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Wnt/Beta-Catenin and EGFR/PI3K/pAKT/mTOR Signaling Pathways and Their Relation with Cervical Cancer

Guerra F[1], *Rocher A*[1], *Díaz L*[2], *Palaoro L*[1*]

[1]Department of Clinical Biochemistry, University of Buenos Aires, Argentina

[2]Department of Pathology, University of Buenos Aires, Argentina

Abstract

The cervical cancer is related to the presence of Human Papillomavirus (HPV) that interacts with the Wnt/Beta-Catenin and the PI3K/pAKT/mTOR signaling pathways. The Epithelial-Mesenchymal Transition (EMT) is frequent in the spread of carcinomas, ensuring mobility of the cells to distant places. 122 cervical surgical pieces were analyzed by immunohistochemistry in order to verify the EMT process *in vivo* in cervical cancer progression and the activation of Wnt/Beta-Catenin and EGFR/PI3K/pAKT/mTOR pathways.

In total, 24% of the invasive cancers showed full activation of EGFR/PI3K/AKT/mTOR pathway. The characteristics of the fully activated Wnt/Beta-Catenin pathway (Beta-Catenin nuclear translocation and Cytoplasmic expression of mesenchymal markers) were not detected in our cases. HPV oncogenic proteins can activate various stages of the EGFR/PI3K/AKT/mTOR pathway and also destabilize key proteins for maintaining the integrity of intercellular junctions. Both processes could contribute to the development of malignancy, at least in a percentage of cases (24% in our series). mTOR is key in the modification of the cytoskeleton which allows the amoeboid mobility of cells in the invasion process of cervical neoplasia. The use of the term EMT may not always be appropriate for describing the diverse processes associated with tumor spread. Thus, the phenomenon of cervical cancer invasion could be considered as a “partial EMT”.

Keywords: Cervical cancer; Epithelial-mesenchymal transition; PI3K/pAKT/mTOR signaling pathway; Wnt/Beta-Catenin signaling pathway

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Correspondence:
Luis Palaoro, Department of Clinical Biochemistry, University of Buenos Aires, Cordoba, 2351(1120), Avda Forest 1318-4°B (1427) CABA, Argentina, Tel: +54-1145520449; E-mail: luispalaoro@yahoo.com.ar

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Introduction

The Epithelial-Mesenchymal Transition (EMT) is a frequent process in the spread of carcinomas, which ensures greater mobility of the cells that must move to distant places in the metastasis [1,2]. During EMT, cells decrease the expression of their epithelial markers (Cytokeratin, E-Cadherin), dissolve adherens junction proteins, break tight junctions [3] and express mesenchymal markers (Vimentin, Alpha smooth muscle act in), as well as powerful transcription factors (Snail, Slug) that repress the gene that encodes for E-Cadherin and activate genes that promote transformation and cell mobility [4,5].

In EMT, the Wnt pathway [6] is frequently activated, with Wnt proteins being the official ligands of this pathway. They are secretory glycoproteins from a wide range of cells, from neurons to fibroblasts. However, several growth factors also stimulate the Wnt pathway (FGF or fibroblast growth factor and Beta-TGF) [7].

Although 4 routes are known for Wnt, the most widespread is the so-called canonical route Wnt/β-Catenin. In this pathway, in the absence of ligand, the free Beta-Catenin of the cytoplasm is continuously degraded in proteasomes by a phosphorylation process mediated by a complex formed by the enzyme Glycogen Synthetase Kinase 3Beta (GSK-3Beta), Polyposis Coli Adenomatous (APC), Axin and Casein Kinase 1α (CK1alpha). (It is important to remember that a part of the Beta-Catenin is bound to E-Cadherin, and together with Alpha-Catenin they connect with the act in cytoskeleton, which gives stability to the epithelial cell).

In the presence of the ligand (Wnt), the Frizzled receptor (Fzd) is activated, which phosphorylates the Disheveled protein (Dvl), blocking the complex that phosphorylates Beta-Catenin. The consequence is the increased concentration of this molecule that Trans locates to the nucleus and binds to Lymphoid stimulating Factor (LEF) and T-cell factor (LEF-1/TCF), to form a powerful gene transcription factor for c-*Myc*, c-Jun, *CCND1*, metalloproteases, Cyclin D1, Vimentin, etc., all related to cell proliferation, invasion and EMT [8].

The main function of Snail, which is also phosphorylated by GSK-3Beta [9], is the inhibition of the expression of the *E-Cadherin* gene, probably carried out by epigenetic mechanisms, by activating his tone deacetylases [10]. In this way, repression of E-Cadherin synthesis is the first step of EMT, marking the beginning of tumor invasion.

Squamous and cylindrical cancers developed in the cervix uteri may be candidates for EMT during its spread. However, it is not clear, so far, if this process takes place *in vivo* or if it occurs totally or partially. Most studies were performed *in vitro* in cell cultures [11-15], and the results are difficult to compare with those obtained with biopsy samples or surgical pieces. EMT is regulated in several ways: by cytoskeleton modulators, such as Rho-GTPase involved in the dissociation of cell junctions and in the remodeling of the cytoskeleton, soluble factors such as Beta1-TGF, fibronectin from the extracellular matrix that through its Alpha-5-beta 1 receptor can activate the EGFR pathway, among others [16].

In cervical cancer, strongly related to the presence of Human Papillomavirus (HPV), High-Risk (HR-HPV) oncogenic proteins (E6, E7, E5) interact with the Wnt/Beta-Cater in signaling pathway and with the PI3K/pAKT/ mTOR pathway [17]. In turn, AKT signaling has been shown to up regulate the Wnt signaling pathway through inhibitory phosphorylation of GSK-3beta, thus stabilizing Beta-catenin and promoting its recruitment to TCF/LEF complexes [18].

In HR-HPV infected cells, viral integration produces mutations in the *PIK3CA* gene, which encodes PI3K, which in turn activates AKT. *PIK3CA* shows the highest frequency of mutations in HPV-related cancers [19] and the highest frequency of mutations in cancer in general [20].

A higher number of Receptors for Epidermal Growth Factor (EGFR) are detected in neo plastic cells of the cervix due to the activity of HR-HPV E5 that stimulates their recruitment to the plasma membrane [21]. HPV16 E5 was shown to promote the activation of EGFR [22], the RAS/RAF/MAPK cascade, c-fos, c-*myc* and c-jun [23] and the inhibition of p21 and p27, thus causing cell cycle progression and DNA synthesis (S phase) [24].

MicroRNAs have been previously reported as cancer-related miRNA that are dysregulated in various cancer types and function either as oncogenic or as tumour suppressive miRNAs, promoting cervical cancer progression [25-27] or inhibiting the metastatic phenotype of cervical cancer cells through regulating Wnt/Beta-catenin signaling pathways [28]. Furthermore, microRNAs can inhibit PI3K/AKT/mTOR signaling pathway in human cervical cancer cell [29]. Reports of the activity of these microRNAs in cervical cancer support the crosstalk and collaboration of the two pathways: Wnt/Beta-Catenin and PI3K/AKT/mTOR in the development of this tumor [15].

In cell cultures, the activity of E6 and E7 of HR-HPV was reported in relation to the Wnt/Beta-Cater in pathway, stabilizing Cytoplasmic Beta-Catenin and favoring nuclear translocation and *TCF*-mediated transcription [6].

HR-HPV E6 activates the AKT/mTOR pathway by binding to Tuber in, which normally blocks protein kinase S6, important in ribosomal RNA synthesis [30] and degrading MAGI 2 and 3 guanylate cyclase homologs, inhibitors of AKT [31]. The destruction of MAGI by HR-HPV E6 leads to the activation of mTOR (mTORC1 and mTORC2), which modulates cell growth through the regulation of protein translation, and is involved in cytoskeleton remodeling and cell mobility. These mechanisms would contribute to the spread of tumors in the cervix, independently or in collaboration with EMT [32].

The objective of the present work was to analyze the Wnt/Beta-Catenin and EGFR/PI3K/pAKT/mTOR pathways, and the expression of epithelial and mesenchymal markers in surgical pieces of cervical cancers, in order to verify the EMT process *in vivo* in cervical cancer progression.

Methods

122 cervical surgical pieces of cervical cancer from the file of the Pathology Department of the Hospital of Clinics, fixed in 5% formalin and preserved in paraffin, were used. 3μ to 5μ thick microtome cuts were made and mounted on slides prepared to perform antigenic retrieval. The smears tested were: Squamous Intraepithelial Lesión (SIL) (24), squamous carcinoma (54), Adenocarcinoma *in Situ* (AIS) (14), Adenocarcinoma (30). After de-paraffinizing with xylene, the slides were hydrated and divided into two groups:

a) Hematoxylin-Eosin stained (H&E), b) immunohistochemistry

Immunohistochemistry

After the antigenic retrieval and blocking of peroxidase and nonspecific antigens, the samples were incubated overnight with the different antibodies:

For the analysis of the EGFR/PI3K/pAKT pathway, the following antibodies were used against the phosphorylated forms of the proteins integrating the pathway.

- P-EGFR (Tyr 1173), rabbit polyclonal IgG, sc-101668-Santa Cruz Biotechnology/Dallas, Texas, USA, 1/30.
- PI3 kinase p85 alpha antibody (M253) ab86714-Abcam/Cambridge, UK, 1/100.
- P-Akt1/2/3 Antibody (C-11): sc-514032-Santa Cruz Biotechnology/Dallas, Texas, USA, 1/50.

For the study of the Wnt/Beta-Catenin signaling pathway, the following antibodies were used:

- Anti-Beta-Catenin antibody - BD Biosciences, Denver CO, USA, 1:100.
- Anti- Vimentin antibody - Dako, Carpentaria, CA, USA, 1:300.
- Anti-alpha Smooth Muscle Act in (αSMA) antibody (ab5694) - Abcam Cambridge, USA, 1:200.

Finally, the slides were incubated with HRP Streptavidin Label (CytoScan™ HRP detection System; Cell Marque, Rocklin, CA), Polyvalent Biotinilated Link (CytoScan™ HRP detection System; Cell Marque, Rocklin, CA), H_2O_2 and diaminobenzidine.

Biopsies of not small cells lung squamous carcinomas were used as positive controls for the components of the EGFR/PI3K/pAKT

Table 1: Expression of Vimentin, Alpha SMA and Beta-Catenin in cervix uteri.

	Vimentin			Alpha SMA			Beta-Catenin		
	Neg	Pos	Pattern	Neg	Pos	Pattern	Neg	Pos	Pattern
Squamous epithelium	6	0	-	6	0	-	0	6	M
Reserve cells	9	0	-	9	0	-	0	9	M+C
Endocervical cells	20	0	-	20	0	-	1	7	M+C
Meta plastic cells	10	0	-	10	0	-	2	6	M+C
LSIL	18	0	-	18	0	-	0	18	M+C
Squamous carcinomas	54	0	-	54	0	-	39	15 ↑	M+C
AIS	11	3	C	14	0	-	9	5	C
Adenocarcinoma	30	0	-	30	0	-	27	3	C

Neg: Negative; Pos: Positive; C: Cytoplasmic; M: Membranous; ↑: only at the centre the cancer nests

pathway.

Positive controls for the study of the Wnt/Beta-Catenin pathway was: for Alpha SMA, vascular smooth muscle from cervical biopsies; for Vimentin, smooth muscle and vascular smooth muscle from cervical biopsies; for Beta-Catenin, Squamous epithelium of the uterine cervix.

The development of brown color in the cells demonstrated the presence of the antigen. A qualitative evaluation of the staining intensity was recorded as negative, low positive and positive. Low positive was considered if less than 20% of cells were immune stained and positive if more than 20% of cells were stained. Membranous or Cytoplasmic expression for all the markers was recorded.

Results

Wnt/β-Catenin pathway

a) Vimentin

- Negative in all cases of squamous carcinoma and Adenocarcinoma.
- Negative in endocervix, squamous metaplasia, reserve cells and stratified Squamous epithelium cells.
- Positive in two cases of AIS (21%).

b) Alpha SMA

- Negative in all cases of squamous carcinoma, Adenocarcinoma, and AIS.
- Negative in endocervix, squamous metaplasia, reserve cells and stratified Squamous epithelium cells.

c) Beta-Catenin

- Positive in differentiated areas of squamous carcinomas (28%), with membranous and Cytoplasmic expression.
- Positive in Adenocarcinoma (11%), with Cytoplasmic expression.
- Positive in AIS (36%), with Cytoplasmic expression.
- Positive in LSIL membranes and cytoplasm's in 100% of cases.
- Positive in exocervix Squamous epithelium membranes in 100% of cases.
- Positive in membranes and cytoplasm's of Endocervical cells (87%), reserve cells (100%) and Meta plastic cells (75%).

Table 2: Expression of pEGFR, PI3K and pAKT in cervix uteri.

	pEGFR		pI3K		pAKT	
	Neg	Pos	Neg	Pos	Neg	Pos
LSIL	10	8	15	3	15	3
HSIL	6	0	5	1	5	1
Squamous carcinoma	38	16	46	8	46	8
AIS	11	3	9	5	9	5
Adenocarcinoma	13	17	16	14	18	12

Neg: Negative; Pos: Positive

- There was no positivity in stroma or in nuclei of any cell type (Table 1).

EGFR/PI3K/pAKT pathway

- 15% of squamous carcinomas, 40% of Adenocarcinoma and 21% of AIS showed activation of this route.
- 7% of Adenocarcinoma, 17% of HSIL and 21% of AIS showed absence of EGFR activation but high expression of PI3K/AKT.
- Cells from exocervix Squamous epithelium weakly expressed membranous EGRF.
- LSILs showed expression of Cytoplasmic and membranous EGFR in 44% of cases, accompanied by PI3K and pAKT in 17% of them (Table 2).

Discussion

The PI3K/pAKT/mTOR signaling pathway plays a critical role in many human cancers. HPV infection accompanied by E6/E7 expression alters multiple cellular and molecular events to drive cervical carcinogenesis.

Our results showed that in 15% of squamous carcinomas, 40% of Adenocarcinoma and 21% of AIS, the PI3K/AKT/mTOR pathway was activated (Figure 1-3).

High expression of pI3k/Akt was detected in 7% of Adenocarcinoma, 17% of HSIL and 21% of AIS, without activation of ERGR. These cases were interpreted as probable mutations of the *PIK3CA* gene, which codes for pI3K, since this mutation is predominant in cervical cancer [19]. In total, 20 of the 84 invasive pathologies studied (Squamous carcinoma and Adenocarcinoma) presented activation of the EGFR/PI3K/pAKT/mTOR pathway (24%).

mTOR is activated in at least 60% of HPV-related cancers

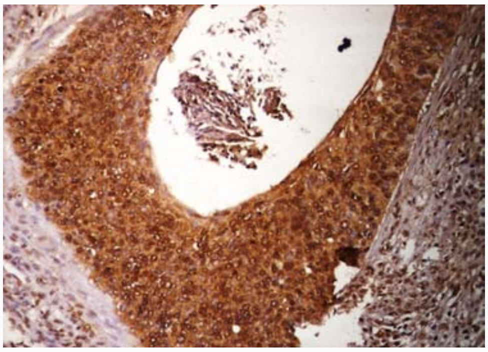

Figure 1: Expression of pEGFR in squamous carcinoma of uterine cervix (Immunohistochemistry with hematoxylin counterstain-400x).

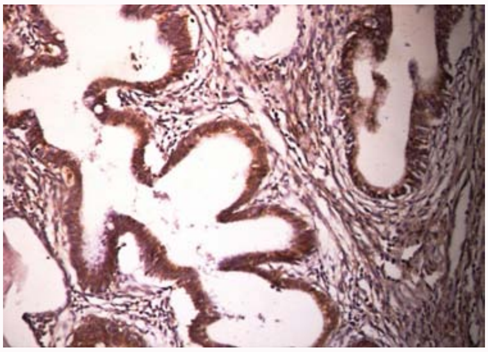

Figure 2: Expression of *pI3K* in adenocarcinoma of uterine cervix. (Immunohistochemistry with hematoxylin counterstain-100x).

(HPV (+) Squamous carcinomas of the head and neck, HPV (+) oropharyngeal cancers, and Squamous of neck cancers), which is consistent with *AKT* activation [33].

mTORC2 regulates the formation of the act in skeleton through PKC phosphorylation [34] and phosphorylates Filamin A, thereby regulating focal adhesion and migration [35]. mTORC2 also activates Rho, Rac and Cdc42, a family of GPTases, which, together with the actin-related protein 2/3 (Arp2/3), are involved in the polymerization of F Actin filaments and the formation of dendritic structure networks in the lamelipodia areas, [36]. The contraction of the actin skeleton, in close contact with beta integrin, makes the cell move in an amoeboidal way [32].

One of the characteristics of cells that undergo the EMT process is the modification of their cytoskeleton, decreasing the expression of cytokeratin and expressing mesenchymal markers such as vimentin and alpha SMA. However, in the case of cervical cancer, the reports on the expression of these markers are confusing.

The increase in vimentin was correlated with migration and invasion of cells from human cervical cancer cell lines (*in vitro*) [37,38]. Yu et al. reported that 75% of squamous carcinomas of the cervix expressed Vimentin, but the mark was detected in stromal cells as well, without specifying what percentage of tumor cells actually expressed vimentin [39]. Other researchers reported that less than 10% of cervical tumor cells expressed vimentin; they attribute to these cells the invasive properties of the tumor [40].

Alpha SMA was reported as one of the EMT markers. However, we have not found works that refer to this marker in epithelial cells in cervical cancer. The few references that relate Alpha SMA to this tumor refer to its expression in fibroblasts, not in cells from the epithelium [41-43].

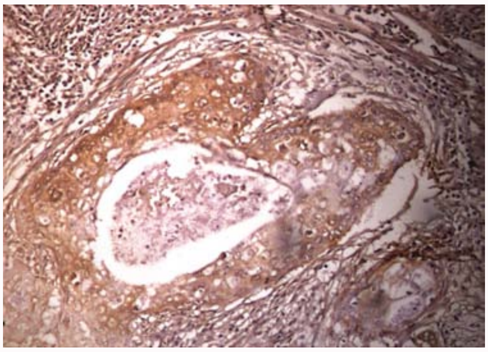

Figure 3: Expression of pAKT in adenocarcinoma of uterine cervix. (Immunohistochemistry with hematoxylin counterstain-400x).

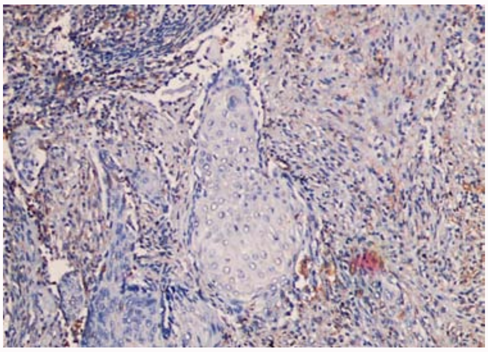

Figure 4: Vimentin negative in squamous carcinoma of uterine cervix (Immunohistochemistry with hematoxylin counterstain-400x).

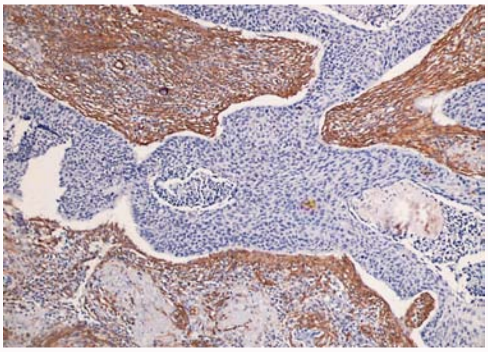

Figure 5: Alpha SMA negative in cancer cells from squamous carcinoma of the uterine cervix, strongly positive in stroma (Immunohistochemistry with hematoxylin counterstain-100x).

As vimentin and Alpha SMA were negative in all our cervical carcinomas analyzed, we believe that in the case of cervical cancers other mechanisms should be involved in the progression of tumors (Figure 4 and 5).

Several lines of evidence suggest that many invasive and metastatic carcinomas have not performed a complete transition to a mesenchymal phenotype, and a complete EMT is observed among cancer cells only under *in vitro* culture conditions [44,45]. For example, transfection into CxWJ cells with HPV 16 E6 and E7 induces the expression of mesenchymal markers such as Alpha-SMA and Vimentin [46].

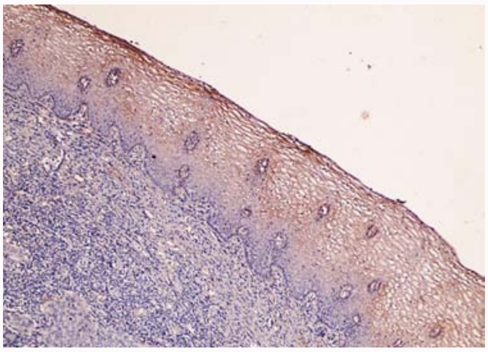

Figure 6: Expression of Beta-Catenin in normal squamous epithelium of the exocervix, showing membranous pattern (Immunohistochemistry with hematoxylin counterstain-100x).

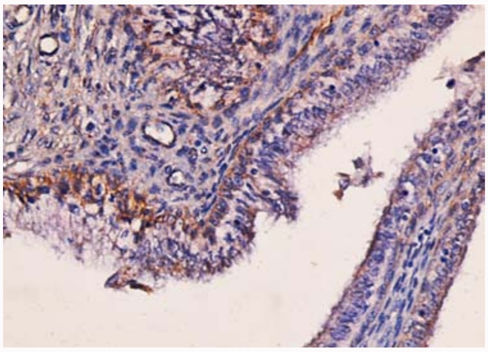

Figure 7: Expression of Beta-Catenin in reserve cells from de endocervix, cytoplasmic pattern (Immunohistochemistry with hematoxylin counterstain-400x).

The Wnt/Beta-Catenin signaling pathway does not appear to be fully activated in our cases, since two important markers (Vimentin and Alpha SMA) were not detected in the surgical specimens. EMT is not the only mechanism for tumor dissemination: In animal and *in vitro* models was reported that podoplanin induces collective cell migration by filopodia formation *via* the down regulation of the activities of small Rho family GTPases. Podoplanin induces an alternative pathway of tumor cell invasion in the absence of a Cadherin switch or epithelial-mesenchymal transition [47].

In our series, Beta-Catenin showed a peripheral (membranous) expression in the cells of the normal Squamous epithelium of exocervix, as it is intimately linked to E- Cadherin at intercellular junctions. Endocervical cells, reserve cells, and metaplastic cells expressed Beta- Catenin in cytoplasm and cell membrane (Figure 6 and 7).

The LSILs tested presented a typical pattern of peripheral beta catenin, and Cytoplasmic expression in the lower third of these epithelia, where the most important histological modifications of these pathologies take place (Figure 8). The appearance of this molecule in the cytoplasms was interpreted as a consequence of the decoupling of the E-Cadherin / Beta-Catenin complex, as a prelude to the invasive process that continues from the SILs. In general, the disappearance of Beta-Catenin from the cell membrane and its increase in the cytoplasm would indicate tumor progression. According to Jiang et al., this process is related to the appearance of EMT in cervical cancer [48].

Squamous carcinoma samples showed Beta-Catenin expression only in cells of differentiated areas with membranous and Cytoplasmic expression in 28% of cases (Figure 9 and 10). Some cases of AIS (36%) (Figure 11) and Adenocarcinoma (11%) (Figure 12) expressed this marker in cytoplasm.

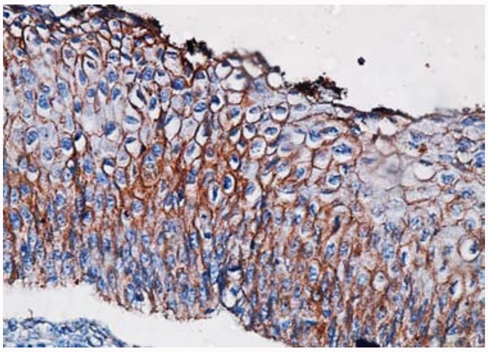

Figure 8: Cytoplasmic/Membranous pattern of Beta-catenin in LSIL). High cytoplasmic expression at the lower third of these epithelium (Immunohistochemistry with hematoxylin counterstain-400x).

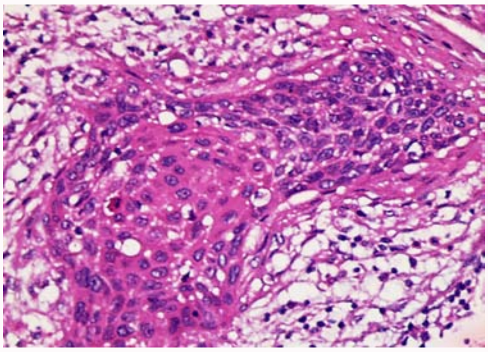

Figure 9: Squamous carcinoma of the uterine cervix, with a differentiated zone (H&E -400x). (Compare with Figure 10).

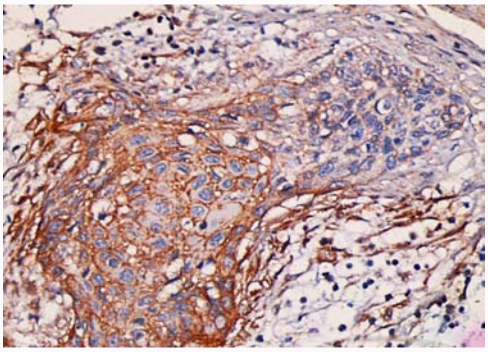

Figure 10: The same zone of the Figure 9 shows expression of Beta-Catenin with a predominantly membranous pattern (Immunohistochemistry with hematoxylin counterstain-400x).

Several investigators reported increased expression of Beta-Catenin in the cytoplasm and/or in cell nuclei in biopsies of pre-invasive and invasive cervical lesions [49,50] and in the cytoplasm of cells in cell cultures (*in vitro*) [51]. Interestingly, a decrease in this epithelial marker was reported in a paper [52].

Pereira-Suárez et al. observed high levels of beta catenin in 9 of the 20 cervical carcinomas analyzed. The expression was membranous, Cytoplasmic, and in some cases nuclear. Importantly,

Figure 11: Expression of Beta-Catenin in AIS of uterine cervix, cytoplasmic pattern (Immunohistochemistry with hematoxylin counterstain-400x).

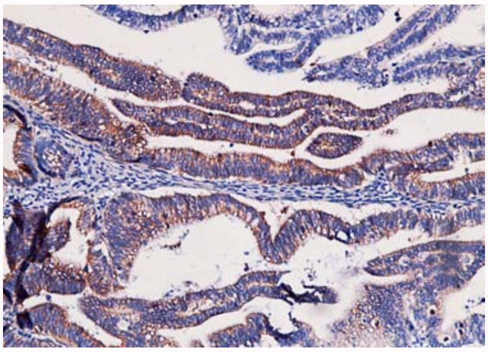

Figure 12: Expression of Beta-Catenin in adenocarcinoma of uterine cervix, cytoplasmic pattern (Immunohistochemistry with hematoxylin counterstain-100x).

PCR-SSCP and sequence analysis showed no mutations in exons 3, 4, and 6 of the Beta-Catenin gene [53], unlike reports on mutations detected in various tumor cell lines isolated from melanoma, colon and breast cancer, as well as in primary tumors: colon, hepatocellular and endometrial carcinomas [54-56]. In the case of Pereira-Suárez's work, the increased expression of Beta-Catenin could be related to the activation of the Wnt/Beta-Catenin pathway, rather than to mutations of this marker.

According to Imura et al., 41% of the cervical cancer samples studied had an altered pattern for Beta-Catenin. The AIS showed peripheral expression and most of the adenocarcinoma were negative for this marker. In this study, however, nuclear translocation was not observed as frequently as in other carcinomas, such as colorectal and hepatocellular carcinomas [57]. Nuclear translocation of this marker was detected in few oropharyngeal cancer cells, in relation to activation of EGFR by E6 and E7 of 16 HPV [58].

None of the samples studied in the present work showed nuclear Beta-Catenin. Thus, the Wnt/Beta-Catenin route would not fully activate. Many advanced carcinomas (esophagus, oral epithelium, lung, and cervix) possess molecular and morphologic characteristics indicative of well-differentiated epithelia, including high levels of E-Cadherin expression, the presence of epithelial junctions, and apical-basolateral plasma membrane asymmetry [59,60]. The sustained synthesis of key epithelial markers has also been shown in invasive colon cancer cells [60], in mammary carcinomas [61], and in a neoplastic epithelial line derived from mammary cells [62].

It is well-known that repression of E-Cadherin is observed in cancer cells at the edges of tumor nests and at the invasion front of multicellular clusters, whereas is almost normally expressed in cancer cells at the center of tumor nests of solid tumors [63]. Beta-Catenin, in 28% of our series, showed a cytoplasmic/membranous pattern at the center of cancer nests, which corresponds to its close union with E-cadherin. Thus, cancer cells at the edges of tumor nests and at the invasion front of multicellular clusters can respond to signals presented in reactive stroma and undergo EMT, possibly leading to collective cancer cell migration [64]. The reactive stroma is composed by Cancer-Associated Fibroblasts (CAFs), myofibroblasts, and Tumor-Associated Macrophages (TAMs), developing in conjunction with cancer, and behaves as a tumor-promoting driver [65]. Other stromal-derived factors, such as FFF, HHH, FGFs, HGF, Alpha-TNF and Beta-TGF induce the EMT of cancer cells [66,67].

Cancer cell with epithelial/mesenchymal phenotype can undergo cell migration in groups, not in the form of isolated cells, through their remaining epithelial character and enhance attachment to the ECM by achieving mesenchymal character. This mechanism can be considered as a partial EMT rather than a complete EMT [68].

The term EMT means that carcinoma cells invariably adopt a mesenchymal phenotype to invade surrounding tissues and metastasize. However, compelling evidence suggests that carcinoma cells may invade or metastasize without losing epithelial morphology or molecular markers, and without inducing expression of mesenchymal genes. Thus, in the case of cervical cancer, we suggest that the appropriate term be "partial EMT" [69,70].

Conclusion

Invasion in cervical cancer does not appear to develop complete EMT. We did not observe the characteristics of the fully activated Wnt/beta-Catenin pathway, since it was not detected Beta-Catenin nuclear translocation and Cytoplasmic expression of mesenchymal markers. The results obtained in cell cultures cannot be extrapolated to cervical cancers *in vivo*, where the transformation processes developed in the epithelial cells are influenced by the signals from the surrounding stroma.

HPV oncogenic proteins (E6/7/5) can activate various stages of the EGFR/PI3K/AKT/mTOR pathway and also destabilize key proteins for maintaining the integrity of intercellular junctions. Both processes could contribute to the development of malignancy, at least in a percentage of cases (24% in our series). mTOR is key in the modification of the cytoskeleton and in the reorganization of the actin filaments, which allow the amoeboid mobility of cells in the invasion process of cervical neoplasia. The use of the term EMT may not always be appropriate for describing the diverse processes associated with tumor spread. Thus, the phenomenon of cervical cancer invasion could be considered as a "partial EMT".

Ethical Aspects

The authors are accountable for all aspects of the work. All samples collected for examination and diagnosis were completely anonymized for use in this study.

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