Pronounced Allelic Imbalance at D9S162 in Skin Squamous Cell Carcinoma of Organ Transplant Recipients

Beda Mühleisen, MD; Ivaylo Petrov, MD; Simona Frigerio, MD; Piotr Dziunycz, MD; Lars E. French, MD; Günther F. L. Hofbauer, MD

Objective: To evaluate chromosomal instability at 9p21-22 with p16 protein expression in organ transplant recipients (OTRs) compared with immunocompetent patients with squamous cell carcinoma (SCC).

Design: In a select population of intraepithelial and subsequent invasive SCC from the same anatomic region of the same patient at different times, we assessed loss of heterozygosity at 3 microsatellites—IFNA, D9S162, and D9S925—in the course of carcinogenesis in OTRs and immunocompetent patients.

Setting: Department of Dermatology, University Hospital Zurich.

Patients: Immunocompetent patients and OTRs with SCC on sun-damaged skin.

Main Outcome Measure: Chromosomal allelic balance in SCC of OTRs and immunocompetent patients.

Results: Reduced allelic balance at IFNA, D9S162, and D9S925 in intraepithelial forms of SCC and similar allelic imbalance in invasive forms of SCC were found. Allelic balance at D9S162 was reduced for SCC in OTRs compared with SCC in immunocompetent patients. The study revealed broadly reduced allelic balance at 9p21-22 in all cutaneous SCCs, and OTRs presented a further reduced allelic balance for D9S162, suggesting a common trait for SCC in OTRs. Actinic keratosis and Bowen disease differed in allelic balance at D9S162, suggesting substantial differences in their carcinogenesis.

Conclusion: Reduced allelic balance around locus D9S162 is a genomic correlate for enhanced carcinogenesis in OTRs.

able p16INK4 expression in invasive SCC. Blokx and colleagues\textsuperscript{16} assessed p16 and p53 protein expression in 23 AK (termed low-grade keratinocytic intraepidermal neoplasia [KIN]), 28 BD (high-grade KIN), and 35 invasive SCCs from 44 OTRs and 42 immunocompetent patients, concluding that p16 expression was independent of immune status and of p53 expression. Other tumor suppressor genes located in the 9p21-22 region include CDKN2A, CDKN2B (also known as p16 and p15), and ARF (alternative reading frame).\textsuperscript{17-19}

The aim of this study was to determine the imprint of field cancerization in the course of carcinogenesis in a highly selected population of intraepithelial and subsequently invasive SCC from the same anatomic region of the same patient at different times. Allelic imbalance on 9p21-22 as a well-known highly variable region in SCC was analyzed. Because OTRs empirically show a greatly increased incidence of SCC and are known to harbor genetic damage in addition to that which is generally incurred by UV damage alone,\textsuperscript{20,21} SCC of both immunocompetent patients and OTRs were included.

The use of clinically indicated biopsy material for the study was approved by the ethical committee of the Canton of Zurich, Switzerland. All experiments were performed at the Departments of Dermatology and Pathology, University Hospital Zurich.

### METHODS

#### PATIENTS AND TUMOR SELECTION

The goal of this study was to obtain tissue material from an area of field cancerization that had first given rise to an intraepithelial SCC and at least 1 year later to an invasive SCC of the skin. Paraffin-embedded archival tissue specimens from 43 immunocompetent patients and 42 immunosuppressed OTRs were randomly selected. All the participants first had an intraepithelial form of cutaneous SCC, such as AK or BD, followed by an invasive SCC of the skin in the same anatomic region at least 1 year later. These intraepithelial forms were then compared with the subsequent invasive SCC in the same individual. Of the 42 organ transplants, 32 were kidney, 4 were lung, and 6 were heart (Table 1). All tumors were located on chronically sun-exposed skin of the head, neck, and upper extremities. The histologic diagnosis was made by a board-certified dermatologist on the basis of criteria defined by the World Health Organization classification of skin tumors.\textsuperscript{22} Actinic keratoses and BD samples were analyzed separately and compared with each other and then were grouped as intraepithelial SCC to compare them with invasive SCC.

#### MICRODISSECTION

From each sample, 3 to 5 adjacent slices of 10-µm thickness were stained with hematoxylin-eosin. Microdissection was performed under a light microscope (magnification ×400). In each sample, 150 tumor cells were selectively removed using a disposable 30-gauge needle. In addition, 150 normal skin cells (apocrine glands and endothelial cells far from the tumor) were harvested from the same slides and served as internal controls.

#### DNA EXTRACTION

Microdissected cells were immediately suspended in 20 µL of digestion solution containing 50mM TRIS-HCl, 1mM EDTA, 1% Tween 20, and 2.5 mg/mL of proteinase K (pH 8.0, P2308; Sigma-Aldrich, Inc) and incubated for 16 hours at 37°C. The mixture was then heated for 10 minutes at 94°C to inactivate proteinase K. Five microliters of this solution was used as the template DNA for polymerase chain reaction (PCR) amplification.

#### PRIMERS AND PCR CONDITIONS

To establish a highly reproducible LOH analysis for cutaneous SCC, 3 microsatellite marker loci on chromosome arm 9 (IFNA, D9S925, and D9S162), previously linked to early tumor development\textsuperscript{24} and flanking the p16 tumor suppressor gene region, were selected. These loci included either dinucleotide or tetranucleotide tandem repeats known for their high degree of allelic variability and, hence, useful source of information (Table 2). Polymerase chain reaction of the 3 different microsatellite markers was performed with separate primer pairs, of which 1 oligonucleotide was labeled at the 5’ sense strand end with the fluorescent dyes 6-FAM, HEX (Molecular Simplicity), or NED (Applied Biosystems). The primer sequences were as follows: IFNA up: GTAAGGTGAAAACC (FAM); IFNA low: TGCGGGTTAAGTTAATTGG; D9S162 up: CCAGAGAAACAGAACCA (NED); and D9S925 u: TGTAGCCAGGCGCCCTTATAG (HEX); and D9S925 low: GTCTGGGTTCCTCCAAAGAAA. Amplification of specific DNA was performed in a reaction volume of 25 µL including 0.2mM dNTPs (deoxynucleotide triphosphates) (Roche Diagnostics), 2.5mM magnesium chloride, and 0.5 U of a DNA polymerase (AmpliTaq Gold; both from Applied Biosystems). Cycling was performed (GeneAmp PCR System 9700; Applied Biosystems) using the following temperature conditions: 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes for 40 cycles, and a final extension for 60 minutes at 60°C. The PCR products were subsequently denatured for 1 minute at 95°C (Hi-Di formamide; Applied Biosystems) and separated on a genetic analyzer (ABI PRISM 3100; Applied Biosystems) equipped with a 36-cm capillary array loaded with performance-optimized polymer 4 (POP-4; Applied Biosystems); ROX-400HD was used as an internal size standard (Applied Biosystems). Artifacts generally can be a concern in LOH analysis. Polymerase chain reaction for each locus was performed twice with similar results. Moreover, several samples were analyzed, which allows for statistical assuredance of true allelic imbalance.

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ICps</th>
<th>OTRs a</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, No.</td>
<td>43</td>
<td>42</td>
<td>85</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>32 (74)</td>
<td>36 (86)</td>
<td>68 (80)</td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
<td>66.0 (0.74)</td>
<td>61.3 (1.07)</td>
<td>63.7 (0.67)</td>
</tr>
<tr>
<td>Range</td>
<td>47.6-81.5</td>
<td>36.1-79.5</td>
<td>36.1-81.5</td>
</tr>
<tr>
<td>Diagnosis, No. (%) b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinic keratosis</td>
<td>34 (40)c</td>
<td>29 (34)c</td>
<td>63 (37)d</td>
</tr>
<tr>
<td>Bowen disease</td>
<td>9 (11)c</td>
<td>13 (15)c</td>
<td>22 (13)d</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>43 (51)c</td>
<td>42 (49)c</td>
<td>85 (50)d</td>
</tr>
</tbody>
</table>

Abbreviations: ICps, immunocompetent patients; OTRs, organ transplant recipient.

a Organ transplants included 32 kidney, 6 heart, and 4 lung.

b Totals for the diagnosis section reflect intraindividual plus combined testing.

c The denominator for calculating the percentage was 170 diagnoses.

d The denominator for calculating the percentage was 85 patients.

Range 47.6-81.5 36.1-79.5 36.1-81.5

Squamous cell carcinoma 43 (51)c 42 (34)c

Bowen disease 9 (11)c 13 (15)c

Actinic keratosis 34 (40)c 29 (34)c

The use of clinically indicated biopsy material for the study was approved by the ethical committee of the Canton of Zurich, Switzerland. All experiments were performed at the Departments of Dermatology and Pathology, University Hospital Zurich.
LOH ANALYSIS

Analysis of the samples was carried out using commercial software (GeneMapper, version 3.7; Applied Biosystems). A case was considered informative for a polymorphic marker if normal tissue DNA showed 2 different alleles. To identify samples with LOH, the allelic balance ratios of heterozygous markers from the cutaneous SCC DNA and from the patient’s control DNA (from normal skin cells) were compared. Briefly, the ratio of the peak areas of the 2 alleles amplified from the tumor sample was divided by the peak areas of the 2 alleles from the control DNA. When the result of this calculation exceeded 1, the reciprocal value was used. Consequently, the calculated numbers varied between 0 (representing complete LOH) and 1 (retained heterozygosity) (eFigure 1; http://www.archdermatol.com). To determine whether the allelic imbalance ratio in a tumor sample met the criterion for LOH, published standard threshold values for these 3 loci were applied.\(^2^4\)

IMMUNOHISTOCHEMICAL ANALYSIS

We used 3- to 5-µm adjacent sections for hematoxylin-eosin staining and immunohistochemistry. The deparaffinized sections were heated in a 100-W household microwave oven at maximum power 3 times at 5 minutes each in 10mM citric acid for antigen retrieval. Primary antibody was then applied for 60 minutes at room temperature. Immunohistochemical staining was performed with monoclonal IgG mouse antibodies specifically binding human p16\(^{\text{INK4}}\) (1:200, G175-405, 550834; PharMingen) and p53 (1:100, DO-7, Pab 1801; Sigma Biosciences). Secondary staining was performed using the alkaline phosphatase antialkaline phosphatase method.\(^2^5\) Normal epidermis and dermal cells served as internal negative controls. Sections of benign melanocytic nevi and breast cancer tissue served as external positive controls. Immunoreactivity was rated as 0% to 5%, 6% to 25%, 26% to 50%, 51% to 75%, and 76% to 100% positive tumor cells. Personnel reviewing the samples were unaware of the patients’ diagnosis and immune status. For exemplary staining of p16 and p53, see eFigure 2.

STATISTICAL ANALYSIS

Allelic balance ratios and immunoreactivity between 2 independent groups were calculated using the Mann-Whitney test, and frequencies of LOH between the 2 groups were evaluated using the Fisher exact test. Correlations between LOH, p16, and p53 were expressed using Spearman rank correlation (\(\rho\)). Statistical significance was set at \(P<.05\). Statistical analyses were performed using commercial software (SPSS for Windows 11.5, SPSS Inc; GraphPad Prism 3.0, GraphPad Software Inc; and Microsoft Excel 2000, Microsoft Corp).

RESULTS

Considerably reduced allelic balance was found on microsatellite loci IFNA, D9S162, and D9S925 on chromosome arm 9p and was similar in intraepithelial and invasive SCC. Median allelic balance in all tumors was 0.80 for IFNA (n=73), 0.76 for D9S162 (n=86), and 0.75 for D9S925 (n=95) (mean, 0.73, 0.69, and 0.71, respectively). At all 3 loci, there was no significant reduction in allelic balance on the course from intraepithelial to invasive SCC (IFNA, 0.79 for intraepithelial SCC and 0.80 for invasive SCC; \(P=.98\); D9S162, 0.72 and 0.77 [n=29], \(P=.47\); and D9S925, 0.80 and 0.73 [n=31], \(P=.58\); Wilcoxon matched-pair test, 2-tailed \(P\) values) (Figure 1A). Loss of heterozygosity, defined as allelic balance below threshold values for these 3 loci (eTable 1), was found in 61.6% of informative samples at locus IFNA, in 41.9% at D9S162, and in 50.5% at D9S925. Loss of heterozygosity was frequent already in intraepithelial SCC (IFNA, 60.0%; D9S162, 39.5%; and D9S925, 40.0%) and did not increase significantly on the course to invasive SCC (63.6%, 44.2%, and 60.0%, respectively; data not shown).

The analysis detected reduced allelic balance at D9S162 in OTRs compared with immunocompetent patients with SCC (0.70 [n=37] vs 0.76 [n=49], \(P=.04\)) (Figure 1B). Allelic balance at IFNA and D9S925 was similar between immunocompetent patients and OTRs (\(P=.12\) and \(P=.98\), respectively). Loss of heterozygosity as defined by threshold values (eTable; http://www.archdermatol.com) was more frequent at D9S162 in OTRs than in immunocompetent patients (34.8% vs 45.0% of informative samples; \(P=.04\)) but similar at IFNA and D9S925 (data not shown).

Actinic keratosis showed reduced allelic balance at D9S162 compared with BD. Allelic balance for all samples of AK together was reduced at D9S162 (0.69) in contrast to BD (0.84; \(P=.004\)). At IFNA and D9S925, AK and BD showed similar allelic balance (0.83 vs 0.75 and 0.84 vs 0.79, respectively) (Figure 2A). Loss of heterozygosity as defined by the respective threshold values was significantly less frequent in BD at D9S162 than in AK (50.0% vs 10.0%; \(P=.03\)) (data not shown).

Subgroup analysis in OTRs revealed that the allelic difference observed for AK and BD overall stems mainly from malignant neoplasms in OTRs: the allelic balance at

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>D9S162</th>
<th>D9S925</th>
<th>p16</th>
<th>p53</th>
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</thead>
<tbody>
<tr>
<td>IFNA</td>
<td>0.320</td>
<td>0.630</td>
<td>0.086</td>
<td>-0.001</td>
</tr>
<tr>
<td>P value</td>
<td>.02</td>
<td>&lt;.001</td>
<td>.47</td>
<td>.99</td>
</tr>
<tr>
<td>D9S162</td>
<td></td>
<td>0.567</td>
<td>0.056</td>
<td>0.105</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>&lt;.001</td>
<td>.61</td>
<td>.36</td>
</tr>
<tr>
<td>D9S925</td>
<td></td>
<td></td>
<td>0.131</td>
<td>-0.132</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td>.21</td>
<td>.22</td>
</tr>
<tr>
<td>p16</td>
<td></td>
<td></td>
<td></td>
<td>.158</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td></td>
<td>.04</td>
</tr>
</tbody>
</table>
The difference in allelic balance found for D9S162 in combined SCC samples from OTRs and immunocompetent patients primarily occurred in the invasive SCC subgroup. In invasive SCC, allelic balance at D9S162 was reduced in OTRs (0.62 [n=17]) compared with immunocompetent patients (0.82 [n=26]; P = .048) (Figure 3A).

There was a higher proportion of p16 immunoreactive tumor cells in invasive SCC in OTRs than in immunocompetent patients. In all SCC samples, p16 protein expression was upregulated compared with normal skin and similar between immunocompetent patients and OTRs (P = .23; data not shown). Subgroup analysis of invasive SCC, however, revealed higher proportions of p16 immunoreactive tumor cells in OTRs than in immunocompetent patients (P < .001, Figure 3B).

Protein expression of p16 and p53 did not correlate with allelic balance at IFNA, D9S162, or D9S925. Because the gene locus for p16 is flanked by microsatellite loci IFNA and D9S162, the correlation between p16 immunoreactivity and allelic imbalance at the 3 microsatellite markers was assessed. Protein expression of p16 did not correlate with allelic balance at the 3 loci (IFNA, p = 0.086; D9S162, p = 0.056; and D9S925, p = 0.131) (Table 2). Expression of p53, which was determined as a tumor suppressor protein not coded on 9p21-22 and frequently upregulated in SCC, was upregulated in all SCC samples to a similar degree without significant differences (data not shown). Protein expression of p53 also did not correlate with allelic balance at the 3 loci (IFNA, p = 0.001; D9S162, p = 0.105; and D9S925, p = 0.132) (Table 2). Previous analysis of human papillomavirus protein expression by immunohistochemistry in these samples also found this expression to be very low.26

![Figure 1. Allelic balance ratios for squamous cell carcinoma (SCC) in immunocompetent patients (ICPs) and organ transplant recipients (OTRs). A. Allelic balance was similarly reduced in all SCC (intraepithelial and invasive) samples. Two-tailed P values were determined with Wilcoxon matched-pairs test. Total number of patients, 80. IFNA informative paired samples, 20 (P = .56); D9S162 informative paired samples, 26 (P = .47); and D9S925 informative paired samples, 31 (P = .58). B. Squamous cell carcinoma in OTRs overall showed reduced allelic balance for D9S162 vs SCC in ICPs. Two-tailed P values were determined with the Mann-Whitney U test. Total number of SCC samples, 170. IFNA informative samples: ICPs, 42; OTRs, 46 (P = .35). D9S162 informative samples: ICP, 49; OTRs, 37 (P = .04). D9S925 informative samples: ICPs, 49; OTRs, 46 (P = .21). Two-tailed P values were determined with Wilcoxon matched-pairs test. The horizontal line in the middle of each box indicates the median, while the top and bottom borders of the box mark the 75th and 25th percentiles. The whiskers represent the 95th and 9th percentiles.](http://archderm.jamanetwork.com/pdfaccess.ashx?url=/data/journals/derm/24221/)
These findings are in line with previously published data on SCC and other malignant neoplasms. Rehman and coworkers found that 20% of AK samples showed LOH of 8 or more alleles, with 39% from 9p. In a study on LOH in SCC, Quinn and colleagues found LOH in a distinct region encompassing 9p21-22 to occur in 41% of the samples. This led to the hypothesis that the tumor suppressor gene CDKN2A may play a critical role in SCC development. Mutational analysis and loss of transcript expression further suggests that inactivation of CDKN2A is important for SCC progression.

In contrast to basal cell carcinoma, the pattern and degree of LOH in SCC were more diverse and widespread. This suggests that increased allelic imbalance on 9p21-22 in early lesions, such as AK and BD, supports the notion that AK, BD, and invasive SCC are part of the same continuum of SCC and that DNA damage at 9p21-22 may be an early event in the carcinogenesis of SCC.

The marker D9S162 in particular showed a loss of allelic balance that distinguished SCC in OTRs from SCC in immunocompetent patients and was the most varied in all comparisons made. Not only did SCC in OTRs show a reduced allelic balance for this marker, but allelic balance of D9S162 was less in OTRs than in BD for all comparisons made.

Invasive SCC again showed allelic balance of D9S162 to be reduced in OTRs compared with immunocompetent patients. Increased and particular patterns of DNA damage have been reported in OTRs and ascribed to, for example, the effect of azathioprine. Azathioprine is an antimetabolite that doubles photosensitivity to UV-A in keratinocytes and enables direct DNA damage by UV-A. Although the underlying factors cannot be differentiated in the studied samples, these results may suggest that pronounced loss of allelic balance for D9S162 seems particular to SCC in OTRs compared with SCC in the general immunocompetent population.

Actinic keratosis was initially grouped with BD as intraepithelial SCC for our analysis. Allelic balance on 9p21-22, however, differed greatly between these 2 conditions, showing a clear reduction of allelic balance for AK.
compared with BD. This suggests that BD may be set apart in its early mutational steps from the continuum of AK and invasive SCC. The gene locus for p16 is flanked by microsatellite loci IFNA and D9S162; thus, allelic imbalance in these markers may affect expression of this tumor suppressor protein. Indeed, AK showed a lower expression of p16 by immunohistochemistry. As for allelic imbalance, the expression difference for p16 was mainly found within intraepithelial lesions of OTRs. Two immunohistochemical studies evaluating p16INK4 expression in AK, BD, and SCC of the skin presented rather controversial results. Hodges and Smoller33 found immuno-reactivity in nearly all patients with invasive SCC and BD but only in 30% of those with invasive AK, whereas Salama and colleagues34 showed a high frequency of immunoreactivity for BD in invasive SCC samples, 85. The box and whisker plot elements are defined in the legend to Figure 1. B, Invasive SCC of OTRs expressed more p16 protein than invasive SCC of immunocompetent patients (P < .001; analysis of variance). The numbers on the x-axis represent the proportions of p16 immunoreactive tumor cells in intraepithelial and invasive SCC in ICPs and OTRs grouped by the percentage of immunoreactive cells within the tumors.

Figure 3. Allelic balance ratios of invasive squamous cell carcinoma (SCC) in immunocompetent patients (ICPs) and organ transplant recipients (OTRs). A, Invasive SCC in OTRs shows reduced allelic balance for D9S162 compared with invasive SCC in ICPs (P = .048; 2-tailed P value was determined with the Mann-Whitney test). IFNA informative samples: ICPs, 19; OTRs, 14. D9S162 informative samples: ICPs, 26; OTRs, 17. D9S925 informative samples: ICPs, 27; OTRs, 23. Total number of invasive SCC samples, 85. The box and whisker plot elements are defined in the legend to Figure 1. B, Invasive SCC of OTRs expressed more p16 protein than invasive SCC of immunocompetent patients (P < .001; analysis of variance). The numbers on the x-axis represent the proportions of p16 immunoreactive tumor cells in intraepithelial and invasive SCC in ICPs and OTRs grouped by the percentage of immunoreactive cells within the tumors.

Nilsson et al33 found weak and cytoplasmic p16INK4 expression in AK, strong nuclear and cytoplasmic p16INK4 expression in carcinomas in situ (BD), and variable p16INK4 expression in invasive SCC. Although the allelic balance in OTRs seems reduced for all samples at 9p21-22, in particular for OTR, the upregulation of p16 expression in SCC of OTRs is at this time difficult to interpret and suggests that a lack of p16 protein expression is not a factor in OTRs; rather, other mechanisms are involved in enhanced cutaneous squamous cell carcinogenesis. The data presented herein on the low correlation of allelic balance at 9p21-22 and tumor suppressor protein p16 and p53 expression underlines this difficulty. We conclude that SCC of OTRs, both intraepithelial and invasive, usually shows a reduced allelic balance on 9p21-22 compared with SCC in the general population. The expression of p16 seems to not be affected by such allelic imbalance at 9p21-22.

In summary, allelic balance at 9p21-22 is broadly reduced in all SCC, and OTRs have an even further reduced allelic balance for D9S162, suggesting a common trait for SCC in OTRs. Actinic keratosis and BD differ in allelic balance at D9S162, in particular among OTRs, suggesting essential differences in their carcinogenesis. Tumor suppressor protein p16, while located close to the observed reduced allelic balance, shows no close relationship to the allelic balance observed, nor does p53 have an effect.

The allelic imbalances found in our study suggest a role for them in the increased incidence and more aggressive carcinogenesis of SCC in OTRs. Our results add to the rationale for close clinical monitoring of OTRs to recognize and treat intraepithelial SCC early, preferably addressing field cancerization.

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Author Contributions: Drs Muhleisen and Hofbauer had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Muhleisen and Hofbauer. Acquisition of data: Muhleisen, Petrov, Frigerio, and Hofbauer. Analysis and interpretation of data: Muhleisen, Frigerio, Dziunycz, French, and Hofbauer. Drafting of the manuscript: Muhleisen, Petrov, and Hofbauer. Critical revision of the manuscript for important intellectual content: Frigerio, Dziunycz, French, and Hofbauer. Obtained funding: Hofbauer. Administrative, technical, and material support: Muhleisen, Petrov, and Dziunycz. Study supervision: French and Hofbauer.

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REFERENCES