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PROGNOSTIC SIGNIFICANCE OF HLA-DR ANTIGEN IN DYSPLASIA, CARCINOMA *IN SITU* AND INVASIVE CARCINOMA OF THE GALLBLADDER
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ABSTRACT

Objective: Prognosis of gallbladder carcinoma is poor. The two most important factors having the greatest effect on survival are pathologic stage of disease and histologic grade of the tumour. Our study points towards the value of HLA-DR antigen in the prognosis of gallbladder carcinoma.

Design: Thirty one cases of dysplasia of the gallbladder, 12 cases of carcinoma *in situ*, and 39 cases of invasive carcinoma for the detection of HLA-DR monoclonal antigen were studied. T helper (T_H) marker (CD4) in the tumour stroma of the relevant cases was also studied, given that it is now known that the dependence of immune responsiveness on the class II antigens reflects the central role of these molecules in presenting antigen to T_H cells.

Setting: Pathology Departments of Drama General Hospital and Ippokraton Hospital of Salonica in twelve years period (1990-2002).

Results: HLA-DR was expressed in 20 of 31 dysplasias (64.5%), four of 12 *in situ* (33.3%), and in 10 of 39 invasive carcinomas (25.6%). CD4 was expressed in nine of 31, dysplasias (29%), five of 12 *in situ* (42%), and in 26 of 39 invasive carcinomas (67%).

Conclusions: The results showed decreased expression of HLA-DR and increased expression of CD4 as the lesion progressed to malignancy. The aberrant expression of HLA-DR by epithelial cells of dysplasias, of carcinomas *in situ* and of invasive carcinomas agrees with the hypothesis of the dysplasia-carcinoma *in situ* sequence as the usual route for the development of invasive carcinoma. The immune attract mechanism by low HLA-DR signalling seems to be of minor importance in the malignant and metastatic potential of the gallbladder, epithelial tumours.

INTRODUCTION

The major histocompatibility complex is a series of genes that participate in the regulation of the immune response. This complex encodes two classes of cell-surface glycoprotein antigens: class I, found in all nucleated cells; and class II antigens, normally found only on a limited number of cells (B lymphocytes, macrophages, Langerhans' cells, dendritic cells, vascular endothelial cells and some epithelial cells)(1 - 3). Class II antigens control cellular interactions between lymphocytes. In man at least three class II antigens (DR, DQ, and DP), each consisting of α and β glycoproteins chains, are encoded by the HLA-D region of chromosome 6(2,4,5).

The majority of pathogens gain access to the body at a mucosal site, and the epithelial cells lining the lumen of the mucosa provide the first barrier against their invasion. In addition to their barrier, absorption and transport functions, epithelial cells play an important role in both innate and adaptive immune responses.

They secrete soluble molecules including defensins (6,7) and complement components(8) that neutralize and inactivate micro-organisms and their toxins. In addition, they can present foreign antigens to T cells affecting their proliferation, cytolytic activity and cytokine production. Typically, antigen presenting cells are bone marrow derived cells such as dendritic cells, macrophages (MO) and monocytes (i.e. professional antigen presenting cells). However, certain other cell types including intestinal epithelial cells(8-10), renal tubular epithelial cells(11), keratinocytes(12) and endothelial cells(13) have been shown to function in a limited context as antigen presenting cells, which are characteristically less efficient at antigen processing and presentation and thus referred to as non-professional antigen presenting cells.

Epithelial cells can transport antigens from the lumen by a process of transcytosis for eventual processing and presentation by professional antigen-presenting cells found in the underlying sub-epithelial stroma. The transcellular transport of antigen by epithelial

cells is generally a slow process but may be enhanced by immunization(14). Studies by Blumberg and co-workers have demonstrated functional MHC class I related IgG receptor (FcRn) on intestinal epithelial cells(15,16). Since both the female reproductive tract (FRT) and the gut have IgG, which increases in disease states, FcRn may facilitate transport of IgG-antigen complexes through epithelial cells into the basolateral sub-epithelium where antigen presenting cells and T cells reside. Previous studies have reported the presence of antigen presenting cells including macrophages (MO) and B cells throughout the FRT (17). Lymphoid aggregates consisting of a central core of B cells surrounded by numerous T cells, which are in turn circumscribed by MO, are shown(18). Other antigen presenting cells including dendritic cells have been observed in the lower tract although their presence in the upper tract remains controversial(19).

Recent studies have established that intestinal epithelial cells can express MHC class II molecules and present antigen directly to CD4+ T cells(9,20,21).

There have been no reported studies examining antigen presentation by isolated epithelial cells from the human gallbladder. We report that there is a loss in the HLA-DR expression by the epithelial cells and a gain in the CD4 expression in the lamina propria and muscular layer of the gallbladder, during the progression from dysplasia to invasive carcinoma.

MATERIALS AND METHODS

Thirty one cases of dysplasia, 12 cases of carcinoma *in situ*, and 39 cases of invasive carcinoma of the gallbladder were studied.

Source and preparation of tissues: All our cases were retrieved from the files of the Pathology Departments of Ippokraton Hospital of Salonica and General Hospital of Drama (Greece). The study was approved by the Regional Committees of Ethics. Written informed consent was obtained from all patients and the procedures followed were in accordance with the institutional guidelines. The samples were fixed in formalin and embedded in paraffin for immunohistochemical study.

Immunohistochemistry: This was performed with the various antibodies used on serial sections. Tissue sections (5µm) were deparaffinized, rehydrated, and treated with 0.3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity. Non-specific binding was blocked with serum for 10 min. Slides were then incubated for 30 min with the monoclonal antibodies (1/40), namely mouse anti-human HLA-DR, Alpha-Chain (DAKO) and CD4 (DAKO). Control slides were incubated for the same period with normal mouse serum. After several 10 min washes in PBS, samples were developed with the peroxidase LSAB kit (labelled streptavidin-biotin method, DAKO; which allows the detection of the first antibody. The slides were briefly counter-stained with Mayer's haematoxylin, mounted, and examined under an Olympus BX40 microscope.

The immunostained sections were examined with a X 40 objective and the distribution of HLA-DR and CD4 within the cell was recorded. Every stained cell was scored as

positive regardless of staining intensity. To count the number of cells with HLA-DR and CD4 stainings, a 10 X 10 square calibrated grid was inserted into the eyepiece of an Olympus binocular microscope.

Five-to-ten fields were examined for each section, and at least 1000 cells were scored, depending on cellularity. The percentage of positive cells was recorded as the HLA-DR and CD4 indices.

$$\text{HLA-DR index} = \frac{\text{no. of positive cells}}{\text{no. total (positive+negative cells)}}$$

$$\text{CD4 index} = \frac{\text{no. of positive cells}}{\text{no. of total (positive+negative cells)}}$$

The indices ranged from 0-100%, with a mean of 18%. The mean index was evaluated in three ranges: low index (under 18%), grade I; moderate index (from 18 to 50%), grade II; and high index (from 51 to 100%), grade III.

RESULTS

The sections were examined independently by two observers, and positive cellular staining for HLA-DR and CD4 antigens were manifested as fine yellow-brown cytoplasmic expression (Figures 1-5). HLA-DR was expressed in 20 of 31 of dysplasias (64.5%), in 4 of 12 carcinomas *in situ* (33.3%), and in 10 of 39 invasive carcinomas (25.6%). Of the 20 positive dysplasias nine were scored as HLA-DR grade II and eleven as HLA-DR grade III. Of four positive carcinomas *in situ* one was scored as HLA-DR grade I, and three as HLA-DR grade II. Of 10 positive invasive carcinomas one was scored as HLA-DR grade I, 7 as grade II, and two as grade III.

Figure 1

Gallbladder epithelial dysplasia: HLA-DR expression. Immunostain X200

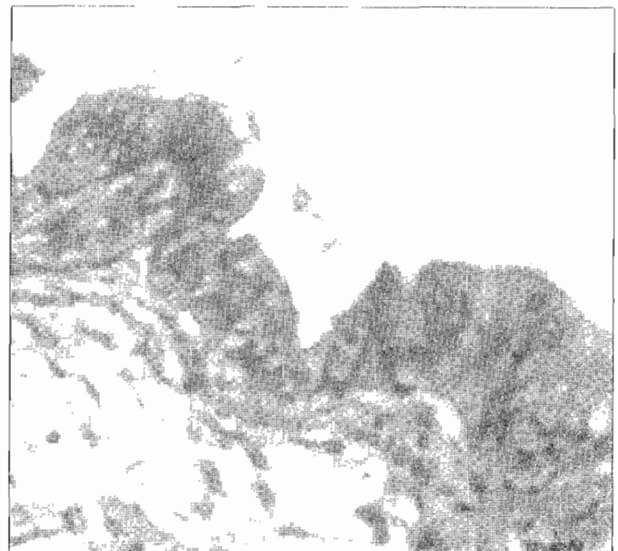
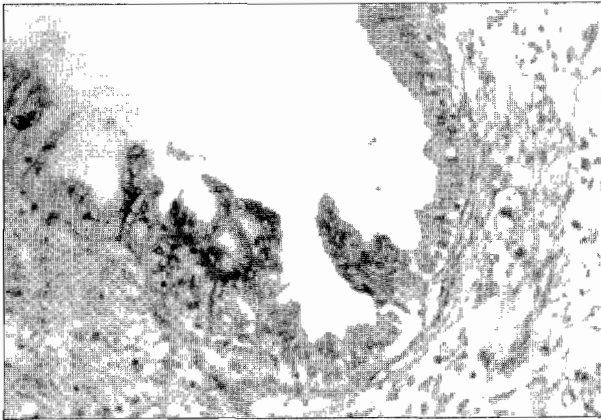
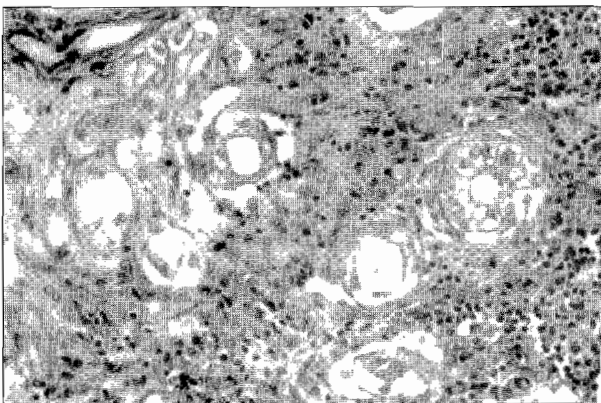


Figure 2

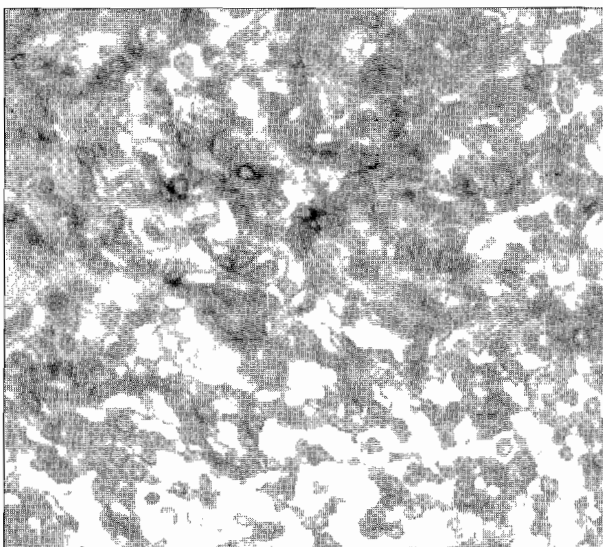
Gallbladder carcinoma in situ: HLA-DR expression. Immunostain X200

**Figure 3**

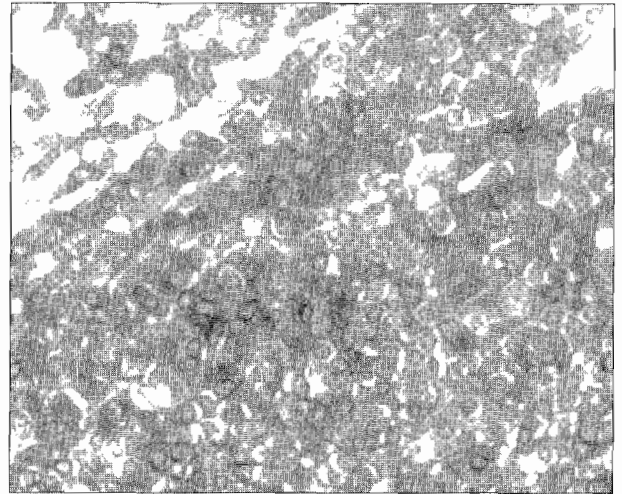
Invasive carcinoma with stromal lymphocytic infiltrate: HLA-DR expression. Immunostain X200

**Figure 4**

Gallbladder epithelial dysplasia: Stromal CD4 expression. Immunostain x200

**Figure 5**

Invasive carcinoma: Stromal CD4 expression. Immunostain X200



CD4 was expressed in nine of 31 dysplasias (29%), in five of 12 carcinomas *in situ* (42%), and in 26 of 39 invasive carcinomas (67%). Of nine positive dysplasias four were scored as CD4 grade II and five as CD4 grade III. Of five positive carcinomas *in situ* one was scored as CD4 grade I, three as CD4 grade II and one as CD4 grade III. Of 26 positive invasive carcinomas four were scored as CD4 grade I, 12 as CD4 grade II, and 10 as CD4 grade III.

DISCUSSION

Major histocompatibility complex antigens (MHC), or human leukocyte antigens (HLA) in humans, are considered to be essential when tumour cells are recognised and attacked by host immune cells. Therefore, the tumour growth may be affected by the states of HLA expression. In various neoplasms, the grade of HLA expression has been clinically reported to be associated with the degree of differentiation and the prognosis regarding both class I (22 - 26,36) and class II antigens (23,26-28,36). However, contradictory results have been also reported (29-33). Such controversy is probably not only due to the different tissue origins of various tumours but also due to the heterogeneous expression of individual tumour cells. It is difficult to quantitatively evaluate the heterogeneity of HLA expression using conventional tissue sections for a histologic examination. The dispersed cells of fresh tumour tissues most likely represent the whole population of tumour cells and are thus advantageous to the quantitative assessment of HLA expression.

It is accepted that most invasive carcinomas of the gallbladder and extrahepatic bile ducts are preceded by dysplasia and carcinoma *in situ*. Only a small proportion of invasive tumours arise from pre-existing adenomas. It is therefore believed that the dysplasia-carcinoma *in*

situ sequence is the usual route for the development of invasive carcinoma (37). The following facts have been offered in support of this hypothesis: dysplasia and carcinoma *in situ* are found in the mucosa adjacent to most carcinomas of the gallbladder and extrahepatic bile ducts; multiple sections of gallbladders removed for cholelithiasis have shown dysplasia and carcinoma *in situ* in 13.5% and 3.5% of the cases respectively; the anatomic distribution of these lesions is similar to that of invasive carcinoma.

To elucidate the clinico-biological significance of HLA expressed on neoplastic cells, we have quantitatively assessed the degrees of the class II expression using paraffin embedded neoplastic cells, and also the grade of T helper lymphocytic infiltration in epithelial dysplasia, and neoplasia of the gallbladder. In the present study, we clearly demonstrated a loss of HLA-DR expression from dysplasia towards invasive carcinoma of the gallbladder.

It is well known that HLA class II antigens are usually expressed on such immune cells as macrophages, B cells and activated T cells and that they are also involved in antigen presentation as well as in the regulation of the helper T cell function. A number of studies have also revealed the expression of class II antigens by both various non-immune normal and malignant cells (23, 26-29,32,33), although the biological significance of the class II expression of such cells remains unclear.

On the other hand, in view of immunological aspects, the class II expression of tumour cells has been reported to correlate with the local infiltration of lymphocytes (34, 35). In the present study, expression of HLA-DR by epithelial neoplastic cells was possibly mediated by stromal T helper lymphocytes as lymphoid cell infiltrates were observed to all biopsy specimens containing HLA-DR positive neoplastic cells. The increased aberrant expression of HLA-DR in tumour cells has been viewed as an important feature to escape tumour recognition by immune cells, and correlates with high grade malignancy and enhanced metastatic potential. In our series of gallbladder epithelial tumours, there was a decreased expression of HLA-DR as the neoplastic process progressed to malignancy and a subsequent increased immune response, providing new insights for a better understanding of the tumour host relationships in this extremely severe form of neoplasia.

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