Matrix metalloproteinases-2, -3, and -9 secreted by explants of benign and malignant lesions of the uterine cervix

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Abstract. Argüello-Ramírez J, Pérez-Cárdenas E, Delgado-Chávez R, Solorza-Luna G, Villa-Treviño S, Arenas-Huertero F. Matrix metalloproteinases-2, -3 and -9 secreted by explants of benign and malignant lesions of the uterine cervix. *Int J Gynecol Cancer* 2004;**14**:333–340.

Elevated expression of matrix metalloproteinases (MMPs) plays a critical role in extracellular matrix (EM) degradation in tumor development and prognosis of different human carcinomas. In cervical carcinoma (Ce Ca), the role of these proteinases in the biological development of this neoplasm is controversial. In the present study, we compared the secretion of MMP-2, MMP-3 and MMP-9 among 29 benign and premalignant cervical lesions (cervicitis and cervical intraepithelial neoplasias) and 46 tumoral explants of Ce Ca. The explants were cultured for 48 h. The gelatinases secreted into conditioned medium were revealed by zymography and quantified by densitometry. The results showed high levels of MMP-3 and MMP-9 in tumoral explants. In contrast, only the pro-MMP-2 was higher in benign cervical lesions, although both active and inactive MMP-2 species are associated with advanced clinical stages in tumoral samples, and only the secretion of MMP-3 was associated with unresponsiveness to radiotherapy. We can conclude that the expression of MMPs is related to the invasive process in Ce Ca and suggest that they may play a role in degradation of the EM during local invasion. In addition, MMP-3 secretion could be a marker of poor prognosis in Ce Ca.

KEYWORDS: cervical carcinoma, invasion, metastasis, MMPs, proteinases.

Basement membranes and the extracellular matrix (EM) are the more important barriers that block tumor invasion[1–3]. The matrix metalloproteinases (MMPs) are the principal class of enzymes responsible for EM degradation associated with tumor invasion[4–6]. Uncontrolled expression and/or loss of their inhibitor activity (tissue inhibitor of metalloproteinases, TIMPs) have been associated in pathological and neoplasic conditions and correlate with poor prognosis in human cancer[7–9].

The ability to degrade tissues is a characteristic of aggressive tumors. This process requires an elevated secretion of MMPs and migration of neoplasic cells[10,11]. MMP-2, MMP-9, and MMP-3 are members of the MMP family[12,13]. These zinc- and calcium-dependent endopeptidases are responsible for

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degradation of type I, II, III, IV, and V collagens, fibronectin, laminin, and gelatins[4,14]. MMP-2, MMP-9, and MMP-3 are of particular importance in tumor progression because of their capacity to degrade type-IV collagen of basement membranes and other components of the EM. In addition, these enzymes have been implicated in tumor invasion and metastasis[4,11].

Many reports have demonstrated the overexpression of several MMPs in different cancers: carcinoma of the oral cavity[15,16], bronchopulmonary carcinomas[17], neuroblastomas[18], prostate carcinoma[19], gastrointestinal adenocarcinoma[20], and breast cancer[21–23]. In gynecological tumors like ovarian and endometrial cancer, the MMPs play a role in cancer invasion and metastasis[24,25]. Ovarian and endometrial cancers may secrete these enzymes, and they are modulated by steroid hormones[26].

Cervical Carcinoma (Ce Ca) is the most common solid tumor in Mexican women and is very common in developing countries[27–29]. Typically, this neoplasm is a loco-regional invasive tumor. Because of the loss of local control, the possibility for development of recurrences and metastasis after radiotherapy is high[30,31]. The role of MMPs in the development of this neoplasm is controversial. Several studies have been carried out by different methods: Reverse transcriptase-polymerase chain reaction *in situ*[32–34] immunostaining of several MMPs in premalignant and tumoral samples[33–37] as well as studies of proteolytic activity in extracts of cervical tissues[38]. These facts have made it difficult to establish a consensus for what the role of MMPs is in Ce Ca. Zymographic analysis permits the evaluation of both forms of each MMP, ie, the active and the latent enzyme. Due to an imbalance between high concentrations of active forms of MMPs and their implications in other carcinomas, in this study, we first proved that tumoral explants secreted more active forms of MMP-2, -3, and -9. This fact correlated with clinicopathological data. From these results, we will evaluate quantitatively these and other MMPs, and also TIMPs, to study more broadly this kind of carcinoma that is characteristic of our country. In addition, we will immunolocalize both MMPs and TIMPs in Ce Ca slides.

Patients and methods

Patients

Benign and premalignant lesions (BPLs). Twenty-six specimens of inflammatory and cervical intraepithelial neoplasia (CIN) lesions of the uterine cervix were obtained from patients who had undergone a hysterectomy at the Hospital General 'Manuel Gea González' (México City, México). All samples were confirmed to have cervicitis and CIN-I and CIN-II.

Tumoral tissues

Forty-nine tumoral biopsies of Ce Ca were collected from patients treated by conventional means in the Gynecology Service at Instituto Nacional de Cancerología (México City. México). All patients had been previously untreated at the moment of the study. Biopsies were taken from macroscopic tumors (a mirror fragment), and a representative fragment was histologically analyzed to prove that the determination of gelatinases came from malignant tissue. The tumoral explants were histopathologically confirmed as representative with more than 70% of the neoplasic epithelium. The patients were at stages I–IV according to the classification of the FIGO. Clinicopathologic information was obtained from the medical records (including disease-free survival and overall survival). The study was approved by the committee of our institution.

Both BPL and Ce Ca samples were collected in ice-cold RPMI-1640 medium (Invitrogen, Carlsbad, CA), without phenol red and fetal bovine serum, and supplemented with L-glutamine (292 µg/ml, Invitrogen), penicillin-G (91 µg/ml, Sigma Chemical, US), streptomycin (150 µg/ml, Sigma Chemical), gentamycin (150 µg/ml, Sigma Chemical St. Louis, MO), and fungizone (750 µg/ml, Sigma Chemical). The samples were processed immediately.

Histoculture of benign and tumoral explants

Necrotic tissue was removed from the biopsies, and remaining viable tissue was sectioned with a sterile surgical blade in 5 mm^3 explants. Each explant was placed on top of 8 mm^3 of previously hydrated RPMI-1640 Gelfoam blocks (UPJOHN, Kalamazoo, MI). Tissues were cultured in 8 ml of the same medium without phenol red and fetal bovine serum. The medium was supplemented with L-glutamine (292 µg/ml, Invitrogen), penicillin-G (30 µg/ml, Sigma Chemical), streptomycin (50 µg/ml, Sigma Chemical), gentamycin (50 µg/ml, Sigma Chemical), fungizone (250 ng/ml, Sigma Chemical), and with nonessential aminoacids (1:100 dilution, Invitrogen). Conditioned media were collected after 48 h, centrifuged at $1500 \times g$ for 10 min and concentrated by ultrafiltration (AMICON, Bedford, MA), using membranes with 10 kDa pore size. The protein content was measured by Bradford's method[39].

Gelatin and casein zymography

The activity of the MMPs secreted by the explants was analyzed by zymography, as described by Arenas-Huertero *et al.*[16]. Briefly, 4 µg of protein from concentrated media were loaded onto an 8% sodium dodecyl sulphate (SDS)-polyacrylamide gel copolymerized with 0.1% gelatin (Sigma Chemical), or on a 10% SDS-polyacrylamide gel containing 0.1% casein (Sigma Chemical). After electrophoresis, gels were rinsed in 2.5% triton X-100 (Sigma Chemical), incubated in TNC buffer (Tris 50 mM, NaCl 150 mM, $CaCl_2$ 20 mM, pH 7.4) at 37°C for 16 h and stained with Coomassie blue R250 (Sigma Chemical). MMPs were detected as clear bands (digested area) on the blue background of the stained gel. The levels of proteolytic activities were quantified by densitometric analysis using a Beckman spectrophotometer (DU650).

Activity analysis of MMPs

The inhibition of gelatin degradation was tested with TNC buffer-5 mM 1,10-phenantroline (Sigma Chemical), and the inhibition of casein degradation was tested in the presence of TNC buffer 10 mM *N*-etylmaleimide (NEM, Sigma Chemical) and −10 mM phenyl-methyl-sulfonyl fluoride (PMSF, Sigma Chemical), using the same electrophoretic conditions described above. The activation of MMPs was tested with 1 mM aminophenyl-mercuric acetate (APMA, Sigma Chemical).

Statistical analysis

The medians of MMPs secreted by each explant were expressed in densitometric units (DU). The differences in the medians of MMPs-2, -3, and -9 were tested using the Mann–Whitney test and the statistical significance was considered when $P < 0.05$. The correlation between the activity of MMPs and the clinical information was analyzed by the Kruskall-Wallis test.

Results

Histopathologic analysis of the BPLs showed chronic cervicitis in 12 cases (46.15%), eight cases of CIN-I (30.76%), and six cases of CIN-II (23.08%). The clinical characteristics of the cancer patients are summarized in Table 1. The most common histological type was squamous cell carcinoma, found in 77.5% of cases, and 57% of the patients were clinical stage III. The mean age of the patients with BPLs was similar to that of the Ce Ca patients (median = 52 years; range 29–83 years).

Table 1. Clinicopathological characteristics of the patients with Ce Ca

Variable	Number	%
Histologic type		
Squamous cell carcinoma	38	77.5
Adenosquamous carcinoma	7	14.3
Adenocarcinoma	4	8.2
Clinical stage		
I	7	14.3
II	11	22.4
III	28	57.1
IV	3	36.1
Menopausal status		
Premenopausal	16	32.6
Postmenopausal	33	67.3

Clearly, more latent MMP-2 expression was detected in BPL samples in relation to Ce Ca samples (190.5 DU versus 18 DU, respectively; $P < 0.02$), but there was no difference in the active enzyme levels between the tumoral and BPL samples (13.5 DU versus 6.0 DU, respectively; $P > 0.05$) (Fig. 1A).

The MMP-9 level in Ce Ca samples was significantly higher than in the BPL samples ($P < 0.005$). The active form of MMP-9 (84 kDa) showed a higher median of activity in Ce Ca explants than in BPLs (44.36 DU versus 0.0 DU, respectively; Fig. 1B). In addition, there was a similar trend in the activity in latent species of MMP-9: 146.3 DU for Ce Ca explants versus 55.2 DU for BPL explants ($P < 0.03$).

Conditioned media from Ce Ca explants showed higher gelatinolytic activity in comparison to explants of BPLs (Fig. 2A and B). Different bands of MMPs were seen with molecular weights ranging from 62 to 200 kDa.

Inhibition with 5 mM of 1,10-phenanthroline indicated the metalloproteinase nature of these gelatinolytic bands. Active forms were visualized with 1 mM APMA. No inhibition of gelatinolytic activity with 10 mM of NEM and 10 mM of PMSF was observed (data not shown). These biochemical tests confirmed these gelatinases as MMPs. In addition, we verified equal amounts of protein in each sample by staining the proteins after denaturing polyacrylamide gel electrophoresis (data not shown).

Because MMP-3 has no gelatinolytic activity, we performed casein zymography to visualize it. Two molecular forms of MMP-3 (52 and 60 kDa, active and inactive species, respectively) were obtained from explants of patients with BPLs and Ce Ca. These bands were inhibited with 5 mM of 1,10-phenantroline, but not with 10 mM PMSF or 10 mM NEM. Higher medians of both forms were observed
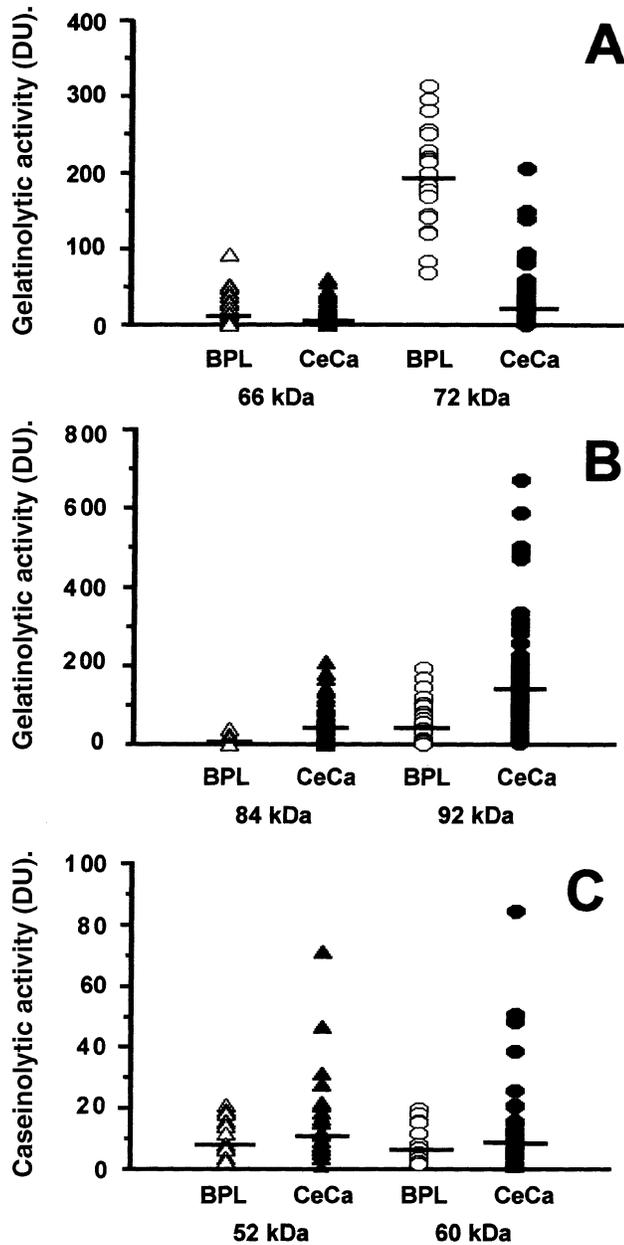
Fig. 1. Analysis of the gelatinolytic and caseinolytic activity by benign and premalignant lesions (BPL) and tumoral explants of cervical carcinoma (Ce Ca). The horizontal bars represent the median. (A). Gelatinolytic activity of matrix metalloproteinase (MMP)-2. Active (66 kDa) and inactive (72 kDa) forms. (B). Gelatinolytic activity of MMP-9. Active (84 kDa) and inactive (92 kDa) forms. (C). Caseinolytic activity of MMP-3. Active (52 kDa) and inactive (60 kDa) forms.

in the Ce Ca explants than in the BPL explants: 52 and 10.6 DU for Ce Ca, and 11.7 and 9.9 DU for BPLs, for active and inactive forms, respectively (Fig. 1C). However, the differences were not significant ($P > 0.05$). Six additional bands were seen in casein zymography with molecular weights ranging from 20 to 46 kDa (data not shown).
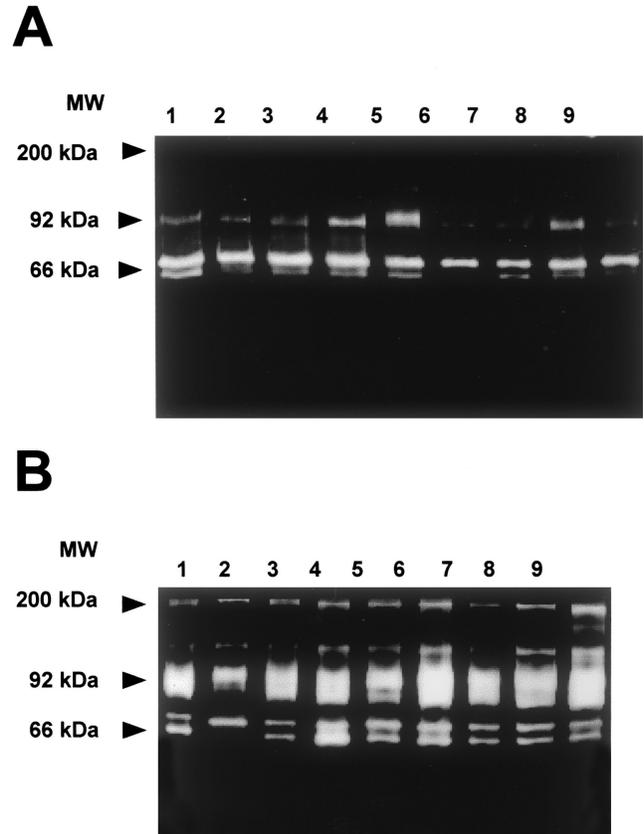

Fig. 2. Gelatin zymography of conditioned medium secreted by benign and premalignant lesions (BPLs) and tumoral explants of cervical carcinoma (Ce Ca). The migration of molecular weight markers is indicated. (A) Zymography of the conditioned medium of BPLs. (B) Zymography of the conditioned medium of Ce Ca explants. The lanes 1–9 in both gels indicate different samples.

Activity medians of MMP-2, -3, and -9 in Ce Ca samples were compared and correlated with clinico-pathological features. Table 2 illustrates the gelatinolytic or caseinolytic activity medians of these three MMPs, according to clinical stage, histological differentiation, and treatment response. Ce Ca explants have a higher median of MMP-9 in comparison with MMP-2 and -3. The gelatinolytic activity of MMP-2 (active and inactive forms) and MMP-9 (active forms) tended to be higher in more advanced clinical stages, but no significant correlation was observed ($P > 0.2$). In adenosquamous carcinomas, we observed an increase in the gelatinolytic or caseinolytic medians of MMP-9 and -3. However, this trend was not statistically significant ($P > 0.1$; Table 2). A significant difference was found only for MMP-3 between nonkeratinizing and keratinizing squamous cell carcinomas ($P = 0.007$). No correlation was observed between MMP levels and other clinicopathological factors.

There was a trend between clinical response to radiotherapy and low medians of both forms of

Table 2. Matrix metalloproteinase (MMP)-2, MMP-9 and MMP-3 medians secreted by Ce Ca explants

Variable	n (%)	MMP-2*		MMP-9*		MMP-3*	
		66 kDa	72 kDa	84 kDa	92 kDa	52 kDa	60 kDa
Clinical stage							
I	7	1.0	5.2	27.0	174.4	0.0	0.0
II	11	2.3	17.5	45.5	198.0	15.0	0.0
III	25	7.8	27.1	44.3	112.0	0.24	8.3
IV	5	23.2	34.0	74.1	162.5	0.0	11.6
Histological type							
Squamous	38	6.5	19.0	43.3	130.2	0.2	0.9
Adenosquamous	7	4.5	10.5	87.1	162.5	4.0	10.0
Adenocarcinoma	4	3.4	28.0	18.0	27.4	3.4	3.2
Response to radiotherapy							
Response	22 (45.5)	6.0	18.4	50.2	188.6	0.0	0.6
No response	11 (22.0)	4.6	10.6	44.3	147.0	0.2	7.0

MMP, matrix metalloproteinase.
*Densitometric units.

MMP-3 (Table 2). The differences in the medians were not significant ($P > 0.05$). Unexpectedly, both species of MMP-2 and -9 (active and inactive forms) were higher in those patients who responded to radiotherapy, but this trend was not statistically significant ($P > 0.1$; Table 2).

Discussion

MMP expression is associated with the invasive potential of cancer cells[5,6]. There is also a close relationship between MMPs expression and the clinical course in different patients[15–25,31–38]. MMPs are the most important family of biomarkers that define the malignant phenotype[6,8,40]. Previous studies have shown an increase in the expression of MMPs in Ce Ca. In particular, MMP-2 has been proven to correlate with the natural history of Ce Ca[36,37]. An increase in the tumoral proteolytic activity correlated with progression in cervical cancer[34,41]. In the present study, MMP-2 was zymographically detected in culture-conditioned media by BPLs and tumoral explants. Both forms of MMP-2 were detected and the inactive form (72 kDa) was strongly expressed in BPLs, in comparison with Ce Ca explants. In other tissues, such as endometrium, the stromal cells adjacent to the tumoral tissue could also produce this enzyme[42]. Our histopathological analysis of BPLs showed stromal tissue surrounding the lesion. Therefore, the higher MMP-2 expression in BPLs may reflect a stromal reaction[34]. However, an increase in MMP-2 medians in Ce Ca explants was observed from more advanced clinical stages, but this trend was not statistically significant (Kruskall–Wallis, $P > 0.12$). Others have described the immunostaining of MMP-2. Garzetti *et al.* observed a significant relationship between the MMP-2 index and the risk of lymphatic spread of Ce Ca[35]. On the other hand, Davidson *et al.* using mRNA *in situ* hybridization of MMP-2 observed that the presence of MMP-2 in tumor cells correlated with advanced stages and poor survival[34]. Thus, the expression of this MMP tended to increase with tumor progression. Talvensaari-Mattila *et al.* did not observe a correlation between MMP-2 staining and the clinical course or prognosis[37]. It is important to denote the differences in the methodologies used in the study of MMPs in this neoplasia, but our results are in agreement with those described by Garzetti *et al.* and Davidson *et al.*[34–36]. Finally, Talvensaari-Mattila *et al.* reported the immunostaining pattern of MMP-2 with high scores only in histologically higher-grade early stages of Ce Ca[37]. We observed a higher median of the latent form of MMP-2 in adenocarcinoma samples. This histologic kind of Ce Ca is more aggressive, and clinically the patients suffer local recidivism or distant metastasis with poor survival. It is possible that MMP-2 has prognostic value in this neoplasia and we want to document this fact to add other biological markers of tumoral progression in Ce Ca.

MMP-9 has been proposed to be a key factor in determining the invasive phenotype in different tumors and in sera samples of patients with breast and prostate cancers[19,22]. In Ce Ca, MMP-9 expression correlated with poor prognosis[32,33]. Our results demonstrate that the levels of MMP-9 (active and inactive forms) in the Ce Ca samples were significantly different from those in the BPLs, and they correlated with the adenosquamous histological type. Moreover, the high gelatinolytic median of the

84 kDa form of this gelatinase in adenosquamous tissues is the active species of this enzyme. We should analyze MMP-9 in more cases to evaluate the histological correlation. It also could explain the more aggressive nature of this carcinoma. Davidson *et al.* revealed an intense positive signal for MMP-9 mRNA in lethal tumors (stages III and IV), when compared to their less aggressive counterparts (CINs and stages I and II)[33]. We observed a higher median of the active form of MMP-9 in stage IV than in stages I, II, and III. This correlation suggests a role for this MMP in the progression of Ce Ca.

MMP-3 has the broadest substrate specificity and is of particular importance in tumor progression[43–46]. In some tumors, this enzyme is associated with an aggressive phenotype[23,47] and is synthesized by stromal fibroblasts[43]. We observed that this enzyme is elevated in adenosquamous cervical cancer, and it presents higher medians of caseinolytic activity in the patient groups that are not responsive to radiotherapy. Our results reveal that this MMP is a preliminary biological marker for predicting the response to therapy of this carcinoma. This is the first study that describes the secretion and the role of MMP-3 in progression and as a possible biological marker of prognosis in Ce Ca.

The presence of high molecular weight forms of MMP-2 and -9 was evident. The relative mobility of these molecular forms were 130, 140, 170, and 200 kDa. Previous studies described oligomeric forms of metalloproteinases with molecular weights over 100 kDa that correspond to the four gelatinase species (active and inactive)[48–50]. Our analysis with different inhibitors, and the activation of gelatinases with APMA, suggests that the presence of these degraded bands corresponds to oligomeric forms of MMP-2 and -9. Currently, we do not know the biological significance of these oligomeric forms of MMPs, but we cannot discard the possibility that oligomerization is another form of MMP regulation.

In this study, we employed an organ culture system using a gelatin matrix (gelfoam). This culture system avoids the dissociation of the cells and preserves the histologic architecture[26,51]. The gelatin matrix gives similar conditions of *in vivo* cellular interactions[26,51]. It is important to note that the proteinases analyzed in this study were obtained under these experimental conditions.

Human papilloma virus infection is an essential step in the development of cervical cancer[52], and the balance of MMP-9 and MMP-2 to TIMP-1 and TIMP-2 expression plays a role in tumoral progres-

sion. Our results confirm the notion that MMP expression is related to the invasive process in cervical cancer and suggest that in tumor development, MMPs may play a role in degradation of the EM by direct action. It is important to describe the possible relationship between HPV infection and MMP secretion, due to the role of HPV in tumoral progression. However, Talvensaari-Mattila *et al.* did not observe a correlation between overexpression of MMP-2 by immunostaining and the presence of HPV33[53].

We conclude that the proportion of BPL lesions expressing MMP-9 and -3 was low. In contrast, the Ce Ca lesions expressed higher levels of these MMPs. Nevertheless, the pro-MMP-2 was higher in BPL tissues in comparison with Ce Ca. Therefore, this MMP may not play an active role in Ce Ca development, although MMP-2 activity is associated with advanced clinical stages. The concentrations of MMP-9 and -3 were higher in adenosquamous carcinoma. In this regard, this type of carcinoma tends to behave more aggressively. Supporting these findings, we also observed that MMP-3 activity is associated with tumors that are not responsive to radiotherapy. In the future, we propose to extend the analysis of MMP-9 and -3 in more cases to confirm these results and also to analyze the expression of TIMPs and to evaluate the imbalance between active forms and TIMPs.

Acknowledgments

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