Single Nucleotide Polymorphism Array Analysis Defines a Specific Genetic Fingerprint for Well-Differentiated Cutaneous SCCs

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Cutaneous squamous cell carcinomas (cSCCs) are the second most frequent cancers in fair-skinned populations; yet, because of their genetic heterogeneity, the key molecular events in cSCC tumorigenesis remain poorly defined. We have used single nucleotide polymorphism microarray analysis to examine genome-wide allelic imbalance in 60 cSCCs using paired non-tumor samples. The most frequent recurrent aberrations were loss of heterozygosity at 3p and 9p, observed in 39 (65%) and 45 (75%) tumors, respectively. Microdeletions at 9p23 within the protein tyrosine phosphatase receptor type D (*PTPRD*) locus were identified in 9 (15%) samples, supporting a tumor suppressor role for *PTPRD* in cSCC. In addition, microdeletions at 3p14.2 were detected in 3 (5%) cSCCs, implicating the fragile histidine triad (*FHIT*) gene as a possible target for inactivation. Statistical analysis revealed that well-differentiated cSCCs demonstrated significantly fewer aberrations than moderately and poorly differentiated cSCCs; yet, despite a lower rate of allelic imbalance, some specific aberrations were observed equally frequently in both groups. No correlation was established between the frequency of chromosomal aberrations and immune or human papillomavirus status. Our data suggest that well-differentiated tumors are a genetically distinct subpopulation of cSCC.

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INTRODUCTION

Keratinocyte skin cancers, consisting basal cell carcinoma and cutaneous squamous cell carcinoma (cSCC), represent an important cause of morbidity in fair-skinned populations and their incidence is increasing worldwide (de Vries *et al.*, 2005). Ultraviolet radiation is the principal carcinogen implicated in keratinocyte skin cancer development (Armstrong and Kricker, 2001). Substantial data indicate that dysregulation of the Sonic Hedgehog pathway is a key event in basal cell carcinoma tumorigenesis (reviewed in Boukamp, 2005). In contrast, cSCC display much more genetic heterogeneity, with several studies reporting recurrent aberrations on many chromosomes (Quinn *et al.*, 1994; Ashton *et al.*, 2003; Clausen *et al.*, 2006; Purdie *et al.*, 2007).

An etiological cofactor role in keratinocyte skin cancer has also been suggested for beta genus human papillomaviruses (HPVs). Beyond a demonstrated role in cSCC tumorigenesis in patients with the rare genetic disease epidermodysplasia verruciformis (Majewski and Jablonska, 1995) their oncogenic potential remains less well defined although the presence of beta HPV in normal skin has been shown to be a risk factor for keratinocyte skin cancers (Harwood *et al.*, 2004). Organ transplant recipients (OTRs) receiving immunosuppression are at 100-fold increased risk of cSCC compared with immunocompetent (IC) individuals and their tumors may be more aggressive (Glover *et al.*, 1997). It has been suggested that OTR cSCCs display fewer chromosomal aberrations than IC tumors (Rehman *et al.*, 1997), possibly reflecting differing etiologies, but this observation remains to be confirmed.

SCC may be categorized as well, moderately and poorly differentiated depending on the degree of keratinization and cellular atypia. A greater percentage of poorly differentiated SCCs metastasize compared with well-differentiated counterparts (17 *vs* 0.6%, respectively) (Breuninger *et al.*, 1990).

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Abbreviations: CGH, comparative genomic hybridization; cSCC, cutaneous squamous cell carcinoma; FHIT, *fragile histidine triad; HPV, human papillomavirus; IC, immunocompetent; LOH, loss of heterozygosity; OTR, organ transplant recipient;* PTPRD, *protein tyrosine phosphatase receptor type D; SNP, single nucleotide polymorphism*

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Previously, in a smaller series of tumors, we reported that well-differentiated cSCC show a lower rate of allelic imbalance than moderately or poorly differentiated tumors although certain specific aberrations occur equally frequently, suggesting that these tumors may constitute a genetically distinct subpopulation of cSCC (Purdie *et al.*, 2007). However, this study was not sufficiently powered to enable stratification for two important potential confounding factors: HPV status and immune status. Here we have used microarray-based single nucleotide polymorphism (SNP) analysis to investigate whether a correlation exists between the genetic profile and immune status and/or HPV status of cSCC. In addition, we have used a larger series of samples to examine our previous hypothesis that well-differentiated tumors may be genetically distinct from other cSCCs.

RESULTS AND DISCUSSION

SNP microarray analysis was performed on a series of 60 primary cSCCs. Tumor DNA was analyzed concomitantly with paired non-tumor DNA to permit the accurate identification of tumor-specific genetic events. Data included those from 16 cSCCs previously analyzed using 10K SNP microarrays (Purdie *et al.*, 2007). Statistical analysis was restricted to gross chromosomal aberrations greater than approximately 600 kb as a comparison of data from two cSCCs analyzed on both 10 and 250K SNP microarrays revealed that aberrations of this size were reliably detected by both microarrays.

Histopathologic evaluation of the 60 cSCCs revealed that 29 were well differentiated, 21 moderately differentiated, and 10 poorly differentiated. Keratoacanthomas were specifically excluded from this study. Allelic imbalance was observed at 1–9 chromosomes (median of 5) in well-differentiated tumors compared with 5–21 chromosomes (median of 9) in moderately and poorly differentiated tumors (Table 1; complete data in Table S1). Well-differentiated cSCC had significantly fewer chromosomes displaying allelic imbalance than moderately or poorly differentiated cSCC ($P<0.001$ in both the cases) and a linear trend was shown to exist with respect to differentiation status ($P<0.001$). No significant difference existed between the numbers of chromosomes with aberrations in moderately and poorly differentiated cSCCs ($P=0.645$) nor was there any significant difference between these two groups when the comparison was restricted to the number of chromosomes showing loss of heterozygosity (LOH) or gain events alone ($P=1$ and 0.092, respectively). Similar results were obtained when the 16 previously reported cSCCs were excluded from the analysis (Table S2). Despite a lower rate of allelic imbalance, certain specific aberrations were as frequent in well-differentiated as in moderately or poorly differentiated tumors (Figure 1). For example, there was no significant difference between well-differentiated and other cSCCs in the frequency of LOH at 3p ($P=0.235$) or gain at 3q ($P=0.815$) or 9q ($P=0.839$). Hierarchical clustering was used to investigate the genetic similarity between individual tumors. A high proportion of well-differentiated cSCC clustered into a separate subset from the majority of moderately and poorly differentiated cSCCs (Figure 2). Taken together, these data support the hypothesis that well-differentiated tumors demonstrate a characteristic genotype and constitute a discrete subpopulation of cSCCs.

One early study using low-resolution microsatellite analysis reported that the rate of LOH in OTR cSCCs was less than half of that observed in IC cSCCs and proposed that this reflected the influence of immune status on the molecular pathogenesis of tumors (Rehman *et al.*, 1997). However, we observed no significant difference ($P=0.396$) between the number of chromosomes showing aberrations in OTR and IC tumors (Table 1; complete data provided in Table S1). As the earlier study focused on LOH alone, we also analyzed LOH and gain events separately: no significant difference existed between the number of chromosomes showing LOH or gain in OTR and IC cSCCs ($P=0.450$ and 0.639, respectively). Similar results were obtained when the 16 previously published cSCC were analyzed separately from the other tumors (Table S2). A probable explanation for the discrepancy between these results and the previous finding lies in the fact that 60% (9/15) of OTR cSCCs from the earlier study were well differentiated compared with only 35% (7/20) of IC cSCCs. Thus, we propose that the finding of an apparently lower frequency of allelic loss in the OTR tumors in this previous study was confounded by the genetic fingerprint associated with differentiation status rather than immune status *per se*.

The oncogenic potential of beta genus HPV, whose DNAs are prevalent in cSCC, remains unclear (reviewed in Nindl *et al.*, 2007). Differences in chromosomal aberrations between HPV-positive and -negative cSCCs could indicate diverse genetic mechanisms of tumor development, providing support for an etiological role for HPV. Statistical analysis established that no correlation existed between the number of chromosomes demonstrating aberrations and HPV status ($P=0.847$). Similar results were obtained when the 16 previously published cSCCs were analyzed separately from the other tumors (Table S2). Earlier data (Stockfleth *et al.*, 2004) suggest that HPV DNA prevalence is lower in IC than OTR cSCC. A previous study from this laboratory using a degenerate nested PCR method detected beta HPV in 80% OTR cSCC, compared with only 30% IC cSCC (Harwood *et al.*, 2000). Conversely, in the current study beta HPV DNAs were identified in similar proportions of IC (78%, 14/18) and OTR (74%, 29/39) tumors. One explanation for the discrepancy here is our use of a new and highly sensitive PCR-reverse hybridization assay capable of identifying HPV DNA at levels below the detection threshold of earlier methods (de Koning *et al.*, 2006), potentially resulting in a disproportionate increase in HPV detection among IC samples where viral copy number may be lower (Pfister, 2003; Stockfleth *et al.*, 2004).

The most frequent aberration detected in the cSCC series was LOH at 9p, observed in 45 of 60 (75%) samples (Figure 1). Analysis of SNP call signal intensity data revealed that 20% (9/45) of 9p LOH events were copy number neutral and hence indicative of acquired uniparental disomy, extending our previous finding that uniparental disomy is a key mechanism of LOH in cSCC (Purdie *et al.*, 2007). In the

Table 1. Allelic imbalance in 60 SCCs

ID[1]	Allelic imbalance[2]	Histological diagnosis	Immune status	HPV
1	15	Poorly differentiated[3]	RT	+
2	3	Well differentiated[4]	CT	–
3	7	Moderately differentiated	RT	+
4	3	Well differentiated	RT	+
5	6	Well differentiated	RT	–
6	3	Well differentiated	RT	+
7	5	Well differentiated	RT	+
8	10	Moderately differentiated	RT	–
9	5	Poorly differentiated	IC	+
10	8	Moderately differentiated	IC	+
11	7	Well differentiated	IC	+
12	6	Moderately differentiated	IC	+
13	9	Moderately differentiated	IC	+
14	11	Moderately differentiated	IC	+
15	12	Moderately differentiated	IC	–
16	10	Moderately differentiated	IC	+
17	9	Well differentiated	RT	+
18	6	Moderately differentiated	RT	+
19	6	Moderately differentiated	RT	+
20	11	Moderately differentiated	IC	–
21	9	Well differentiated	RT	+
22	9	Moderately differentiated	RT	+
23	6	Moderately differentiated	IC	+
24	9	Moderately differentiated	IC	–
25	8	Well differentiated	IC	+
26	9	Poorly differentiated	PUVA	–
27	2	Well differentiated	RT	+
28	3	Well differentiated	RT	–
29	8	Moderately differentiated	RT	–
30	3	Well differentiated	RT	+
31	9	Poorly differentiated	RT	+
32	11	Moderately differentiated	RT	–
33	6	Well differentiated	CT	+
34	7	Moderately differentiated	RT	–
35	11	Moderately differentiated	PUVA	–
36	5	Well differentiated	IC	+
37	16	Moderately differentiated	RT	+
38	3	Well differentiated	IC	–
39	8	Moderately differentiated	IC	+
40	13	Poorly differentiated	IC	+
41	6	Well differentiated	RT	–
42	8	Well differentiated	RT	+
43	11	Poorly differentiated	IC	+
44	6	Poorly differentiated	RT	+
45	7	Well differentiated	RT	+
46	6	Well differentiated	RT	–
47	2	Well differentiated	RT	+
48	14	Moderately differentiated	CLL	–
49	2	Well differentiated	RT	+
50	7	Well differentiated	CT	+
51	6	Well differentiated	RT	+
52	17	Poorly differentiated	RT	+
53	5	Well differentiated	RT	+
54	1	Well differentiated	RT	–
55	2	Well differentiated	RT	+
56	4	Well differentiated	RT	+
57	21	Poorly differentiated	RT	+
58	4	Well differentiated	RT	+
59	5	Poorly differentiated	IC	+
60	5	Well differentiated	RT	+

CLL, chronic lymphocytic leukemia; CT, cardiac transplant recipient; IC, immunocompetent individual; PUVA, patients receiving psoralen and UVA photochemotherapy; RT, renal transplant recipient.
[1]Sample numbers 1–16 were published previously (Purdie *et al.*, 2007).
[2]Number of chromosomes with allelic imbalance
[3]All tumors were invasive SCC.
[4]Well-differentiated SCC did not include keratoacanthomas.

initial series of 16 cSCCs, we identified a frequent homozygous microdeletion at 9p23 within the protein tyrosine phosphatase receptor type D (PTPRD) locus. These data implicated *PTPRD* as a candidate tumor suppressor gene in cSCC and raised the possibility that, in addition to the established *CDKN2A* tumor suppressor locus (Kubo *et al.*, 1997), 9p LOH may be important for the inactivation of *PTPRD*. We have extended this finding by identifying *PTPRD* microdeletions in 6 of the 44 additional cSCCs analyzed in the current study, making a total of 9 of 60 (15%) cSCCs (Table 2). Two of the nine cSCCs demonstrated metastatic potential and four of the remaining seven cSCCs with *PTPRD* microdeletions were diagnosed as poorly differentiated, although poorly differentiated tumors comprised a minority of our sample series (10/60 cSCCs); these data suggested that *PTPRD* inactivation may be associated with more aggressive tumors. We also observed homozygous microdeletions within the *CDKN2A* locus at 9p21.2 or 9p21.3 in three samples, confirming the previous finding that this tumor suppressor gene may be inactivated by both genetic and epigenetic mechanisms (Brown *et al.*, 2004).

LOH at 3p was another frequent aberration, observed in 39 (65%) tumors and involving the whole of 3p in 23 (59%) of these. Two cSCCs from patients 26 and 52 displayed a homozygous microdeletion at 3p14.2 on a background of LOH across 3p (Figure 3) whereas tumor T20 exhibited extensive LOH at the telomeric end of 3p (3pter-3p21.31) in

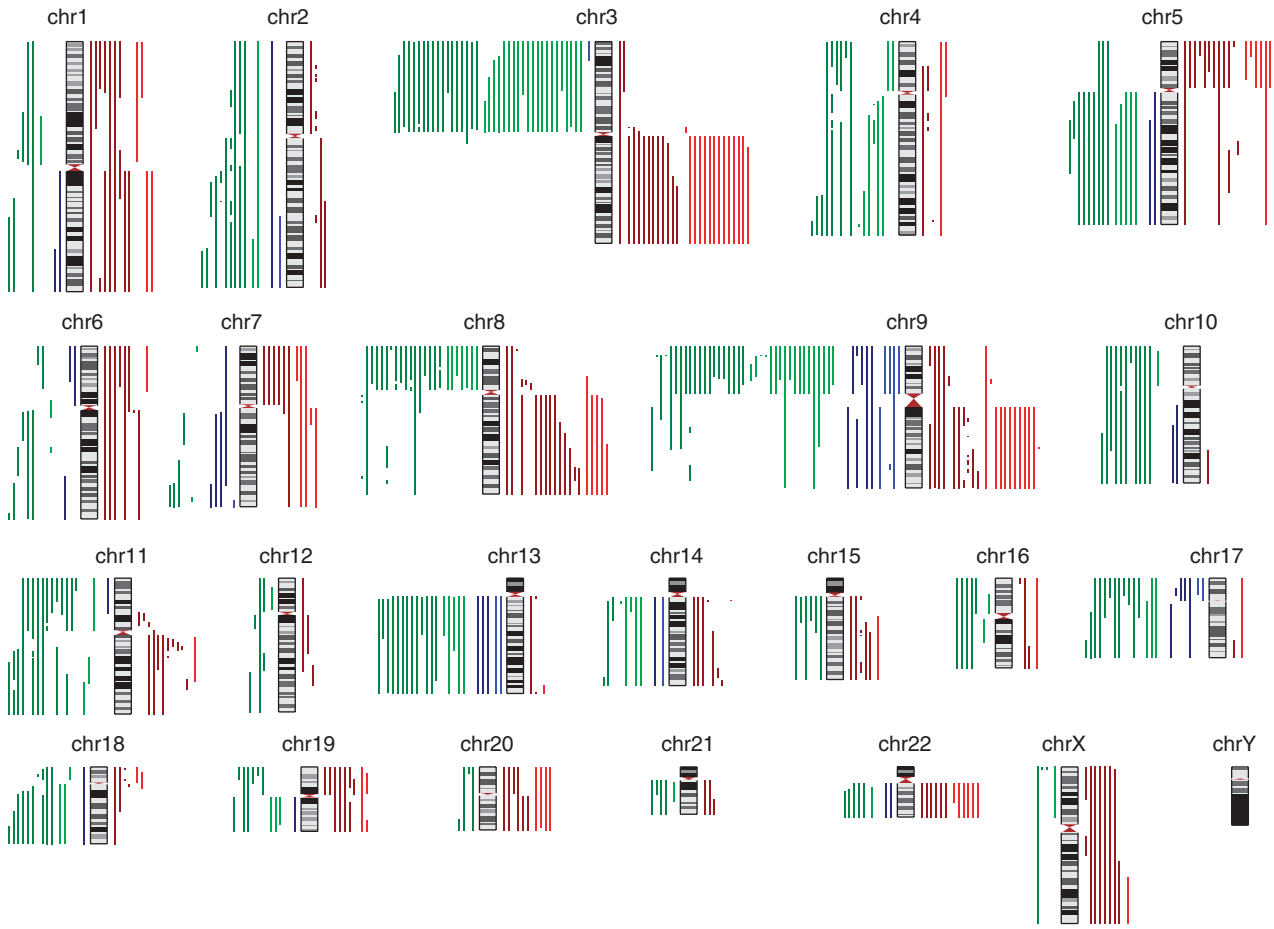

Figure 1. Ideogram summarizing allelic imbalance in well-differentiated cSCCs (light colored lines) and moderately and poorly differentiated cSCCs (dark colored lines). LOH events are indicated to the left of chromosomes with deletion shown in green and uniparental disomy in blue and gains are indicated to the right in red.

conjunction with a homozygous 3p14.2 microdeletion in a heterozygous region of 3p. All microdeletions mapped to a region at 60.41–61.16 Mb, within the locus of fragile histidine triad (*FHIT*) gene. *FHIT* is a recognized tumor suppressor gene that is inactivated in several epithelial cancers including lung cancer (Sozzi *et al.*, 1996) and breast cancer (Negrini *et al.*, 1996), although its role in cSCC remains unclear. One previous study (Sikkink *et al.*, 1997) reported that two of five cSCC tumors showed abnormal *FHIT* transcripts whereas another (Popp *et al.*, 2002) found that three cSCC lines with 3p loss expressed normal *FHIT* transcripts, albeit at much reduced levels in two cases. However, the microsatellite marker analysis and comparative genomic hybridization (CGH) used did not permit fine mapping of the genetic changes underlying these expression data. Our data from high-resolution SNP microarray analysis provide the first reported example of a homozygous microdeletion within this locus in cSCC. Although it is possible that expression analysis may reveal abnormal *FHIT* transcripts in a larger subset of tumors, the low frequency of the 3p14.2 microdeletion within our samples, together with the observation that T20 displayed extensive LOH on 3p separate from the 3p14.2 microdeletion, suggests that *FHIT* is unlikely to be the only target on 3p. Indeed, early 3p deletion mapping studies in other cancers (Hibi *et al.*, 1992; Maestro *et al.*, 1993) have demonstrated the presence of three distinct deleted regions in several tumors, 3pter-3p24, 3p21, and 3p 14.2–3p12, implying that 3p loss may inactivate multiple tumor suppressor genes.

Other recurrent events included LOH at 13q and 8p, each observed in 23 (38%) tumors and chromosomal gain on 3q (26 tumors; 43%), 8q (23 tumors; 38%), and 9q (21 tumors; 35%). Our data are consistent with the results of earlier research using polymorphic microsatellite markers or CGH to examine the genetics of cSCC (Quinn *et al.*, 1994; Ashton *et al.*, 2003), where allelic imbalance on chromosomes 3, 9, and 13 was among the predominant aberrations reported. In contrast, a recent study (Clausen *et al.*, 2006) using CGH to examine cSCC including 11 tumors from our laboratory found that 9p loss was a minor aberration, occurring in only 3.2% (1/31) SCCs. Loss of 3p and 13q was also observed less frequently, in 19.4% (6/31) and 12.9% (4/31) tumors, respectively. The most common chromosomal aberration was gain at 1p, identified in 41.9% (13/31) SCCs compared

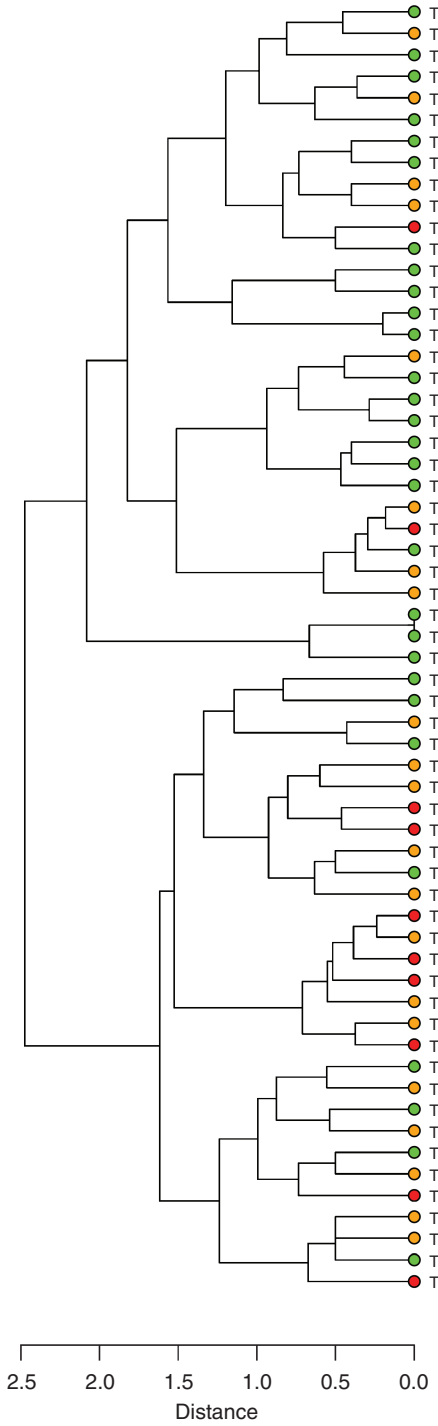

Figure 2. Cluster dendrogram of cSCCs. Distances between samples relate to agreement in aberration as a ratio of total number of aberrations. Well-differentiated cSCCs are indicated in green, moderately differentiated in orange, and poorly differentiated in red.

Table 2. *PTPRD* microdeletions in nine SCCs

SCC ID	Start position[1]	End position	Histological diagnosis
3	9.08[2]	9.22	Moderately differentiated[3]
	10.42	10.51	
9	9.16	9.76	Poorly differentiated
12[4]	8.55	9.38	Moderately differentiated
21	8.98	9.87	Well differentiated
25[4]	9.22	9.83	Well differentiated
26	9.13	9.99	Poorly differentiated
39	9.25	9.58	Moderately differentiated
40	9.47	9.86	Poorly differentiated
52[4]	9.37	9.48	Poorly differentiated

SCC, squamous cell carcinoma; PTPRD, protein tyrosine phosphatase receptor type D.
[1]Start and end positions of the microdeletion are indicated in Mb.
[2]Tumor demonstrated two regions of microdeletion.
[3]SCC T3 and T12 demonstrated metastatic potential as reported previously (Purdie *et al.*, 2007).
[4]Microdeletions in these tumors were hemizygous, all others were homozygous.

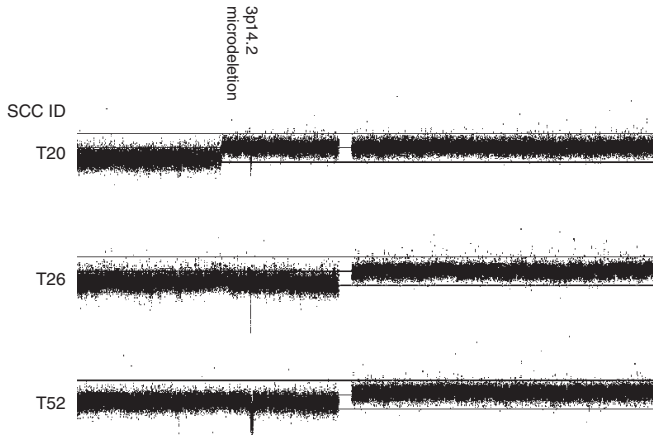

Figure 3. Display of copy number ratios on chromosome 3 reveals a 3p14.2 microdeletion within the *FHIT* locus. A running average of two consecutive tumor/non-tumor signal value ratios is plotted on a $\log_2$ scale according to chromosomal position. Upper line represents $\log_2(2)$ and lower line represents $\log_2(0.5)$. Microdeletions mapped to the following regions: SCC T20, 60.46–60.61 Mb; SCC T26, 60.41–60.58 Mb; SCC T52, 60.41–61.16 Mb.

with only 9/60 (15%) samples in the present study. Overall more gains than losses of chromosomal material (ratio of 1.65:1) were reported using CGH (Clausen *et al.*, 2006) whereas we observed that LOH (deletion and uniparental disomy) predominated over gain events by a ratio of 1.5:1 (Table S1). These discrepancies may in part reflect the limitations of CGH for identifying LOH and possible preferential detection of amplifications: deleted regions must be as large as 5–10 Mb to be detected whereas amplifications of 1 Mb can be identified (Forozan *et al.*, 1997). Furthermore, CGH is unable to detect copy number neutral LOH (uniparental disomy), which accounted for 20% of 9p LOH and 22% 13q LOH in the current study. Conversely, the lower frequency of 1p gain observed here combined with the overall predominance of LOH over amplifications detected implies that SNP microarray analysis may display greater sensitivity for identifying LOH than gain events. Methodological differences are likely to have been another contributory factor: in the two earlier investigations that reported a high

frequency of LOH at 9p and other chromosomes (Quinn *et al.*, 1994; Ashton *et al.*, 2003) tumors were microdissected before DNA extraction as in our protocol, whereas the more recent CGH study (Clausen *et al.*, 2006) performed analysis on a central section of the entire lesion. Depending on the proportion of stromal tissue and hence non-tumor DNA present within samples, it seems possible that some LOH events may have been masked, whereas gains of chromosomal material might still be detected if the degree of amplification were sufficiently large.

In summary, we have used genome-wide SNP microarray analysis to confirm our previous hypothesis that well-differentiated tumors are a genetically distinct subpopulation of cSCC. Contrary to earlier research (Rehman *et al.*, 1997), we found no significant difference in the frequency of allelic imbalance in OTR and IC cSCCs nor were we able to establish any correlation between the frequency of chromosomal aberrations and HPV status. Extensive LOH at 3p and 9p were observed in a majority of samples, consistent with previous data. Loss of 9p may entail the inactivation of *PTPRD*, recently implicated as a candidate tumor suppressor gene in cSCC (Purdie *et al.*, 2007). Here, we demonstrate *PTPRD* microdeletions in 9 of 60 (15%) cSCCs and identify *FHIT* on 3p14.2 as another possible target for inactivation. Our findings demonstrate the effectiveness of genome-wide SNP microarray analysis as a means of unraveling the genetic complexity of cSCC.

MATERIALS AND METHODS

A total of 60 primary cSCCs were analyzed, including 16 primary cSCCs described in an earlier study (Purdie *et al.*, 2007). Of these tumors, 42 were from immunocompromised individuals (39 were receiving immunosuppressive therapy (3 cardiac and 36 renal transplant recipients), 2 were receiving psoralen and UVA photochemotherapy for psoriasis, and 1 had chronic lymphocytic leukemia). Ethical approval for this investigation was obtained from the East London and City Health Authority local ethics committee and the study was conducted according to the Declaration of Helsinki Principles. All patients participating in the study provided written, informed consent.

All histopathologic diagnoses were confirmed by a single experienced dermatopathologist (RC). Samples were enriched for cSCC keratinocytes, DNA was extracted from cSCC and non-tumor control blood samples and subjected to 250K Nsp SNP array analysis as previously described (Purdie *et al.*, 2007). The presence of beta HPV was investigated in cSCC DNA samples using the RHA kit skin (beta) HPV (Diassay BV, Rijswijk, the Netherlands) according to the manufacturer's instructions.

Statistical analyses were carried out using Poisson regression. We used a generalized linear model with a Poisson distribution imposed on the number of chromosomes per patient with at least one event, and a log link function. Sequential analysis of deviance was carried out on the model that was fitted with terms representing differentiation effect, immune effect, and HPV effect, respectively, using a χ^2-test on the deviances. To test for a linear trend with respect to differentiation status, *P*-values were calculated against the null hypothesis that the linear trend of count against differentiation status had zero slope. This analysis was all carried out using the ''glm'' function within the statistical environment R (R Development Core Team, 2007). For individual comparisons among the differentiation subgroups, we used Tukey's contrasts between the pairwise comparisons, using the multcomp (Hothorn *et al.*, 2008) package within R to adjust for the multiple comparison situation. Hierarchical clustering was carried out in R using hclust with an asymmetrical binary metric between samples, where the asymmetric binary distance between pairs of samples was calculated as the number of aberrations observed in just one sample expressed as a proportion of the number of aberrations in one or both of the samples. Intercluster similarities were calculated using Ward's agglomeration method (Ward, 1963).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Chromosomal aberrations in 60 cSCCs.

Table S2. Statistical analysis of cSCC sample series.

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