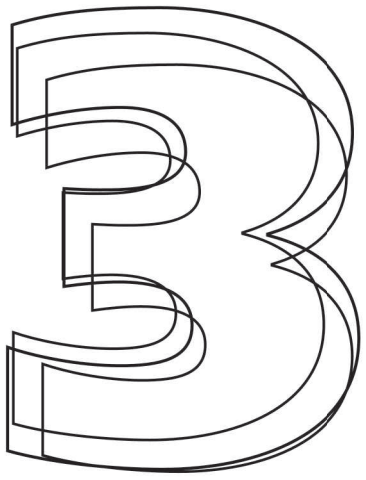

REDUCED HUMAN LEUKOCYTE ANTIGEN EXPRESSION IN ADVANCED-STAGE EWING SARCOMA: IMPLICATIONS FOR IMMUNE RECOGNITION

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J Pathol 2009; 218 (2): 222

ABSTRACT

Ewing sarcoma (EWS) is a tumor most commonly arising in bone, though on occasion in soft tissue as well, with poor prognosis in patients with refractory or relapsed disease, despite multimodal therapy. Immunotherapeutic strategies based on tumor-reactive T- and/or natural killer cells may improve treatment of advanced-stage EWS. Since cellular immune recognition critically depends on Human Leukocyte Antigen (HLA) expression, knowledge about HLA expression in EWS is crucial in the design of cellular immunotherapeutic strategies.

Constitutive and IFNγ-induced HLA class I expression was analyzed in EWS cell lines (n=6) by flow cytometry, using antibodies against both monomorphic and allele-specific antigens. Expression of Antigen Processing Pathway-components and beta-2 microglobulin (β2m) were assessed by Western blot. Expression of class II transactivator (CIITA), and its contribution to HLA class II expression, was evaluated by qRT-PCR, transduction assays and flow cytometry. β2m/HLA class I- and class II expression were validated in EWS tumors (n=67) by immunofluorescence.

Complete or partial absence of HLA class I expression was observed in 79% of EWS tumors. Lung metastases consistently lacked HLA class I and sequential tumors demonstrated a tendency towards decreased expression upon disease progression. Together with absent or low constitutive expression levels of specific HLA class I loci and alleles, and differential induction of identical alleles by IFNγ in different cell lines, these results may reflect the existence of an immune escape mechanism. Inducible expression of TAP-1/-2, tapasin, LMP-2/-7 and the β2m/HLA class I complex by IFNγ suggests that regulatory mechanisms are mainly responsible for heterogeneity in constitutive class I expression. EWS lack IFNγ-inducible HLA class II, due to lack of functional CIITA.

The majority of EWS tumors, particularly if advanced-stage, exhibit complete or partial absence of both classes of HLA. This knowledge will be instrumental in the design of cellular immunotherapeutic strategies for advanced-stage EWS.

Key words: Ewing sarcoma, bone tumor, soft tissue tumor, Human Leukocyte Antigen, Antigen Processing Pathway, immune recognition, immunotherapy, flow cytometry, immunohistochemistry

INTRODUCTION

Ewing sarcoma (EWS) is a malignant sarcoma, most commonly arising in bone and less frequent in soft tissue, that mainly affects children, adolescents and young adults [1]. This tumor belongs to the Ewing Family of Tumors, a family of round cell sarcomas characterized by specific EWS/ETS gene fusions [2]. Despite multimodal therapy including surgery, high-dose chemotherapy and radiotherapy, patients with refractory or relapsed disease have poor prognoses. Furthermore, no significant improvement has been achieved during the past decade with regard to survival of patients presenting with localized disease [3]. During recent years, (pre)clinical studies in various tumor models have shown that immunotherapeutic strategies, including adoptive cell transfer therapies using tumor-specific cytotoxic T lymphocytes (CTL) or natural killer (NK) cells, may represent novel therapeutic approaches [4-6]. These approaches may offer advantageous and less toxic treatment modalities for advanced-stage EWS as well. Human Leukocyte Antigen (HLA) expression plays a crucial role in recognition of tumors by tumor-reactive T- and/or NK cells, influencing immunity or tolerance against these tumors as well as the susceptibility to cellular immunotherapeutic approaches [7]. Knowledge about HLA expression in EWS is crucial in the design of cellular immunotherapeutic strategies.

The human major histocompatibility complex (MHC) encodes HLA class I and -II molecules. The classical HLA class I molecules HLA-A, -B and -C are, together with beta-2 microglobulin (β2m), expressed by almost all nucleated cells and present peptides derived from intracellular proteins to CTL. Peptides are generated in the cytoplasm by the (immuno-) proteasome and transported into the endoplasmic reticulum by the heterodimeric transporter associated with antigen processing (TAP-1/-2) complex. Assembly of the HLA class I heavy chain – β2m heterodimer with peptide is orchestrated by chaperones, including tapasin. After peptide loading, HLA class I molecules are transported to the cell surface [8,9]. HLA class II molecules (HLA-DR, -DP, -DQ) are constitutively expressed only by 'professional' antigen presenting cells and present peptides derived mainly from processed extracellular antigens to helper T lymphocytes. However, via activation of the class II transactivator (CIITA) by immune regulators, mainly interferon-γ (IFNγ), HLA class II gene transcription can be induced in non-immune- and some tumor cells as well [10,11].

Aberrant HLA class I expression has been demonstrated in human tumors of distinct histology and has been attributed to structural defects and/or dysregulation of several components involved in HLA class I surface expression (reviewed in [12]). Impaired HLA class I expression may have significance, since it has been associated with histopathological characteristics of lesions and patients' prognoses [13-15]. Furthermore, lack of constitutive and/or IFNγ-inducible HLA class II expression has been observed in malignancies of hematopoietic origin as well as in solid tumors [16,17].

In this study, we report original data on HLA class I and -II expression, regulation and correlations with clinicopathological characteristics in EWS cell lines and tumors.

METHODS

Cell lines

EWS cell lines A673, SK-N-MC, SK-ES-1, RD-ES (ATCC, Manassas, VA), EW3[18] and L1062[19] were cultured in RPMI-1640 (Invitrogen, Scotland) supplemented with 10% fetal bovine serum (FBS; Greiner Bio-One, Netherlands), 100 IU/ml streptomycin/penicillin (Invitrogen). Molecular HLA typing of these cell lines, performed at Leiden University Medical Center (LUMC), was converted to serological equivalents (no serological equivalents exist for HLA-Cw*16 and -Cw*12): A673 (A1/A2/B7/Cw7), SK-N-MC (A1/A25/B8/Cw7), SK-ES-1 (A2/A11/B7/B44/Cw5/Cw7), RD-ES (A24/A29/B15/B44/Cw3/ Cw*16), EW3 (A2/A31/B27/B35/Cw2/Cw4), L1062 (A2/A3/B8/B39/Cw7/Cw*12). EBV B-LCL cell line was generated from a healthy blood bank donor. Human glioblastoma cell line U251-MG (ATCC) was cultured in Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 10% FBS, 100 IU/ml streptomycin/penicillin. For induction of HLA expression, EWS and U251-MG cells were cultured for 48 and 24 hours in the presence of 100 and 500 IU/ml IFNγ (Boehringer, Germany), respectively. For construction of LZRS retroviral vector containing CIITA, 4.5 kb cDNA encoding CIITA was excised from a pEBO-sfi vector (kindly provided by dr. B. Mach, University of Geneva, Switzerland) after digestion with SalI and inserted into the multiple cloning site of pLZRS-mcs-IRES-GFP digested with XhoI. Correct sequence orientation was confirmed after restriction enzyme digestion with BamHI and NotI. Retroviral supernatant from the LZRS-CIITA-IRES-GFP retroviral vector was produced and used as previously described [20].

Monoclonal antibodies (mAbs)

The mAbs used for staining of antigens for flow cytometric analysis, western blot analysis and immunofluorescence/-histochemistry are described in Supplementary Table 1.

Flow cytometric evaluation of HLA surface expression

Flow cytometric analysis of surface HLA expression was performed on a FACScalibur (Beckton Dickinson, Germany) and results were analyzed using Cellquest software. In short, cells were collected, centrifuged, washed in 1% BSA/PBS, stained with primary mAbs and, if necessary, stained with fluorochrome-labeled secondary antibodies. Isotype control stainings were included as indicated in the results section. HLA expression was represented as fold increase in Mean Fluorescence Intensity over control staining.

Western blot analysis for expression of HLA, β2m and Antigen Processing Pathway components

Preparation of cell lysates, gel electrophoresis, blotting and blocking were performed as previously reported [21]. Blots were exposed to primary mAbs. After washing, HRP-labeled secondary antibodies were added. Proteins were visualized with enhanced chemiluminescence (ECL plus, GE Healthcare, Germany). Equal protein loading was verified by immunodetection of β-actin. For TAP-1/-2 Western blotting, 0.5% NP-40

lysis buffer (0.5% NP-40, 50mM Tris HCl, pH 7.5, 5mM $MgCl_2$, 10µM leupeptin, 1mM 4-(2-aminoethyl)benzenesulfonyl fluoride) and polyvinylidene difluoride membranes (GE Healthcare) were used. Blocking was performed using 0,6% BSA/TBST.

Quantitative RT-PCR analysis for detection of HLA-DR and CIITA transcripts

Total RNA was isolated from cell lines using TRIzol (Invitrogen), according to the manufacturer's protocol. 1µg total RNA was transcribed into cDNA using avian myeloblastosis virus-reverse transcriptase (Roche). Quantification of panCIITA, HLA-DRA and GAPDH transcripts was performed on an iCycler iQ Real-time Detection system (Bio-Rad Laboratories) using the IQ SYBR Green Supermix and primers: panCIITA sense, 5'-CCGACACAGACACCATCAAC-3'; panCIITA antisense, 5'-CTTTTCTGCCCAACTTCTGC-3'; HLA-DRA sense, 5'-CAAAG AAGGAGACGGTCT GG-3'; HLA-DRA antisense, 5'-GGCTCTCTCAGTTCCACAGG-3'; GAPDH sense, 5'-GG TCGGAGTCAACGGATTTG-3'; GAPDH antisense, 5'-ATGAGCCCCAGCCTTCTCCAT-3'. For comparison of relative amounts of target transcripts between samples, values were normalized to GAPDH.

EWS tumor samples and patients' clinical characteristics

Archival formalin-fixed, paraffin-embedded tumor samples were retrieved from the Department of Pathology, LUMC (twenty-seven diagnostic biopsies, four lung metastases and two bone metastases obtained from twenty-eight EWS patients; for five patients, sequential material was available) and a tissue array containing forty-nine 2mm-diameter diagnostic EWS tissue-cores (Heinrich Heine University; thirty-four samples were eligible for analysis of immunostainings). Mean age at diagnosis of all patients was 18.7 years. Follow-up until 2007 (mean/median duration of follow-up: 34.4/29.0 months, respectively) provided information concerning recurrence rate and performance state. Only patients that were treated according to the EURO-E.W.I.N.G.99 study [22] were included in survival analyses. From LUMC and the tissue array, fifteen respectively twenty patients were eligible for inclusion. All patient material was coded, such that code breaking and correlation with clinical data were only possible for physicians involved in treatment of the patients. Subsequent research was conducted following the ethical guidelines of the national organization of scientific societies (FEDERA).

Immunostainings for validation of HLA expression in EWS tumors

4-µm sections containing representative tumor were deparaffinized and citrate antigen retrieval was performed. Three-color immunofluorescent staining was performed after blocking (10% normal goat serum/PBS) by overnight incubation with primary mAbs, followed by incubation with fluorochrome-labeled secondary antibodies (Alexa Fluor, Invitrogen) and mounting in Mowiol (3.62M glycerine, 0.87M Mowiol 40-88, 0.13M Tris, 34 mM DABCO, pH 8.5). Slices were scanned on a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Germany). Enzymatic immunohistochemistry was performed as previously reported [23]. Staining was scored semi-quantitatively by the quality control system proposed by Ruiter et al.[24]. The intensity of staining was

scored as 0, 1, 2 or 3 indicating absent, weak, clear or strong expression, respectively. Percentages of positive cells were scored as 0 for 0%; 1 for 1-5%; 2 for 5-25%; 3 for 25-50%; 4 for 50-75% and 5 for 75-100%. The sum of both scores was used to identify three categories of expression: homogeneous (total score 7-8), partial (3-6) and absent (0-2).

Statistical analysis

Statistical analyses were performed with SPSS version 12 software package. Mann-Whitney- and chi-square or, where appropriate, Fisher's exact tests were used for comparison of expression between samples and associations between expression and clinicopathological parameters. Five-year survival rates were calculated according to the Kaplan-Meier method using the log rank test. Overall survival was defined as survival till death due to disease, whereas disease-free survival was defined as time to disease recurrence or disease-specific death. All tests were two-sided, the significance level was set to 5%.

RESULTS

Heterogeneous constitutive HLA class I expression in Ewing sarcoma cell lines

Surface expression of HLA class I antigenic determinants was investigated by flow cytometry in six EWS cell lines, after staining with an anti-HLA-A/B/C mAb and allele-specific mAbs, respectively. Levels of constitutive HLA-A/B/C surface expression varied among the cell lines, ranging from undetectable (SK-ES-1) to relatively high (SK-N-MC and RD-ES) (table 1 and figure 1-A). Absence of detectable constitutive expression of at least one allele, most often within the HLA-B locus, was observed in all cell lines. Five cell lines exhibited complete lack of detectable constitutive expression of either one or two loci, in all cases including the HLA-B locus (table 1). Due to limited availability of HLA-C alloantigen specific mAbs, no data were obtained regarding the expression of most HLA-C alleles. Western blot analysis confirmed the heterogeneity in constitutive HLA class I expression levels and excluded intracellular retention of HLA class I molecules in the SK-ES-1 cell line to be responsible for lack of constitutive HLA class I surface expression (figure 1-B).

Invariable induction of HLA class I expression by IFNγ

In addition to the analysis of constitutive expression levels, IFNγ-induced expression of HLA class I was investigated. Without exception, stimulation of cell lines with IFNγ induced HLA-A/B/C expression (table 1 and figure 1-A/-B), suggesting that the observed absent or relatively low constitutive expression levels are not due to genetic defects. Analysis of allele-specific expression supported this notion: all alleles were inducible by IFNγ, except for HLA-B7 in SK-ES-1 and HLA-A3/-B39 in L1062 (table 1). Interestingly, differential IFNγ responses were observed for identical alleles among the different cell lines (e.g. limited induction of HLA-A2 in SK-ES-1 and A673, while more robust induction of this allele in L1062 and, particularly, EW3) (figure 1-C).

Table 1. Flow cytometric evaluation of constitutive and IFNγ-induced, monomorphic and allele-specific HLA class I expression in Ewing sarcoma cell lines

Cell line	HLA-A/B/C*	HLA class I alleles					
A673		A1	A2	B7		Cw7	
Constitutive	±	±	-	-		n.d.	
+ IFNγ	++	++	±	+		n.d.	
L1062		A2	A3	B8	B39	Cw7	Cw12
Constitutive	±	-	±	-	-	n.d.	n.d.
+ IFNγ	++	+	±	±	-	n.d.	n.d.
SK-N-MC		A1	A25	B8		Cw7	
Constitutive	+	±	±	-		n.d.	
+ IFNγ	++	+	++	±		n.d.	
RD-ES		A24	A29	B44	B62	Cw3	Cw16
Constitutive	+	+	+	±	-	±	n.d.
+ IFNγ	++	++	++	+	±	++	n.d.
SK-ES-1		A2	A11	B7	B44	Cw5	Cw7
Constitutive	-	-	-	-	-	n.d.	n.d.
+ IFNγ	+	±	±	-	±	n.d.	n.d.
EW3		A2	A31	B27	B35	Cw2	Cw4
Constitutive	±	-	±	-	-	n.d.	-
+ IFNγ	++	++	++	±	++	n.d.	±

Legend: - = Mean Fluorescence Intensity (MFI) ratio specific staining/isotype staining < 2; ± = MFI ratio between 2 and 5; + = MFI ratio between 5 and 10; ++ = MFI ratio >10; n.d. = not determined, due to absence of specific antibodies. * anti-HLA-A/B/C, clone G46-2.6.
Representative results of at least four experiments.

Simultaneous induction of Antigen Processing Pathway components, HLA class I and beta-2 microglobulin by IFNγ in Ewing sarcoma cell lines

Since deficient expression of several Antigen Processing Pathway (APP) components contributes to reduced HLA class I expression in various human tumors, expression of APP components TAP-1/-2, tapasin and LMP-2/-7 was investigated by Western blot analysis in three cell lines, selected on the basis of differences in constitutive HLA class I expression levels. As shown in figure 1-B, constitutive TAP-1/-2 and tapasin expression levels were heterogeneous among the cell lines, though congruent with those observed for HLA class I. IFNγ stimulation induced expression of these proteins, including the immunoproteasome components LMP-2 and LMP-7, similar to HLA class I. B2m was constitutively expressed in all three cell lines and, similar to the APP components and HLA class I, inducible by IFNγ. Together, these data demonstrate

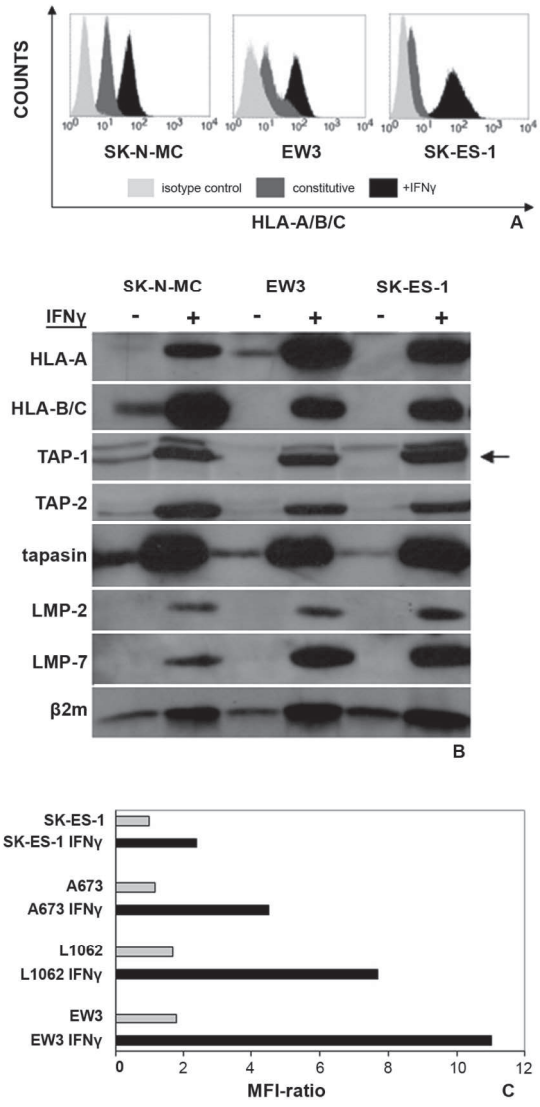

Figure 1. Constitutive and IFNγ-induced expression of HLA class I, beta-2 microglobulin and components of the Antigen Processing Pathway in Ewing sarcoma cell lines. **A.** Flow cytometric evaluation of HLA class I (anti-HLA-A/B/C, clone G46-2.6) surface expression in unstimulated (dark grey) and IFNγ-stimulated (black) EWS cells. Representative results are shown for EWS cell lines SK-N-M-C, EW3 and SK-ES-1. Similar results were obtained for EWS cell lines A673, L1062 and RD-ES (not shown). Isotype-matched control stainings are shown in light grey. **B.** Western blot analysis for expression of HLA-A (HCA2 mAb), HLA-B/C (HC10 mAb), the APP components TAP-1/-2, tapasin, LMP-2/-7 and β2m in unstimulated and IFNγ-stimulated EWS cell lines SK-N-MC, EW3 and SK-ES-1. Arrow: TAP-1. β-actin was used as control for protein loading (not shown). **C.** Differential IFNγ responses for the HLA-A2 allele among Ewing sarcoma cell lines SK-ES-1, A673, L1062 and EW3. Mean Fluorescence Intensity-ratio (MFI-ratio) of HLA-A2 specific staining/isotype-matched control staining.

heterogeneous constitutive expression, but IFNγ-inducibility of all APP components and the HLA class I/β2m complex in EWS cell lines.

HLA class I expression in primary and metastatic Ewing sarcoma tumor samples

To determine whether the observed heterogeneity in HLA class I expression *in vitro* reflected the *in vivo* situation, expression of β2m, HLA-A and HLA-B/C was evaluated in a large cohort EWS tumor samples by three-color immunofluorescent staining. Variation between as well as within individual tumors was observed, ranging from complete lack of detectable expression of both β2m and HLA class I to homogeneous expression of these proteins (figure 2). As shown in table 2, forty-eight of sixty-one (79%) biopsies exhibited aberrant HLA class I expression (defined as absent or partial expression). Seventeen biopsies (28%) showed complete lack of detectable HLA class I. In eleven of these seventeen cases (17% of all biopsies), absence of HLA class I was accompanied by complete lack of detectable β2m. Within a cohort of uniformly treated EWS patients (n=35), neither β2m nor HLA class I expression was significantly associated with overall- or event free survival (data not shown).

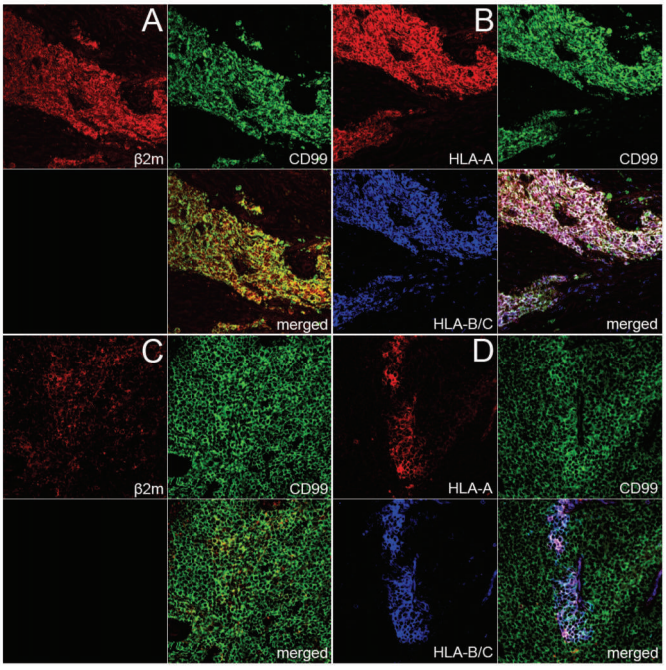

Figure 2. Heterogeneous HLA class I expression in Ewing sarcoma tumors. Representative examples of three-color immunofluorescent staining on formalin-fixed, paraffin-embedded EWS tumors. mAbs against CD99, β2m and HLA class I heavy chains (HLA-A (HCA2 mAb); HLA-B/C (HC10 mAb)) were used. **A+B**: diagnostic biopsy, homogeneous expression of β2m and HLA class I. **C+D**: diagnostic biopsy, focal (partial) expression of β2m and HLA class I. Stromal cells, including infiltrating leukocytes, were used as internal positive controls.

Table 2. Expression of beta-2 microglobulin, HLA class I heavy chains and overall HLA class I in diagnostic and metastatic Ewing sarcoma tumors

	β2m			HLA-A			HLA-B/C			HLA class I (overall)		
Expression*	*absent (%)*	*partial (%)*	*homogeneous (%)*	*absent (%)*	*partial (%)*	*homogeneous (%)*	*absent (%)*	*partial (%)*	*homogeneous (%)*	*absent (%)*	*partial (%)*	*homogeneous (%)*
Biopsies (n=61)†	11 (18)	26 (43)	24 (39)	24 (39)	19 (31)	18 (30)	20 (33)	20 (33)	21 (34)	17 (28)	31 (51)	13 (21)
Metastases (n=6)#	4 (67)	2 (33)	0 (0)	4 (67)	1 (17)	1 (17)	5 (83)	1 (17)	0 (0)	4 (67)	2 (33)	0 (0)

* Expression according to [24]. † biopsies, obtained at diagnosis; # metastatic lesions showed significantly more often total loss of β2m (p=0.017, Fisher's Exact Test) and HLA-B/C heavy chain expression (p=0.024, Fisher's Exact Test).

Table 3. Tendency towards decreased beta-2 microglobulin and overall HLA class I expression levels upon progression of disease

UPN	sample type (years after preceding sample)	β2m	HLA class I (overall)
1	lung metastasis	○	○
	bone metastasis (4)	●	●
2	diagnostic biopsy	●	●
	lung metastasis (5)	○	○
3	diagnostic biopsy	●	●
	lung metastasis (4)	○	○
4	diagnostic biopsy	●	●
	bone metastasis (1)	●	●
5	diagnostic biopsy	○	○
	lung metastasis (1)	○	○

Expression according to [24]. Legend: ○ = absent expression; ● = partial expression. UPN: patient number.

Compared to diagnostic biopsies, however, metastatic lesions showed significantly lower levels (Mann-Whitney Test, p=0.001 and p=0.01) and more often total loss (Fisher's Exact Test, p=0.017 and p=0.024) of detectable β2m and HLA-B/C heavy chain expression, respectively. This was due to complete absence of detectable β2m and HLA class I expression in lung metastases (n=4). Bone metastases (n=2), however, expressed both β2m and HLA class I. This difference between lung- and bone metastases was observed even within a single patient, presenting with β2m- and HLA class I-positive bone metastases four years after resection of a β2m- and HLA class I-negative lung metastasis (table 3, UPN 1). Sequential patient material demonstrated a tendency towards decreased levels of β2m and HLA class I expression upon disease progression, particularly when relatively long periods of latency existed between occurrences of consecutive events (table 3, UPN 2-3).

Absence of functional endogenous CIITA causes lack of HLA class II expression in Ewing sarcoma

Since HLA class II expression may contribute to immune recognition of tumors, expression of HLA class II was investigated in EWS cell lines and tumors. Flow cytometric analysis showed lack of IFNγ-induced expression of HLA-DR/DP/DQ

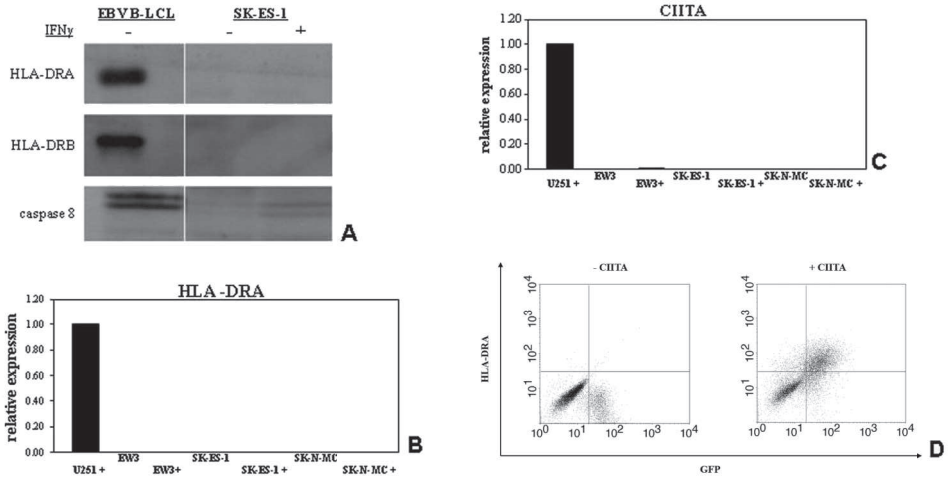

Figure 3. Ewing sarcoma cell lines lack constitutive and IFNγ-induced expression of HLA class II, due to lack of functional endogenous class II transactivator (CIITA). **A.** Western Blot analysis for expression of HLA-DR (HLA-DRA (clone DA6.147) and HLA-DRB (clone HB10-A)) in unstimulated and IFNγ-stimulated EWS cell line SK-ES-1. Induction of caspase-8 expression was used as a positive control for effectiveness of IFNγ stimulation. β-actin was used as control for protein loading (not shown). EBV B-LCL was used as a positive control. **B+C.** Quantitative RT-PCR analysis of HLA-DRA (B) and panCIITA (C) mRNA transcript levels in unstimulated and IFNγ-stimulated EWS cell lines EW3, SK-ES-1 and SK-N-MC. Values were normalized to GAPDH and the amount of transcripts in IFNγ-stimulated U251 was set at 1. **D.** Flow cytometric evaluation of HLA-DRA (clone G46-6) surface expression in EWS cell line SK-N-MC upon retroviral transduction by virus containing either no cDNA (- CIITA) or full-length CIITA cDNA (+ CIITA). Similar results were obtained for EWS cell lines SK-ES-1 and EW3 (not shown).

in six constitutively HLA class II-negative cell lines (data not shown). Western blot analysis confirmed absence of expression and excluded intracellular retention of HLA class II proteins in SK-ES-1 cells (figure 3-A). Immunohistochemical stainings demonstrated lack of HLA class II expression in EWS tumor samples as well (data not shown). Quantitative RT-PCR in three cell lines demonstrated absence of IFNγ-induced HLA-DRA expression at mRNA level as well (figure 3-B) and identified absence or very low levels of transcripts for endogenous CIITA as a potential cause of lack of HLA class II expression (figure 3-C). Indeed, retroviral transduction of these cells by CIITA-containing virus restored HLA-DRA surface expression in GFP-expressing cells (figure 3-D). Together, these data indicate lack of functional endogenous CIITA rather than HLA class II gene defects to be responsible for absent HLA class II expression in EWS.

DISCUSSION

Our results demonstrate heterogeneous constitutive expression levels of monomorphic HLA class I molecules, ranging from undetectable to relatively high, in both EWS cell lines and tumor samples. These results are consistent with previous studies in EWS involving either cell lines or very small numbers of tumors [25-28]. So far, most published studies on HLA class I expression in tumors, including EWS, have used antibodies to monomorphic HLA class I antigens and were, therefore, not suitable for evaluation of locus- or allele-specific expression. This study, however, expands knowledge by showing differential expression of individual HLA class I loci and alleles as well. Five of six cell lines lacked constitutive expression of either one or two loci. According to the concept of cancer immuno-editing, which proposes tumors to interfere with immune surveillance by so-called immune escape mechanisms, tumors may escape from recognition by tumor-specific CTL by down-regulating one or more HLA class I loci or alleles [29,30]. Expression of remaining alleles may protect the cells from NK cell-mediated cytotoxicity [31,32]. In uveal melanoma, a high frequency of allele-specific HLA class I down-regulation has been reported and suggested to contribute to tumor immune escape [33]. Moreover, persistent downregulation of major alleles contributing to antigen presentation has been reported in melanoma [34]. The absence or relatively low constitutive expression of individual HLA class I loci and alleles together with differential induction of identical alleles by IFNγ in different cell lines (e.g. HLA-A2 and HLA-B7), as observed in this study, may reflect the existence of a similar immune escape mechanism in EWS as well. Further support for this concept of cancer immuno-editing in EWS is provided by our *in vivo* observations of (i) significantly lower levels of β2m and HLA expression in lung metastases compared to diagnostic biopsies and (ii) a tendency towards reduced HLA expression upon disease progression.

Seventeen percent of all biopsies and 4/4 lung metastases exhibited lack of both detectable HLA class I and β2m expression. Mutations of HLA-, APP component- and β2m-genes resulting in impaired HLA class I expression have been described in human tumors [12,35]. Such mutations are not excluded in this cohort of EWS tumors by means of gene sequencing. However, the observed intact IFNγ-inducibility of all APP

components, β2m and HLA class I molecules *in vitro,* which can be explained by the presence of Interferon Stimulated Response Elements in the promoters of these genes, indicates that regulatory mechanisms are mainly responsible for absent or low constitutive expression levels. Equivalent patterns of expression of APP components, β2m and HLA class I have been reported in other human tumors [36-39]. Although we can not exclude that insufficient expression of a single APP component (as described by, among others, Lou et al.[40]), rather than coordinated regulation of several APP components, restricts constitutive HLA class I expression, the observed IFNγ-induced expression indicates that structural aberrations are an unlikely cause for lack of expression in EWS tumors. Support for this statement is provided by publications demonstrating lack of secondary genetic aberrations, acquired in addition to the EWS-specific chromosomal translocations, of chromosome 6 (TAP1/-2, LMP-2/-7, tapasin and HLA class I genes) and chromosome 15 (β2m gene) in localized and metastatic EWS tumors [41,42].

Our results demonstrate lack of (inducible) HLA class II expression in EWS, both *in vitro* and *in vivo,* which appeared to be due to lack of endogenous CIITA expression rather than HLA class II gene defects. Epigenetic modifications of gene promoters, including CIITA promoter, have been described to influence HLA expression in cancer [43-45]. However, pilot experiments in which EWS cells were pre-incubated with epigenetic modulators (Trichostatin A or 5-aza-2'deoxycytidine) and IFNγ neither induced HLA class II nor increased HLA class I expression (data not shown). Structural CIITA gene defects seem unlikely to cause the observed lack of expression, since such defects have seldom been described other than for patients with specific MHC-II deficiencies [46]. Defects in the IFNγ-signaling pathway are proven unlikely by our observation of IFNγ-induced HLA class I expression. In developmental tissues, lack of inducible HLA class II expression is assumed to reflect the silent state of these genes during early development, creating immune privilege for these cells to evade maternal immune recognition. EWS are primitive, undifferentiated tumors, considered to be derived from either neuro-ectodermal or mesenchymal stem cells [28,47]. Whether such a silent state, rather than the processes of neoplastic transformation and cancer immuno-editing, accounts for the absence of HLA class II as well as the observed constitutive HLA class I expression levels in EWS, remains to be established. However, irrespective the underlying mechanism, the obtained knowledge about HLA expression in EWS may direct future immunotherapy studies using either (xenografted) murine or human tumor models [48,49]. The observed heterogeneity in HLA class I expression may affect immune recognition of these tumors by tumor-reactive T- or NK cells and, therefore, influence the efficacy of cellular immunotherapeutic strategies using these cytotoxic effector cells.

ACKNOWLEDGMENTS

The authors like to thank dr. B. Mach (University of Geneva, Geneva, Switzerland) for provision of the vector pEBO-CIITA; dr. G. Nolan (Stanford University, Stanford, California) for provision of the retroviral vector pLZRS-pBMN; prof. dr. J. Neefjes (Dutch

Cancer Institute, Amsterdam, Netherlands), dr. R. Tampé (Institute of Biochemistry, Johann Wolfgang Goethe University, Frankfurt, Germany), dr. P. van Endert (Hôpital Necker, Paris, France) and dr. P. Cresswell (Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT) for provision of mAbs; M.C. Verweij (Dept. of Medical Microbiology and Center for Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands), G. Alblas (Dept. of Pediatrics, Leiden University Medical Center, Leiden, Netherlands), dr. K.B.J. Scholten (Dept. of Pathology, VU University Medical Center, Amsterdam, Netherlands), K.G. van der Ham (Dept. of Pathology, Leiden University Medical Center, Leiden, Netherlands) and M.J. Ronkes for technical assistance.

This study was financially supported by: Dutch Cancer Society Nederlandse Kankerbestrijding/Koningin Wilhelmina Fonds (grant RUL 2003-2800 to A.S.K. de H. and S.J.S., RUL 2005-3259 to D.H.); European Commission (EuroBoNeT, grant no. 018814); Stichting The Quality of Life Gala 2007.

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SUPPLEMENTAL TABLE

Supplemental table 1. Primary monoclonal antibodies used for the different applications, as described in the Methods section

application	mAb	clone	source
flow cytometry			
	anti-HLA-A/B/C	G46-2.6	Pharmingen, San Diego, CA
	anti-HLA-DR/DP/DQ	TÜ39	Pharmingen, San Diego, CA
	anti-HLA-DRA	G46-6	Pharmingen, San Diego, CA
	mAbs with HLA alloantigen specificity		Leiden University Medical Center [1]
western blot			
	anti-tapasin	7F6	kind gift of dr. R. Tampé, Frankfurt, Germany
	anti-TAP1	148.3	kind gift of dr. R. Tampé, Frankfurt, Germany
	anti-TAP2	435.4	kind gift of dr. P. van Endert, Hôpital Necker, France
	anti-HLA-DRA	DA6.147	kind gift of dr. P. Cresswell, New Haven, Connecticut
	anti-HLA-DRB	HB10-A	kind gift of dr. P. Cresswell, New Haven, Connecticut
	anti-caspase 8	8CSP03	Abcam, United Kingdom
	anti-LMP2	#3328	Abcam, United Kingdom
	anti-LMP7	#3329	Abcam, United Kingdom
	anti-CD99	H036-1.1	Abcam, United Kingdom
	anti-β2m	#A0072	Dako, Denmark
	anti-β actin	AC-15	Sigma-Aldrich, Netherlands
	HCA2		prof. dr. J. Neefjes, Dutch Cancer Institute, Netherlands [2]
	HC10		prof. dr. J. Neefjes, Dutch Cancer Institute, Netherlands [2]
immunostaining			
	anti-CD99	H036-1.1	Abcam, United Kingdom
	anti-HLA-DR/DP/DQ	CR3/43	Dako, Denmark
	anti-β2m	#A0072	Dako, Denmark
	mAbs with HLA alloantigen specificity		Leiden University Medical Center [1]
	HCA2		prof. dr. J. Neefjes, Dutch Cancer Institute, Netherlands [2]
	HC10		prof. dr. J. Neefjes, Dutch Cancer Institute, Netherlands [2]

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