EXPRESSION OF β -DEFENSIN-1 AND 2 IN HPV-INDUCED EPITHELIAL LESIONS

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ABSTRACT

α , β , θ defensins represent a family of small antimicrobial peptides expressed predominantly by a series of cells, including neutrophils, monocytes-macrophages and epithelial cells that are involved in defense mechanisms against viral infections. β -defensins are the most widespread in this family being encountered in oral, digestive, urogenital mucosa and cutaneous lesions. β -defensins directly inactivate certain viruses, including the human papillomavirus (HPV) suppressing viral replication by altering target cells. Considering these aspects, we investigated the immunohistochemical expression of β -defensin-1 and 2 in HPV-induced epithelial lesions. For this study, tumoral and normal mucosal tissue fragments were collected from 10 patients aged between 31-60 years, with previously confirmed HPV infection, diagnosed clinically and histopathologically with cervical carcinoma. Patients did not receive any chemotherapy or radiotherapy before the biopsy procedure. The tissue fragments were processed by the standard immunohistochemistry technique using anti- β -defensin-1 and 2 antibodies (Bioss Antibodies). The samples examination revealed weak positive(+) membrane, cytoplasmic and nuclear IR for h β D-1 in basal layer of normal cervical mucosa and moderate positive(++) membrane and cytoplasmic IR in squamous epithelium. For dysplastic HPV-associated tissues we highlighted a nuclear moderate positive(++) IR. For h β D-2, IR in basal layer of the normal mucosa was lower(+) compared with dysplastic cells IR and showed a strong expression(+++) at membrane, cytoplasmic and nuclear level in koilocytes of patients with HPV-associated dysplasia. It was also observed a moderate positive(++) IR in basal layer of dysplastic cells of patients without HPV. The obtained results are in agreement with some literature data, which highlighted the fact that h β D-1 and h β D-2 are very important components of the molecular pattern in HPV-induced lesions.

Keywords: defensins, HPV, immunohistochemistry

Introduction

Alfa, beta and theta defensins (α , β , θ) represent a family of small antimicrobial peptides expressed predominantly by a series of cells, including neutrophils, monocytes-macrophages and epithelial cells that are involved in defense mechanisms against viral and bacterial infections. α -defensin-1 to 4 (also known as human neutrophil peptides- HNP) and β -defensin-1 (h β D-1) show a constant expression during bacterial infection.

α -defensin -5 and 6 as well as, h β D-2 to 4 can be induced by proinflammatory cytokine, bacteria and viruses (1,2). Currently it is unanimously accepted that β -defensins are involved in tumor evolution and it has been demonstrated a change of their expression in tumors of the oral cavity, esophagus, thyroid, liver, skin, prostate, cervix, vulvar region and could have an overexpression or not depending on the tissue type (2). It has been demonstrated that there are more than 30 defensin-like sequences, but only the first 6 were included in the β -defensin family.

The role of h β D-1 and h β D-2 in modulating the immune response in oncogenesis, the association with some cells signaling pathways and potential therapeutic applications determined some researchers to study their expression in various tissues. The expression of h β D-1 in epithelial cells is altered in multiple types of neoplasms, for example in patients with oral squamous cell carcinoma, the h β D-1 expression is deficient, which might indicate an increased risk of lymph node metastasis (3,4).

The h β D-2 expression may be induced by proinflammatory cytokines, interleukins and is mediated by the transcription of the kappa-B nuclear factor (NF- κ -B), which is having broad spectrum bactericidal activity (4). Also, there are studies that claim, that one of the most recent discovered β -defensin, h β D-3, is not only involved in antibacterial activities, but also, it may act as a chemoattractant.

β -defensins directly inactivate certain viruses, including the human papillomavirus (HPV), suppressing viral replication by altering target cells (5,6). HPV is commonly associated with certain types of cancer such as cervical, vulvar, vaginal neoplasia or anus, penis or even head and neck region (5).

Currently, more than 205 different HPV genotypes have been detected and classified into five classes, of which Alphapapillomavirus, Betapapillomavirus and Gammapapillomavirus are the largest (7). Alpha class of human papillomavirus have a major clinical importance, affecting particularly the genital mucosa. Low-risk mucosal HPV, such as HPV-6 and HPV-11, causes benign papilloma / condyloma, while high-risk HPV, such as HPV-16 and HPV-18, causes squamous intraepithelial lesions that may progress into squamous cell carcinoma (8).

The urgent need to stop and eradicate these types of cancers associated with HPV infection raises an alarm about awareness of numerous HPV-associated neoplasms to encourage the research and detection of new targeted therapeutic strategies.

The clinical aspects in the genital area are diverse and might develop from simple infections into invasive neoplasia. Thus, there was described warts, veruciform epidermodysplasia, focal epithelial hyperplasia, bowenoid papulosis,

genital cancers (9). The appearance of the lesions also depends on the area which was affected: for example, localization on areas with well-keratinized epithelium may lead to occurrence of keratosis areas; localization on different zone with loose tissues may lead to condyloma acuminata; localization on wet areas (like urethral meatus, vaginal or anal mucosa) could form a soft red condyloma (9,10,11).

Given all these data, as well as, the fact that approximately 20% of malignancies are correlated with inflammatory processes that most often precede and / or accompany tumor development, the molecular components of inflammation are identified in the tumor microclimate and an important proportion is attributed to the presence of HPV, it was considered necessary to start the research with the identification and quantification of some proinflammatory molecules expression, which are part of the response of the cells in the host tissues in which HPV is present.

Considering the fact that are few studies about the implication of beta-defensins in cervical carcinoma, we investigated the immunohistochemical expression of h β D-1 and 2 in dysplastic tissues with and without HPV compared with normal tissues.

Materials and methods

For this study, the patients enrolled were from "Cuza Vodă" Obstetrics and Gynecology Clinical Hospital. We harvested mucosal dysplastic tissue fragments from 10 patients aged between 31-60 years: 2 with previously confirmed HPV type 16 infection (+HPV) and 2 with HPV type 18 (+HPV), diagnosed clinically and histopathologically with cervical carcinoma and other 6 with cervical carcinoma without HPV infection (-HPV). As well as, we harvested normal mucosal tissue fragments from these patients. They did not receive any chemotherapy or radiotherapy before the biopsy procedure. Patients received all the information needed and the informed consent was obtained from each of them. The obtained tissues were harvested through large loop excision of the transformation zone (LLETZ) or conization technique.

For this study, some tissue fragments were processed using the standard staining

with haematoxylin and eosin (H&E) for histopathological examination and others through standard immunohistochemistry technique using anti- β -defensin-1 and 2 antibodies (Bioss Antibodies) and 1:250 Novolink (Leica Biosystems). The samples were examined with an Olympus BX40 microscope with an Olympus E330 camera attached at Cell and Molecular Biology department from Medicine and Pharmacy University "Grigore T.Popa", Iași.

Assessment of β -defensin-1 and -2 immunoreactivity

Immunohistochemical expression of β -defensin-1 and -2 was considered positive after detected immunoreactivity (IR) in more than 10% of tumoral cells, and we used the lymphocytes as a reference of IR intensity as following: strong IR (+++), moderate IR (++) , weak IR (+) , and negative (–) when we had IR less than 10% from tumoral cells.

Results

The tissue fragments stained with H&E revealed on dysplastic cervical tissues associated with HPV type 16 a koilocytic atypia, cells with voluminous nuclei, hyperchromia with nuclear pleomorphism, and polarity loss in 1/3 basal layer. For HPV type 18 we remarked also cell proliferation, polarity loss and structural disruption in 2/3 of exocervical squamous epithelium, nuclear hyperchromia, atypical mitoses in 2/3 basal layer and koilocytic atypia. The cervical dysplasia without HPV presented structural disorganization in 1/3 of exocervical squamous epithelium, mild nuclear atypia in 2/3 of metaplastic squamous epithelium, nuclear hyperchromia and atypical mitoses. These aspects are suggestive for cervical carcinoma previously established clinically.

On the tissues samples processed by immunohistochemical technique we analysed and evaluated the intensity of IR for h β D-1 and 2 at membrane, cytoplasm and nuclear level (Table 1 and Table 2).

Table 1. Expression of β -defensin-1 in normal cells and cervical carcinoma with and without HPV

Tissue		No. of cases	β -defensin -1 expression		
			Membrane	Cytoplasm	Nuclear
Normal tissue	Normal tissue	10	+	+	+
Cervical carcinoma	+ HPV	4	++	++	++
	- HPV	6	++	++	+

Table 2. Expression of β -defensin-2 in normal cells and cervical carcinoma with and without HPV

Tissue		No. of cases	β -defensin -2 expression		
			Membrane	Cytoplasm	Nuclear
Normal tissue	Normal tissue	10	+	+	+
Cervical carcinoma	+ HPV	4	+++	+++	+++
	- HPV	6	++	++	++

The samples examination revealed weak positive (+) membrane, cytoplasmic and nuclear IR for h β D-1 in basal layer of normal cervical mucosa and moderate positive (++) membrane and cytoplasmic IR in squamous epithelium. For dysplastic HPV-associated tissues we highlighted a nuclear moderate positive (++) IR (Figure 1) compared with dysplastic tissues without HPV which has a weak IR (+) (Figure 2).

Figure 1. hβD-1 on HPV-associated dysplasia show nuclear moderate (++) IR

Figure 2. hβD 1 - dysplastic tissue without HPV show weak (+) membrane and cytoplasmic IR

For hβD-2, IR in basal layer of the normal mucosa was lower (+) compared with dysplastic cell's IR which showed a strong expression (+++) at membrane, cytoplasmic and nuclear level in koilocytes of patients with HPV-associated dysplasia (Figure 3). We identified a moderate IR (++) in squamous epithelium and also strong positive IR (+++) in cytoplasm of inflammatory cells. It was observed a moderate positive (++) IR in basal layer of dysplastic cells of patients without HPV (Figure 4). In HPV-associated dysplastic tissue the overexpression of hβD- 2 in

basal layer may be considered a marker which can indicate a higher risk for malignity.

Figure 3. hβD 2 on HPV-associated dysplasia reveal strong expression (+++) at membrane, cytoplasmic and nuclear level

Figure 4. hβD 2- dysplastic tissue without HPV show a moderate (++) IR in basal layer

Discussions

This research continues our previous studies of the hβD -1 and - 2 involvement in carcinogenesis of female reproductive system. Because there are few studies upon this subject, our purpose was to evaluate the immunoreactivity of hβD-1 and 2 on dysplastic tissues and to clarify its possible function of this disease pathogenesis.

The obtained results which indicated the

h β D-2 overexpression in the lesion areas, confirm the correlation that exists between this intense IR and the evolution to squamous cell carcinoma, probably by presence of inflammation and changes in tissue microenvironment. It was also observed that the IR is higher in the dysplastic tissues than in the normal ones.

Similar to our findings, Erheart et al. (12) confirmed that the expression of h β D-2 is very strong (+++) in HPV-associated vulvovaginal lesions compared with normal tissues and they highlighted the importance of β -defensin in modulation of immune response of HPV-associated genital neoplasia.

Another finding of our results is that the inflammation observed on several lesions, which were associated with the presence of HPV, it induced an increased expression of h β D-2 on the examined tissues. Patients with an inflammatory infiltrate on squamous epithelial lesions, developed an increased expression of beta-defensin -1 and 2 even in the absence of HPV.

In some lesions the positive IR appeared in the basal layers, the place affected especially by HPV.

According to another meta-analysis, Markeeva N et al. reported a weak positive (+) expression of h β D-2 in cytoplasm of cells from basal layer of normal epithelium comparative with tumoral cells where IR for h β D-2 was stronger (+++) in all layers.

Our results showed an intense expression of h β D- 1 in the membrane and cytoplasm of cells from basal layer of patients with HPV 16 and 18 type, which suggest an inhibitory action of this molecule on transduction of these HPV types (4).

The results of this study are in concordance with Erheart et al. and Chong et al. (5) analysis which reported in HPV-associated lesions of respiratory tract an upregulation of h β D-1 by interferon-gamma (IFN γ) concluding that β -defensins plays an essential role in defending against HPV, so that could have a therapeutic benefit.

In another study, Hubert et al. (13) found that h β D-2 chemoattracts dendritic cells in high-risk HPV types-associated cervical cancer and link both adaptive and innate immune system targeted against HPV infection.

Positive IR in inflammatory cells corroborated with literature data confirm that h β D-1 and 2 expression increase in cytoplasm of leukocytes and could be considered a marker for malignant transformation and proliferation.

Conclusions

The obtained results are in agreement with some data from literature, which highlight the fact that h β D-1 and h β D-2 are very important components of the molecular pattern in HPV-induced lesions.

The presence of β -defensins suggests that they may serve as signaling molecules that facilitate the generation of adaptive immune responses against viral infections with papillomaviruses that cause minor inflammatory events. This observation is relevant to the development of a potential vaccine and could provide a method for developing defensin-based therapy in patients with HPV, using exogenous defensin preparations or by increasing the production of defensins on target epithelial surfaces.

Further immunohistochemical study for these molecules, as well as for other types of defensins on this tissue, or other types of mucosa with and without HPV, doubled by a statistical analysis of the data will bring valuable information regarding the involvement of HPV in the appearance and evolution of epithelial lesions.

Considering all these results is important to continue the research to clarify if the β -defensins expression is HPV -induced or is just a response of infection.

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