Disrupted regulation of serpinB9 in circulating T cells is associated with an increased risk for post-transplant skin cancer

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Summary
Cutaneous squamous cell carcinoma (cSCC) is a serious complication after organ transplantation and patients benefit from an early risk assessment. We hypothesized that functional differences in circulating T cells may represent risk factors for post-transplant cSCC development. Here, we analysed genome-wide DNA methylation of circulating T cells of kidney transplant recipients before the clinical onset of cSCC, to identify differences associated with post-transplant cSCC development. This analysis identified higher DNA methylation of SERPINB9, which is an intracellular inhibitor of granzyme B, a protein that induces apoptosis in target cells. High DNA methylation of SERPINB9 in circulating T cells was confirmed in a second patient cohort during recurrent cSCC, indicating that high SERPINB9 methylation represents a persistent risk factor for cSCC development. At the functional level, the inverse correlation between DNA methylation and messenger RNA expression present in non-cSCC patients was absent in the cSCC patients. Also, a significant difference in serpinB9 protein expression between cSCC patients and non-cSCC patients was observed. It was concluded that disturbed regulation of serpinB9 in circulating T cells represents a novel risk factor for post-transplant cSCC in kidney transplant recipients.

Keywords: cutaneous squamous cell carcinoma, DNA methylation, epigenetics, kidney transplantation, PI-9

Introduction
Imunosuppression after organ transplantation is associated with a higher prevalence of cancer [1,2]. Non-melanoma skin cancer, in particular, such as cutaneous squamous cell carcinoma (cSCC), can occur up to 200 times more in transplant patients compared to the general population [3–5]. Transplant recipients also experience more metastasis and more than 70% of the patients develop a subsequent cSCC within 5 years [6]. Although immunosuppressive treatment is recognized as an important risk factor for the development of cSCC after solid organ transplantation, little is known on the immune regulation leading up to formation of cSCC.

Most cSCCs are surrounded by immune cell infiltrates; however, these cells are incapable of mounting an effective immune response directed against the cSCC [7]. The role and phenotype of T cells surrounding an cSCC lesion has been studied extensively [8–10]; high numbers of forkhead box protein 3 (FoxP3)+ regulatory T cells are associated with higher metastasis of cSCCs [11,12], whereas increased activity of effector and cytotoxic T cells often associates with better prognostic outcomes [13,14].

The function of cells, including T cells, is regulated by epigenetic mechanisms such as DNA methylation, which is the addition of a methyl group to a cytosine (C) followed by a guanine (G; CpG dinucleotide) in the DNA. DNA methylation controls gene expression, is a dynamic feature and can be influenced by environmental cues [15]. DNA methylation is also known to be dysregulated in disease such as cancer; however, it is often difficult to determine whether it is a driver or a consequence of the disease [16,17].
Here, we hypothesized that functional differences in circulating T cells represent risk factors in the development of a de-novo post-transplant cSCC. To address this hypothesis, we took an unbiased approach and performed genomewide DNA methylation analysis of circulating T cells after kidney transplantation but before the clinical onset of cSCC (discovery phase). DNA methylation profiles of kidney transplant recipients with a future cSCC were compared to those of matched kidney transplant recipients without cSCC. The prominent finding of this analysis was higher methylation of a region within SERPINB9 in cSCC patients. SerpinB9 is an intracellular serine protease inhibitor that inhibits granzyme B [18,19], which is an important protease in the effector function of cytotoxic T cells by inducing apoptosis in target cells [20]. Cytotoxic T cells express serpinB9 to protect themselves against the activity of granzyme B, and studies have shown that high expression of serpinB9 in cytotoxic T cells makes them more potent killers [21,22]. Given these data, the finding on SERPINB9 DNA methylation prompted us to further study SERPINB9 methylation in a second cohort of kidney transplant recipients with recurrent cSCC, as well as the functional role of SERPINB9 in cSCC on the level of mRNA and protein expression.

Materials and methods

Study design

Anonymized retrospective biobank samples were used in the discovery phase of the study; this included kidney transplant recipients before the diagnosis of their first post-transplant cSCC. A second cohort of patients was used to confirm findings from the discovery phase, and this included kidney transplant recipients during recurrent post-transplant cSCC. The use of biobank material and the inclusion of new patients was approved by the local medical ethical committee (MEC-2015-642). All kidney transplant recipients during recurrent cSCC were matched to kidney transplant recipients who did not develop an cSCC within a similar time-period after the first transplant. Matching criteria included gender, age (± 4 years), ethnicity, cytomegalovirus (CMV) status and type of immunosuppressive drugs directly after transplantation. We included patients with at least one cSCC after transplantation and patients with cSCC in situ (Bowen’s disease). Patients with another donor organ such as liver, heart or lung were excluded, as well as patients with a history of malignancy prior to transplantation. Non-cSCC patients with actinic keratosis, a precancerous lesion, were also excluded.

T cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll-Paque procedures. T cells were isolated from the PBMCs using fluorescence-activated cell sorting (FACS) with the BD FACSaria™ ll (BD Biosciences, San Jose, CA, USA). Total PBMCs were stained with CD3 Brilliant Violet 510 (Biolegend, San Diego, CA, USA), CD4 Pacific Blue (BD Biosciences), CD8 allophycocyanin-cyanin 7 (APC-Cy7) (BD Biosciences), CD45RO APC (Biolegend), CCR7 phycoerythrin (PE)-Cy7 (BD Biosciences), CD25 PE (BD Biosciences), CD127 fluorescein isothiocyanate (FITC) (eBioscience, Waltham, MA, USA) and to exclude non-viable cells Via-Probe 7-aminoactinomycin D (7AAD) (BD Biosciences) was used. After cell sorting the purities were > 96% for CD3+ cells; samples below 95% were excluded for further analysis.

Genomewide DNA methylation arrays

Before isolating DNA from the T cells in the discovery cohort, all patient samples were randomized to minimize batch effects. DNA was isolated using the QIAamp DNA Micro kit (Qiagen, Venlo, the Netherlands), according to the manufacturer’s protocol. Purity and concentration of the isolated DNA was assessed with the NanoDrop ND-8000 (Isogen Life Science, Utrecht, the Netherlands). DNA degradation was determined by gel electrophoresis. None of the samples showed a significant degradation.

The Infinium Human Methylation 450 arrays (Illumina, San Diego, CA, USA) were performed as described previously [23]. Data quality was examined using the MethylAid R package [24,25] and all samples passed quality controls using the default MethylAid thresholds. Probes with a detection \( P \)-value > 0.01, probes containing single nucleotide polymorphisms and probes on the sex chromosome were removed from the data set. A between-array normalization was applied to the types I and II probes separately using the DASEN method within the watermelon Bioconductor R package [25–27]. The methylation level of a CpG site is presented as a beta-value, a value between 0 (unmethylated) and 1 (fully methylated). After the quality controls and normalization, beta-values of 423 289 CpG sites remained for further analysis. Both the raw and normalized data are available via the NCBI Gene Expression Omnibus (GEO) database with Accession number GSE117050.

Data analysis DNA methylation arrays

To identify DNA methylation differences between the future cSCC and non-cSCC patients, we performed the data analysis as previously described [23]. First, a linear
mixed-effect model was performed using the lme4 package [28]. The fixed effects included age, percentage of CD4, percentage of CD8 and CMV status. Percentages of CD4 and CD8 were included to correct for differences in T cell composition between individuals, and CMV is known to affect DNA methylation at specific genes [29]. Array IDs were included as a random effect to account for technical variation between the arrays. This resulted in single site-specific P-values and these P-values, together with their genomic location, were used as input into comb-p [30] to find differentially methylated regions (DMRs). A sliding window of 500 base pairs (bp) was used and the seed was set at \( P < 0.01 \). A stringent multiple testing correction was applied using a Šidák correction (Šidák < 0.05) [31].

**DNA methylation analysis by pyrosequencing**

After the discovery phase we continued measuring DNA methylation of T cells with bisulphite pyrosequencing, an easy technique to quantitatively measure single-site DNA methylation [32], but first we tested whether pyrosequencing resulted in the same methylation values as the microarrays. Therefore, CpG sites within DMR 1 (SERPINB9) and DMR 2 (VTRNA2-1) were analysed in the same DNA samples that were used for the array analysis of 10 patients, a mixture of cSCC and non-cSCC patients.

Pyrosequencing was performed as described previously [23,33]. The polymerase chain reaction (PCR) primers, melting temperatures and amplicon sizes for the different PCR products can be found in the Supporting information, Table S1 together with the specific PCR programmes. For SERPINB9, 12 CpG sites, five of which were array probes, were sequenced in two separate reactions and DNA methylation was averaged per sequence reaction (regions 1 and 2). For VTRNA2-1, five CpG sites, three of which were array probes, were measured and an average of those five sites is presented in the results.

**mRNA analysis**

Total RNA was isolated from T cells using the High Pure RNA Isolation kit (Roche Applied Science, Pennsburg, Germany), according to the manufacturer’s protocol. The quality and purity of the RNA was assessed using the NanoDrop ND-8000 (Isogen Life Science). Samples with a 260/280 ratio below 1.8 and a 260/230 ratio above 1 were excluded for further analysis. Messenger RNA (mRNA) of SERPINB9 and GRANZYM E B (GZMB) was quantified by real-time quantitative PCR (qPCR) using a Taqman gene expression assay. Primers used were Hs00394497_m1 (SERPINB9; Thermo Fisher Scientific, Waltham, MA, USA) and Hs01554355_m1 (GZMB; Thermo Fisher Scientific), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1, Thermo Fisher Scientific) was used as housekeeping gene. qPCR was performed on the StepOnePlus Real-Time PCR system (Applied Biosystems). Gene expression was then calculated by transforming the cycle threshold (Ct) to cDNA copies \((2^{ΔCt})\). Dividing the number of SERPINB9 and GZMB copies by the number of GAPDH copies resulted in a relative gene expression value.

**Protein analysis**

Protein levels of serpinB9 and granzyme B within T cells were assessed in cells before and after stimulation for 6 h at 37°C with or without α-CD3/CD28-coated Dynabeads® (Gibco, Waltham, MA, USA). The cells were measured by flow cytometry directly after defrosting the PBMCs. Monensin was added after 1 h of stimulation, whereby this newly synthesized granzyme B could be measured. CD107a APC (BD Biosciences) was added to the cell cultures to assess degranulation of the cells. Cells were stained with the following surface antibodies: CD3 Brilliant Violet 510 (Biolegend), CD4 APC-Cy7 (Biolegend), CD8 PE (Thermo Fisher Scientific) and Via-Probe 7AAD (BD Biosciences) was used to exclude non-viable cells. After surface staining the cells were fixed, permeabilized and stained for intracellular serpinB9 labelled with Alexa Fluor 488 (Bio-rad, Hercules, CA, USA) and granzyme B labelled with Brilliant Violet 421 (BD Biosciences). Isotype controls for AF488 (Bio-Rad) and BV421 (BD Biosciences) were used as negative controls for serpinB9 and granzyme B expression. The cells were then analysed on the FACS Canto ll (BD Biosciences) with FACSDiva software. Data were analysed blind, without knowledge of cSCC status of the samples, using Kaluza software 1.5a (Beckman Coulter, Brea, CA, USA).

**Statistical analysis**

Differences in clinical characteristics, DNA methylation, mRNA and protein expression between the cSCC and non-cSCC patients were statistically tested using spss version 21.0 (IBM Corp., Armonk, NY, USA). The Mann–Whitney U-test was used for the continuous variables and \( \chi^2 \) test for the categorical variables. Data processing and statistical analysis of all the microarray data was performed in RStudio version 1.0.136 (RStudio Inc., Boston, MA, USA) with r version 3.2.5 [26]. Multiple testing correction of the microarray data was performed using a Šidák correction (Šidák < 0.05) [31]. Correlation between the DNA methylation levels quantified by pyrosequencing and the beta-values of the Illumina 450k arrays was calculated using Spearman’s rank correlation coefficient using spss, as well as correlations between DNA methylation and mRNA expression. All statistical tests were two-tailed and a \( P < 0.05 \) was considered statistically significant.
Results

Patients
The discovery cohort was comprised of 19 future cSCC and 19 non-cSCC patients who had been transplanted between 1997 and 2012. All patients in the discovery cohort were Caucasian European. No statistical differences were found between the patient characteristics of the future cSCC and non-cSCC patients (Table 1). The T cell subpopulations measured during FACS sorting were also not different between future cSCC and non-cSCC patients (data not shown). A detailed overview of the time between transplantation, sample and first diagnosis of cSCC can be found in the Supporting information, Fig. S1a.

The second cohort consisted of 37 non-cSCC and 45 cSCC patients during recurrent cSCC, who had been transplanted between 1976 and 2014. Six cSCC patients and five non-cSCC patients received a second kidney transplant. All patients in the second cohort were Caucasian European. There was a small statistical difference in the end-stage renal disease (ESRD) diagnosis between the cSCC and non-cSCC patients (P = 0·04; Table 2); no other statistical differences were found. The T cell subpopulations measured during FACS sorting were not different between cSCC and non-cSCC patients (data not shown). A detailed overview of the time between transplantation, sample and first diagnosis of cSCC can be found in the Supporting information, Fig. S1b.

Discovery of significant DMRs in circulating T cells before cSCC
To identify differentially methylated regions (DMRs) in circulating T cells associated with future cSCC development, we compared genomewide DNA methylation of kidney transplant recipients with and without a post-transplant cSCC, before the clinical onset of the cSCC. None of the single-site CpGs were statistically significant after multiple testing correction. However, we found seven regions significantly differentially methylated. In Table 3 the different DMRs, the genes annotated to these DMRs based on genomic location, the genomic location of the DMRs according to the hg19 genome build (UCSC Genome Browser), the number of probes (CpG sites on the array) within the regions and the effect size are presented. Of the significant DMRs, five were hypermethylated and two were hypomethylated in the future cSCC patients.

Genomic characteristics of DMR 1, 2 and 3
To understand the potential regulatory effect of the DMRs in T cells, the genomic location of the DMR and characteristics of that location are important. In Fig. 1, we visualized the top three DMRs with DNA methylation characteristics of that location are important. In Fig. 1, we visualized the top three DMRs with DNA methylation

| Table 1. Patient characteristics of the discovery cohort before cSCC |
|-----------------|-----------------|-----------------|
|                 | cSCC  n = 19    | Non-cSCC  n = 19 |
| Age (years)                 | 64·8 (45–77)    | 63·5 (45–80)    | P = 0·49 |
| Gender (male)                | 14 (73·7%)      | 14 (73·7%)      | P = 1    |
| Years post-Tx                | 1·5 (0·1–6·9)   | 1·3 (0·1–6·3)   | P = 0·93 |
| Years between Tx and first cSCC | 5·4 (0·9–12·5)  |      –         |       |
| Biopsy-proven rejection      |                  |                |         |
| Calcineurin inhibitors       |                  |                |         |
| Proliferation inhibitors     |                  |                |         |
| Anti-metabolites             |                  |                |         |
| Corticosteroids              | 18 (94·7%)      | 19 (100%)      |         |
| HLA mismatches               | 3·11 (0–6)      | 3·11 (0–6)     | P = 0·94 |
| CMV serostatus acceptor      |                  |                | P = 1    |
| Negative                     | 4 (21·1%)       | 4 (21·1%)      |         |
| Positive                     | 15 (78·9%)      | 15 (78·9%)     |         |
| CMV serostatus donor         |                  |                | P = 0·11 |
| Negative                     | 12 (63·2%)      | 7 (36·8%)      |         |
| Positive                     | 7 (36·8%)       | 12 (63·2%)     |         |
| ESRD diagnosis               |                  |                | P = 0·26 |
| Polycystic kidney            | 7 (36·8%)       | 2 (10·5%)      |         |
| Hypertension                 | 3 (15·8%)       | 6 (31·6%)      |         |
| Diabetic nephropathy         | 1 (5·3%)        | 1 (5·3%)       |         |
| Glomerulonephritis           | 1 (5·3%)        | 0 (0%)         |         |
| Other                        | 7 (36·8%)       | 10 (52·6%)     |         |
| Dialysis pretransplantation  |                  |                | P = 0·49 |
| Yes (PD/HD)                  | 14 (73·7%)      | 12 (63·2%)     |         |
| No                           | 5 (26·3%)       | 7 (36·8%)      |         |

*Median and range.

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Table 2. Patient characteristics of the second cohort during cSCC

|                      | cSCC     | Non-cSCC  |
|----------------------|----------|-----------|
|                      | n = 45   | n = 37    |
| Age (years)*         | 66–4 (34–84) | 64–0 (28–75) | P = 0·20 |
| Gender (male)        | 30 (66·7%) | 25 (67·6%) | P = 0·93 |
| Years post-Tx        | 8·5 (0·4–40·5) | 9·5 (0·1–35·9) | P = 0·89 |
| Years between Tx and first cSCC* | 4·7 (0–33) | – |
| Biopsy proven rejection | 12 (26·7%) | 13 (35·1%) | P = 0·41 |
| Immunosuppressive treatment | 1 (2·2%) | 6 (16·2%) |
| Induction therapy    | 37 (82·2%) | 34 (92%)  |
| Proliferation inhibitors | 27 (60%) | 22 (59·5%) |
| Anti-metabolites     | 9 (20%)   | 4 (10·8%) |
| Corticosteroids      | 44 (97·8%) | 37 (100%) |
| HLA mismatchesa      | 3·0 (0–6)  | 3·0 (0–6) | P = 0·74 |
| CMV serostatus acceptor | 17 (37·8%) | 11 (29·7%) |
| Positive             | 22 (48·9%) | 20 (54·1%) |
| Negative             | 6 (13·3%)  | 6 (16·2%) |
| CMV serostatus donor | 15 (33·3%) | 14 (37·8%) |
| Positive             | 18 (40%)   | 11 (29·7%) |
| Unknown              | 12 (26·7%) | 12 (32·4%) |
| ESRD diagnosis       | 11 (24·4%) | 5 (13·5%)  |
| Polycystic kidney    | 8 (17·8%)  | 7 (18·9%)  |
| Diabetic nephropathy | 7 (15·6%)  | 4 (10·8%)  |
| Glomerulonephritis   | 19 (42·2%) | 14 (37·8%) |
| Dialysis             | 22 (48·9%) | 19 (51·4%) |

*Median and range.

cSCC = cutaneous squamous cell carcinoma; Tx = transplantation; ATG = anti-thymocyte globulin; MMF = mycophenolate mofetil; HLA = human leucocyte antigen; CMV = cytomegalovirus; ESRD = end-stage renal disease; PD = peritoneal dialysis; HD = haemodialysis.

Spearman’s r was 0·96 (P < 0·0001). As a result of this strong correlation, we measured DNA methylation with pyrosequencing throughout the rest of the study.

High intragenic SERPINB9 methylation during cSCC

To assess the stability of the DNA methylation profiles identified before the development of cSCC, we included a second patient cohort during recurrent cSCC (Table 2). VTRNA2-1 was measured and was not significantly different between cSCC and non-cSCC patients (data not shown). When SERPINB9 was measured, it was significantly different between cSCC and non-cSCC patients. Median DNA methylation of SERPINB9 was 58·7% (range: 32·5–81·3%) for region 1 and 54·4% (30·0–78·5%) for region 2 in the cSCC patients and 50·2% (21·8–77·5%) for region 1 and 46·4% (22·1–74·0%) for region 2 in the non-cSCC patients (region 1: P = 0·004; region 2: P = 0·008) (Fig. 2).

Similarly to our discovery cohort, cSCC patients demonstrated higher SERPINB9 methylation values than non-cSCC patients. In addition, serpinB9 has a strong relation to T cell function as a regulator of cytotoxicity [21,22]. Together, these findings warranted further investigation into the role of SERPINB9 in controlling the cytotoxic T cells that are key for immunosurveillance in post-transplant cSCC.

mRNA expression negatively correlates to DNA methylation only in the non-cSCC patients

To study the translation from DNA to protein of serpinB9, we analysed mRNA expression of SERPINB9 in T cells. Relative mRNA expression of SERPINB9 was not significantly different between cSCC (n = 30) and non-cSCC patients (n = 27; Fig. 3a). When we zoomed in and studied the correlation between DNA methylation and mRNA expression of SERPINB9 in the total patient population this was statistically significant (P = 0·004, Fig. 3b). However, when we stratified the data by cSCC status, the correlation remained significant only in the non-cSCC patients (P = 0·0003; Fig. 3c,d) and not in the cSCC patients, indicating a disrupted transcriptional regulation in the cSCC patients.

Lower serpinB9 expression in circulating T cells of cSCC patients

To investigate the functional impact of differentially methylated SERPINB9 on cytotoxicity, we analysed the expression of the following markers: granzyme B (inhibited by serpinB9) and degranulation of T cells by CD107a expression before and after stimulation. Gating strategies for granzyme B and CD107a are presented in the Supporting information, Fig. S3. The percentage of CD3+ granzyme

Confirmation of microarray methylation values by pyrosequencing

To confirm that the above-described findings can also be found with a different technique, DNA methylation of DMRs 1 and 2 was measured by pyrosequencing in the same DNA samples. Correlation between DNA methylation values obtained with the microarray and pyrosequencing was strong in both DMRs (Supporting information, Fig. S2). Spearman’s r for SERPINB9 was 0·86 (P < 0·0001) and for VTRNA2-1
observed that the percentage of CD4+ serpinB9+ cells was not significantly different between the cSCC patients and non-cSCC patients (Supporting information, Fig. S4a), nor was degranulation of the T cells, as determined by CD107a staining (Supporting information, Fig. S4b). SerpinB9 expression was also measured in the T cells. Gating strategy for serpinB9 is presented in the Supporting information, Fig. S5. The percentage of CD3+ serpinB9+ cells before stimulation was not significantly different between cSCC and non-cSCC patients (Fig. 4a). After stimulation, serpinB9 expression in all T cells was up-regulated to 98.2% (97.2–99.7%) for the cSCC patients and 99.1% (97.0–99.0%) for the non-cSCC patients, and this was significantly different even though the differences were small (P = 0.006; Fig. 4b). When analysing serpinB9 expression in the CD4+ and CD8+ population separately, we observed that the percentage of CD4+ serpinB9+ cells was significantly lower in the cSCC patients than in the non-cSCC patients after stimulation (Fig. 4c). In the CD8+ population this difference was not observed (Fig. 4d). These results show that the CD4+ population is the main contributor to the difference observed in the total T cell population.

Discussion

In this study, we demonstrate high DNA methylation of SERPINB9 in circulating T cells before the clinical onset of cSCC in kidney transplant recipients and, in a different patient cohort, during recurrent post-transplant cSCC. These data identify high DNA methylation of SERPINB9 as a novel risk factor for development of both de-novo and subsequent post-transplant cSCC. In addition, T cells of cSCC patients were unable to fully up-regulate serpinB9 expression in vitro, which might provide insight into the role of the peripheral immune system in the development of an cSCC in kidney transplant recipients.

In a previous study, where we identified DMRs associated with post-transplant cSCC before transplantation [23], SERPINB9 was not significantly different between T cells of future cSCC patients and non-cSCC patients. Thus, differences in SERPINB9 DNA methylation that identify patients at risk for cSCC arise after kidney transplantation. Kidney transplantation and the use of immunosuppressive therapy probably affect DNA methylation profiles of the T cells [35]. However, as we demonstrated high SERPINB9 methylation in patients before and after the development of a de-novo post-transplant cSCC, it seems a persistent risk factor for cSCC after transplantation.

Based on the differential DNA methylation of SERPINB9, one could expect differences in mRNA expression of SERPINB9. Nevertheless, when SERPINB9 mRNA expression was measured in the T cells, this was not significantly different between cSCC patients and non-cSCC patients. This is comparable to findings by Ryer et al. [36], who identified higher methylation of the same region within SERPINB9 in PBMCs of patients with abdominal aortic aneurysm. Despite the differential DNA methylation, they also did not detect a difference in mRNA expression. This is due most probably to the intragenic location of the DMR, outside the promoter region of SERPINB9. The effect of intragenic DNA methylation on gene expression is still debated [37], although in this study we demonstrated an inverse correlation between intragenic SERPINB9 DNA methylation and mRNA expression in the T cells of non-cSCC patients. Surprisingly, this inverse correlation was absent in the T cells of cSCC patients. This illustrates a disturbed transcriptional regulation of SERPINB9 in cSCC patients and a clear difference between these two patient groups.

| Annotated to: | Genomic location (hg19) | Length DMR | No. of probes | Function | Regional P-value | Effect size | DMR state |
|---------------|-------------------------|------------|---------------|----------|-----------------|------------|----------|
| 1 SERPINB9    | chr6:2891973–2892153    | 180 bp     | 5             | Granzyme B inhibitor | 1.09 × 10^-11 | 0.14     | Hyper    |
| 2 VTRNA2-1    | chr5:135415948–135416614| 666 bp     | 12            | Inhibitor of protein kinase R | 1.40 × 10^-10 | 0.11     | Hyper    |
| 3 VTRNA2-1    | chr5:135414858–135415259| 401 bp     | 4             | Inhibitor of protein kinase R | 1.90 × 10^-8 | 0.07     | Hyper    |
| 4 PIF1        | chr15:65116194–65116558 | 364 bp     | 3             | ATP metabolism | 1.36 × 10^-7 | –0.05    | Hypo     |
| 5 APC2        | chr19:1465962–1466163   | 201 bp     | 2             | Signalling pathway regulation | 1.48 × 10^-7 | 0.08     | Hyper    |
| 6 RPH3AL      | chr17:151914–152351     | 437 bp     | 6             | Tumour suppressor | 3.39 × 10^-7 | –0.07    | Hypo     |
| 7 AC144450.2  | chr2:1609660–1609833    | 173 bp     | 2             | LncRNA      | 3.15 × 10^-6 | 0.08     | Hyper    |

DMR = differentially methylated region; chr = chromosome; bp = base pair.

In vitro regulation of serpinB9 expression, which might provide insight into the role of the peripheral immune system in the development of an cSCC in kidney transplant recipients.

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Previous studies have shown that higher expression of serpinB9 increased the potency of cytotoxic T cells [21,22]. We observed a slightly lower expression of serpinB9 in the T cells of our cSCC patients, which was due mainly to the CD4 compartment of the T cells, although it is questionable whether a difference
between 98 and 99% serpinB9-positive cells is biologically relevant. In addition, the regulation of serpinB9 seems independent from the regulation of cytotoxic markers of T cells, as we did not identify differences in the expression of granzyme B and CD107a between cSCC patients and non-cSCC patients. SerpinB9 is an intracellular protein inactivating granzyme B once it is released into the cytoplasm [38], and therefore serpinB9 exerts its effect on cytotoxicity only after granzyme B is synthesized to its active form. The absence of differences in cytotoxicity which, in most cases, is restricted to the CD8⁺ T cells, and the SERPINB9 DNA methylation difference in the CD4⁺ T cells may lead to the conclusion that the CD4⁺ T cells are the population of interest in post-transplant cSCC.

DNA methylation of SERPINB9 might represent a future treatment target for cSCC in transplant recipients. It would be interesting to decrease SERPINB9 DNA methylation in cSCC patients to the level observed in non-cSCC patients and study whether that affects future cSCC development in those patients. DNA methylation can be edited by use of the CRISPR/cas9 system, a technique called 'epigenetic editing' [39]. Although this novel technology is far from a clinical application, it is a promising concept for the future. Additionally, this approach will reveal whether

Fig. 3. Relative mRNA expression of SERPINB9 (a) in cutaneous squamous cell carcinoma (cSCC) versus non-cSCC patients, (b) as correlated to SERPINB9 DNA methylation (x-axis) within all patients, (c) within the cSCC patients and (d) within the non-cSCC patients.
SERPINB9 DNA methylation plays a causal role in cSCC development or whether it is a consequence of another, as yet unknown, mechanism leading to post-transplant cSCC development.

We are aware that the single-centre design and small sample size may be a limitation of this study. Details such as sun exposure were unknown, and dosages of immunosuppression were often adjusted or immunosuppressive regimens were changed during the course of post-transplant treatment. It was therefore not possible to take these factors into account. Nevertheless, we show a promising proof-of-concept that studying DNA methylation of SERPINB9 in peripheral T cells can identify kidney transplant recipients at risk for cSCC. The disturbed transcriptional regulation of SERPINB9 and the lower protein expression of serpinB9 warrant further investigation to fully understand the relation with cSCC development in kidney transplant recipients.

Together, these findings demonstrate that DNA methylation, transcriptional regulation and protein expression of serpinB9 differ between cSCC and non-cSCC patients. This identifies a novel risk factor for the development of post-transplant cSCC and may provide mechanistic insight into the role of circulating T cells in cSCC development. Future studies will identify whether serpinB9 plays a causal role in cSCC development and if it is a suitable treatment target to prevent cSCC development after kidney transplantation.

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Disclosures

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1. Detailed time schedule of (a) the discovery patient cohort and (b) the second patient cohort. On the Y-axis are all the cSCC patients and their matched controls and on the X-axis is the time in months from the transplantation (Tx) onwards.

Fig. S2. Correlation between methylation values obtained by pyrosequencing and microarray. (a) XY plot of DMR 1 (SERPINB9) with array values on the X-axis and pyro values on the Y-axis. (b) Detailed graph for each of the 5 sites within DMR 1 (SERPINB9) with in red the cSCC patients and in green the non-cSCC patients. (c) XY plot of DMR 2 (VTRNA2-J) with pyro values on the X-axis and array values on the Y-axis. (d) Detailed graph for each of the 3 sites within DMR 2 (VTRNA2-J) with in red the cSCC patients and in green the non-cSCC patients.

Fig. S3. Gating strategy for granzyme B expression and CD107a by T cells. Representative examples of (a) lymphocyte gate from forward scatter (FSC) and sideward scatter (SSC), (b) living T cells gated from 7AAD-CD3 staining, (c) granzyme B+ cells gated within the living T cells at 0 hours, (d) granzyme B+ cells gated within the living T cells at 6 hours, (e) CD107a+ cells gated within the living T cells at 0 hours, (f) CD107a+ cells gated within the living T cells at 6 hours, (g) isotype control in green and granzyme B stained sample in red at 0 hours and (h) isotype control in green and granzyme B stained sample in red at 6 hours.

Fig. S4. Quantified flow cytometry data on granzyme B and CD107a expression by T cells. (a) Percentage of T cells that expressed granzyme B before and after stimulation in the cSCC and non-cSCC patients. (b) Percentage of T cells that expressed CD107a before and after stimulation in the cSCC and non-cSCC patients.

Fig. S5. Gating strategy for serpinB9 expression by T cells. Representative examples of (a) lymphocyte gate from forward scatter (FSC) and sideward scatter (SSC), (b) living T cells gated from 7AAD-CD3 staining, (c) serpinB9+ cells gated within the living T cells at 0 hours, (d) serpinB9+ cells gated within the living T cells at 6 hours, (e) isotype control in green and serpinB9 stained sample in red at 0 hours and (f) isotype control in green and serpinB9 stained sample in red at 6 hours.

Table S1. PCR and sequence primers of genes for validation.