ORIGINAL ARTICLE

Loss of human leucocyte antigen class I and gain of class II expression are early events in carcinogenesis: clues from a study of Barrett's oesophagus

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Background: Human leucocyte antigen (HLA) expression is altered in oesophageal carcinomas compared with normal tissue. It is unclear, however, whether this phenotype precedes malignant transformation or results as a consequence of it.

Aim: To investigate HLA class I and II expression in Barrett's oesophagus and normal squamous oesophageal tissue.

Methods: Asian patients with Barrett's oesophagus (n=64) and a control group (n=60) with a normal oesophagus but without reflux symptoms were recruited using endoscopic and histopathological criteria. Tissue samples were stained with monoclonal antibodies specific for HLA-ABC, HLA-DR α chain or HLA-DP/DQ/DR, and scored semiquantitatively. The results of immunohistochemical staining were correlated with clinical and histopathological characteristics of patients.

Results: Marked expression of HLA-ABC was observed in 50% of Barrett's oesophagus sections as compared with 68.3% of controls (p=0.038). HLA-DR staining was seen in 51.6% of Barrett's oesophagus samples versus 11.7% of controls (p<0.001). Expression of HLA-DP/DQ/DR was evident in 73.4% of oesophageal intestinal metaplasia tissue as opposed to 18.3% of controls (p<0.001). Importantly, a total loss of HLA-ABC and a concomitant gain of HLA-DP/DQ/DR expression were seen in 37.5% of patients with Barrett's oesophagus but in none of the controls (p<0.001). Interestingly, this phenotype was associated positively with dysplasia (adjusted p, p*=0.031) but negatively with non-steroidal anti-inflammatory drug use (p*=0.004).

Conclusions: HLA class I expression is down regulated and class II expression is up regulated in Barrett's oesophagus. As these changes predate malignant transformation, altered major histocompatibility complex expression may be a key event in disease progression, possibly in facilitating evasion from immune surveillance.

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Chronic gastro-oesophageal reflux (gastroesophageal reflux disease) predisposes to Barrett's oesophagus, which is a precursor lesion for oesophageal adenocarcinoma.¹ It has been hypothesised that Barrett's metaplasia develops as an adaptive epithelium in response to chronic damage by gastro-oesophageal refluxate.² The relative increase in interleukin 4 in Barrett's oesophagus compared with that in non-inflamed squamous oesophagus supports this postulate of a cytoprotective anti-inflammatory type response.³ The immune profiles that characterise the varied phenotypic responses to gastroesophageal reflux disease³ in response to a common antigenic stimulus may be important in modulating disease. Tumour cells often express new antigens as a result of the multiple genetic changes that are associated with cell transformation,⁴ and immune recognition of these neo-antigens is a key event in the history of a cancer.⁵ Host cytotoxic (cytotoxic T lymphocyte) cells and helper T cells can potentially recognise these neo-antigens presented by human leucocyte antigen (HLA) class I or II molecules, respectively.⁶ We recently reported that HLA B7 may be a susceptibility locus for Barrett's oesophagus,⁷ although the precise mechanism of association remains elusive. HLA molecules perform a crucial function in the regulation of the immune response. The observed association with major histocompatibility complex (MHC) class I genes may reflect either linkage disequilibrium to other MHC or non-MHC genes or a role for MHC class I molecules in disease pathogenesis.

HLA expression is often altered in tumours compared with the tissue from which they originate.⁸ Tumours often exhibit loss of expression when derived from HLA⁺ mucosa, but may also up regulate HLA expression when derived from HLA⁻ or weakly expressing tissues.⁹ Similarly, HLA class II antigen-positive tumours can be derived from negative mucosa.¹⁰ This may be a mechanism for the evasion of immune surveillance by malignant cells.¹¹ Most glandular or squamous epithelia, as well as the surrounding connective tissue, express HLA class I antigens, although the intensity of expression varies between different localisations. By contrast, HLA class II antigens are not normally expressed in most epithelia but appear in pathological circumstances, particularly in inflammation, tumour transformation and autoimmunity.¹²

To assess whether change in MHC expression is a critical early event in the multistep origin of oesophageal adenocarcinoma, HLA expression in the premalignant precursor lesion must be examined. Thus far, HLA expression in Barrett's oesophagus has been examined only once, but the results were drawn from as few as three informative samples.¹³ In this study, we investigate the expression of HLA-ABC, DP, DQ and DR in a well-characterised cohort of 64 multi-ethnic Asian patients with Barrett's oesophagus and 60 controls with a normal oesophagus and no reflux symptoms.

Abbreviations: HLA, human leucocyte antigen; MHC, major histocompatibility complex; NSAID, non-steroidal anti-inflammatory drug; TBS, Tris-buffered saline

MATERIALS AND METHODS

Patients

Patients and controls were prospectively recruited from Ipoh Hospital (Perak, Malaysia) between May 2003 and November 2004. The demographic details of all patients were recorded. Written informed consent was obtained from all patients for this study. The study protocol was reviewed and approved by the Medical Research and Ethics Committee, Ministry of Health, Malaysia.

Sixty-four Asian patients with an endoscopic and histological diagnosis of Barrett's oesophagus were prospectively recruited. The presence of Barrett's oesophagus, defined as columnar epithelium with specialised intestinal metaplasia obtained from any length segment of the tubular oesophagus, was recorded and proven by histological examination (stained with Alcian Blue, pH 2.5) of the associated mucosa. In addition to surveillance of Barrett's oesophagus, paired biopsy specimens were obtained from the midpoint of the Barrett's segment for research. The length of columnar lined segments containing specialised intestinal metaplasia was documented, as was the presence and grade of dysplasia. Exclusion criteria included those patients with oesophageal disease other than Barrett's oesophagus. Patients with secondary causes of gastro-oesophageal reflux disease or with any contraindication for endoscopic biopsies were also excluded.

The 60 Asian controls consisted of people undergoing upper gastrointestinal endoscopy for reasons other than reflux symptoms, Barrett's oesophagus surveillance or any form of dyspepsia. This group included those who were being investigated for anaemia or faecal occult blood positive stools, chronic diarrhoea requiring small bowel biopsy, irritable bowel syndrome and familial adenomatous polyposis. Exclusion criteria included those who had consumed antacids or had undergone antisecretory treatment in the past 2 years, prior gastric or oesophageal surgery, and those who currently had or had a history of peptic ulcers or gastric cancer. Two biopsy specimens of macroscopically normal oesophageal tissue, 2 cm above the gastro-oesophageal junction, were obtained from this group. Squamous oesophageal samples were subjected to microscopic examination to exclude oesophagitis.

HLA-ABC immunohistochemistry

Frozen 3- μ m sections mounted on slides were air dried at room temperature (25°C) and then dipped in ice-cold acetone for 10 min. After drying for 45 min, the slides were washed three times with Tris-buffered saline (TBS). Protein block (Dako, Glostrup, Denmark) was used to cover the specimen for 15 min and then tapped off. Then, primary antibody—that is, monoclonal mouse anti-human HLA-A, HLA-B and HLA-C (Dako)—in a dilution of 1 in 300 was added to cover the specimens for 10 min. Subsequently, the slides were rinsed with TBS (3 $\times$ 3 min) and anti-mouse antibody (Dako) labelled with alkaline phosphatase was applied to the specimens, after which they were incubated for 10 min. Slides were once again washed with TBS (3 $\times$ 3 min) and treated with the substrate chromogen, fast red (Dako) for 10 min, after which it was rinsed off with distilled water. The slides were counterstained with haematoxylin for 30 s, washed in distilled water and mounted. For all experiments, a negative control was prepared by substitution of the primary antibody with TBS. The intensity of staining relative to that observed on mononuclear cells was also noted and served as an internal control. Parallel positive controls were prepared by using sections of tonsil.

HLA-DP/DQ/DR and DR α immunohistochemistry

Sections mounted on glass slides were dewaxed at 37°C in the oven overnight. Pretreatment with target retrieval solution

(pH 6.0, Dako) was carried out at 98°C for 30 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water. Two kinds of primary antibodies were used. One was a mouse monoclonal antibody that was specific for HLA DR α (Dako), and the other was an antibody polyvalent for HLA DP/DQ/DR (Dako). In both cases, the primary antibody was applied for 30 min. After washing with TBS, sections were incubated with a biotin-labelled secondary antimouse immunoglobulin (Dako) for 15 min. After rinsing with TBS, sections were treated with peroxidase-labelled streptavidin for 15 min at room temperature. The colour was developed with a 10-min incubation with 3,3'-diaminobenzidine hydrochloride solution (Dako). The sections were subsequently counterstained with haematoxylin, dehydrated, cleared and mounted. Similar positive, negative and internal controls were used as described for HLA-ABC immunohistochemistry.

Semiquantitative analysis

All biopsy specimens were evaluated independently by two experienced histopathologists (NK and MKK). Each slide was initially examined at low magnification for an overall view and 10 high-power fields of representative areas were evaluated. The degree of HLA expression was graded semiquantitatively with a score that was calculated by adding the scale of intensity of staining to the area of staining (score = intensity + area).¹⁴ The intensity of staining was graded according to the following scale: 0, no staining of the mucosal epithelial cells; 1+, mild staining; 2+, moderate staining; 3+, marked staining (comparable with HLA-ABC levels on control mucosa). The area of staining was evaluated using the following scale: 0, no staining of the mucosal epithelial cells in any field; 1+, <25% of the epithelium stained positive; 2+, 25–50% stained positive; 3+, >50–75% stained positive; 4+, >75% stained positive. The two scores were added to give a final score of 0–7. A final score of <3 was regarded as negative.

Statistical analysis

Statistical analysis was performed using SPSS V.11.0. Pearson's χ^2 test (for categorical data) and t test for independent groups (for continuous data) were used to determine differences in HLA expression between the group with Barrett's oesophagus and the controls. Patients with Barrett's oesophagus with a concomitant loss of HLA class I and gain of class II expression and those without were compared for a variety of risk factors. Known risk factors for which on univariate analysis the p value was <0.250 (dysplasia, age >50 and family history of heartburn) were entered into a forward stepwise logistic regression model. p Values <0.05 were considered to be significant. p* Refers to the p values adjusted after multivariate analysis.

RESULTS

A total of 64 patients with Barrett's oesophagus were evaluated, together with 60 controls. The mean age (SD) of patients with Barrett's oesophagus was 56.3 (10.6), as compared with 50.1 (12.2) for the controls (p = 0.003). The male to female sex ratio was 1.8 in the group with Barrett's oesophagus versus 0.8 in controls (p = 0.021). Both these demographic features are consistent with previously published data.¹⁵ Among those with Barrett's oesophagus, 27 were Indians, 20 Chinese and 17 Malays. Among the controls, there were 20 Indians, 21 Chinese and 19 Malays. In the group with Barrett's oesophagus, 62 had columnar epithelium with specialised intestinal metaplasia, one had low-grade and another high-grade dysplasia. The length of Barrett's oesophagus ranged from 1.5 to 6 cm (mean 2.9).

Table 1 Human leucocyte antigen class I and class II expression in normal squamous epithelium and Barrett's oesophagus (positive: total score ≥ 3 and negative: total score < 3).

	Barrett's oesophagus, n = 64 (%)	Controls, n = 60 (%)	p Value
HLA-ABC positive	32 (50.0)	41 (68.3)	0.038
HLA-DR positive	33 (51.6)	7 (11.7)	< 0.0001
HLA-DP/DQ/DR positive	47 (73.4)	11 (18.3)	< 0.0001

HLA, human leucocyte antigen.

HLA-ABC positivity resulted in a purplish pink stain in tissue as opposed to a brown stain for HLA-DR α and HLA-DP/DQ/DR. Variations in intensity of staining were noted in tissue samples. HLA expression was transepithelial in the squamous tissue of controls, although it was of greater intensity in the basal and suprabasal layers. In Barrett's oesophagus, the stromal and glandular elements were markedly positive for HLA class I and II staining. No appreciable difference was seen in staining intensity between the earliest and the latest specimens. Table 1 shows significant differences in the immunohistochemical expression of HLA between the two histological groups.

Total loss of expression of HLA-ABC was found in 22 (34.4%) patients with Barrett's oesophagus and in 1 (1.6%) control ($p < 0.0001$). HLA-DR expression was completely absent in 12 (18.8%) patients with Barrett's oesophagus versus 45 (70.3%) controls ($p < 0.0001$). HLA-DP/DQ/DR expression was totally absent in 5 (7.8%) patients with Barrett's oesophagus as opposed to 42 (70%) controls ($p < 0.0001$). Interestingly, a total loss of HLA-ABC expression and a concomitant gain of HLA-DP/DQ/DR (or HLA-DR α) expression were seen in 24 (37.5%) patients with oesophageal intestinal metaplasia and dysplasia, but in none of the controls ($p < 0.0001$).

Patients with Barrett's oesophagus with a concomitant loss of HLA class I and gain of class II expression and those without were compared for a variety of risk factors (table 2). Although none of these patients had oesophageal adenocarcinoma, it was interesting to observe that this phenotype was positively associated with dysplasia ($p^* = 0.031$), but negatively associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs; $p^* = 0.004$).

DISCUSSION

We have shown for the first time that HLA-class I expression is down regulated and class II activity up regulated in Barrett's metaplasia, the premalignant precursor lesion of oesophageal adenocarcinoma. In addition, both patients with dysplastic oesophageal epithelium in our series (one with low grade and the other with high-grade dysplasia) showed a total loss of HLA class I and gain of class II expression. The only other report of HLA class I down regulation preceding tumorigenesis is in premalignant lesions of the cervix,^{16, 17} where the loss of HLA-B44 in premalignant lesions was associated with tumour progression.¹⁷ In contrast, HLA class I down regulation of between 4% and 35% has been reported in a large number of human tumours.^{11, 18} HLA-ABC is absent in a high proportion of oesophageal carcinomas (41–89%)^{13, 19} and may partly explain the aggressive nature of oesophageal malignancies. Invasive tumours totally or partially lose HLA antigens at a very high frequency (between 40% and 90%),²⁰ as is the case with metastases when compared with the primary tumour.²¹ These figures are probably underestimates, as the use of reliable monoclonal antibodies that have the capacity to recognise all HLA-ABC alleles in tissue sections is still very limited. Furthermore, the results of immunohistochemical analyses are based on a subjective evaluation of staining and lack standardised scoring criteria. The present study was designed to deal with some of these issues.

Considerable research has been devoted to analysing how HLA class I down regulation may promote tumorigenesis. HLA class I is a surface molecule recognised by CD8+ T cells and NK cells. Hence, HLA class I antigen down regulation is likely to disrupt tumour recognition by the CD8 cytotoxic T lymphocytes and NK cells, thereby allowing the tumour cells

Table 2 Comparison of clinical and histopathological characteristics of patients with Barrett's oesophagus with and without loss of major histocompatibility complex class I and gain of class II expression

	MHC class I negative, class II positive patients, n = 24 (%)	Non-MHC class I negative, class II positive patients, n = 40 (%)	p Value	Adjusted p value (p*)
Age > 55	15 (62.5)	18 (45)	0.175	
Male sex	16 (66.7)	25 (62.5)	0.737	
Indian race	9 (37.5)	18 (45)	0.699	
BMI (mean)	25.4	25.4	0.977	
Family history of heartburn	4 (16.7)	12 (30)	0.233	
Proton pump inhibitor use	12 (50)	19 (47.5)	0.846	
NSAID use	6* (25)	24 (60)	0.007	0.004
Smoking	7 (29.2)	15 (37.5)	0.497	
Excess alcohol consumption	4 (16.7)	5 (12.5)	0.718†	
Long-segment Barrett's oesophagus	12 (50)	12 (30)	0.560	
Dysplasia	2 (8.3)	0 (0)	0.137†	0.031

MHC, major histocompatibility complex; NSAID, non-steroidal anti-inflammatory drug.

p values were derived from χ^2 analysis. p*, adjusted p value. *One of these MHC class I negative and class II positive NSAID users had low-grade dysplasia. †Fisher's exact test.

Figure 1 (A) Oesophageal squamous transepithelial positivity for HLA-ABC with intense staining mainly confined to basal and suprabasal layers (purplish-pink stain); original magnification $\times 100$ (B) higher power view; original magnification $\times 400$. (C) Marked expression of HLA-ABC in predominantly stromal areas of Barrett's oesophagus; original magnification $\times 100$ (D) higher power view; original magnification $\times 400$. (E) No HLA Class I expression in Barrett's oesophagus; original magnification $\times 100$. (F) Lack of HLA-DR expression in squamous oesophageal epithelium. Only intraepithelial mononuclear cells are stained by the monoclonal antibody (brown stain); original magnification $\times 100$. (G) HLA-DR expression in a patient with Barrett's oesophagus. The glands and the stroma are intensely labelled by the monoclonal antibody (brown stain); original magnification $\times 100$. (H) Immunohistochemical staining for HLA-DP/DQ/DR in Barrett's oesophagus showing intense brown staining of stromal and glandular elements. Adjacent squamous epithelium does not take up the stain except for inflammatory cells; original magnification $\times 40$. [BM=basement membrane, L=lumen].

to escape immune surveillance. Different mechanisms may potentially lead to total or partial loss of HLA expression. These include heavy chain and B2-microglobulin gene mutations,^{22, 23} aberrations in regulatory factors,²⁴ changes in glycosylation and transport,²⁵ and deletions or promoter

methylation of the HLA genes.²⁶ Currently, we do not know which of these mechanisms account for the HLA class I down regulation in Barrett's oesophagus.

The expression of HLA-DR antigens is considerably more complex. This study shows that squamous epithelium of the

Take-home messages

- Human leucocyte antigen (HLA) expression is altered in oesophageal carcinoma compared with normal tissue.
- HLA-class I down regulation and class II up regulation in Barrett's oesophagus support the notion that changes in major histocompatibility complex (MHC) expression precede tumorigenesis.
- Given the central role of MHC products in T cell or natural killer cell recognition, this change in MHC expression may be a strategy for the evasion of immune surveillance by metaplastic cells. Thus, these cells have an increased chance to follow the Barrett's metaplasia–dysplasia–adenocarcinoma sequence.
- The negative association noted for non-steroidal anti-inflammatory drugs and class I down regulation and class II up regulation suggests immunomodulatory effects that might reduce the risk of malignant progression in Barrett's oesophagus.

oesophagus is devoid of HLA class II expression, like most other epithelia. Weak expression, however, has been found in lung, stomach and breast epithelium.²⁷ Most premalignant lesions of the colon, cervix, larynx and breast acquire de novo expression or increase their HLA class II expression. The proportions of HLA class II positive tumours from these tissues, however, are significantly lower than those in the corresponding premalignant lesions,²⁷ as is the case with Barrett's oesophagus. In this report, just over half the samples with Barrett's oesophagus were positive for HLA-DR, but only 16% of oesophageal carcinomas expressed this molecule.¹³ The presence of HLA class II expression in premalignant lesions and on some tumour cells appears to confer an advantage to the host in terms of restricted tumour growth²⁸ and survival²⁸ through their role as initiators of CD4 T helper cell responses against the tumour.

HLA-DR expression is not restricted to patients with Barrett's oesophagus, but is also found in the oesophageal tissue of patients with Crohn's disease.²⁹ Aberrant oesophageal HLA-DR expression in patients with Crohn's disease is an indicator of ongoing inflammation in the oesophageal lamina propria and submucosa as a result of cytokine release. Marked expression of HLA-DR is also seen in other inflammatory conditions such as graft versus host disease,³⁰ ulcerative colitis³¹ and coeliac disease.³² Again, chronic inflammation in the context of high concentrations of cytokine-secreting lymphocytes and eosinophils has been implicated. Barrett's oesophagus is characterised by a distinct cytokine profile, which is thought to result in a cytoprotective anti-inflammatory response to chronic damage by gastro-oesophageal refluxate.³ Whether the up regulation of HLA-DR in Barrett's oesophagus is a consequence of this altered cytokine milieu is presently unclear.

Interestingly, the use of NSAIDs in patients with Barrett's oesophagus in our study was inversely proportional to the simultaneous loss of HLA-ABC and gain of HLA-DP/DQ/DR expression. This finding lends biological plausibility to the published data showing a decreased rate of oesophageal cancer in patients who use NSAIDs.³³ A recent meta-analysis of nine clinical studies has shown a 43% decreased rate of oesophageal cancer in patients who use NSAIDs.³³ A 50% reduction of oesophageal malignancy was seen with aspirin use. Inhibition of the enzyme cyclooxygenase-2 by NSAIDs has been postulated as the mechanism for the prevention of progression of Barrett's oesophagus to adenocarcinoma. Our

study suggests that modulation of HLA expression may be another mode of action of NSAIDs in their chemopreventive role in Barrett's oesophagus.

In summary, we have shown that almost half the samples with Barrett's oesophagus show HLA-ABC down regulation and a greater proportion gain HLA-DR/DP/DQ expression, supporting the notion that differences in expression of MHC precede tumorigenesis. Given the central role of MHC products in T cell or NK cell recognition, this change in MHC expression may be a strategy for the evasion of immune surveillance by metaplastic cells, which therefore have an increased chance of following the Barrett's metaplasia–dysplasia–adenocarcinoma sequence. Further studies are required to assess the usefulness of these genetic aberrations and related molecular changes in disease prognostication. An enticing possibility is that HLA expression may help stratify patients into low-risk and high-risk groups for progression to oesophageal adenocarcinoma.

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