Award Number:  DAMD17-00-1-0288

TITLE:  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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CONTRACTING ORGANIZATION:  Henry Ford Health System
Detroit, Michigan  48202

REPORT DATE:  June 2001

TYPE OF REPORT:  Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

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**ABSTRACT (Maximum 200 Words)**

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 outlined studies are expected to better define the clinical significance of abnormal MHC class I molecules in precancerous and invasive breast lesions as markers of immunological events that could affect survival, selection, and outgrowth of precancerous cells, and their subsequent progression to breast cancer. These MHC losses could also mark more aggressive tumors and thus contribute to selection of appropriate treatments in individual cases.
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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 loss and its interaction with other histo-pathologic and epidemiological factors that can serve as risk indicators in the progression of primary breast cancer to metastatic disease.

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 (3000 cases). The Pathologist Dr. Raju and the P.I will begin screening breast cancer cases for delineation into Stage 1-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

PROGRESS (JANUARY 1, 2001-JUNE 30, 2001):

1. The Pathology Review Form (PRF) has been finalized (Appendix A)
2. Thus far 1,115 pathology reports have been obtained
3. H&E slides have been retrieved from the pathology archives for 250 cases
4. 200 cases have been reviewed by Dr. Raju on PRF forms
5. Selection of tumor blocks and sectioning of tissue for immunohistochemistry assays: completed for 170 cases
6. HLA class I immunoassays: completed for 60 cases
7. Medical abstraction form finalized (Appendix A)
8. Medical abstraction form: completed in 100 cases

TASK 2

Final analysis and report writing (months 56-60)
a: Final analysis of epidemiological risk factor data, histopathological and clinical data and HLA expression results will be performed.
b: A final report and additional manuscripts on the breast cancer cohort will be prepared

Progress: PENDING

KEY RESEARCH ACCOMPLISHMENTS

• Begun the compilation of a 3000 subject cohort of primary breast cancer patients
• Completed a multicenter validation of HLA immunoassays (Appendix B)
• Pilot study of 12 cases
REPORTABLE OUTCOMES

1. ABSTRACTS/PRESENTATIONS/MANUSCRIPTS

a: HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS. R Nanavati, U Raju, S Ferrone, M J. Worsham. Department of Pathology, Henry Ford Health System, Detroit, Department of Immunology, Roswell Park Cancer Institute, Buffalo, 15th European Histocompatibility Conference, Granada Spain, March, 2001


CONCLUSIONS:

HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS

In the present study we have compared the expression of HLA antigens in breast carcinoma epithelial cells and in autologous normal epithelial cells utilizing 12 formalin-fixed, paraffin-embedded lesions. HLA class I antigen expression, measured by staining with mAb HC-10, was downregulated in 5 lesions, upregulated in 3, heterogeneous in 2, and similar to that in the autologous epithelial cells in 2. The results of the staining with mAb L368 were similar, but not superimposable, to those obtained with mAb HC-10. The 5 lesions which were stained by mAb L368 with a pattern similar to that of normal epithelial cells included 3 lesions with reduced staining by mAb HC-10 and 1 with an enhanced staining. The 3 lesions with an enhanced staining by mAb L368 displayed also an enhanced staining by mAb HC-10. The 4 lesions with a reduced staining by mAb L368 included 3 lesions with a reduced staining by mAb HC-10. A relationship was found between HLA class I antigen expression and the degree of differentiation of malignant cells. Neither breast carcinoma cells nor normal mammary epithelial cells were stained by anti-HLA class II mAb LGII-612.14. The only cells to be stained by mAb LGII-612.14 in the tissue sections analyzed were lymphocytes and dendritic cells, which were also identified by staining with an anti-CD45 mAb. These findings suggest that abnormalities in HLA class I antigen
expression are frequently associated with malignant transformation of mammary epithelial cells.

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

Despite the possible clinical significance and potential for T-cell based immunotherapy, 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 IHC assays. Little information is available about the usefulness of formalin-fixed paraffin-embedded tissues (FFPT) as substrates in IHC assays to evaluate tissue expression of HLA antigens. We therefore undertook a multicentric study to develop and standardize an IHC protocol using FFPTs and anti-HLA mAbs. To determine if loss of expression of MHC Class I molecules at the protein level reflect alterations at the gene level, DNA from microdissected normal and tumor tissue were evaluated with microsatellites at the MHC class I 6p21.3 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for concordance of expression. 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 the anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, no staining of normal and malignant mammary cells was detected by the four laboratories in the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class I was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus. 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.

**REFERENCES**


APPENDICES

A: Study Instruments
   a: Pathology Review Form
   b: Medical Record Abstraction Form

B: Presented Abstracts:
   a: HLA class I and II antigen expression in breast carcinomas
   b: HLA antigen expression in breast cancer: a multicentric study utilizing formalin-fixed paraffinized tissues
CANCER PATHOLOGY REVIEW FORM

PLACE LABEL HERE:
MRN
Pathology # Specimen #
Date of Pathology Report

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<th>Maria J. Worsham</th>
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FORM COMPLETION DATE

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Size of Largest Mass/Cyst ______ cm

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| Number Sampled | Number Positive |

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*ADH: Atypical Ductal Hyperplasia
*ALH: Atypical Lobular Hyperplasia
*LCIS: Lobular Carcinoma In Situ
*DCIS: Ductal Carcinoma In Situ
BENIGN BREAST DISEASE STUDY

MEDICAL RECORD ABSTRACT

MRN ____________________________

Index Date ______ / ______/ ______

Date Abstracted ______ / ______/ ______

Follow-up Complete Yes No

Abstractor Status: 1. Complete/Finalized
2. Incomplete/Finalized
3. Chart Not Received

Abstractor's Initials ______

DEMOGRAPHICS at time of chart abstraction:

1. Name: __________________________ [last] __________________________ [first] __________________________ [mi]

2. Social Security Number: _______ - _______ - _______ _______

3. Date of Birth: _______ / _______ / _______ _______

4. Sex: 1=Female
2=Male

5. Race: 1=White/Caucasian
2=Black/African American
3=Hispanic/Latino
4=Asian/Pacific Islander
5=Middle Eastern
6=Native American/American Indian
7=Other, specify________________________
9=Unknown

6. Current Marital Status: 1=Divorced
2=Married
3=Single
4=Widowed
5=Legally separated
9=Unknown
7. Spouse's Name, if applicable: ________________________________

8. Maiden Name: ________________________________

9. Former Last Name: ________________________________

10. Vital Status:  
    0 = Deceased  
    1 = Alive  

11. Date of Vital Status Assessment: __ __ / __ / _____

12. Insurance at Index Date:  
   1 = HAP  
   2 = Other HMO  
   3 = Blue Cross/Blue Shield  
   4 = Medicare  
   5 = Medicaid  
   6 = Other  
   7 = None  
   9 = Unknown
   Date documented: __ __ / __ / _____

13. Previous Insurance:  
    (w/in 10 yrs prior to index)  
   1 = HAP  
   2 = Other HMO  
   3 = Blue Cross/Blue Shield  
   4 = Medicare  
   5 = Medicaid  
   6 = Other  
   7 = None  
   9 = Unknown
   Date documented: __ __ / __ / _____

14. Highest Education:  
   1 = Grade School (< 8 years)  
   2 = Some High School (8 - 11 years)  
   3 = Completed High School/GED  
   4 = Vocational School  
   5 = Some College  
   6 = Completed College  
   7 = Post-graduate School  
   9 = Unknown

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Revised 10-15-99  
C. Spoutz
MEDICAL HISTORY

1. Hormonal Contraceptive Use from beginning of chart up to index date: 0=No
   1=Yes
   9=Unknown

   Start date of use: __/__/____
   Length of time (years): _______________

   Type:
   1=Birth Control Pills
   2=Shots or Injections
   3=Subdermal Implants

   Start date of use: __/__/____
   Length of time (years): _______________

   Type:
   1=Birth Control Pills
   2=Shots or Injections
   3=Subdermal Implants

2. Hormone Replacement Therapy Use from beginning up to index date: 0=No
   1=Yes
   9=Unknown

   Date mentioned in chart: __/__/____
   Start date: __/__/____
   Stop date: __/__/____

   Type:
   1=Estrogen Alone
   2=Estrogen plus Progesterone
   3=Progesterone Alone

   Date mentioned in chart: __/__/____
   Start date: __/__/____
   Stop date: __/__/____

   Type:
   1=Estrogen Alone
   2=Estrogen plus Progesterone
   3=Progesterone Alone
3. Other Medical Conditions diagnosed/mentioned up to 10 years prior to index date:

Allergies:
- Drug allergy
- Food allergy
- Hay fever
- Other allergies

- Anemia or other blood disorder
- Arthritis (Non-inflammatory)
- Arthritis (Rheumatoid)

Cardiovascular Diseases:
- Heart disease
- Hypertension (high blood pressure)

Cerebrovascular Diseases:
- Stroke
- Transient Ischemic Attack (TIA)

- Diabetes ('sugar')
- Folate deficiency
- Hyperthyroid disease
- Hypoglycemia
- Hypothyroid disease
- Immune system disorder

Infectious Diseases:
- Chicken pox
- Encephalitis
- Herpes simplex
- Measles
- Meningitis
- Mononucleosis (mono)
- Mumps
- Pneumonia

Infectious Diseases (cont.):
- Poliomyelitis (polio)
- Shingles zoster
- Toxoplasmosis
- Tuberculosis (TB)
- Typhoid

- Kidney disease
- Liver disease

Neurologic/Psychiatric Disorders:
- Clinical depression
- Epilepsy/Seizures/Convulsions
- Migraine headaches
- Multiple Sclerosis (MS)
- Psychiatric conditions requiring medication

- Parathyroid disease
- Pituitary disease

Respiratory Diseases:
- Asthma
- Emphysema
- Other respiratory disease

- Stomach or other digestive disorder
- Vitamin B1 Deficiency
- Vitamin B12 Deficiency

- Other Medical Conditions (specify):

________________________________________

________________________________________

Index Date ___ / ___ / ______

0=No
1=Yes
9=Unknown

Page 4

Revised 10-15-99
C. Spoutz
4. Mammography History from beginning up to one year after index date:

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5. Breast Biopsy History from beginning up to one year after index date:

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1=Benign Breast Disease (BBD)  
2=Ductal or Lobular Carcinoma In Situ (DCIS or LCIS)  
3=Cancer (Carcinoma)  
4=Both BBD and CIS/Cancer  
5=Lumpectomy or Mastectomy (unilateral or bilateral)  
6=Cosmetic Breast Reduction or Enlargement  
7=Other Breast Biopsy (non-mammary epithelial biopsy of skin, nipple, fat, axillary lymph nodes, etc.)  
8=Incomplete/Inconclusive  
9=Unknown
BODY SIZE INFORMATION

1. Maximum Height (inches): __________ Date: __ __/ __ __/ ______

2. Weight closest to index date (pounds): __________ Date: __ __/ __ __/ ______

3. Weight during previous decade (pounds): __________ Date: __ __/ __ __/ ______

4. Weight during previous decade (pounds): __________ Date: __ __/ __ __/ ______

5. Weight during previous decade (pounds): __________ Date: __ __/ __ __/ ______

REPRODUCTIVE HISTORY from beginning of chart up to index date:

1. Age at Menarche (years): __________ [99=Unknown]

2. Age at First Birth (years): __________ [88=No Children; 99=Unknown]

3. Number of Pregnancies up to index date: __________ [99=Unknown]

4. Total Number of Pregnancies (gravida): __________ [99=Unknown]

5. Total Number of Births (para): __________ [99=Unknown]

6. Menopausal Status at index date: 1=Pre-menopausal
2=Peri-menopausal
3=Post-menopausal
9=Unknown

7. Year of Menopause: (skip if not post-menopausal) ________

8. Hysterectomy: 0=No
1=Yes
9=Unknown

   Number of ovaries removed: __________

   Date of surgery: __ __/ __ __/ ______
## CANCER HISTORY

1. Subject's History of Primary Cancer from beginning of chart up to index date:

<table>
<thead>
<tr>
<th>Yes/No</th>
<th>Date of Dx</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Breast</td>
<td></td>
</tr>
<tr>
<td>B. Endometrial</td>
<td></td>
</tr>
<tr>
<td>C. Colorectal</td>
<td></td>
</tr>
<tr>
<td>D. Ovarian</td>
<td></td>
</tr>
<tr>
<td>E. Cervical</td>
<td></td>
</tr>
<tr>
<td>F. Other: site</td>
<td>code:</td>
</tr>
<tr>
<td>site</td>
<td>code:</td>
</tr>
</tbody>
</table>

   0=No  
   1=Yes  
   9=Unknown

2. Family History of Cancer from beginning of chart up to index date:

<table>
<thead>
<tr>
<th>Relative</th>
<th>Rel. Code</th>
<th>Cancer</th>
<th>Cancer Code</th>
<th>Age at Dx</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LIFESTYLE HISTORY

1. Smoking Status starting with beginning of chart up to index date:
   0=No
   1=Yes
   9=Unknown

<table>
<thead>
<tr>
<th>Date</th>
<th>Status:</th>
<th>Packs/Day*</th>
<th># of Years at this Packs/Day</th>
<th>If Past Smoker, Calendar Year Quit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1=Current Smoker</td>
<td>2=Past Smoker</td>
<td>3=Never Smoker</td>
<td></td>
</tr>
<tr>
<td><em>/__/</em>___</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>/__/</em>___</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>/__/</em>___</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Cigarettes/day Packs/day
1 - 5 0.25
6 - 10 0.50
11 - 15 0.75
16 - 20 1.0

2. Occupational History within 10 years of index date:
   0=No
   1=Yes
   9=Unknown

<table>
<thead>
<tr>
<th>Date</th>
<th>Name of Occupation</th>
<th>Years in Occupation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>/__/</em>___</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>/__/</em>___</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>/__/</em>___</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>/__/</em>___</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
IF INCOMPLETE FOLLOW-UP:

Index Date ____ / ____ / ____

1. Telephone numbers: Home: (___) __________ Work: (___) __________
   Emergency: (___) __________

2. Current address: Street Address ___________________________________
   City, State, Zip Code _____________________________________________

3. Address at index date, if different: __________________ Date: ___ / ___ / ____
   ____________________________________________________________

4. Previous address, if different: __________________ Date: ___ / ___ / ____
   ____________________________________________________________

5. Spouse's employer: Name ___________________________ Phone #: (___) __________
   City, State _________________________________________________

6. Name and address of next-of-kin: _________________________________
   ___________________________________________________________
   ___________________________________________________________
   Relationship: 1. Current spouse  
   2. Former spouse  
   3. Offspring  
   4. Parent  
   5. Sibling  
   6. Other, specify _____________________________

7. Date of last physician visit or hospital admission: ___ / ___ / ____

8. Name of primary care physician: _________________________________

9. Location of primary care physician: _______________________________
HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS.
R Nanavati, U Raju, S Ferrone, M J. Worsham. Department of Pathology, Henry Ford Health System, Detroit, Department of Immunology, Roswell Park Cancer Institute, Buffalo

Abstract
Immunohistochemical staining of a large number of frozen sections of surgically removed lesions has shown that malignant transformation of cells may be associated with changes in their HLA phenotype. Since the use of frozen tissue sections as a substrate in immunohistochemical reactions has hindered the routine analysis of HLA antigen expression in malignant lesions, we have been evaluating the expression of HLA antigens in formalin-fixed, paraffin-embedded lesions. The anti-HLA class I heavy chain mAb HC-10, the anti-ß2m mAb L368 and the anti-HLA class II mAb LGII-612.14 have been used as probes. In the present study we have compared the expression of HLA antigens in breast carcinoma epithelial cells and in autologous normal epithelial cells utilizing 12 formalin-fixed, paraffin-embedded lesions. HLA class I antigen expression, measured by staining with mAb HC-10, was downregulated in 5 lesions, upregulated in 3, heterogeneous in 2, and similar to that in the autologous epithelial cells in 2. The results of the staining with mAb L368 were similar, but not superimposable, to those obtained with mAb HC-10. The 5 lesions which were stained by mAb L368 with a pattern similar to that of normal epithelial cells included 3 lesions with reduced staining by mAb HC-10 and 1 with an enhanced staining. The 3 lesions with an enhanced staining by mAb L368 displayed also an enhanced staining by mAb HC-10. The 4 lesions with a reduced staining by mAb L368 included 3 lesions with a reduced staining by mAb HC-10. A relationship was found between HLA class I antigen expression and the degree of differentiation of malignant cells. Neither breast carcinoma cells nor normal mammary epithelial cells were stained by anti-HLA class II mAb LGII-612.14. The only cells to be stained by mAb LGII-612.14 in the tissue sections analyzed were lymphocytes and dendritic cells, which were also identified by staining with an anti-CD45 mAb. These findings suggest that abnormalities in HLA class I antigen expression are frequently associated with malignant transformation of mammary epithelial cells.

Supported by DOD DAMD 17-00-1-0288 and the Fund for Henry Ford Hospital

BACKGROUND
Complete loss as well as allele- and locus-specific downregulation of the Major Histocompatibility Complex (MHC) class I expression occurs frequently in human tumors of different origins. Tumor cells can only be recognized by cytotoxic T cells if they express the same tumor antigen in conjunction with a MHC class I molecule. Reduced MHC class I expression may present an "escape route" for neoplastic cells constituting a selective advantage for survival of precancerous cells, and permitting their subsequent outgrowth or metastasis. This lack of recognition of the tumor cell could result in diminished returns for T cell-based immunotherapy for malignant diseases.

Approximately half (51%) of human breast cancers have an abnormally low content of HLA-A, B and C determinants (MHC class I). Abnormalities of MHC class I appear to be related to breast tumor invasiveness and with clinical and pathological parameters such as stage and degree of differentiation.

Over the past two decades the availability of HLA-specific monoclonal antibodies (mAb) suitable for immunohistochemical staining and technical advances in
immunohistochemical staining techniques have allowed extensive analysis of MHC class I antigen expression in cryopreserved tumors. These studies have shown conclusively that MHC class I antigen down regulation occurs in a number of malignant lesions, although with marked differences in frequency (for review, see 1).

Testing of surgically removed malignant lesions for HLA Class I antigen expression has not become standard-of-care for evaluation of patients with malignancies, even those to be enrolled in trials of T cell-based immunotherapy. This attitude reflects, at least in part, pathologists’ reluctance to utilize frozen tissue sections in immunohistochemical assays, since very few anti-HLA class I mAb have been reported to stain formalin-fixed tissues.

We evaluated 12 breast carcinomas to assess HLA class I and II antigen abnormalities utilizing immunohistochemical staining in formalin-fixed paraffin tissues.

MATERIAL AND METHODS

Antibodies

In this study we evaluated three HLA antibodies that bind to respective epitopes in a background of formalin fixation (Table 1). Two HLA class I antibodies, HC10 and LG368, and one class II antibody, LGII, were evaluated in this study. Anti-HLA Class I mAbs, HC-10 (2, 3) and L368 (4) stain formalin-fixed tissue sections. mAb, HC-10 recognizes distinct determinants expressed on the heavy chains of many, but not all HLA Class I alleles. mAb L368 recognizes one determinant of beta2 microglobulin.
Table 1  

<table>
<thead>
<tr>
<th>HLA Class I</th>
<th>HLA Class II</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC10</td>
<td>LGII</td>
<td>CD45</td>
</tr>
<tr>
<td>L368</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tumor Specimens

Formalin fixed paraffin tissue sections (5 microns in thickness) from 12 breast cancer cases with normal breast epithelium and cancer in the same tissue section were evaluated for HLA class I and class II expression.

Immunohistochemistry (IHC) Methodology

An IHC methodology, validated for HLA antigen assays in formalin fixed tissues in four laboratories was adopted. In brief, dilutions were as follows: mAb HC-10: 1/100 dilution, mAb L368: 1/25, mAb LGII1612.14: 1/25, with overnight incubation for all three. The standardized manual methodology utilizes antigen-retrieval with endogenous peroxidase inactivation using 3% hydrogen peroxide, with normal horse serum as the blocking agent (Vector Laboratories, 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 is visualized with 3′,3-diaminobenzidine tetrahydrochloride (Vector laboratories, Inc.) and the sections are counterstained with Mayer’s Hematoxylin.

At the present time we have converted to an automated immunoperoxidase staining system (Ventana Medical Systems, Inc, Tucson, Az). Antigen retrieval is accomplished by steaming in an Black and Decker steamer, followed by titration using the same dilutions as the manual method and counterstained with DAB.

IHC Interpretation

Scoring of stain intensity in the normal/benign areas and the tumor are done with reference to staining intensity of normal breast epithelium present in the same section with the breast tumor. Stromal lymphocytes in lymphocytic infiltrations which stain intensely can also serve as a reference for normal “staining intensity”.

Scoring parameters include 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 is done by two independent reviewers. Complete absence of staining as compared to the presence of staining in the normal breast epithelium is scored as “negative’. Marked decrease in staining intensity is scored as “+”, presence of some
staining intensity receives a score of "++", moderate stain intensity a score of "+++", and a score of "++++" indicates intensity of stain seen in normal breast tissue or stromal or lymphocytes in infiltrates.

RESULTS

HLA Expression

Table 2  
ANTIBODY: HC10

<table>
<thead>
<tr>
<th>Tissue area</th>
<th>NORMAL</th>
<th></th>
<th>TUMOR</th>
<th></th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heterogenous</td>
<td></td>
<td>Heterogenous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staining score</td>
<td>&lt; 25%</td>
<td>&gt; 25%</td>
<td>&gt; 50%</td>
<td>&gt; 75%</td>
<td>&lt; 25%</td>
</tr>
<tr>
<td>% cells stained</td>
<td>&lt; 25%</td>
<td>&gt; 25%</td>
<td>&gt; 50%</td>
<td>&gt; 75%</td>
<td>&lt; 25%</td>
</tr>
<tr>
<td>Case 1</td>
<td>•</td>
<td>•a</td>
<td>•b</td>
<td>upregulation/ downregulation</td>
<td></td>
</tr>
<tr>
<td>Case 2</td>
<td>•</td>
<td></td>
<td></td>
<td>upregulation</td>
<td></td>
</tr>
<tr>
<td>Case 3</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>downregulation</td>
<td></td>
</tr>
<tr>
<td>Case 4</td>
<td>•</td>
<td>•</td>
<td></td>
<td>upregulation</td>
<td></td>
</tr>
<tr>
<td>Case 5</td>
<td>•</td>
<td></td>
<td></td>
<td>upregulation</td>
<td></td>
</tr>
<tr>
<td>Case 6</td>
<td>•</td>
<td></td>
<td></td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td>Case 7</td>
<td>•</td>
<td>•a</td>
<td>•b</td>
<td>upregulation/ downregulation</td>
<td></td>
</tr>
<tr>
<td>Case 8</td>
<td>•</td>
<td>•</td>
<td></td>
<td>downregulation</td>
<td></td>
</tr>
<tr>
<td>Case 9</td>
<td>•</td>
<td></td>
<td></td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td>Case 10</td>
<td>•</td>
<td>•</td>
<td></td>
<td>downregulation</td>
<td></td>
</tr>
<tr>
<td>Case 11</td>
<td>•</td>
<td>•</td>
<td></td>
<td>downregulation</td>
<td></td>
</tr>
<tr>
<td>Case 12</td>
<td>•</td>
<td>•</td>
<td></td>
<td>downregulation</td>
<td></td>
</tr>
</tbody>
</table>

a: poorly differentiated areas of the tumor  
b: well differentiated areas of the tumor
**HLA Expression Results**

### Table 3

<table>
<thead>
<tr>
<th>Tissue area</th>
<th>ANTIBODYL368</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NORMAL</td>
</tr>
<tr>
<td>Staining score</td>
<td>Heterogenous</td>
</tr>
<tr>
<td>% cells stained</td>
<td>&lt;25% &gt;25% &gt;50% &gt;75%</td>
</tr>
<tr>
<td>Case 1</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 2</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 3</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 4</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 5</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 6</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 7</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 8</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 9</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 10</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 11</td>
<td>⋅</td>
</tr>
<tr>
<td>Case 12</td>
<td>⋅</td>
</tr>
</tbody>
</table>

a: poorly differentiated areas of the tumor  
b: well differentiated areas of the tumor
Table 4  Summary of HLA class I Antibodies

<table>
<thead>
<tr>
<th>Case</th>
<th>Outcome HC10</th>
<th>Outcome L368</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>upregulation/downregulation</td>
<td>upregulation</td>
</tr>
<tr>
<td>Case 2</td>
<td>upregulation</td>
<td>upregulation</td>
</tr>
<tr>
<td>Case 3</td>
<td>downregulation</td>
<td>no change</td>
</tr>
<tr>
<td>Case 4</td>
<td>upregulation</td>
<td>upregulation</td>
</tr>
<tr>
<td>Case 5</td>
<td>upregulation</td>
<td>no change</td>
</tr>
<tr>
<td>Case 6</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>Case 7</td>
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</tr>
<tr>
<td>Case 8</td>
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<td>downregulation</td>
</tr>
<tr>
<td>Case 9</td>
<td>no change</td>
<td>downregulation</td>
</tr>
<tr>
<td>Case 10</td>
<td>downregulation</td>
<td>no change</td>
</tr>
<tr>
<td>Case 11</td>
<td>downregulation</td>
<td>no change</td>
</tr>
<tr>
<td>Case 12</td>
<td>downregulation</td>
<td>downregulation:heterogeneous</td>
</tr>
</tbody>
</table>

SUMMARY

- Aberrant class I expression in breast tumors appears to be a more frequent phenomenon than previously reported.
- Upregulation of L368 appears to occur in conjunction with upregulation of HC10.
- Downregulation of HC10 (6p21 loci) may be a more frequent event occurring together with and independent of L368 (15q21 loci).

DISCUSSION

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 outlined studies are expected to better define the clinical significance of
abnormal MHC class I molecules in invasive breast lesions as markers of immunological events that target more aggressive tumors and thus contribute to selection of appropriate treatments in individual cases.

REFERENCES


ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTRIC STUDY UTILIZING FORMALIN FIXED TISSUES.
Maria J. Worsham, Reshma Nanavati, Usha Raju, Sandra R. Wolman, Teresa Cabrera, Federico Garrido, Elizabeth Reparsky, Bonnie Hylander, Manita Feenstra, Marina Verdaasdonk, Marguerite Schipper, Marcel Tilanus, Soldano Ferrone. Cancer Genetics Research, Department of Pathology, Henry Ford Health Systems, Detroit, MI; Hospital Universitario Virgen de las Nieves, Granada, Spain; Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY; University Hospital, Utrecht, The Netherlands; Uniformed Services University of the Health Sciences, Bethesda, MD

ABSTRACT

Immunohistochemical (IHC) staining of frozen malignant lesions has shown that abnormalities in HLA class I antigen expression are frequent in malignant cells. Although these abnormalities appear to have clinical significance, evaluation of malignant lesions for HLA class I antigen expression is not a routine procedure, even for those patients to be treated with T cell-based immunotherapy. This reflects, at least in part, pathologists’ reluctance to utilize frozen tissue sections in IHC assays.

Scanty information is available about the usefulness of formalin fixed tissues as a substrate in IHC assays to evaluate HLA antigen expression in tissues. We therefore undertook a multicentric study to develop and standardize an IHC protocol using formalin fixed tissues and anti-HLA mAbs.

HLA class I antigen downregulation in two of three breast carcinoma lesions in conjunction with heterogeneity was concordantly scored by the four participating laboratories with the anti-HLA class I mAb HC-10 and with the anti-beta 2 microglobulin mAb L368. Furthermore, no staining of normal and malignant mammary cells was detected by the four laboratories in the lesions stained with the anti-HLA class II mAb LGII-612.14. In contrast, infiltrating lymphocytes were strongly stained by mAb LGII. These results indicate that formalin fixed tissue sections 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.

Supported by DAMD17-00-1-0288

BACKGROUND

Tumor immunology has been a subject of great interest for over a century. Improved molecular understanding of human tumor immunity and recent technical advances have made immunotherapy (e.g., peptide vaccination, adoptive immunotherapy) a promising modality of treatment.

Over the past two decades the availability of HLA-specific monoclonal antibodies (mAb) suitable for immunohistochemical staining and technical advances in immunohistochemical staining techniques have allowed extensive analysis of MHC class I antigen expression in cryopreserved tumors. These studies have shown conclusively that MHC class I antigen down regulation occurs in a number of malignant lesions, although
with marked differences in frequency (for review, see 1). The frequency of HLA Class I antigen down regulation is significantly higher (p<0.01) in breast carcinoma (51%) and prostate carcinoma (85%) than it is in head and neck squamous cell carcinoma, lung carcinoma, colon carcinoma, cervical carcinoma or melanoma (1). In human breast cancers, MHC class I antigen expression appears to be related inversely to tumor invasiveness (2) and is strongly associated with clinico-pathological parameters such as lower tumor stage and degree of differentiation (3, 4). Furthermore, in a limited number of patients, MHC class I antigen down regulation in metastatic lesions appears to have a negative impact on the outcome of T cell-based immunotherapy of malignant diseases. Reduction or loss of HLA Class I antigen expression in metastatic lesions is associated with disease progression (5) or with recurrence of the disease in patients treated with T cell-based immunotherapy (6, 7). In spite of these results, testing of surgically removed malignant lesions for HLA Class I antigen expression has not become standard-of-care for evaluation of patients with malignancies, even those to be enrolled in trials of T cell-based immunotherapy. This attitude reflects, at least in part, pathologists’ reluctance to utilize frozen tissue sections in immunohistochemical assays, since very few anti-HLA class I mAb have been reported to stain formalin-fixed tissues.

We therefore undertook a multicenter study to standardize methodology and reagents in order to assess the validity of immunohistochemical staining in formalin-fixed paraffin tissues for HLA class I antigen abnormalities.

MATERIAL AND METHODS

Antibodies

In this study we evaluated three HLA antibodies that bind to respective epitopes in a background of formalin fixation (Table 1). Two HLA class I antibodies, HC-10 and LG-368, and one class II antibody, LGII, were evaluated in this study. The same batch of antibodies was provided by Laboratory 1 (Ferrone) to the three other laboratories: Laboratory 2 (Garrido), Laboratory 3 (Tilanus), and Laboratory 4 (Worsham). Anti-HLA Class I mAbs, HC-10 (14, 15) and L368 (16) stain formalin-fixed tissue sections. mAb, HC-10 recognizes distinct determinants expressed on the heavy chains of many, but not all HLA Class I alleles and may underestimate the expression of HLA Class I alleles in malignant lesions or even yield false negative results. mAb L368 recognizes one determinant of the beta2-microglobulin at the 15q21 locus.

Anti-CD45 staining was also done to confirm the presence of lymphocytes and dendritic cells in tumor infiltrates.

Tumor Specimens

Formalin fixed paraffin tissue sections (5 microns in thickness) from three breast cancer cases that spanned different years (to test the consistency of the methodology where formalin fixation might be a variable) were provided by Laboratory 4 to the other
participants. In all cases, normal or benign breast epithelium was present in addition to tumor on the sections.

Immunohistochemistry (IHC) Methodology

A validated IHC methodology was employed. In brief, dilutions were as follows: mAb HC-10: 1/100 dilution, mAb L368: 1/25, mAb L368:1/25, 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, 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 is visualized with 3',3-diaminobenzidine tetrahydrochloride (Vector laboratories, Inc.) and the sections are counterstained with Mayer’s Hematoxylin.

IHC Interpretation

Scoring of stain intensity in the benign lesion areas and the tumor are done with reference to staining intensity of normal breast epithelium present either in the same section with the breast tumor, or processed concurrently with the tumor section. Stromal lymphocytes in lymphocytic infiltrations which stain intensely can also serve as a reference for normal “staining intensity”.

Scoring parameters include 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 is 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 is scored as “negative”. Marked decrease in staining intensity is scored as “+”, presence of some staining intensity receives a score of “+++”, moderate stain intensity a score of “++++”, and a score of “++++” indicates intensity of stain seen in normal breast tissue or lymphocytes in infiltrates.

RESULTS

DISCUSSION

The potential predictive value of HLA expression in evaluation and therapy planning has been under-utilized. The importance of this marker in evaluation of breast cancer patients prompted our undertaking of a multicentric study approach to develop and standardize an IHC protocol using formalin-fixed, paraffin-embedded tissues and anti-HLA mAbs. HC-10, a mAB to a determinant expressed on free HLA class I heavy chains (chromosome 6p21) and L368, an anti-human beta2- microglobulin (chromosome 15q21).
were evaluated in archival formalin-fixed paraffin embedded tissue section. 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 and malignant mammary cells was detected by the four laboratories in lesions stained with LGII, although infiltrating lymphocytes were strongly stained. 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.

The results indicate that formalin fixed paraffin tissues 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. Lack of staining of a lesion with one mAb does not always reflect loss of the molecule expressing the tested determinant. Assessment of the HLA expression with either HC-10 or L368 may yield false negative results. To overcome the limitations posed by false negatives, mAbs to distinct monomorphic determinants on the heavy chains of all HLA Class I alleles should provide additional evidence for HLA expression in formalin fixed tissues.

REFERENCES


Table 1: HLA Antibodies

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Table 2  Summary of Validation Results

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† = complete concordance
† = incomplete concordance
† † = complete concordance, see text
† † † = incomplete concordance, see text
† † † † = stromal cells and lymphocytes
Figure legends HLA

Figure 1: Normal breast epithelium from case 1 illustrating uniform membrane “+++” stain intensity with HC-10

Figure 2: Concurrent invasion lesion from Case 1 showing reduced expression of HC-10. Note stromal cells and scattered lymphocytes with “+++” staining intensity.