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Modulation of repair of ultraviolet damage in the host-cell reactivation assay by polymorphic *XPC* and *XPD/ERCC2* genotypes

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DNA repair capacity (DRC) plays an important role in genetic susceptibility to cancer. Polymorphisms of a number of DNA repair genes involved in several distinct pathways have been identified. However, their effects on repair function have not been well characterized. We demonstrated previously that DRC for removal of benzo[*a*]pyrene diol epoxide-induced DNA damage measured by a host-cell reactivation assay was modulated by two *XPD/ERCC2* polymorphisms in lung cancer. In this report, we investigated the association between the repair phenotype of ultraviolet (UV)-induced damage and genotypes of three DNA repair genes, *XPC* and *XPD* [involved in nucleotide excision repair (NER)] and *XRCC1* [involved in base excision repair (BER)]. We measured DRC for removal of UV photoproducts by the host-cell reactivation assay in cryopreserved lymphocytes from 102 healthy non-Hispanic white subjects. We also typed these subjects for five polymorphisms in these three DNA repair genes (at intron 9 of *XPC*; exons 6, 10 and 23 of *XPD* and exon 10 of *XRCC1*). Compared with wild-type homozygotes, subjects homozygous for polymorphisms of the two NER genes consistently had suboptimal DRC. The DRC was consistently lower in subjects homozygous for *XPC*, *XPD* or both than in subjects with other genotypes, although the difference was not statistically significant for *XPD* variants. In contrast, the polymorphic allele of the BER gene, *XRCC1*, had no consistent effect on DRC. We concluded that these NER polymorphisms may modulate DRC and may be useful biomarkers for identifying individuals at risk of developing cancer.

Introduction

DNA repair capacity (DRC) plays a role in genetic susceptibility to cancer both in hereditary cancer syndromes (1) and in sporadic cancer (2,3). A number of DNA repair genes participate in several distinct pathways. Nucleotide excision repair (NER) is responsible for removing a variety of DNA damage, including ultraviolet (UV)-induced photoproducts, bulky monoadducts, cross-links and oxidative damage (4). The defect in NER, seen in patients with xeroderma pigmentosum (XP) who are not able to repair UV-induced DNA damage efficiently, is associated with a 1000-fold increased risk of sunlight-related skin cancer (1). In addition, there is inter-individual variation in DRC among both cancer patients and healthy subjects, and the ability to repair UV-induced photoproducts and benzo[*a*]pyrene diol epoxide (BPDE)-induced adducts in peripheral lymphocytes is a significant predictor of risk of sun-induced skin cancer (2) and smoking-related head and neck (5) and lung cancer (3), respectively.

To understand inter-individual variation in DRC and to provide a tool for risk assessment of genetic susceptibility to cancer, the search for polymorphisms, particularly single nucleotide polymorphisms, of DNA repair genes has been intensified. To date, two polymorphisms of *XPC* (6), 13 of *XPD/ERCC2* and nine of *XRCC1* (7) have been identified. Although mutations in *XPC* and *XPD* result in defective NER and the XP phenotypes (8,9), the functional relevance of the polymorphisms has not been determined. Studies of the effects of these polymorphisms on the functions of the proteins are the first step to understanding their association with complex diseases such as cancer in the general population (10). However, understanding the correlation between DNA repair genotypes and phenotypes is also an important step towards determining how the polymorphic genotypes are associated with cancer in the general population. In a previous study, we found that two *XPD* polymorphisms modulate DRC measured by the host-cell reactivation (HCR) assay using the ultimate tobacco carcinogen BPDE in lung cancer patients (11).

To further investigate the correlation of the polymorphisms of XP genes with the DNA repair phenotype, we genotyped 102 healthy subjects for a new polymorphism of *XPC* [poly(AT) in intron 9] that is in close linkage disequilibrium with a single nucleotide polymorphism in exon 15 (6). We also examined three polymorphisms of *XPD*: the A→C silent polymorphism (C22541A in exon 6), Asp312Asn (exon 10) and Lys751Gln (exon 23) (8). Other *XPD* polymorphisms were not examined because they do not cause amino acid changes or because the frequencies of their least common alleles were too small (<0.10) to be useful in this study. The Arg399Gln polymorphism of the base excision repair (BER) gene *XRCC1* was also examined to determine the damage-repair pathway involved, because this polymorphism has been linked to risk of cancer (12,13).

We also measured in short-term cultures of undamaged, cryopreserved primary peripheral lymphocytes of these subjects the NER capacity to remove UV-induced DNA damage using the HCR assay with plasmids harboring chloramphenicol acetyltransferase (*CAT*). The HCR assay is relatively fast and an objective way of measuring intrinsic cellular DRC (14). We hypothesized that these *XPC* and *XPD* polymorphisms

Abbreviations: BER, base excision repair; BPDE, benzo[*a*]pyrene diol epoxide; CAT, chloramphenicol acetyltransferase; DRC, DNA repair capacity; HCR, host-cell reactivation; NER, nucleotide excision repair; PCR, polymerase chain reaction; UV, ultraviolet; XP, xeroderma pigmentosum.

may modulate the host cells' DRC for removing bulky lesions induced by UV. If so, then we might be able to detect differences in DRC in individuals by genotyping these genes. These functional assays require viable lymphocytes and so are not currently suitable for large-scale population-based epidemiological studies of cancer susceptibility. Our objective was to identify genotypes that predict DRC phenotype and therefore could be used in future high-throughput analysis.

Materials and methods

Study subjects

The subjects were 102 healthy controls from an ongoing case-control study of skin cancers at The University of Texas M. D. Anderson Cancer Center. These healthy subjects were genetically unrelated visitors or companions of patients seen at M. D. Anderson clinics and none were included in our previous report (11). The research protocol was approved by the M. D. Anderson Institutional Review Board.

Blood collection, isolation of lymphocytes and cell cuture

Each subject donated 30 ml of blood collected in heparinized tubes. The lymphocytes were isolated by Ficoll (Pharmacia Biotech, Piscataway, NJ) gradient centrifugation and suspended in freezing medium containing 50% fetal bovine serum, 40% RPMI 1640 medium (Gibco BRL, Grand Island, NY) and 10% dimethyl sulfoxide (at 10^7 cells/ml), and 2.0 ml aliquots were stored in a –80°C freezer. They were later thawed in batches for the HCR assay.

UV treatment and transfection of plasmids

The plasmid expression vector pCMV*cat* was used for the CAT assays (14). One batch of the plasmids was treated with UVC and used for all assays in this study. For the UV treatment, plasmid stock (500 mg/ml) was diluted with TE (pH 7.8) to 50 mg/ml, and 2 ml was pipetted into a 60 mm culture dish on ice. The plasmids were then irradiated with 0 and 800 J/m^2 UVC (254 nm) with a 15 W germicidal lamp (Sankyo Denki Co., Japan). After UV treatment, the plasmids were stored in aliquots at –80°C.

The cells in each frozen vial were thawed quickly at 37°C in a water bath and mixed (before the last trace of ice disappeared) with 7 ml of thawing medium (50% fetal bovine serum, 40% RPMI 1640 medium and 10% dextrose). Cell viability was determined microscopically with 0.4% trypan blue stain (Sigma Chemical Co., St Louis, MO) and a hemocytometer. Then, the cells were centrifuged at 900 r.p.m. for 10 min and resuspended at 0.3×10^6/ml in RPMI 1640 medium (supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin). The cells were then cultured in RPMI 1640 supplemented with 20% fetal calf serum (Gibco BRL) and 56.25 mg/ml phytohemagglutinin (Murex Diagnostics, Norcross, GA) at 37°C and 5% CO_2 and incubated for 72 h. The lymphoblasts were then counted to calculate the blastogenic rate. The lymphoblasts from each subject were divided into four aliquots, each containing $0.5–1.0\times10^6$ cells. The diethylaminoethyl-dextran (Pharmacia Biotech) method (14) was used to transfect two aliquots with undamaged pCMV*cat* and two with pCMV*cat* damaged with 800 J/m^2 UV. The cultures were then incubated for 40 h after transfection.

DRC phenotype

DRC phenotype was measured by the CAT assay. The procedures for this assay were basically the same in terms of cell culture, cell harvesting and transfection as described previously (14). The radioactivity of the CAT assay products was measured with a liquid scintillation counter (Beckman Instruments, Fullerton, CA). The CAT activity, measured by counts per minute, was recorded for the cells with undamaged (background reading) and UV-damaged (repair reading) plasmids. DRC (%) was calculated as a ratio of the damaged plasmid values to the undamaged plasmid values and multiplied by 100%.

Polymerase chain reaction (PCR)-based genotyping

All blood samples used for the DNA repair assays were also used for DNA extraction. A published protocol for non-radioactive PCR was used to type the intron 9 polymorphism of *XPC* [an 83 bp poly(AT) insertion with a 5 bp deletion of GTAAC] (6). The primers for *XPC* intron 9 generated a 266 bp fragment (the wild-type or PAT– allele) and a 344 bp fragment (the polymorphic or PAT+, allele). PAT+/– heterozygotes have both bands. Published protocols for PCR-restriction fragment length polymorphism assays were used to type three polymorphisms of *XPD*: the C→A silent polymorphism of exon 6 (13) and Asp312Asn of exon 10 and Lys751Gln of exon 23 (11). In the *C22541A* of *XPD* exon 6 polymorphism, the wild-type allele has a single *Tfi*I-restriction site resulting in two bands (587 and 57 bp), the polymorphic allele produces three fragments (474, 113 and 57 bp) and the heterozygote has four bands (587, 474, 113 and 57 bp). In the Asp312Asn exon 10 polymorphism, wild-type (Asp/Asp) homozygotes are identified by two DNA bands (507 and 244 bp), Asn/Asn homozygotes produces three bands (474, 244 and 33 bp) and Asp/Asn heterozygotes have all four bands (507, 474, 244 and 33 bp). In the Lys751Gln exon 23 polymorphism, the homozygous wild-type allele (751Lys) produces two DNA bands (290 and 146 bp), whereas the polymorphic allele (751Gln) produces three DNA bands (227, 146 and 63 bp) and heterozygotes have all four bands (290, 227, 146 and 63 bp). The *XRCCI* Arg399Gln polymorphism was typed according to published protocols (12).

Statistical methods

Pearson's χ^2 was used to test the differences in the distributions of qualitative variables. Hardy–Weinberg equilibrium was tested by a goodness-of-fit χ^2 test to compare the observed genotype frequencies to the expected genotype frequencies among the subjects. DRC data were analyzed as continuous variables before and after natural logarithmic transformation. Student's *t*-test was used to compare DRC by genotypes. Pearson's correlation analysis was performed for continuous variables. The linkage disequilbrium parameter δ was computed for two-locus (15) and three-locus (16) models. These normalized disequilibrium coefficients are denoted by δ'. We compared DRC for each genotype and combined genotypes for the same gene. A P value of 0.05 for any test or model was considered to be statistically significant. All statistical tests were two-sided and were performed with Statistical Analysis System software (version 6.2; SAS Institute, Cary, NC).

Results

DNA repair phenotype and genotypes of the subjects

This analysis included 50 male and 52 female healthy non-Hispanic white subjects aged 19–78 years (mean age, 51.7; median age, 52.5). The allele frequencies for *XPC* PAT+, *XPD* 156Arg, 312Asn and 751Gln and *XRCC1* 399Gln were 0.387, 0.485, 0.294, 0.363 and 0.387, respectively (Table I), which are consistent with the results of published studies with larger sample sizes (11,13,17,18). The distributions of the variant genotypes of all three genes were very close to the values predicted from the Hardy–Weinberg equilibrium model (data not shown).

We then compared the results of the CAT assays by *XPC*, *XPD/ERCC2* and *XRCC1* genotypes. As shown in Table I, the DRC for *XPC* PAT+/+ was significantly lower (23.4% less; $P = 0.020$) than that of the wild-type homozygotes, but the heterozygous *XPC* genotype did not appear to have an effect on DRC. Homozygosity of *XPD* polymorphic alleles was also consistently associated with lower DRC (7.9, 5.4 and 14.6% less for exons 6, 10 and 23, respectively) than wild-type homozygosity was, although these differences were not statistically significant. It is interesting that those heterozygous for the two polymorphisms at exons 10 and 23, which cause amino acid changes, consistently had DRC levels similar to the wild-type homozygotes (Table I), suggesting that these polymorphic alleles may have a recessive effect on DRC phenotype. The exon 6 polymorphism, which does not cause an amino acid change, seemed to have an allelic effect on DRC that may be due to disequilibrium with other functional polymorphisms.

Because there were fewer *XPC* and *XPD* polymorphic-allele homozygotes than of individuals of other genotypes (except for *C22541C* homozygotes), we combined these variant homozygotes into one group, i.e. those homozygotes for one or more polymorphic XP alleles. This group ($n = 45$) had lower DRC (11.7% less) than the other genotypes ($n = 57$) in the CAT assay, although this difference did not approach statistical significance ($P = 0.119$). In contrast, there was no evidence that the homozygotes for the *XRCC1* polymorphic allele had a lower DRC than wild-type homozygotes (Table I).

We also tested for linkage disequilibrium and associations

Table I. Differences in DRC phenotype by *XPC, XPD* and *XRCC1* genotypes

Genotype	No. (%)	DRC (%) (mean ± SD)	Relative reduction (%)	P^a
All subjects	102 (100)	8.54 ± 3.37		
XPC intron 9				
PAT–/–	36 (35)	8.79 ± 2.42	Reference	Reference
PAT–/+	53 (52)	8.81 ± 3.86	0.2	0.978
PAT+/+	13 (13)	6.73 ± 3.18	–23.4	0.020
PAT+ allele frequency	0.387			
XPD exon 6 C22541A, Arg156Arg				
AA	22 (22)	9.34 ± 4.65	Reference	Reference
AC	55 (54)	8.18 ± 3.13	–12.4	0.208
CC	25 (24)	8.60 ± 2.47	–7.9	0.492
A allele frequency	0.485			
XPD exon 10 G23591A, Asp312Asn				
Asp/Asp	53 (52)	8.51 ± 3.60	Reference	Reference
Asp/Asn	38 (37)	8.71 ± 3.26	2.4	0.786
Asn/Asn	11 (11)	8.05 ± 2.78	–5.4	0.691
A allele frequency	0.294			
XPD exon 23 A35931C, Lys751Gln				
Lys/Lys	46 (45)	8.30 ± 2.22	Reference	Reference
Lys/Gln	38 (37)	9.51 ± 4.22	14.6	0.096
Gln/Gln	18 (18)	7.09 ± 2.49	–14.6	0.063
C allele frequency	0.363			
One or more homozygous XP polymorphic alleles				
No	57 (56)	9.00 ± 3.65	Reference	Reference
Yes	45 (44)	7.95 ± 2.92	–11.7	0.119
XRCC1 exon 10 G28152A, Arg399Gln				
Arg/Arg	38 (37)	8.36 ± 4.13	Reference	Reference
Arg/Gln	49 (48)	8.66 ± 2.97	3.6	0.695
Gln/Gln	15 (15)	8.58 ± 2.56	2.6	0.849
A allele frequency	0.387			

[a]Two-sided *t* test.

of the variant genotypes and variant alleles of these three genes, respectively. In the two-locus analysis, the normalized disequlibrium coefficient (δ') was 0.694 for *XPD* Lys751Gln and *XPD* Arg156Arg ($\chi^2 = 34.89$; $P = 3.5\times10^{-8}$), 0.819 for *XPD* Arg156Arg and *XPD* Asp312Asn ($\chi^2 = 34.73$; $P = 3.8\times10^{-8}$) and 0.711 for *XPD* Lys751Gln and *XPD* Asp312Asn ($\chi^2 = 53.48$; $P = 2.6\times10^{-12}$) (data not shown). Hence we concluded that these pairs of loci were in linkage disequilibrium. In the three-locus analysis, δ' for these three loci was 0.174 with $\chi^2 = 100.55$, with the corresponding *P* value being very close to zero. Hence we concluded that these three loci were in linkage disequilibrium. Because the *XPD* C22541A mutation did not cause an amino acid change (156Arg), lower DRC in *XPD* 156Arg(cc) homozygotes (Table I) may have been due to the linkage disequilibrium with the other two *XPD* polymorphisms. Because the *XPD* A35931C mutation, which results in an amino acid change (Lys751Gln), was strongly linked to the other two *XPD* polymorphisms, we used it for the linkage analysis with the other polymorphisms of *XPC* and *XRCC1*.

However, the linkage disequilibrium between pairs of polymorphisms of the three genes (*XPD* Lys751Gln versus *XPC*-PAT, *XPD* Lys751Gln versus *XRCC1* Arg399Gln and *XPC*-PAT versus *XRCC1* Arg399Gln) was not statistically significant by the two-loci test ($P = 1.000$, 0.578 and 0.791, respectively) or by the three-loci test ($P = 0.463$) (data not shown). While *XPC* is on chromosome 3p25, *XPD* is on chromosome 19q13.2-13.3 ~250 kb from *XRCC1* at the same locus. The associations between these five polymorphisms of the three genes are summarized in Table II. Consistent with the results of linkage disequilibrium analysis, the distributions of the genotypes of the three *XPD* polymorphisms were significantly associated with each other ($P < 0.0001$), whereas the distribution of the *XPC* polymorphic genotypes was only borderline significantly associated with that of the *XPD* Asp312Asn polymorphism, and the *XRCC1* polymorphism was not associated with any other genotype.

Discussion

In this study, we demonstrated that post-UV DRC for NER can be modulated by genetic polymorphisms of NER genes such as *XPC* and *XPD*. Because UV damage is repaired by NER and not by the BER pathway, the specific repair pathway of the post-UV HCR assays used in this study was also demonstrated by the lack of correlation between DRC phenotype and a BER gene, *XRCC1*. Although our sample was rather small, *XPC* PAT+/+ homozygous subjects exhibited lower DRC than did those with other *XPC*-PAT genotypes. However, heterozygosity for PAT–/+ was not associated with suboptimal DRC, suggesting that *XPC* PAT+/+ is an adverse genotype rather than *XPC* PAT+ itself having an effect on DRC. Indeed, we found in a previous study that the homozygous *XPC* genotype, PAT+/+, is associated with increased risk of developing head and neck cancer (17).

We consistently observed a lower DRC for UV-induced DNA damage in those homozygous (but not heterozygous) for polymorphic *XPD* alleles than in those homozygous for the wild-type allele. Our results are consistent with our earlier reports that the *XPD* polymorphisms contribute to the risk of developing cancers of the head and neck (13) and the lung (11). In our case-control study of 316 lung cancer cases and 316 healthy controls (11), we demonstrated that the reduction in post-BPDE DRC was more significant in lung cancer

Table II. Associations between *XPC*, *XPD* and *XRCC1* genotypes in 102 healthy subjects

Variant	χ^2 value / *P* value			
	XPC intron 9 Poly-AT	*XPD* exon 6 Arg156Arg	*XPD* exon 10 Asp312Asn	*XPD* exon 23 Lys751Gln
XPD exon 6 Arg156Arg	7.104 / 0.131			
XPD exon 10 Asp312Asn	9.714 / 0.046	36.206 / <0.0001		
XPD exon 23 Lys751Gln	4.141 / 0.387	36.267 / <0.0001	53.718 / <0.0001	
XRCC1 exon 10 Arg399Gln	4.684 / 0.321	8.486 / 0.075	6.677 / 0.154	3.738 / 0.443

patients who were homozygous for two *XPD* polymorphisms, Asp312Asn of exon 10 and Lys751Gln of exon 23 (–12.3 and –18.3%, respectively), than in the controls (–3.3 and –5.4%, respectively). Among these lung cancer patients, those homozygous for the *XPD* Asn312Asn and Gln751Gln had an increased risk of having suboptimal DRC phenotypes (ORs = 1.57 and 3.50, respectively) compared with wild-type homozygotes (11). In the present study with 102 healthy controls who were not included in this previous report (11), we further demonstrated that the homozygous genotypes of these two *XPD* polymorphisms plus another *XPD* silent polymorphism, Arg156Arg, were similarly associated with lower post-UV DRC (14.6, 5.4 and 7.9% less, respectively) compared with the homozygous wild-type genotypes. These results, combined with the *XPC* results in this report, suggest a NER pathway-specific association between the genotypes and phenotype, because both BPDE and UV-induced DNA damage is effectively repaired by NER (4). The consistency of the findings is highlighted by our use of different populations and different DNA damage induced by different carcinogens.

Several other studies, with relatively small sample sizes and using different assays for assessing DNA repair phenotype, have examined the correlation between the repair phenotype and the newly identified polymorphisms of *XPD* (7). Using the comet assay, which is not specific for particular repair pathways, Moller *et al*. (19) investigated the formation of DNA strand breaks after UVC irradiation in lymphocytes from 20 psoriasis patients with and 20 without basal cell carcinoma. Their results suggested that high break levels were correlated with the AA or AC genotypes of polymorphisms in exons 6 and 23 of *XPD*, supporting their original finding that the A alleles of exons 6 (156Arg) and 23 (Lys751) were risk alleles in patients with psoriasis. Because the positive findings were in subjects with psoriasis, the results cannot be extended to the general population.

Lunn *et al*. (20) compared the *XPD* genotypes of codons 199 (exon 8), 312 (exon 10) and 751 (exon 23), but not C22541A of exon 6, with the DNA repair proficiency reflected by repair of chromosomal aberrations induced by X irradiation in 31 women. Too few individuals had the codon 199 variant (Ile/Met) for a meaningful analysis, but individuals who had the codon 312 variant (Asp/Asn) did not differ in X-ray-induced DNA damage from those with other genotypes, and individuals with the codon 751 variant (Lys/Lys) had more chromatid aberrations than those with a 751Gln allele, which was suggested to be the result of suboptimal repair of X-ray-induced DNA damage. The numbers in each subgroup of this study were also small (four to 12). Because this study included only women who had a high risk for breast cancer because they had a family history of breast cancer, and because cells from XPD patients with mutations in *XPD* are not sensitive to ionizing radiation, including X-rays (21), the involvement of *ATM* rather than *XPD* in breast cancer may partly explain the hypersensitivity to X-rays (22).

Our subjects did not have any known medical conditions. The discrepancies between our findings and those of other investigators may be due to (i) use of the HCR assay, which does not involve damage to cells, whereas the assays used in previous studies measured repair of chromosomal damage (the G2 sensitivity assay) and DNA damage (the Comet assay) as a result of direct exposure of the cells to carcinogens; (ii) the fact that repair of chromosomal aberrations is not related to NER measured by the HCR assay, or most importantly, (iii) that the previous studies examined subjects with pre-existing medical conditions.

Our data did not show any impact of *XRCC1* polymorphism on DRC. In a study of 76 subjects, Duell *et al*. (12) found that the *XPD* polymorphism was not related to sister chromatid exchange frequency or DNA adduct level. However, the mean sister chromatid exchange frequencies among current smokers who were homozygous for the *XRCC1* codon 399Gln allele were greater than those in codon 399 Arg/Arg current smokers, and there were significantly more adducts in older subjects with the 399Gln allele than in younger subjects with the 399 Arg/Arg genotype. These results are consistent with an earlier report that the *XRCC1* 399Gln allele is significantly associated with higher levels of both aflatoxin B1–DNA adducts and glycophorin A NN mutations (20) and risk of developing cancer (18,23–28). However, we did not find evidence for a reduction in DRC due to the variant *XRCC1* allele. While BER plays a major role in reducing the damage to DNA from direct exposure to chemical carcinogens, NER deals with more bulky DNA lesions induced by BPDE or UV. It is probable that the effect of *XRCC1* polymorphism on the DNA repair phenotype cannot be evaluated by the post-UV HCR assay, which measures NER but not BER. However, the HCR may be adapted for BER by introducing oxidative damage into the plasmids (29).

In summary, our results revealed a correlation between DNA repair genotypes and phenotype in 102 healthy subjects. However, our findings, particularly for the *XPD* genotypes, may also be due to chance. Because the variation in the HCR assay was relatively large and the differences were modest, larger studies are needed to detect a statistically significant difference in the DRC phenotype among individuals with different DNA repair genotypes. However, the results of this study further support our hypothesis that each variant NER allele may contribute to the NER phenotype and therefore to genetic susceptibility to cancer. Because more polymorphisms of DNA repair genes will be identified, this type of study is a step towards our objective of identifying variant DNA repair genotypes that predict DRC and therefore can be used in future

high-throughput analysis in molecular epidemiological studies. It is clear that simultaneously genotyping many of these polymorphisms will enhance both the probability of identifying individuals with suboptimal DRC and our ability to perform risk assessment, particularly assessment of genetic susceptibility to cancer.

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