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Expression of MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 in Intraepithelial and Invasive Cervical Neoplasia

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Abstract

Aim: To evaluate the expression of MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 in Cervical intraepithelial neoplasia grade 3 (CIN 3) and invasive squamous cell carcinoma.

Methods and Results: This study comprised three groups: Group 1: 55 cases with CIN 3; Group 2: 30 cases with CIN 3 and invasive carcinoma components; Group 3: 46 cases with invasive carcinoma. Protein expression was investigated in tumor and stromal cells by immunohistochemistry and percentages of immunostained cells were determined. The mean percentage of MMP-14 tumor cells was significantly higher in stromal cells in all groups. TIMP-2 and MMP-9 expression was significantly higher in stromal cells than in tumor cells. TIMP-1 had a significantly higher expression in stromal cells of carcinoma and tumor cells of CIN 3. Stromal cells expression of MMP-2, MMP-14, TIMP-1 was increased with the severity of cervical neoplasia. The expression of MMP-2 in stromal cells that was higher in CIN 3 component of Group 2 than in CIN 3 of Group 1.

Conclusions: The increase in MMP-2 expression from CIN 3 to invasive cervical cancer reinforces their role in cervical cancer progression. From CIN 3 to invasive cervical carcinoma, TIMP-1 expression increased in stromal cells and decreased in tumor cells.

Introduction

Cervical intraepithelial neoplasia grade 3 (CIN 3) is the precancerous lesion that may progress to invasive carcinoma [1,2]. Transformation from preinvasive neoplasia to invasive carcinoma starts by a focal disruption of the subepithelial basement membrane. Potent proteolytic enzymes termed matrix proteinases (MMPs) are known to play a key role in this process [3].

Tumor cells secrete MMPs and induce stromal cells to produce MMPs [4,5]. MMP-2 and MMP-9 are proteolytic enzymes with a major role in type IV collagen digestion, an important event in vascular invasion and metastasis. MMP-14 is a key enzyme in tumor cell invasion. This enzyme seems to be implicated in both breaching the basement membrane by tumor cells and cell invasion through interstitial type-I collagen tissues [6].

Tissue inhibitors of matrix metalloproteinases (TIMPs 1 to 4) are natural inhibitors of MMPs. TIMPs are multifunctional proteins that not only inhibit MMPs but also promote cell growth, induce apoptosis, and inhibit angiogenesis [5,7]. However evidence suggests that TIMPs, in particular TIMP-1 and TIMP-2, may have unique biological properties, independent of their ability to inhibit MMPs [8].

Expression of MMP and TIMP markers has been studied mostly in tumor cells. In stromal cells, expression of these markers has been less frequently evaluated. It has been shown that cell–cell contact between cervical carcinoma cells and peripheral stromal fibroblasts augments the production and activation of MMPs. Therefore, the subsequent imbalance between MMPs and TIMPs may result in the gradually increasing invasive potential of cervical cancer cells [9-11].

A better understanding of the mechanisms by which CIN 3 acquires the ability to invade the cervical stroma may have a significant clinical relevance. Therefore, the aim of this study was to describe and analyze the expression of MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 in CIN 3 and invasive carcinoma of the cervix, in tumor cells and stromal cells.

Materials and Methods

Ethics statement

All tissue samples were collected for histologic examination and diagnostic purposes and were thoroughly anonymized for the use in this study. This study was approved by the State University of Campinas ethics committee (Approval No. 858/2007 - November 11th, 2007).

Tissue samples

This comparative observational study of women with cervical neoplasia consisted of three groups. The first group comprised 55 cases of histologic diagnosis of unique CIN 3 (Group 1), the second group comprised 30 cases with both components: CIN 3 (Group 2) and invasive carcinoma (Group 2) and the third group was composed of

46 cases with a histologic diagnosis of invasive carcinoma (Group 3). The study design was constructed assuming that pure CIN 3 (Group 1), CIN 3 associated with invasive carcinoma (Group 2) and pure cervical invasive carcinoma (Group 3) represent progressive steps from CIN 3 to invasive carcinoma.

Case selection was made from records of pathology reports obtained from women consecutively managed in the Women's Hospital, State University of Campinas, Brazil, between 2002 and 2008. Hematoxilin-eosin paraffin-embedded sections were reviewed and the best representative sample was identified considering tumor tissue and the underlying stroma. Specimens were obtained by punch biopsies, conizations or hysterectomies. The number of cases varied in each analysis, in some cases lesions were not represented for all markers, due to serial section of the tissue block. Despite some limitations of immunohistochemistry analysis of the stroma cells, this tissue compartment was analyzed because there is evidence that the stroma has a relevant role in cervical cancer progression [9].

Immunohistochemistry assay

Expressions of MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 were investigated in paraffin-embedded sections using the avidinbiotin-peroxidase complex method. The paraffin sections (5 μm thick) were deparaffinized and immersed in 3% hydrogen peroxidase in methanol to block endogenous peroxidase activity. Next, an antigen retrieval procedure was performed by immersing the slides in 10 mM citrate buffer (pH 6.0) and autoclaving at 121°C for 10 min. After washing in PBS, the tissue sections were preblocked using 10% normal goat serum for 15 min. The protocol for the Dako LSAB 2 peroxidase kit (Dako, Kyoto, Japan) was followed. The sections were incubated overnight with primary antibodies in a humidity chamber at 4°C.

The primary antibodies used were polyclonal mouse antihuman MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 (Nemarkers). The working dilutions for each primary antibody were: MMP-2 (1:100), MMP-9 (1:200), MMP-14 (1:100), TIMP-1(1:100) and TIMP-2 (1:100). Sections were rinsed with PBS for 15 min and incubated for 1h with the secondary antibody (biotinylated goat anti-mouse and rabbit immunoglobulin G secondary antibody; Dako). The sections were then incubated with streptavidin-peroxidase complex using 3,3'-diaminobenzidine as a chromogen. The sections were counterstained with Mayer's hematoxylin. Specificity of the immunohistochemical reactions was checked by omitting the primary antibody.

Immunohistochemistry assay and image acquisition

The methodology for interpretation of the immunostaining sections consisted in the following steps:

1 – Identification of representative regions of the lesions selected, according to group (CIN 3, invasive carcinoma and underlying stromal lesion); selection criteria for these representative areas were based on: image sharpness, areas of higher intensity of cellular immunoreaction (but any intensity of immunostaining was considered positive), a similar proportion of stromal and tumor region in the same picture, excluding necrotic regions. Stromal regions with a high concentration of inflammatory cells were excluded.

2 – Images of these regions were captured under a magnification of 400X. In Group 2, which included cases of invasive cell carcinoma associated with CIN 3, pictures were taken separately.

3 - Selection of one photograph per lesion and its underlying stroma was made for quantitative and qualitative analysis.

4 - Calculation of total cell number and number of immunostained cells (stromal and tumor cells separately) was made by two observers using morphometric software (Image Pro Plus®, version 6.3, Olympus). To determine the percentage of immunostained cells, at least 1,000 tumor cells and stromal cells per case were counted (Figures 1-3).

Statistical analysis

Statistical analysis was carried out, considering the mean percentage of immunopositive tumor cells and stromal cells within each group and between diagnostic groups. The CIN 3 and invasive carcinoma components of Group 2 were analyzed separately. The
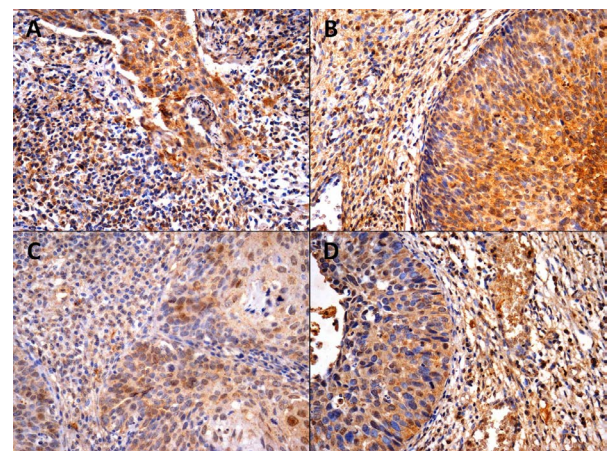

Figure 1: Percentage of tumor and stromal cells expressing MMP-2. **A:** Invasive carcinoma (Group 3): Stromal cells 97.5%, Tumor Cells 97.8%; **B:** CIN 3 (Group 1): Stromal cells 89.6%, Tumor Cells100%; **C:** Invasive carcinoma compartment (Group 2): Stromal cells-88.2%, Tumor Cells-82.4%; **D:** CIN 3 compartment (Group 2): Stromal cells-83.8%, Tumor Cells-84.5%. MMP-Metalloproteinases, Cervical Intraepithelial Neoplasia
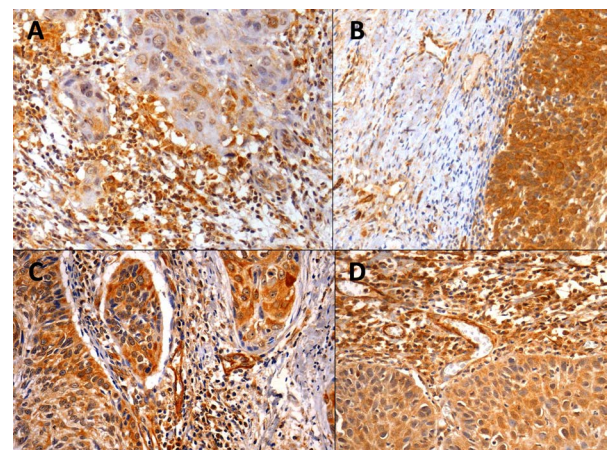

Figure 2: Percentage of tumor and stromal cells expressing TIM- 1. **A:** Invasive carcinoma (Group 3): Stromal cells 89.6%, Tumor Cells 56,2%; **B:** CIN 3 (Group 1): Stromal cells 98.7%, Tumor Cells-19.8%; **C:** Invasive carcinoma compartment (Group 2): Stromal cells-87.2%, Tumor Cells-98.2%; **D:** CIN 3 compartment (Group 2): Stromal cells-88.8%, Tumor Cells-82.0%. TIMP-Tissue inhibitors of metalloproteinases, Cervical Intraepithelial Neoplasia
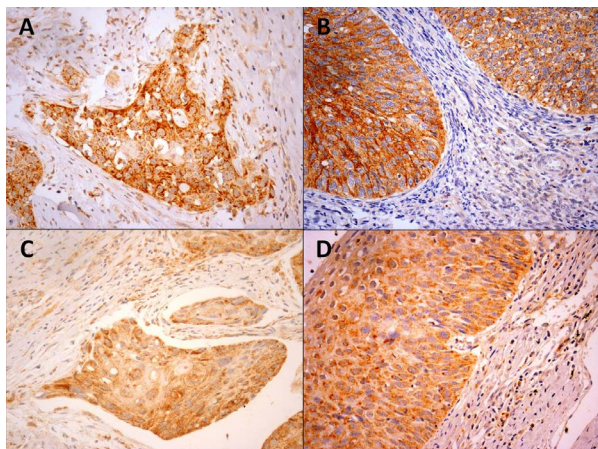
Figure 3: Percentage of tumor and stromal cells expressing MMP-14. A: Invasive carcinoma (Group 3): Stromal cells 67%, Tumor Cells 100%; B: CIN 3 (Group 1): Stromal cells 21.3%, Tumor Cells-100%; C: Invasive carcinoma compartment (Group 2): Stromal cells-62.2%, Tumor Cells-100%; D: CIN 3 compartment (Group 2): Stromal cells-49.9%, Tumor Cells-100%
MMP-Metalloproteinases, CIN- Cervical Intraepithelial Neoplasia

T-paired test was used to compare the mean percentage of tumor cells and stromal cells within each diagnostic group. The percentages of tumor and stromal cells between two diagnostic groups were analyzed by the Mann-Whitney and Wilcoxon Signed Rank tests. Differences were considered significant when p value was less than 0.05.

Results

The MMP-14, MMP-2 and TIMP-1 proteins were markedly expressed in tumor cells and stromal cells most cases.

MMP-14

In all groups, the mean percentage of cells expressing MMP-14 was significantly higher in tumor than in stromal cells. For tumor cells, the mean percentage ranged from 80.2% (Group 2 - invasive carcinoma) to 89.1% (Group 2 – CIN 3); for stromal cells, the mean percentage ranged from 51.9% (Group 1 - CIN 3) to 66.1% (Group 3 – invasive carcinoma). Intergroup analysis of tumor cells showed that MMP-14 expression did not vary. Nevertheless, the percentage of stromal cells positive for MMP-14 was significantly higher (p=0.0212) in invasive carcinoma (Group 3) (median=69.8%) than in CIN 3 (Group 1) (median=57.2%) (Tables 1-4 and Figure 4).

MMP-2

In all diagnostic groups, there was no statistically significant difference in the mean percentage of cells expressing MMP-2 in both tumor and stromal cells. The percentages of both tumor and stromal cells immunopositive for MMP-2 were significantly higher (p=0.0066 and p=0.0014, respectively) in invasive carcinoma (Group 3) compared to CIN 3 (Group 1). The percentage of stromal cells immunopositive for MMP-2 was significantly higher (p=0.0141) in the CIN 3 component (Group 2) than in CIN 3 (Group 1) (Tables 1-4 and Figure 4).

MMP-9

The mean percentage of cells expressing MMP-9 was significantly higher in stromal than in tumor cells in the three groups, i.e.: CIN 3 (Group 1), CIN 3 component (Group 2) and invasive carcinoma (Group 3). For stromal cells, the mean percentage was 58.8% (p=0.0295) in

CIN 3 (Group 1), 65.9% (p=0.0357) in the CIN 3 component (Group 2), and 53.2% (p=0.0009) in invasive carcinoma (Group 3). For tumor cells, the mean percentage was 49.9%, 52.3% and 42.0%, respectively. When tumor and stromal cells were analyzed, MMP-9 expression did not vary between groups (Table 1-4 and Figure 4).

TIMP-1

In groups at opposite ends (of the range of severity), the mean percentage of cells expressing TIMP-1 was statistically higher in tumor cells (mean=83.8%; p=0.0002) from CIN 3 (Group 1). Inversely, it was statistically higher in stromal cells (mean=83.7%; p=0.0076) from invasive carcinoma (Group 3). There was a significantly higher percentage of tumor cells expressing TIMP-1 (p=0.0119) than stromal cells, when CIN 3 (Group 1) was compared to invasive carcinoma (Group 3). Inversely, there was a significantly higher percentage of stromal cells expressing TIMP-1 (p=0.0082) than tumor cells, when invasive carcinoma (Group 3) was compared to CIN 3 (Group 1) (Tables 1-4 and Figure 4).

TIMP-2

In all groups, the mean percentage of cells expressing TIMP-2 was significantly higher in stromal than in tumor cells. In stromal cells, the mean percentage ranged from 59.0% (p<0.0001) (CIN 3- Group 1) to 65.1% (p<0.0001) (invasive carcinoma - Group 3). In tumor cells, the mean percentage ranged from 37.7% (invasive carcinoma - Group 3) to 51.1% (CIN 3 component - Group 2).. However, TIMP-2 expression in tumor or stromal cells showed no statistical difference between the diagnostic groups examined (Tables 1-4 and Figure 4).

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	*p-value*
MMP2	Tumor	51	58.42	26.42	11.66	38.82	50.64	82.33	100.0	0.3245
	Stromal	51	55.32	21.48	21.44	34.31	52.78	71.31	99.15	
MMP9	Tumor	55	49.87	29.85	1.62	26.14	47.71	77.65	99.65	0.0295
	Stromal	55	58.78	29.58	0.62	41.0	62.49	85.15	97.98	
MMP14	Tumor	51	86.25	20.41	8.43	78.91	97.84	100.0	100.0	< 0.0001
	Stromal	51	51.89	28.53	1.25	28.8	57.17	75.68	99.79	
TIMP1	Tumor	53	83.75	14.81	42.49	77.1	84.29	96.98	100.0	0.0002
	Stromal	53	72.31	20.3	19.77	56.64	71.67	88.46	100.0	
TIMP2	Tumor	52	42.08	25.73	0.77	23.29	37.53	56.22	96.39	< 0.0001
	Stromal	52	59.04	25.58	5.72	35.0	67.77	80.17	94.34	
Comparisons between means were performed using the paired t-test										

Table 1: Comparison of mean percentages of tumor and stromal cells immunopositive in CIN 3 (Group 1).

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	*p-value*
MMP2	Tumor	30	63.81	30.13	4.9	38.5	75.46	90.48	99.36	0.5244
	Stromal	30	67.8	26.15	6.9	51.54	77.86	88.3	98.2	
MMP9	Tumor	26	52.28	32.32	1.73	15.87	54.03	84.57	94.29	0.0357
	Stromal	26	65.94	24.13	9.77	43.35	74.17	85.23	93.56	
MMP14	Tumor	27	89.13	17.48	35.79	84.17	100.0	100.0	100.0	< 0.0001
	Stromal	27	56.46	24.95	11.39	40.29	56.93	81.46	92.67	
TIMP1	Tumor	18	81.58	16.73	45.74	67.3	86.73	95.78	99.79	0.7674
	Stromal	18	82.81	15.62	48.1	71.84	84.82	96.37	100.0	
TIMP2	Tumor	19	51.11	23.96	5.19	27.27	60.97	68.59	79.48	0.0123
	Stromal	19	63.91	22.04	18.39	42.73	64.24	79.18	96.46	
Comparisons between means were performed using the paired t-test										

Table 2: Comparison of mean percentages of tumor and stromal cells immunopositive in CIN 3 (Group 2).

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	p-value
MMP2	Tumor	30	64.85	26.87	12.1	45.63	72.71	85.99	99.27	0.7785
	Stromal	30	66.54	20.39	13.57	55.08	67.81	81.35	100.0	
MMP9	Tumor	26	54.68	31.59	0.71	28.61	55.49	85.62	100.0	0.2659
	Stromal	26	63.19	28.87	0.17	35.37	75.36	86.33	100.0	
MMP14	Tumor	27	80.18	26.26	17.21	54.27	100.0	100.0	100.0	0.0224
	Stromal	27	64.96	27.93	14.58	45.3	74.57	87.32	98.14	
TIMP1	Tumor	18	82.3	15.12	54.07	74.85	88.41	93.32	99.51	0.9072
	Stromal	18	82.76	16.03	33.94	76.74	86.15	94.26	98.96	
TIMP2	Tumor	19	44.3	19.65	14.69	28.59	42.47	53.01	78.76	0.0174
	Stromal	19	61.15	16.9	8.43	55.24	61.9	71.33	89.4	
Comparisons between means were performed using the paired t-test										

Table 3: Comparison of mean percentages of tumor and stromal cells immunopositive in Invasive Carcinoma (Group 2)

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	p-value
MMP2	Tumor	37	76.05	20.14	28.58	64.81	82.99	91.39	99.96	0.1953
	Stromal	37	69.99	18.87	6.51	62.21	72.99	84.9	97.81	
MMP9	Tumor	46	42.01	38.56	0.07	3.45	35.74	81.42	100.0	0.0009
	Stromal	46	53.2	35.51	0.07	17.89	66.23	85.74	95.41	
MMP14	Tumor	35	85.75	25.15	3.39	83.23	100.0	100.0	100.0	< 0.0001
	Stromal	35	66.06	23.79	10.9	45.64	69.83	87.49	100.0	
TIMP1	Tumor	46	76.53	15.08	40.26	68.03	77.66	88.45	99.65	0.0076
	Stromal	46	83.72	11.64	59.55	76.37	84.52	93.81	100.0	
TIMP2	Tumor	38	37.69	24.58	0.0	17.42	37.44	49.46	88.57	< 0.0001
	Stromal	38	65.11	21.73	3.13	49.08	64.21	86.38	100.0	
Comparisons between means were performed using the paired t-test										

Table 4: Comparison of mean percentages of tumor and stromal cells immunopositive in Invasive Carcinoma (Group 3)

Discussion

The analyzed markers were similarly or more highly expressed in stromal cells than in tumor cells, except for MMP-14 expression, that was more expressed in tumor cell. In all groups, MMP-2 expression was similar in tumor and stromal cells. The marker TIMP-1 was the unique that changed the distribution between tumor and stromal cells, since TIMP-1 expression was higher in tumor cells of CIN 3, and higher in stromal cells of invasive carcinoma.

Similarly to this study, Nair et al. [10] observed that MMP-2, MMP-9 and TIMPs showed intense immunostaining in both stromal and tumor cells of invasive carcinoma in virtually all cases. Neoplastic changes occur in epithelial cells, but there is also a significant change in MMP and TIMP expression in stromal cells, reinforcing that stromal cells play an important role in the carcinogenic process. Under normal physiological conditions, the stroma acts as an important barrier to epithelial cell transformation. Nevertheless, the stromal compartment undergoes changes in response to emerging epithelial lesions. This compartment has a key role in cancer initiation and progression, including the recruitment of new stromal cells that provide factors involved in cell growth and matrix remodeling [11,12].

Although this study showed that MMP-2, MMP-14 and TIMP-1 were highly expressed in all groups, major differences in marker expression were found between CIN 3 (Group 1) and invasive carcinoma (Group 3). MMP-2 expression was higher in tumor and stromal cells of invasive carcinoma than in these cells of CIN 3. This marker also showed higher expression in stromal cells of CIN 3 of Group 2 than in stromal cells of CIN 3 of Group 1. MMP-14 was more expressed in stromal cells of invasive carcinoma than in stromal cells of CIN 3. There was a different trend in TIMP-1 expression from CIN

3 to invasive carcinoma in tumor and stromal cells. TIMP-1 expression was higher in tumor cells of CIN 3 and, inversely, was higher in stromal cells of invasive carcinoma. There was a similar expression of MMP-9 and TIMP-2 in invasive carcinoma and CIN 3.

Although MMP-2 and MMP-9 are almost identical proteinases, their contribution to biological or pathological processes can be very different. At present, it is still unclear which of these enzymes is more important in tumor progression and metastasis [13]. Evidence shows that MMP-2 is especially important in ECM degradation, cancer cell invasion and metastasis. Therefore, MMP-2 is a determinant of cancer cell behavior [10,13,14]. This study demonstrated a higher MMP-2 expression in tumor and stromal cells of invasive cervical carcinoma than in the corresponding cells of CIN 3. In addition, MMP-2 was statistically more expressed in CIN 3 stromal cells of Group 2.
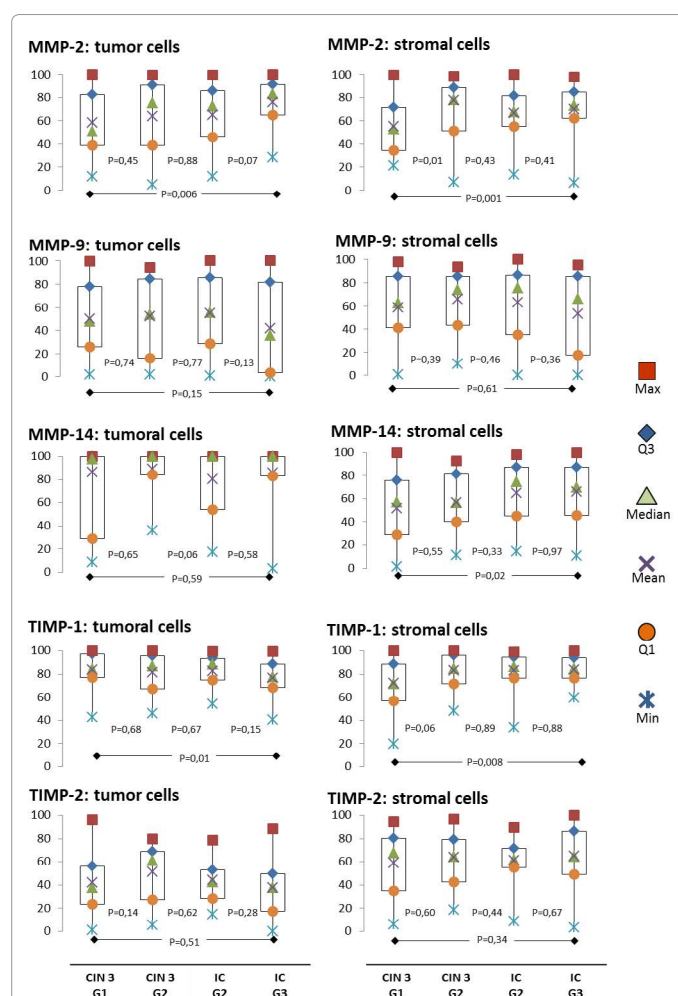

Figure 4: MMP-2, MMP 9, MMP 14, TIM-1 and TIMP 2 expressions in Cervical Neoplasia MMP- Metalloproteinases, TIMP- Tissue inhibitors of metalloproteinases, CIN – Cervical Intraepithelial Neoplasia, IC – Invasive carcinoma, Group 1-G1, Group-G2, Group 3-G3, Min-Minimum, Max-Maximum, Q1- 25th percentile, Q3-75th percentile.
Higher percentages of stromal and tumoral cells expressing MMP 2 were observed in invasive carcinoma (Group 3). Higher percentages of stromal cells expressing MMP-2 were detected in CIN 3 of Group 2 when compared with CIN 3 of Group 1. There was a significantly higher percentage of tumor cells expressing TIMP-1 than stromal cells, when CIN 3 of Group 1 was compared to invasive carcinoma of Group 3. There was a significantly higher percentage of stromal cells expressing TIMP-1 than tumor cells, when invasive carcinoma of Group 3 was compared to CIN 3 of Group 1.

However, no difference was demonstrated when the expression of this protein in tumor and stromal cells within the same group was analyzed. In contrast, no difference in MMP-9 expression was demonstrated between CIN 3 and invasive carcinoma, however, this marker showed a different pattern of expression, which was higher in stromal than in tumor cells in almost all groups. These findings suggest that MMP-2 could have a role in the neoplastic progression of the lesion and MMP-9 would act through an interaction between tumor and stromal cells.

In agreement with results of this study, Nair et al. [10] also found a progressive increase in MMP-2 positivity related to the severity of cervical neoplasia. Fernandes et al. [15] also showed that MMP-2 expression was higher in stromal cells of invasive carcinomas than of CIN 3 and indicated that stromal cells play an important role in tumor invasion and progression, mediated by the progressive enhancement of MMP-2 expression from CIN 3 to advanced invasive tumor. Brummer et al. [16] suggested that MMP-2 expression, when focally observed in high-grade intraepithelial lesions of the cervix, may indicate tumor regions with an increased risk for invasive growth.

MMP-14 may have a very important role in tumor cell progression. Cell invasion is a multistep process, involving degradation of the extracellular matrix (ECM) and cell mobility. While ECM degradation can be orchestrated collectively by MMPs, cell migration is likely to be predominantly associated with MMP-14 [17]. This understanding is considered concordant with findings in this study which showed that MMP-14 expression was higher in stromal cells in invasive carcinoma than in stromal cells of CIN 3.

Recent studies have demonstrated that TIMP-1 possesses additional functions, including enhancement of malignant transformation, stimulation of cell growth and inhibition of apoptosis, as well as promotion of migration, invasion and angiogenesis. This indicates a potential tumor-promoting role of TIMP-1 in the early stages of tumorigenesis [18]. Elevated TIMP-levels have been reported in association with cancer progression and were identified as poor prognostic indicators in several human types of tumor. This study showed a higher TIMP-1 expression in CIN 3 tumor cells, where malignant transformation, stimulation of cell growth and inhibition of apoptosis occur. In invasive carcinoma, this study showed a higher TIMP-1 expression in stromal cells, where the promotion of migration, invasion and angiogenesis take place. While MMPs are important in the late stage of tumor progression leading to metastasis, the anti-apoptotic effects of some TIMPs may favor tumor growth during tumor onset and early primary tumor growth [19].

The power of this study was the design that analyzed three steps of cervical cancer progression ranging from CIN 3 to invasive carcinoma. However, findings would be more conclusive, if normal cervical tissue had been included as control group. Limitations of the study were subjectivity and variability in immunostaining evaluation.

Conclusions

The progression of CIN 3 to invasive carcinoma seems be determined by complex interactions between tumor and stromal cells. These findings reinforce the evidence of the role of stromal cells in tumor invasiveness.

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