

# Hypermutable of UV-treated Plasmids in Dysplastic Nevus/Familial Melanoma Cell Lines

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## ABSTRACT

Members of cutaneous melanoma (CM) families with dysplastic nevi (DN) are at high risk of developing CM. Using a shuttle vector plasmid, pSP189, cell lines from three patients with CM plus DN were previously found to have elevated post-UV plasmid mutability. To investigate familial occurrence of this cellular phenotype, we examined post-UV plasmid mutability in 31 lymphoblastoid cell lines from 6 familial CM kindreds. In comparison to 16 normal control lines, we found an abnormally elevated post-UV plasmid mutability in cell lines from 13 of 13 patients with CM plus DN ( $P = 1.5 \times 10^{-8}$ ) and from 5 of 8 patients with DN only ( $P = 0.001$ ). Elevated spontaneous plasmid mutation frequency (MF) was also present in cell lines from six of the CM plus DN patients ( $P = 0.002$ ) and three of the DN-only patients ( $P = 0.028$ ). However, cell lines from two patients with CM without DN had normal post-UV plasmid MF. Although not specific for CM patients, of 27 cell lines with elevated post-UV plasmid MF, only 8 were from donors who did not have CM + DN or DN (19 of 24 versus 8 of 28;  $P = 0.0003$ ). This study indicates that post-UV plasmid hypermutability is a laboratory marker for members of melanoma-prone families and suggests that patients with familial CM have a defective mechanism for handling UV-induced DNA damage.

## INTRODUCTION

Approximately 8–12% of melanoma cases occur in people with a history of CM<sup>3</sup> in a blood relative (1, 2). DN are morphologically atypical moles that are more irregular and often larger than common pigmented moles and have a characteristic histological appearance with nuclear atypia and a disorderly growth pattern of melanocytes (3–5). These lesions are both markers of people with increased risk of melanoma and are precursors of melanoma. Members of CM families with DN are at extremely high risk of developing CM (relative risk elevated about 100-fold; Refs. 2 and 6). However, the mechanism of this increased melanoma risk is not understood.

Sunlight has been implicated in induction of CM and DN (7, 8), and there is clinical evidence for sun sensitivity in patients with familial CM. There have been occasional reports of hypersensitivity to killing by UV in cells from patients with familial CM and DN (9, 10), but this has not been a consistent finding (11, 12). Earlier studies have shown that several cultured cell lines from CM patients with CM and DN are hypermutable to UV (10, 11). Recently, shuttle vector plasmids have been developed that permit the assessment of the extent of induction of post-UV mutagenesis in DNA replicated in human cells (13, 14). In pilot studies, we demonstrated UV hypermutability in a shuttle vector plasmid replicated in cell lines from three patients with CM who had severe involvement with CM plus DN (15, 16). The present study was designed to investigate familial occurrence of this cellular phenotype.

Received 6/4/97; accepted 8/16/97.

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<sup>3</sup> The abbreviations used are: CM, cutaneous melanoma; DN, dysplastic nevi; MF, mutation frequency; XP, xeroderma pigmentosum.

We examined cell lines from 6 kindreds comprising a total of 31 blood-line family members with different clinical features (CM plus DN, CM alone, DN alone, clinically unaffected, or clinically indeterminate). We found post-UV plasmid hypermutability in all cell lines from family members with CM plus DN and from many family members with DN alone.

## PATIENTS AND METHODS

**Subjects.** Families with at least two living cases of CM in first-degree relatives (6, 17) were studied (Table 1). All CM diagnoses were confirmed using histological review, local pathology reports, medical records, or death certificates. Both invasive CM and noninvasive lesions (melanoma *in situ*) were designated as melanomas. DN were diagnosed using previously published clinical and histological criteria (2, 4, 5). Prepubertal subjects (less than 16 years of age) were classified as indeterminate unless they had definite clinical and/or histological evidence of DN because these often do not develop before the onset of puberty (18). Families 479, 562, 928, and 1017 had germ-line mutations in *p16* (19), family 342 had a germ-line mutation in *CDK4* (20), and family 373 had no detectable disease-specific germ-line mutation.

**Cells.** Lymphoblastoid cell lines were established by EBV transformation of blood lymphocytes from members of six families with CM and DN (Table 1; Refs. 11 and 19). All blood-line family members and their spouses whose cell lines were studied were examined clinically. Sixteen normal control lines from donors ranging in age from 4 to 98 years were studied (21). These normal control lines were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ) and from Dr. Alan Bale (Yale University, New Haven, CT), and the donors were not examined. Cells were cultured in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) supplemented with 15% fetal bovine serum (S&S Media, Rockville, MD) and 2 mM L-glutamine (Life Technologies, Inc.) at 37°C in a 5% CO<sub>2</sub> atmosphere as described (11, 16).

**Plasmid.** The shuttle vector plasmid, pSP189, (14) was UV treated, transfected, recovered, and assayed as described previously (16). Briefly, the 5.5-kb pair plasmid contained an SV40 origin of replication, an enhancer, large-T antigen sequences, which permit replication in human cells, and the pBR327 origin of replication, which permits growth in bacteria. An approximately 150-bp bacterial *supF* suppressor tRNA gene serves as a marker for mutations, and a gene for ampicillin resistance permits selection in bacteria. Our pilot study (16) showed a linear relationship between the UV dose to the plasmid and the plasmid MF in lymphoblastoid cells from normal and CM donors. On the basis of these results (16), pSP189 was treated with 1000 J/m<sup>2</sup> UV from a germicidal lamp and transfected into the human cells by use of DEAE-dextran. Untreated plasmid pZ189K (with the gene for kanamycin resistance) was cotransfected as an internal standard. After 2 days, the replicated plasmids were harvested from the cells and used to transform indicator bacteria. The bacteria were plated on agar containing ampicillin or kanamycin and colorless dye (isopropyl-1-thio-β-D-galactopyranoside plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Bacteria containing a plasmid with the wild-type *supF* gene yield blue colonies, whereas those containing plasmids with inactivating mutations in the *supF* gene (22) yield white or light blue colonies. The total number of colonies reflect plasmid survival, whereas the proportion of white or light blue colonies is a measure of the plasmid MF.

**Statistical Analysis.** Plasmid MF and survival were calculated as described (16), and the mean and SE were calculated from three or more independent transfections with each donor line. Comparisons of differences of means were made with the Student's *t* test. The data were fitted to least-squares linear regression curves, and the 95% prediction intervals (repeated results from a single experiment have a 95% chance of falling within the 95% prediction interval) were plotted against age using the SlideWrite Plus computer program

Table 1 Lymphoblastoid cell lines studied from melanoma-prone families

Family no.	No. of cell lines						
	Blood-line family members				Spouses <sup>a</sup>		
	CM + DN	CM only	DN only	Clinically unaffected	Clinically indeterminate	Normal	DN only
342	1		1				
373	1						
479	2	1	2	2		1	1
567	1						
928	5	1	4	3	1	2	
1017	3		1	1	1	1	
Total	13	2	8	6	2	4	1

<sup>a</sup> Spouses of blood-line family members.

(Advanced Graphics Software, Inc., Carlsbad, CA). Comparisons of proportions of cell lines with elevated plasmid mutation frequencies were performed with Fisher's exact test. Two-tailed *P*s are reported throughout.

**RESULTS**

**Post-UV Plasmid Mutation Frequency.** The post-UV plasmid MF was determined for cell lines from 13 donors who had both CM and DN (Fig. 1). These donors, from all six families studied (Table 1), ranged in age from 17 to 64 years. All 13 cell lines showed elevated post-UV plasmid MF compared to the 95% prediction interval for the normal donors in the same age range (21). This difference is highly significant ( $P = 1.5 \times 10^{-8}$ ; Table 2).

Post-UV plasmid MF was determined for cell lines from two donors with CM who were not observed to have DN (Fig. 1 and Table 2). Both donors were more than 70 years old, and their cells lines showed normal post-UV plasmid MF.

Eight donors in four families had DN only (Table 1). They ranged in age from 17 to 53 years. Cell lines from all four donors with DN from family 928 showed elevated post-UV plasmid MF, as did one of two cell lines from family 479 (Fig. 1). Thus, cell lines from five of eight donors with DN only showed elevated post-UV plasmid MF ( $P = 0.001$ ; Table 2).

Eight blood-line family members in three kindreds who had neither melanoma nor DN were studied. Six of these were postpubertal (ages 17–64 years) and were considered clinically unaffected, whereas two had not passed puberty (ages 16 and 8 years) and were classified as indeterminate (Table 1). Cell lines from four of the unaffected individuals and one of the indeterminate individuals had elevated post-UV plasmid MF (Table 2). The cell line with the highest post-UV plasmid MF (12.8%) came from a 40-year-old family member who had approximately 100 moles. None of his moles was considered to be

dysplastic on clinical examination, and the four moles examined histologically showed no evidence of dysplasia.

Cell lines from four normal spouses of blood-line family members and from one spouse who had DN (Table 1) were examined. These donors from three kindreds ranged in age from 39 to 87 years. Post-UV plasmid MF was elevated in three of the cell lines from the normal spouses and from one spouse with DN (Table 2).

As shown in Table 2, a total of 52 cell lines was examined. Of 27 cell lines with elevated post-UV plasmid MF, 19 were from the 24 donors with CM plus DN or DN, whereas only 8 were from the 28 donors who did not have CM or DN ( $P = 0.0003$ ).

**Spontaneous Plasmid Mutation Frequency.** Spontaneous plasmid MF was determined in the cell lines from normal donors (21). The calculated spontaneous plasmid MF at birth was 0.14% and increased to 0.21% at age 100 years. Thus, the spontaneous MF increased approximately 0.4% per year of age, although the increase was not statistically significant. Fig. 2 shows the 95% prediction interval for the spontaneous plasmid MF. All 16 normal donors tested had values within this 95% prediction interval. Six of the cell lines from donors who had CM and DN and three of the cell lines from donors who had DN only showed elevated spontaneous plasmid MF (Fig. 2 and Table 2). Six of these were from donors in family 928 (three with melanoma plus DN and three with DN only). All of the cell lines with elevated spontaneous plasmid MF also had elevated post-UV plasmid MF.

Cell lines from none of the spouses and from only one of the clinically unaffected blood-line family members showed elevated spontaneous plasmid MF (Table 2). Of 10 cell lines with elevated spontaneous plasmid MF, only 1 was from a donor who did not have CM or DN (9 of 24 versus 1 of 28;  $P = 0.003$ ).

**Plasmid Survival.** The post-UV normalized relative plasmid survival for the 16 cell lines from normal donors was  $4.7 \pm 0.6\%$

Fig. 1. Post-UV plasmid MF versus donor age in cells from six CM kindreds. Plasmid pSP189 was treated with 1000 J/m<sup>2</sup> UV and transfected along with the untreated internal standard plasmid (pZ189K) into triplicate cultures of lymphoblastoid cell lines from different donors. After 2 days, the replicated plasmids were harvested and used to transform indicator bacteria to resistance to ampicillin or kanamycin. The bacterial colonies obtained were analyzed for plasmids with wild-type *supF* gene (dark blue colonies) or mutated *supF* gene (white or light blue colonies). The mutation frequencies of the plasmids are plotted as a function of the age of the donor. Data points, mean for each donor; bars, SE. (Data for three of the patients with CM plus DN are from Ref. 16). Donors were from six familial CM kindreds with melanoma only (▲), DN only (▼), or melanoma and DN (◆). —, least squares linear regression line (correlation coefficient  $r = 0.77$ ); - - - - , 95% prediction range for the normal donors (21).

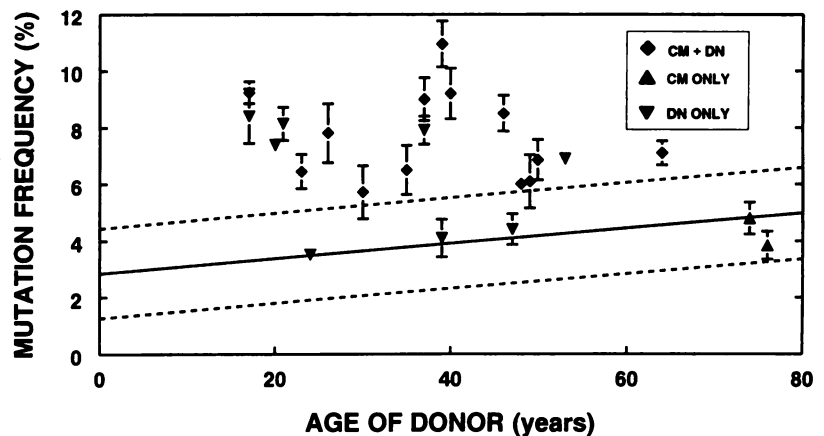


Table 2. Summary of plasmid MF data in lymphoblastoid cell lines from blood-line family members and controls

Clinical diagnosis	Total no.	No. of cell lines					
		Elevated post-UV plasmid MF			Elevated spontaneous plasmid MF		
		No.	%	$P^a$	No.	%	$P^a$
<b>Blood-line family members</b>							
Melanoma + DN	13	13	100	$1.5 \times 10^{-8}$	6	46	0.004
Melanoma only	2	0	0	— <sup>b</sup>	0	0	— <sup>b</sup>
DN only	8	5	63	0.001	3	38	0.03
Clinically unaffected	6	4	66	0.002	1	17	0.5
Clinically indeterminate	2	1	50	0.1	0	0	— <sup>b</sup>
<b>Controls</b>							
Normal donors	16	0 <sup>c</sup>	0	— <sup>b</sup>	0	0	— <sup>b</sup>
Normal spouses <sup>d</sup>	4	3	75	0.003	0	0	— <sup>b</sup>
DN spouse <sup>d</sup>	1	1	100	0.1	0	0	— <sup>b</sup>

<sup>a</sup> Two-tailed  $P$  using Fisher's exact test comparing indicated group to normal donors.  
<sup>b</sup>  $P$  undefined.  
<sup>c</sup> Post-UV plasmid mutation frequency data from Ref. 21.  
<sup>d</sup> Spouses of blood-line family members.

(mean ± SE). There was no evidence of a change in plasmid survival with age. The 95% prediction interval ranged from 0 to 10.8%. The post-UV relative plasmid survival was within the 95% prediction interval for all 16 normal cell lines (data not shown).

As in the studies with the replicating shuttle vector plasmid (15, 16) or with a nonreplicating plasmid (23), post-UV relative plasmid survival was not lower than normal with cell lines from patients with CM or DN (data not shown). The post-UV relative plasmid survival was elevated with cell lines from two patients with CM from family 479 (35-year-old woman with CM plus DN, 19%; 74-year-old man with CM only, 20%) and with one cell line from a patient with DN only in family 928 (17-year-old woman, 10.8%). Cell lines from two clinically unaffected blood-line family members also showed elevated post-UV plasmid survival (17-year-old man in family 1017, 22.5%; 28-year-old woman in family 928, 11.7%). The post-UV relative plasmid survival was within the 95% prediction interval for all of the other cell lines examined. The proportion of cell lines from blood-line family members with elevated post-UV relative plasmid survival was not significantly different from the normal controls (5 of 31 versus 0 of 16;  $P = 0.15$ ).

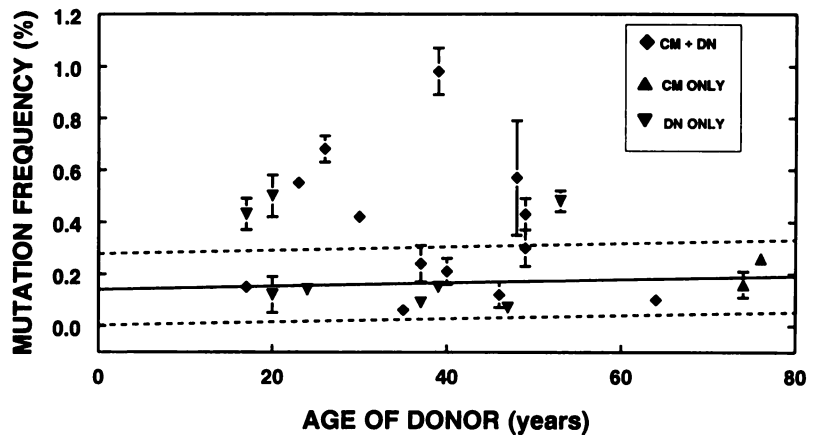
**DISCUSSION**

**Shuttle Vectors and DNA Repair.** Shuttle vectors have been used to measure the ability of cells to repair damaged DNA. This host cell reactivation assay is dependent on cellular systems to repair the damage in plasmid DNA. Plasmids have been developed to measure DNA repair, UV hypersensitivity, and UV mutagenesis in human cells (13, 14, 24). Use of plasmids that are damaged *in vitro* ensures that the

DNA damage is the same in all cells and permits a rapid assessment of survival and mutagenesis. Abnormalities have been reported in cells from patients with XP (24–29), Cockayne syndrome (30), and trichothiodystrophy (31, 32) and in apparently normal individuals with nonmelanoma skin cancer (33). Earlier studies reported normal post-UV plasmid reactivation (23) and increased plasmid mutability in lymphoblastoid cell lines from three patients with CM plus DN (15, 16). The present study examines whether this plasmid post-UV hypermutability phenotype is present in cell lines from members of melanoma-prone families with different degrees of clinical involvement, including less severely affected family members and normal controls.

**XP and Familial CM.** We used XP as a model for human melanoma susceptibility with post-UV hypermutability (34). XP patients have increased melanoma frequency, multiple pigmented lesions, and clinical sun sensitivity (34, 35). The diagnosis of XP is based on clinical features plus the laboratory detection of abnormalities, including cellular and plasmid UV hypersensitivity, hypermutability, and defective DNA repair. The “variant” form of XP is characterized by the clinical features of XP, including melanoma, along with normal or nearly normal post-UV cell survival, elevated cellular and plasmid post-UV MF, and normal unscheduled DNA synthesis (27, 36). Many of the clinical and laboratory features present in XP variants are found in familial CM. Familial CM patients have increased melanoma frequency, multiple pigmented lesions (which are usually different histologically from those in XP), and sun sensitivity. Like XP variant patients, unscheduled DNA synthesis is normal in familial CM. However, unlike the XP variant, there is no abnormality in postreplication

Fig. 2. Spontaneous plasmid MF versus donor age in cells from six CM kindreds. Cells were transfected with untreated plasmid and analyzed as indicated in the legend to Fig. 1. Donors were from six familial CM kindreds with melanoma only (▲), DN only (▼), or melanoma and DN (◆). Data were obtained from untreated control plasmids in the same experiments as in Fig. 1. —, least squares linear regression line; - - - - - , 95% prediction range for 16 normal control donors. Correlation coefficient,  $r = 0.32$ . (Normal control data were obtained from untreated control plasmids in the same experiments as in Ref. 21.)



repair (11). Cells from some patients with familial CM have been reported to be hypersensitive to killing by UV, but others had normal post-UV cell survival. Perera *et al.* (11) and Howell *et al.* (10) showed normal post-UV cell survival with elevated post-UV MF in familial CM. Seetharam *et al.* (15) and Moriwaki *et al.* (16) showed normal post-UV plasmid survival with elevated plasmid post-UV MF in three cell lines from patients with CM and DN. The present study demonstrates that this laboratory abnormality is present in a familial context with involvement in cell lines from 13 of 13 patients with familial CM and DN in 6 kindreds and many of their relatives (Table 2).

**Specificity of Post-UV Plasmid Hypermutability Assay.** Post-UV plasmid hypermutability appears to be a marker for cell lines from melanoma-prone family members who have CM or DN. As shown in Table 2, a total of 52 cell lines were examined. Of 27 cell lines with elevated post-UV plasmid MF, only 8 were from donors who did not have CM or DN (19 of 24 *versus* 8 of 28;  $P = 0.0003$ ). In addition, all 10 cell lines with elevated spontaneous plasmid MF (Table 2) also had elevated post-UV plasmid MF, and only 1 was from a donor who did not have CM plus DN or DN (9 of 24 *versus* 1 of 28;  $P = 0.003$ ). Post-UV plasmid hypermutability was present in cell lines from all 13 individuals with CM plus DN in the 6 families studied (Table 2) and was also seen in cell lines from 5 of 8 family members with DN alone, a melanoma risk factor. Post-UV plasmid hypermutability thus appears to be more closely associated with CM plus DN than with CM alone because cell lines from 2 individuals with CM without DN had normal post-UV plasmid MF (13 of 13 *versus* 0 of 2,  $P = 0.01$ ; Table 2). However, these two patients were both in their 8th decade (Fig. 1) and might have had DN earlier in life.

Post-UV plasmid hypermutability was also found in cell lines from four of six clinically normal blood relatives (Table 2). These six individuals were in families 479, 928, and 1017, but they did not have the *p16* germ-line mutations that were found in their relatives with melanoma (19). One of these clinically normal family members had more than 100 nevi, a recognized melanoma risk factor (37), and his father was the spouse with DN and elevated post-UV plasmid MF (Table 2). The detection of post-UV plasmid hypermutability in cell lines from one of two indeterminate blood relatives may indicate an increased melanoma risk in that individual. The finding of post-UV plasmid hypermutability in cell lines from 3 of 4 normal spouses but in 0 of 16 normal controls ( $P = 0.003$ ) is unexplained. Perhaps acquired factors may also affect post-UV plasmid hypermutability. Post-UV plasmid hypermutability is not specific to familial CM. As indicated above, previous studies of cell lines from patients with XP in complementation groups A (25), C (29), D (26), F (28), and variant (27), as well as from a patient with Cockayne syndrome (30), also showed elevated post-UV plasmid hypermutability.

**Melanoma Gene.** There is evidence for genes for melanoma susceptibility on chromosomes 9p21 (*p16*; Refs. 19, 38, and 39), 12q13 (*CDK4*; Refs. 20 and 40), and 1p36 (41–44). Previous analyses of the same kindreds that we examined showed significant evidence of linkage to chromosomes 1p, 12q, and 9p as well as genetic heterogeneity (19, 20, 41, 42, 45). Post-UV plasmid MF was elevated in cell lines from individuals with CM plus DN in all six kindreds that we studied. We also examined a lymphoblastoid cell line from a sporadic melanoma patient who had a translocation of chromosome 9 (38, 39). This cell line also showed elevated post-UV plasmid MF.<sup>4</sup> Thus the elevated post-UV plasmid MF appears to be associated with CM patients who have abnormalities on different chromosomes.

**Hypermutability: Mechanism.** Our studies indicate that cells from patients with familial CM have normal post-UV plasmid sur-

vival with increased plasmid post-UV mutability. These observations suggest that the cells have a defective mechanism for handling DNA damage. Cells from patients with XP complementation groups A–G have post-UV plasmid hypermutability with decreased post-UV plasmid survival associated with defects in DNA excision repair (34), whereas XP variant cells have a similar phenotype to the familial CM cells in association with defects in postreplication repair (34). Cockayne syndrome cells with elevated post-UV plasmid mutability (30) have decreased post-UV plasmid survival and normal excision repair of the bulk DNA but a defect in repair of actively transcribing genes. Neither these defects or other mechanisms have been identified in cells from patients with familial CM (9–12). Studies with *Escherichia coli* have demonstrated that defects in polymerase III (46) or RecA (47–49) may lead to post-UV hypermutability with normal cell survival. Abnormalities in CDC7, a protein kinase involved in cell cycle regulation, have been associated with cellular hypermutability and normal post-UV cell survival in yeast (50). The melanoma susceptibility genes *p16* and *CDK4* are also involved with cell cycle regulation (19, 20). A related protein kinase involved in cell division control, CDC2L1 (*p58*; Ref. 51), is located on chromosome 1p36 near a gene for melanoma susceptibility. Abnormalities in recovery of the integrity of chromosomes of cultured cells exposed to x-irradiation in the G<sub>2</sub> phase of the cell cycle have been reported in cells from patients with a large number of cancer-prone disorders, including familial CM and XP (52, 53). This study suggests that patients with familial CM have a defective mechanism for handling UV-induced DNA damage.

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