.3 × $10^{-5}$; df = 2, 21; P<.001, ANOVA). In cells treated with millimolar doses of acrylamide, the cII mutation frequency was lower, i.e., similar to that of untreated cells (i.e., baseline) (Fig. 3).

To provide a perspective for the mutagenic potency of acrylamide in our system, we treated a separate set of cells with various concentrations of BPDE, a well-known carcinogen (22). The BPDE-treated cells showed a linear dose-dependent increase in cII mutation frequency relative to control-treated cells ($r = .97$ and $P = .03$, simple regression analysis). The mutation frequency in cells treated with $0.01 \mu M$ BPDE was $14.3 \times 10^{-5}$ (95% CI = 10.4 to $18.2 \times 10^{-5}$), which was comparable to that induced with acrylamide at $320 \mu M$.

To analyze the mutational spectra (i.e., the kinds of mutational events), we sequenced the DNA isolated from the verified mutant cII plaques induced by acrylamide at $320 \mu M$ (number of sequenced plaques = 232) and derived spontaneously in the control-treated group (number of sequenced plaques = 173) for the entire length of the cII gene. Overall, eight (4.6%) samples in the control group and six (2.6%) samples in the acrylamide-treated group did not show any mutation throughout the cII gene. Four samples from each group harbored multiple mutations, all of these double- or triple-base substitutions or deletions. The total numbers of mutations in the control and acrylamide-treated groups were 170 and 231, respectively.

We next characterized the spontaneous and the acrylamide-induced cII mutational spectra. In both spectra, we observed three “jackpot” mutations at nucleotide positions 179–184 [G insertion/deletion], 211 [G → C transversion], and 221 [T → G transversion]. These jackpot mutations, already found in the cII gene in previous studies by us and by others (17,23–25), are assumed to occur in the early development of the transgenic rodent and to undergo clonal expansion such that many cells from a single tissue harbor the same type of mutation (26). Methodologically, therefore, it is appropriate to exclude these jackpot mutations from the comparative spectral analysis. The jackpot mutations accounted for 21% of the spontaneous and 17% of the acrylamide-induced cII mutations (Fig. 4, A and B).

Fig. 2. Mapping of DNA adducts along the lower strand of the cII gene in Big Blue mouse embryonic fibroblasts treated with $3.2 mM$ and $16 mM$ acrylamide or control solvent (double-distilled water, lane C) for 4 hours. Genomic DNA was extracted, and a terminal transferase-dependent polymerase chain reaction was used to map DNA lesions (16). Numbers indicate the nucleotide positions. M = sizing standard; bp = base pair; nt = nucleotide.

Fig. 3. Mutation frequency of the cII transgene in Big Blue mouse embryonic fibroblasts treated with various concentrations of acrylamide for 4 hours. Mutations were determined after 8 days with the λ Select-cII Mutation Detection System for Big Blue Rodents (Stratagene), a phage-based assay that permits detection of mutations within the transgene on the basis of plaque formation (nonmutant cII = no plaque formation; mutant cII = plaque formation). Mutation frequency was determined from a minimum of $3 \times 10^5$ plaques. Results are expressed as means of three independent experiments with 95% confidence intervals. Statistical significance between acrylamide-treated and control (double-distilled water-treated) groups was determined by analysis of variance.

$*P = .01$, $†P = .001$, $‡P < .001$, df = 2, 21.
The remaining nonjackpot mutations were predominately single-base substitutions, which constituted 73% and 81% of the spontaneous and acrylamide-induced cII mutations, respectively.

Because the cII gene is almost certainly not transcribed after being integrated into the genome (27), it is unlikely that the transgene is susceptible to “strand-dependent mutation,” a phenomenon that occurs in endogenous mammalian genes as a consequence of transcription-coupled DNA repair (28–30). Accordingly, we combined the strand mirror counterparts of all transitions (i.e., G → A + C → T) and transversions (i.e., G → T + C → A and G → C + C → G) and compared the spectra of the acrylamide-induced and spontaneous cII mutations (Fig. 5). The two mutational spectra were statistically significantly different from one another (P = .024, 95% CI = .016 to .031; Adams and Skopek test). Most notably, the percentages of all combined T → C plus A → G transitions and G → C plus C → G transversions induced by acrylamide showed 2.6-fold and 2.2-fold increases over those induced spontaneously (13.5% versus 5.2%, df = 1, P = .025 and 13.0% versus 6.0%, df = 1, P = .05, respectively; \( \chi^2 \) test).

Although some of the frequently mutated sites in the acrylamide-induced cII mutational spectrum co-localized with the preferential acrylamide–DNA adduction sites, there was no direct relationship between the pattern of induced mutations and the mapping of DNA adducts.
Fig. 5. Mutational spectra of the cII transgene in Big Blue mouse embryonic fibroblasts treated with 320 μM acrylamide (total number of sequenced mutant plaques = 232) or control solvent (double-distilled water; total number of sequenced mutant plaques = 173) for 4 hours. The strand mirror counterparts of all transitions (e.g., G → A + C → T) and transversions (e.g., G → T + C → A and G → C + C → G) were combined and used to compare the spectra of the induced and spontaneous cII mutations. Ins = insertion; Del = deletion. Statistical significance was compared by the chi-square test; *P = .05; †P = .025; df = 1.

DISCUSSION

Although the carcinogenicity of acrylamide in rodents is well established, whether acrylamide is mutagenic in mammalian and/or bacterial cells is still controversial (8,9). Moreover, the known organ-specific tumorigenicity of acrylamide is not concordant with its extent of DNA binding in the corresponding tissues (8,9). To address these issues, we investigated whether acrylamide could trigger DNA adduct formation and induce mutations in the cII gene in transgenic Big Blue mouse embryonic fibroblasts.

We found that, compared with the potent mutagen and known carcinogen BPDE (22), acrylamide was indeed a weak yet distinguishable mutagen in this test system. Our results showed that acrylamide could statistically significantly induce cII mutations only at relatively high doses (in the micromolar range) and that it produced a different spectrum of mutations from that derived spontaneously. At millimolar doses of acrylamide, however, the cII mutation frequency was at the baseline level (Fig. 3). The decrease in cII mutation frequency in cells treated with millimolar doses of acrylamide may reflect the mechanism of acrylamide mutagenicity. Glycidamide, an inhibitor of CYP2E1 (the isozyme involved in the epoxidation of acrylamide), acts directly with DNA via a Michael-type reaction, forming acrylamide-induced DNA adducts is unclear. Acrylamide intercalates of glycidamide than millimolar doses, thereby more frequently inducing cII mutations. We should, however, acknowledge that this possible scenario deserves further investigation because the exact metabolic machinery of the embryonic mouse fibroblasts may differ from that of an organ (i.e., the liver) in the fully grown animal.

To elucidate the importance of acrylamide-induced DNA adducts in the mutagenicity of acrylamide, we sought an association between the preferential adduct formation sites and the mutational hotspots produced by this compound in the cII gene. The profile of acrylamide-induced DNA adducts did not perfectly match that of acrylamide-induced mutations. Although some of the frequently mutated sites in the cII gene co-localized with the sites of preferential DNA adduction, there was no direct relationship. This may imply that not all DNA adducts formed by acrylamide are necessarily promutagenic. The imperfect correspondence between preferential DNA adduct sites and mutational hotspots might to some extent be methodologic, because the TD-PCR is a polymerase arrest-based assay, which indiscriminately quantifies all lesions regardless of their mutagenicity (16). It needs to be taken into account that the relatively low sensitivity of the TD-PCR for quantification of DNA adducts formed at the micromolar doses of acrylamide did not permit parallel analyses of the adduct mapping and the mutational spectrum produced at the same dose of acrylamide. Therefore, we could only establish an association between DNA adduct formation data obtained at the cytotoxic (millimolar) dose of acrylamide with the mutational data found at the non-cytotoxic (micromolar) dose of this compound.

Whether the mutagenicity of acrylamide is associated with acrylamide-induced DNA adducts is unclear. Acrylamide interacts directly with DNA via a Michael-type reaction, forming various adducts, including 1-carboxyethyl adenine, 3-carboxyethyl cytosine, 7-formamidoethyguanine, 1-carboxyethyl guanine, and N^2-carboxyethyl adenine (33). Moreover, glycidamide can alkylate DNA primarily at the N^1 position of guanine, thereby giving rise to N^1-2-carbamoyl-2-hydroxyethylguanine (9,34). Although the mutagenicities of the different DNA adducts are yet to be determined, the glycidamide adduct may well be promutagenic, especially in light of the positive results obtained with glycidamide in some mutagenesis assays (8,9). In our
study, the non-dose dependency of DNA adduct formation at specific nucleotide positions, e.g., 161, 167, and 176–187, may indicate that at least some of the formed adducts are glycidamide-derived. However, the contribution of such presumed glycidamide–DNA adducts to the observed mutagenicity of acrylamide is unclear. It remains to be seen if glycidamide–DNA adducts indeed gave rise to the herein induced cII mutations because the more specific mutational events ascribed to acrylamide (T → C + A → G transitions and G → C + C → G transversions) were not detected at nucleotide positions 161, 167, and 176–187 in the treated samples. To explore the involvement of glycidamide in acrylamide mutagenicity, we are currently investigating the mutagenic effects of glycidamide on the Big Blue mouse embryonic fibroblasts. It is also worth mentioning that several of the above-identified acrylamide DNA adducts are labile DNA bases, which can be converted to abasic sites. This fact, together with the diverse pattern of mutations induced by acrylamide, supports the idea that more than one type of DNA adduct might be involved in acrylamide mutagenicity.

In summary, our data show a distinct pattern of mutagenicity for acrylamide in mammalian cells, which may potentially be ascribed to its DNA adduct-inducing property. Given a limitation of our study—that is, it was conducted at a transgene rather than a native gene level—we must be cautious in interpreting our results, particularly in generalizing their biologic significance to humans. To this end, our findings warrant further investigations into the carcinogenicity of acrylamide in humans. Such investigations will require careful study design because acrylamide is relatively ubiquitous in the diet, is found in high concentration in tobacco smoke, and is formed endogenously. All of these properties may easily act as confounding factors and complicate the interpretation of the data (35). The interfering role played by such confounders may be best reflected in the conventional epidemiologic studies in which occupational exposure to acrylamide could not be linked to mortality from any types of cancer (36,37).

REFERENCES


NOTES

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