Metastatic cutaneous squamous cell carcinoma shows frequent deletion in the protein tyrosine phosphatase receptor Type D gene

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Abbreviations: BAF: B allele frequency; cSCC: cutaneous squamous cell carcinoma; FFPE: formalin-fixed paraffin-embedded; HPRT: hypoxanthine phosphoribosyltransferase; LOH: loss of heterozygosity; MD: moderately differentiated; NMSC: nonmelanoma skin cancer; OPA: Oligo Pool All; PD: poorly differentiated; PTPRD: protein tyrosine phosphatase receptor Type D; UTR: untranslated region; UVR: ultraviolet radiation; WD: well differentiated

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Nonmelanoma skin cancer (NMSC) has a predicted prevalence equal to that of all other cancers combined and its incidence is increasing. There are more than 81,500 new cases of NMSC diagnosed annually in the United Kingdom alone, placing a heavy burden on both patients and healthcare resources. Despite accounting for only 20% of total NMSC cases, cutaneous squamous cell carcinoma (cSCC) is responsible for the majority of NMSC deaths, largely as a result of metastatic disease. A subset of immunosuppressed individuals, such as organ transplant recipients on long-term immunosuppressive drugs, develop particularly aggressive tumors with an increased risk of metastasis. Risk factors for metastasis include poor differentiation of the tumor cells, large tumor size, tumor depth > 5 mm, immunosuppression and localization on the ear and lip. Metastatic cSCC is currently treated by surgical intervention and/or chemotherapy or radiotherapy and is associated with a poor outcome.

Despite the well-established role of ultraviolet radiation (UVR) in the etiology of cSCC, the molecular events underlying its development remain largely undefined. Inactivation of the tumor suppressor genes TP53 and p16INK4A is a common and early event in cSCC pathogenesis and is characteristic of all histological grades of tumor. Recently, genotyping of 60 cSCC identified high rates of loss of heterozygosity (LOH)
of 3p and 9p in 65% and 75% of cases, respectively. Furthermore, reduced karyotypic complexity was observed in well-differentiated (WD) cSCC, suggesting that low-grade tumors are a distinct subset of cSCC.\(^1\) Histologically, WD tumors show less nuclear atypia and a higher degree of keratinization than moderately (MD) and poorly differentiated (PD) tumors. However, there are no established molecular pathways that separate these subtypes of cSCC.

The genotyping analysis cited above also identified small deletions (typically <1 Mb) in the protein tyrosine phosphatase receptor Type D (PTPRD) gene in 40% (4/10) of PD tumors, 16% (3/19) of MD tumors and only 6.5% (2/31) of WD tumors, suggesting an association with the more aggressive tumors. Furthermore, all three primary cSCCs that had metastasized had deletions within this locus.\(^1\) PTPRD is one of 21 tyrosine phosphatase receptors and has emerged as a putative tumor suppressor in a number of different cancers. Homozygous deletion of PTPRD has been reported at a frequency of 8–20% in lung cancer, melanoma, neuroblastoma, glioblastoma multiforme, laryngeal SCC and lymphoma.\(^1\)–\(^9\)

Mutations of the coding exons of PTPRD and methylation at the promoter region have also been reported in lung cancer, melanoma, glioblastoma, breast and colorectal cancers, indicating that multiple mechanisms of disruption affect this gene in cancer.\(^1\)–\(^2\) The overexpression of PTPRD in vitro causes transient growth arrest and an increase in apoptotic cells, with the converse demonstrated for the knockdown of PTPRD.\(^1\)\(^8\)\(^1\)\(^9\) Furthermore, PTPRD has been shown to de-phosphorylate the tyrosine 705 residue of pStat3 in vitro, which is the first putative target of its catalytic phosphatase activity.\(^1\) Collectively, these studies suggest that PTPRD has growth-suppressive activities in human cancer cells.

On the basis of the previous findings, we hypothesized that deletions within PTPRD may segregate with metastatic cSCC and that alternative mechanisms of disruption may also affect the gene, as demonstrated in other cancer types. To investigate this, we have extended the analysis of PTPRD in primary and metastatic cSCCs and found a positive association between the frequency of deletion and metastasis. We have also identified novel mutations in the coding exons of PTPRD and confirmed by qRT-PCR that PTPRD mRNA levels are lowest in high-grade cSCC. The association of this gene with metastasis may provide a potential prognostic biomarker, particularly for high-risk patients who present with multiple and/or large tumors.

Material and Methods
Sample collection
For the Illumina SNP microarray, 54 formalin-fixed paraffin-embedded (FFPE) primary, recurrent and metastatic cSCC from 19 patients were obtained from the Department of Pathology, Barts and The Royal London NHS Trust. These included all cases of metastatic cSCC from the last 15 years for which the blocks were available for analysis. Five of these patients had an additional PD primary cSCC that was not associated with metastatic disease and was included for analysis. Fifteen recurrent or PD primary cSCC were obtained from a further 12 patients from the same time period. All had at least 3 years follow-up and no presentation of metastatic disease (see Supporting Information Table 1 for clinicopathological details). For the melanoma microarray, 36 primary, recurrent and metastatic FFPE tumors were obtained from 18 patients with metastatic disease and 40 primary and recurrent melanoma from 38 patients without metastatic disease. Melanoma FFPE samples were chosen retrospectively from the last 15 years. A range of Breslow thickness from <1 mm (good prognosis) to >4 mm (poor prognosis) were included. Venous blood samples were obtained from 22 of the patients with corresponding cSCC and 20 of the patients with melanoma.

Fresh-frozen tumor biopsies and short-term cultures for mutation and methylation analyses \((n = 41)\) are detailed in Supporting Information Table 2. All tumors had been previously analyzed for PTPRD genotype status.\(^1\) Punch biopsies were obtained at the time of surgical resection and immediately snap frozen. Biopsies obtained for short-term culture were prepared directly from patient material as previously described.\(^2\) The study was undertaken in accordance with ethical approval from the East London and City Health Authority Local Research Ethics Committee.

**DNA extraction**
To enrich for \(\geq 70\%\) tumor cell populations, DNA was microdissected from 10 \(\mu\)m FFPE sections using a reference H&E slide. DNA was extracted using the Qiagen DNA Mini Kit (Qiagen, UK) with an extended proteinase K digestion between 48 and 72 hr. After extraction, DNA was concentrated using YM-30 Microcon columns according to the manufacturer’s instructions (Millipore, UK). DNA was extracted from 1 ml of venous blood using the Nucleon BACC kit (Amersham Biosciences, UK).

**Custom Illumina array**
A 384 SNP Oligo Pool All (OPA) was designed using the Illumina Assay Design Tool, with \(\sim 6\) Kb spacing across the PTPRD locus. Each SNP was chosen according to the manufacturer’s recommendations (see Supporting Information). DNA (250 ng) was prepared with the OPA according to the GoldenGate Assay and hybridized to the 96-sample Universal Array Matrix.\(^2\) Gene Calls were extracted using the Bead Scanner, and data were analyzed using BeadStudio Genotyping Module.\(^2\) Five SNPs failed to meet the standard GenCall cutoff score of 0.4 and were excluded from further analysis, along with one metastatic cSCC sample and four melanoma samples that had call rates lower than 50%. Call rates of \(\geq 70\%\) were obtained for the remaining samples.

Eleven cSCC (from a total of eight patients) and 17 melanoma (from 15 patients) were excluded from LOH analysis because of scattered B allele frequency (BAF) distribution.
Deletions were only considered genuine events if there was a reduction in the log $R$ ratio that was concomitant with an altered pattern of the BAF, as described by Assie et al. The coordinates for deletion were taken as the first and last SNP in a run of at least five consecutive SNPs that met the above criteria. Statistical significance for the comparison of $PTPRD$ deletion between groups was determined using the $2 \times 2$ Fish-er’s test function in the “R” programming environment.

### Table 1. Deletion events detected in cSCC samples by the Illumina SNP microarray

| Patient no. | Primary/Metastatic/PD cSCC | Differentiation status | Site                | Deletion at $PTPRD$ locus | LOH |
|-------------|-----------------------------|------------------------|----------------------|---------------------------|-----|
| PM1         | Primary (SCC12)             | MD                     | Right temple         | Het: 8528919-9380450      | N   |
|             | Metastasis                  | Not reported           | Parotid              | Het: 8528919-9380450      | N   |
|             | Metastasis                  | MD                     | Lymph node           | Het: 8714264-9338102      | N   |
| P2          | Primary                     | PD                     | Left ear             | Homo: 9092409-9884322     | N   |
| P3          | Primary                     | PD                     | Right cheek          | Het: 9044621-9206908      | N   |
| PM8         | Primary                     | PD                     | Right wrist          | Het: 9063311-9807514      | N   |
|             | Skin metastasis 1           | PD                     | Right forearm        | Het: 9109229-9807514      | N   |
|             | Skin metastasis 2           | PD                     | Right forearm        | Het: 9199719-9807514      | N   |
| P9          | Primary                     | PD                     | Right thumb          | Het: 9589450-9771039      | N   |
| PM10        | Primary 1 (SCC23)           | MD                     | Left cheek           | N                         | Y   |
|             | Primary 2 or recurrence     | MD                     | Left temple/cheek    | N                         | N   |
|             | Recurrence                  | MD                     | Left cheek           | Homo: 9435004-9559081     | Y   |
|             | Recurrence                  | Left mandible          | N                     | N                         | N   |
|             | Recurrence                  | WD                     | Left cheek           | N                         | N   |
|             | Metastasis                  | Parotid                |                       |                           |     |
| PM11        | Primary (SCC9)              | PD                     | Scalp                | Homo: 8927116-10035049    | Y   |
|             | Skin metastasis             | MD                     | Right preauricular   | Homo: 8927116-10021987    | Y   |
| P12 (PUVA)  | Primary (SCC26)             | PD                     | Buttock              | Homo: 9135908-1009302     | Y   |
| PM13        | Primary                     | M-PD                   | Right knee           | Homo: 8899574-10057853    | Y   |
|             | Metastasis                  | Lymph node (groin)     | Homo: 8853644-10013288| Y   |
| PM14        | Primary                     | PD                     | Pinna right ear      | Homo: 10160958-10381988   | Y   |
|             | Recurrence                  | MD                     | Right ear            | Homo: 10151681-10389727   | Y   |
|             | Metastasis                  | MD                     | Parotid              | Undetermined              | Y   |
|             | Recurrence                  | MD                     | Right ear            | Homo: 10147409-10351438   | Y   |
| PM17        | Primary                     | WD                     | Left outer canthus eye| N                         | N   |
|             | Metastasis                  | PD                     | Parotid              | Homo: 9290973-9735530     | Y   |

*Patient codes are “PM” for primary–metastatic and “P” for poorly differentiated tumors that did not metastasize. Dark gray shading indicates tumors in primary–metastatic series that were not available for analysis. Sample IDs in brackets are those assigned in Ref. 11. PUVA is psoralen ultraviolet A treatment and indicates a cSCC arising in a patient who had been treated with PUVA for a pre-existing skin condition. LOH indicates loss of heterozygosity across the entire locus.

1Both these tumors occurred in close proximity to each other, and it was not possible to distinguish clinically between two separate primary tumors or a primary and recurrence.*

### Table 2. Frequency of $PTPRD$ deletion by differentiation status and metastatic potential, as used for statistical analysis

|                 | WD (n = 33) | MD (n = 28) | PD (n = 35) |
|-----------------|-------------|-------------|-------------|
| Deletion within $PTPRD$ | Nonmetastatic | Metastatic | Nonmetastatic | Metastatic | Nonmetastatic | Metastatic |
| PM1             | 2           | 0           | 1           | 3           | 6            | 5           |
| No deletion within $PTPRD$ | 30          | 1           | 18          | 6           | 19           | 5           |

*Abbreviations: WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated.*
**Sequencing**

DNA extracted from 12 microdissected fresh-frozen cSCC was subjected to whole genome amplification using Repli G (Qiagen) and used in PCR as a template for 35 primer sets designed to amplify all coding exons of PTPRD (for primer sets, see Ref. 12). DNA extracted from seven short-passage cultured cSCC was also amplified in PCR without undergoing prior whole genome amplification. PCR products were purified using ExoSap and used directly in sequencing with BigDye v3.1 and the Applied Biosystems 96 capillary Prism 3700 sequencer. Mutations were confirmed by sequencing a second PCR product from the original nonamplified DNA sample and determined to be somatic by sequencing matched blood DNA.

**Pyrosequencing**

Genomic DNA (200 ng) from 25 fresh-frozen cSCC and 15 cell lines was sodium bisulfite modified using the Zymo EZ DNA methylation kit (Zymo, USA) according to the manufacturer’s instructions. Unmethylated human DNA extracted from normal peripheral blood and CpGenome Universal Methylated DNA (Chemicon International, USA) were included as negative and positive controls, respectively. Approximately 10 ng of modified DNA was amplified in a 20 μl PCR reaction as previously described.28 Primer pairs were designed using the Pyrosequencing Assay Design Software (Biotage, Sweden), see Supporting Information for primer sequences. Primer sets were demonstrated to amplify only bisulfite-modified DNA using nonmodified genomic DNA as a template. Amplified products were analyzed for methylation using the PyroMark ID pyrosequencer with PyroGold Q96 reagents according to the manufacturer’s instructions (Qiagen, UK). Data were analyzed using PyroMarkID software (Qiagen, UK) and assessed using the percentage of T and C incorporation at each CpG dinucleotide.

**RNA extraction and qRT-PCR**

Fifteen 8 μm sections were cut onto 1.0 mm PEN membrane slides (Zeiss, UK), stained using cresyl violet and dehydrated in an increasing alcohol series. Tumor cells were identified from a reference H&E slide and laser capture microdissected using the Zeiss Palm Microbeam Microscope (Zeiss, UK). Cells were collected into 350 μl RLT buffer (Qiagen, UK) and extracted using the RNeasy Mini Kit (Qiagen, UK). RNA was extracted from 4 mm punch biopsies of normal skin that were ground under liquid nitrogen and processed using the RNeasy Mini kit (Qiagen). All RNA extractions were performed in triplicate for each PCR master mix as well as RT-negative controls in duplicate for each cDNA. PCR reactions were performed on the Applied Biosystems 7500. Each sample was measured in triplicate, and PTPRD levels were normalized to the housekeeping gene HPRT. The relative expression level of PTPRD was determined using a baseline panel of six normal skin samples according to the ΔΔCt method.29

**Results**

To investigate the proposed association between deletions within PTPRD and metastatic cSCC, we developed a custom Illumina SNP microarray designed to cover the locus at high resolution. The mean age of patients with metastatic disease upon presentation of the primary tumor was 68.4 years (range: 54–91), and an approximately equal number of patients were immunosuppressed (n = 10) and immunocompetent (n = 9). By using this array, we were able to successfully analyze 73 of 74 FFPE cSCC tumors for deletion within PTPRD, indicating high compatibility for this assay with FFPE material. Four control FFPE tumors, for which matched fresh-frozen or cultured cell equivalents had been previously genotyped using the Affymetrix 250K SNP microarrays,11 were included as controls for the detection of copy number change. One control sample displayed LOH across the entire locus, one had a heterozygous deletion and two had homozygous deletions. In all four cases, an identical pattern and location of copy number/deletion was detected in the FFPE samples, suggesting good reliability (Figs. 1a and 1b).

**PTPRD is frequently deleted in primary-metastatic and aggressive cSCC**

In total, deletions within PTPRD were found in primary and/or metastatic tumors from 37% (7/19) of patients with metastatic disease (Table 1). Five of the deletions were homozygous, occurring on a background of LOH and two were heterozygous deletions with a normal copy number across the rest of the locus. The primary–metastatic series from a further seven patients showed LOH at the locus, with an overall LOH rate for this sample subset at 63% (12/19; Supporting Information Table 1). Of the PD cSCC included on the array, 22% (4/18) showed a deletion within PTPRD, of which two were homozygous on a background of LOH and two were heterozygous deletions with no LOH (Table 1). Three PD cSCC also showed LOH in the absence of deletion (Supporting Information Table 1). For statistical analysis, SNP microarray data of the PTPRD locus from the 60 cSCC analyzed by Purdie et al.11 were combined with the series of tumors analyzed in our study to give a total of 96 tumors from 80 patients (Table 2). This included one additional patient with metastatic disease. This analysis revealed a significant association between primary tumors that metastasized and deletion at the PTPRD locus (p = 0.007). To exclude the possibility that patient effect may be skewing the data for the 13
patients from whom multiple primary tumors were analyzed, the test was repeated with these patients excluded. This still showed a significant association between deletion within \textit{PTPRD} and metastasis ($p = 0.002$). There was also a significant association between PD tumors and \textit{PTPRD} deletion when compared to WD and MD tumors ($p = 0.01$), which is consistent with the more aggressive nature of PD tumors.

**Deletions of \textit{PTPRD} in metastatic cSCC are clonal**

Including the additional metastatic tumor from the series analyzed by Purdie et al.,\textsuperscript{11} six of eight (75\%) patients with metastatic disease and deletion of \textit{PTPRD} had an identical pattern of deletion throughout the individual tumor series. This included the primary tumor and subsequent recurrent and metastatic tumors (Fig. 1c). In the remaining two primary–metastatic series (PM10 and PM17), deletion of \textit{PTPRD} was detected in the metastatic tumors only and not in the corresponding primary tumor that was analyzed.

**\textit{PTPRD} deletions were not detected in melanoma tissue samples**

To investigate if deletions within \textit{PTPRD} may also be associated with metastasis in other skin tumors, DNA was extracted from a series of 76 FFPE primary and metastatic melanoma samples and analyzed by the custom SNP microarray. This revealed no deletions within \textit{PTPRD} in any of the melanoma samples, regardless of metastatic potential or Breslow thickness. There was also a lower rate of LOH than...
seen in cSCC, with tumors from 39% (16/41) of melanoma patients displaying loss of copy number.

**PTPRD deletions frequently target the 5’ untranslated region**

PTPRD deletions have been reported in a wide range of different cancers. Figure 2 shows the location of deletions from the primary–metastatic cSCC series reported here as well as those cited in the literature from seven studies on lung cancer, neuroblastoma, glioblastoma multiforme, melanoma, cSCC and follicular lymphoma. In total, 15% (7/46) of deletions extend into the protein-coding region, with the remainder located exclusively in the 5’ untranslated region (UTR). Furthermore, 60% (28/47) of the deletions include exon B7 of the 5’ UTR, suggesting that this exon is a common target for deletion.

**PTPRD mutations are common in cSCC**

There was insufficient material from the FFPE cSCC tumor samples to sequence the entire 35 coding exons of the PTPRD gene. Therefore, sequencing was performed in 12 fresh-frozen microdissected cSCC tissue samples and six short-term passage cSCC cultures that we had previously analyzed for PTPRD genotype status. Samples with a homozygous deletion of PTPRD were not included. The samples included three primary tumors from the metastatic series analyzed by the Illumina microarray, four PD, six MD and five WD tumors that had not metastasized. In addition, sequencing data from PM1 that we had previously analyzed were included. In total, 37% (7/19) of tumors had a novel coding region mutation and all were found to be somatic upon sequencing matched blood DNA (Fig. 3, Supporting Information Fig. 1). This is the highest frequency of PTPRD mutation reported to date in any cancer type. The majority of mutations (7/10) were missense changes spread throughout the PTPRD protein, including one in the active phosphatase subunit of the catalytic domain (Table 3). One further change was a stop mutation—W775X—predicted to truncate the protein in a fibronectin subunit upstream of the catalytic domain. The remaining two changes were either synonymous or in the 3’ UTR.

Of the seven tumors that showed a PTPRD mutation, four were from PD cSCC or primary tumors that had metastasized. The remainder were WD (2/7) or MD (1/7) cSCC that had not metastasized. Furthermore, the mutations in two of the seven tumors (SCC10 and SCC33) revealed biallelic disruption of PTPRD, combining LOH with mutation of the remaining allele. Two additional primary tumors that had metastasized also showed potential biallelic disruption with “two-hits” within PTPRD; however, it was not determined if
the two events were on separate alleles. PM1 had a coding sequence mutation combined with a heterozygous deletion in the 5' UTR that extended into the coding sequence and PM6 had two mutations.

Promoter methylation is not a common mechanism of PTPRD inactivation in cSCC

PTPRD has two distinct 5' UTR isoforms that arise from different promoters. Pyrosequencing was used to interrogate part of each promoter region for methylation changes relative to normal skin. The longer (L) isoform has a canonical 698-bp CpG island located at the transcription start site and has been previously shown to be methylated in breast, colorectal cancer and glioblastoma, whereas the shorter (S) isoform does not have a defined promoter region and has not been previously investigated. In the absence of a CpG island at the designated start site of the S isoform, a 1,223-bp CpG island for which EST evidence supports the generation of PTPRD transcripts was analyzed. Primers were designed for the L isoform promoter region to overlap with the region previously reported as methylated in other cancer types. A panel of 25 microdissected fresh-frozen tumors, eight normal skin samples and 15 cell lines were tested for methylation at both promoter regions. This included 8 of 12 fresh-frozen tumors and all cell lines that had been used for sequence analysis of PTPRD (Supporting Information Table 2). No methylation was identified in the normal skin samples or tumor tissue, indicating that this is not an important mechanism of disruption in this tumor type. Similarly, the cSCC cell lines did not show methylation. The only cell line to show positive methylation at both CpG islands was the HeLa cell line.

PTPRD is expressed at lower levels in high-grade tumors

The expression level of PTPRD was measured in 16 laser capture microdissected cSCC, which included seven WD tumors, seven MD or PD tumors and two lymph node metastases. Fold change differences were determined relative to the average level of PTPRD expression across six normal skin samples. Expression levels were twofold increased in WD cSCC when compared to normal skin, whereas MD and PD showed a decrease in the levels of mRNA (Fig. 4). Intriguingly, tumors with a deletion showed an increase in expression of PTPRD when compared to tumors of the same histological grade without a deletion.

Discussion

Taken together, these data show that PTPRD is disrupted in cutaneous SCC by multiple mechanisms and that deletions within PTPRD are associated with the clinically more aggressive tumors. This is the first study to specifically associate deletions of PTPRD with metastatic tumors and is consistent with previous reports showing reduced mRNA expression of PTPRD in higher grade neuroblastoma, breast and colorectal carcinomas.

Figure 3. Map of PTPRD mutations identified by our study and previous publications. (a) Schematic representation of the location of mutations in the PTPRD protein; (b) sequencing chromatograph of the nonsense mutation (W775*) in SCC10 tumor (top panel) and corresponding blood DNA (lower panel); (c) T931I mutation in PM 6 tumor (top panel) and corresponding blood DNA (lower panel).
Table 3. Summary of samples used for mutation analysis of PTPRD

| Sample ID | Differentiation status | PTPRD deletion | LOH | Mutation | Mutation effect | Heterozygous or homozygous | Domain | Methylation |
|-----------|------------------------|----------------|-----|----------|-----------------|-----------------------------|--------|-------------|
| SCC1²     | PD                     | N              | N   | N        | –               | –                           | –      | N           |
| SCC2²     | WD                     | N              | Y   | N        | –               | –                           | –      | N           |
| SCC5²     | WD                     | N              | Y   | N        | –               | –                           | –      | N           |
| SCC10     | MD                     | N              | Y   | G/A      | G450E           | Heterozygous                | Fibronectin, Type III | N           |
|           |                        |                |     | G/A      | W775Stop        | Heterozygous                | Fibronectin, Type III | N           |
| PM 1 (SCC12) | MD             | Y (heterozygous)| N   | G/A      | G554A           | Heterozygous                | Fibronectin, Type III | N           |
| SCC16     | MD                     | N              | Y   | N        | –               | –                           | –      | N           |
| SCC20²    | MD                     | N              | N   | N        | –               | –                           | –      | N           |
| SCC24²    | MD                     | N              | Y   | N        | –               | –                           | –      | N           |
| SCC28     | WD                     | N              | N   | G/A      | E1078K          | Heterozygous                | Fibronectin, Type III | N           |
| SCC29     | MD                     | N              | Y   | N        | –               | –                           | –      | N           |
| SCC31     | PD                     | N              | N   | G/A      | S1471N          | Heterozygous                | Protein tyrosine phosphatase C1 | N           |
| SCC33     | WD                     | N              | Y   | C/T      | T13I            | Heterozygous                | Signal peptide             | N           |
| SCC34     | MD                     | N              | N   | N        | –               | –                           | –      | N           |
| SCC54     | WD                     | N              | Y   | N        | –               | –                           | –      | N           |
| SCC57     | M-PD                   | N              | N   | N        | –               | –                           | –      | N           |
| SCC59²    | PD                     | N              | T/C | V340 (Synon) | Homozygous      | Fibronectin, Type III      | N      |             |
| PM3       | MD                     | N              | Y   | N        | –               | –                           | –      | N/A         |
| PM5       | MD                     | N              | Y   | N        | –               | –                           | –      | N/A         |
| PM6       | PD                     | N              | C/T | T849I    | Heterozygous    | Fibronectin, Type III      | N/A    |             |
|           |                        |                |     | T931I    | Heterozygous    | Fibronectin, Type III      | N/A    |             |

Sample IDs are those assigned in Ref. 11, and PM indicates tumors from primary–metastatic patients.
²Indicates samples where cultured cells were used for the analysis.
Abbreviations: WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated; N/A: not analyzed; Y: yes; N: no.
demonstrated a PTPRD deletion in a metastatic tumor of the bone marrow that was not present in the primary tumor from which it was derived, suggesting that the link with metastasis may also extend to additional cancer types. In summary, deletions within PTPRD were identified in 37% (7/19) of patients with metastatic disease, and mutations were present in 37% (7/19) of tumors analyzed. Methylation of the promoter regions was not observed in cSCC tissue samples or cell lines. No deletions were identified in either primary or metastatic melanoma samples, suggesting that the association of PTPRD deletion and metastasis may be specific to cSCC, rather than skin cancers per se. This finding is in contrast to a study by Stark and Hayward that found deletion of PTPRD in 9% of melanoma cell lines. The difference in the rate of deletion reported by the two studies could reflect the use of primary melanoma tissue in our study when compared to cell lines in the previous publication and could indicate either an acquired change in the cell lines or masking of the deletions in our study by stromal contamination of the tumor tissue. However, the latter is unlikely as all samples were crudely microdissected in an identical manner to the cSCC in which the deletions were readily identified.

PTPRD belongs to a family of 21 transmembrane protein tyrosine phosphatase receptors (PTPRs) that are implicated in ligand-controlled protein dephosphorylation, cell–cell adhesion and cell–matrix adhesion. Many different family members have been implicated as putative tumor suppressor genes, including PTPRG, PTPRK and PTPRJ, which are disrupted in nasopharyngeal carcinoma, lymphoma and pancreatic cancer, respectively. Conversely, PTPRA has growth-promoting effects and is overexpressed in oral SCC, colorectal and gastric carcinomas, suggesting a diverse role for this phosphatase family in cancer. The role of PTPRD has not been fully characterized, and determining its function in both normal skin and cSCC is essential to understand its part in cSCC progression. A recent publication has shown that PTPRD dephosphorylates the pStat3 protein in vitro, suggesting a potential mechanism through which PTPRD inactivation may contribute to tumorigenesis. The effect of the deletion within PTPRD is currently unknown. As illustrated in this publication, only 15% of deletions described in the literature extend into the protein-coding region, with the remainder located in the 5' UTR of the gene. In addition, a study by Nair et al. revealed aberrant splicing of exons within the 5' UTR of PTPRD in the absence of deletion. Exon B7 was one of the most frequently spliced exons, mimicking the effect of genomic deletions. This suggests that the 5' UTR is the target of multiple mechanisms of disruption in cancer and may play a key role in regulating the expression of PTPRD. Upstream noncoding regions are important regulators of protein expression affecting the localization, efficiency of translation and stability of mRNA. Long 5' UTRs (>200 bp) usually have a higher degree of secondary
structure and are frequently found in genes that encode tightly regulated proteins such as transcription factors, tumor suppressors and proto-oncogenes. Deletion of exons within long 5′ UTRs can decrease the complexity of mRNA and is typically associated with increased protein expression. The higher mRNA expression level seen in cSCC with a deletion suggests that the mRNA may be stabilized in these tumors, which would be predicted to increase protein expression. Unfortunately, the commercial antibodies that are currently available are not effective for visualization of PTPRD in vivo, so we were not able to continue these observations at the protein level.

Despite the unknown function of the 5′ UTR deletions, the association with metastatic cSCC is of clinical relevance, as they could potentially be used as a genetic marker to identify risk of disease progression in high-risk patients. Currently, only clinicopathological criteria are available for assessing metastatic risk, and the only suggested biomarker for metastasis—the epidermal growth factor receptor—shows inconsistent protein expression that does not correlate with tumor grade or progression. The metastatic rate for cSCC is between 0.1 and 9.9% and represents the main cause of death for patients suffering from NMSC. For tumors with a depth >6 mm, the metastatic rate rises to 16% and high-risk patients such as those on immunosuppression are at an increased risk of developing aggressive cSCC with a corresponding higher rate of metastasis. For these patients in particular, a biomarker to distinguish primary tumors with an increased risk of metastasis would be a highly desirable clinical tool, enabling more efficient targeted management such as sentinel lymph node surveillance. Given that the majority of the deletions found within individual metastatic series were clonal, a cytogenetic-based approach using probes within the most frequently deleted region would detect the majority of primary cSCC with deletion. Such cytogenetic-based methods are routinely used for diagnostic purposes in hematological and solid malignancies, where they represent an integral part of the clinical management of some cancers.

In conclusion, the data presented here show PTPRD to be an important gene that warrants further investigation to define its role in cSCC progression. The association between deletion and metastasis indicates its potential as a diagnostic biomarker, particularly for high-risk patients.

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References
1. Madan V, Hoban P, Strange RC, Fryer AA, Lear JT. Genetics and risk factors for basal cell carcinoma. Br J Dermatol 2006;154 (Suppl 1):5–7.
2. Cancer Research UK (2009). Skin cancer statistics - UK [Online]. Available: http://info.cancerresearchuk.org/cancerstats/types/skin/?script=true. Date accessed: 2010
3. Euvrad S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. N Engl J Med 2003;348:1681–91.
4. Lund HZ. How often does squamous cell carcinoma of the skin metastasise? Arch Dermatol 1965;92:635–7.
5. Rowe DE, Carroll RJ, Day CL, Jr. Prognostic factors for local recurrence, metastasis, and survival rates in squamous cell carcinoma of the skin, ear, and lip. Implications for treatment modality selection. J Am Acad Dermatol 1992;26: 976–90.
6. Tavin E, Persky M. Metastatic cutaneous squamous cell carcinoma of the head and neck region. Laryngoscope 1996;106:156–8.
7. Brantsch KD, Meiner C, Schombuch B, Trilling B, Wehner-Carol J, Rocken M, Breuninger H. Analysis of risk factors determining prognosis of cutaneous squamous-cell carcinoma: a prospective study. Lancet Oncol 2008;9: 713–20.
8. Kraus DH, Carew JF, Harrison LB. Regional lymph node metastasis from cutaneous squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 1998;124:582–7.
9. Tornioleti S, Rozek D, Pfeifer GP. Mapping of UV photoproducts along the human P53 gene. Ann NY Acad Sci 1994;726:324–6.
10. Brown VL, Harwood CA, Crook T, Cronin JG, Kelsell DP, Proby CM. p16INK4a and p14ARF tumor suppressor genes are commonly inactivated in cutaneous squamous cell carcinoma. J Invest Dermatol 2004;122:1284–92.
11. Purdie KJ, Harwood CA, Galati A, Chaplin T, Lambert SR, Cerio R, Kelly GP, Cazier JB, Young BD, Leigh IM, Proby CM. Single nucleotide polymorphism array analysis defines a specific genetic fingerprint for well-differentiated cutaneous SCCs. J Invest Dermatol 2009;129: 1562–8.
12. Purdie KJ, Lambert SR, Teh MT, Chaplin T, Molloy G, Raghavan M, Kelsell DP, Leigh IM, Harwood CA, Proby CM, Young BD. Allelic imbalances and microdeletions affecting the PTPRD gene in cutaneous squamous cell carcinomas detected using single nucleotide polymorphism microarray analysis. Genes Chromosomes Cancer 2007;46: 661–9.
13. Sato M, Takahashi K, Nagayama K, Ariai Y, Ito N, Okada M, Minna JD, Yokota J, Kohno T. Identification of chromosome arm 9p as the most frequent target of homozygous deletions in lung cancer. Genes Chromosomes Cancer 2005;44: 405–14.
14. Zhao X, Weir BA, Laframboise T, Lin M, Beroukhim R, Garraway L, Beheshti J, Lee JC, Naoki K, Richards WG, Sugarbaker D, Chen F, et al. Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. Cancer Res 2005;65:5561–70.
15. Stark M, Hayward N. Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays. Cancer Res 2007;67: 2632–42.
16. Stallings RL, Nair P, Maris JM, Catchpoole D, McDermott M, O’Meara A, Breitnach F. High-resolution analysis of chromosomal breakpoints and genomic instability identifies PTPRD as a candidate tumor suppressor gene in neuroblastoma. Cancer Res 2006;66:3673–80.
17. Solomon DA, Kim JS, Cronin JG, Shibahara Z, Ryken T, Rosenberg SA, Resom H, Jean W, Bigner D, Yan H, Samuels Y, Waldman T. Mutational inactivation of PTPRD in glioblastoma multiforme and malignant melanoma. Cancer Res 2008;68:10300–6.
18. Kohno T, Otsuka A, Girard L, Sato M, Iwakawa R, Ogawa H, Sanchez-Cespedes M, Minna JD, Yokota J. A catalog of genes homozygously deleted in human lung cancer and the candidacy...
of PTPRD as a tumor suppressor gene. *Genes Chromosomes Cancer* 2010;49:342–52.
19. Giefering M, Zemke N, Brauße D, Kostrewska-Poczekaj M, Luccak M, Staumkessel M, Pelinska K, Kiwerska K, Tonnie H, Grenman R, Figlerowicz M, Siebert R, et al. High resolution ArrayCGH and expression profiling identifies PTPRD and PCDH17/PCDH68 as tumor suppressor gene candidates in laryngeal squamous cell carcinoma. *Genes Chromosomes Cancer* 2011;50:154–66.
20. Chan TA, Glickner S, Yi JM, Chen W, Van Neste L, Cope I, Herman JG, Velculescu V, Schuebel KE, Ahuja N, Baylin SB. Convergence of mutation and epigenetic alterations identifies common genes in cancer that predict for poor prognosis. *PLoS Med* 2008;5:e114.
21. Veeriah S, Brennan C, Meng S, Singh B, Fagin JA, Solit DB, Paty PB, Rohde D, Vivanco I, Chmielecki J, Pao W, Ladanyi M, et al. The tyrosine phosphatase PTPRD is a tumor suppressor that is frequently inactivated and mutated in glioblastoma and other human cancers. *Proc Natl Acad Sci USA* 2009;106:9435–40.
22. Weir BA, Woo MS, Getz G, Pernar S, Ding L, Berman RM, Lin WM, Province MA, Kraja A, Johnson LA, Shah K, Sato M, et al. Characterizing the cancer genome in lung adenocarcinoma. *Nature* 2007;450:893–8.
23. MacKeigan JP, Murphy LO, Blenis J. Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and cell death. *Cancer Research* 2008;68:1137–48.
24. Proby CM, Purdie KJ, Sexton CJ, Purkis P, Nsvairska HA, Stables JN, Leigh IM. Spontaneous keratinocyte cell lines representing early and advanced stages of malignant transformation of the epidermis. *Exp Dermatol* 2009;18:104–17.
25. Fan JB, Gunderson KL, Bibikova M, Yealey JM, Chen J, Wickham Garcia E, Lebruska LL, Laurent M, Shen R, Barker D. Illumina universal bead arrays. *Methods Enzymol* 2006;410:57–73.
26. Howarth K, Ranta S, Winter E, Teixeira A, Schaschl H, Harvey JJ, Rowan A, Jones A, Spain S, Clark S, Guenther T, Stewart A, et al. A mitotic recombination map proximal to the APC locus on chromosome 5q and assessment of influences on colorectal cancer risk. *BMC Med Genet* 2009;10:54.
27. Assie G, LaFrambboise T, Platzner P, Bertherat J, Stratakis CA, Eng C. SNP arrays in heterogeneous tissue: highly accurate collection of both germline and somatic genetic information from unpaired single tumor samples. *Am J Hum Genet* 2008;82:903–15.
28. Lee S, Syed N, Taylor J, Smith P, Griffin B, Baens M, Bai M, Bourantas K, Stebbing J, Naresh K, Nelson M, Tuthill M, et al. DUSP16 is an epigenetically regulated determinant of JNK signalling in Burkitt’s lymphoma. *Br J Cancer* 2010;103:265–74.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402–8.
30. Nair P, De Preter K, Vandesompele J, Speleman F, Stallings RL. aberrant splicing of the PTPRD gene mimics microdeletions identified at this locus in neuroblastomas. *Genes Chromosomes Cancer* 2008;47:197–202.
31. Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat Rev Mol Cell Biol* 2006;7:833–46.
32. Trapasso F, Yendamuri S, Dumon KR, Iuliano R, Cesari R, Feig B, Seto R, Infante L, Ishii H, Vecchione A, During MJ, Croce CM, et al. Restoration of receptor-type protein tyrosine phosphatase eta function inhibits human pancreatic carcinoma cell growth in vitro and in vivo. *Carcinogenesis* 2004;25:2107–14.
33. Xu Y, Tan LJ, Grachtchouk V, Voorhees JJ, Fisher GJ. Receptor-type protein-tyrosine phosphatase-kappa regulates epidermal growth factor receptor function. *J Biol Chem* 2005;280:20694–700.
34. Flavell JR, Baumforth KR, Wood VH, Davies GL, Wei W, Reynolds GM, Moogan S, Boyce A, Kelly GL, Young LS, Murray PG. Down-regulation of the epidermal growth factor receptor in HIV-1 infected cells. *Nature* 2004;426:700–7.
35. Cheung AK, Lung HL, Hung SC, Law EW, Wu CW, Kao HL, Li AF, Chi CW, Lin WC. Protein tyrosine-phosphatase expression profiling in gastric cancer tissues. *Cancer Lett* 2006;242:95–103.
36. Chen SY, Takeuchi S, Moroi Y, Hayashida S, Kido M, Chen SJ, Tomoeda H, Venoutsou T, Tu YT, Furie M, Urabe K. Overexpression of phosphorylated-ATF2 and STAT3 in cutaneous squamous cell carcinoma, Bowen’s disease and basal cell carcinoma. *J Dermatol Sci* 2008;51:210–15.
37. Bito T, Sumita N, Ashida M, Budiyanto A, Ueda M, Ichihashi M, Tokura Y, Nishigori C. Inhibition of epidermal growth factor receptor and PI3K/Akt signaling suppresses cell proliferation and survival through regulation of Stat3 activation in human cutaneous squamous cell carcinoma. *J Skin Cancer* 2011;2011:874571.
38. Pesole G, Mignone F, Gissi C, Grillo G, Licciulli F, Liuni S. Structural and functional features of eukaryotic mRNA untranslated regions. *Gene* 2001;276:73–81.
39. Vartanian JG, Carvalho AL, de Araujo Filho MJ, Cerezo N, de Souza R, Coutinho G, Watson JD, Monforte H, Haas R, Bedogni G, et al. Expression of the transmembrane protein tyrosine phosphatase RPTPalpha in human oral squamous cell carcinoma. *Histone Cell Biol* 1999;111:399–403.