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TITLE: Population Based Assessment of MHC Class I Antigens Down Regulation as Marker of Increased Risk for Development and Progression of Breast Cancer From Benign Breast Lesions

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14. ABSTRACT Despite advances in chemotherapy and radiation therapies, advanced breast cancer still carries a high mortality rate. The need for effective therapies is urgent. The overall aim of this research proposal is to recognize early markers of disease and their interaction with other epidemiological risk factors that can serve as risk indicators for subsequent development of breast cancer from precancerous lesions and as prognostic markers for progression from primary to metastatic disease. The major histocompatibility complex (MHC) class I molecules are found on the cell membrane of all cells in the body and are involved in intercellular communications and in complex interactions with the immune system. Cancer cells with reduced or aberrant MHC molecules have been shown to evade immune surveillance and become selected for cancer progression and spread of disease to distant sites of the body. About half of all breast cancers have complete loss or reduced level of MHC class I molecules and this finding has been associated with increased tumor invasiveness and more aggressive cancers with poorer outcome. The results of our investigation to assess the role of HLA class 1 in breast cancer progression are as follows: 1) formalin-fixed paraffin tissues represent a useful substrate upon which to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells; 2) there was a significant correlation of expression levels of HC10 and LGII and breast lesion type pointing in the direction of upregulation in tumor cells, rather than downregulation as commonly reported; 3) The results do not point to an association between HC10 abnormalities and stage; 4) There is a significant trend towards lesion severity and high lymphocytic infiltration.					
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INTRODUCTION

It has been known for some time that malignant transformation of cells is frequently associated with abnormalities in the expression of MHC class I antigens (1). These abnormalities appear to play a role in the clinical course of the disease (1) and to have a negative effect on the outcome of T cell-based immunotherapy for malignant diseases (2, 3). In breast lesions examined for expression of MHC class I, approximately half (51%) of carcinomas had an abnormally low content of HLA-A, -B, and -C determinants (4). Down regulation of HLA class I antigens in breast carcinomas may be more frequent than previously reported suggesting that alterations of HLA class I could represent an important step associated with tumor invasion providing tumor cells with the ability to escape recognition by T-lymphocytes (5). The overall aim of our research is to better define the role of MHC class I antigen abnormalities in the progression of primary breast cancer.

BODY: Statement of Work

TASK 1:

In women with primary and metastatic lesions of the breast to determine whether HLA Class I antigen loss and down regulation is greater in those with late stage and metastatic disease than in women with early stage disease (months 1-48); to determine whether among women with concurrent preneoplastic lesions and breast tumors HLA Class I antigen loss or down regulation is more frequent in the tumor than in the pre-neoplastic lesion (1-54); association with histopathologic characteristics of the lesions, including estrogen and progesterone receptor status (months 1-54); and disease survival (1-58).

- a: Begin construction of the breast cancer cohort. The Pathologist Dr.Raju and the P.I will begin screening breast cancer cases for delineation into Stage I-IV, and for the presence of concurrent lesions of benign proliferative and cancer lesions, together with normal breast tissue. We will design appropriate forms to record histopathological and clinical data based on our current NIH project forms and instruments (Instruments section in original grant)
- b: Retrieval of H & E slides for cases
- c: Review of slides
- d: selection of tumor blocks and sectioning of tissue for immunohistochemistry assays
- e: begin HLA class I immunoassays, as slides become available
- f: Continue construction of the breast cancer cohort, the concurrent lesion cohort and the histopathologic data gathering. See Pathology Review Form (PRF) (Instruments Section) for histopathologic parameters.
- g: continue HLA class I immunoassays as additional cases are entered into the cohort
- h: Annual reports will be written
- i: Initial manuscripts on the PBD cohort will be written

Task 1 PROGRESS (January 1, 2001- July 31, 2006):

- 1: A total of 1967 records from 385 patients were recorded in the final dataset.
- 2: For each record, expression of HC10, CD45, and LGII were determined for multiple

lesions present at the time of the primary breast cancer diagnosis. Lesions types included the following: presence of lymphocytes of lymphocytic infiltration (L), normal breast epithelium (N), benign breast disease (BBD), carcinoma in situ (CIS) lesions of ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), tumor (T).

TASK 2:

Final analysis and report writing (months 56-60)

- a: Final analysis of HLA expression results will be performed.
- b: A final report and additional manuscripts on the breast cancer cohort will be prepared

Task 2 Progress: Data analysis for a total of 1967 records from 385 patients has been completed for the following and detailed in “Key Research Accomplishments”

KEY RESEARCH ACCOMPLISHMENTS: See Appendix A

- REPORTABLE OUTCOMES

- 1. MANUSCRIPTS/REPORTS

A: DOWNREGULATION OF HLA-A AND BW6, BUT NOT BW4, ALLOSPECIFICITIES IN LEUKEMIC CELLS. AN ESCAPE MECHANISM FROM CTL AND NK ATTACK?

Demanet C, Mulder A, Deneys V, Worsham MJ, Maes P, Claas FH, Ferrone S.
Down- regulation of HLA-A and HLA-Bw6, but not HLA-Bw4, allospecificities in leukemic cells: an escape mechanism from CTL and NK attack? *Blood*. 2004;103(8):3122-30. Manuscript attached: Appendix A-1

B: HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTRIC STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES. M J.

Worsham¹, R. Nanavati¹, U. Raju¹, S.R. Wolman², T. Cabrera ³, F. Garrido ³, E. A. Repasky⁴, B. Hylander⁴, M. Feenstra ⁵, M.Verdaasdonk⁵, M.Schipper⁵, M.Tilanus⁵, S.Ferrone⁴. ¹ Cancer Genetics Research, Department of Pathology, Henry Ford Health Systems, Detroit, MI, 48202, USA ² Uniformed Services Univ., of the Health Sciences, Bethesda, MD 20814, USA, Hosp., Univ., Virgen de las Nieves, Granada, Spain, ⁴ Roswell Park Cancer Institute, Buffalo, NY 14263, ⁵ Univ., Hosp., Utrecht, The Netherlands.
Pending revision: Manuscript attached: Appendix A-2

C: EXPRESSION OF MHC CLASS I AND II EXPRESSION SIGNIFICANTLY DISCRIMINATES AMONG BENIGN, CARCINOMA IN SITU, AND MALIGNANT LESIONS OF THE BREAST. Submitted abstract to 2005 Era of Hope DOD Meeting

Abstract attached. Manuscript in preparation: Appendix A-3

- D: The association between the expression levels of HC10 and LGII and tumor stage
Summary: Stage data were verifiable on 268 patients. There were 61 cases with stage 0 comprising either CIS or BBD; there were 66 cases in stage 1, 65 in stage 2, 31 in stage 3 and 45 in stage 4. The p-values of the chi-squared test for independence between stages and expression levels of HC10 and LGII are 0.15 and 0.32, respectively. These data while pointing to a slight trend in the direction of increasing class I expression of HC10 and increasing stage, are not significant. Concordant with lack of LGII expression in epithelial cells, 201 of the 265 cases had low or absent LGII expression as compared to 41 with some expression (25-50%) and 25 cases with > than 50% expression.
Manuscript in preparation: Statistical Data: Appendix A-4

- E: The relationship between lymphocytic infiltration and lesion type based on CD45 Expression:
Summary: The analysis is based on the most severe lesion in the categories of benign (BBD), CIS, and tumor, observed in each patient. This qualification is relevant because of the presence of multiple lesions in a primary breast biopsy. We, therefore, considered only one record per patient as follows: tumor when tumor is present, CIS when only CIS is present and in the absence of any tumor, BBD when only a benign lesion was present and in the absence of CIS and tumor. Based on this, of the 313 patients, 5 had only BBD, 92 had CIS and 216 had a tumor lesion. Lymphocytic infiltrate levels were scored as low, medium, and high. The p-value for the chi-squared test of independence between lymphocytic infiltrate and lesion type is less than 0.001. Thus, there is a significant trend towards lesion severity and high lymphocytic infiltration.
Manuscript in preparation. Statistical Data: Appendix A-4

CONCLUSIONS:

- A: **DOWNREGULATION OF HLA-A AND BW6, BUT NOT BW4, ALLOSPECIFICITIES IN LEUKEMIC CELLS. AN ESCAPE MECHANISM FROM CTL AND NK ATTACK?** The results suggest that the selective downregulation of HLA-A and HLA-Bw6 allospecificities associated with HLA-Bw4 preservation provides leukemic cells with an escape mechanism not only from CTL, but also from NK cells. As a result T cell-based immunotherapeutic strategies for leukemia should utilize HLA-Bw4 alloantigens as restricting elements since a selective HLA-Bw4 allele loss would provide leukemic cells with an escape mechanism
- B: **HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTER STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES:** The results indicate that FFPTs represent a useful substrate upon which to monitor HLA antigen expression in

malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

- C: EXPRESSION OF MHC CLASS I AND II EXPRESSION SIGNIFICANTLY DISCRIMINATES AMONG BENIGN, CARCINOMA IN SITU, AND MALIGNANT LESIONS OF THE BREAST. Maria J. Worsham, James J. Yang , Xia Sheng, Roberta Sheffer, Jingfang Cheng, Usha Raju: The results suggest that there was a significant correlation of expression levels of HC10 and LGII and breast lesion type pointing in the direction of upregulation in tumor cells, rather than downregulation as commonly reported. The implications of the latter with respect to immune surveillance status surrogates of HC10 and LGII and patient outcome are underway.
- D: THE ASSOCIATION BETWEEN THE EXPRESSION LEVELS OF HC10 AND LGII AND TUMOR STAGE: The results do not point to an association between HC10 abnormalities and stage.
- E: THE RELATIONSHIP BETWEEN LYMPHOCTIC INFILTRATION AND LESION TYPE BASED ON CD45 EXPRESSION: There is a significant trend towards lesion severity and high lymphocytic infiltration.

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Maiorana AM, Cesinaro AM, Fano RA, Collina G. Expression of MHC class I and class II antigens in primary breast carcinomas and synchronous nodal metastases. *Clin Exp Metastasis* 13: 43-48, 1995

ATTACHMENTS:

Appendix A

- 1: Demanet C, Mulder A, Deneys V, Worsham MJ, Maes P, Claas FH, Ferrone S. Down-regulation of HLA-A and HLA-Bw6, but not HLA-Bw4, allospecificities in leukemic cells: an escape mechanism from CTL and NK attack? *Blood*. 2004;103(8):3122-30.
- 2: HLA antigen expression in breast cancer: a multicenter study utilizing formalin-fixed paraffinized tissues
- 3: Abstract to 2005 Era of Hope DOD Meeting
- 4: Statistical data for stage and HLA IHC expression

Appendix A

- 1: Demanet C, Mulder A, Deneys V, Worsham MJ, Maes P, Claas FH, Ferrone S. Down-regulation of HLA-A and HLA-Bw6, but not HLA-Bw4, allospecificities in leukemic

**Selective downregulation of HLA class I BW4 negative
antigens in human leukemia suggest a combined escape from
T- and NK cell attack.**

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Abbreviations:

ALL: acute lymphoblastic leukemia
AML: acute myeloid leukemia
CLL: chronic lymphoblastic leukemia
HLA: human leukocyte antigen
NK: Natural Killer cell
CDC: complement dependent cytotoxicity
MFI: mean fluorescence index

Keywords: HLA class I, leukemia, downregulation, Bw4, Bw6, Natural Killer cell, immunosurveillance

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Introduction

The classical HLA class I molecules (HLA-A, HLA-B, and HLA-C) are expressed on the surface of most nucleated cells as a heterodimer composed of a 45 kDa polymorphic heavy chain (α -chain) and a 12 kDa monomorphic light chain (β 2-microglobulin), presenting an 8 to 11 amino acid peptide to cytotoxic/cytolytic CD8 expressing T-lymphocytes. Every individual expresses two alleles for each locus for a total of six alleles. These cells are triggered to kill virus-infected cells or tumor cells presenting cytosolically degraded foreign viral or tumoral peptides on class I molecules. HLA class I molecules also interact with receptors on natural killer (NK) cells. They are equipped with a set of inhibitory and activatory killer immunoglobulin-like receptors that specifically recognize groups of HLA-A, -B, or -C alleles (1). When the predominant inhibitory signal on NK cells is lost due to downregulation or loss of one or more HLA class I alleles the NK cell becomes activated and will eliminate the viral-infected or malignant cell. Accordingly, downregulation or loss of HLA class I expression would not only lead to impaired T-cell recognition and escape of T-cell immunity but should also make these cells susceptible to NK attack.

Down-regulation of HLA class I expression seems to be a common event in solid tumors with various underlying mechanisms (2,3). Reduced expression has been observed in basal cell, breast-, bladder-, cervical-, colorectal-, head and neck-, laryngeal-, oesophageal-, lung-, renal cell- and pancreas carcinomas (4-13, 14, 15, 16) as well as in neuroblastoma, melanoma, prostate cancer and other tumor types (17-19, 20).

According to these studies on solid tumors 4 major types of changed HLA class I expression were recognized: 1) total loss of HLA-molecules, 2) haplotype loss, 3) HLA locus loss and 4) allele loss or a combination of these (2). Thus, it appeared that important changes occurred allowing the tumor cells to escape from cytolytic/cytotoxic T-cell activity. Because these studies were nearly exclusively done on solid tumors with immunohistochemistry techniques only a limited idea on quantitative changes of HLA molecules could be gathered.

The interest of studying HLA expression in solid tumors was mainly induced by the discovery of tumor specific tumor antigens (melanoma), the concomitant start of peptide vaccination protocols and the need for selection of patients. Indeed, eligible patients entering these vaccination studies should be positive for the restrictive peptide-binding HLA determinant on the tumor cell.

During the last years numerous leukemia-specific translocations or overexpressed genes were described both in myeloid and lymphoid leukemia's that could eventually serve as tumor specific antigen in leukemia vaccination programs (21). A natural or induced immune response against some of these translocations or genetic abnormalities was already described in several reports (22,23).

Over time however, only a few limited studies on HLA class I expression have been done on hematological malignancies such as in B cell and Hodgkin lymphoma's (24,25), CLL (26), ALL and AML (27-29). Total lack of HLA class I molecules in non-Hodgkin lymphoma has been associated with an aggressive clinical course (30). In AML, Savoia et al (31) reported an increased leukocyte concentration at diagnosis with loss of HLA class I molecules.

The aim of the present study was to map the HLA class I abnormalities on acute and chronic leukemic cells and to compare the results with those of normal patient or donor cells. Therefore we used both qualitative and quantitative techniques involving serology, flow cytometry and molecular biology. Moreover, a series of allospecific human monoclonal antibodies was extensively tested and used to study HLA expression at the allelic level.

Materials and methods

Samples

Leukemia patients

EDTA anticoagulated blood or bone marrow samples were obtained from 64 leukemia patients at diagnosis. The only selection criterion was CD19+ of CD33+ membrane expression for lymphoid or myeloid tumors respectively. All patients' samples were obtained from the Clinical Hematology Department of the Academic Hospital of the Vrije Universiteit Brussel, Brussels and the Université Catholique de Louvain, Brussels, Belgium.

Control individuals

Normal blood or bone marrow was obtained from regular blood donors or from allogeneic bone marrow donors at the time of collection. The institutional Ethics Committee approved this study.

These samples were used to isolate normal, cellular counterparts to serve as reference material for the flow cytometry studies.

Mononuclear cells from peripheral blood and bone marrow were separated by Ficoll-Hypaque density gradient centrifugation. The isolated cells were further washed and diluted in PBS FACSflow (Becton Dickinson, San Jose, CA, USA) supplemented with 0.5% bovine serum albumin.

Magnetic cell sorting system (MACS)

The MACS-system and the microbeads were obtained from Miltenyi (Sanvertech, Boechout, Belgium) and applied as described by the manufacturer. In brief, a starting population of mononuclear cells was incubated for 15 min with the appropriate mouse anti-CD antibodies (see further). The cells were washed in buffer and incubated in 100 μ L of a 1:10 dilution of the anti-mouse labeled magnetic microbeads for 15 minutes on ice.

The cell suspension was then applied to a MIDIMACS column in a magnetic collar. The column was washed thrice to obtain the untouched cells. The bound cells (touched fraction) were recovered by removing the magnetic collar and passing 5 mL of buffer through the column using a plunger.

It was decided at the start of the study that all HLA-expression experiments would be done on negative (untouched) isolated cell suspensions, primarily to exclude the possible interference from antibody-coated magnetic beads on cells in further procedures and to examine the HLA expression on the tumor cells and normal cells from a patient in identical conditions.

Therefore, two individual magnetic bead separations were performed on each peripheral blood or bone marrow sample to obtain untouched normal or tumor cell fractions. In addition, different microbeads were used depending on the type of tumor.

To isolate tumoral B cells from CLL-B or ALL-B samples the patients normal cells were magnetically labeled by a cocktail of anti-CD2, -CD4, -CD11b, -CD16 and -CD36 antibodies.

The unlabeled CD19⁺ B cells were recovered by simple washing. In a second procedure the patient's normal cells were obtained after the tumoral B cells were retained on the column by anti-CD19 microbeads. In the case of myeloid tumors the patient's normal cells were isolated after the tumor cells were bound to the column through anti-CD33 microbeads. Untouched CD33⁺ tumor cells were obtained by retaining the normal cells on the column by a combination of anti-CD2 and anti-CD19 microbeads. The purity of the isolated cells, as tested by flow cytometry, was always higher than 95 %.

All touched, positive fractions (patient tumor- or normal cells) were cryopreserved in complete medium containing 10% DMSO for eventual DNA isolation purposes.

An identical procedure was used to isolate normal B-cells and normal T-cells from peripheral blood or bone marrow from healthy donors.

No attempt was made in this study to isolate the small fraction of NK cells (5%) out of the major T-cell fraction (95%). These cells are further called “normal cells” in the study.

The purified, untouched cells were preferentially used for general flow cytometric purposes. The remaining cells were further used for complement-dependent cytotoxicity assays and eventually cryopreserved for later on staining with allele specific mAb's.

Flow cytometry

The following unlabelled, mouse mAb's against differentiation antigens were used for leukemic cell type assignment: anti-CD2 (clone leu 5b), anti-CD3 (clone leu 4), anti-CD5 (clone leu1), anti-CD16 (clone leu 11), anti-CD19 (clone leu 12), anti-CD45 (leuco) and anti-CD56 (clone leu19) were obtained from Becton Dickinson. Anti-CD13 (clone My7) and anti-CD33 (clone My9) were obtained from Analis (Namur, Belgium).

A second panel of mouse anti human mAb's was directed against general HLA antigens. Anti-locus A (clone LGIII-147-4) and anti-β2 microglobulin (clone NAMB-1) were obtained from Prof. S. Ferrone and anti-locus B+C (clone B1.23.2) from Dr. A. Mulder. Anti HLA total class I (clone W6/32) and anti HLA total class II (clone Tu-39) were obtained from ATCC. Anti HLA locus DR (clone HLA-DR) was obtained from Becton Dickinson.

In addition, a panel of human mAb's (Dr. A. Mulder) recognizing single HLA alleles were used to study individual allospecific expression levels in normal healthy individuals and leukemic patients and to confirm allelic losses found by cytotoxicity assays.

Cell suspensions of 4×10^6 cells/mL were made in PBS supplemented with 5% BSA. Twenty-five μL of this suspension was incubated for 20 min with 25 μL of each mAb at room temperature. The cells were rinsed with PBS + 1% BSA.

All mouse antibodies were detected with F(ab')₂ fragments of goat anti-mouse isotype specific antibodies coupled to fluorescein isothiocyanate (FITC). Human allele-specific monoclonal antibodies were detected with F(ab')₂ fragments of rabbit anti-human isotype specific antibodies coupled to FITC. In each case, at least two mAb's against single HLA

antigens, not present on the patient's cells, were used as negative controls. All secondary reagents were obtained from Prosan (Merelbeke, Belgium).

After washing 500 μ L of FACS flow solution was added (Becton Dickinson) and the samples examined consecutively. Data were acquired, analyzed and displayed by a Coulter Epics II – MCC cytometer using the Coulter System II – software v 3.0 (Coulter, Miami, Florida, USA). The mean fluorescence intensity (MFI) from samples was noted and used for further analysis.

HLA typing

Serological HLA typing

The HLA-A and HLA-B phenotype was established on the different isolated cell fractions using the conventional complement-mediated microlymphocytotoxicity test (CDC-testing). Each plate contained at least two sera identifying individual HLA-antigens. In individual cases (weak or extra reactions) other commercially available, serological plates were used (Biotest Seralc, Kortenberg, Belgium). Allelic losses were considered when no reaction was observed on the tumor cells compared to the patient's normal cells.

All microcytotoxicity assays were supervised by the same experienced technician ensuring reproducibility and reliability of the assays during the study.

Molecular HLA typing

Reverse hybridization technique

In all cases where locus A or B showed homozygosity by serology, molecular typing was performed based on the reverse hybridization technique (Innogenetics, Ghent, Belgium).

DNA was isolated using a routine salting-out method (DNA E-Z Prekit, Orchid Diagnostics Europe, St Katelijne Waver, Belgium).

Sequence based typing

In cases where complete HLA-losses on tumor cells were detected by serology, DNA was extracted from cryopreserved normal and leukemic cells (touched fractions).

First, a 2 KB PCR amplicon spanning exons 1 to 5 from locus A and B was generated using a Gene Amp PCR 9700 (Applied Biosystems, Foster City, Ca, USA). The PCR product was always cleaned-up using Centricon YM-100 filters (Millipore, Brussels, Belgium) and visualized in agarose gel electrophoresis using ethidiumbromide to show the single 2 KB band before sequencing.

Next, sequencing reactions were performed separately for exons 2, 3 and 4 both in forward and reverse directions for each sample using Big Dye Terminator Chemistry (Applied Biosystems). Sample electrophoresis was done on an ABI Prism 310 sequencer. Sequencing reactions were analyzed using ABI Match Tools PPC v 1.0. Both the PCR amplification and sequencing reagents were obtained from Applied Biosystems.

Cell lines

The following human leukemic cell lines molecular typed for HLA class I, were used in testing the specificity and reactivity of the human mAb's: MONO-MAC-6 (A03, B07, B51), NALM-6 (A01, A02, B08, B15), RPMI-8402 (A01, A29, B07, B38), ML-2 (A02, B44, B51), NB-4 (A11, B35, B40), 697 (A02, A25, B07, B15), KARPAS-299 (A03, A11, B07, B35) and K562 (A11, A31, B18, B40).

Statistics

The non-parametric (two-sided) Mann-Whitney U test was used to calculate probabilities between groups. $P \leq 0.05$ was considered significant.

Results

Reactivity and specificity of the human allele-specific antibodies

Before using the allele-specific human mAb's in our study they were first extensively tested for reactivity and specificity on HLA class I typed donors and cell lines. The results are shown separately for HLA locus A (Table 1) and locus B (Table 2). The reactivities of the Ab's tested in flow cytometry always showed expression levels ranging from 1 till 3 logs stronger than the negative controls. The results for the NALM-6 and MONO-MAC-6 cell lines are shown (Fig 1a,b). Overlapping profiles were never observed. Some antibodies did react with an epitope present on distinct HLA antigens. For example, clone SN66E3 reacted with A2 but also with A28. In these cases, the donors or cell lines were selected for having only one of these antigens. Other mAb's, such as clone VDK1D12, are restricted in the recognition

of only one HLA antigen. The overall results showed that all mAb's, except clone BR011F6, reacted as predicted by the HLA type of all the cells under study confirming their specificity. Clone BR011F6 did not recognize A1104. The HLA class I negative K562 cell line remained negative with all relevant mAb's (not shown).

Locus-specific analysis of HLA antigens

In a first attempt to detect HLA expression abnormalities 46 leukemic patients, including CLL-B (n=17), ALL-B (n=9) and AML (n=20), have been studied with common anti-HLA mAb's. The expression level of the untouched leukemic B cells was compared to the expression on normal B cells from healthy donors. In CLL-B total class I, as measured by the W6/32 mAb, was significantly lower than on normal B cells (Table 3). No significant difference was however noted for locus A or B. Total HLA class II was also significantly lower on the tumoral B cells.

In ALL-B the decrease in total HLA class I was even more pronounced than in CLL-B. In all individual cases (data not shown) a severe decrease for locus A and B was observed. No significant changes were noted for class II expression. Furthermore, all CLL-B and ALL-B cases showed β 2 microglobulin expression.

In contrast, in CD33+ AML cells total levels of class I and II were increased when compared to normal CD33+ bone marrow derived progenitors (Table 4). Significance could however not be shown for individual locus A or B. Individual data (not shown) revealed that only two cases showed a moderate decrease in total HLA class I expression. All showed expression of β 2 microglobulin. Expression of total HLA class II, and more specially locus DR, was increased.

Detection of HLA class I total loss phenotypes

Immunomagnetically separated normal and tumor cells from 37 leukemic cases were analyzed by CDC (Table 5). The high purity and general HLA class I expression of two representative cases is shown (Fig 2a,b). In 5 cases complete HLA losses were detected. One patient (case 1) showed a complete locus A loss and four others showed a complete loss of one HLA-A (case 29 and 38) or HLA-B (case 7 and 44) allele.

In a number of cases too few normal cells could be isolated to allow serological typing. To avoid false conclusions on allelic losses, the HLA homozygosity of case 21 was therefore confirmed on the tumor cells by molecular typing.

Confirmation of HLA-A or -B allelic losses by flow cytometry.

Monoclonal antibodies against single HLA alleles allow the study of HLA antigens by flow cytometry. Cryopreserved patients normal and tumor cells were available from 4 cases where a complete single allele loss was detected by CDC. Allele-specific mAb's were available against all HLA-A and -B antigens from the 4 cases. Figure 3 shows that the

expression of HLA A10 and A31 in case 1, B8 in case 7, HLA A28 in case 29 HLA A1 in case 38 are completely negative. The contour plots are identical to those from the negative isotype controls. On the other hand HLA-B8 (case 38) expression is nearly negative on flow but scored positive in CDC. This illustrates that the CDC serological HLA class I testing is extremely sensitive and is therefore only useful in detection of complete allelic or locus losses and not for incomplete downregulations. The expression of HLA B8 is approximately 10-times lower (1 log) on the tumor cells than on the patients normal cells. On the same tumor it can be observed that a small fraction of the tumor cells didn't express HLA A2 anymore. This could be an example of an emerging variant tumor clone. HLA total losses were never detected on the patient's normal cells illustrating the relation of HLA losses with tumor cells.

Mutation analysis

In an attempt to explain the HLA allele losses on the tumor cells the HLA-A (cases 1, 29, 38) or -B (case 7) genes were sequenced in forward and reverse direction for exons 2, 3 and 4 from the tumor cells and the patients normal cells. The results for all cases on both cell fractions were completely identical excluding a mutational problem for the exons under study.

The β 2-microglobulin gene was not sequenced since other HLA class I alleles on the tumor cells were present on the cell's surface excluding a mutation, or transcriptional problem of the β 2-microglobulin gene.

Allele-specific expression levels on leukemic cells

During this part of the study the expression levels of individual alleles were studied on 32 leukemic samples using previously well characterized human mAb's. The expression levels were compared to those obtained from the patients normal cells and not to those from unrelated donors. In our opinion is comparison at the allelic level indeed more appropriate and accurate with cells having the same set of HLA alleles. Indeed, differential expression of HLA-A and -B antigens is genetically predetermined and inherited according to Mendelian laws (32). A drawback is that these cells are not identical cellular counterparts.

The results for the tumor cells and the patient's normal cells are given expressed as MFI and presented separately for locus A and B (Table 7). A ratio R was then calculated ($R = \text{MFI tumor cell} / \text{MFI T cells}$). We concluded for downregulation if the R-value was lower than 1. Data on the quantitative expression of individual alleles in healthy individuals are scarce in the literature. During the characterization of the human mAb's, individual analysis of normal T

and B cells allowed us to investigate this point. In normal, healthy individuals expression of total HLA class I was always higher on B cells than on T cells (n=10; data not shown). Similar to total HLA class I levels, individual expression levels of locus A or B were higher on B than on T cells. No single exception was observed. The mean MFI value was approximately double on B cells than on T cells. These results were confirmed very recently (33).

For the HLA-A locus, 56 alleles could be studied in 32 patients and according to our definition 20 alleles showed a downregulated expression (35 %). For the HLA-B locus 50 alleles were studied and 19 showed a downregulation (38 %). Thus, a total number of 106 alleles were studied. Combining the locus A and B results makes, that in 21 out of the 32 patients (65 %), one or more allelic downregulation(s) were observed.

Some HLA-A and -B antigens could not be studied because the relevant antibody was lacking. Locus C was also not studied. Thus, it is possible that the exact number of patients with a downregulation on the tumor cells of HLA locus A, -B, -C or a combination of these would even be higher. The patients that showed a complete allelic loss on the tumor cells and described earlier, were not included here.

Downregulation according to the presence of the Bw4 or Bw6 epitope

Allelic downregulation was therefore further analyzed according to the presence or absence of the Bw4 or Bw6 epitope (34). From the 36 HLA-B alleles expressing Bw6, 16 showed downregulation (44 %). In contrast, on the 21 HLA-A and -B alleles having the Bw4 epitope, only 3 showed a downregulation (14%). Forty % (20/49) of the BW4/BW6 double-negative antigens also showed downregulation. The difference in frequency in downregulation of alleles expressing Bw4 or Bw6 is statistically significant ($p < 0.03$). Since Bw4-positive HLA class I antigen expression is under the control of NK cells these results suggest that preferentially those alleles are downregulated that are not under NK cell control. This finding suggests that emerging tumor cells, by downregulating HLA class I alleles (but not BW4-positive antigens) not only escape from T-cell but also from NK killing.

Discussion

Our first objective was to investigate whether HLA class I alterations, such as those observed in solid tumors were detectable in leukemia. In this study HLA expression on leukemic cells was compared to donor-derived normal cellular counterparts (first part of the study) or to the patients own remaining normal cells (second part). This methodology allowed comparison of identical cells but equipped with a different set of HLA antigens versus non-identical counterparts but completely HLA matched cells. Throughout the study we only used untouched, immunomagnetically

purified leukemic or normal cells. This was mainly to exclude any interference with antibody coated magnetic beads or it was necessary for the technique itself. Indeed, CDC analysis on the leukemic cells would score positive, even with an HLA class I loss, if normal cells would grossly contaminate the leukemic test sample.

In a first part of the study we investigated by flow cytometry the general expression of HLA class I on leukemic subtypes by measuring total class I, locus A, locus B and β 2 microglobulin. This revealed a decrease of total HLA class I in CLL-B and ALL-B. A pronounced HLA-A and-B locus-specific decrease was especially demonstrated in ALL-B. Expression of β 2 microglobulin was detected in all leukemic samples excluding a total HLA class I loss as described in solid tumors (35). Additionally, HLA class II was decreased in CLL-B, but not in ALL-B.

In contrast, malignant CD33+ AML cells show an upregulated class I and II expression when compared to normal cellular counterparts. In agreement with the study of Wetzler et al (28) abnormalities in HLA class I are infrequent in AML, but it was surprising that in AML the phenomenon of overexpression could co-exist with a complete absence of a single allele (case 29 and 44). Indeed, a complete allelic loss was only detected in 5 out of 35 (13%) leukemic cases by CDC (Table 5).

The AML and CLL samples with a complete allelic loss were further analysed, but mutation analysis of the polymorphic regions of locus A or -B antigens did not reveal any abnormality. These results are in agreement with those from Brouwer et al (27). They also didn't detect HLA class I genetic abnormalities. Moreover, they could restore HLA class I allelic expression by interferon- γ treatment and subsequent T-cell recognition and lysis of the target cells. Real et al (35) also showed that suppression of single alleles could be restored by cytokine treatment. Further DNA or RNA- based analysis could however not be done in our study due to the lack of sufficient material. A new bloodsample could not be obtained since all patients with a complete allelic loss died very soon after diagnosis.

In the second part of the study the expression of individual alleles was assessed with the use of highly specific mAb's. The comparison of the allelic expression on the leukemic cells and on the patient's normal cells made a detailed analysis possible. On 32 leukemic samples 116 individual allelic expression profiles could be studied. Downregulation was observed in 35 % and 38 % of HLA-A and HLA-B alleles respectively. This means that in the majority of the leukemic patients (65%) downregulation was observed for the HLA-A and/or HLA-B locus. HLA-B has been divided by serology into two groups: Bw4 and Bw6. In contrast to HLA-C, only one group of HLA-B allotypes is involved in inhibition of NK cells. Whereas HLA-B allotypes that carry the Bw4 serological epitope provide protection from NK attack, those

with the Bw6 epitope do not. Further study of our leukemic samples made clear that preferentially Bw6 positive and non-Bw4/non-Bw6 containing alleles were the subject of downregulation. In solid tumors, other investigators also noticed the preferential downregulation of the HLA-B locus or B alleles but it was apparently never analyzed according to the Bw4 and Bw6 allotypes (37,38).

In conclusion, in leukemic samples a complete allelic loss was seen in a modest number of samples (10 %) but downregulation seems to be a common event in leukemia. In our opinion downregulation will finally end-up in a complete allelic loss during tumor progression. All the patients that we detected with a complete allelic loss quickly died after diagnosis suggesting a higher degree of malignant behavior of these tumors. Moreover, this could be one of the reasons why a total loss is rather infrequently detected during our and other studies (27). It is tempting to speculate that this high degree of downregulation is the result of selective pressure due to an upcoming natural anti-leukemic T-cell immunity. This activity was already described by others (39). If evasion of T-cell immunosurveillance is the case, than the escaping leukemic clone, by downregulating HLA class I antigens, should or could become a target for NK attack unless the relevant NK receptors are not available in the patients repertoire, NK receptor expression is disturbed (40) or downregulation is limited to those alleles that are not under the control of autologous NK cells. These are HLA-A alleles not expressing the Bw4 epitope (none-Bw4/none-Bw6) and HLA-B Bw6 positive alleles. Together our findings point to the conclusion that leukemic cells, especially those of lymphoid origin, developed strategies to escape from both T-cell and NK-cell attack.

If preferential downregulation of non-Bw4 class I alleles proves to be a general phenomenon also observed in solid tumors, than vaccination protocols should preferentially include tumor-specific peptides that can bind to Bw4 containing alleles. An eventual escape from T-cell immunity would render them susceptible to autologous NK cells.

Total HLA class I loss, haplotype loss or locus loss are frequently seen in various solid tumors but not in leukemia. From our study allelic downregulation seems to be the most common abnormality in human leukemia. Therefore, future studies on these malignant cells should preferentially include reagents able to dissect expression at the allelic level (allele-specific antibodies) over those to backbone structures (anti-locus A and B, β 2m, total anti-class I).

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Legends figures and tables

Figure 1. HLA expression on cell lines

Flow cytometric histograms of HLA class I and II antigens on the NALM-6 (Figure 1a) and MONO-MAC-6 (Figure 1b) cell lines as detected by different specific mAb's described in materials and methods. Fluorescence intensity with anti-HLA mAb's (black) was compared to that of irrelevant, isotype-matched controls (white).

Figure 2. HLA class I expression on immunomagnetic isolated malignant and normal cells.

In Figure 2a untouched, normal cells were incubated with anti-CD3 (black) and anti-CD19 (white) (panel A), with anti-locus A (white), anti locus-B (black) or control mAb (dotted) (panel B), and anti-W6/32 (black) or control mAb (white) (panel C). The untouched malignant cells were incubated with anti-CD3 (white), anti-CD19 (black) (panel D), with anti-locus A (white), anti-locus B (black) or with control mAb (dotted) (panel E) and anti-W6/32 (black) or control mAb (white) (panel F). The expression levels of HLA locus A and B and total class I are lower on the malignant cells than on the patients normal cells.

Figure 2b shows the results on the leukemic cells and normal cells of an AML patient. The mAb's used were identical as described under Figure 2a except that anti-CD33 was used instead of anti-CD19. Expression levels show that HLA locus A and B and total class I are higher on the tumor cells than on the patients normal cells.

Forward/side scatter dot plots of the different isolated cell populations are shown on the left.

Figure 3. Confirmations of complete HLA class I allelic losses on leukemic cells.

The patients malignant and normal cells were stained with the relevant human allele-specific anti HLA mAb's (black) and compared to irrelevant, isotypic control mAb's (white). The complete losses detected by CDC were all confirmed: A10 and A31 (case 1), B8 (case 7), A28 (case 29) and A1 (case 38). The weak reaction for B8 (case 38) scored clearly positive in CDC.

Table 1. Expression of locus A alleles on donor lymphocytes and cell lines.

HLA-A allele specific antibodies were tested against HLA class I typed donors and cell lines. Two isotype-matched controls were always included in every case tested. All antibodies reacted with all cells tested, except clone BR011F6. This antibody did not react with HLA A1104 (2 donors out of 4 tested). Reactivity is expressed as + (1 log), ++ (2 logs), +++ (3 logs) stronger than the negative controls used.

Table 2. Expression of locus B alleles on donor lymphocytes and cell lines.

HLA-B allele-specific antibodies were tested against HLA class I typed donors and cell lines. Conditions and interpretation of reactivity are identical as described under Table 1.

Table 3. Total HLA class I and locus-specific expression on lymphoid leukemic cells.

HLA expression on CLL-B and ALL-B at diagnosis was compared to the expression on normal peripheral blood B cells derived from healthy donors. All cells tested were untouched fractions derived from immunomagnetic isolation procedures. The results are expressed as mean fluorescence intensity values +/- SD. Comparison between patients and donor values was performed by means of the non-parametric Mann-Whitney U test.

Table 4. Total HLA class I and locus-specific expression on AML cells.

HLA expression on AML cells at diagnosis was compared to the expression on bone marrow derived CD33+ progenitors from healthy donors. All cells tested were untouched CD33+ cells obtained from immunomagnetic isolation procedures. Results and statistics as described in Table 3.

Table 5. Serological HLA-A and HLA-B typing on leukemic and normal cells.

Untouched leukemic and normal cells from patients at diagnosis were obtained using immunomagnetic procedures and tested for HLA class I expression by CDC.

(-: homozygous sample; ND: not done, not enough normal cells available; ■: no reaction with the respective anti-HLA antisera)

Table 6. Expression of locus A and B allospecificities on leukemic cells.

Allelic expression levels were tested by flow cytometry on malignant cells and compared to the patients remaining normal cells. All cells tested were untouched cell fractions obtained from immunomagnetic procedures. The results are expressed as mean fluorescence intensity (MFI) after background correction. The ratio R was derived from MFI malignant cells / MFI normal cells. An R value lower than 1 was considered as downregulation of that particular allele.

(H: homozygous; ND: not done, no specific antibody available)

Appendix A

2: HLA antigen expression in breast cancer: a multicenter study utilizing formalin-fixed paraffinized tissues

HLA antigen expression in breast cancer: a multicenter study utilizing formalin-fixed, paraffin embedded tissues

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Running title: HLA antigen expression in formalin-fixed tissues

Key words: breast cancer, formalin-fixed tissues, HLA antigen expression, loss of heterozygosity

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Summary

HLA class I and class II antigen expression was examined in formalin-fixed, paraffin embedded tissue from three breast cancer patients. To determine if loss of expression of MHC Class I molecules at the protein level with immunohistochemistry reflects alterations at the gene level, DNA from microdissected normal and tumor tissue was evaluated with microsatellites at the MHC class I 6p21 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin ($\beta 2m$) locus for loss of heterozygosity (LOH).

HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with anti-HLA class I heavy chain monoclonal antibody (mAb) HC-10 and with the anti- $\beta 2m$ mAb L368. Furthermore, the four laboratories detected no staining of normal or malignant breast tissue for the lesions stained with the anti-HLA-DR, DQ and DP mAb LGII-612.14. In contrast, infiltrating lymphocytes were strongly stained by mAb LGII-612.14. Furthermore, LOH was detected in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus.

These results validate the use of formalin fixed, paraffin embedded tissue sections as substrates in immunohistochemical reactions with mAb to measure the expression of HLA antigens. Furthermore these results show for the first time that immunostaining of formalin fixed, paraffin embedded tissue sections with anti-HLA mAb yields reproducible results, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

Introduction

HLA class I antigen expression in surgically removed malignant lesions has been extensively investigated in recent years (1-5). Frozen tissue sections have been used as a substrate in immunohistochemical reactions with mAb, because most of them do not detect the corresponding determinants in formalin fixed, paraffin embedded tissues. These studies have convincingly shown that abnormalities in HLA class I antigen expression are frequently associated with malignant transformation of cells (6-8)). Furthermore an increasing body of evidence is compatible with the possibility that abnormalities in HLA class I antigens in malignant lesions may have a negative impact on the clinical course of the disease and on the outcome of T cell-based immunotherapy (2)). In spite of this evidence analysis of HLA class I antigen expression in malignant lesions represents a criterion neither to evaluate patients with malignancies nor to select those to be treated with T cell-based immunotherapy. This phenomenon is likely to reflect, at least in part, pathologists' reluctance to use frozen tissue sections as a substrate in immunohistochemical reactions. To overcome this limitation in the routine analysis of HLA class I antigen expression in malignant lesions, the HLA and Cancer component of the 13th International Histocompatibility Workshop made one of its goals the analysis of HLA class I antigen expression in formalin fixed malignant lesions and the assessment of its clinical significance.

Distinct mechanisms have been found to underlie the multiple tumor HLA class I immuno-surveillance escape phenotypes which have been identified by immunohistochemical staining of frozen tissue sections with mAb^s which recognize monomorphic, locus-specific and allele-specific determinants of HLA class I antigens. HLA haplotype loss in tumor cells has been shown to be generated frequently by total or partial deletion of chromosome 6, which carries the major histocompatibility complex in humans (9-11)). The detection of these losses has been facilitated by the recent identification of highly polymorphic microsatellite markers (short tandem repeats). Utilizing short tandem repeats mapped on chromosome 6 (6p21, HLA locus) and on chromosome 15 (5 q21 locus, β 2m), loss of heterozygosity differences have been described in the frequency of HLA class I genes and of β 2m genes in various types of malignancies. The values range from about 15% in colorectal carcinoma lesions to about 50% in head and neck squamous cell carcinoma (9, 10)- and in cervical carcinoma lesions (11). Whether these differences reflect the use of different markers, lack of standardization in the methodology used, and/or different characteristics of the malignancies and/or patients investigated remains to be determined.

The aim of the present study was to optimize and assess the reproducibility of the assays to measure the expression of HLA class I antigens and to detect LOH at the HLA locus (6p21) and at the β 2m locus (15q21) in formalin fixed, paraffin embedded normal breast epithelium and corresponding malignant breast lesions.

Methods

Four laboratories (Lab) participated in this evaluation (1-Garrido, 2-Tilanus, 3- Ferrone, 4-Worsham), using shared antibodies (prepared in Lab 3) and sections cut from the same tissue blocks (prepared in Lab 4).

Monoclonal antibodies and conventional antisera

The anti-HLA class I heavy chain mAb HC-10, the anti- β_2m mAb L368 and the anti-HLA-DR, DQ, DP mAb LGII-612.14 were developed and characterized as described (12-14). Peroxidase-conjugated anti-mouse IgG xenoantibodies were purchased from Vector Laboratories, Inc (Burlingame, CA).

Immunohistochemistry methodology, validated in Lab 4 with recommended dilutions for the three antibodies was provided to the other participating centers. In brief, dilutions were as follows: mAb HC-10: 1/100 dilution, mAb L368: 1/25, mAb LGII-612.14: 1/25, with overnight incubation for all three. The standardized methodology utilizes antigen-retrieval with endogenous peroxidase inactivation using 3% hydrogen peroxide, with normal horse serum as the blocking agent (Vector Laboratories Inc., Burlingame, CA). Following incubation with monoclonal dilutions, the slides are washed and incubated with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Inc.), followed by incubation with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit: Vector Laboratories, Inc.). Immunoreactivity was visualized with 3', 3-diaminobenzidine tetrahydrochloride (Vector laboratories, Inc.) and the sections are counterstained with Mayer's Hematoxylin.

Immunohistochemistry Interpretation

Scoring of stain intensity in the tumor areas were performed with reference to staining intensity of normal breast tissue (control) present either in the same section with the breast tumor, or processed concurrently with the tumor section. Stromal lymphocytes in lymphocytic infiltrates surrounding the tissue, which stain intensely, are also considered as a reference for normal staining intensity.

Scoring parameters included cell membrane localization. Staining interpretation is derived as follows: 0-25% of cells = negative; >25%-<50% = heterogeneous; >50%-<75% = heterogeneous; (>25%-<75% = heterogeneous); >75% = positive staining. Scoring was done by two independent reviewers in each of the four centers in a blinded fashion. Complete absence of staining as compared to the presence of staining in the normal breast epithelium and in surrounding lymphocytes is scored as "complete loss". Marked decrease in staining intensity is scored as "+", presence of some staining intensity receives a score of "++", a score of "+++" indicates intensity of stain seen in normal breast tissue or surrounding lymphocytes.

Molecular methods: loss of heterozygosity (LOH)

Processing Tumor Specimens for DNA Analysis

DNA from normal and tumor tissue was extracted from 4-5 micron sections from archival paraffin embedded tissue blocks.

DNA was extracted using the QIAamp Kit (Qiagen Inc. Chatsworth, CA) according to the manufacturer's protocol.

Microsatellite analysis

A minimal microsatellite panel to detect LOH at the HLA locus at 6p and the β_2m locus at 15q included D6S1618, D6S291, D6S273, D6S265 and D6S311, and D15S126 and D15S209, respectively (Table 1). Fluorescent-labeled primers were obtained from Perkin-Elmer Applied Biosystems (Foster City, CA). 2 μ l-4 μ l of purified paraffin DNA from a total elution volume of 200 μ l from normal paraffin tissue and tumor in each case was used for each PCR reaction. PCR was performed in a 1X PCR buffer (10mM Tris-HCL pH 8.3, 50 mM KCl), 2.5mM MgCl₂, 200 μ M each dNTPs, 1 μ M of each primer and 0.8 units of Ampli Taq Gold polymerase (Perkin-Elmer) in a 10 μ l reaction volume, using a Perkin Elmer GeneAmp 9600 followed by 35 cycles, 12 min 95 °C initial denaturation, an additional 50sec denaturation at 94 °C, 60 sec annealing at 57 °C and 60 sec extension at 70 °C. Amplification was completed with a final incubation step at 72 °C for 30 minutes.

The amplified PCR products were analyzed using an automated ABI PRISM sequencer (model 310, PE Applied Biosystems), using GeneScan Rox 400 size standard (PE Applied Biosystems). In brief, 12 μ l deionized formamide were combined with 0.5 μ l GeneScan Rox 400 size standard (PE Applied Biosystems) and 2 μ l of PCR product in a Genetic Analyzer sample tube. The tubes were closed with Genetic Analyzer septa and, after short mixing (vortex), the samples were denatured in a heat block for 4 minute at 90 °C, chilled on the ice, and spun briefly in a microcentrifuge in order to collect the contents. The samples were loaded on the ABI 310 Genetic Analyzer and the run in accordance with the manufacturer's protocol (15).

Interpretation Criteria

A sample was defined as heterozygous when there were two distinct alleles for a given marker, i.e., maternal and paternal alleles were distinguishable (informative for LOH). A sample was defined as homozygous when only one size allele for a given marker was detected, i.e., maternal and paternal alleles were indistinguishable (uninformative for LOH). Loss of one of the two constitutive alleles in the tumor was scored as loss of heterozygosity (LOH). LOH was assigned when the fluorescent signal of one of two alleles was reduced by more than 30% as compared with the heterozygous control normal DNA sample (Figure 1).

Results

Laboratory 2 employed a negative control and followed the provided protocol and dilutions exactly. Laboratory 1 made the following changes to the provided protocol: no avidin/biotin block. Furthermore, antigens were retrieved by incubating tissue sections in

antigen retrieval solution in the microwave. Lastly, tissue sections were stained with anti-CD45 mAb to identify lymphocytes and dendritic cells in tumor infiltrates.

HLA expression

The staining of malignant cells in Case 1 by anti-HLA class I heavy chain mAb HC-10 was described as heterogeneous by the four laboratories (Figure 2, Table 2). The heterogeneity of staining was attributed to varying degrees of tumor cell differentiation and to the presence of heavy lymphocytic infiltrate and CD45-positive dendritic cells within tumor areas. The latter resulted in an increased staining intensity in the tumor when compared with the normal/benign epithelium. For anti β 2m mAb L368, normal ducts were scored as negative for Case 1 by Laboratories 1 and 2 and heterogenous by Labs 3 and 4. The staining intensity was scored as low as compared to the staining intensity of stromal and lymphocytic cells. The staining of malignant cells by anti β 2m mAb L368 in Case 1 was scored as negative by the four laboratories. As far as the results obtained with the anti-HLA class II mAb LGII-612.14 is concerned, the agreement among the four laboratories was complete.

For Case 2, for HC10, Labs 1, 2 and 3 scored normal epithelium as positive, with a heterogeneous interpretation by Lab 4. All four laboratories scored tumor areas as negative. For L368, laboratories 2, 3 and 4 had positive staining in normal ducts. Laboratory 1 reported weak staining in normal ducts with L368. Within tumor areas, all four laboratories indicated loss of staining for L368 with heterogeneity of staining in more well-differentiated areas of the tumor. While there was complete concordance for lack of staining with LGII-612.14, Lab 1 noted some staining within the luminal epithelium of the normal ducts.

In Case 3, there was concordance for staining of the tumor areas with all 3 antibodies among the four centers. Tumor areas for HC10, L368 and LGII-612.14 were negative in undifferentiated areas of the tumor, with heterogeneous staining in the well-differentiated areas (25%) for HC10 and L368. Normal breast epithelium was scored as weakly staining by Lab 1. Normal breast epithelium was not present in the tissue sections examined by Labs 2, 3, and 4. Stromal cells were present and scored as positive among all four laboratories.

The quality of the staining and the overall results were similar despite variations to the protocol. Microwave and citrate buffer antigen-retrieval substitutions yielded comparable results. Lab 2 demonstrated that a negative control is useful as an internal standard for reagent optimization and for noting any non-specific reactivity. CD45 staining as employed in Lab 1 may be helpful in identifying the infiltrating lymphocytes in germinal centers and dendritic cells as seen in case 1.

Microsatellite Analysis

Labs 1, 2, and 4 performed microsatellite analysis. Loss of heterozygosity was observed at the β 2m locus in Case 1 and 3, concordant with down regulation at the level of HLA expression. This locus was either normal or

uninformative in Case 2. For the HLA class I loci at 6p21, LOH for three 6p21 markers suggested concordance with down regulation of class I antibodies, HC-10 and L368 in Case 1. For Case 2, 6p21 markers were either uninformative, normal or did not yield an interpretable result. For Case 3, 6p21 markers indicated LOH for D6S105 and microsatellite instability (MSI) for marker D6S265 by Lab 4, as compared to normal and uninformative results, respectively, by Lab 1 (Table 3).

For an interpretation of LOH, there was concordance, except for marker D6S105 in Case 1, which was scored as LOH by Lab 1 and 4, and homozygous by Lab 2. In case 3, LOH for D6S105 and MSI for marker D6S265 by Lab 4 were scored as normal and uninformative respectively, by Lab 1. Among laboratories, for Case 1, all three indicated LOH for markers D6S265 and D6S276 (Figure 1), and LOH for D15S209 by Labs 1 and 4. For all three cases, Labs 1 and 4 concordantly interpreted marker D6S1618 as homozygous (uninformative). Labs 1 and 4 noted homozygosity for marker D15S126 in case 1; Lab 2 did not generate an interpretable result.

Discrepant results were noted in Case 2, for marker D6S105; Lab 1 indicated heterozygosity in the normal and tumor, whereas Laboratories 2 and 4 indicated a homozygous result. This was also the case for marker D15S209 in Case 2, which was scored as heterozygous (normal) by Lab 1 and homozygous (uninformative) by Lab 4. In Case 3, LOH was indicated by Lab 4 for marker D6S105, and microsatellite instability (gain of an allele as compared to a homozygous status in the normal) for D6S276, an interpretation that was not shared by Lab 1.

Discussion

Immunohistochemical staining of frozen sections of malignant tumors has shown that abnormalities in HLA class I antigen expression are frequent in malignant cells (1-5)). Using formalin fixed, paraffin embedded tissues, HLA class I antigen down-regulation in primary maxillary sinus SCC lesions appears to have clinical significance, because it is correlated with a statistically significant decrease in patient survival (16). Despite the possible clinical significance and potential for T cell-based immuno-therapy (2), evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in immunohistochemistry assays.

The potential predictive value of HLA expression in evaluation and therapy planning has been under-utilized. The importance of this marker in the evaluation of breast cancer patients prompted our undertaking of a multicenter study approach to develop and standardize an immunohistochemistry protocol using formalin-fixed, paraffin-embedded tissue and anti-HLA mAbs. Anti-HLA HC-10, a mAb to a determinant expressed on HLA class I heavy chains (chromosome 6p21), LGII-612.14, anti-HLA class II mAb (chromosome

6p21), and L368, an anti-human β 2m (chromosome 15q21) were evaluated in sections of archival formalin-fixed paraffin embedded breast tumors. Positive staining with CD45 was useful in accounting for lymphocyte and dendritic cell populations in tumor areas that were otherwise predominantly negative with all three HLA antibodies.

HLA class I antigen downregulation in conjunction with and without heterogeneity was concordantly scored in tumor regions of all cases with HC10 and mAb L368. Furthermore, no staining of either normal or malignant breast tissue was detected by the four laboratories in lesions stained with LGII-612.14, however, lymphocytes as expected were strongly stained in the same section. While there was some lack of agreement with staining interpretations in normal breast epithelium for L368, lymphocytes and stromal cells were judged positively stained by all four laboratories.

DNA analysis from archival tissue permits retrospective characterization of disease and has facilitated molecular data gathering from large population-based epidemiological study cohorts. It can also serve as surrogate tissue material for genetic testing of diseases or syndromes in the absence of fresh or frozen tissue or peripheral blood lymphocytes. In retrospective studies for DNA marker characteristics, tissue is mostly available in the form of formalin-fixed paraffin tissue blocks. For the detection of allelic imbalance in tumor DNA, paired normal and corresponding tumor tissue is necessary. Lack of or scarcity of normal or tumor tissue in the paraffin sections or poor quality of the DNA due to the use of inadequately buffered formaldehyde during the fixation process presents serious limitations to studies that rely on archival patient tissue resources.

Discordant results and lack of interpretable results in some instances reflect in part low DNA yields and poor quality DNA that become factors in interpretation of low signal allele peaks, stutter peaks and difficulty with amplification of larger size alleles due to degradation of DNA inherent in formalin-fixed archival tissue resources. For this study, limited resources that required a four-way distribution hampered availability of DNA for repeat sampling in some cases. Despite these challenges, for markers D6S276 and D6265, an interpretation of LOH was validated by all three participating centers, and D15S209 by at least two laboratories.

The results indicate that formalin fixed paraffin embedded tissue represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are also used to differentiate malignant cells from lymphocytes and dendritic cells. Loss of heterozygosity for markers representative of the HLA loci at 6p21 and 15q21 agreed with down regulation of expression of class I gene expression. LOH associated for loss of the HLA locus at chromosome 6p supports an extended mechanism that may contribute to HLA haplotype loss previously described in different histological tumor types (9-11)). Contaminating stroma can mask LOH results and microscopic or laser microdissection to separate stroma and tumor would facilitate an interpretation of LOH in cases with unclear results.

Acknowledgements

Maria J. Worsham: DOD Final Report: 1/30/06 "Population Based Assessment of MHC Class I Antigens Down Regulation as Markers of Increased Risk for Development and Progression of Breast Cancer from Benign Breast Lesions"

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FIGURE LEGENDS

Figure 1: Loss of heterozygosity in Case 1 with marker D6S276. The A2 allele is significantly reduced in the tumor as compared to the normal

Figure 2: Normal breast epithelium in Case 1 (Figure 2A) and corresponding tumor (Figure 2B), low power (10X) representations on the left side, high power (40X) on the right.

Figure 2A: Normal breast epithelium (Case 1) is uniformly stained with HC-10 and with some degree of heterogeneity with β 2m. Note complete lack of staining with class II LGII-612.14 and with CD45 in normal breast epithelia areas, with intense staining of infiltrating lymphocytes.

Figure 2B: Tumor epithelium (Case 1) indicates heterogeneous staining with class I mAb HC-10. Note complete loss of β 2m, LGII-612.14 and CD45 in tumor areas, with intense staining of infiltrating lymphocytes and dendrite cells (CD45).

Appendix A:: Abstract to 2005 Era of Hope DOD Meeting

EXPRESSION OF MHC CLASS I AND II EXPRESSION SIGNIFICANTLY DISCRIMINATES AMONG BENIGN, CARCINOMA IN SITU, AND MALIGNANT LESIONS OF THE BREAST

Malignant transformation of cells is frequently associated with abnormalities in the expression of MHC class I antigens. These abnormalities appear to play a role in the clinical course of the disease and to have a negative effect on the outcome of T cell-based immunotherapy for malignant diseases. Approximately half (51%) of breast carcinomas have altered regulation of HLA class I, which may represent an important step associated with tumor invasion providing tumor cells with the ability to escape recognition by T-lymphocytes. We report on the expression behavior of MHC class I (anti-HLA class I heavy chain mAb HC-10) and class II (anti-HLA-DR, DQ, DP mAb LGII-612.14) in formalin-fixed paraffin-embedded tissue from 189 invasive breast cancer cases. Tumor areas concurrent with benign proliferative lesions, normal breast epithelium, normal "other" (skin, lymph node), lymphocytes in lymphocytic infiltrates, and in situ lesions (DCIS, LCIS) within a 5 micron section were marked by the study pathologist as part of the pathology review process and immunohistochemically evaluated with the anti-HLA class I heavy chain mAb HC-10, and the anti-HLA-DR, DQ, DP mAb LGII-612.14. The lymphocyte specific anti-CD45 antibody marked the presence of infiltrating lymphocytes

and dendritic cells, and served as in internal control, particularly when normal breast epithelium was absent. Three staining categories were defined as follows: absent expression: 0-<25%, heterogeneous: >25% <50%; positive staining: >50%. Of the 189 cases, 149 had multiple records, yielding a total of 338 records, where each record is derived from either a different tissue block (of the same biopsy) or a subsequent biopsy.

To assess differences in expression of HC-10 and LG-II in the normal breast epithelium (N), benign proliferative (BPL), carcinoma in situ (CIS), and tumor (T), log-linear models were employed. For LGII, the uniform associated model (UA) model was an excellent fit underscoring lack of expression of LGII in epithelial lesions. The odds ratio analyses (for N, BPL, CIS, T) however, indicate that when LGII is expressed (presence of staining of >25%), it is significantly more likely to occur in the tumor as compared to BBD and CIS lesions, using normal epithelium as a reference, with CIS having a higher likelihood than BBD, and BBD a higher likelihood than normal epithelium. For HC10, although goodness-of-fit for the UA model was weaker suggesting a more variable expression of HC10 among the four lesion types, the HC10 expression trend was similar to LGII. Thus, there was a significant correlation of expression levels of HC10 and LGII and breast lesion type pointing in the direction of upregulation in tumor cells, rather than downregulation as commonly reported. The implications of the latter with respect to immune surveillance status surrogates of HC10 and LGII and patient outcome are underway.

Appendix A:: Statistical data for stage and HLA IHC expression

8/22/06

MHC class 1 expression levels discriminate among breast lesions along a morphological continuum to breast cancer. or

Hypothesis: Altered MHC class 1 expression is predictive of breast lesion severity along a progressive continuum to malignancy

Expression of MHC class I and II expression significantly discriminates among benign, carcinoma in situ, and malignant lesions of the breast

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Malignant transformation of cells is frequently associated with abnormalities in the expression of MHC class I antigens. These abnormalities appear to play a role in the clinical course of the disease and to have a negative effect on the outcome of T cell-based immunotherapy for malignant diseases. Approximately half (51%) of breast carcinomas have altered regulation of HLA class I, which may represent an important step associated with tumor invasion providing tumor cells with the ability to escape recognition by T-lymphocytes. We report on the expression behavior of MHC class I (anti-HLA class I heavy chain mAb HC-10) and class II (anti-HLA-DR, DQ, DP mAb LGII-612.14) in formalin-fixed paraffin-embedded tissue from **385 invasive breast** cancer cases. Tumor areas concurrent with benign proliferative lesions, normal breast epithelium, normal "other" (skin, lymph node), lymphocytes in lymphocytic infiltrates, and in situ lesions (DCIS, LCIS) within a 5 micron section were marked by the study pathologist as part of the pathology review process and immunohistochemically evaluated with the anti-HLA class I heavy chain mAb HC-10, and the anti-HLA-DR, DQ, DP mAb LGII-612.14. The lymphocyte specific anti-CD45 antibody marked the presence of infiltrating lymphocytes and dendritic cells, and served as in internal control, particularly when normal breast epithelium was absent. Three staining categories were defined as follows: absent expression: 0-<25%, heterogeneous: >25% <50%; positive staining: >50%. Of the 189 cases, 149 had multiple records, yielding a total of 338 records, where each record is derived from either a different tissue block (of the same biopsy) or a subsequent biopsy.

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Statistical methods:

4 categories and 3 risk levels:

- 1: normal: either lymphocytic infiltrates or normal breast epithelium/other (e.g., skin)
- 2: BBD= premalignant: less severe: Risk Level 1
- 3: CIS: stage 0: in situ ; more severe: Risk Level 2
- 4: Tumor: stages 1-4: malignant or breast carcinoma: Risk Level 3

The measurements of HC10, LG2 and CD45 were collected on each tissue types as the expression status in 5 levels

Level 1: 0% of cells stained

Level 2=<10%

Level 3=10 to 25%

Level 4=25+ to 50%

Level 5=50%

Class I Antibody: HC10

Class II antibody: LG2

Control for lymphocyte staining: CD45

Total number of women: **385**

Total tissue records: **1964**: 333 : Tumor (T); 265: CIS; 178: BBD; 1190 Normal: Lymphocyte normal records: 797; normal breast epithelium or normal other: 393

The number of observed tissue records per patients was in a range of 1 to 22 records.

Statistical Analysis

The GEE approach was considered adjusting for multiple concurrent breast lesions within a subject (patient) and their corresponding staining measurements.

We used a multinomial logistic regression model to deal with the ordinal tissue outcome from low as normal to the high as tumor.

Analysis began testing for individual measurement (expression levels of HC10 and LG2) effect (a univariate analysis), followed by multivariable modeling.

The final model included variables with $p < 0.05$ along with calculation of the odds ratio for each expression level (level 1=0%; level 2= $<10\%$; level 3=.10%-25%; level 4= $>25\%$ -50%) versus the reference group (level 5: $>50\%$) and its 95% of confidence interval.

We also assessed the goodness-of-fit for the multivariable model with calculation of C-index value, area under the study Receiver Operating Characteristic curve (AUROC) for model accuracy. The accuracy of the model depends on how well the predictor(s) separate the tissue groups from less (normal) to high severity breast lesions (BBD, CIS, T) in question, where c-value as 1 represents a perfect separation; 0.5 represents a worthless separation, and >0.80 is considered as good predictive accuracy.

Analysis Results:

- 1) The number of observed tissue records per patients was in a range of 1 to 22 records.
- 2) The univariate analysis showed that each individual measurement was associated with lesion tissue severity ($p < 0.01$).
- 3) HC10 and LG2 remained in the final multivariable models.
- 4) C-index value of the multivariable model was 0.816 (See Table 1).
- 5) HC10 was correlated with LG2 with correlation coefficient, r , as 0.73.
- 6) Breast tissues with decreased HC10 expression were more likely to be more advanced or breast lesions of increased risk, compared to those with HC10 expression levels of over 50%;
- 7) HC10 absence in tissues (level 1 expression or 0%) were 10.8 times likely to predict lesions of risk when compared to those tissues with over 50% HC10 expression;
- 8) Level 2 or $<10\%$ HC10 expression was 4.8 times more likely to predict have a sever lesion types, compared to the over 50% expressed HC10 tissues.
- 9) On the other hand, decreased LG2 expression was less likely to be associated with lesions of risk when compared those over 50% expressed LG2 tissues.

Lesions	N Obs	MHC Variable	Expression level Mean	Median	Minimum	Maximum
Normal: Lymphocyte records=797 Breast epithelium/other=393	1190	HC10	4.1361345	5.0000000	1.0000000	5.0000000
		LG2	3.6420168	5.0000000	1.0000000	5.0000000
BBD	178	HC10	2.6123596	3.0000000	1.0000000	5.0000000
		LG2	1.0224719	1.0000000	1.0000000	5.0000000
CIS	265	HC10	3.2830189	3.0000000	1.0000000	5.0000000
		LG2	1.1358491	1.0000000	1.0000000	5.0000000
Tumor	334	HC10	3.9071856	4.0000000	1.0000000	5.0000000
		LG2	1.3952096	1.0000000	1.0000000	5.0000000

Table 1. Multivariable Model Results: model #1

Odds Ratio Estimates			
Effect	Point Estimate	95% Wald Confidence Limits	
HC10 1 vs 5	9.929	6.314	15.614
HC10 2 vs 5	4.427	2.995	6.545
HC10 3 vs 5	2.891	1.994	4.194
HC10 4 vs 5	1.629	1.066	2.489
LG2 1 & 2 vs 5	0.012	0.007	0.020
LG2 3 & 4 vs 5	0.009	0.005	0.016

Conclusion /discussion:

- 1) The model predictive accuracy is very good.
- 2) Decreased HC10 expression was associated with more advanced breast lesions
- 3) Decreased LG2 favored lesions in the 3 risk categories. This observation reflects the fact that the majority of normal tissues were lymphocytes, which stain with LG2, as opposed to breast epithelium, which lacks MHC class II epitopes.