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Jong, E. de; Quint, K.D.; Ghalbzouri, A. el; Verdijk, R.M.; Goeman, J.J.; Heidt, S.; ... ; Bavinck, J.N.B.

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Research article

HLA expression as a risk factor for metastases of cutaneous squamous-cell carcinoma in organ- transplant recipients

Estella de Jong [a,*], Koen D. Quint [a], Abdoelwaheb El Ghalbzouri [a], Robert M. Verdijk [b,c], Jelle J. Goeman [d], Sebastiaan Heidt [e], Frans H.J. Claas [e], Johan W. de Fijter [f], Roel E. Genders [a], Maarten H. Vermeer [a], Jan Nico Bouwes Bavinck [a]

[a] Department of Dermatology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, the Netherlands
[b] Department of Pathology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, the Netherlands
[c] Department of Pathology, Erasmus University Medical Center Rotterdam, PO Box 2040, 3000 CA Rotterdam, the Netherlands
[d] Department of Biomedical Data Sciences, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, the Netherlands
[e] Department of Immunology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, the Netherlands
[f] Department of Internal Medicine (Nephrology), Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, the Netherlands

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ABSTRACT

Background: Solid organ-transplant recipients (SOTR) have an increased risk of cutaneous squamous-cell carcinoma (cSCC), metastasis and death from cSCC. In immunocompetent patients with mucosal SCC, downregulation of HLA class I is associated with poor prognosis. Since the degree of HLA expression on tumor cells could play a role in immunogenicity and pathophysiology of cSCC metastasis, we hypothesized that decreased HLA expression is associated with an increased risk of metastasis.

Methods: We compared HLA expression between primary metastasized cSCCs, their metastases, and non-metastasized cSCCs from the same patients. Samples were stained for HLA-A, HLA-B/-C and quantified by calculating the difference in immunoreactivity score (IRS) of the primary cSCC compared with all non-metastasized cSCCs.

Results: The mean IRS score for HLA-B/C expression was 2.07 point higher in metastasized compared to non-metastasized cSCCs ($p = 0.065$, 95 % CI −0.18–4.32). 83.3 % of the primary metastasized cSCCs had an IRS score of 4 or higher, compared to 42.9 % in non-metastasized cSCCs. Moderately to poorly differentiated cSCCs had more HLA class I expression compared to well-differentiated cSCCs.

Conclusion: Contrary to immunocompetent patients, HLA-B/C expression tends to be upregulated in metastasized cSCC compared to non-metastasized cSCC in SOTR, suggesting that different tumor escape mechanisms play a role in SOTR compared to immunocompetent patients.

1. Introduction

Cutaneous squamous-cell carcinoma (cSCC) accounts for approximately 20 % of all skin cancers, with cumulative exposure to UV-radiation as its most important risk factor.[1,2] In 5–10 %, metastases of cSCC can occur and in the majority leads to death of the patient.[3] Immunocompromised patients, e.g. solid organ-transplant recipients (SOTR) are at increased risk for developing cSCC compared to immunocompetent patients.[4–6] Clinical as well as histological risk factors (e.g. tumor size, invasion depth, differentiation grade, perineural growth, vaso-invasive growth) for cSCC metastases have been investigated extensively in literature.[3,7–10] Furthermore, there is literature stating that organ transplantation itself may be a risk factor for cSCC metastasizing as well. [9,11]

cSCC has been reported to have the highest mutational burden of any malignancy[12,13] and an adequate immune response is therefore

Abbreviations: APC, Antigen presenting cell; cSCC, Cutaneous squamous-cell carcinoma; HLA, Human leukocyte antigen; HNSCC, Head and neck squamous cell carcinoma; ICC, Intraclass correlation; IRS, Immunoreactivity score; MHC, Major histocompatibility complex; NK, Natural killer; OR, Odds ratio; sOTR, Solid organ-transplant recipients; UV, Ultraviolet.

* Corresponding author at: Albinusdreef 2, 2333ZA Leiden, the Netherlands.
E-mail address: e.de_jong.huid@lumc.nl (E. de Jong).

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essential in the recognition and elimination of cSCC. The adaptive immune response is critically dependent on the expression of human leukocyte antigen (HLA) class I and II for the recognition of presented peptides by CD8 + and CD4 + T lymphocytes, respectively.

The HLA system describes constitutes of a group of genes encoding major histocompatibility complex (MHC) proteins. The classical class I HLA genes (A, B, and C) encode proteins on the surface of all nucleated cells, while class II HLA genes (DR, DQ, and DP) encode proteins on the surface of antigen-presenting cells (APCs). β2-microglobulin is an invariant component of all HLA class I molecules. Contrary to melanoma and basal cell carcinoma, in which complete loss of function of class I HLA has been observed (due to for example β2-microglobulin deficiency), a more heterogenous expression is found in cSCC.[14–17].

In the last years, evidence has been acquired concerning the involvement of HLA in the development of cSCC.[18] Downregulation of HLA class I molecules on tumor cells is commonly described in literature as a mechanism for immune evasion. Urosevic et al. suggested that downregulation of HLA class I prevents presentation of tumor antigens to CD8 + T lymphocytes, and, therefore, the destruction of cSCC cells. On the other hand, downregulation of HLA results in an increased risk of lysis by natural killer (NK) cells.[19] It is thought that cSCCs have heterogeneous class I HLA expression with selective or partial downregulation, which decreases the presentation to T lymphocytes, but in which NK cell inhibition is still present.[18]

HLA class II genes play a role in the activation of CD4 + T lymphocytes. Multiple studies have found high cell surface expression of HLA class II proteins on cSCC.[16,20] However, cSCC cells lack costimulatory molecules, and therefore the immune response of the CD4 + T lymphocytes may be suppressed rather than activated.[21,22]

Little is known about the exact role of the HLA system in the prognosis of cSCC and the development of metastasis. A few studies showed that a high level of HLA class II expression (mostly HLA-DR) was more frequently present in undifferentiated tumors.[16,23] One study, in which HLA class I expression was compared between the primary metastasizing SCC and the metastasis, showed downregulation in primary tumors as well as in the metastases, but downregulation of the metastases was significantly lower than in the primary tumors.[24] Another study found significant lower percentages of CD4 + T lymphocytes expressing HLA-DR in metastases high-risk SCC.[25] In both studies mucosal SCCs were investigated instead of cutaneous SCCs. Furthermore, it was unknown which SCC caused the metastasis. Similar results have been found in other studies for mucosal SCC.[26–28] To the best of our knowledge, studies comparing metastasized and non-metastasized cSCCs within the same patient have not been conducted yet. We believe these data will contribute to a better understanding in the differentiation between cSCCs that are at risk for metastasizing and cSCCs that are not. Since SOTR are especially at risk for cSCC development, we decided to investigate differences in HLA class I expression in the prediction of cSCC metastases development in these patients with cSCC.

2. Materials and Methods

2.1. Study design and patient selection

A comparative study was performed to evaluate differences in HLA expression in metastatic cSCCs compared to cSCCs from the same patients that did not metastasize. The study population consisted of SOTR from the Leiden University Medical Hospital (LUMC) who developed cSCC metastases between 2013 and 2019.

For study inclusion, all patients needed to have a metastasized cSCC and at least three histologically proven cSCCs that did not metastasize to serve as control cSCCs. Patients were excluded from the study if metastases were caused by malignancies other than cSCC, or when the primary tumor location was unknown for the metastasis.

With knowledge of the localization and time relation of the tumors, it was possible to identify which cSCC most likely caused the metastasis. For all patients, a meeting was organized between two dermatologists (JNB and REG) and a resident (EJ) to reach consensus. Tissue from the metastasized cSCC, control cSCCs, the metastasis itself and a normal skin sample were obtained. The normal skin was acquired from residual material from excisions. All tissue blocks were obtained from the archives of the Department of Pathology of the LUMC. Information on patient and tumor characteristics, as well as histology reports were collected from electronic patient files (HiX Chipsoft) and verified (by EJ and KDQ).

In total, 42 tissue samples were processed and evaluated, from 6 patients with 6 primary cSCCs, 23 control (non-metastasized) cSCCs, 7 metastases, and 6 normal skin tissue samples. Two control cSCCs were later excluded since not sufficient tissue was present. All 6 patients were men.

This study was approved by the LUMC Medical Ethical Committee (P12-117 and P19-020).

2.2. Immunohistochemical staining

Sectioning and staining of the tissue was performed using an inhouse protocol in the Erasmus University Medical Center, as described before.[29] Briefly, the formalin-fixed paraffine-embedded tissue blocks were sectioned at 4 μm. Immunohistochemistry was performed with an automated, validated and accredited staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, Tucsen, AZ, USA) using ultraview universal alkaline phosphatase red detection Kit (#760–501). Following deparaffinization and heat-induced antigen retrieval, the tissue samples were incubated according to their optimized time with mouse-anti-HCA2 in a dilution of 1:500 (for HLA-A expression) and mouse-anti-HC10 in a dilution of 1:3200 (for HLA-B/C expression), produced by the Netherlands Cancer Institute and obtained from the Immunology department of the LUMC, as described before.[30] Incubation was followed by hematoxylin II counter stain for twelve minutes and then a blue coloring reagent for eight minutes according to the manufactures instructions (Ventana). To confirm the initial diagnosis, an additional section was stained with hematoxylin and eosin for each tumor. As a positive control, tonsils were used from healthy individuals and negative controls were obtained from liver tissue. These sections were treated in exactly the same manner as the tumor samples. All the relevant controls have been performed under ISO15189:2012 certification.

2.3. Imaging and scoring

The slides were evaluated using an Olympus CX41 microscope (Olympus, U.S.A.) and intensity and percentage of the HLA-positive tumor cells were scored blinded using a specific earlier validated semi-quantitative scoring system.[26,31–34] Intensity of the staining was scored as: 0, absent; 1, weak staining; 2, clear staining; and 3, strong staining. The percentage of positive tumor cells was scored as: 1, <10 %; 2, 10–50 %; 3, 50–80 %; and 4, >80 % of cells stained. The total score (Immunoreactivity score or IRS) was calculated by multiplying the intensity and the percentage of the HLA-positive cells. The number of HLA-positive cells was determined at a 50x magnification and expressed as a percentage of the total tumor cells. Stromal cells and infiltrating immune cells that were HLA-positive were ignored. The percentage and intensity of stained tumor cells in each lesion were evaluated independently by three trained experts (EJ, KDQ and AG) and discrepancies were discussed to reach consensus. Photos were taken with an Olympus SC100 high-resolution digital color camera with a picture magnification of 50x.

2.4. Data analysis

Data were analyzed using SPSS version 25. The primary outcome of the study was to measure the effect of HLA class I expression on cSCC cells on the metastatic outcome. Differences in HLA expression between primary and control cSCCs were analyzed by calculating the difference in IRS score of the primary cSCC and the mean IRS of all control cSCCs per patient. The assumption was that the difference between the IRS scores of metastasized and non-metastasized cSCCs would be equal to zero. Since we studied primary and control cSCCs from the same patients, a One Sample *t*-test was used to determine whether the difference in the mean IRS between the groups was statistically different from the hypothesized zero. The inter-rater reliability was calculated by a two-way random intraclass correlation (ICC).

Logistic regression analyses were used for our secondary objectives for determining possible confounders. An alpha value of < 0.05 was considered as significant.

3. Results

All six primary cSCCs, 21 control (non–metastasized) cSCCs, seven metastases, and six normal skin tissue samples were colored for HLA-A and HLA-B/C. Patient and tumor characteristics are presented in Table 1.

Fig. 1 shows examples of HLA expression in a metastasized cSCC (1A, 1B and 1C), a non-metastasized cSCC from the same patient (2A, 2B and 2C) and the metastasis (3A, 3B and 3C) which are representative for all six patients. Unaffected skin of the patients showed little HLA-B/C expression, which was only present in the most basal layer of the epidermis (Fig. 2). Some cSCCs were surrounded by numerous HLA positive inflammatory cells (Fig. 3). HLA-A and HLA-B/C followed the same staining pattern in tumor cells. Furthermore, it was remarkable that tumor cells of more poorly differentiated cSCC had higher HLA expression than well-differentiated cSCC.

The mean IRS score for HLA-B/C expression was 2.07 point higher in the metastasized cSCC group compared to the non-metastasized cSCCs, which was almost statistically significant ($p = 0.065$, 95 % CI −0.18–4.32). 83.3 % of the primary metastasized cSCCs had an IRS score of 4 or higher, compared to 42.9 % in the non-metastasized cSCCs, as displayed in Table 2 and Fig. 4.

No significant difference in mean IRS for HLA-A expression was found between metastasized cSCCs and non-metastasized cSCCs (mean difference 1.19, $p = 0.32$, 95 % CI −1.61–4.00). Likewise, no correlation of HLA expression (both A and B/C) between metastasized cSCCs and the metastases (nodal, parotid, lung or in-transit) was found.

Table 1
Overview of all patients included in this study. All patients were men.

Pt	Age SCC*	Age*	Tissue*	Location	Size*	Diff.*	Inv.*	Perin.*	IRS* HCA2	IRS* HC10	AJCC*	BWH*
1	70	† 70	Primary	Cheek	25	Poor	No	–	5	5	T2	T2b
			Control 1	Arm	7	Good	Yes	Yes	5	5	T3	T2b
			Control 2	Forehead	5	Good	No	–	4	4	T1	T1
			Control 3	Forehead	10	Good	No	–	4	2	T1	T1
			Metastasis	Leg	–	–	–	–	5	3	–	–
2	56	59	Primary	Cheek	20	Moderate	No	Yes	5	5	T3	T2b
			Control 1	Temple	7	Good	No	–	1	1	T1	T1
			Control 2	Face	5	Good	No	–	2	3	T1	T1
			Control 3	Skull	22	Good	Yes	–	4	4	T3	T2b
			Metastasis	Parotid gland	–	–	–	–	2	3	–	–
			Metastasis	Lung	–	–	–	–	2	2	–	–
3	62	† 67	Primary	Ear	30	Good	Yes	Yes	6	5	T3	T2b
			Control 1	Hand	15	Good	No	–	4	2	T1	T1
			Control 2	Cheek	8	Good	No	–	1	3	T1	T1
			Control 3	Finger	15	Good	No	No	3	3	T1	T1
			Control 4	Cheek	8	Good	No	No	7	3	T1	T1
			Metastasis	Parotid gland	–	–	–	–	7	5	–	–
4	52	58	Primary	Ear	15	Moderate	Yes	No	3	5	T3	T2a
			Control 1	Chest	13	Good	No	No	2	3	T1	T1
			Control 2	Leg	–	Good	No	Yes	2	2	T3	T2a
			Control 3	Hand	10	Good	No	No	4	5	T1	T1
			Control 4	Temple	32	Poor	Yes	Yes	4	4	T3	T3
			Metastasis	Parotid gland	–	–	–	–	6	6	–	–
5	73	† 73	Primary	Pre-auricular	15	Poor	No	Yes	2	4	T3	T2b
			Control 1	Forehead	4	Good	No	–	3	4	T1	T1
			Control 2	Forehead	8	Good	No	No	3	2	T1	T1
			Control 3	Cheek	8	Moderate	No	No	3	4	T1	T1
			Metastasis	Lymph node neck	–	–	–	–	4	6	–	–
6	72	† 77	Primary	Forehead	17	Good	Yes	–	2	4	T3	T2a
			Control 1	Temple	14	Good	No	No	4	5	T1	T1
			Control 2	Temple	20	Good	No	No	2	3	T2	T1
			Control 3	Ear	16	Good	No	Yes	4	6	T3	T1
			Control 4	Face	13	Good	No	–	3	4	T1	T1
			Metastasis	Parotic gland	–	–	–	–	4	4	–	–

*Age: current age or age at death †.
*Age cSCC: age at time presence metastasized cSCC.
*Tissue: primary = metastasized cSCC, control = cSCC that did not metastasize.
*Size: horizontal size in mm.
*Diff.: differentiation grade.
*Inv.: invasion beyond the subcutaneous fat or > 6 mm.
*Perin.: perineural invasion.
*IRS: Immunoreactivity score, calculated by the sum of the intensity and the percentage of the HLA-positive cells.
*AJCC: American Joint Committee on Cancer tumor classification system, eighth edition.
*BWH: Brigham and Women's Hospital tumor Classification system.

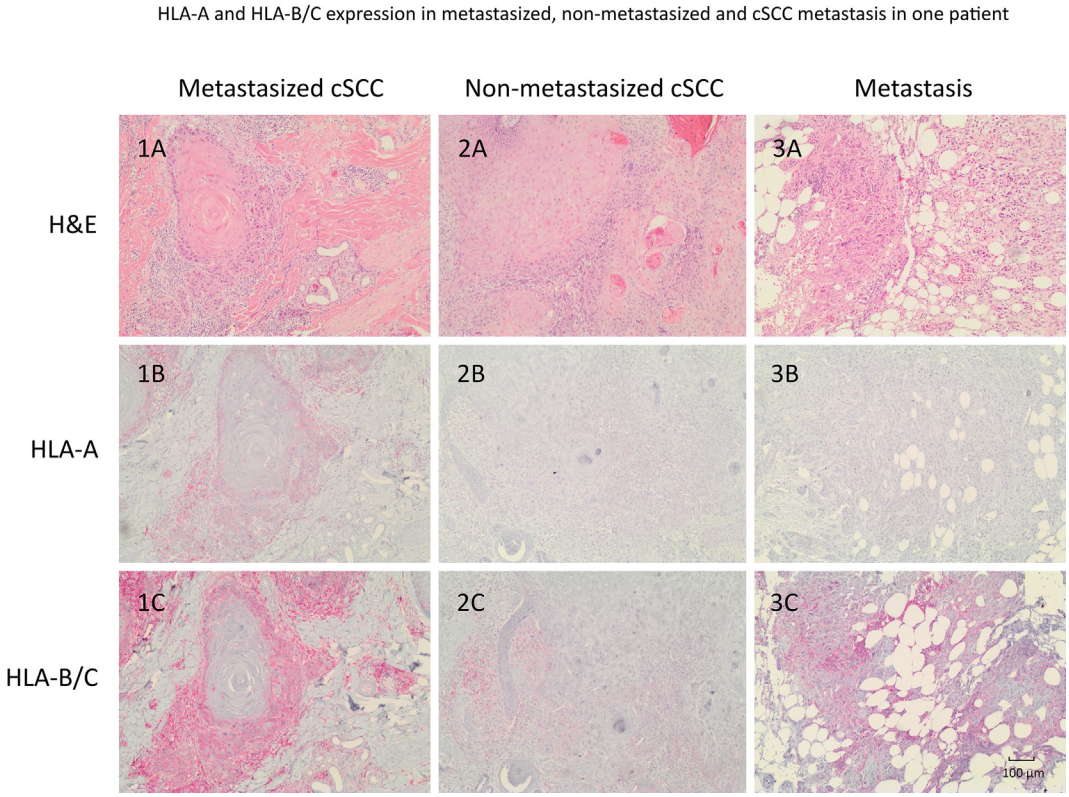

Fig. 1. Representative examples of: 1. A cSCC of the cheek that metastasized during follow-up, stained with hematoxylin and eosin (1A), a pink color representing mouse-anti-HCA2 staining for HLA-A expression (1B), and a pink color representing mouse-anti-HC10 staining for HLA-B/C expression (1C). More poorly differentiated tumor cells had higher expression of HLA than well differentiated tumor cells. 2. A cSCC located just beneath the ear that did not develop a metastasis during follow-up, stained with hematoxylin and eosin (2A), a pink color representing mouse-anti-HCA2 staining for HLA-A expression (2B), and a pink color representing mouse-anti-HC10 staining for HLA-B/C expression (2C). 3. A parotid gland metastasis, caused by the cSCC on the cheek, stained with hematoxylin and eosin (3A), a pink color representing mouse-anti-HCA2 staining for HLA-A expression (3B), and a pink color representing mouse-anti-HC10 staining for HLA-B/C expression (3C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

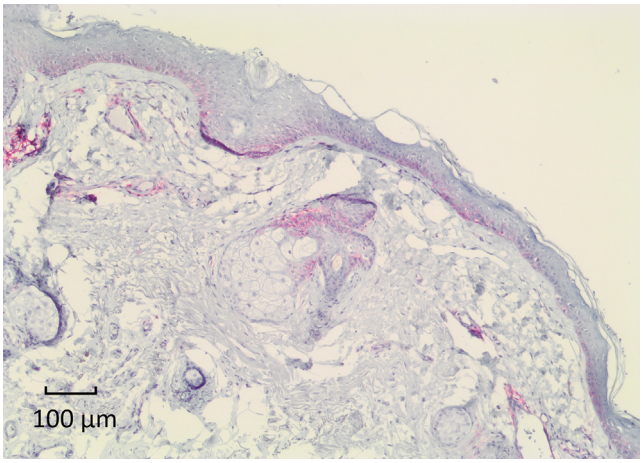

Fig. 2. HLA-B/C expression in basal keratinocytes, follicular keratinocytes and perivascular lymphocytes of normal skin. The section is stained with hematoxylin II and a blue coloring reagent. The pink color represents mouse-anti-HC10 staining (for HLA-B/C expression). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

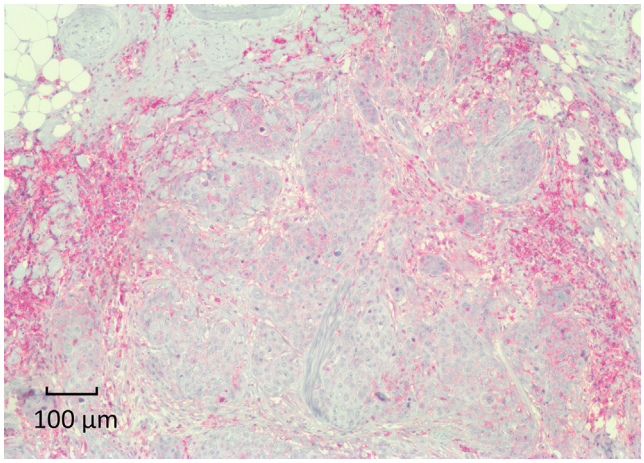

Fig. 3. Inflammatory infiltrate surrounding tumor cells. The section is stained with hematoxylin II and a blue coloring reagent. The pink color represents mouse-anti-HC10 staining (for HLA-B/C expression). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Odds ratios for HLA expression in metastasized versus non-metastasized cSCC are presented in Table 3. The non-adjusted odds was 8.5. All metastasized cSCC were located in the head and neck area compared to 71.4 % in the control group. After adjustment for tumors located in the head and neck versus elsewhere the odds ratio increased to 13.0. Adjustment for differentiation grade and perineural invasion lowered the odds ratios to statistically non-significant values, but one should notice that, because of the low statistical power all confidence intervals were very broad.

Table 2
IRS scores of HLA-B/C expression in non-metastasized and metastasized cSCCs.

IRS score	*non-metastasized* cSCC, n (%)	*metastasized* cSCC, n (%)
0	1 (4.8)	0 (0.0)
1	4 (19.0)	0 (0.0)
2	6 (28.6)	0 (0.0)
3	1 (4.8)	1 (16.7)
4	5 (23.8)	1 (16.7)
6	3 (14.3)	4 (66.7)
9	1 (4.8)	0 (0.0)
Total	21 (100)	6 (100)

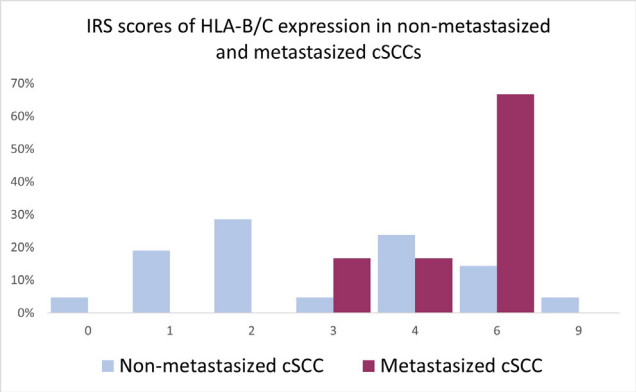

Fig. 4. IRS scores of HLA-B/C expression in non-metastasized and metastasized cSCCs, correlating with Table 2.

Table 3
HLA-B/C expression in metastasized cSCCs vs non– metastasized cSCCs. HLA expression categorized in IRS score 6 or 9 vs <6.

Possible confounders	OR	95 % CI
Non-adjusted	8.5	1.13–63.9
Adjusted for location (head vs rest)	13.0	1.36–124.3
Adjusted for horizontal size	6.3	0.66–60.7
Adjusted for differentiation grade	4.9	0.25–96.6
Adjusted for thickness	8.1	0.15–439.3
Adjusted for perineural invasion	4.9	0.33–71.4
Adjusted for horizontal size and differentiation grade	4.6	0.22–94.7

No significant difference was found between primary metastasizing cSCCs and their metastases (mean difference 0.71, p = 0.64, 95 % CI −2.82–4.24).

The inter-rater agreement was calculated between the three observers to provide a measure of reliability for the IRS scores. For HLA-A expression, an inter-rater agreement (for intensity and percentage combined) of 0.86 (p = 0.000, 95 % CI 0.78–0.92) was calculated between the three observers. This correlated with a good agreement. [35–37] An inter-rater agreement of 0.88 (p = 0.000, 95 % CI 0.81–0.93) was found for HLA-B/C expression which correlates with a good to excellent agreement.

4. Discussion

In this study we aimed to investigate differences in HLA class I expression between metastasized and non-metastasized cSCCs to examine its possible use as a marker of metastases caused by cSCCs.

We did not find significant differences in HLA-A and HLA-B/C expression between metastasized and non-metastasized cSCCs in our population, although a trend towards significance was found for HLA-B/C, with higher expression in metastasized cSCCs than in non-metastasized cSCCs. 83.3 % of the primary metastasized cSCCs had an IRS score of 4 or higher, compared to 42.9 % in the non-metastasized group. We found no correlation between HLA expression of the metastasized cSCC and nodal, parotid gland, lung or in-transit metastases for the tested HLA class I antigens. Comparing HLA-A with B/C expression, all tumor cells showed a similar staining pattern, but moderately to poorly differentiated tumor cells tended to have more HLA class I expression than well-differentiated tumor cells.

Literature on HLA expression and the metastatic risk of cSCC is scarce. In contrast to the results of Bandoh et al., we did not find that metastases had significantly lower HLA-B/C expression than the metastasized SCCs connected to these metastases.[24] These conflicting results could be due to the fact that Bandoh. et al. studied mucosal SCCs of the head and neck (HNSCC) instead of cSCC. There are some small studies investigating the differentiation grade of cSCC, suggesting that there was higher HLA class II expression in undifferentiated tumors. [16,23] Other studies were not able to confirm this association.[38].

In immunocompetent patients, downregulation of HLA class I has been described as an escape mechanism to evade the adaptive immune response, and as a risk factor for poor prognosis.[24] The underlying mechanism has been extensively investigated. Garrido et al. described a phenomenon called *T*-cell mediated immunoselection, in which tumor cells are initially HLA class I positive, but later become HLA-class I negative, in order to escape T lymphocyte infiltration.[39] In this study, we did not find this escape mechanism, and in fact, a big proportion of the metastasized cSCCs had higher HLA B/C expression compared to non-metastasized cSCCs within the same patient, although this was not significant due to the small study population. A possible explanation for the higher expression of HLA B/C in these patients may be the fact that they are receiving immunosuppressive medication. The main focus of maintenance immunosuppression in SOTR recipients is to prevent *T*-cell clonal expansion, resulting in decreased levels of T lymphocytes. We hypothesize that in SOTR, cSCCs with high HLA B/C expression evade tumor destruction because of immunosuppression-induced *T*-cell inhibition, combined with less lytic activity of NK cells, due to the presence of inhibitory NK cell ligands expressed on HLA class I molecules.[38,40] Hopefully, in future research it will be able to include a larger study population to test this hypothesis and see whether there are in fact significant differences between HLA-expression in metastasized and non-metastasized tumors in OTR. Bigger groups may also allow for specific HLA class I combinations where inhibitory motifs may be present/absent, which would serve as definitive proof for the hypothesis. Furthermore, HLA-C is the most important ligand for NK-cells, and therefore it might be useful to distinguish HLA-B and C in future research.

There are many more escape mechanisms described in literature that could play a role in these tumors, for instance alterations of the apoptosis program, loss of tumor-specific antigens and production of immunosuppressive factors, such as TGF-B, VEGF and IL-10.[41] The HLA system is an elegant, yet very complex system that has not been fully understood yet and there are multiple other factors that may play a role in cSCC development and prognosis.

A strength of this study is that we compared metastasized with non-metastasized cSCCs from the same patients, which results in more objective measurements on cSCC prognosis.

A limitation of our study is that we identified the metastasized cSCCs only based on their location and time relation with the metastases, which may have led to some misclassification. Misclassification can bias the outcome to the null, i.e. an apparent association may disappear. The determination of DNA mutation profiles in cSCC and metastases is a more reliable method to identify the metastasized cSCC and could have even led to a stronger association.

5. Conclusion

In conclusion, although not statistically significant, this study shows that HLA-B/C expression may be increased in metastasized com-

pared to non-metastasized cSCC, which offers new perspectives to studying the underlying pathogenesis of cSCC metastatic potential in SOTR. CSCCs have a heterogenous cell surface expression of HLA class I antigens and future research is needed to determine if high HLA class B/C expression on the surface of tumor cells will indeed increase the risk of metastasizing.

6. Authorship

Estella de Jong, Maarten H. Vermeer and Jan N. Bouwes Bavinck participated in the research design, writing of the paper, the performance of the research and the data analysis. Koen D. Quint, Abdoel El Ghalbzouri and Rob M. Verdijk participated in the performance of the research. Sebastiaan Heidt, Frans Claas and Johan W. de Fijter contributed to the research design. Jelle Goeman helped with the statistical analyses. Roel E. Genders participated in the patient selection and writing of the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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