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Original Contribution

Evidence that oxidative stress is a risk factor for the development of squamous cell carcinoma in renal transplant patients

Marcus S. Cooke [a,b,*], Joy E. Osborne [a,c], Rajinder Singh [a,d], Vilas Mistry [a], Peter B. Farmer [a,d], Mark D. Evans [a], Peter E. Hutchinson [a,c]

[a] *Radiation and Oxidative Stress Group, Department of Cancer Studies and Molecular Medicine, Leicester LE2 7LX, UK*
[b] *Department of Genetics, Robert Kilpatrick Clinical Sciences Building, University of Leicester, Leicester LE2 7LX, UK*
[c] *Department of Dermatology, University Hospitals of Leicester NHS Trust, Leicester LE1 5WW, UK*
[d] *Cancer Biomarkers and Prevention Group, Biocentre, University of Leicester, University Road, Leicester LE1 9HN, UK*

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Abstract

Renal transplant patients are at a greatly increased risk of skin malignancy, particularly squamous cell carcinoma (SCC), a tumor closely associated with UV exposure. There is also significant interindividual skin cancer risk among transplant patients, with evidence suggesting that this derives from variation in response to oxidative stress. Our aim was to assess urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), by liquid chromatography–tandem mass spectrometry, in renal transplant patients with and without SCC. The relationships between SCC and urinary 8-oxodG were analyzed by conditional logistic regression and those between 8-oxodG and other candidate variables by linear regression, correcting for the effect of SCC. In SCC patients, urinary 8-oxodG was significantly elevated ($p=0.03$), both pre- and post-tumor development, compared to non-SCC transplant patients. Secondary analyses indicated that 8-oxodG was related to current heavy smoking ($p=0.02$) and darker skin type ($p=0.02$), but not measures of previous chronic sun exposure or current age and gender. Although subject numbers were limited, immunosuppression with azathioprine was positively associated with 8-oxodG in all patients combined ($p=0.02$). These results demonstrate, for the first time, that a subpopulation of renal transplant patients is under greater oxidative burden, and it is this population that is particularly predisposed to skin cancer.

Keywords: Oxidative stress; Skin cancer; Urine; 8-Oxo-7,8-dihydro-2′-deoxyguanosine; Renal transplant; Immunosuppression; Free radicals

Transplant patients are at a high risk of developing malignancy, most frequently nonmelanoma skin cancer (NMSC), which is largely attributed to decreased tumor surveillance arising from immunosuppression, in conjunction with ultraviolet radiation (UVR) exposure [1]. Genetic disposition [2] and a history of pretransplant malignancy are further risk factors [3]. The likelihood of developing skin cancer, in Caucasians in a temperate climate, during the first 10 years after renal transplantation is 14%, rising to 30–40% 20 years after transplantation (reviewed in Hardie [4]). It is now therefore considered to be the "gold standard" of care to provide regular dermatological screening and surveillance to transplant patients. Squamous cell carcinoma (SCC) represents a minority of skin cancers in nontransplant patients but is the most frequent tumor after renal transplantation [5,6]. It seems that certain renal transplant patients are at particular risk of developing SCC and may acquire multiple tumors. This could be a function of increased exposure to known risk factors for SCC, or decreased cellular defense mechanisms, including genetic defects, or both.

Exposure to UVR is a major risk factor for the development of SCC in all Caucasians, including renal transplant patients, and to a greater extent than for other skin cancers, i.e., basal cell carcinoma (BCC) and melanoma [6]. The carcinogenic effect of UVR is therefore particularly relevant to renal transplant pa-

Abbreviations: SCC, squamous cell carcinoma; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; NMSC, nonmelanoma skin cancer; UVR, ultraviolet radiation; BCC, basal cell carcinoma; ROS, reactive oxygen species; LC-MS/MS, liquid chromatography with tandem mass spectrometry.

* Corresponding author.
E-mail address: msc5@le.ac.uk (M.S. Cooke).

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tients, for whom there is a distinct propensity to SCC. Although the predominant DNA modifications induced by UVB and UVA seem to be dimeric photoproducts, such as cyclobutane pyrimidine dimers [7], UVR can also generate reactive oxygen species (ROS), principally singlet oxygen [8], leading to oxidative stress and oxidative modification of various cellular molecules, including DNA [9]. ROS-induced DNA damage is of particular interest [10,11], as over 70 products have been described [12], many of which have proven cellular effects related to carcinogenesis, such as mutation, alteration of cell signaling and gene expression, promotion of microsatellite instability, and telomere shortening [13]. The induction of oxidative stress is therefore widely considered to be an important factor in UVR-induced carcinogenesis [14–16,44], capable of acting as both an initiator and a promoter of carcinogenesis [11,17], not least due to the wide range of possible effects these oxidatively generated DNA products have upon cell function. This is further evidenced by reported differences in antioxidant defense, arising from genetic variation and contributing to the variability in skin cancer incidence in both transplant [18–20] and nontransplant patients [21]. Possible genetic variation in other protective systems has not been investigated.

Products of oxidatively damaged DNA, for example, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), are frequently used as biomarkers of oxidative stress [22], although there is an appreciable risk of the formation of artifactual damage in cellular DNA during sample workup [23]. However, measurement of 8-oxodG in urine is also a useful oxidative stress marker [24], being noninvasive, with a minimal risk of artifact (reviewed in Cooke et al. [25]). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is increasingly used for the analysis of such urinary markers of oxidative stress [26,27], owing to the existence of well-established methods, plus the sensitivity and specificity of the technique. We have recently reported a method for the assessment of urinary 8-oxodG, which uses a simple solid-phase extraction step to clean up the urine before LC-MS/MS [28].

In the present study, we used this method to examine urinary 8-oxodG in SCC and control renal transplant patients, closely matched or controlled for predisposing factors for development of SCC. The aim was to examine whether oxidative stress, a significant factor in tumorigenesis [29,30], contributed to the interpersonal differences in propensity to SCC development in renal transplant patients.

Material and methods

Patients

During the past 5 years, renal transplant patients have been routinely referred from the Leicestershire renal transplant service to the Dermatology Department at the Leicester Royal Infirmary for screening and regular surveillance for signs of cutaneous malignancy. All patients were seen and examined by one dermatologist (J.E.O.). Basic demographic data, details of age at transplantation, immunosuppression regimes, and personal and family history of skin cancer were recorded. A history of previous and current cigarette smoking habits was noted in terms of smoking rate (number smoked per day) and cumulative amount smoked (pack years). The patient's skin type was determined by asking about the reaction of their skin to 30 min of unprotected sun exposure at midday in June in the United Kingdom (skin erythema or not) and the ability to tan (minimal, light, or deep tan). Skin type was classified using our modification of the Fitzpatrick scale (I, always burn, never tan; IIa, initially burn, subsequently tan moderately; IIb, initially burn, subsequently tan well; III, always tan, rarely burn; IV, Asian or black skin). A detailed history of sun exposure variables both pre- and posttransplantation was also collected. These included outdoor occupation and hobbies, details of sunny holidays spent abroad and in the United Kingdom, sunbathing habits including the use of sun beds, and a history of childhood sunburn.

A spot urine sample was collected from each patient at the first attendance. All patients were reviewed at least annually, and more frequently if clinically indicated. They were also able to self-refer with any new or changing lesions between scheduled visits. Details of any histologically confirmed skin cancers identified during surveillance were noted. All data were recorded in a database, which was updated at each attendance.

All patients who had a previous history of, or subsequently developed, SCC and for whom a urine sample was available were identified, and each subject was individually matched for the conditional logistic regression analysis, in terms of sex, current age, age at transplantation, skin type, and outdoor occupation, with a control renal transplant patient who had not developed SCC. These control subjects were selected from 253 white Caucasian patients entered on our database referred to above, and the closest matching patient to each SCC patient was identified.

Patients who had developed Bowens disease (intraepithelial SCC), keratoacanthoma, or BCC were also excluded from the control population. As spot urine samples were collected on first attendance for dermatological screening, some samples were collected from individuals who subsequently developed SCC postsampling. Written, informed consent was obtained before inclusion into the study; no patient refused consent.

Urine samples

Urine samples were stored at −80°C until analysis. In order to provide a correction factor for urine concentration, aliquots of urine were also assayed for creatinine (Department of Chemical Pathology, University Hospitals of Leicester NHS Trust), and urinary 8-oxodG measurements were corrected accordingly.

Solid-phase extraction (SPE) of 8-oxodG from urine and LC-MS/MS analysis

All urine samples were spiked with 12 pmol $^{15}N_5$-labeled 8-oxodG, as an internal standard, before manipulation. The synthesis of $^{15}N_5$-labeled 8-oxodG and the method for extraction of urinary 8-oxodG have been described in detail elsewhere [28]. Briefly, after spiking, the urine samples (1.2 ml) were applied to SPE columns (3 ml, 60 mg, Waters Oasis HLB; Waters Ltd.,

Elstree, UK) to isolate 8-oxodG. After elution, samples were stored at −20°C, until being dried under vacuum. The dried, purified urine samples were reconstituted in 50 μl of HPLC grade water for LC-MS/MS analysis. A 10 μl aliquot of the purified sample was injected onto a HyPurity C_{18} column (3 μm, 2.1×150 mm; Thermo Electron Corp., Runcorn, UK). The column was eluted isocratically with 0.1% acetic acid/methanol (92/8, v/v) at a flow rate of 120 μl/min. The LC-MS/MS system consisted of a Waters Alliance 2695 separation module connected to a Micromass Quattro Ultima Platinum (Waters-Micromass Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. Selected reaction monitoring analysis was performed and the level of 8-oxodG in each urine sample was determined from the ratio of the peak area of 8-oxodG to that of the internal standard.

Statistical analysis

Graphs were plotted using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA). The relationship between 8-oxodG and the occurrence of SCC was investigated by conditional logistic regression (Stata software package, version 7.0; Stata Corp., College Drive, TX, USA). In addition a multivariate model was constructed to correct for other variables we have found to influence susceptibility to SCC development in this population, which were age at transplantation, current cigarette smoking >20/day, outdoor worker for >4 years, holidays abroad for and >30 weeks in toto, and history of previous frequent sunbathing. A number of these variables have potential relevance to oxidative stress and any relationship with 8-oxodG was investigated by logistic regression separately in patients and controls and, where appropriate, in the combined population. Differences, in the form of the relationship of the test variable and 8-oxodG, between SCC patients and controls were first assessed in a multivariate model via the significance of the interaction term of the test variable and presence of SCC. If there was no difference in the relationship of the test variable between SCC patients and controls, then the test variable and 8-oxodG relationship was further investigated in the combined group, correcting for differences in mean levels of 8-oxodG between SCC and controls by including presence of SCC in the regression model.

Results

There were 17 SCC patients with matched controls. Nine patients had developed more than one SCC. Eight patients developed the first SCC before the urine was sampled [mean (±SD, standard deviation)=3.6 (±3.3) years], and 9 patients developed the first SCC at a mean of 2.8 (±1.6) years post-sampling. Matched demographic data of the control and SCC populations are shown in Table 1 and unmatched data in Table 2. The 7 patients whose current immunosuppressive drugs are not shown received prednisolone and one or more of tacrolimus and mycophenolate mofetil and there were no more than 2 patients on any of these regimes. One patient was receiving no immunosuppression at the time of urine sampling. It is possible that 8-oxodG concentration in spot urine samples may be affected by renal function, and as all these patients had received a renal transplant, differences in postoperative function could form the basis of the results seen. However, there was no significant effect of urinary creatinine concentration on 8-oxodG in the combined SCC and control data, correcting for the effect of SCC ($p=0.6$), and also mean urinary creatinine was very similar in the two groups [cases mean (SD)=6.4 (±5.8) vs controls 7.2 (±5.5) mmol/L ($p=0.9$)], indicating that renal function was similar in the two groups.

Table 1
Determinants of urinary 8-oxodG excretion (pmol/μmol creatinine) that were matched between the renal transplant patients who had been diagnosed with SCC and those that had not (Controls)

	Controls		SCC		Combined	
	N	8-oxodG (pmol/μmol) [median (IR)]	*N*	8-oxodG (pmol/μmol) [median (IR)]	*N*	8-oxodG (pmol/μmol) [median (IR)]
All	17	1.9 (1.2)	17	3.1 (2.8)[a]	34	2.4 (2.6)
Gender						
Female	6	1.8 (2.1)	6	3.4 (5.4)	12	2.3 (4.7)
Male	11	1.9 (1.1)	11	3.1 (2.4)	22	2.5 (2.3)
Skin type						
1/2a	7	1.9 (3.1)	7	2.1 (1.8)	14	2.0 (1.8)
2b	6	2.1 (1.6)	8	4.8 (4.6)	14	2.2 (1.4)
3	4	1.8 (0.8)	2	4.7 (3.3)	6	3.7 (4.9)
Outdoor worker						
Not an outdoor worker	10	1.7 (2.0)	9	3.1 (2.9)	19	2.1 (3.1)
Outdoor worker (covered)	5	1.9 (0.5)	1	2.1	6	3.7 (3.7)
Outdoor worker (uncovered)	2	4.7 (5.4)	7	3.7 (3.7)	9	2.0 (0.5)
Age (mean, years)						
At urine sampling		56.6		55.8		
At renal transplantation		45.0		43.1		

[a] Merged data for both groups (Combined) are also shown. IR, interquartile range.

The distributions of the urinary 8-oxodG concentrations were markedly skewed to the right in both controls and SCC patients and both differed significantly from the normal distribution ($p=0.009$, in both, Shapiro–Wilk normality test). Log_{10} transformation of the data resulted in distributions which did not differ from the normal, controls ($p=0.5$), SCC ($p=1.0$), and patients combined ($p=0.9$).

The relationship between SCC and 8-oxodG

Fig. 1A shows urinary 8-oxodG concentrations (log_{10} transformed) in SCC patients and controls. Urinary 8-oxodG levels were significantly higher in the SCC patients ($p=0.03$). Multiple regression including variables known to be associated with the development of SCC in renal transplant patients, but not included in the matching process, had little impact on the SCC/8-oxodG relationship compared to that of univariate analysis (relative odds ratio (OR) multiple regression 20, univariate OR 26). In fact these three variables combined had no significant

Table 2
Determinants of urinary 8-oxodG excretion (pmol/μmol creatinine) that were unmatched between the renal transplant patients who had been diagnosed with SCC and those that had not (Controls)

	Controls		SCC		Combined	
	N	8-oxodG (pmol/μmol) [median (IR)]	*N*	8-oxodG (pmol/μmol) [median (IR)]	*N*	8-oxodG (pmol/μmol) [median (IR)]
Smoking status						
Never smoked	7	1.9 (3.2)	8	3.7 (4.8)	15	2.4 (5.1)
Previous but not current smoking	7	2.0 (1.1)	4	3.1 (0.9)	11	2.5 (1.4)
Current smoking <20 cigarettes/day	2	4.2 (6.3)	3	2.1 (3.6)	5	2.1 (3.6)
Current smoking ≥20 cigarettes/day	1	1.9	2	8.9 (10.5)	3	3.7 (12.3)
Holidays abroad (total for life)						
<30 weeks	14	2.2 (2.0)	10	2.8 (2.7)	24	2.4 (2.3)
≥30 weeks	3	1.8 (0.3)	7	4.2 (4.8)	10	2.8 (4.6)
Sunbathing						
Infrequently	14	2.2 (1.6)	12	2.8 (3.0)	26	2.4 (1.9)
Frequently	3	1.2 (1.2)	5	4.8 (0.7)	8	3.3 (3.4)
Current immunosuppressants						
Prednisolone and cyclosporin	5	1.2 (0.5)	5	2.6 (0.6)	10	1.8 (1.4)
Prednisolone and azathioprine	2	2.8 (0.8)	4	3.9 (2.9)	6	3.2 (2.3)

IR, interquartile range.

relationship with the presence of SCC in this group of patients ($p=0.4$). Whether the SCC patients had >1 SCC had no impact on 8-oxodG levels ($p=0.4$, *t* test pooled variance). Fig. 1B shows urinary 8-oxodG levels in samples taken before development of the first SCC and samples taken after the development of the first SCC, which were very similar ($p=0.8$, t test pooled variance).

The relationship between 8-oxodG and other variables in the study

Levels of 8-oxodG are shown in Tables 1 and 2. Comparing 8-oxodG in SCC and control patients, there were significant differences in the 8-oxodG relationship with skin type and frequent sunbathing only ($p=0.03$ in both). On univariate analysis there was no significant relationship for most variables. Skin types IIb and III were associated with higher levels of 8-oxodG in the SCC patients ($p=0.003$ and $p=0.08$, respectively, and $p=0.02$ combined) but not in controls. Working outdoors, with skin uncovered, was associated with higher 8-oxodG levels in both controls and SCC patients but not significantly in patients combined ($p=0.08$). There was an apparent increase in 8-oxodG in SCC patients smoking >20 cigarettes/day but this was not statistically significant (compared to all other smoking situations) on univariate analysis ($p=0.1$) nor was smoking at this level in the combined population ($p=0.2$). There were sufficient numbers of patients to compare the effects of immunosuppressive therapy on urinary 8-oxodG only for patients taking azathioprine and cyclosporin (Table 2). The prednisolone and cyclosporin combination was associated with lower 8-oxodG levels than prednisolone and azathioprine in both control ($p=0.01$) and SCC patients ($p=0.2$) and in patients combined ($p=0.02$). Using the whole model, analyzing 8-oxodG versus SCC/control and azathioprine/cyclosporin was highly significant ($p=0.004$).

Multiple regression analysis of 8-oxodG on all variables investigated in the study, except immunosuppressants, in SCC patients gave a significant effect of smoking and >20 cigarettes/day ($p=0.04$), skin type IIb ($p=0.02$), and skin type III ($p=0.04$), whereas similar analysis in the control population disclosed no significance ($p=0.3$ to 0.9). Backward hierarchical elimination ($\alpha=0.05$) in the SCC population resulted in a final model ($p=0.002$) which included smoking and >20 cigarettes/day ($p=0.02$), skin type IIIb ($p=0.001$) and skin type III ($p=0.02$), and skin types IIb and III combined ($p=0.02$).

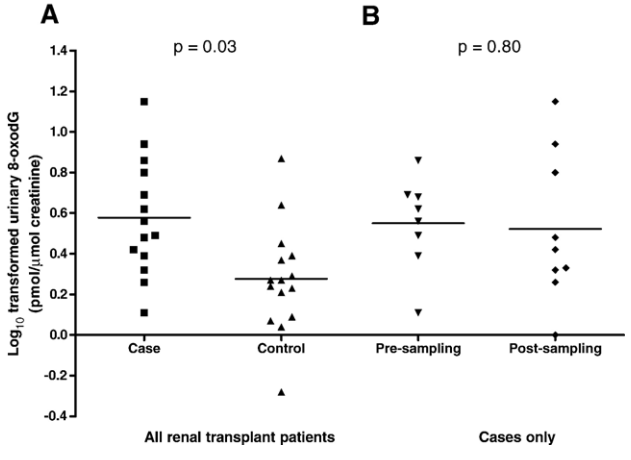

Fig. 1. (A) Log_{10} transformed levels of urinary 8-oxodG in renal transplant patients who have developed SCC (■) versus renal transplant patients who have not developed SCC (▲). (B) Effect of sampling time, relative to diagnosis of SCC, upon log_{10}-transformed levels of urinary 8-oxodG. Pre-sampling (▼), sample was taken before a later diagnosis of SCC; postsampling (⧫), SCC had already been diagnosed and removed at time of urine sampling. Means are indicated.

Discussion

The basis for the interindividual differences in skin cancer susceptibility in renal transplant patients is unclear. Although

drug-induced immunosuppression is an obvious factor in the increased susceptibility of renal transplant patients to skin cancer, compared to a nontransplant population, this is unlikely to explain the differences within the transplant group. There is emerging evidence that suggests that genetic variation in enzymes involved in protection against oxidative stress may predispose certain renal transplant patients to NMSC [18–20]. We report, for the first time, an association between the development of SCC, in renal transplant patients, and urinary 8-oxodG level, a well-established marker of oxidative stress, and propose that differential responses to oxidative stress are a possible determinant of SCC susceptibility in these patients. The increased levels of urinary 8-oxodG in the SCC group seemed independent of other variables known to influence susceptibility to SCC development, which were age at transplantation, skin type, current heavy cigarette smoking, and chronic sun exposure variables. The mean levels of urinary 8-oxodG in the control transplant group, 2.62 (±2.02) pmol 8-oxodG/μmol creatinine, were of an order similar to the values for healthy individuals, determined by chromatographic techniques, discussed by Lin et al. [31], e.g., 2.13 (±0.99) pmol 8-oxodG/μmol creatinine. These patients' values tend to be at the higher end of this range, reflecting some degree of oxidative stress, which is unsurprising for patients having received a renal transplant and undergoing immunosuppressive therapy, both of which have been reported previously to be associated with oxidative stress [32,33].

Does the oxidative stress occur pre- or post-development of the tumor? As no patient in the SCC group had an existing tumor at the time of urine collection (urine samples were either prediagnosis or post-tumor removal), we would conclude that the oxidative stress either occurs before tumorigenesis and persists, even after removal of the tumor, or is induced during tumorigenesis and continues after tumor removal. Our evidence strongly supports the former, as 8-oxodG concentration was very similar in urine samples taken before diagnosis of SCC and in samples taken after tumor removal, and also multiple tumors did not increase urinary 8-oxodG. Further prospective investigation in a larger group of renal transplant patients is therefore required to clarify whether urinary 8-oxodG levels might have a predictive role in this instance and better define the distribution of 8-oxodG in these patients.

There is some evidence for the involvement of oxidative stress in the etiology of both SCC and BCC in nontransplant patients [34–36]. However, SCC is the predominant NMSC seen in renal transplant patients [5,6] and, to our knowledge, there have been no attempts to explore the reason for this, although, from the literature, SCC does seem more closely linked to oxidative stress [34]. It would be of interest to explore oxidative stress in renal transplant patients developing exclusively BCC. In the present study BCC was deliberately excluded to give a clear indication of whether any changes were linked specifically to SCC.

This study has also afforded the opportunity to investigate any relationship between urinary 8-oxodG excretion and the demographic variables known to influence propensity to SCC development in renal transplant patients and which may affect oxidative stress. A limitation is that, for the noncontinuous variables, it was necessary to consider substrata, resulting in small numbers, and therefore these results need verification in a larger patient base. There was no apparent association with patient age or gender. There was, however, an effect of patient skin type, which was surprising. Darker skinned individuals tended to have higher urinary 8-oxodG concentrations. This was not the case with propensity to SCC development, in which skin types I, IIa, and IIb were at greater risk. A possible explanation is that darker skin types are less likely to be protected from sunlight and therefore receive greater UVR doses, which it would have to be argued is not compensated for by the darker pigmentation, and this produces increased oxidatively generated damage. That the darker skinned individuals do not go on to develop SCC implies greater cellular defense against such damage once formed, e.g., DNA repair, compared to lighter skinned individuals, a phenomenon discussed further in [37]. We also noted that it was only in the SCC patients that the relationship between 8-oxodG and skin type was evident ($p=0.01$) and there was a significant difference between SCC and control patients. A possible interpretation is that less effective antioxidant defense, rather than increased production of ROS, is responsible for the increased levels of 8-oxodG noted in the SCC-prone patients, for which there is some precedence [18–20,38]. Indeed, a report by Sander et al. [39] links decreased antioxidant defense with NMSC development, in contrast to increased ROS production in melanoma. These data, combined with our evidence above, further support the suggestion that oxidative stress is present before development of SCC and is probably a consequence of attenuated antioxidant defense.

The measures of chronic UVR exposure, working outdoors with skin unprotected, longer cumulative time on holiday in sunny destinations, and history of frequent sunbathing, were not related to urinary 8-oxodG levels. This is in apparent conflict with the skin type findings. However, these markers are essentially of historical, cumulative UV exposure, important in development of SCC, but it is likely that it is recent exposure which affects 8-oxodG formation. There was a trend toward higher 8-oxodG in current, heavy smokers. This did not reach statistical significance on univariate analysis but was significant on multiple regression of all variables in the study (except immunosuppressants, final model), consistent with other reports [45]. The final multiple regression model included skin type and cigarette smoking and was highly significant in SCC patients, but not in the controls, further implying defective defense against ROS in the SCC group.

Due to the small numbers of patients because of the multiplicity of drug regimes, analysis of the effect of the individual immunosuppressive agents was limited. However, a significant effect of azathioprine over cyclosporin was evident and this was independent of the relationship between 8-oxodG and SCC. This trend is entirely consistent with a recent report describing the in vitro formation of ROS after exposure of azathioprine-treated cells to UVA radiation [40]. In a subsequent study the authors speculate singlet oxygen to be the primary ROS [41] for which 2′-deoxyguanosine moieties are a primary target, irrespective of initiator [42,43], and hence a possible source of

8-oxodG. Our data provide the first in vivo evidence linking azathioprine use and oxidative stress. It is worth noting that azathioprine use is not restricted solely to transplantation, as it is used as an immunosuppressive agent to treat inflammatory conditions such as variants of lupus erythematosus, autoimmune hepatitis, and inflammatory bowel disease. However, a literature search revealed very few reports of SCC development in conjunction with any nontransplantation use of azathioprine.

Our results extend earlier findings linking genetic variation in enzymes associated with cellular defense with skin cancer predisposition in renal transplant patients [18–20]. We demonstrate specifically that a subgroup of transplant patients is under an increased oxidative stress which, these data suggest, arises from defective antioxidant defense, having perhaps a genetic basis. We hypothesize that this then renders the patients sensitive to a subsequent genotoxic insult, such as UVR exposure, in some cases in conjunction with azathioprine use, which may initiate the carcinogenic process and corresponds to an increased risk of SCC. The evidence that oxidative stress is therefore a predisposing factor is tantalizing and warrants further investigation in a larger population of renal transplant patients with SCC.

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