Pronounced allelic imbalance at D9S162 in skin squamous cell carcinoma of organ transplant recipients

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Abstract: 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.

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Pronounced allelic imbalance at D9S162 in skin squamous cell carcinoma of organ transplant recipients

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Key words: Loss of heterozygosity (LOH), organ transplant recipient, squamous cell carcinoma, actinic keratosis, Bowen’s disease, 9p, IFNA, D9S162, D9S925, p16, p53

Running title: Allelic balance in skin cancer

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Abstract

Objective: Cutaneous squamous cell carcinoma (SCC) is the second most frequent skin tumor with 60 to 100-fold higher incidence in immunocompromised patients particularly organ transplant recipients (OTR). Chromosomal instability on 9p21-22 encompassing the tumor suppressor gene p16 is a frequent hallmark of carcinogenesis in a variety of tumors. To date there is no data comparing chromosomal instability at 9p21-22 with p16 protein expression in SCC and no data in OTR.

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

Settings: Department of Dermatology. University Hospital Zurich

Patients: Immunocompetent patients and OTR with SCC on sun damaged skin

Main outcome measure: Chromosomal allelic balance in SCC of OTR and immunocompetent patients

Results: Reduced allelic balance at IFNA, D9S162 and D9S925 in intraepithelial forms of SCC and similar allelic imbalance in invasive forms has been found. Allelic balance at D9S162 was reduced for SCC in OTR compared to SCC in immunocompetent patients. The study revealed broadly reduced allelic balance at 9p21-22 in all cutaneous SCC while OTR presented a further reduced allelic balance for D9S162, suggesting a common trait for SCC in OTR. AK and BD differed in allelic balance at D9S162, suggesting substantial differences in their carcinogenesis.

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

Squamous cell carcinoma of the skin (SCC) is a common cutaneous neoplasm of keratinocytes mainly in Caucasians. In the setting of chronic immunosuppression such as in organ transplant recipients (OTR), SCC incidence rises dramatically 65- to a 100-fold compared to the general population \(^1\). Chronic UV damage is a major risk factor and results in keratinocyte DNA damage, both initiating and propagating SCC formation. Intraepithelial lesions such as actinic keratosis (AK) and Bowen’s disease (BD) are increasingly considered *in situ* SCC \(^2,3\).

The occurrence of SCC is not an isolated event, but it indicates field cancerization, i.e. the presence of wide-spread DNA damage in keratinocytes of sun-exposed skin which keeps giving rise to intraepithelial and invasive SCC in affected patients \(^4\). Genomic instability is a hallmark of many different neoplasias. The resultant allelic imbalance or loss of heterozygosity (LOH) at particular loci can be used as marker for an ongoing neoplastic process. Mutations on chromosome 9p have been described in a variety of tumors \(^5-9\). In SCC, LOH encompassing 9p21-22 occurred in 41% \(^10\). Rehman and co-workers found 20% of AK with LOH of eight or more alleles, 39% of which on chromosome 9p \(^11\).

Cyclin-dependent kinase inhibitor p16\(^{\text{INK4}}\) specifically inhibits progression through G1 phase of the cell cycle by blocking the cyclin-dependent kinase 4 from phosphorylating the retinoblastoma protein \(^12\). LOH as well as loss of parts or the entire short arm of chromosome 9, where p16\(^{\text{INK4}}\) maps to 9p21, are frequently observed in SCCs \(^13,14\). Nilsson et al. \(^15\) found weak and cytoplasmatic p16\(^{\text{INK4a}}\) expression in AK, strong nuclear and cytoplasmatic p16\(^{\text{INK4a}}\) expression in carcinomas.
in situ (BD) and variable p16\textsuperscript{InK4a} expression in invasive SCC. Blokx and colleagues assessed p16 and p53 protein expression in 23 AK (termed low-grade KIN), 28 BD (high-grade KIN) and 35 invasive SCCs from 44 OTR and 42 immunocompetent patients (ICP) concluding that p16 expression was independent of immune status and of p53 expression\textsuperscript{16}. Other tumor suppressor genes located in the 9p21-22 region include INK4b and ARF (Alternative Reading Frame)\textsuperscript{17-19}.

The aim of the study was to recognize the imprint of field cancerization in the course of carcinogenesis in a highly select population of first intraepitelial and subsequently invasive SCC from the same anatomical region of the same patient at different time points. Allelic imbalance on 9p21-22 as a well-known highly variable region in SCC has been analyzed. Because OTR empirically show a greatly increased SCC incidence and are known to harbor genetic damage in addition to the one generally incurred by UV damage alone\textsuperscript{20, 21}, SCC of both ICP and OTR were included.
Material and Methods

Ethical considerations

The use of clinically indicated biopsy material for the study was approved by the ethical committee of the Canton of Zurich, Switzerland.

Patients and tumor selection

The goal was to obtain tissue material from an area of field cancerization which had first given rise to an intraepithelial and at least one year later to an invasive squamous cell carcinoma of the skin. Following approval by the local ethical committee, paraffin-embedded archival tissue specimens from 43 immunocompetent patients and 42 immunosuppressed organ transplant recipients were randomly included, all of which first had an intraepithelial form of cutaneous squamous cell carcinoma such as actinic keratosis or Bowen’s disease, followed by an invasive squamous cell carcinoma of the skin in the same anatomical region at least one year later. These intraepithelial forms were then intraindividually compared to the subsequent invasive squamous cell carcinoma. Of the 42 organ transplant recipients, 32 were kidney, 4 lung, and 6 heart recipients (table 1). All tumors were located on chronically sun-exposed skin of the head, neck and upper extremities. The histological diagnosis was made by a board-certified dermatopathologist based on the criteria defined by the WHO classification of skin tumours. Actinic keratoses and Bowen’s disease were on the one hand analysed separately and compared to each other, and on the other hand and grouped as “intraepithelial SCC” the cases of actinic keratosis and Bowen’s disease to compare them to invasive SCC.

Microdissection
From each sample 3-5 adjacent sections of 10 µm thickness were stained with hematoxylin and eosin. Microdissection was performed under a light microscope (magnification 400X). 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 in far distance from the tumor) were harvested from the same slides and served as internal controls.

**DNA extraction**

Microdissected cells were immediately suspended in 20 µl digestion solution containing 50 mM Tris-HCl, 1 mM EDTA, 1% Tween 20 and 2.5 mg/ml proteinase K (pH 8.0, Sigma-Aldrich, P2308, Inc., Saint Louis, MO, USA) and incubated for 16 hours at 37°C. The mixture was then heated for 10 min at 94°C to inactivate proteinase K. 5 µl 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 squamous cell carcinoma, 3 microsatellite marker loci on chromosome 9 (IFNA, D9S925, D9S162) previously linked to early tumor development and flanking the p16 tumor suppressor gene region have been selected. These loci included either di- or tetra-nucleotide tandem repeats known for their high degree of allelic variability and hence, informativity (table 2). PCR of the 3 different microsatellite markers was performed with separate primer pairs of which 1 oligonucleotide was labeled at the 5'-end with the fluorescent dyes 6-FAM, HEX (Microsynth, Balgach, Switzerland) or NED (Applied Biosystems, Rotkreuz, Switzerland). The primer sequences were as follows: IFNA up: GTAAGGTGGAAACCCCC (FAM), IFNA low:
Amplification of specific DNA was done in a reaction volume of 25 µl including 0.2 mM dNTPs (Roche Diagnostics), 2.5 mM MgCl2, 0.5 U AmpliTaq Gold (both from Applied Biosystems). Cycling was performed with a GeneAmp PCR System 9700 (Applied Biosystems) using the following temperature conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 2 min for 40 cycles; final extension 60 min at 60°C. The PCR products were subsequently denatured for 1 min at 95°C in HiDi formamide (Applied Biosystems) and separated on an ABI PRISM 3100 Genetic Analyzer equipped with a 36 cm capillary array loaded with POP-4 polymer. ROX-400HD was used as internal size standard (Applied Biosystems). Generally artefacts can be a concern in LOH analysis. PCR for each locus has been performed twice with similar results. Moreover a large number of samples was analyzed which allows for statistical assurance of true allelic imbalance.

**Loss of heterozygosity (LOH) analysis**

Analysis of the samples was carried out with the GeneMapper software version 3.7 (Applied Biosystems). A case was considered informative for a polymorphic marker if normal tissue DNA showed two 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 surmounted 1, the reciprocal value was used instead. Consequently, the calculated numbers varied between 0 (representing complete loss) and 1 (retained
heterozygosity). To determine if allelic imbalance ratio in a tumor sample met the
criterion for LOH, previously published standard threshold values for these three loci
were applied.24

**Immunohistochemistry**

3- to 5-μm adjacent sections were used for hematoxylin and eosin (HE) staining and
immunohistochemistry. The deparaffinized sections were heated in a 100-W
household microwave oven at maximum power for three times 5 minutes each in 10
mmol/L citric acid for antigen retrieval. Primary antibody was then applied for 60
minutes at room temperature. Immunohistochemical stainings were performed with
monoclonal IgG mouse antibodies specifically binding human p16\textsuperscript{INK4a} (G175-405;
550834, PharMingen, San Diego, CA, 1:200) and p53 (DO-7, Pab 1801, Sigma
Biosciences, St Louis, MO, USA, 1:100). Secondary staining was performed using
the alkaline phosphatase anti-alkaline phosphatase method.25 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 0-5%, 6-25%, 25-50%, 51-75%, 76-100% positive tumor cells. All
samples were reviewed unaware of the patients’ diagnosis or immune status.

**Statistical analysis**

Allelic balance ratios and immunoreactivity between two independent groups were
calculated using the Mann Whitney U Test, frequencies of LOH between two groups
using Fisher’s Exact Test. Correlations between LOH, p16 and p53 were expressed
using Spearman’s rho. P-values <0.05 were considered statistically significant.
Statistical analyses were performed using SPSS for Windows 11.5 (SPSS Inc.,
Chicago, IL, USA), GraphPad Prism 5.0 and Microsoft Excel 2000.
Results

Considerably reduced allelic balance on microsatellite loci IFNA, D9S162 and D9S925 on chromosome 9p is similar in intraepithelial and invasive SCC. Considerable allelic imbalance at all three microsatellite loci has been found. 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 three loci, there was no reduction in allelic balance on the course from intraepithelial to invasive SCC (IFNA 0.79 for intraepithelial SCC, 0.80 for invasive SCC, n=20, p=0.56; D9S162 0.72, 0.77, n=29, p=0.47; D9S925 0.80, 0.73, n=31, p=0.58, Wilcoxon matched pair test, two-tailed p-values) (Figure 1(a)). Loss of heterozygosity (LOH) defined as allelic balance below threshold values for these three loci (supplementary table 1) was found in 61.6% of informative samples at locus IFNA, in 41.9% at D9S162 and in 50.5% at D9S925. LOH was frequent already in intraepithelial SCC (IFNA 60.0%, D9S162 39.5%, D9S925 40.0%) and did not increase significantly on the course to invasive SCC (63.6%, 44.2%, 60.0%, respectively; data not shown).

Allelic balance at D9S162 is reduced for SCC in OTR compared to SCC in immunocompetent patients. The analysis detected reduced allelic balance at D9S162 in OTR compared to ICP (0.70 vs. 0.76; p=0.04, n=37, n=49, respectively) (Figure 1(b)). Allelic balance at IFNA and D9S925 was similar between ICP and OTR. LOH as defined by threshold values (supplementary table 1) was more frequent at D9S162 in OTR than in ICP (34.8% vs. 45.0% of informative samples, p=0.04, but similar at IFNA and D9S925 (data not shown).
*Actinic keratosis shows reduced allelic balance at D9S162 compared to Bowen’s disease.*

Allelic balance for all samples of actinic keratosis together is reduced at D9S162 (0.69) in contrast to Bowen’s disease (0.84; p=0.004). At IFNA and D9S925 AK and BD show similar allelic balance (0.83, 0.75 and 084, 0.79, respectively) (Figure 2(a)). LOH as defined by the respective threshold values is significantly less frequent in BD at D9S162 than in AK (50% vs. 10%, p=0.03) (data not shown).

*The difference in allelic balance for D9S162 between AK and BD is primarily found in OTR neoplasms*

Subgroup analysis in OTR reveals that the allelic difference observed for AK and BD overall stems mainly from neoplasms in OTR: the allelic balance at D9S162 is higher in BD than in AK (0.93, n=10, 0.61, n=13, respectively; p=0.01) (Figure 2(b)). In the ICP subgroup there is no significant difference between AK and BD at D9S162 (p=0.09, data not shown). Intraepithelial SCC overall did not differ between OTR and ICP in allelic balance at any of the three loci (data not shown).

*Pronounced p16 expression in Bowen’s disease (BD) compared to actinic keratosis (AK) is primarily found in OTR, not ICP.*

p16 immunoreactive tumor cells were found in a higher proportion in BD (n=22 samples) than in AK (n=63) (p=0.0001 for all BD versus AK). This was primarily due to the expression difference in OTR intraepithelial SCC (BD n=13, AK n=29, p=0.0002), while p16 expression did not differ between BD and AK in ICP (p=0.70) (figure 2(c)).
Invasive SCC in OTR shows reduced allelic balance for D9S162 compared to invasive SCC in immunocompetent patients

The difference in allelic balance found for all SCC between OTR and ICP is primarily due to the invasive SCC subgroup: In invasive SCC, allelic balance at D9S162 was reduced in OTR (0.62) compared to ICP (0.82, p=0.04, n=17, n=26, respectively) (Figure 3(a)).

Higher proportion of p16 immunoreactive tumor cells in invasive SCC in OTR than in ICP.

In all SCC together, p16 protein expression was upregulated compared to normal skin and similar between ICP and OTR (p=0.23; data not shown). Subgroup analysis of invasive SCC, however, revealed higher proportions of p16 immunoreactive tumor cells in OTR than in ICP (p=0.0001, Figure 3(b)).

p16 and p53 protein expression do not correlate with allelic balance at IFNA, D9S162 and D9S925.

As the gene locus for p16 is flanked by microsatellite loci IFNA and D9S162, the correlation between p16 immunoreactivity and allelic imbalance at the three microsatellite markers has been assessed. p16 protein expression did not correlate with allelic balance at the three loci (IFNA rho=0.09, D9S162 rho=0.056, D9S925 rho=0.131) (table 2). p53 expression, which was determined as a tumor suppressor protein not coded on 9p21-22 and frequently upregulated in SCC, was found upregulated in all SCC to a similar degree without differences (data not shown). P53 protein expression also did not correlate with allelic balance at the three loci (IFNA rho=-0.001, D9S162 rho=0.105, D9S925 rho=-0.132) (table 2). We previously also
analysed HPV protein expression by immunohistochemistry in these samples and found it to be very low\textsuperscript{26}. 
Discussion

A highly selected SCC sample group has been analysed: the samples were all paired from the same patient and the same anatomical region with first an intraepithelial and at least a year later an invasive SCC which were available for study. Such careful selection allows closer observation of allelic imbalance in the course of SCC development, in particular in the transition of SCC across the dermo-epidermal junction and may yield meaningful insight with a limited number of samples 26, 27.

The analysed region of the chromosome 9p21-22 is a well-known highly variable region in SCC of different organs. One of the genes located in this region is cyclin-dependent kinase inhibitor p16\textsuperscript{INK4}. The P16 protein is known to specifically inhibit progression through the G1 phase of the cell cycle by blocking the cyclin-dependent kinase 4 from phosphorylating the retinoblastoma protein. Thus this gene may play the role of an antioncogene. We aimed to study the allelic imbalance and putative consequence in p16 protein expression levels. Our study shows a difference in both allelic imbalance at 9p21-22 and p16\textsuperscript{INK4} expression between the AK and BD, which suggests a role of this particular chromosomal locus for SCC carcinogenesis.

The chromosomal region 9p21-22 in our SCC samples is broadly impaired in its allelic balance, a common finding for all samples assessed. These findings are in line with previously published data on SCC and other neoplasms 5-11. Rehman and co-workers found, that 20% of AK showed LOH of eight or more alleles, 39% of which from 9p 11. In a study on LOH in SCC, Quinn and colleagues found LOH in a distinct region encompassing 9p21-22 to occur in 41% 10. 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 28-31. In contrast to basal
cell carcinoma, the pattern and degree of LOH in SCC were more diverse and widespread. This suggests that increase 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 \(^2, ^3\) 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 OTR from SCC in immunocompetent patients and was most varied in all comparisons made. Not only did SCC in OTR show a reduced allelic balance for this marker, but allelic balance of D9S162 distinguished AK from BD for all intraepithelial lesions with OTR contributing primarily to this difference. Invasive SCC again showed allelic balance of D9S162 reduced in OTR compared to immunocompetent patients. Increased and particular patterns of DNA damage have been reported in OTR and ascribed to e.g. the effect of azathioprine \(^1, ^2\). Azathioprine is an antimetabolite which doubles photosensitivity to UVA in keratinocytes and enables direct DNA damage by UVA \(^2\). While 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 OTR compared to SCC in the general, immunocompetent population.

Actinic keratosis was initially grouped with Bowen’s disease as intraepithelial SCC for our analysis. Allelic balance on 9p21-22, however, differed greatly for these two conditions, yielding a clear reduction of allelic balance for AK compared to 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 OTR. Two immunohistochemical studies, evaluating p16^{Ink4} expression in AK, Bowen’s disease and SCC of the skin presented rather controversial results. Hodges and co-workers\textsuperscript{33} found immunoreactivity in about all AK and Bowen’s disease but only in 30% of invasive SCC whereas Salama and colleagues\textsuperscript{34} showed a high frequency of immunoreactivity for BD but very little for AK and none for SCC. From this they concluded that p16^{Ink4} is a selective and specific marker to distinguish BD from SCC. This study shows that AK in OTR is set apart from BD by reduced allelic balance on 9p21-22 and reduced p16 expression, suggesting different paths of carcinogenesis for AK and BD in OTR.

Drug-induced immunosuppression in OTR dramatically increases SCC 60- to 100-fold\textsuperscript{35}. Immunosuppressants also act directly on keratinocytes\textsuperscript{1}, some on the transcriptional level such as cyclosporine A on ATF3\textsuperscript{36} and some on the genomic level such as azathioprine in conjunction with UVA\textsuperscript{20}. Blokx and colleagues assessed p16 and p53 protein expression in 23 AK (named it low-grade KIN), 28 BD (high-grade KIN) and 35 invasive SCCs from 44 OTRs and 42 immunocompetent controls and came to the conclusion, that p16 expression was independent of immune status and also independent of p53 expression\textsuperscript{16}. This study shows that invasive SCC in OTR exhibits reduced allelic balance for D9S162, but in contrast to intraepithelial SCC, p16 expression seems increased in SCC of OTR compared to SCC of the general population.
Nilsson et al. 15 found weak and cytoplasmatic p16\textsuperscript{INK4a} expression in AK, strong nuclear and cytoplasmatic p16\textsuperscript{INKa} expression in carcinomas in situ (BD) and variable p16\textsuperscript{INK4a} expression in invasive SCC. While the allelic balance in OTR seems reduced for all samples at 9p21-22, in particular for OTR, the upregulation of p16 expression in SCC of OTR is at this time difficult to interpret and suggests that in OTR not a lack of p16 protein expression but other mechanisms are involved in enhanced cutaneous squamous cell carcinogenesis. The presented data 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 OTR, both intraepithelial and invasive, show a reduced allelic balance on 9p21-22 compared to the general population's SCC as a common trait. The expression of p16 seems not affected by such allelic imbalance at 9p21-22.

In summary, allelic balance at 9p21-22 broadly reduced in all SCC has been found, while OTR presented even further reduced allelic balance for D9S162, suggesting a common trait for SCC in OTR. AK and BD differ in allelic balance at D9S162, in particular among OTR, 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. The allelic imbalances found in our study suggest a role for them in the increased and more aggressive carcinogenesis in OTR. Our results add to the rationale of a tight clinical follow-up for OTR to recognize and treat intraepithelial SCC early, preferably addressing field cancerization.


Figure legends

Figure 1. (a) Allelic balance is similarly reduced in all SCC (intraepithelial and invasive). Allelic balance ratios in intraepithelial (black box plots) and invasive SCC (white box plots). Whiskers of box plots represent 9- and 95-percentiles. Two-tailed p-value from Wilcoxon matched pairs test.  n.s.: non-significant. Total number of patients n=85. IFNA informative paired samples: n=20, p=0.56, D9S162 informative paired samples: n=29, p=0.47, D9S925 informative paired samples: n=31, p=0.58.  
(b) SCC in OTR overall show reduced allelic balance for D9S162 compared to SCC in immunocompetent patients. Allelic balance ratios in SCC in immunocompetent (ICP, black box plots) and immunosuppressed (OTR, organ transplant recipients, white box plots) patients. Whiskers of box plots represent 9- and 95-percentiles. Two-tailed p-value from Mann Whitney U test.  n.s.: non-significant. Total number of SCC n=170. IFNA informative samples: Immunocompetent=42, OTR=31, D9S162 informative samples immunocompetent=49, OTR=37, D9S925 informative samples: immunocompetent=49, OTR=46. Two-tailed p-value from Wilcoxon matched pairs test. n.s.: non-significant.

Figure 2. (a) Overall, AK show reduced allelic balance for D9S162 compared to BD. Allelic balance ratios in actinic keratosis (AK, black box plots) and Bowen’s disease (BD, white box plots). Bowen’s disease shows significantly higher allelic balance at D9S162 than actinic keratosis (p=0.0038). IFNA informative samples: AK=30, BD= 10, D9S162 informative samples AK=32, BD=11, D9S925 informative samples: AK=31, BD=14. Two-tailed p-value from Mann Whitney U test. Total number of AK=63, Bowen’s disease=22. (b) AK in OTR show less allelic balance
for D9S162 than BD in OTR. (p=0.01, Mann Whitney U test). Allelic balance ratios in actinic keratosis (AK, black box plots) and Bowen’s disease (BD, white box plots) in OTR. IFNA informative samples: AK=12, BD=5, D9S162 informative samples: AK=13, BD=7, D9S925 informative samples: AK=13, BD=10. Total number of intraepithelial SCC in OTR: AK=29, Bowen’s disease=13. Fisher’s exact test. (c) AK in OTR express less p16 protein than BD in OTR (p<0.005, ANOVA). Y-axis: SCC samples in absolute numbers. X-axis: Proportion of p16 immunoreactive tumor cells grouped by percent of immunoreactive cells within tumor.

Figure 3. (a) Invasive SCC in OTR shows reduced allelic balance for D9S162 compared to invasive SCC in immunocompetent patients (p=0.048, two-tailed p-value from Mann Whitney U test). Allelic balance ratios in invasive SCC in immunocompetent patients (ICP, black box plots) and organ transplant recipients (OTR, white box plots). IFNA informative samples: Immunocompetent=19, OTR=14, D9S162 informative samples immunocompetent=26, OTR=17, D9S925 informative samples: immunocompetent=27, OTR=23. Total number of invasive SCC n=85. (b) Invasive SCC of OTR express more p16 protein than invasive SCC of immunocompetent patients (p=0.001, ANOVA). Organ transplant recipients (OTR) compared to immunocompetent patients (ICP). Y-axis: absolute number of SCC samples. X-axis: Proportions of p16 immunoreactive tumor cells in intraepithelial and invasive SCC in ICP and OTR grouped by percent of immunoreactive cells within tumor.
Table legends

Table 1
Patient characteristics. Immunocompetent: Immunocompetent patients. OTR: organ transplant recipients. Transplanted organs were: 32 kidney, 6 heart, 4 lung.

Table 2
Correlation (Spearman’s rho) between allelic balance ratio for IFNA, D9S162 and D9S925, p16 and p53 protein expression and corresponding p-value.
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<td>- Bowen’s disease</td>
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<td>- Squamous cell carcinoma</td>
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* kidney: 32, heart: 6, lung: 4
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