Analysis of gene status in cervical dysplastic lesions and squamous cell carcinoma using tissue microarrays

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Summary. Cervical dysplasia are classified as CIN-I, CIN-II and CIN-III. It has been observed that in at least 60% of CIN-I and CIN-II, the pathology disappears spontaneously, while around 30% persist at 24 months, 10% progress to CIN-III and 1% develops as a SCC. The factors involved in the evolution of the pathology are not defined, although infection of HPV is a necessary condition, but not the only one. For this reason, the identification of genetic changes is an essential element for understanding the carcinogenic process. It can also serve as a helpful tool for identifying patients who may be susceptible to its evolution and treatment, from patients whose lesions could regress spontaneous and for whom periodic follow-ups would be enough.

Fifty three cervical biopsies from patients with dysplasia and ISCC were included in the study. These biopsies were set into nine macroarrays. Eight genes and five proteins were examined in each samples (hTERT, PIK3CA, hTERC, MYC, CCND1, BCL2, ZNF217 and p16) by fluorescence in situ hybridization (FISH) and/or immunohistochemistry (IHC).

The results reflected that the genetic alterations of PIK3CA, ZNF217 and CCND1 were associated with the evolution of normal tissue to CIN I, those of hTERC and ERBB with the evolution of LSIL to HSIL, those of hTERT and MYC with the evolution of CIN-II/CIN-III to ISCC, and those of BCL-2 with the inception of ISCC. With regards to proteins, the expression of MYC and CCND1 in the initial stages of the illness would help in the acquisition of the altered cellular phenotype.

Key words: Cervical lesions, Fish, IHC and progression

Introduction

Cervical dysplasia is an abnormal growth of the squamous epithelium of the uterine cervix, classified into mild (CIN-I), moderate (CIN-II) and severe or in situ carcinoma (CIN-III). This histological classification is used in the study of biopsies and surgical specimens. The appearance of these lesions is closely related to sexual activity, being found most often in those women with numerous partners or those who began sexual intercourse at an early age. HPV infection has been found to be essential in the development of this pathology, being most common in women who smoke and who do not consume sufficient folic acid in their diets (Castellsague and Muñoz, 2003).

Sánchez et al. (2004) assert that in 60% of all patients with CIN-I and CIN-II, the pathology disappear spontaneously, thirty percent persist at 24 months, 10% progress to CIN-III, and one percent end up developing an infiltrating squamous carcinoma (ISCC). The factors involved in the evolution of this process have not yet been identified, although it is known that infection by HPV is a necessary factor, though not sufficient on its own. Several other possibilities of genetic and epidemiological character have been proposed.

With conventional cytogenetic techniques it has been possible to describe a great number of chromosomal alterations related to cervical dysplasia. The main alterations can be localized at chromosome 1 (losses of 1p, gains of 1q), chromosome 3 (losses of 3p or 3q), chromosome 5 (iso 5p or iso 5q) and losses of 5q) chromosome 11 (losses of 11q and translocations) and chromosome 17 (Atkin, 1997).

Comparative genomic hybridization (CGH) has permitted the description of more concrete alterations related to the evolution of the disease. The first studies, dating from 1996, concluded that the gain of 3q (3q25-qter) is an important and determining factor in the...
progression of the illness (Heselmeyer et al., 1996). In the mentioned region, the genes PIK3CA, hTERC, ETS1, ETV5 and OPA1 are found, which are involved in other types of tumors such as breast, ovarian, pulmonary, head and neck, hematological and dominant optic atrophy (Dellas et al., 1999; Allen et al., 2000; Yang et al., 2001; Umayahara et al., 2002).

Other studies corroborated the findings of Heselmeyer et al. (1996) and postulated the existence of other alterations associated with the process, such as losses in 2q (2q34-q36) and 3p (3p14-p22) or amplifications at 11q13 (Heselmeyer et al., 1996, 1997; Aubele et al., 1998; Dellas et al., 1999; Kirchhoff et al., 1999; Allen et al., 2000; Hidalgo et al., 2000; Matthews et al., 2000; Kirchhoff et al., 2001; Umayahara et al., 2002; Rao et al., 2004; Halder et al., 2005; Costa et al., 2006).

Using FISH technique, Zhang et al. (2002a) described gains of PIK3CA, hTERT, 20q13.2, ERBB2, MYC and CCND1 genes, though with a low frequency of amplification (3-7 copies of the gene). A later study observed the gain of hTR (hTERC) (3q26) in all of the cases of ISCC that were studied, and the gain of 20q11.21-q13.33 in 89%, where the localization of the REM1, DNMT3B, E2F1 and TOP1 genes has been proposed (Wilting et al., 2006).

Heselmeyer-Haddad et al. (2003) identified the amplification of hTERC as a definitive factor in the progression of LSIL to HSIL, later affirming its diagnostic value above that of routine cytological samples (Heselmeyer-Haddad et al., 2005). On the other hand, Cortés-Gutiérrez et al. (2005) suggested that aneusomy of chromosome 1 was related to the evolution of CIN-I/CIN-II to CIN-III.

In the clinical setting, risk factors related to the origin and development of dysplasias and cases of ISCC have been described. The main factors are infection by HIV and/or hepatitis, tobacco use, the use of oral contraceptives, sexual promiscuity, precocity in the first intercourse, the presence of other sexually transmitted diseases and infection by HPV (Castellsague and Muñoz, 2003).

The present study was designed based on the literature and with the goal of establishing a base that would permit the separation of patients with low-grade lesions who need surgical treatment from those who only require periodic follow-ups.

Material and methods

Patients characteristics

Fifty three biopsies from patients diagnosed as dysplasia of different grades were selected (22 CIN-I, 10 CIN-II and 14 CIN-III and 7 ISCC). Ten controls were added corresponding to cervical biopsies diagnosed with chronic cervicitis without alterations in epithelial maturation or morphological signs of HPV infection. This series did not include glandular lesions.

The samples came from the Department of Pathology, Hospital del Mar, Barcelona, Spain. Their selection was carried out by an expert pathologist using the usual histopathological criteria (Blaustein's Pathology of the Female Genital Tract (Kurman, 2001)).

The following clinical data were collected from each patient: age, HIV and/or B-hepatitis infection, contraceptives, number of sexual partners, parity, age of first sexual intercourse, tobacco use, history of previous illnesses, and the evolution and treatment of the cervical disease.

Immunohistochemistry (IHC) Assay

The IHC technique was performed using an automated system (Tech Mate 500, Dako Cytomation, Golstrup, Denmark) after antigen retrieval at 110°C for 1 minute in autowave. The method involved the application of different specific primary antibodies (rabbit anti-human ERBB2 oncoprotein, DAKO; mouse anti-human MYC oncoprotein, DAKO; mouse anti-human CCND1 oncoprotein, DAKO; mouse anti-human BCL2 oncoprotein, DAKO; mouse anti-human p16INK4a suppressor gene, DAKO). All were evaluated using the dextran/peroxidase technique (DAKO Envision).

In order to assess the results obtained using this technique, the area of interest was previously determined with the help of Hematoxylin-Eosin (H/E) staining. Two independent observers evaluated the positivity or negativity in each case, basing their observations on the localization of the staining (nuclear, cytoplasmic or membranous) and the results obtained in the controls cases.

FISH assay

After deparaffination, tissue sections were treated with EDTA in a microwave three times for 15 minutes each. Pretreated slides were incubated in a solution of proteinase K for nine minutes at 37°C. Then the slides were post-fixed in buffered formalin.

Pre-treated tissue sections and probes were denatured at 78°C for five minutes and hybridized overnight at 37°C in a Hybrite chamber (Vysis). Post-hybridization washes were performed three times in a 0.2xSSC solution for ten minutes and in a 0.2xSSC/0.1% NP-40 solution for five minutes at 45°C. Tissue sections were counterstained with ten microliters of 4.6-diamino-2-phenylindole (DAPI Counterstain) (Vysis).

The probes used for the study were the following: the LSI IGH/MYC/CEP 8 Probe labeled with Spectrum green/Spectrum orange/Spectrum aqua (Vysis), the LSI IGH/CCND1 Probe labeled with Spectrum green/Spectrum orange (Vysis), the LSI IGH/CCND1 Probe labeled with Spectrum green/Spectrum orange (Vysis), the PathVysion ERBB2/CEP17 Probe labeled with Spectrum orange/Spectrum green (Vysis), the LSI 20q (ZNF217) Probe labeled with Spectrum orange (Vysis),
the LSI 3q26 (hTERC)/Alphasatellite 3 Cocktail Probe, the Dual-color Probe labeled with Spectrum red/Spectrum green (Oncor), and the LSI 5p15 (hTERT) Probe labeled with Spectrum green (Oncor).

Results were analyzed under a fluorescent microscope (Olympus Optical España, Barcelona, Spain) using the CytoVision Software (Applied Imaging, Santa Clara, CA). Tissue sections were scanned at low magnification (100x) with DAPI excitation to localize those areas where histopathological characteristics had been established through the examination of serially-sectioned H/E stained tissue sections from the same patient.

A minimum of 200 nuclei per case were scored, and the cut-off was defined according to the levels obtained with the controls (Table 1).

### Statistical analysis

The statistical analysis was carried out using the Chi-Square Test for comparison of observed and expected frequencies.

### Results

The results of the study of the eight genes conducted on the 53 pathological samples are reflected in Figure 1. The results obtained in the study through IHC can be seen grouped in Figure 2.

#### FISH assay

It was possible to observe an increase in the percentage of cases with gains of hTERC, hTERT, MYC, CCND1, ERBB2 and BCL2, in relation to the increase of the grade of the dysplasia. Among PIK3CA, in the groups of CIN-I and CIN-II it was possible to observe an increase in the percentage of cases with gains, but in the CIN-III group the percentage decreased. Finally, in the case of ZNF217 it was possible to observe a higher number of cases with gains in the CIN-I group. This percentage decreased in the CIN-II group, but gradually increased in the other two groups (Fig. 1).

#### IHC assay

In the study of protein expression, no pattern of positivity for the five antibodies analyzed was observed. In the case of MYC the percentage of cases with positive immunoreactivity was quite elevated in all of the groups, with the exception of the CIN-III. The immunoreactivity of CCND1 and ERBB2 decreased related to the increase of the grade of the dysplasia, and in the case of P16INK4 the percentage of cases with immunoreactivity increased with the grade of dysplasia, diminishing in those cases with SCC (Fig. 2).

#### Comparison of FISH versus IHC results

In comparing, for CCND1 it was possible to note that in 12 of the 22 CIN-I cases (54,54%), 3 of the 10 CIN-II cases (30%), 6 of the 14 CIN-III cases (42,85%) and 4 of the 7 cases of SCC (57,14%), the results of both techniques were positive, while no correlation among the rest of the cases was observed.

With regards to MYC, in the CIN-I group, four cases presented gains of the gene and protein immunoreactivity. In both the CIN-II and the CIN-III groups two

<table>
<thead>
<tr>
<th>Gene</th>
<th>Losses (%)</th>
<th>Gains (%)</th>
<th>Monosomy (%)</th>
<th>Polisomy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTERC</td>
<td>34</td>
<td>2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>26.8</td>
<td>4.2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>hTERT</td>
<td>25.2</td>
<td>3</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MYC</td>
<td>10.8</td>
<td>3</td>
<td>33.8</td>
<td>3</td>
</tr>
<tr>
<td>CCND1</td>
<td>41.8</td>
<td>3.5</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>ERBB2</td>
<td>13.5</td>
<td>3.4</td>
<td>11.8</td>
<td>2.2</td>
</tr>
<tr>
<td>BCL-2</td>
<td>25</td>
<td>11.2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>ZNF217</td>
<td>31.8</td>
<td>4.4</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 1. Levels of cut-off for FISH samples defined with the analysis of the controls cases.

![Fig. 1. Results of the FISH technique grouped by probe and pathology. Cases where genic gains and polysomy were encountered are reflected.](image1)

![Fig. 2. Results of the IHC technique grouped by antibody and pathology. Cases where immunoreactivity was encountered are reflected.](image2)
Cervical lesions, fish, ihc and progression

When comparing the results of BCL2, there were no instances of correlation between FISH and IHC either in CIN-I or CIN-II. Furthermore, in each of CIN-III and ISCC groups, there was only one instances of such correlation (positive un both techniques).

Cervical dysplasia and evolution

Upon selection of the patients included in each of these groups, some were gathered who presented evolution of the disease after treatment. In the CIN-I group, four patients were included who later developed CIN-II and three who evolved to CIN-III. In all of these cases gains of ZNF217, PIK3CA and hTERC were observed. Similarly, with the exception of one case, all of the patients with evolution and persistence of the disease showed immunoreactivity for CCND1 and MYC (Fig. 4).

Three patients were included in the CIN-II group evolved to CIN-III. These cases coincided in that all of them showed immunoreactivity for MYC. In addition, gains or amplification of the CCND1, ERBB2 and PIK3CA genes could be observed (Fig. 4).

Table 2. Statistical results of the comparison (using the Chi-Square Test) of FISH results with the grade of dysplasia or carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>hTERC</th>
<th>PIK3CA</th>
<th>hTERT</th>
<th>C-MYC</th>
<th>CCND1</th>
<th>ERBB2</th>
<th>BCL-2</th>
<th>ZNF217</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN-I</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>CIN-II</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>CIN-III</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>CEI</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

CG: with gains; SG: without gains.
In the group of patients with CIN-III, only one patient presented persistence of the disease after treatment, and another developed CIN-I over time. In both cases, gains of hTERC and CCND1 were observed (Fig. 4).

**Statistical study**

The statistical analysis described the existence of an association between grade of dysplasia and the FISH results for the hTERT, MYC, ERBB2 and BCL2 genes (Table 2). When the results were analyzed for the IHC, no significant association among the results was obtained, with the exception of P16INK4a (Table 3a). For P16INK4a if we consider CIN I versus CIN II/III significant statistic association was observed \( (p=0.007) \) (Table 3b).

In order to complete the results, statistical studies were carried out relating the findings obtained through FISH and IHC to all of the collected clinical parameters. Significant associations between HPV and the immunoreactivity of p16INK4a and between HPV and the hTERT FISH results were observed.

Although no significant statistical results were obtained upon study of lesion evolution, it was possible to observe that in the cases without immunoreactivity for MYC, negativity of the lesions was produced in 83.3% of the cases. In contrast, in those cases where immunoreactivity had been observed, negativity of the lesion was produced in 56% of the cases. Something similar occurred with BCL2 and CCND1 when there was no immunoreactivity. Negativity of the lesion was produced in 72.7% and 91.8% of the cases, respectively. In addition, when immunoreactivity was positive, negativity of the lesion was produced in 33.3% and in 54.5% of the cases, respectively.

**Discussion**

Our results revealed that in the initial stages of dysplasia the genes that presented gains with greatest frequency were PIK3CA, ZNF217 and CCND1.

Different authors have indicated that the 3q region seems to be involved in the process of the appearance of dysplasia (Heselmeyer et al., 1996, 1997; Kirchoff et al., 1999; Allen et al., 2000; Hidalgo et al., 2000; Kirchoff et al., 2001; Umayahara et al., 2002; Wilting et al., 2006). However, while some authors confirm that the responsible gene is PIK3CA, others affirm that it is hTERC. Even though, both genes are closely localized in the close chromosomal region, our results reflect that PIK3CA may be more related to the appearance of dysplasia (Zhang et al., 2002b; Goto et al., 2006; Heselmeyer-Haddad et al., 2003). With regards to the mechanism, gains of this gene could suppose an increase in the activity of PI3-K, and as a result, deregulation of the cell cycle (Shayesteh et al., 1999).

In the case of hTERC, we observed gains in the majority of the cases of HSIL, which we related to the step from LSIL to HSIL, just as proposed by Heselmeyer-Haddad et al. (2003). The acting mechanism of the gene ZNF217 is not well defined. It is believed that it intervenes in the processes of transcriptional repression and attenuation of...
the apoptotic signal (Nonet et al., 2001). The genetic gains in the first phases of the disease could be related to the inhibition of the apoptotic capacity of cells. The only published study at this time dealing with ZNF217 in ISCC is of Zhang et al. (2002a). They observed that a percentage of cases with gains or amplification of the gene was found in 30% of their cases compared to the 100% observed in our study. The difference in the obtained results varies due to the cut-off points utilized. In the present study, if the cut-off point used by Zhang et al. (2002a) were used, none of our cases with ISCC would have shown gains of the gene.

Studies on other tumors, specifically on breast tumors, associated a gain of the 20q region, where ZNF217 is located, with the acquisition of an immortal phenotype and a worse prognosis for the disease (Tanner et al., 1995; Nonet et al., 2001). Our results showed that the patients that presented CIN-I and who later developed CIN-III showed gains in this gene. For this reason, and for the elevated frequency of observed gains in the initial studies of the disease, ZNF217 could be found to have a predictive and prognostic value in cervical dysplasias, and this should be confirmed through more studies using considerably larger numbers of cases.

The protein CCND1 has a role in the regulation of the cell cycle in the step from G-phase to S. The over-expression is related to the hyperphosphorylation of Rb and the constant activation of the transcription factor E2F. Southern and Herrington (1998) directly related CCND1 to the infection of high risk HPV, observing that only those cases of CIN-I infected by high risk HPV expressed this protein. Bae et al. (2001) found that there was no significant statistical relationship between HPV and the expression of CCND1, but they believed that dysplasias present little expression and that this expression increases along with the evolution of the illness. Our results contradicted these findings. Our patients with CIN-I had an elevated protein expression, which in its turn coincided with the elevated percentage of cases showing genic gains, but the expression diminished as the grade of the dysplasia increased. This could have been due to the fact that the expression of P16INK4a continued to increase in the CIN-II and CIN-III cases, and it is a potent inhibitor of the expression of CCND1 (Saqj et al., 2002; Sahebali et al., 2004; Kalof and Cooper, 2006).

In addition, in the CIN-I group the protein expression of CCND1 that was observed in all of the biopsies, except for one, was nuclear. In contrast, in the CIN-II and CIN-III groups 50% were nuclear and cytoplasmic and the other 50% were only cytoplasmic. Carreras et al. (2007) described that in relation to the increased intensity of the lesion, the nuclear expression decreases and the cytoplasmic expression increases. Our results confirmed those findings. This fact could be the result of the process of protein degradation, which abandons the nucleus and passes to the cytoplasm, where it loses its activity and ends up being degraded in the proteasomes (Burgués et al., 2005). In our study, considering the positive cases that presented nuclear staining, the percentage of immunoreactivity decreased considerably, correlating even more to the increased expression of P16INK4a. It would be interesting to study the phenotypical repercussions of this event.

In the group of studied patients, amplification of ERBB2 was not observed, although there were gains of the gene in an elevated number of cases. Rosty et al. (2004) presented similar results and spoke of the lack of efficiency of treatment with anti-cerbB2 in these types of patients. Upon study of the expression of the protein, we observed that the results between FISH and IHC did not coincide, the cases with genic gains or polysomy not necessarily presenting immunoreactivity. It has been described that the ERBB2 receptor can suffer a proteolytic rupture that can lead to the liberation of the extracellular domain. The transmembrane part and the intracellular domain remain intact and functional in an independent manner of the ligand (Kong et al., 2006). This could explain the results obtained, since if the process of proteolytic degradation of ERBB2 were greater in cervical lesions, immunoreactivity would not be observed against antibodies directed against the extracellular domain. That could also provide a basis for explaining the bad response to treatment using anti-cerbB2 described by Rosty et al. (2004).

The results showed that the percentage of cases with gains of MYC increases with the grade of dysplasia, similar to descriptions in the literature (Golijow et al., 2001; Abba et al., 2004). However, in our study there existed an elevated discrepancy between the FISH and IHC. Our findings did not show a significant statistical correlation between the expression of MYC, infection by HPV and the grade of dysplasia described by some authors (Dellas et al., 1998). On the other hand, a statistically significant correlation was observed between the results of FISH and the grade of the disease. This could be explained based on the observations of Wentzensen et al. (2004) regarding the integration of high risk HPV close to some gene promoters, among MYC, above all in cases of HSIL and some carcinomas (Ferber et al., 2003).

In the group of patients whose disease persisted or progressed, an elevated expression of MYC was observed. This could lead us to believe that this event might be useful as a prognostic factor for the evolution of the disease, even though statistically significant results were not observed. However, there were visible differences among the group of patients with persistence/progression and the group without persistence/progression.

There is controversy regarding whether or not hTERT can be used as a prognostic marker for the disease. According to our results, alterations of this gene were observable with greater frequency in the CIN-III and in the ISCC groups, but no statistically significant correlation between the grade of the dysplasia and the gains of the gene were able to be shown. Analyzing
These results, it is possible to affirm that there was a relationship between MYC and hTERT gains with the infection of high risk HPV.

None of the cases of CIN-I presented genetic alterations of BCL2, but in an elevated percentage it was possible to observe expression of the protein. As the disease continued to develop, the number of cases with genic gains increased. In contrast, the protein expression decreased. For this reason, BCL2 could be considered a gene for late onset. Ozalp et al. (2002) and Grace et al. (2003) observed a greater protein expression in a group of dysplasias than in a group of carcinomas and concluded that BCL2 is related to the apoptotic process and with the progression of the disease. We also found that in the CIN-I and CIN-II groups the expression of BCL2 was greater to that of the ISCC group. We could propose that the protein could help in the anti-apoptotic process of the tumoral cells and that its elevated expression could be related to P53, and indirectly to infection by HPV. Fonseca-Moutinho et al. (2004) described that an increase in the expression of BCL2 could confer a good prognosis for dysplasias. Our findings do not coincide with that study since we did not observe a statistically significant relationship between the evolution of the disease and the expression of this protein. However, we observed that the negativity of the lesion was more related to the non-expression of BCL2.

Finally, the results regarding P16INK4a remain to be discussed. We observed significant differences between the immunoexpression and the different grades of dysplasia. There also existed a significant statistical correlation between P16INK4a and infection by HPV. These findings coincide with those published by Ishikawa et al. (2006), who found that as the grade of dysplasia increases, the percentage of cases with IHC results positive for P16INK4a also increases. The principal cause for this is infection by high risk HPV, specifically the expression of the viral protein E7, which degrades Rb and, as a result, liberates E2F, provoking the cell to cycle constantly. The cell, to counter this effect, increases its expression of P16INK4a (Saqi et al., 2002; Sahebali et al., 2004; Kalof and Cooper, 2006). Because of this, we agree that it would be useful to employ the expression of P16INK4a as a viral marker.

In the clinical field, there are several publications where infection by HIV and/or hepatitis (type of virus), smoking, contraceptive use, number of sexual partners, parity and the age of the first sexual intercourse are considered risk factors in the inception and development of cervical lesions (Castellsague and Muñoz, 2003). In the group of selected patients, and in each subgroup, HIV positive and HIV negative patients were included. When comparing the FISH and IHC results, no significant differences were observed. This leads us to believe that, considering the number of samples included in our series, the gains or amplification of this group of genes and the expression of these five proteins did not appear to be related to infection by HIV. We believe that the clinical differences observed in the group of HIV positive patients with respect to those who were HIV negative would be more related to factors such as might be published by Castellsague et al. (2002).

Castellsague and Muñoz (2003) described the habit of smoking in women infected by HPV as a risk factor. Prior articles have not established a relationship between this fact and the development of cervical dysplasias (Muñoz et al., 1992; Eluf Neto, 1994). We did not observe a relationship between these events either. It would be valuable if infection by HPV, along with the habit of smoking, could be found to jointly affect the development of the disease.

In conclusion, the results obtained in this study have allowed us to formulate a hypothesis regarding some of the genes that are altered in the early stages of the disease and some in the more advanced stages (Fig. 4). This hypothesis must be confirmed or rejected based on a larger series of cases.
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