HMFG-2 as a prognostic indicator in superficial bladder cancer

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SUMMARY A series of transitional cell carcinomas and mucosal biopsy specimens of bladder were stained immunohistochemically with the monoclonal antibody HMFG-2. Staining characteristics ranged from luminal staining in well differentiated, superficial lesions to staining of all cells in invasive carcinomas. Invasive tumour nests also stained strongly with the antibody. There was good correlation between the staining pattern and histological assessment of both tumours and mucosal biopsy specimens. Correlation with clinical course showed that it was also a good predictor of subsequent disease progression of superficial tumours.

It is suggested that HMFG-2 staining is a sensitive indicator of malignant transformation and that it may have a role as an adjunct to conventional histological examination in the assessment of urothelial carcinomas.

Superficial bladder cancers vary widely in their propensity to recur or invade deeply. Once diagnosed as having superficial bladder cancer, a patient has about a 70% chance of developing recurrences, either at the site of the primary lesion or at another site in the urinary tract, and about a 10–20% chance of progression to muscle invasive disease.¹² Several characteristics of the initial tumour have been correlated with clinical course and eventual outcome. Stromal invasion, poor histological grade, large and multiple tumours have all been associated with an increased risk of recurrence and progression.^{3–5} Although these clinical and pathological variables can give some indication of prognosis and are of value in the planning of follow up requirements, more accurate methods of identifying the “high risk” bladder are required if prophylaxis, such as intravesical chemotherapy or early radical surgery, is to be considered.

An antiserum raised against epithelial membrane antigen (EMA), a large glycoprotein component of human milk fat globule (HMFG) membrane, has previously been shown to exhibit a changed staining pattern in urothelial malignancy.⁶ The monoclonal antibody HMFG-2 is directed against a much smaller carbohydrate residue on the HMFG membrane which, although present as a normal feature of the luminal surface, also seems to be a result of changed cell surface glycosylation in malignant transformation.⁷

This study was performed to assess the value of immunohistochemical staining with HMFG-2 as an aid to standard histological techniques in the assessment of superficial bladder tumours and urothelial mucosal biopsy specimens.

Material and methods

RETROSPECTIVE STUDY

Eighty four transitional cell carcinomas of the bladder (69 superficial, 15 invasive), which were initially resected in the Queen Elizabeth Hospital, Birmingham between 1980 and 1982, were studied. All the blocks had been fixed in 10% formol saline, wax embedded, and were archived in the pathology department. Sections of 3 µm thickness were cut from each tumour block and processed for immunohistochemical staining as described below. Hospital case records and cancer registry follow up notes were scrutinised to obtain five year follow up information on recurrence and progression for all the patients. Progression was defined as an increase in pT category from pTa or pT1 to muscle invasive disease (pT2+), or the development of metastatic disease.

PROSPECTIVE STUDY

Twenty six patients attending for cystoscopy under general anaesthetic either as follow up for superficial bladder tumours before transurethral resection of the prostate were studied. Either a random mucosal biopsy or, if a tumour was present, near-far biopsy

specimens from macroscopically normal mucosa⁸ were taken using cold-cup biopsy forceps, fixed in 10% formaldehyde in phosphate buffered saline and embedded in paraffin wax. Sections of 3 μ m thickness were then cut from each block for immunohistochemical analysis.

IMMUNOCHEMISTRY

The HMFG-2 mouse monoclonal antibody was obtained from Oxoid-Unipath Ltd, Viking Industrial Estate, Bedford, United Kingdom. After initial dewaxing and rehydration the sections were stained by the indirect immunoperoxidase technique.⁹ Briefly, they were incubated with the HMFG-2 antibody, diluted 1:100 in phosphate buffered saline with 1% bovine serum albumin and then with rabbit anti-mouse peroxidase conjugate for 30 minutes each. Peroxidase activity was visualised using diaminobenzidine.

CLASSIFICATION AND SCORING OF HISTOLOGICAL SECTIONS

Sections stained with haematoxylin and eosin were examined and each tumour classified according to stromal or muscular invasion¹⁰ and degree of histological differentiation.¹¹ The sections stained for HMFG-2 were given a score of 1–3, depending on the depth of penetration of stain from the luminal surface to the basal cells of the epithelium: 1 = staining of luminal cells alone (fig 1); 2 = staining of luminal and

intermediate cells (fig 2); 3 = staining of all epithelial cells (fig 3).

Statistical analysis was performed using programs

Fig 2 Moderately differentiated (G2) pTa transitional cell carcinoma of bladder. Staining of superficial and intermediate cells (HMFG-2).

Fig 1 Well differentiated (G1) pTa transitional cell carcinoma of bladder. Staining of luminal cells alone (HMFG-2).

Fig 3 Moderately differentiated (G2) pTa transitional cell carcinoma of bladder. Staining of all epithelial cells (HMFG-2).

from the BMDP (Biomedical Programs, Los Angeles, California) statistical package on the Systime VAX 11/730 minicomputer in the West Midlands Cancer Research Campaign Clinical Trials Unit. Actuarial survival curves for time to progression were drawn using the method of Kaplan and Meier¹² and the difference between groups calculated using the log-rank test.¹³

Results

RETROSPECTIVE STUDY

Of the 84 tumours, sections from eight were either too small or fragmented to permit adequate assessment of grade, stromal, or muscular invasion. The study was therefore performed on the remaining 76 tumours. All of the sections stained positively for HMFG-2. Staining characteristics ranged from luminal staining alone (score = 1) in 16, staining of superficial and intermediate cells (score = 2) in 38, to staining of all the cells (score = 3) in 22. There was a significant association between pT category and HMFG-2 score (table 1). Of 36 pTa tumours, 13 (36%) had a score of 1 and only seven (19%) had a score of 3. Where there was histological evidence of stromal invasion (pT1) only two of 27 (7%) had a score of 1 and the remainder had some degree of penetration of stain towards the basement membrane. Of 13 muscle invasive tumours, only one had a score of 1; eight (62%) had a score of 3. Table 2 shows that there was also a significant association between HMFG-2 score and histological grade, with poorly differentiated lesions having deeper penetration of stain towards the basement membrane. Strong staining was also seen in nests of invasive tumour cells in the submucosa (fig 4). In three tumours, which were regarded as pTa by conventional

histological criteria, this showed probable stromal invasion.

The association between HMFG-2 staining and clinical course was further examined for the 63 superficial tumours in the study. Although there was poor correlation between HMFG-2 staining and recurrence (χ^2 (2df) = 4.7 p = 0.09), there was a significant correlation between staining pattern and subsequent progression to muscle invasive or

Fig 4 Moderately differentiated (G2) pT1 transitional cell carcinoma of bladder. Strong staining of invasive nests (HMFG-2).

Table 1 HMFG-2 staining and pT category

	HMFG-2 score		
	1	2	3
pTa	13	16	7
pT1	2	18	7
pT2+	1	4	8

(χ^2 (4df) = 16.0, p = 0.003).

Table 2 HMFG-2 staining and histological grade

	HMFG-2 score		
	1	2	3
G1	9	9	6
G2	7	16	5
G3	0	13	11

(χ^2 (4df) = 13.2, p = 0.01).

Fig 5 Effect of HMFG-2 staining on disease progression: score = 1/0/15 (0%) progressed; score = 2/6/34 (18%) progressed; score = 3/5/14 (36%) progressed; χ^2 (2df) = 6.1 p = 0.047.

Table 3 HMFG-2 staining of mucosal biopsy specimens

	HMFG-2 score		
	1	2	3
Normal	9	8	0
Hyperplasia	0	1	2
Mild dysplasia	6	3	1
Severe dysplasia	1	1	0
Carcinoma in situ	0	0	3
	16	13	6

Fig 6 Mild dysplasia. Staining of luminal and intermediate cells (HMFG-2).

Fig 7 Carcinoma in situ. Staining of all epithelial cells (HMFG-2).

metastatic disease (fig 5). Of 15 tumours with staining of the luminal cells alone (score = 1), none progressed; six of 34 (18%) with stain penetrating towards the basement membrane (score = 2) and five of 14 (36%) with staining of all the epithelial cells (score = 3) progressed.

PROSPECTIVE STUDY

Of 43 mucosal biopsy specimens examined, eight were unsuitable for analysis because they were too small or traumatised. The study was therefore performed on the remaining 35 specimens (table 3). Seventeen were histologically normal and of these, nine had luminal staining alone; eight had some penetration of stain through the epithelium (fig 6). None had staining of all the epithelial cells. The remaining 18 specimens showed some degree of premalignant change and, of these, 11 had a score of 2 or 3. All three biopsy specimens of carcinoma in situ had staining throughout the epithelial layer (fig 7).

Discussion

The fat globules released into milk during lactation are surrounded by a membrane which is representative of the terminally differentiated mammary epithelial cell. These membranes are easily obtainable from the cream fraction of milk and have been widely used to produce both polyclonal antisera and monoclonal antibodies which are specific for epithelial cells.^{14,15} The most immunogenic component of HMFG is a large mucin-like glycoprotein with molecular weight of around 440 000. Heyderman *et al* first described EMA, identified by an antiserum raised against HMFG membrane, which was localised to membranes with secretory function.¹⁶ They showed positive staining with this antibody in bladder, prostatic, and colorectal neoplasms.¹⁷ Further work showed that EMA was confined to the luminal surface of normal epithelial tissues but, in inflammatory and neoplastic conditions, staining increased and penetrated more deeply into the epithelium. Micrometastases also stained positively with the antibody.¹⁸ The potential usefulness of EMA staining in urothelial carcinoma was suggested by Pocock *et al*, who correlated its staining pattern with pT category, grade, and subsequent clinical course.⁶ They showed luminal staining only in normal epithelium but penetration down towards the submucosa in many cases of neoplasia.

HMFG-1 and HMFG-2 are monoclonal antibodies raised against components of the HMFG membrane. These have been used to differentiate carcinomas of epithelial origin from mesotheliomas and reactive mesothelium.¹⁹ The HMFG-1 determinant is a large glycoprotein molecule with complex carbohydrate side chains whereas HMFG-2 is active against smaller carbohydrate molecules. Its expression is thought to be associated with incomplete or changed glycosylation in malignant change.⁷

Our results show a strong correlation between HMFG-2 staining and both extent of invasion and histological grade. A high HMFG-2 score was also associated with a significantly higher risk of progression to muscle invasive or metastatic disease ($p = 0.047$). Greater penetration of the stain towards the basement membrane therefore seems to be associated with an increasing degree of malignant transformation of the epithelial cells. The mucosal biopsy specimens also expressed a similar relation, with most normal biopsy specimens having luminal staining alone; dysplastic or neoplastic epithelium had deep penetration of staining.

In developing malignancy, therefore, intermediate and then basal cells progressively express this antigen which is a normal feature of the luminal membrane of urothelial cells. One explanation for this appearance is that the changed cell surface glycosylation associated with malignant transformation is similar to the change which develops in the terminally differentiated superficial cell. Another possible explanation, however, is that the HMFG-2 antibody is directed against a secretory mucin on the luminal cell surface. Intercellular adhesion has been shown to decrease with malignant change²⁰ and this would allow penetration of mucin in between the superficial and intermediate cells to give the characteristic appearance described above. In the poorly differentiated tumour both loss of polarity and decreased cell to cell adhesion may contribute to the diffuse HMFG-2 staining throughout the epithelial layer. The fact that the antibody is raised against a secretory product—the milk fat globule—strengthens this argument.

HMFG-2 is immunoreactive in routinely fixed, paraffin wax embedded material without the need for protease pretreatment. In this study HMFG-2 staining identified a group of patients who were at higher risk of disease progression and, as such, may have benefited from an early aggressive treatment policy. A low risk group was also identified in whom follow up requirements could possibly have been reduced. A range of staining was also seen in mucosal biopsy specimens, possibly reflecting early malignant change.

We conclude that HMFG-2 staining is a sensitive indicator of malignant transformation in urothelial carcinoma. A larger, prospective study would, however, be required to assess the implications of HMFG-2 staining on patient management.

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