Allelic Imbalances and Microdeletions Affecting the *PTPRD* Gene in Cutaneous Squamous Cell Carcinomas Detected Using Single Nucleotide Polymorphism Microarray Analysis

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Abstract

Cutaneous squamous cell carcinomas (SCC) are the second most commonly diagnosed cancers in fair-skinned people; yet the genetic mechanisms involved in SCC tumorigenesis remain poorly understood. We have used single nucleotide polymorphism (SNP) microarray analysis to examine genome-wide allelic imbalance in 16 primary and 2 lymph node metastatic SCC using paired non-tumour samples to counteract normal copy number variation. The most common genetic change was loss of heterozygosity (LOH) on 9p, observed in 13 of 16 primary SCC. Other recurrent events included LOH on 3p (9 tumors), 2q, 8p, and 13 (each in 8 SCC) and allelic gain on 3q and 8q (each in 6 tumors). Copy number-neutral LOH was observed in a proportion of samples, implying that somatic recombination had led to acquired uniparental disomy, an event not previously demonstrated in SCC. As well as recurrent patterns of gross chromosomal changes, SNP microarray analysis revealed, in 2 primary SCC, a homozygous microdeletion on 9p23 within the protein tyrosine phosphatase receptor type D (*PTPRD*) locus, an emerging frequent target of homozygous deletion in lung cancer and neuroblastoma. A third sample was heterozygously deleted within this locus and *PTPRD* expression was aberrant. Two of the 3 primary SCC with *PTPRD* deletion had demonstrated metastatic potential. Our data identify *PTPRD* as a candidate tumor suppressor gene in cutaneous SCC with a possible association with metastasis.

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INTRODUCTION

Nonmelanoma skin cancers (NMSC), comprising basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), have become a significant health problem in fair-skinned people worldwide and their incidence is rising rapidly (de Vries et al., 2005). UV radiation (UVR) is the principal carcinogen implicated in the development of SCC (Armstrong and Kricker, 2001). Although UVR-induced genetic changes in potential target genes including *TP53* (Brash et al., 1991), *CDKN2A* (Soufir et al., 1999) and *RAS* (Pierceall et al., 1991) have been demonstrated in cutaneous SCC, other key molecular events in tumor initiation and progression are yet to be defined. Microarray-based single nucleotide polymorphism (SNP) analysis facilitates the accurate and rapid identification of genome-wide allelic changes in tumor DNA samples. It has been used to investigate the molecular pathogenesis of various human cancers, including BCC (Teh et al., 2005) and acute myeloid leukemia (Raghavan et al., 2005). Here, this technique has been applied to cutaneous SCC for the first time.

MATERIALS AND METHODS

Sixteen primary cutaneous SCC and 2 lymph node metastatic SCC were obtained from 16 patients, 8 of whom were immunosuppressed (1 cardiac transplant and 7 renal transplant recipients). Ethical approval for this investigation was obtained from the East London and City Health Authority local ethics committee and all patients provided informed consent. Samples were enriched for SCC keratinocytes by short-term culture as previously described (Proby et al., 2000) or by microdissection of frozen lesional tissue sections using a H&E-stained section as a reference. Non-tumour control DNA was obtained from blood or from dermal fibroblasts isolated from perilesional skin by incubation in 0.05 mg/ml collagenase D (Roche, Applied Science, Indianapolis, IN) for 16 hr at 37°C.

DNA was extracted from cultured cells and blood samples using the Nucleon BACC3 Genomic DNA Extraction kit and from lesional tissue using the Nucleon HT Genomic DNA Extraction kit (both from Amersham Biosciences, Buckinghamshire, UK). Samples were subjected to the GeneChip Human Mapping 10K (V2.0) Xba or 250K Nsp assay (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol with the modification that PCR products were purified using the Ultrafree-MC filtration column (Millipore, Watford, UK). Processing was performed as previously described (Teh et al., 2005) using the Genome Oriented Laboratory File (GOLF) system for the analysis and display of SNP call data.

The Qiagen RNeasy Mini kit (Qiagen, Crawley, UK) was used to extract RNA from primary and metastatic SCC tissue and from normal skin samples removed during cosmetic surgery from 7 different individuals with no known skin cancer history. cDNA was synthesized from RNA using Superscript II Reverse Transcriptase and oligo $(dT)_{12-18}$ primers (both from Invitrogen, Paisley, UK). Samples were subjected to RT-PCR using the *PTPRD* primers described previously (Sato et al., 2005). Poly (A+) RNA (Ambion, Austin, TX) from brain tissue, which expresses both S and L *PTPRD* isoforms (Sato et al., 2005), was used as a positive control.

RESULTS AND DISCUSSION

SNP microarray analysis was performed on 16 primary and 2 lymph node metastatic SCC with corresponding non-tumour samples analyzed concomitantly to overcome the recently highlighted problem of normal copy number variation (Redon et al., 2006) thereby allowing accurate identification of acquired genome-wide genetic events that may be important in SCC tumorigenesis. Allelic loss: Of 16 primary SCC, 13 exhibited extensive loss of

heterozygosity (LOH) at 9p (Fig. 1a, Table 1). Analysis of SNP call signal intensity data (Fig. 1b) revealed that in 23% (3/13) of samples, LOH regions were not accompanied by loss of copy number, suggesting that LOH was due to acquired uniparental disomy (UPD). UPD occurs as a result of a somatic recombination event, and has recently been recognized as a key mechanism of LOH in several types of solid tumors including BCC (Teh et al., 2005) but our data provide the first evidence of UPD in SCC tumorigenesis. Previous studies have linked the acquisition of UPD in leukemia with homozygosity for certain gene mutations (Fitzgibbon et al., 2005) suggesting that it may play a similar role in the evolution of SCC. Other frequent genetic events including 3p LOH (observed in 9 primary SCC) and LOH on 2q, 8p, and 13 (each in 8 primary tumors) are listed in Table 2. Allelic gain: Analysis of SNP call signal intensity data also revealed recurrent regions of increased allelic copy number in the absence of LOH (Table 1). The most frequent of these occurred on 3q and 8q, each in 6 of 16 primary tumors. Other recurrent regions of allelic gain are listed in Table 2.

Paired primary and lymph node metastatic SCC were available for two patients in this study, thereby presenting an opportunity to investigate genetic changes associated with acquisition of metastatic potential. The primary and metastatic tumors from patient 3 displayed identical patterns of LOH accompanied by allelic loss on 5q, 8p, 11p, and 17 together with LOH due to UPD on 7q, 9, and 17q and allelic gain on 7p, 8q, and 17q (Table 1, Fig. 1c). However, only the metastatic lesion exhibited allelic loss on chromosome 10 while the 3p deletion observed in the primary lesion extended to 3q21.3 in the metastatic sample. Conversely, allelic gain at 5p was observed in the primary SCC alone together with increased regions of allelic gain on 3q and 11q in comparison to the metastatic tumor. The primary and metastatic tumors from patient 12 demonstrated identical patterns of allelic loss on 2q, 9p, and 18q with allelic gain on 8q, 11p, and X but the metastatic sample alone showed allelic loss on chromosome 4 while 18p loss was observed only in the primary SCC. These data indicate that, in both patients, primary and metastatic SCC were of common origin, with further genetic events occurring in each tumor after metastasis.

Although gross chromosomal changes constitute the majority of the aberrations revealed by this technique, we have identified a microdeletion on 9p23 in both primary-metastatic SCC pairs as well as the primary SCC from patient 9[*] (Table 1). The microdeletion was detected by comparison of SNP genotypes in tumors and paired non-tumor samples and therefore was clearly somatically acquired. This region was homozygously deleted in the samples from patients 3 and 9 whereas both primary and metastatic SCC from patient 12 contained a heterozygous deletion. Using 10K SNP microarray analysis all microdeletions were mapped to a region spanning 8.45–11.0 Mb, within the locus of the recently identified PTPRD (protein tyrosine phosphatase receptor type D) L isoform (Sato et al., 2005). The physiological function of PTPRD has not been fully characterized, although it is known to interact with the putative metastasis suppressor protein MIM to mediate actin cytoskeletal reorganization (Gonzalez-Quevedo et al., 2005). To date, a tumor suppressor role for *PTPRD* remains speculative, although several other protein tyrosine phosphatase receptors have been postulated as tumor suppressor genes including *PTPRO* in lung cancer (Motiwala et al., 2004) and *HPTP*η, implicated in several carcinomas, most recently pancreatic cancer (Trapasso et al., 2004). Interestingly, *PTPRD* has been revealed as a recurrent target of homozygous deletion in lung carcinoma (Sato et al., 2005; Zhao et al., 2005) as well as in neuroblastoma (Stallings et al., 2006), where a possible association with metastasis was suggested. Furthermore, cancer genome mutation analysis has recently identified *PTPRD* as

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[*]The primary SCC from patient 9 was particularly aggressive, despite apparent complete excision two recurrences occurred within 12 months. Furthermore, the presence of metastatic SCC cannot be excluded; a suspicious pulmonary nodule was observed by CT scan 21 months after excision of the primary tumor but not confirmed as the patient died shortly afterwards of chest infection.

a candidate cancer gene in colorectal carcinoma (Sjöblom et al., 2006). To compare microdeletions in the two primary SCC with demonstrated metastatic potential, samples were subjected to higher resolution 250K *Nsp* SNP microarray analysis (Fig. 2a). The sample from patient 3 demonstrated two regions of homozygous deletion at 9.08–9.22 Mb and 10.42–10.51 Mb, while an overlapping region of deletion at 8.55–9.38 Mb was observed on one allele in the patient 12 sample. DNA sequencing (primers listed in supplementary information (Supplementary material for this article can be found at http:// www.interscience.wiley.com/jpages/1045-2257/suppmat)) revealed the second copy of *PTPRD* to be wild type. RT-PCR was performed to compare *PTPRD* expression in the primary and metastatic SCC from patient 12 with the pattern observed in normal skin. Two primer sets were used to amplify sequences common to S and L isoforms, with the forward primer located either within or outside the region of heterozygous deletion (Fig. 2b). Products of the expected size were observed in all cases (Fig. 2c), suggesting that wild type *PTPRD* transcripts were present in the SCC samples. Additional RT-PCR reactions with an L-isoform-specific forward primer located within the region of heterozygous deletion yielded no products in any samples, whereas the use of an L-isoform-specific forward primer located outside the region of heterozygous deletion yielded products solely in the SCC samples (Fig. 2d), implying that normal skin expresses the S isoform alone. In both SCC samples, only a single band of smaller size than the predicted full-length product was detected, pointing to the absence of wild type *PTPRD* L isoform transcripts. Sequencing revealed that exon B8 was fused to exon 5 within this amplimer (Fig. 2d), thereby corresponding to the region of heterozygous deletion observed by SNP analysis. A similar *PTPRD* deletion involving part of the 5′-UTR together with coding sequence has been reported previously in lung cancer (Sato et al., 2005), but our data provide the first reported example of transcription of this deleted gene. Although the wild type *PTPRD* start codon in exon 2 is located within the deleted region, the shortened transcripts contain another start codon within exon 5. Translation from this codon would yield a truncated product in frame with full-length PTPRD, suggesting a possible dominant negative mechanism. Further study is clearly required to investigate this hypothesis.

Our data show similarities with the results of early research where polymorphic microsatellite markers have revealed widespread allelic loss in SCC. In one study (Quinn et al., 1994) of 47 tumors frequent LOH of markers was observed on 9p (41%), 13q (46%), 17p (33%), 17q (33%), and 3p (23%). More recently, comparative genomic hybridization (CGH) has been used to identify genome-wide chromosomal imbalances within SCC (Popp et al., 2002; Ashton et al., 2003; Clausen et al., 2006). Despite considerable variation, the combined data from these three studies suggest a characteristic pattern of genetic aberrations within SCC. Recurrent allelic loss was reported on 3p, 4q, 5q, 8p, 9p, 11p, 13q, 17p, and 18 while recurrent allelic gain occurred on 1q, 3q, 5p, 7, 8q, 9q, 14q, 17q, 20q and Xq. In general, the allelic imbalance frequencies detected in the current study fall within the ranges of these earlier data. However, LOH was observed on 2q, 7q and 13q at higher frequencies than previously reported. This suggests that SNP microarray mapping may provide greater sensitivity than the other techniques, possibly due to its ability to detect copy-number-neutral LOH. Despite the small sample number, all of the allelic imbalances reported previously were detected in at least 1 of the 16 primary SCC examined here.

Organ transplant recipients (OTR) receiving immunosuppression are at a 100-fold increased risk of SCC compared with immunocompetent (IC) individuals, and tumors are frequently more aggressive (Glover et al., 1997). One early study (Rehman et al., 1997) suggested a different molecular pathogenesis for OTR SCC based on a rate of LOH less than half of that observed in IC tumors. In the current study, we observed allelic imbalance at 3–13 chromosomes (median value 5.5) in the 8 primary OTR SCC compared with 5–15 chromosomes (median value 9) in the 8 IC samples. Although numbers were small, our

results suggested that the frequency of aberrations may be correlated with the degree of differentiation rather than immune status of the tumor: overall, allelic imbalance was observed at 3–7 chromosomes (median of 4) in well-differentiated tumors compared with 5–15 chromosomes (median of 10) in moderately or poorly differentiated tumors. Furthermore, despite a lower rate of allelic imbalance, some specific aberrations were more frequent in well differentiated than in moderately or poorly differentiated tumors. For example, 3p LOH was observed in 83% (5/6) of well-differentiated SCC compared with only 40% (4/10) of other SCC and allelic gain at 9q occurred in 50% (3/6) of well-differentiated but only 10% (1/10) of moderately and poorly differentiated tumors. These data suggest that well differentiated tumors may constitute a genetically distinct subpopulation of cutaneous SCC.

Sample purity is of particular importance when performing SNP microarray analysis as this technique is reliant on high-level PCR amplification. Indeed, it has been shown that normal DNA contamination of only 10% may decrease the accuracy of LOH detection in tumor samples, and that accuracy drops markedly once contamination approaches 30% (Lindblad-Toh et al., 2000). Normal DNA contamination is a major issue for solid epithelial tumors such as SCC where cancer cells are generally intermixed with stroma and inflammatory cells. In this study we have examined the efficacy of using short-term cell culture or lesional tissue microdissection to increase the purity of the tumor DNA sample. For one pair of primary and lymph node metastatic SCC samples from patient 12, analysis was performed in triplicate using DNA extracted from whole lesional biopsy, microdissected lesional tissue and short-term (passage 3) cultured cells. A comparison of the tumor to blood SNP signal ratios revealed that, while the method of tumor DNA preparation did not affect the pattern of allelic copy number changes observed for either primary or metastatic lesion, the cultured cell DNA sample yielded the clearest results and the use of microdissection also reduced the level of "noise" in comparison with the whole biopsy sample. Hence, the use of short-term culture did not change the composition of the original tumor sample by selecting for a subpopulation of cells. Furthermore, we found no evidence for the acquisition of culture-specific genetic aberrations in our samples. This is in line with the results of an earlier study (Popp et al., 2000), where few cell-line specific aberrations were observed after much longer periods of culture. Overall, a higher call rate was obtained with the DNA sample extracted from cultured cells rather than microdissected lesional tissue, suggesting that, where possible, this may be the better method of obtaining a pure tumor sample.

In summary, we have used SNP microarray analysis to reveal UPD in SCC for the first time and to show frequent 3p and 9p loss, consistent with previous microsatellite and CGH studies. While 9p loss may involve the *CDKN2A* locus, epigenetic events seem more common than genetic mechanisms for *CDKN2A* inactivation (Brown et al., 2004). Consequently, the identification in this study of a microdeletion at 9p23, within the *PTPRD* locus, possibly associated with acquisition of metastatic potential may be relevant. Our findings highlight the power of SNP array genotyping for investigating the global genetic changes underlying tumorigenesis.

Acknowledgments

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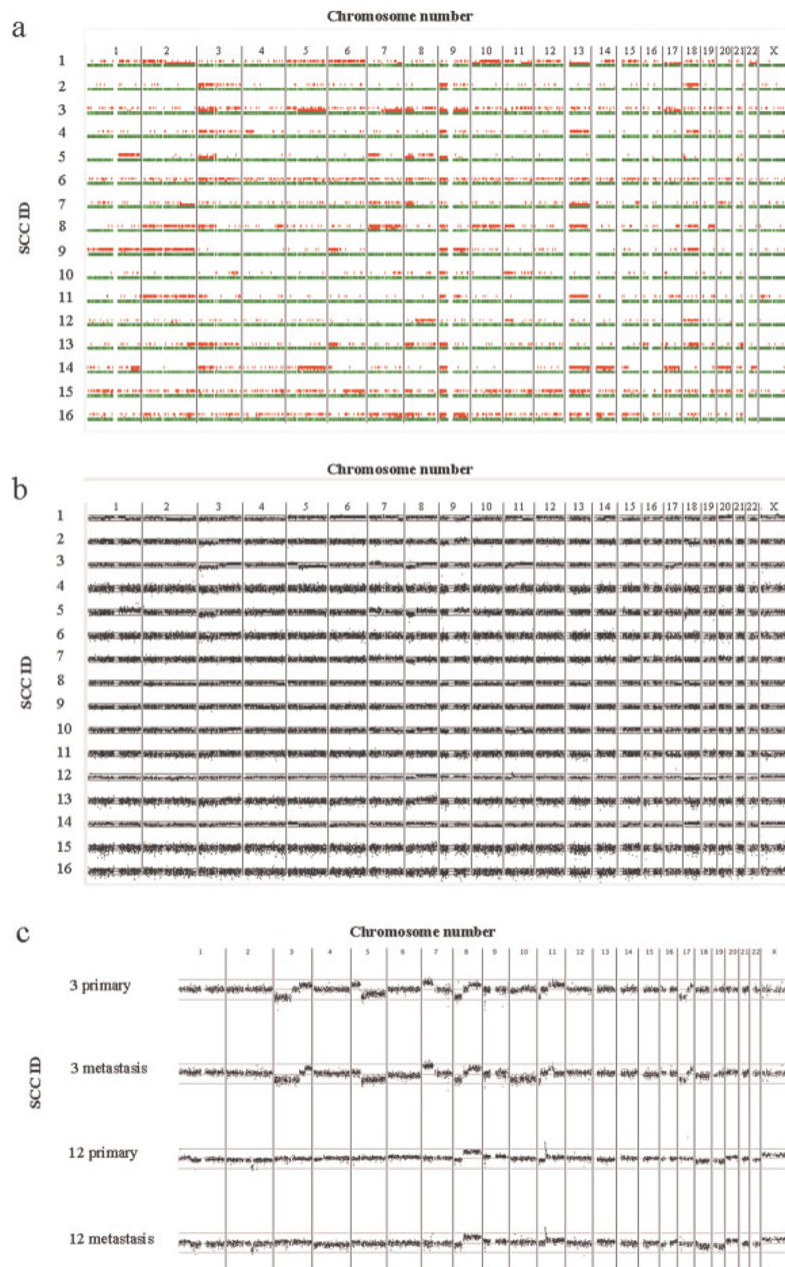
Figure 1.
Allelic imbalances demonstrated by 10K SNP microarray analysis. (a) Comparison of SNP genotypes in 16 primary SCC and paired non-tumour samples. SNP loci shown in green indicate calls unchanged between tumor and non-tumour samples, loci shown in red immediately above indicate LOH in the tumor sample and loci shown in red on the top row indicate SNPs not called in the tumor samples. Localized areas of low call rate in the tumor sample may imply LOH in a tumor subpopulation or allelic gain. (b,c) DNA copy number alterations in 16 primary SCC (b) and in 2 primary-metastatic SCC pairs (c). A running average of 2 consecutive ratios of signal values is plotted on a $\log_2$ scale according to chromosomal position. Upper line represents $\log_2(2)$ and lower line represents $\log_2(0.5)$.
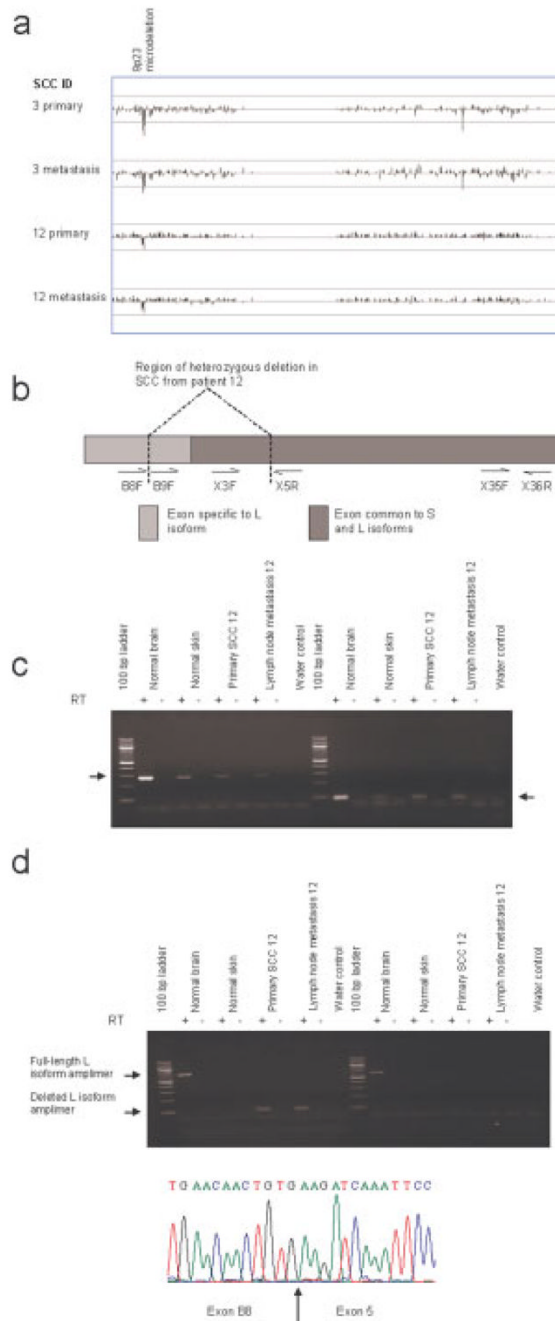
Figure 2.
Chromosome band 9p23 microdeletion and aberrant *PTPRD* expression observed in SCC
with metastatic potential. (a) Display of copy number values on chromosome 9 reveals 9p23
microdeletion within the *PTPRD* locus. A running average of 2 consecutive tumor:non-
tumour signal value ratios is plotted on a $\log_2$ scale according to chromosomal position.
Upper line represents $\log_2(2)$ and lower line represents $\log_2(0.5)$. For clarity each value is
represented by a vertical line from $\log_2(1)$. (b–d) Aberrant *PTPRD* expression in patient 12
SCC with heterozygous 9p23 microdeletion. One representative set of data summarizing the
pattern of *PTPRD* expression observed in 7 normal skin samples is shown. RT indicates
RNA was subjected to reverse transcription. Arrows indicate *PTPRD*-specific amplimers.

(b) Schematic representation of location of RT-PCR primers as described previously (Sato et al., 2005). (c) RT-PCR amplification of *PTPRD* using forward primer in exon 3 within the region of heterozygous deletion and reverse primer in exon 5 (left panel) or primers in exons 35 and 36 outside the deleted region (right panel). (d) L isoform-specific RT-PCR with forward primer located in exon B8 outside (left panel) or in exon B9 within (right panel) the region of heterozygous deletion. The reverse primer was located in exon 5. Sequence trace shows splicing within the deleted L isoform amplimer. Arrow marks junction between B8 and exon 5. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

TABLE 1

Clinicohistopathogical Data and Chromosomal Aberrations of the 18 Squamous Cell Carcinomas

ID	Immune status	Sex	Age	Location	Histological diagnosis	Aberrations		
						Loss	Uniparental disomy	Gain
1[a]	RT	M	67	ear	poorly differentiated[b]	1q25.1-qter	13	1p31.1-1q25.1
						2q11.2-2qter	17	6
						7q33-7qter		9q33.2-9qter
						9qcen-9q33.2		14q
						10pter-10pcen		20q
						11q14.1-11qter		Xpter-Xp21.1
						14p		Xq13.1-Xq21.31
						22		
2[a]	CT	M	66	hand	well differentiated[c]	3pter-3pcen	–	3qcen-3qter
						9pter-9p13.2		9qcen-3qter
						18qcen-18qter		
3[a]	RT	M	55	1. hand	moderately differentiated	3pter-3p12.1	7q11.21-7qter	3q22.1-3qter
						5q11.2-5qter	9	5pter-5p12
						8pter-8p21.3	17q21.31-17q24.1	7pter-7pcen
						8p21.2-8p11.21		8q21.11-8qter
						9p23		11p11.12-11qter
						11pter-11p15.3		17q24.1-17qter
						17pter-17q21.31		
					2. axillary lymph node · metastatic[d]	3pter-3q21.3	7q11.21-7qter	3q25.33-3qter
						5q11.2-5qter	9	7pter-7pcen
						8pter-8p21.3	17q21.31-17q24.1	8q21.11-8qter
						8p21.2-8p11.21		11p11.12-11q14.1
						9p23		17q24.1-17qter
						10		11pter-11p15.3
						17pter-17q21.31		

ID	Immune status	Sex	Age	Location	Histological diagnosis	Aberrations		
						Loss	Uniparental disomy	Gain
4	RT	M	66	ear	well differentiated	3pter-3pcen 9pter-9pcen 13	–	3qcen-3qter 9qcen-9qter
5[a]	RT	M	61	arm	well differentiated	3pter-3p12.1 8p23.1-8p11.21 18pter-18p11.21	8pter-8p23.1 9pter-9pcen	1qcen-1qter 3p12.1-3p11.1 7pter-7p14.3 7p14.1-7p11.1 8q11.21-8qter 9qcen-9qter
6	RT	F	71	scalp	well differentiated	3pter-3pcen 8pter-8p12 9pter-9pcen	–	3qcen-3qter
7	RT	M	42	scalp	well differentiated	8pter-8p11.21	2q31.1-2qter 13	7 20
8	RT	F	66	wrist	moderately differentiated	2 3pter-3p12.1 4q31.3-4qter 7q34-7qter 9pter-9p13.2 10 11pter-11pcen 13pter-13q33.1 17q24.1-17qter 18q12.1-18qter	7pter-7q34	–
9	IC	M	83	forehead	poorly differentiated	1 9p23	2 6pter-6p11.2 9	

ID	Immune status	Sex	Age	Location	Histological diagnosis	Aberrations		
						Loss	Uniparental disomy	Gain
10	IC	M	75	forehead	moderately differentiated	7q31.1-7qter	18	1qcen-1qter
						8pter-8p11.21	–	3q23-3qter
						9pter-9pcen		7pter-7p21.1
						11pter-11p11.12		7q21.11-7q31.1
						11q21-11qter		8q11.1-8qter
						19pter-19p12		11q12.1-11q14.2
								15
11	IC	F	79	forehead	well differentiated	2	3q28-3q29	19p13.3-19p12
						3pter-3p21.31		19q13.32-19qter
						9pter-9q22.33		
						9q33.2-9qter		
						13		
						17q11.1-17qter		
						Xpter-Xp11.22		
12[a]	IC	M	77	1. temple	moderately differentiated	2q14.3-2q22.1	–	8q11.1-8qter
						2q24.1		11p13-11p12
						9p24.1-9p23		X
						18		
						19		
				2. pre-auricular lymph node	metastatic	2q14.3-2q22.1	–	8q11.1-8qter
						2q24.1		11p13-11p12
						4		X
						9p24.1-9p23		
						18q11.2-18qter		
						19		
13	IC	M	64	scalp	moderately differentiated	2q33.3-2qter	17p13.1-17p11.2	3q12.1-3qter
						3pter-3p12.1		8q11.21-8qter

ID	Immune status	Sex	Age	Location	Histological diagnosis	Aberrations		
						Loss	Uniparental disomy	Gain
						6pter-6p21.1		12pter-12p11.1
						8pter-8p11.21		19pter-19p31.12
						9pter-9p13.2		19q13.41-19q13.43
						16pter-16p11.2		21q22.11-21qter
						18q12.1-18qter		
14	IC	M	92	ear	moderately differentiated	3pter-3p11.1	1q32.2-1q43	5pter-5pcen
						6pter-6p22.3	5qcen-5qter	18
						9pter-9p13.2	13	X
						10	14	
						15pter-15q12		
						16p		
						17		
						20		
						22q11.22-22qter		
15	IC	M	84	scalp	moderately differentiated	1p31.1-1p22.2	2q33.1-2qter	1p12-1q32.1
						4q31.3-4qter	10q21.1-10qter	2q24.1-2q33.1
						6q12-6qter	11q24.2-11qter	8q24.12-8qter
						8pter-8p21.1	19q13.2-19q13.32	15
						9pter-9p13.2		19p13.2-19q13.12
						11pter-11p13		20q11.21-20qter
						13		21q22.3
16	IC	M	70	scalp	moderately differentiated	2q33.2-2qter	7q11.22-7qter	1
						8pter-8q12.3	9q	2pter-2p21
						9pter-9p21.1		7pter-7q11.22
						13pter-9p21.1		12q21.32-12q23.3
						14q11.2-14q21.1		19p13.2-19p13.12
								X

RT, renal transplant recipient; CT, cardiac transplant recipient; IC immunocompetent individual.

[a] Indicates that short-term cultured cells were used for the tumour DNA sample.

[b] All primary tumours were invasive SCC.

[c] Well differentiated SCC did not include keratoacanthomas.

[d] Both metastatic SCC were lymph node metastases.

TABLE 2

Recurrent Aberrations in 16 Primary Squamous Cell Carcinomas

Chromosomal location	Frequency	Copy number
1q	5	gain
2q	8	deletion, UPD
3p	9	deletion
3q	6	gain
7p	5	gain
7q	5	deletion, UPD
8p	8	deletion, UPD
8q	6	gain
9p	13	deletion, UPD
9q	4	UPD, deletion
9q	4	gain
11p	4	deletion
13	8	deletion, UPD
17q	4	deletion, UPD
18q	5	deletion, UPD
X	4	gain