Targets for personalized treatment of conjunctival melanoma

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Chapter 6

HLA class I antigen expression in conjunctival melanoma is not associated with PD-L1/PD-1 status

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Abstract

Purpose: Anti-tumor T cells need expression of human leukocyte antigen (HLA) class I molecules, but can be inhibited by ligands such as PD-L1. We determined expression and regulation of these molecules in human conjunctival melanoma (CM) samples, cell lines, and murine xenografts.

Methods: Immunofluorescence staining was performed to examine the expression of HLA-A, HLA-B/C and beta-2-microglobulin (B2M) in 23 primary CM samples. HLA class I expression was compared with clinicopathological characteristics, the presence of tumor-infiltrating leukocytes and PD-L1/PD-1 status. The effect of interferon gamma (IFN-γ) on HLA class I expression was tested on three CM cell lines using qPCR and flow cytometry. Furthermore, HLA class I expression was determined in CM cell line-derived murine xenografts.

Results: One third of tumors had positive HLA-A, HLA-B/C and B2M expression. A positive expression was especially seen in thin and epibulbar tumors, but not associated with recurrences. HLA class I expression was correlated with M2 macrophage density, and tended to associate with $CD8^+$ T cell density, but was independent of PD-L1 or PD-1 expression. IFN-γ upregulated HLA class I expression and genes involved in HLA transcription and transportation on CM cell lines. Murine xenografts showed a comparable HLA class I expression as their respective cell lines.

Conclusions: Our data indicate that subsets of CM have positive HLA class I expression, and HLA class I and PD-L1/PD-1 are expressed independently. When one considers immunotherapy to a CM patient, one should keep the variable HLA class I expression in mind.

Key words: conjunctival melanoma, HLA class I, immune escape, animal model, tumor infiltrating lymphocytes.

Introduction

Currently, a lot of attention is given to the role of the immune system in tumor growth, control and treatment. The theory of immune surveillance, which states that the immune system can recognize and destroy tumor cells, has been widely accepted and provides targets for therapy[1]. The discovery of immune checkpoint regulators has transformed the treatment of many malignancies: cancer often protects itself from T cell attack by blocking T cell signals. Binding of programmed death ligand 1 (PD-L1) on the cancer cell to PD-1 on the T cell will suppress the T cell's function, impair cytokine production, and induce T cell apoptosis. To reverse this immunologically-tolerant state, antibodies against these immune checkpoints have been developed, which target e.g. PD-1 or cytotoxic T-lymphocyte-associated protein 4 (CTL-A4). The results in cutaneous melanoma, bladder cancer, non-small cell lung cancer and mucosal melanoma are promising, but restricted to subsets of patients[2-5].

Expression of the human leukocyte antigen (HLA) class I molecules is needed to recognize and lyse tumor cells by cytotoxic T cells: cytotoxic T cells (CTL) can destruct cells which express the appropriate peptide/MHC class I complex[6]. HLA class I molecules consist of a glycoprotein heavy chain (coded in the HLA region on chromosome 6p21) and a beta-2-microglobulin (B2M) light chain (gene located on chromosome 15)[7]. Defects in HLA class I antigen-processing machinery (APM) components impair effective immune responses to cancer, although tumor-infiltrating lymphocytes may still present. A lack of HLA class I expression which may be associated with a worse survival[8], has been described in many malignancies, and can either be "soft" (reversible) or "hard" (irreversible), due to various mechanisms[9]. A low HLA expression can often be corrected by interferon-gamma (IFN-γ), thereby restoring a tumor cell's susceptibility to be lysed by T cells.

Although a lot of immunological research in melanoma has focused on cutaneous lesions, little is known about melanoma of the conjunctiva. Conjunctival melanoma (CM) is a rare type of mucosal melanoma that originates in the melanocytes of the basal layer of the conjunctiva and accounts for 5% of all ocular

melanoma[10]. The incidence in Caucasians is 0.8/million[11], and in recent years has been increasing in Western countries[12,13]. The appearance of CM ranges from amelanotic to heavily pigmented and CM can occur in any part of the epibulbar or non-epibulbar conjunctiva. Wide local excision with adjuvant treatment (cryotherapy, chemotherapy, brachytherapy) is the most common therapy, while more extensive surgical procedures are reserved as a last resort therapy[14]. Despite treatment, recurrence rates of 26-61% 5-year post treatment have been reported[15-18], with metastases occurring in 11-16% of patients[15,19]. Since limited chemotherapeutic options are available for extended or metastasized CM, these patients may benefit from the new T-cell-mediated treatments. We recently described that PD-1 and PD-L1 positive cells are present in CM[20], making treatment with PD-L1/PD-1 inhibitors potentially interesting. Very recently, Nivolumab (PD-1 inhibitor) led to a successful clinical response in a CM patient with breast metastases[21].

However, effective T cell immune responses also need a proper expression of the HLA class I antigens. Currently, very little is known regarding the expression of the HLA class I antigens in CM. We studied the degree of HLA class I expression in human CM samples, CM cell lines and animal xenografts. We found out that HLA class I expression was not associated with PD-L1/PD-1 status, and detected positive HLA class I expression in only one third of primary CM.

Methods

Patient data and selection

Patients were treated at the Leiden University Medical Center, Leiden, The Netherlands, between 1996 and 2014. The tumour cohort included 23 primary CM for which sufficient material was available (Table 1). Tumors were examined by an experienced ophthalmic pathologist (SD). Tumor stage was assessed according to the 7th edition of the AJCC TNM cancer staging manual[22]. Local recurrence was defined as a histologically-proven recurrence. Metastasis was verified by histological examination or imaging analysis. Total follow-up time was from the

time of diagnosis to the last known status. The study adhered to the tenets of the Declaration of Helsinki, and the Medical Ethical Committee of LUMC agreed with this retrospective study.

Immunohistological staining and scoring

Immunofluorescence (IF) staining of HLA-A, HLA-B/C and B2M on human paraffin-embedded CM sections was performed as previously described[19,23]. For immunohistochemistry (IHC) on mouse xenografts, the procedures were performed as described[24]. In short, primary antibodies HCA2 (mouse, anti-HLA-A, IgG1, MUB2036P, 1:50; Nordic Mubio), HC10 (mouse, anti-HLA-B/C, IgG2a, MUB2037P, 1:100; Nordic Mubio) and B2M (rabbit, Z0311, 1:2000 (IHC), 1:1000 (IF); DAKO) were used for both IF and IHC staining. Isotype control staining was obtained by replacing primary antibodies with control antibodies with respective isotypes antibodies. The detailed information of secondary antibodies is in Supplementary Table 1. IF images were taken using a Zeiss LSM 700 confocal scanning microscope. Based on the tumor size, one to five representative images at high power (250X) were randomly selected from different areas for scoring. Tumor areas were distinguishable from normal tissue by DAPI (4',6-diamidino-2-phenylindole; H-1200; Vector Laboratories, USA) staining. Two independent observers (JC and NB or EJ), blinded to the clinicopathological data, evaluated the intensity and percentage of HC10, HCA2 and B2M expression separately. In case of discrepancies, a consensus was reached by simultaneous analysis. The semi-quantitative scoring system was obtained from prior studies[23,25-27]: intensity was scored as 0 (absent), 1 (weak), 2 (moderate) and 3 (strong), and the percentage of positive cells as 0 (0%), 1 (1-5%), 2 (6-25%), 3 (26-50%), 4 (51-75%) and 5 (76-100%). A single value was reached by adding intensity and percentage, and was used to create three categories: negative (0-2), weak (3-6) and positive expression (7-8). Stromal cells served as an internal positive control. The B2M staining of one tumor was not scorable, and was therefore excluded. The images of hematoxylin and eosin (HE) staining were captured using the Philips Image Management System 2.2. The staining and scoring method of tumor-infiltrating lymphocytes (CD3, $CD3^+CD8^+$,

$CD3^+CD8^-$, $CD3^+CD8^-Foxp3^+$ and $CD3^+CD8^-Foxp3^-$ T cells, and CD68 and $CD68^+CD163^+$ macrophages) was performed as previously described[20]. Expression of PD-L1 on tumor or stromal cells and of PD-1 was determined as described[20].

HLA typing

HLA typing was performed at the Laboratory of the Department of Immunohematology and Blood Transfusion (IHB, LUMC) using the Gen-Probe LIFECODES HLA-SSO HLA Typing assay for HLA class I typing.

Cell lines and treatment

Three CM cell lines (CRMM1, CRMM2 and CM2005.1) derived from primary tumors, kindly provided by Michele Madigan (Sydney, Australia) and Sander Keijser (Leiden University Medical Center, Leiden, The Netherlands) were used[28,29]. To examine the influence of IFN-γ on HLA class I expression, CM cells were cultured with or without 100 international units (IU)/ml of IFN-γ (ImmunoTools, Germany) for 48h[30].

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

The isolation of total cellular RNA, complementary DNA conversion and qPCR was performed based on a standard protocol of our laboratory, as described previously[31]. Primer sequences are in Supplementary Table 2. CFX Manager 3.1 (Bio-Rad) software was used to analyse the data: the Ct values of each sample were normalized against the geometric mean Ct values of housekeeping genes (RPS11 and RPL13).

Flow cytometry

Cells were incubated with anti-HLA class I antibody (W6/32, 311414, Alexa Fluor 647; BioLegend) to detect total HLA class I expression. HLA-allele specific monoclonal antibodies were obtained from the IHB Department, LUMC (Supplementary Table 3)[32,33], and were applied for detecting HLA allele-specific expression. Appropriate FITC (F0315, DAKO) or RPE (R5111, DAKO) secondary antibodies were selected. Cells were collected (10,000-50,000 cells per live gate)

using Accuri C6 or FACSCalibur cytometer (Beckton Dickinson). Results were analysed with FlowJo software (V10.0.7, Flowjo LLC).

In vivo xenografts

Mice were used to grow xenografts of CM cell lines[24]. Briefly, non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) IL-2 receptor gamma chain null mice were subconjunctivally injected with one of three CM cell lines. Subsequently, cells (CRMM1 and CM2005.1) obtained from established CM tumor xenografts were reinjected into the subconjunctival space of the second set of NOD/SCID mice. This *in vivo*-passaging technique led to lung metastases. The animal experiment was maintained under the guidelines of the Schepens Eye Research Institute, Boston, USA, and experiments were carried out based on the guidelines for the use of animals in research of the Association for Research in Vision and Ophthalmology.

Statistical analysis

Data were analyzed with SPSS software version 22.0 (SPSS, Inc., Chigaco, IL, USA). Statistical significance was defined as $p \leq 0.05$. Fisher-Freeman-Halton test and Kruskal-Wallis test were used.

Results

Frequent downregulation of HLA class I expression in primary CM

HCA2 and HC10 recognize HLA-A and HLA-B/C, respectively[34]. B2M is a component of the HLA class I complex[35]. We used triple immuno-fluorescence staining to determine the percentage of positively-staining tumor cells and the intensity of staining on formalin-fixed, paraffin-embedded sections of 23 human primary CM (Figure 1). Expression of HLA-A, HLA-B/C and B2M correlated strongly (Table 2). Four (17%) cases showed no staining of HLA-A, three (13%) cases no expression of HLA-B/C, and three cases (14%) no staining of B2M (Table 1). Twelve (52%), 13 (57%) and 11 (50%) cases exhibited weak expression (scores 3-6) of HLA-A, HLA-B/C, and B2M, respectively, with the remaining tumors

showing a positive expression of B2M, HLA-A and HLA-B/C. Heterogenous HLA class I expression was seen in three cases, as presented in Figure 2.

Patient characteristics

We correlated expression levels of HLA-A, HLA-B/C, and B2M with clinical and histopathological data (Table 1, supplementary Table 4). The mean age of the patients at diagnosis was 62.6 (SD 14.3) years, and 13 of the 23 patients (57%) were women. The median follow-up time was 42 months (range 3-249 months). At the end of the follow-up, 10 (43%) patients had developed local recurrences, three (13%) had died from CM metastases, two (9%) from unknown causes, while 18 (78%) patients were alive. Local recurrence was only related to tumor largest basal diameter (p=0.046), not to tumor thickness (p=0.24), or TNM stage (p=0.089). HLA-A, HLA-B/C or B2M expression was not correlated with largest basal diameter, local recurrence, metastasis formation, or melanoma-related survival. Epibulbar/T1 tumors had a higher HLA class I expression than non-epibulbar/T2 tumors (p=0.03 HCA2, p=0.046 B2M, Table 1). Thicker tumors showed a lower HLA-A as well as B2M expression than the thinner tumors (p=0.03 and 0.046, respectively), with a trend for downregulation of HLA-B/C (p=0.08). Additionally, thick tumors had fewer $CD68^+CD163^+$ macrophages (r=-0.56, p=0.01). Expression of HLA class I was associated with the number of $CD68^+CD163^+$ macrophages in the tumor area (p=0.03 HCA2, p=0.07 HC10, p=0.02 B2M, Table 2) and tended to correlate with $CD8^+$ T cell density (p=0.09 HCA2, p=0.10 HC10, p=0.14 B2M, Table 2). These data suggest that macrophages and T cells play a role in stimulating HLA class I expression in CM (Table 2). Expression of PD-L1 on tumor or stromal cells, and of PD-1 occurred independently of HLA class I expression (Table 1 and Cao et al.[20]). Figure 2.

mRNA and membrane expression of HLA class I in CM cell lines

To better understand the regulation of HLA class I expression in CM, we studied

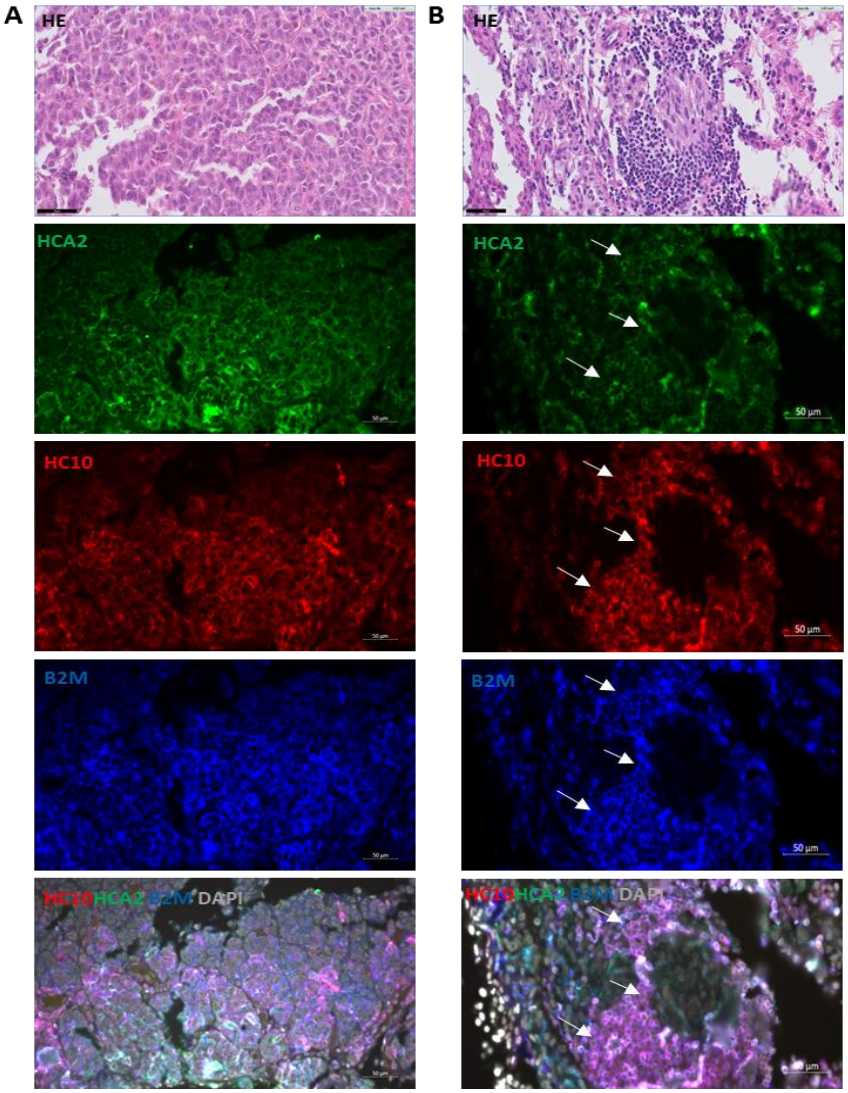

Figure 1. **Immunofluorescence analysis of HLA class I expression**. Representative pictures of triple staining show expression on CM cells in one tumor (**A**) and on T cells in another (**B**): similar membrane expression patterns are seen for the antibodies HCA2 (HLA-A, green), HC10 (HLA-B/C, red), and B2M (HLA class I, blue), respectively. White arrows indicate T cells surrounding the tumor area. Scale bar is 50µm.

Table 1. Clinicopathological characteristics and correlation with HLA-A, HLA-B/C and B2M expression.

Characteristic	All cases	HLA-A (HCA2)				HLA-B/C (HC10)				B2M			
		negative	weak	positive		negative	weak	positive		negative	weak	positive	
	Cases (%)	*Cases (%)*	*Cases (%)*	*Cases (%)*	*p*	*Cases (%)*	*Cases (%)*	*Cases (%)*	*p*	*Cases (%)*	*Cases (%)*	*Cases (%)*	*p*
Overall	23 (100)	4 (17)	12 (52)	7 (30)		3 (13)	13 (57)	7 (30)		3 (14)	11 (50)	8 (36)	
Sex													
Male	10 (43)	1 (25)	4 (33)	5 (71)	0.28*	1 (33)	4 (31)	5 (71)	0.25*	1 (33)	3 (27)	5 (63)	0.30*
Female	13 (57)	3 (75)	8 (67)	2 (29)		2 (67)	9 (69)	2 (29)		2 (67)	8 (73)	3 (38)	
Age at diagnosis, yr													
⩽60	11 (48)	2 (50)	6 (50)	3 (43)	1.00*	1 (33)	8 (62)	2 (29)	0.42*	1 (33)	7 (64)	3 (38)	0.51*
>60	12 (52)	2 (50)	6 (50)	4 (57)		2 (67)	5 (38)	5 (71)		2 (67)	4 (36)	5 (63)	
Tumor thickness	***N=21***	***N=4***	***N=11***	***N=6***		***N=3***	***N=13***	***N=5***		***N=3***	***N=11***	***N=7***	
Median [range], mm	1.2 [0.1-16.0]	1.9 [0.8-16.0]	1.4 [0.2-6.0]	0.2 [0.1-2.5]	*0.03*†	2.5 [0.8-16.0]	1.2[0.2-6.0]	0.2 [0.1-2.5]	0.08†	2.5 [0.8-16.0]	1.4 [0.2-6.0]	0.2 [0.1-2.5]	*0.046*†
Tumor LBD	***N=20***	***N=4***	***N=10***	***N=6***		***N=3***	***N=12***	***N=5***		***N=3***	***N=10***	***N=7***	
Median [range], mm	11.0 [2.0-30.0]	12.0 [7.0-30.0]	12.0 [5.0-20.0]	6.5 [2.0-12.0]	0.14†	16.0 [7.0-30.0]	12.0 [5.0-20.0]	6.0 [2.0-12.0]	0.06†	16.0 [7.0-30.0]	11.0 [5.0-20.0]	7.0 [2.0-12.0]	0.17†
Location													
Epibulbar	16 (70)	1 (25)	8 (67)	7 (100)	*0.03* *	1 (33)	9 (69)	6 (86)	0.30*	1 (33)	7 (64)	8 (100)	*0.046* *
Non-epibulbar	7 (30)	3 (75)	4 (33)	0 (0)		2 (67)	4 (31)	1 (14)		2 (67)	4 (36)	0 (0)	
cTNM													
T1	16 (70)	1 (25)	8 (67)	7 (100)	*0.03* *	1 (33)	9 (69)	6 (86)	0.30*	1 (33)	7 (64)	8 (100)	*0.046* *
T2	7 (30)	3 (75)	4 (33)	0 (0)		2 (67)	4 (31)	1 (14)		2 (67)	4 (36)	0 (0)	
Local recurrence													
No	13 (57)	4 (100)	7 (58)	2 (29)	0.08*	3 (100)	8 (62)	2 (29)	0.18*	3 (100)	7 (64)	3 (38)	0.20*
Yes	10 (43)	0 (0)	5 (42)	5 (71)		0 (0)	5 (38)	5 (71)		0 (00)	4 (36)	5 (63)	
Distant metastasis													
No	20 (87)	4 (100)	10 (83)	6 (86)	1.00*	3 (100)	12 (92)	5 (71)	0.53*	3 (100)	10 (91)	7 (88)	1.00*
Yes	3 (13)	0 (0)	2 (17)	1 (14)		0 (0)	1 (8)	2 (29)		0 (0)	1 (9)	1 (13)	
Tumoral PD-L1													
No	19 (83)	3 (75)	11 (92)	5 (71)	0.46*	2 (66)	12 (92)	5 (71)	0.31*	2 (66)	10 (91)	6 (75)	0.43*
Yes	4 (17)	1 (25)	1 (8)	2 (29)		1 (33)	1 (8)	2 (29)		1 (33)	1 (9)	2 (25)	
Stromal PD-L1													
No	9 (39)	1 (25)	6 (50)	2 (29)	0.62*	0 (0)	8 (62)	1 (14)	0.051*	0 (0)	6 (55)	3 (38)	0.25*
Yes	14 (61)	3 (75)	6 (50)	5 (71)		3 (100)	5 (38)	6 (86)		3 (100)	5 (45)	5 (63)	
PD-L1													
No	7 (30)	1 (25)	5 (42)	1 (14)	0.59*	1 (66)	5 (38)	1 (14)	0.69*	1 (66)	5 (45)	1 (13)	0.27*
Yes	16 (70)	3 (75)	7 (58)	6 (86)		2 (33)	8 (62)	6 (86)		2 (33)	6 (55)	7 (88)	

LBD = largest basal diameter. cTNM = clinical TNM stage, based on the first occurring conjunctival melanoma. * = Fisher-Freeman-Halton test; † = Kruskal-Wallis test. Italic *p* values are ≤ 0.05. Some columns/rows do not add up to 100 due to rounding. HLA scoring system is based on previous studies[23]: intensity was scored as 0 (absent), 1 (weak), 2 (moderate) and 3 (strong), and the percentage of positive cells as 0 (0%), 1 (1-5%), 2 (6-25%), 3 (26-50%), 4 (51-75%) and 5 (76-100%). A single value was given by adding intensity and percentage, and was used to evaluate HLA class I expression in three categories: negative (0-2), weak (3-6) and positive (7-8). B2M: beta-2-microglobulin.

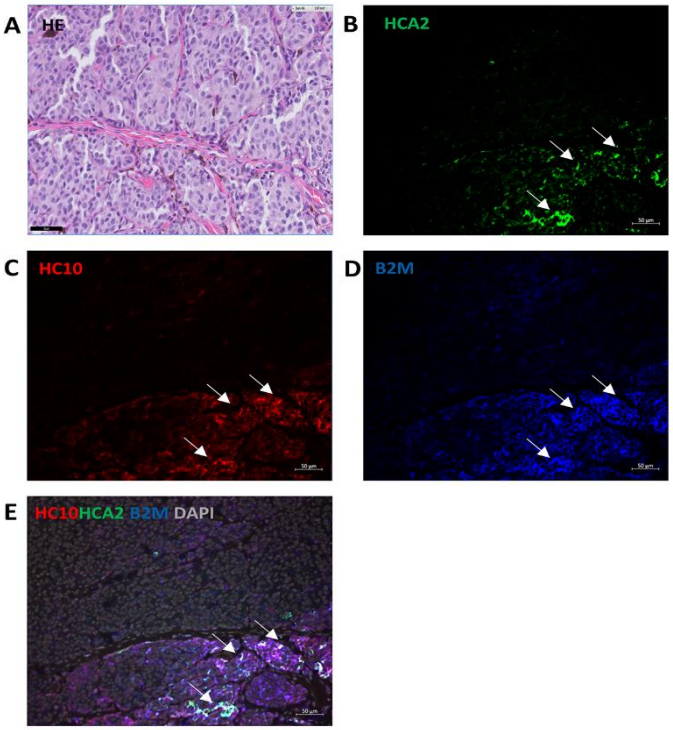

Figure 2. Heterogeneous expression of HLA class I antigens in a CM. Representative IF staining shows membranous HCA2 (HLA-A, green), HC10 (HLA-B/C, red), and B2M (HLA class I, blue) expression only in the lower part of the tumor area. White arrows indicate areas of positive expression. Scale bar is 50μm.

Table 2. Correlation between thickness, HLA expression and different types of infiltrating immune cells.

		HLA-A (HCA2)	HLA-B/C (HC10)	B2M	CD3	$CD3^+CD8^+$	$CD3^+CD8^-$	$CD3^+CD8^-$ $Foxp3^-$	$CD3^+CD8^-$ $Foxp3^+$	CD68	$CD68^+C$ $D163^+$
Thickness	*r*	-0.49	-0.5	-0.53	-0.35	-0.39	-0.32	-0.33	-0.27	-0.35	-0.5
	P	*0.02*	*0.02*	*0.01*	0.12	0.08	0.16	0.15	0.23	0.12	*0.02*
HLA-A (HCA2)	*r*	-	0.94	0.97	0.25	0.36	0.17	0.18	0.22	0.4	0.45
	P	-	*<0.01*	*<0.01*	0.25	0.09	0.45	0.41	0.31	0.06	*0.03*
HLA-B/C (HC10)	*r*	-	-	0.97	0.29	0.36	0.18	0.15	0.33	0.3	0.38
	P	-	-	*<0.01*	0.18	0.1	0.4	0.51	0.13	0.16	0.07
B2M	*r*	-	-	-	0.31	0.33	0.24	0.25	0.28	0.43	0.49
	P	-	-	-	0.17	0.14	0.27	0.25	0.21	*0.04*	*0.02*

r =two-tailed Spearman correlation coefficient, with 23 observations. $P \leq 0.05$ are in italics. The staining and scoring method is previously described16. B2M: beta-2-microglobulin.

three CM cell lines. For this, we first performed HLA-DNA typing (Table 3). CRMM1 genetically carries the HLA-A*02:01 allele, which is a very attractive genotype, because so far, most of the clinical T-cell receptor (TCR) cancer therapies are HLA-A*02:01 restricted.

Table 3. Results of HLA typing in three CM cell lines.

Cell line	HLA alleles
CRMM1	A*02:01/02:07/02:09+, A*02:01/02:07/02:09+, B*44:03/44:07/44:13+, B*44:03/44:07/44:13+, C*04:01/04:07/04:09N+, C*04:01/04:07/04:09N+, DRB1*07:01/07:03/07:04+, DRB1*07:01/07:03/07:04+ DQB1*02:01/02:02/02:04+, DQB1*02:01/02:02/02:04+
CRMM2	A*03:01/03:37/03:03N+, A*30:01/30:14L/30:15+, B*40:01/40:22N/40:54+, B*44:02/44:23N/44:27+, C*03:04/03:24/03:35+, C*05:01/05:05/05:07N+, DRB1*11:01/11:04/11:02+, DRB1*13:01/13:02/13:05+, DQB1*06:03/06:07/06:14+, DQB1*06:03/06:07/06:14+
CM2005.1	A*11:01/11:05/11:07+, A*24:02/24:06/24:09N+, B*14:02/14:09/14:16+, B*35:01/35:41/35:07+, C*02:02/02:09/02:11+, C*04:01/04:09N/04:28+, DRB1*01:01/01:07/01:04+, DRB1*14:54/14:01/14:07+, DQB1*05:01/05:03/05:02, DQB1*05:03/05:02/05:07+

Next, we determined expression of HLA-A, HLA-B, B2M on the three cell lines and analyzed the effect of IFN-γ, which is a known HLA stimulator. HLA allele-specific antibodies were selected according to the results of the HLA DNA typing. Flow cytometry analysis showed that most genetically-determined HLA class I antigens were expressed, except for HLA-A2 and HLA-B44 on CRMM1, and HLA-B44 on CRMM2 (Figure 3A). IFN-γ treatment increased the expression of most alleles, except for HLA-A2 on CRMM1 and HLA-B44 on CRMM2, indicating a hard loss of HLA antigens.

When loss of expression was observed, we set out to determine whether there was a normal activity of the transcriptional regulators (CIITA, IRF1, NLRC5) and

genes encoding the peptide-loading machinery (TAP1, TAP2), using specific primers and qPCR. NLRC5 is essential for HLA class I transcription, whereas CIITA and IRF1 are involved in HLA class I as well as class II transcriptional regulation. TAP1 and TAP2 transport peptides from the cytoplasm to the endoplasmic reticulum and present these peptides to MHC class I molecules[35]. Figure 3B shows that the three CM cell lines presented various baseline mRNA levels of the regulator genes. mRNA expression of not only HLA-A, HLA-B, B2M, but also of CIITA, IRF1, NLRC5, TAP1, and TAP2 was increased after IFN-γ stimulation, indicating that these molecules were normally responsive.

HLA class I expression was maintained in animal xenografts

We previously established a CM metastasis model using *in vivo* passaging[24], and wondered whether *in vivo* placement of the tumor cell lines affected HLA expression. Because immunodeficient mice were used, the HLA class I expression on xenografts would not be influenced by local infiltration with T lymphocytes and environmental cytokines, thus showing the tumor's "natural" expression. IHC staining using HCA2, HC10, and B2M antibodies showed that HLA class I expression of CM cells was maintained in the murine models in the same manner as on the cell lines *in vitro* (Figure 4 and Table 4). There seemed a trend towards a decrease of HLA class I expression during tumor progression (primary-in vivo passaged-metastasis, Figure 4); however, HLA class I was expressed at all stages.

Discussion

We investigated expression of HLA class I molecules in primary CM and compared HLA class I expression with clinical parameters. In only a third of CM did we observe a positive expression of HLA-A (~30%), HLA-B/C (~30%), and B2M (~36%). The presence of a negative or weak HLA class I antigen expression in about 70% of CM is similar to that of bladder cancer, but higher than of cutaneous mela-

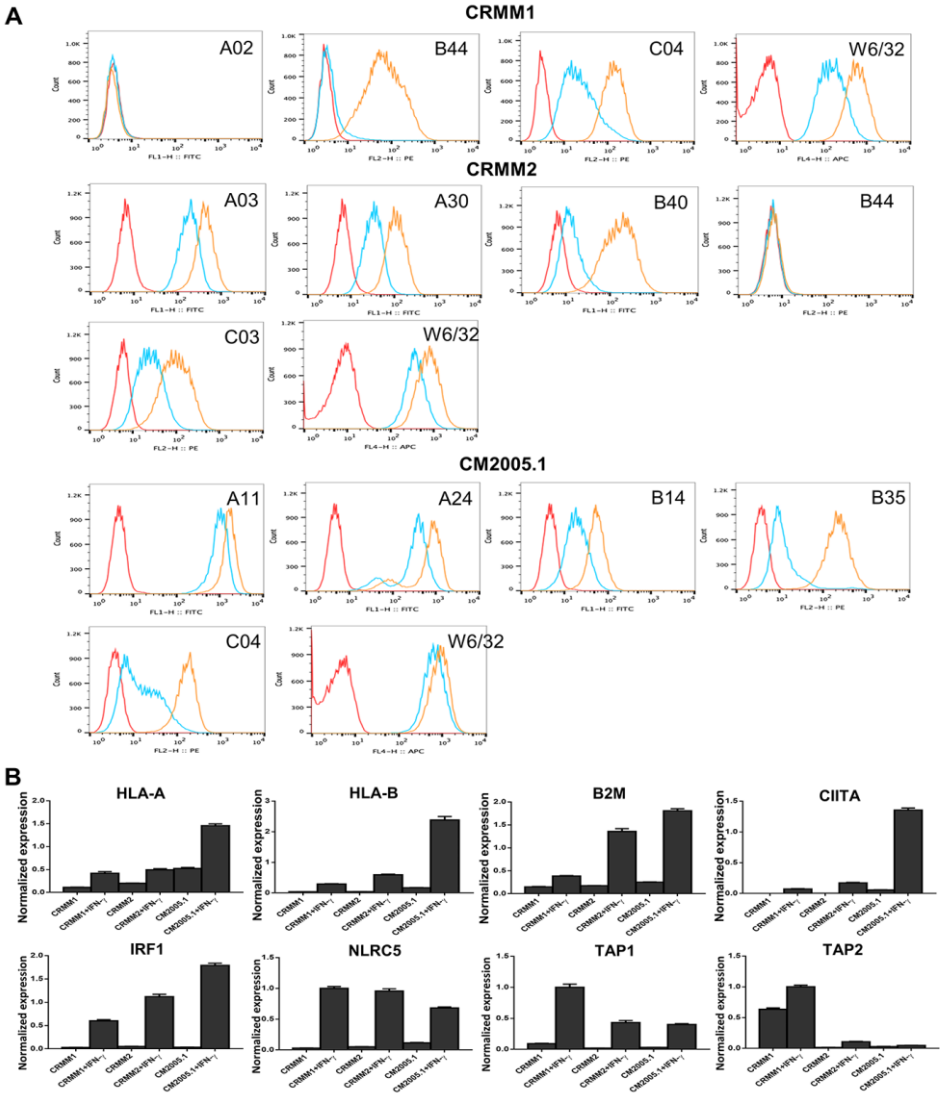

Figure 3. IFN-γ upregulates protein and gene expression of HLA class I, and increases mRNA levels of several HLA regulators in CM cell lines. Cells were treated with or without IFN-γ (100IU/ml) exposure for 48h. (**A**) Representative histograms show the surface expression of HLA class I (W6/32) and specific HLA allele antigens of CRMM1, CRMM2 and CM2005.1 cell lines. Histograms with red, blue and brown line represent unstained, baseline expression, and expression after IFN-γ stimulation, respectively. (**B**) The mRNA expression of HLA genes (HLA-A, HLA-B, B2M), HLA transcriptional regulators (CIITA, IRF1, NLRC5) and genes encoding the peptide-loading machinery (TAP1, TAP2) were variably increased, after IFN-γ stimulation.

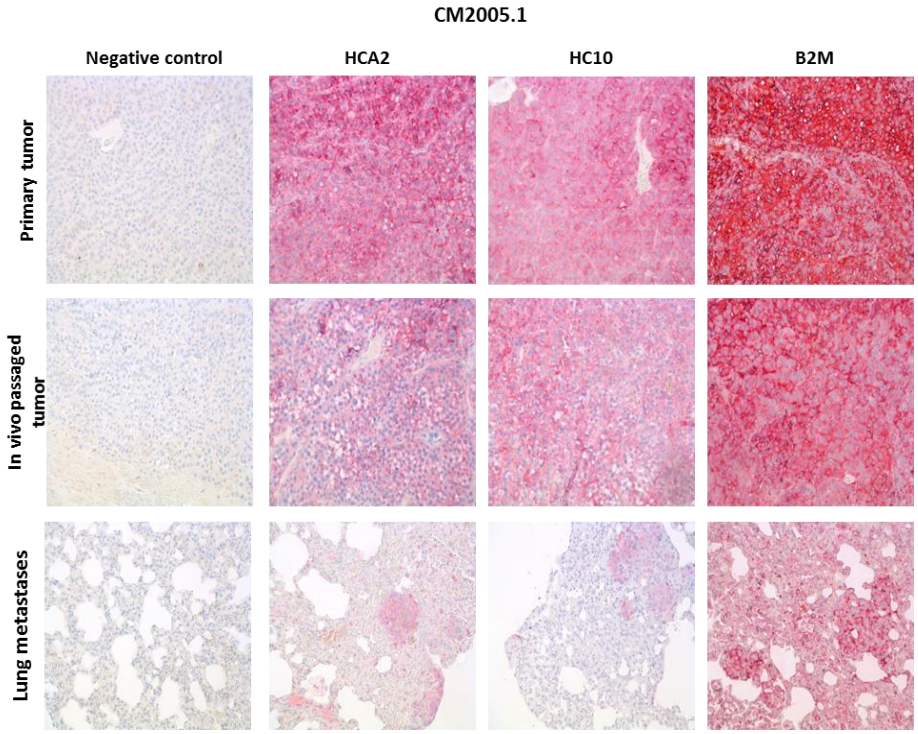

Figure 4. Murine xenografts maintain their HLA class I expression. Representative images present the membranous expression of HLA-A (HCA2), HLA-B/C (HC10) and B2M in a CM2005.1 murine model. Cells from cell line CM2005.1 were first placed subconjunctivally, transferred to the subconjunctival space of a second set of mice, and spread systemically, giving rise to lung metastases. Images were taken at ×200 magnification.

noma and intrahepatic cholangiocarcinoma[37,38]. Several factors may contribute to this observed lack of HLA class I antigen expression: mutations or loss of heterozygosity (LOH) in chromosomes 6 or 15 (where B2M is located), or prior immune selection, leaving only HLA class I negative tumor cells[39]. We did indeed find some tumors that lacked B2M, but do not have information on mutations in this gene. An absent or low expression of HLA class I has been associated with a worse survival in patients with advanced cutaneous melanoma, cervical cancer and rectal cancer[26,40,41]. We did not find a correlation between HLA class I expression and melanoma-related survival in primary CM. However, we did notice that an aberrant

Table 4. HLA class I expression in CM murine xenografts.

	Staining intensity/% Positive tumor cells		
Material	**HLA-A (HCA2)**	**HLA-B/C (HC10)**	**B2M**
CRMM1			
Primary tumor	2/2 (Weak)	2/2 (Weak)	3/3 (Weak)
In vivo-passaged tumor	0/0 (Negative)	2/1 (Weak)	2/2 (Weak)
Lung metastasis	0/0 (Negative)	0/0 (Negative)	0/0 (Negative)
CRMM2			
Primary tumor	2/3 (Weak)	1/2 (Weak)	3/4 (Positive)
CM2005.1			
Primary tumor	3/3 (Weak)	3/2 (Weak)	3/5 (Positive)
In vivo-passaged tumor	3/1 (Weak)	3/2 (Weak)	3/4 (Positive)
Lung metastasis	1/3 (Weak)	2/3 (Weak)	2/3 (Weak)

The staining intensity scoring system is described in the material and methods. B2M: beta-2-microglobulin.

HLA class I expression was significantly correlated with one of the known prognostic factors, i.e. tumor thickness, which is concordant with previous investigations in cervical cancer and cutaneous melanoma[42,43]. The association of HLA class I expression with M2 macrophages and $CD8^+$ T cells suggests a connection of IFN-γ with HLA class I expression in the tumor. The cytokine IFN-γ, mainly secreted by CD4 Th1, CD8 T lymphocytes, macrophages and NK cells[44], is known to be a key modulator of HLA expression. The data on expression and regulation of most of the HLA antigens on three unique CM cell lines, using HLA DNA typing, qPCR and flow cytometry, indeed confirmed this theory: after IFN-γ treatment, mRNA expression levels of CIITA, IRF1, NLRC5, TAP1 and TAP2 were increased, together with HLA class I molecules. Our finding is similar to previous investigations using UM cell lines[36]. Interestingly, the flow cytometry data showed that not all alleles were expressed on the cell surface: although the HLA-A*02:01

and HLA-B*44:02 alleles were detected by HLA DNA typing, they were not expressed on the cell surface of CRMM1 and CRMM2, respectively. This result may be explained by allele-specific mutations, or by post-translational instability due to a missing disulfide bridge[45]. This remains to be investigated.

To further test our theory that the presence of infiltrating immune cells plays a role in HLA expression, we looked at murine xenografts, that were obtained by injection of three CM cell lines into the subconjunctiva of NOD/SCID-mice (with impaired T, B and NK cells). We noticed that primary tumors of the xenograft showed a similarly low HLA class I expression as the cell lines (Figure 3 and Figure 4). In addition, a trend towards gradually decreased HLA class I expression in the xenografts after *in vivo* passaging and on a lung metastasis was seen (Figure 4). A lack of IFN-γ production may be responsible for this finding, as lack of lymphocytic infiltration leads to low IFN-γ production. In a study on uveal melanoma, HLA class I expression was similarly weaker in SCID murine xenografts than in their original freshly-obtained tumors, which had shown high HLA class I expression[35]. However, another study shows an opposite outcome: in a mouse fibrosarcoma model, a HLA class I negative tumor clone led to HLA class I-positive spontaneous lung metastasis in immunodeficient mice which lacked T cells[46].

TCR-based immunotherapies against cutaneous melanoma have also been studied extensively in the last decade, using $CD8^+$ T cells which recognize the tumor-associated antigens of amongst others, tyrosinase, MAGE-A1 (melanoma-associated antigen A1), MART-1 (melanoma antigen recognized by T cells), glycoprotein 100 (gp100)[47]. In this context, a proper HLA class I expression is needed because metastatic cutaneous melanoma with a low HLA class I expression was resistant to T cell-based immunotherapy and progressed[48,49]. A recent study shows that mutations in B2M and IFN genes contribute to the recurrences of four metastatic cutaneous melanoma patients after anti-PD-1 (pembrolizumab) therapy[50]. These findings strongly suggest that both "soft" (reversible) and "hard" (irreversible) types of absence of HLA class I expression can influence the efficacy of T cell-mediated immunotherapy. Our FACS tests showed that some of the HLA antigens were not

expressed, even in the presence of IFN-γ, while other antigens showed a higher expression following exposure to IFN-γ. This indicates that both the soft as well as the hard type of downregulation of HLA expression occurs in CM. PD-L1/PD-1 was not expressed on the same tumors as the HLA antigens, therefore HLA expression cannot be used to predict the effect of PD-L1/PD1 checkpoint inhibitors.

Although our study contains only a small cohort from one medical centre, to the best of our knowledge, this is the first comprehensive view of HLA class I expression of CM *in vivo* and *in vitro*. The downregulation of HLA class I may contribute to the mechanisms of immune escape, and limit effective T-cell immune responses. When designing the immune-therapeutic strategies to treat CM, one should evaluate tumor cell HLA class I expression and potentially enhance it to improve the efficacy of T cell-based immunotherapy.

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Supplementary information

Supplementary Table 1. Secondary antibodies for IF and IHC.

Antibody	Specificity	Isotype	Company	Catalogue	Dilutions
AlexaFluor 488	mouse	IgG1	Life Technologies	A-21121	1：300
AlexaFluor 546	mouse	IgG2a	Life Technologies	A-21133	1：300
AlexaFluor 647	rabbit	IgG	Life Technologies	A-21245	1：300
Biotinylated	mouse	IgG	DAKO	E0433	1：200
Biotinylated	rabbit	IgG	Vector Laboratories	BA-1000	1：300

Supplementary Table 2. An overview of the primers used in qPCR.

Primers	Forward	Reverse
RPL13	GTACGCTGTGAAGGCATCAAC	GGTTGGTGTTCATCCGCTTG
RPS11	AAGCAGCCGACCATCTTTCA	CGGGAGCTTCTCCTTGCC
HLA-A	TGTGTTCGTGTAGGCATA	TTGAGACAGAGATGGAGAC
HLA-B	CTCCATCTCTGTCTCAACTT	CATCAACCTCTCATAGCA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
HLA-DR	CAAAGAAGGAGACGGTCTGG	GGCTCTCTCAGTTCCACAGG
HLA-DQ	TGATGGAGATGAGCAGTTC	GCAGCGGTAGAGTTGTAG
CIITA	GCTGGGATTCCTACACAATGC	TCTCCAGCCAGGTCCATCTG
IRF1	TCACCAAGAACCAGAGAA	TCCATCAGAGAAGGTATCAG
NLRC5	CTGGAGGAGTTGATGCTT	GATGGCTGAATGGTAGGT
TAP1	CGAAGCCCAGAAGTTTAG	CCACCAATGTAGAGGATTC
TAP2	CTATTCTGGTCGTGTGATTG	CTGTCTTAGTCTCCTGGAA

Supplementary Table 3. An overview of the HLA allele-specific antibodies used in the FACS analysis.

Cell line	Antibody clone	HLA-specificity defined by CDC	Isotype
CRMM1			
	SN607D8	A2/A28	IgG
	SN230G6	A2/B17	IgG
	DMS1B10	B62/B21/B37/B56/B72/B52/B45/A23/B44	IgM
	TRA2G9	Cw1/Cw3/Cw4/Cw*1402	IgM
CRMM2			
	MUL4C8	A3/A11	IgG
	WIM8E5	A1/A10/A11/A9/A29/A30/A31/A33/A28	IgG
	ROU9A6	B12/B13/B40/B21/B41	IgG
	DMS1B10	B62/B21/B37/B56/B72/B52/B45/A23/B44	IgM
	WK4C11	Cw1/Cw3/Cw*0801/Cw*1202/Cw*1402	IgM
CM2005.1			
	BRO11F6	A11	IgG
	MUS4H4	Bw4/A24/A32/A25	IgG
	KG30A7	B27/B12/B14/B49	IgM
	HDG8D9	B51/B35	IgG
	TRA2G9	Cw1/Cw3/Cw4/Cw*1402	IgM

CDC= Complement dependent cytotoxicity.

Supplementary Table 4. Information and data of the studied cohort.

Study number	Sex	Age at diagnosis (yr)	Follow-Up (months)	Local Recurrence	Time to recurrence (months)	Metastases	Time to metastasis (months)	Location of Metastases	Status	Location on Conjunctiva	Thickness (mm)	Largest Basal Diameter (mm)	cTNM (7th ed)
1	Female	59	43.6	No		No			Alive	Non-epibulbar	1.2	8	2
2	Female	47	246.8	Yes	48.9	No			Alive	Epibulbar	0.6	12	1
3	Male	64	78.6	Yes	24.6	No			Alive	Epibulbar	0.3	7	1
4	Female	84	42.3	No		Yes	27	Distant skin	Death due to metastases	Non-epibulbar	6	20	2
5	Male	61	51.9	No		No			Alive	Non-epibulbar	1.4	17	2
6	Male	46	6.7	No		No			Alive	Non-epibulbar	2.5	30	2
7	Male	33	107.6	Yes	37.8	No			Alive	Epibulbar	0.2	6	1
8	Female	69	35	No		No			Alive	Non-epibulbar	16	16	2
9	Male	58	64.9	No		No			Alive	Epibulbar	4	13	1
10	Female	49	37.3	No		No			Alive	Non-epibulbar	4.2	10	2
11	Female	84	3.3	No		No			Death due to unknown cause	Epibulbar	4	15	1
12	Female	73	37.2	No		No			Alive	Epibulbar	0.8	7	1
13	Male	60	98.3	Yes	70.3	Yes	91.9	Brain. visceral	Death due to metastases	Epibulbar	Unknown	5	1
14	Female	67	45.7	No		No			Alive	Epibulbar	1.2	12	1
15	Female	56	23.8	No		No			Alive	Epibulbar	0.2	5	1
16	Male	56	34.2	Yes	32.7	No			Alive	Epibulbar	0.2	12	1
17	Female	56	27.1	Yes	8.5	No			Alive	Epibulbar	2.5	Unknown	1
18	Female	75	41.9	Yes	8.4	No			Death due to unknown cause	Epibulbar	5	Unknown	1
19	Female	40	58.1	Yes	34.8	No			Alive	Epibulbar	1	9	1
20	Female	86	12.8	No		No			Alive	Epibulbar	0.1	6	1
21	Male	68	46.4	Yes	17.8	No			Alive	Epibulbar	0.2	2	1
22	Male	68	29.5	No		No			Alive	Epibulbar	0.2	12	1
23	Male	80	52.4	Yes	42.1	Yes	51.4	Unknown	Death due to metastases	Non-epibulbar	Unknown	Unknown	2

Supplementary Table 4. Information and data of the studied cohort (Continued).

Study number	CD4 T helper (cells/mm2)	CD4 (cells/mm2)	Tregs (cells/mm2)	CD68+ (cells/mm2)	CD68+CD163+ (cells/mm2)	CD3 (cells/mm2)	CD8 (cells/mm2)	HLA-A score	HLA-A category	HLA-BC score	HLA-BC category	B2M score	B2M category
1	32.1	70	37.9	57.3	45.6	124.5	54.5	0	total loss	3	partial loss	3	partial loss
2	52.7	94.4	41.7	63.4	58.1	264.1	169.7	6	partial loss	6	partial loss	6	partial loss
3	39.1	70.9	31.8	75	51.2	173.1	102.2	7	normal expression	7	normal expression	7	normal expression
4	12.6	29.5	16.9	46.6	22.1	109.6	80.1	6	partial loss	6	partial loss	6	partial loss
5	35.5	62.9	27.4	63.1	58.8	120	57.1	5	partial loss	6	partial loss	6	partial loss
6	51.4	76.2	24.9	19.1	11.9	144.1	67.8	0	total loss	0	total loss	0	total loss
7	45.2	64.5	19.3	24.8	10.9	129.5	65	5	partial loss	6	partial loss	6	partial loss
8	42.2	63.4	21.2	47.8	18.1	113	49.6	1	total loss	1	total loss	1	total loss
9	30.2	64	33.8	20.9	7.7	91.9	27.9	3	partial loss	3	partial loss	3	partial loss
10	38.7	64	25.3	27.2	11.3	121.5	57.5	3	partial loss	5	partial loss	5	partial loss
11	29.7	39.6	9.9	59.9	46.7	85.4	45.8	4	partial loss	4	partial loss	4	partial loss
12	46	67.8	21.8	85.3	50.7	158.8	91	2	total loss	2	total loss	2	total loss
13	91.8	132.7	41	27.3	19	206.6	73.9	7	normal expression	7	normal expression	7	normal expression
14	115.9	225.2	109.4	95.9	74.8	278.6	53.3	6	partial loss	6	partial loss	7	normal expression
15	99.6	157.3	57.7	51.8	40.6	290.9	133.6	3	partial loss	4	partial loss	4	partial loss
16	15	25.9	10.9	73.2	58.8	84.6	58.8	7	normal expression	6	partial loss	7	normal expression
17	57.7	83.3	25.7	66.8	38.4	171.2	87.9	8	normal expression	8	normal expression	8	normal expression
18	67.3	89.7	22.4	62.8	31.7	157.8	68.1	3	partial loss	3	partial loss	3	partial loss
19	38.8	55.1	16.3	22	14.9	97.8	42.7	3	partial loss	3	partial loss	4	partial loss
20	189.4	313.8	124.4	188.7	161.1	616.5	302.7	8	normal expression	7	normal expression	8	normal expression
21	201.5	301.8	100.3	248.3	219.9	636.6	334.8	7	normal expression	7	normal expression	7	normal expression
22	32.7	45	12.3	18.6	29.6	70.8	25.8	7	normal expression	7	normal expression	7	normal expression
23	12.9	60.8	47.8	40.3	38.8	123.2	62.5	6	partial loss	7	normal expression		Not-Scorable