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Brief Communication

Alteration of Na/H exchange regulatory factor-1 protein levels in anogenital lesions positive for mucosal high-risk human papillomavirus type 16

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ABSTRACT

Mucosal high-risk (HR) human papillomaviruses (HPV) are associated with anogenital carcinogenesis. The products of two early genes, E6 and E7, act as major viral oncoproteins. Functional studies in experimental models showed that HPV16 E6 induces degradation of the PDZ protein, the Na+/H+ exchanger regulatory factor-1 (NHERF-1). Here, we determined NHERF-1 protein levels by immunohistochemistry (IHC) in (i) benign anogenital warts (n = 8) (ii) premalignant lesions (L-SIL and H-SIL) (n = 43) and (iii) invasive cervical squamous cell carcinomas (SCC) (n = 17). A decrease of NHERF-1 protein level was not observed in genital warts in comparison to healthy epithelium. Conversely, a clearly decrease in NHERF-1 protein levels was observed in HPV16-positive pre-malignant and malignant lesions, while the phenomenon was much attenuated in lesions induced by other HR HPV types. In conclusion, these findings show that mucosal HPV types differently impact on NHERF-1 protein level in benign and malignant anogenital lesions.

1. Introduction

Cervical cancer has been clearly associated with persistent infection of some mucosal human papillomavirus (HPV) types, namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. They are referred to as high-risk (HR) HPV types and are classified by the IARC as group 1, carcinogens to humans (IARC WGotEoCRtH, 2012). In addition, mucosal HPV6 and HPV11 have been classified as low-risk (LR) types that are mainly associated with benign lesions of the anogenital tract (IARC WGotEoCRtH, 2012). Regarding the HR HPV types, they clearly show different oncogenicity, being HPV16 and HPV18 responsible approximately of 50% and 20% of all worldwide cervical cancers, respectively (Serrano et al., 2018). However, the reason for this difference in the oncogenic potential of the different HR HPV is still unclear. A large number of independent studies have conclusively proven that the product of the early genes, E6 and E7, plays a key role in cancer development (Tommasino, 2014). Both viral oncoproteins lack any enzymatic activity and exert their oncogenic functions by interacting with a large number of cellular proteins, including key tumor suppressor proteins, such as p53 and pRb (Tommasino, 2014). Such virus-host interactions lead to alterations of cellular protein properties, increasing the susceptibility of infected cells to progress towards malignancy. HR HPV E6 oncoproteins are able to bind several cellular proteins containing the PSD-90/Dlg/ZO-1 homology (PDZ) domain, via a 4-amino--acid motif located at the C-terminus, referred to as the PDZ-binding motif (PBM) (Ganti et al., 2015). A comparative study showed that E6 PBMs from HR HPV types and some non-cancer-associated HPV types can differ substantially in their ability to interact with PDZ proteins (Thomas et al., 2016). In agreement with these findings, it was previously shown that HPV16 E6 is more efficient than HPV18 in degrading the Na+/H+ exchanger regulatory factor-1 (NHERF-1), a multi-domain scaffolding protein, which has two PDZ domains in the N-terminal

Abbreviations: low-risk or high-risk human papillomavirus, (LR or HR HPV); Na^+/H^+ exchange regulatory factor-1, (NHERF-1); PSD-90/Dlg/ZO-1, (PDZ); formalin-fixed paraffin-embedded, (FFPE); low or high grade squamous intraepithelial lesion, (L-SIL or H-SIL); Cervical cancer, (CC); MFI, (Median Fluorescence Intensity); Immune-reactive Score, (IRS); squamous cell carcinomas, (SCC).

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region and which regulates the trafficking and signalling of several G protein-coupled receptors (Accardi et al., 2011; Wang et al., 2019). However, another study showed that E6 from LR and HR HPV types as well as from non-primate mammalian papillomaviruses retains the ability to degrade NHERF-1 (Drews et al., 2019). Thus, it is still unclear whether the efficiency in targeting NHERF-1 by E6 is shared among all HPV types or correlates with the HPV oncogenic potential.

To corroborate the data obtained in experimental models on NHERF-1 and HR HPV interaction, we evaluated NHERF-1 protein levels in different types of genital specimens collected from women with benign or malignant genital lesions.

2. Material and methods

Eight specimens collected from women with anogenital warts, 43 with premalignant cervical lesions (L-SIL and H-SIL), and 17 with a diagnosis of invasive cervical squamous cell carcinomas (SCC), were consecutively retrieved from the archive of the IRCCS-Istituto Tumori "Giovanni Paolo II" of Bari during the period January 2016–December 2020. After DNA extraction from the formalin fixed paraffin embedded (FFPE) tissues (Gheit et al., 2014), HPV genotyping was performed by type-specific E7-MPG assay using specific primers targeting the E7 region of 2 LR HPV types (HPV6 and 11) and 19 HR HPVs (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82) as well as primers for the amplification of a β-globin (Schmitt et al., 2010).

For the NHERF-1 immunostaining, 3 μm sections slides were processed and stained by the indirect immunoperoxidase method using the BenchMark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA). The slides were incubated at 37 °C with anti-NHERF-1 antibody (rabbit polyclonal NHERF-1 antibody ThermoFisher Scientific, Rockford, IL, USA) using 1:250 dilution. The UltraView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA) was used to detect protein expression. Finally, tissues were counterstained with hematoxylin and a bluing reagent for 8 min and 4 min, respectively. Positive and negative controls were included in each staining run. NHERF-1 antibody has been validated, and the procedures standardized for IHC. The omission of the primary antibody was used as negative control. Three distinct visual fields (400x magnification) were selected to evaluate for histology and NHERF-1 staining. Discordant scores were reviewed and resolved by discussion. NHERF-1 was scored according to the signal intensity and distribution. An Immuno-reactive Score (IRS) was calculated multiplying the staining intensity (Intensity Score: IS) and the proportion of stained cells in percentage (Staining Proportional Score: PS). The immunostaining was assessed as follows: IS was graded on four-point scale: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The PS was graded on four-point scale as 1 (1%–25% stained cells), 2 (26%–50% stained cells), 3 (51%–75% stained cells) and 4 (76%–100% stained cells) (Meyerholz and Beck, 2018; Specht et al., 2015; Su et al., 2019). An IRS≥3 was considered positive. Three samples were considered unsuitable for IHC in the pre-analytic phase. The $p16^{INK4}$ expression in FFPE sections was evaluated by IHC using the CINtec p16 Histology kit (Ventana-Roche) together with the Dako Omnis (Agilent) automatized stainer, following the manufacturer's instructions. A $p16^{INK4a}$ CINtec positive status was defined by as diffuse, continuous staining in basal and parabasal cells of the cervical epithelium, as indicated in the manufacturer's instructions. While a focal or no staining for $p16^{INK4a}$ was considered negative. All stained specimens were blinded to clinic pathological data and independently assessed by two researchers, including one pathologist. Stained sections were analyzed by a bright field microscope (Zeiss, Oberkochen, Germany).

For the expression analysis of NHERF-1, two-tailed non-parametric Kruskal–Wallis and Mann–Whitney U-tests were used. Statistical significance and 95% confidence interval were calculated, a $p < 0.05$ was considered statistically significant. Statistical analyses were performed using the prism version 5.00 software package (Graph- Pad Software, San Diego, CA, USA).

Table 1
Summary of HPV status and NHERF-1 expression level in specimens collected from women with pre-malignant cervical lesions, SCC and Condyloma.

Type of Lesion		HPV Status/ Type (n =)	Total NHERF-1 expression	HPVStatus/ NHERF-1 expression level
Pre-malignant cervical lesions (n = 42) (L-SIL and H-SIL)	Single infection	HPV16 (21) HPV31 (6) HPV58 (3) HPV52 (2) HPV51 (1) HPV66 (1)	Positive expression (≥3 IRS): 26 Negative expression (<3 IRS): 13 Not evaluable: 3	HPV16+/ NHERF-1+: 9 HPV16+/ NHERF-1-: 11 HPV31+/ NHERF-1+: 6 HPV58+/ NHERF-1+: 2 HPV52+/ NHERF-1+: 2 HPV51+/ NHERF-1+: 1
	Multiple infection	HPV16/31/ 52 (1) HPV31/51 (1) HPV16/35 (1) HPV16/39/ 66 (1)		HPV16/31/52/ NHERF-1-: 1 HPV31/51/ NHERF-1+: 1 HPV16/35/ NHERF-1-: 1 HPV16/39/66/ NHERF-1+: 1
	HPV-negative infection	No HPVs-tested infection (4)		No HPVs-tested infection/ NHERF-1+: 4
SCC (n = 17)	Single infection	HPV16 (10) HPV33 (1)	Positive expression (≥3 IRS): 12 Negative expression (<3 IRS): 5	HPV16+/ NHERF-1+: 6 HPV16+/ NHERF-1-: 4 HPV33+/ NHERF-1+:1
	Multiple infection	HPV16/26 (1) HPV16/18 (1)		HPV16/26/ NHERF-1-:1 HPV16/18/ NHERF-1+:1
	HPV-negative infection	No HPVs-tested infection (4)		No HPVs-tested infection/ NHERF-1+:4
Condyloma (n = 8)	Single infection	HPV6 (2) ()	Positive expression (≥3 IRS): 8 Negative expression (<3 IRS): 0	All samples were positive
	Multiple infection	HPV6/16 (2) HPV11/16 (1) HPV6/45/ 53/59 (1) HPV6/16/ 53 (1)		
	HPV-negative infection	No HPVs-tested infection (1)		

Human papillomavirus (HPV); Na+/H+ exchanger regulatory factor-1 (NHERF-1); NHERF-1+: NHERF-1 positive; NHERF-1-: negative; Invasive cervical squamous cell carcinomas (SCC). Not evaluable.

3. Results

To evaluate the impact of mucosal HR HPV16 type on NHERF-1 protein levels, we analyzed specimens collected from women with different anogenital lesions by IHC using a specific NHERF-1 antibody. Extracted DNA from anogenital warts (n = 8), pre-malignant cervical lesions (n = 43) and cervical SCC (n = 17) samples were genotyped using the E7-MPG assay (Schmitt et al., 2010). One FFPE tissue specimen resulted β-globin negative and was excluded from further analyses (Table S1).

Genital warts resulted positive for HPV6 alone (n = 2) or in coinfection with other HPV types (n = 4), while HPV11 (n = 1) in coinfection with other HPV types, and one was negative for any tested HPV type (Table 1). In pre-malignant cervical lesions, the majority of single HPV infections were related to HPV16 (n = 21), followed by HPV31 (n = 6),

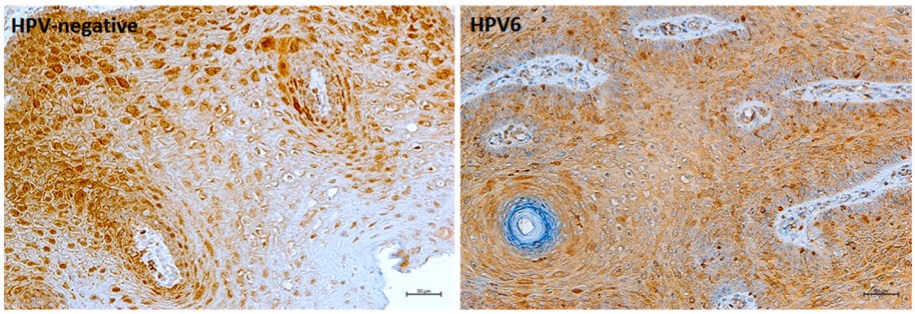

Fig. 1. Representative NHERF-1 immunohistochemical staining of HPV-negative and HPV6-positive Condylomas (Original magnification x200). Images were obtained on an Axion Image 2 upright microscope (Zeiss, Oberkochen, Germany) with an Axiocam 512 color camera.

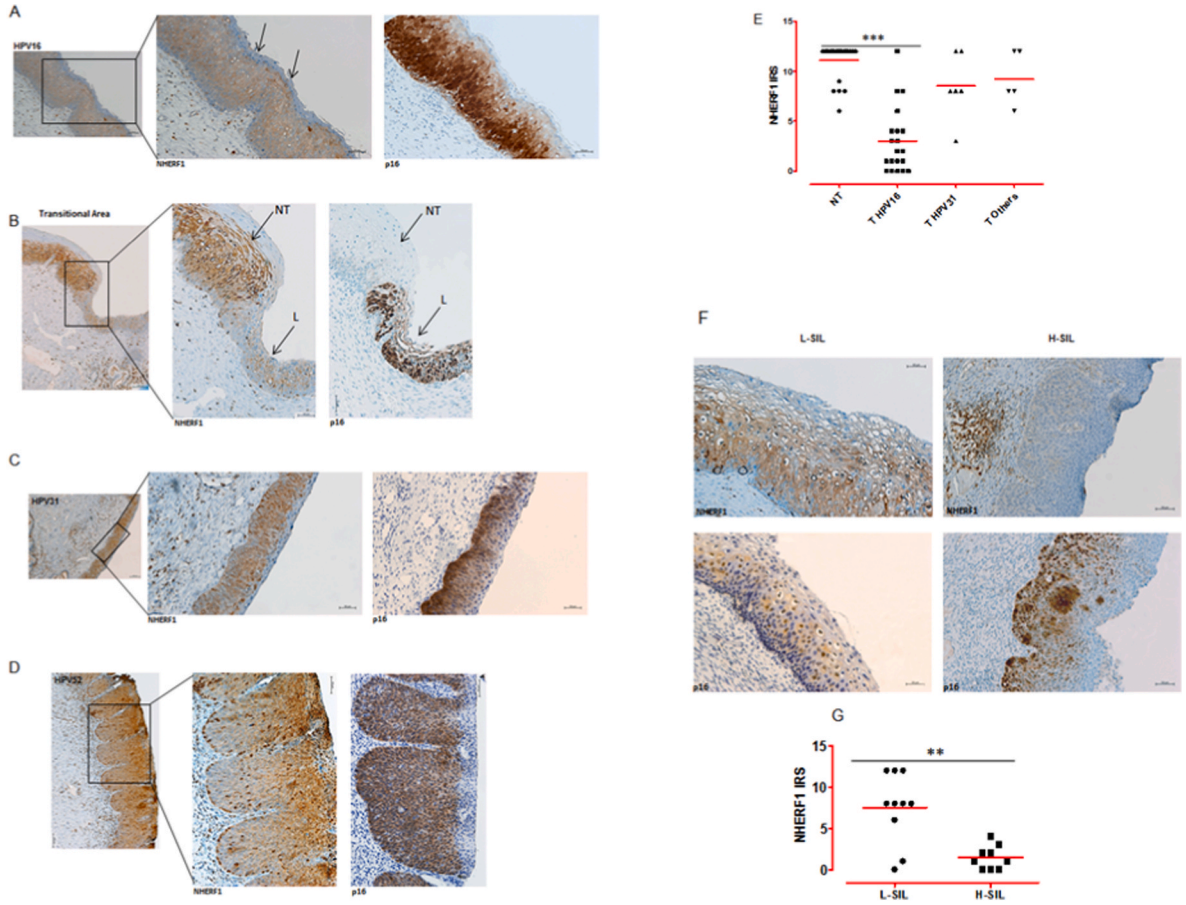

Fig. 2. NHERF-1 and p16^{INKa} expression levels in HR-HPV pre-malignant lesions. NHERF-1 protein levels in L-SIL or H-SIL was determined by IHC using an anti NHERF-1 antibody (ThermoFisher Scientific, dilution 1:250). Original magnification of images on the left × 200, enlargement on the right × 400. (A) Representative NHERF-1 IHC staining of HPV16-positive H-SIL. Arrows indicate the dysplastic regions (right panel). (B) NHERF-1 expression is significantly down-regulated in the dysplastic tissue in comparison to the normal epithelium. Arrows indicate the normal tissue on the left and the dysplastic regions on the right. (C) Representative NHERF-1 IHC staining of HPV31-positive H-SIL. (D) Representative NHERF-1 IHC staining of HPV52 H-SIL. (E) Overall quantification of NHERF-1(+) cells on Non Tumoral area (NT), and HPV16- positive, HPV31-positive and other HR-HPV-positive pre-malignant lesions (T). IRS: immune-reactive score. (Dot indicates outlier; Horizontal red line = median value; ***P < 0.0001). (F) Representative NHERF-1 IHC staining of HPV16-positive L-SIL and H-SIL (Original magnification x200). (G) Quantification of NHERF-1(+) cells on HPV16-positive L-SIL and H-SIL lesions. IRS: immunoreactive score. (Dot indicates outlier; Horizontal red line = median value; **P < 0.01). Images were obtained on an Axion Image 2 upright microscope (Zeiss, Oberkochen, Germany) with an Axiocam 512 color camera.

HPV58 (n = 3), HPV52 (n = 2), HPV51 (n = 1), and HPV66 (n = 1) (Table 1). A few multiple viral infections were also detected, namely HPV16/31/52 (n = 1), HPV31/51 (n = 1), HPV16/35 (n = 1) and HPV16/39/66 (n = 1). Four evaluable samples resulted negative for any tested HPV type by E7-MPG.

Re-evaluation of the histology revealed that majority of the pre-malignant lesions were categorized in either low grade squamous intraepithelial lesion (L-SIL) (n = 2) or high grade SIL (H-SIL) (n = 28), and 12 specimens showed the concomitant presence of both L-SIL and H-SIL lesions.

NHERF-1 expression by IHC was determined in L-SIL positive for HPV16 (n = 10) and HPV31 (n = 1) and in H-SIL positive for HPV16 (n = 20), and other additional HR types, namely HPV31 (n = 6), for HPV51 (n = 1), HPV52 (n = 2) and HPV58 (n = 2). Among the 17 SCC, 10 were positive for HPV16, 1 for HPV33, 1 for HPV16/26 and 1 for HPV16/18. Four cervical SCC resulted negative for any HPV tested type.

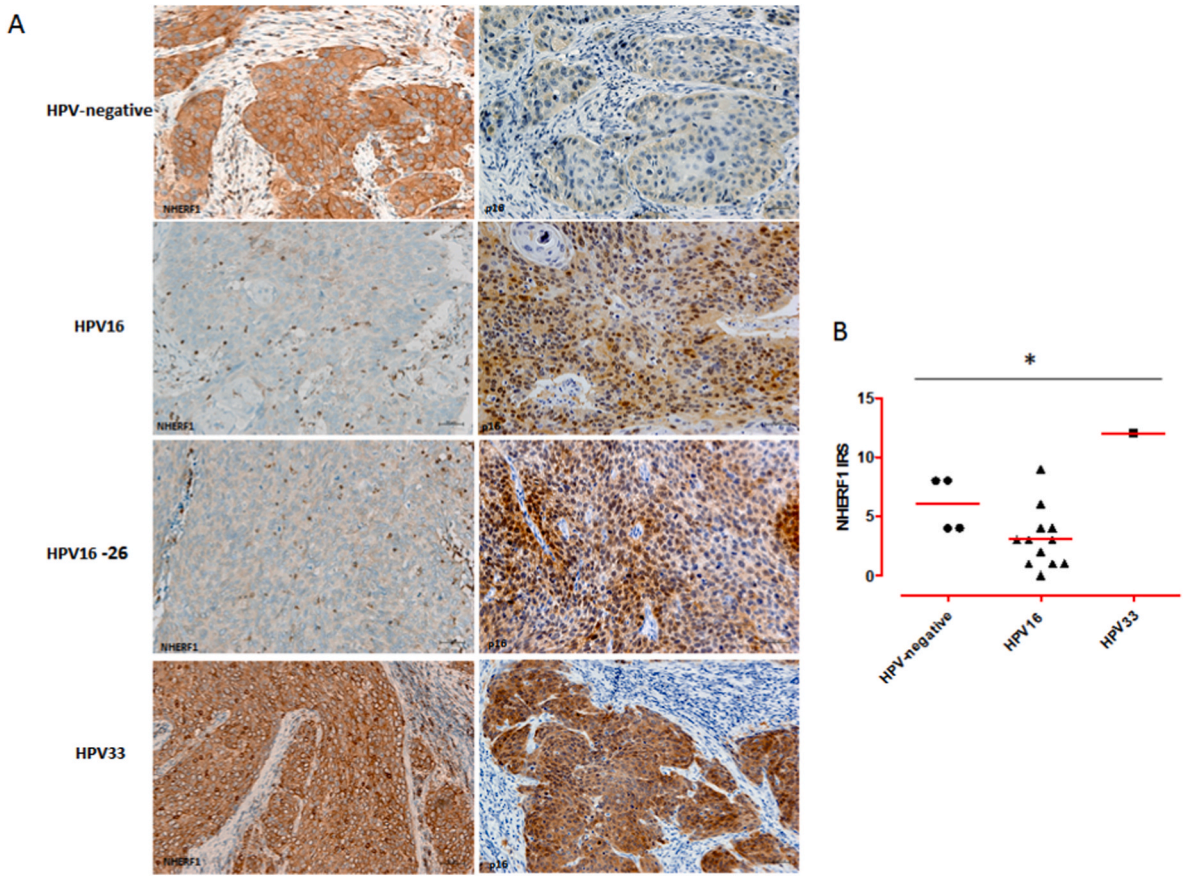

Fig. 3. NHERF-1 and p16^{INKa} expression levels in HR-HPV invasive cervical squamous cell carcinomas (SCC). NHERF-1 protein levels was determined by IHC using an anti NHERF-1 antibody (ThermoFisher Scientific, dilution 1:250), (Original magnification of images × 200). (A) Representative NHERF-1 IHC staining of HPV-negative lesion, HPV16, HPV33 and multiple infection HPV16/26. NHERF-1 expression is significantly down-regulated in HPV16-positive SCC. (B) Quantification of NHERF-1(+) cells on HPV16-positive (n = 12), HPV33-positive (n = 1) and HPV-negative lesions (n = 4). IRS: immunoreactive score. (Dot indicates outlier; Horizontal red line = median value; *P < 0.05).

In HPV6-positive genital warts specimens, no decrease of NHERF-1 protein levels was observed in comparison to tissue surrounding the lesion (Fig. 1).

In HPV16-positive L-SIL, NHERF-1 protein levels were comparable to the ones detected in adjacent normal epithelium. By contrast, NHERF-1 protein levels were decreased in HPV16-positive H-SIL lesion in comparison to the normal epithelium surrounding the lesion (Fig. 2A and B). Decreased protein levels of NHERF-1 was less evident in HR types 31, 52 and 58-positive H-SIL in comparison to HPV16-positive H-SIL (Fig. 2A–D, and data not shown). Although HPV31 is closely phylogenetically related to HPV16, belonging to the same alpha-9 species, it appears that its efficiency in targeting NHERF-1 significantly differs from HPV16 type (Fig. 2 E), suggesting the concept that an efficient NHERF-1 degradation is a specific property of HPV16 (Accardi et al., 2011; Wang et al., 2019) (Fig. 2A and C and E). However, this finding needs to be corroborated in a large cohort of samples. To confirm that loss of NHERF-1 occurs in cells expressing viral proteins, we have determined the accumulation of p16^{INK4a} by IHC in all specimans included in the study. The results show that only in HPV16-positive HSIL or cervical SCC the expression of NHERF-1 and p16^{INKa} are mutually exclusive (Figs. 2 and 3).

NHERF-1 level correlates with the severity of HPV16-positive cervical lesion (Fig. 2F and G), being lower in H-SIL than L-SIL. A possible explanation of these findings is that the levels of NHERF-1 degradation correlate with HPV E6 expression, which is known to increase in H-SIL versus L-SIL.

In cervical SCC, NHERF-1 protein levels were decreased in HPV16-positive in comparison to the tissue surrounding the lesion (Fig. 3A). Multiple infections with HPV16 also showed a decreased expression of NHERF-1 (Fig. 3 A). Quantification of the NHERF-1 signal in SCC confirmed that HPV16 appears to be more efficient in decreasing its intracellular protein levels (Fig. 3B).

4. Discussions

It has been well demonstrated that the E6 oncoprotein from mucosal HR HPV types, via the PDZ-binding motif, are able to alter the regulation of cell polarity and cell proliferation by targeting certain MAGUK family members (Thomas et al., 2008). In contrast to the HR HPV types, E6 from the LR HPV types lacks the PDZ-binding motif and is not able to target the MAGUK family members. Previous independent studies have shown that HPV16 E6 interacts with a PDZ cellular protein NHERF-1 via the E6 PDZ binding motif, leading to degradation of the cellular protein (6). Interestingly, the same study shown that the HR HPV18 E6 was not able to target NHERF-1, despite the presence of the PDZ-binding motif, providing a possible explanation for the different oncogenicity of HPV16 versus HPV18 (Accardi et al., 2011). This functional difference between HPV16 and HPV18 E6 has also been described in another independent study (Wang et al., 2019). In line with these findings, it has been shown that small differences in the PDZ binding motif of E6 proteins from different HR HPV types confer the ability to bind different subgroups of PDZ proteins (Thomas et al., 2016). In contrast, another study reported that HPV16 and HPV18 E6 are able to induce NHERF-1 degradation with similar efficiency (Drews et al., 2019). In addition, the same study showed that also E6 from the low-risk HPV type as well as from non-primate mammalian PVs display the same ability to target NHERF-1

(Drews et al., 2019). Here, the analysis of cervical lesions shows the decrease of NHERF-1 protein levels in presence of HPV16 infection. Although, we have analyzed a limited number of genital lesions positive for LR or HR HPV types other than HPV16, the data indicate that NHERF-1 protein levels are not altered in presence of several HPV types. Thus, the IHC staining support the initial observation that some mucosal HR HPV types do not share the same capacity of HPV16 to decrease NHERF-1 intracellular levels. Alternatively, these findings could be due to specific genetic features of the host but the fact the there is a tight correlation between HPV16 infection and low NHERF-1 protein levels makes this hypothesis unlikely. The limitation of the study is that the analyzed cervical lesions were positive only for a limited number of HPV types (i.e. HPV52, HPV51, HPV66, HPV33) and that we focused on HPV16. In addition, due to the retrospective design of the study we did not include detailed clinical information. However, according to our knowledge, our study includes the largest number of *ex-vivo* genital specimens analyzed for NHERF-1 protein levels and different mucosal HPV types. In future studies, it will be important to compare the ability among all 12 HR HPV types to promote NHERF-1 degradation.

Disclaimer

Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization and the Istituto Tumori Giovanni Paolo II, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy, or views of the two Institutes.

Ethics approval

This study was approved by the Ethic Committee of Istituto Tumori "Giovanni Paolo II" with the reference number 1066/CE 15-03-2022.

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CRediT authorship contribution statement

Concetta Saponaro: Data curation, Formal analysis, Investigation, Writing – original draft. **Luisa Galati:** Formal analysis, Investigation, Writing – original draft. **Tarik Gheit:** Formal analysis, Investigation. **Susanna Anita Pappagallo:** Investigation, Methodology. **Milena Zambetti:** Methodology. **Francesco Alfredo Zito:** Data curation, Supervision. **Rosa Angela Cardone:** Conceptualization. **Stephan Joel :** Conceptualization, Supervision. **Massimo Tommasino:** Writing – original draft, Supervision, Final approval of the manuscript: all authors.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2022.09.004.

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