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RESEARCH

Expression of adhesion molecule epithelial cadherin and matrix metalloproteinase-9 in squamous neoplasia of the uterine cervix

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Background: The objective of the study was to evaluate the expression of cell adhesion molecules [epithelial cadherin (E-cadherin)] and extracellular matrix protease [matrix metalloproteinase (MMP)-9] in preinvasive and invasive lesions of squamous neoplasia of the uterine cervix.
Method: The study included 14 cases of cervical intraepithelial neoplasia (CIN) and 43 cases of squamous cell carcinoma (SCC). Immunohistochemistry (IHC) testing was performed for E-cadherin and MMP-9 using the polymer technique. The pathological prognostic parameters, like tumour grade, stage, lymphovascular space invasion and mitosis were compared with the expression of these markers.
Results: The mean age of the patients with CIN was 40.1 years, and 50.9 years for those with SCC. Complete uniform membranous expression of E-caherin was demonstrated in the normal epithelium and most CIN (79%). MMP-9 was not expressed in the normal epithelium, whereas 43% of CIN (of which 67% were CIN grade III) were positive. Loss of membranous E-cadherin was shown in 88% of SCC, and MMP-9 with variable intensities expressed in 74%. A statistically significant association was established between the expression of these immune markers in preinvasive and invasive lesions, although there was no significant association with the prognostic parameters. In addition, the loss of E-cadherin was evident in patients with recurrence, while the expression of MMP-9 was demonstrated in 60%.
Conclusion: The loss of membranous E-cadherin and the gain of cytoplasmic MMP-9 are markers of neoplastic transformation in squamous neoplasia of the uterine cervix. However, the expression of these immune markers in our study did not relate to the prognostic parameters, indicating the importance of these markers in early neoplastic transformation.

Keywords: cervical cancer, CIN, E-cadherin, MMP-9, squamous neoplasia, SCC

Introduction

Squamous cell carcinoma (SCC) of the uterine cervix is the second most common cancer (15%) in women worldwide, whereas in India it is the most common cancer in women (20–30%). Based on the population-based cancer registries under the 2007 National Cancer Registry Programme of the Indian Council of Medical Research, the estimated number of new cancer cases in 2007 in India was 90 708.[1] It is preceded by a premalignant condition, known as cervical intraepithelial neoplasia (CIN), which is subdivided into three grades, i.e. CIN I, CIN II and CIN III. Certain properties that permit and enhance the capacity for tumour invasiveness and metastasis are required for the progression of CIN III to invasive cervical SCC.

Human cancer arises from a protracted sequence of multiple genetic and epigenetic alterations which involve the growth regulatory genes. Many types of cancer show altered expression of the cell adhesion molecules in the process of tumour invasion and metastasis. Epithelial cadherin (E-cadherin) is the primary cell adhesion proteinaceous molecule and maintains cell-to-cell contact. The loss of E-cadherin is the initial step which helps the primary malignant cells to detach from their neighbours and aids in metastasis.[2] Stromal invasion and migration of the cancer cells from the tissue of origin to surrounding or distant organs is essential for tumour progression. This involves the degradation of the basement membrane and remodelling of the extracellular matrix (ECM).[3] Matrix metalloproteinases (MMPs), a family of endogenous proteases, are zinc- and calcium-dependent enzymes which are capable of degrading the ECM components and aid in tumour invasion and metastasis. Of the different MMPs, the role of MMP-2 and MMP-9 has been exploited in studies on tumour biology.

Cervical cancer provides a useful model with which to study the role of E-cadherin and MMP in tumour invasion and metastasis progression, since there are well defined preneoplastic conditions in cervical squamous neoplasia. The aim of the present study was to understand the role of these markers in mechanisms by which preinvasive lesions acquire the ability to invade the cervical stroma.

These markers may act as prognostic indicators, and help to influence therapeutic decisions with regard to adjuvant treatment, in addition to predicting response to treatment. Hence, the aim of this study was to analyse the immunohistochemical expression of MMP-9 and E-cadherin in CIN and invasive SCC, and their association with pathological prognostic factors.

Method

Cervical biopsies, or conisation, and hysterectomy specimens received for diagnostic purposes at the Department of Pathology, Division of Gynecological Oncology, St John's Medical College and hospital, Bangalore, India, during the period of June 2008 to June 2011 were included in this retrospective observational study. The normal cervical epithelium of patients with other gynaecological complaints undergoing hysterectomy was included as age-matched controls. Patients with a history of human immunodeficiency virus infection and any other

malignancies were excluded. The clinical information, together with the demographic data, socio-economic history and International Federation of Gynecology and Obstetrics stage and follow-up data were collected by reviewing the case records. Pathology records and haematoxylin- and eosin-stained slides were reviewed for tumour size, tumour grade, lymphovascular space invasion (LVSI), pelvic lymph node status and mitotic count. Histological slides of the tumours were independently studied by two pathologists.

Immunohistochemical staining

Cervical epithelium sections of 4–5 µm in thickness, floated on sialinised slides and incubated overnight at 60° C were used. Immunohistochemistry (IHC) testing was performed by polymer technique on the deparaffinised sections. Antigen retrieval was carried out by steam treatment in a citrate buffer, and the sections were quenched for 10 minutes in hydrogen peroxide. The slides were coated with the primary antibody, E-cadherin (clone NCH-38®, Dako, California, USA) and MMP-9 (clone EP® 12557, Biogenex, San Roman, USA), incubated at room temperature for 30 minutes. Reactivity was detected using diaminobenzidine as chromogen and Harris's haematoxylin as the counterstain. The primary antibody was omitted for the negative controls. A positive control was included with each batch of staining.

Assessment of staining

Immunostaining with E-cadherin provides a bright membranous staining of the cells. The proportion of cell staining and the intensity of staining were considered in order to obtain a semi-quantitative score. Intensity was graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The percentage of cell staining in each category was multiplied by its intensity and added together to obtain a semi-quantitative score, with a maximum of 300. The preserved expression of E-cadherin was defined as bright membranous staining with a score of ≥ 200. A score less than 200 was considered to represent impaired expression.[4] Cytoplasmic localisation was also recorded and scored.

The cytoplasmic immunoreactivity in the malignant cells was graded on a scale of 0–3 for MMP-9. Negative staining was scored as 0 (–), whereas scores of 1 (+), 2 (++), and 3 (+++), represented weak, moderate and intense staining, respectively.[5] The case was considered positive when a positive staining was demonstrated in > 1% of the tumour cells. Positive staining in > 50% of the tumour cells was considered to be a strong expression of MMP-9. MMP-9 immunostaining was also assessed in the stromal cells surrounding the tumour and the inflammatory cells in the stroma.

Statistical analysis

Statistical analysis was performed using SPSS® for Windows® version 16.0. The association between the expression of E-cadherin and tumour grade, stage and LVSI was assessed by an independent-sample *t*-test. The association between E-cadherin and mitosis was assessed using Pearsons's product-moment correlation coefficient. The association of MMP-9 with prognostic parameters was determined using Fischer's exact test. The association between the biomarkers in preinvasive and invasive lesions was assessed using the chi-square test. The relationship between the loss of membranous expression of E-cadherin and its delocalisation to the cytoplasm was gauged using Pearson's product-moment correlation coefficient. Similarly, the relationship between the stromal expression of MMP-9 versus its expression in the tumour cells was assessed using Fischer's exact test. A p of < 0.050 was considered to be statistically significant.

Results

The study included cervical biopsies of five cases of CIN I, four cases of CIN II, five cases of CIN III and 43 cases of SCC. The ages of the patients with SCC ranged from 31–75 years (a mean of 50.9), while the ages of those with CIN ranged from 30–48 years (a mean of 40.1). The clinicopathological characteristics of SCC are summarised in Table 1. Most of the CIN cases were detected on routine screening with conventional cytology.

The distribution of E-cadherin staining in normal cervical epithelium, cervical intraepithelial neoplasia and carcinoma

Uniform, complete membranous staining of the squamous, as well as the endocervical, epithelium, was demonstrated in the normal cervical epithelium. The staining was more intense in the basal layer than in the surface epithelium (Figure 1 a). Complete membranous staining with a score of > 200 (Figure 1 b) was demonstrated in 11 of the 14 CIN cases. However, there was focal loss of membranous staining in two cases each of CIN II and CIN III. Loss of membranous staining of E-cadherin at the cell-to-cell interface was shown in 38 of the 43 SCC cases, with the score ranging from 0–190. Incomplete membranous staining or cytoplasmic localisation of immunostaining (Figures 1 c and 2 b–c) was noted in these cases. Either weak staining or cytoplasmic staining was demonstrated in the invasive fronts of the tumours, highlighting the role of loss or abnormal localisation of E-cadherin in tumour invasion and progression. Abnormal localisation of E-cadherin in the cytoplasm of the cells was observed in 32 cases. (The cytoplasmic score ranged from 0 to a maximum of 200). A correlation between membranous staining of the E-cadherin and cytoplasmic localisation was trended to be negatively correlated (Pearson's product-moment correlation coefficient of –0.291, with two-tailed significance of 0.058). The difference in immunostaining of E-cadherin in preinvasive and invasive squamous neoplasia and its localisation in the neoplastic cells was statistically significant (p < 0.001) (Table 3). The

Table 1: The clinicopathological characteristics of patients with squamous cell carcinoma

Age (31–75 years)*	Number of patients
Tumour grade (*n* = 41)	
Grade 2	29
Grade 3	12
≤ Grade 1 b	5
Tumour stage (*n* = 39)	
Stage II	11
Stage III	22
Stage IV	1
Mitosis (average 6.38/10 hpf) (*n* = 39)	
≤ 6/10 hpf	28
> 6/10 hpf	15
LVSI (*n* = 39)	
Absent	30
Present	9

Notes: hpf: high power field, LVSI: lymphovascular space invasion
*: A mean of 50.9 years

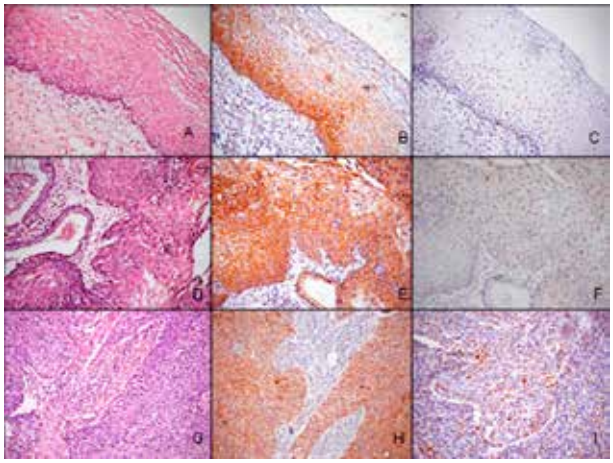

Figure 1: A normal ectocervical epithelium, with complete membranous staining of the epithelial cadherin and negative staining with matrix metalloproteinase-9 (a–c) (20 x); cervical intraepithelial neoplasia grade II, showing complete membranous staining with epithelial cadherin, and negative with matrix metalloproteinase-9 (d–f) (20 x); invasive squamous cell carcinoma, showing incomplete membranous staining with cytoplasmic delocalisation of the epithelial cadherin, and focal cytoplasmic staining of matrix metalloproteinase-9 in tumour and in stroma (g–i) (20 x)

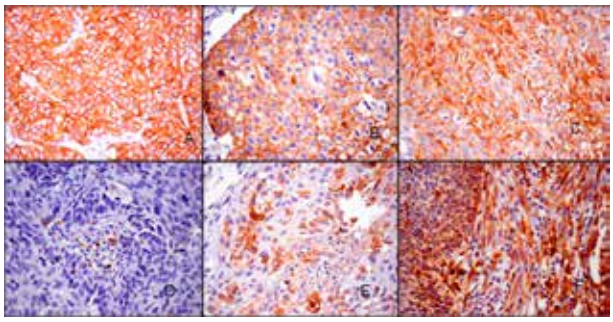

Figure 2: Patterns of epithelial cadherin and matrix metalloproteinase-9 staining in squamous cell carcinoma. Complete membranous (a), incomplete membranous (b) and cytoplasmic delocalisation (c) of epithelial cadherin. Negative matrix metalloproteinase-9 staining (d), intense cytoplasmic staining in the tumour cells (e) and intense cytoplasmic staining at the invasive front and stromal cells (f) (40 x)

downregulation of E-cadherin did not show association with any of the prognostic factors, as indicated in Table 2.

Matrix metalloproteinase-9 staining in the normal cervical epithelium, preinvasive and invasive carcinoma

The normal cervical epithelium was negative for MMP-9 (Figure 1 c). Positive staining was noted in the stromal inflammatory cells. Six of the 14 CIN cases were positive for MMP-9, with four of the six being CIN grade III. The stromal staining seen in these cases was noted in close approximation with the dysplastic epithelium and below the basement membrane, highlighting its role in invasion. Positive staining for MMP-9 was observed in 32 of the 43 SCC cases, with variable staining intensity ranging from weak to strong (Figures 1 i and 2 e–f). Strong cytoplasmic staining of the neoplastic cells was seen at the invasive front and the basal layer. Stromal staining was noted in the desmoplastic intratumoural stroma, indicating that MMP-9 facilitates invasiveness (Figure 2 f). The difference in immunostaining of MMP-9 in preinvasive and invasive squamous neoplasia was statistically significant ($p < 0.050$) (Table 3). The difference in the expression of MMP-9 between the tumour cells and stromal cells did not reach statistical significance (chi-square test, $p = 0.066$). The association of MMP-9 staining with prognostic factors is shown in Table 2.

Epithelial cadherin and matrix metalloproteinase-9 staining

The association between loss of cell adhesion marker, E-cadherin, and the expression of biomarker of invasion, MMP-9, in squamous neoplasms of the uterine cervix was statistically significant (Table 4).

Survival data

Follow-up was available in 22 cases, and the period of follow-up ranged from 9–69 months, with a mean of 31 months. There was no evidence of disease in 17 patients at the last follow up, three cases experienced recurrent disease followed by death, and two a short survival of a few months followed by death due to disease. Loss of the membranous expression of E-cadherin was evident in all of the patients who experienced recurrence and died due to disease, while the moderate expression of MMP-9 was evident in 60%.

Table 2: Association of tumour grade, stage, lymphovascular space invasion and mitosis in squamous cell carcinoma of the uterine cervix with epithelial cadherin and matrix metalloproteinase-9 immunostaining

Clinicopathological parameters	E-cadherin		*p*	MMP-9		*p**
	Positive	Negative		Positive	Negative	
Grade (*n* = 41)						
Grade 2	4	25	0.3024**	22	7	0.7017
Grade 3	0	12		10	2	
Stage (*n* = 39)						
≤ I b	1	4	0.4362**	5	0	0.5628
> I b	3	31		26	8	
LVSI (*n* = 39)						
Absent	4	26	0.5558**	25	5	0.3548
Present	0	9		6	9	
Mitosis (*n* = 39) (Mean of 6/10 hpf)						
≤ 6	4	20	0.1458*	22	2	0.0357
> 6	0	15		9	6	

Notes: E-cadherin: epithelial cadherin, hpf: high power field, LVSI: lymphovascular space invasion, MMP: matrix metalloproteinase
*: Fischer's exact test
**:Independent-sample *t*-test

Table 3: Association between loss of expression of epithelial cadherin and the acquisition of matrix metalloproteinase-9 in squamous cell carcinoma and cervical intraepithelial neoplasia

Biomarkers	CIN (cases)	SCC (cases)	*p* (Fischer's exact test)
E-cadherin positive (*n* = 16)	11	5	0.0000*
E-cadherin negative (*n* = 41)	3	38	
MMP-9 positive (*n* = 38)	5	33	0.0080*
MMP-9 negative (*n* = 19)	9	10	

Notes: CIN: cervical intraepithelial neoplasia, E-cadherin: epithelial cadherin, MMP: matrix metalloproteinase, SCC: squamous cell carcinoma
*: indicates statistical significance

Table 4: Association between the expression of matrix metalloproteinase-9 and the loss of expression of epithelial cadherin in squamous neoplasia

	MMP-9 positive	MMP-9 negative	*p* (Fischer's exact test)
E-cadherin positive	7	9	0.0307*
E-cadherin negative	31	10	

Notes: E-cadherin: epithelial cadherin, MMP: matrix metalloproteinase-9
*: indicates statistical significance

Discussion

An infectious agent is associated with the carcinogenesis of cervical cancer, one of the few human cancers in which this occurs. The epidemiological and laboratory data suggest that persistent infection with specific human papillomavirus (HPV) subtypes, and the integration of their HPV DNA into the genome, are important factors in the development of cervical neoplasia. However, because the majority of patients with HPV infection or CIN do not develop invasive lesions, HPV infection alone is probably insufficient for the complete neoplastic transformation of the cervical cells. HPV infection appears to be an early event. Additional abnormalities are required if biological transformation is to take place.

In order to progress from preinvasive to invasive carcinoma, the neoplastic epithelial cells must acquire the ability to penetrate the basement membrane and degrade the underlying ECM, which is composed of several components, including collagen, elastin and fibronectin.[6] The importance of tumour invasion, and subsequent dissemination via the bloodstream and lymph vessels, are critical steps in the progression of malignant tumours, including those of cervical cancer.

Many established prognostic markers in uterine SCC are used in risk grouping for the purpose of developing treatment protocols. However, the identification of biomolecules, which determine tumour invasiveness and the capacity to metastasise, may improve the prognostic models.

E-cadherin is a 120 kD transmembrane adhesion protein which plays an important role in cell adhesion by binding with catenins through its intracellular domain.[7] The downregulation of E-cadherin in CIN is an early and critical step in acquiring epithelial-mesenchymal transition by the neoplastic cells, which leads to the disruption of intercellular junctions formed by E-cadherin and β-catenin complexes. Silencing by DNA methylation is a possible mechanism for the downregulation of E-cadherin expression.[8] The complete membranous expression of E-cadherin was significantly different between preinvasive and invasive squamous lesions of the uterine cervix in the present study (79% versus 12%). In addition, the cytoplasmic relocalisation of E-cadherin observed in SCC had a statistically significant negative correlation with membranous expression.

There was decreased expression of membranous E-cadherin and focal cytoplasmic delocalisation in CIN, indicating that cytoplasmic delocalisation and the downregulation of E-cadherin is an early event in the tumour progression of cervical squamous neoplasia. Varied results have been obtained for the expression of E-cadherin in different studies. The low expression of E-cadherin in invasive tumours (34–100%) has been reported in most studies.[9–12] However, the high expression thereof has also been reported in a few studies.[13] These differences may partly be explained by technical divergence, such as tissue fixation, the methodologies employed and the staining assessment. A semi-quantitative scoring system was employed in our study, as opposed to the categorical approach of negative, partially positive and positive scoring, as seen in other studies. The downregulation of E-cadherin during the progression of a squamous intraepithelial lesion to invasive cancer has been reported in several studies, together with the relocalisation of E-cadherin in the cytoplasm.[14–16]

MMPs play a central role in ECM degradation and its turnover in various benign and malignant conditions. MMPs degrade the basement membrane and ECM, which facilitates local invasion and metastasis of the neoplastic cells. The upregulation of MMP-9 protein expression in the present study was observed in invasive squamous tumours, as compared to preinvasive lesions, indicating that this protein is required for basement membrane degradation and invasion of the stroma. MMP-9 is produced by the tumour cells, or the tumour cells have a paracrine effect on the surrounding cells, to synthesise these proteases. This is evidenced by localisation of this protein, both in the tumour cells and in the stroma, especially at the invasive fronts. Similar observations were reported in a previous study.[17] It has been suggested that tumour stroma, especially the fibroblasts, play an important role in invasiveness.[18] Even though a relationship between MMP-9 expression in invasive carcinoma and clinical prognostic factors (except for mitosis) was not found in the present study, the upregulation of MMP has been reported in cases with lymphnode metastases, indicating its role in invasion and metastasis.[19] The acquisition of several genetic alterations by the neoplastic cells is required for tumour progression and metastasis, either synchronously or in sequence.

This was a retrospective study, and a relationship between the downregulation of E- cadherin expression or the upregulation of

MMP-9, and clinical prognostic factors and their effect on recurrence or survival, could not be found. This was not the case in other studies.[10] This is probably because of the small sample size in our study, together with the short follow-up duration. In addition, the loss of adhesion molecules and the expression of proteases by the tumour cells and stromal cells occur in the early stages of carcinogenesis.

We observed an inverse relationship between the downregulation of membranous E-cadherin and its cytoplasmic relocalisation, and increased expression of MMP-9, in the present study, which is in accordance with our understanding of the mechanisms of invasion and metastasis. This study highlights that several molecular alterations, not only in the neoplastic cells, but also in the stroma, are required for tumour progression in squamous neoplastic lesions of the uterine cervix. Therefore, we conclude that the upregulation of stromal proteases, together with loss of cell adhesion molecules, is an important step in the acquisition of epithelial mesenchymal transition, and that this plays an important role in the process of local invasion and distant metastasis.

Conflict of interest – The authors declare no conflict of interest.

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