Chapter

Role of Extracellular Vesicles in Human Papillomavirus-Induced Tumorigenesis

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Abstract

Emerging evidence demonstrates a role of extracellular vesicles (EVs) in a variety of fundamental physiological and pathological processes ranging from antigen presentation to T cell to neurodegenerative diseases. In several types of malignancies, a variety of EVs can be isolated from bodily fluids of cancer patients, and it has been reported that the number of circulating EVs seems to be higher than in healthy subjects. This increase correlates with poor prognosis. Data obtained from different groups clearly point out a role of EVs in the transfer of bioactive molecules such as microRNAs and viral oncoproteins in human papillomavirus-induced malignancies of genital and oral tracts. This study summarizes these data in the context of relevant literature considering the EVs as carriers of oncogenic signatures in human cancer as well as their therapeutic potential in HPV-related tumors.

Keywords: extracellular vesicles, exosomes, microvesicles, human papillomavirus, tumorigenesis

1. Introduction

The extracellular vesicles (EVs) are nano- to micro-sized, cell-derived structures delimited by a double-layer lipid membrane. Even if the first reports describing the existence of "particles" derived from platelets with procoagulant effects and the presence in serum of the so-called platelets dust date back to 1946 and 1967, respectively [1], exosomes have been considered for a long time just as cell "rubbish" materials. Spotlight on EV functions have been turned on in the early 1980s when two different groups convincingly demonstrated the role of EVs in the transfer and recycling of transferrin receptor in reticulocytes [2, 3]. Since then, a clear role in several physiological and pathological processes has been ascribed to EVs. For instance, B lymphocyte-derived EVs present antigens and induce antigen-specific response in T cells, suggesting a role in adaptive immune responses [4]. EVs have also been implicated in almost all the neurodegenerative diseases through the spread of aberrant pathogenic peptides/proteins. This is the case of β-amyloid peptides and Tau protein in Alzheimer's disease [5, 6], prion proteins in Creutzfeldt-Jakob disease [7], αB-crystallin proteins in both Alzheimer's disease and multiple sclerosis [8], mutant

superoxide dismutase 1 and transactive response DNA-binding Protein 43 (TDP-43) in amyotrophic lateral sclerosis [9, 10], and α-synuclein in Parkinson's disease [11]. Furthermore, a variety of EVs can be isolated from bodily fluids of cancer patients in several types of hematological and non-hematological malignancies, and it has been reported that in these patients, the number of circulating EVs seems to be higher than in healthy subjects and correlates with poor prognosis [12].

Human papillomaviruses (HPVs) are responsible for around 33% of all human cancers related with infections. To date, more than 200 types of HPVs are classified into three main genera (α, β, and γ genus) and several species in the HPV phylogenetic tree. High-risk (HR) HPVs are grouped into a subgroup within the α genus. HR-HPVs are the etiological agents of cervical carcinomas and anal cancers and are involved in other genital tumors as well as in some head and neck tumors [13]. Twelve HPV types (i.e., HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59) were classified as oncogenic by the International Agency for Research on Cancer (IARC), being the HPV16 prevalent in HPV-positive malignancies. Among the HR-HPV-associated tumors, the large majority is represented by cervical carcinomas (i.e., more than 50,000 over 60,000 cases per year, data from GLO-BOCAN 2012; http://globocan.iarc.fr). Of note, the occurrence of HPV-positive oropharyngeal cancers is incrementing in the last decades [14].

Mucosal HR-HPV types belonging to the alpha genus are the only recognized to be related to human carcinogenesis by large epidemiological studies. Similar studies were performed on the involvement of cutaneous beta HPVs in cancer without achieving unequivocal results because these viruses, present in skin cancer cells only in small amount, are also present in the healthy skin. However, many functional studies on the E6 and E7 of several beta HPVs demonstrated their oncogenicity in vivo systems and in transgenic mouse models. These results lead to consider HPV5, HPV8, HPV20, HPV36 (β1 species), HPV22, HPV23, HPV38 (β2), and HPV49 (β3) as high-risk genotypes. In fact, it is well known that these beta HPVs are involved in disseminated infection and in squamous cell carcinoma (SCC) of immunocompromised subjects [15, 16]. However, the mechanism of beta HPV carcinogenesis is different from that of alpha HPVs; in fact, it has been shown that beta HPV types induce tumors in cooperation with other carcinogens such as UV ray and chemicals [17].

2. Extracellular vesicles

Previously defined in several ways as oncosomes, prostasomes, ectosomes, and microparticles, just to mention a few, EVs are currently named basing on both the biogenesis and particles size. According to these criteria, three main classes of EVs are identified: apoptotic bodies (ABs), microvesicles (MVs), and exosomes (Exos) (**Figure 1**). Both ABs and MVs are generated by plasma membrane blebbing; ABs are produced following cellular shrinking and fragmentation upon induction of programmed cell death (PCD) and are the larger vesicles with size ranging between 500 nm and 1 μm. Conversely, MVs are physiologically released during the cellular lifespan and are smaller than ABs (i.e., between 100 and 500 nm up to 1 μm). On the other side, Exos are generated by membrane invagination of intracellular corpuscles defined multivesicular bodies (MVBs) where they are stored as intraluminal vesicles (ILVs). Once MVBs fuse with the plasma membrane, ILVs are secreted in the extracellular space where they are called Exos. Giving the intracellular genesis of these EVs, Exos are the smallest class of vesicles being in size between 50 and 150 nm.

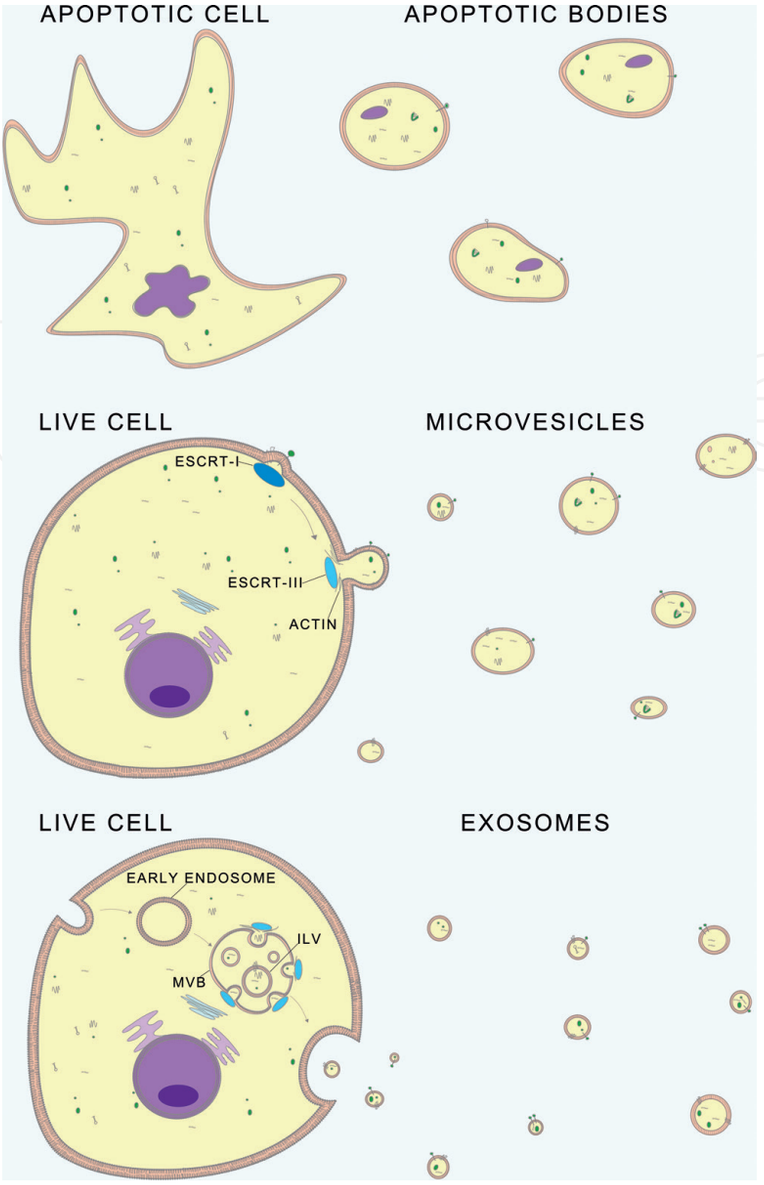

Figure 1.
EV biogenesis. Apoptotic bodies and microvesicles are generated by plasma membrane blebbing. Exosomes are generated by membrane invagination of intracellular corpuscles defined MVBs where they are stored as ILVs. ESCRT, endosomal sorting complexes required for transport; MVBs, multivesicular bodies; ILVs, intraluminal vesicles.

MVs and Exos biogenesis share common elements, particularly the endosomal sorting complex required for transport (ESCRT) proteins. Nevertheless, in the case of Exos, an ESCRT-dependent and ESCRT-independent machinery exists [18].

Depending on their biogenesis routes, the three classes of EVs carry different types of cargos even if all EVs contain lipids, proteins, as well as nucleic acid (i.e., RNA and DNA). Furthermore, specificity of EV cargoes depends on cell type and is associated with the physiological or pathological condition of the producer cell [19]. However, as a general rule, it could be estimated that ABs contain more DNA than MVs and Exos due to their release after PCD induction, whereas MVs are enriched in mRNA surface proteins and, owing to their synthesis, membranes enriched in cholesterol and saturated fatty acid lipids. Among the different classes of EVs, Exos are a very peculiar class because of their size and their intracellular synthesis. These features are of special interest for researchers because they allow the loading of smaller molecules (for instance, microRNAs and other small and long noncoding RNAs rather than mRNAs) and the cargo of proteins able to interact with a

specialized machinery [20]. Nevertheless, at present it is unclear if the elements of the machinery used to sort cargo into Exos are dedicated or rather shared with MVs.

As the three classes of EVs have overlapping size, it is not possible to isolate a single class of EVs in spite of the different methods utilized, which are based on differential centrifugation, density gradients of sucrose or iodixanol, size exclusion chromatography, or ultrafiltration. This is especially true in the case of Exos [21]. In fact, affinity chromatography-based methods that utilize antibodies or molecules able to bind to the phosphatidylserine overexpressed on EV surface [22] are useless as well. Indeed, the possible existence of surface proteins or other molecules specifically expressed by one class of EVs is already matter of debate. The International Society for Extracellular Vesicles has just issued some minimal experimental requirements for definition of extracellular vesicles and their functions, to allow standard assignment of specific biological cargo or functions to a single class of EVs [23, 24].

2.1 Role of EVs in tumorigenesis

EVs serve as shuttle vehicles for intercellular and intratissue communication by transferring a discrete and complex "data packet" consisting of proteins, lipids, and nucleic acids; as a consequence, cancer cells are able to shape tumor microenvironment (TME) through the release not only of soluble factors such as cytokines, chemokines, and growth factors but also of EVs. As previously reported, proliferation, angiogenesis, metastasis, inflammation, limitless replicative potential, resistance to apoptosis, and suppressive signal are considered hallmarks of cancer [25], and a clear role of EVs has been demonstrated in all these processes [26]. Surprisingly enough, this "flow of data" is bidirectional, going not only from transformed to normal cells (i.e., fibroblast, stromal, and immune cells) but also from normal to transformed cells. Exos released from cancer cells, for instance, induce neo-angiogenesis and increase vascular permeability, thereby facilitating metastasis, through the modulation of endothelial cell activities [27]. Fibroblasts are as well a target of tumor-derived EVs that trigger their differentiation into cancer-associated fibroblasts with pro-angiogenic and pro-tumorigenic properties [28]. Several in vitro tumor models demonstrated the ability of tumor cell-derived EVs to transform normal cells. For instance, stromal cells were transformed by Exos derived from colorectal cancer cells [29], adipose stem cells were transformed by EVs derived from prostate cancer cells [30], and nonmalignant fibroblast murine cells isolated from tumor of immunocompromised mice were transformed by breast cancer-derived MVs [31]. Further, Exos isolated from sera of breast cancer patients induce tumorigenicity of nonmalignant human breast cells when injected into immunocompromised mice [32]. However, it is a matter of debate if such a phenomenon occurs also during tumorigenesis. Tumor EV cargo can be represented by mutated oncoproteins [33, 34], mRNA codifying for fusion transcripts [35], oncogenic long noncoding RNAs (lncRNAs) [36], and miRNAs associated with chemotherapy resistance [37].

Conditions associated with tumor growth, like hypoxia, influence both quantitatively and qualitatively the EVs released from cancer cells. When co-injected into SCID mice with human glioblastoma cell line, hypoxic EVs increase both tumor growth and angiogenesis [38], whereas breast cancer cell lines preincubated with hypoxic EVs and injected into mice develop more metastases than the same cells preincubated with normoxic EVs [39].

When cancer cells leave the site of primary growth, they travel through the blood stream acquiring the ability to colonize other sites, thereby generating metastases. It is now evident that these circulating tumor cells (CTCs) are not able to colonize all tissues but only specific sites called pre-metastatic niches, where a favorable TME

has been pre-generated [40]. Due to their peculiar nature, EVs are one of the tools most used by tumors to create pre-metastatic niches. Interestingly, EVs derived from different types of tumor show different tissue tropism. Melanoma-derived EVs target the lung, liver, bone, and brain, whereas colorectal cancer-derived EVs form predominantly liver metastasis. EVs derived from most tumors show a tropism for the bone marrow, thereby generating pre-metastatic bone marrow niches [41, 42]. Although the surface receptors determining the specificity of EVs tropism are unknown to date, the resulting effect is a reprogramming of local target cells that produce soluble factors and extracellular matrix remodeling, necessary for the settlement of CTCs. In other cases, metastasis is dependent not on EVs derived from primary tumor but on EVs released by cells of the target site. For instance, it has been demonstrated that in the brain, but not in other tissues, breast cancer loses tumor suppressor PTEN; this effect is mediated by miR-19a contained in astrocyte-derived Exos [43]. Aging [44] and infection [45] are as well conditions able to create a favorable niche, called in that case active metastatic niche, independent of EVs derived from primary tumor but dependent on EVs produced in loco. This influence of EVs derived from normal cells on transformed ones could even have an opposite effect. Indeed, in some cases, EVs from normal tissues generate an unfavorable field for CTCs, thus inducing the so-called cancer cell dormancy. This is the case of bone marrow mesenchymal stem cell-derived Exos which induce dormancy of breast cancer cells through a miR-23b-mediated mechanism [46].

Another important feature of tumor-derived EVs is their immunomodulatory ability to subvert or evade immune recognition. To accomplish this task, EVs interact with surface immunoreceptors or are internalized within immune cells, thereby hindering activation and polarization into effector or cytotoxic T lymphocytes. Ligands of death receptors, as TNF-R1 and Fas, are engaged on CTL surface by ligands expressed on EVs, leading to the induction of apoptosis in these cells [47, 48]. Tumor-derived EVs also play a role in both T- and myeloid cell differentiation by inducing the generation of regulatory T cells and myeloid-derived suppressor cells, respectively [47, 49]. Finally, NK cells are also targets of tumor-derived EVs [50]. Immunomodulatory activity of tumor-derived EVs on NK cells is also improved by hypoxic conditions through a TGFβ and miR-23a mechanism [51].

3. HPV-induced tumorigenesis

The infection by HPVs that is very frequent in sexually active women can have a driving role in the improvement of tumor injuries of the uterine cervix. The uterine cervical carcinoma (CC) is the third most frequent tumor in women, and the high-risk (HR) HPV is found in about the totality of this tumor [52].

HPV can reach the deep layers of the epithelium through cervical microwounds and enter immature, multiplying cells, where the viral DNA is kept up as an episome and replicates through the host cell genome. As a consequence, the infected immature cervical cells remain in a proliferative state, obstructing their terminal differentiation [53].

During the HPV DNA replication, the HPV early genes, including those coding for the E6 or E7 proteins, are transcribed, but their expression is kept low by the HPV-E2 protein. However, E6 and E7 are produced at levels acceptable to impair the factors that regulate the growth and the differentiation of the host cell. HPV-E7 binds to and inactivates the retinoblastoma tumor suppressor protein (pRb), hampering infected cells to leave the cell cycle and differentiate. Meanwhile, HPV-E6 guides the host cell tumor suppressor protein p53 toward degradation through the proteasome of the cell with the consequence that E6 upregulates the intracellular

levels of the anti-apoptosis Bcl-2 protein, normally blocked by p53, and triggers the activity of telomerase that represses replicative senescence by stabilizing the length of the chromosomes end. Overall, the E6 and E7 activities block the apoptosis due to p53 in the HPV-infected cells, necessary to control cellular proliferation [54].

In around 80% of the cases, HPV-induced cell proliferation stays at subclinical level, developing cervical epithelium thickness or causing benign flat warts. In the remaining cases, HPV-E6 or HPV-E7 stimulates the growth rate of immature cervical basal and parabasal epithelial cells and their transfer to the superficial layer. This prompts the improvement of squamous intraepithelial injuries.

Usually, an immune reaction to HPV happens in few months after infection, with the consequence of viral clearance. From that point forward, the p53 and pRb function is restored in the basal and parabasal layers, and epithelial cell growth and differentiation return to normal. This event usually occurs in infections with low-risk HPV types, e.g., HPV6 or HPV11, whose DNA remains episomal.

On the other hand, persistent infection with HR-HPV, including HPV16 and HPV18, can be followed by the integration of viral DNA into the host cell genome. HPVs are kept up as episomes in precancerous lesions, while in some high-grade lesions, genomes can integrate into the host chromosome [55]. While there is no accord about the exact role of viral integration in HPV-induced cancer progression, it appears that the deregulation of E6 and E7 expression during viral integration contributes to the development of high-grade lesions. In this particular circumstance, cell key mitotic checkpoints are impeded, bringing to genomic instability, accumulation of mutations, and aneuploidy in infected cells. Subsequently, the entire cervical epithelium is replaced by poorly differentiated cells showing anomalous nuclei and atypical mitoses. The E6 and E7 proteins support this process by their ability to induce genetic instability of the host cell DNA and by deregulating cell factors related with epigenetic reprogramming. Recently, a number of studies have enhanced the information of phenotypic effects induced by the HPV-E6 and HPV-E7 oncoproteins. It was demonstrated that oncogenic HPVs contribute to all the main phenotypic changes of cancer cells defined as "hallmarks of cancer" [25], from sustained proliferative signaling, sidestepping growth suppressors, and activating tissue invasion and metastasis to empowering replicative immortality, initiating inflammation and angiogenesis, and repressing cell death.

In any case, the expression of the HPV-E6 and HPV-E7 oncogenes is necessary but not sufficient for HPV-induced carcinogenesis. This statement is supported by the proof that only few women infected with an oncogenic HPV type will develop cervical cancer. Likewise, this process requires a very long time after infection and is characterized by the presence of premalignant precursor lesions with expanding grades of cell dysplasia (cervical intraepithelial neoplasias, CIN, stages 1–3), which either spontaneously relapse or, in the minority of cases, progress to invasive cancer [56].

The risk to develop invasive CC is increased by the use of oral contraceptives, smoking, early sexual practice, different sexual partners, and coinfections.

In addition, HPV utilizes several mechanisms to contrast the host immune response, for example, the suppression of innate immunity, inhibition of T-cell effector function, frequent loss of human leukocyte antigen (HLA) expression, and genetics events, all of which can lead to immune evasion. If the immune system fails to clear persistent HPV infections, after several decades, progression to cervical cancer appears [57].

It is important to underline that the extra alterations required for HPV tumor progression are not restricted to epigenetic changes inside the cell but also with the crosstalk with external cofactors. For example, the microbiome at individual body sites can affect tumor development [58]. Surprisingly, during progression of HPV-positive lesions to cervical tumor, microorganisms resident in cervicovaginal microbiome increase, and *Lactobacillus* spp. diminish [59].

Moreover, different investigations have been performed to analyze the microRNA (miRNA) expression profile in cervical cancer, and important relationships have been found between miRNA patterns and cervical tumor.

MicroRNAs are short RNAs that control the transcriptome and proteome at posttranscriptional level. To deeply understand the role of miRNAs in cervical cancer progression, meta-analysis and gene set enrichment analysis have been utilized to examine studies already published on miRNAs in cervical cancer [60].

It has been demonstrated that some of the dysregulated miRNAs are connected with specific phases of cervical growth development. To study the impact of miRNAs on the pathogenesis of cervical cancer, a miRNA-mRNA interaction network on chosen pathways was created by incorporating viral oncoproteins, dysregulated miRNAs, and their predicted/validated targets. The study has demonstrated that miRNAs deregulated at the different stages of cervical cancer are functionally associated with several key cancer-related pathways, for example, cell cycle, p53, and Wnt signaling pathways. These dysregulated miRNAs may have an important role in tumor development. Some of the stage-specific miRNAs can also be utilized as biomarkers for tumor characterization and checking of cancer development.

It has been demonstrated that miRNAs are discharged into the extracellular space or in the circulation framework in either microvesicles or exosomes [5]. In particular, exosomes have the ability to convey their load to recipient cells through receptor-mediated interactions. Unexpectedly, some studies have shown that some miRNAs are not transported by exosomes, but rather can be fairly recognized in a free soluble form, protected by RNA-binding proteins [61, 62]. The type of delivery of circulating miRNAs could depend on the type of tissue damage, suggesting an alternative role for every kind of transport and demonstrating that different types of cells, for instance, the endothelial cells, could contribute to the delivery of circulating inflamma-miRNAs.

3.1 Role of EVs in HPV-induced tumorigenesis

In spite of the fact that a connection between tumorigenesis and synthesis/release/function of EVs had been shown before, the role of EVs in the pathogenesis of HPV-induced malignancies began to be observed just lately (**Table 1**). The first confirmation of the involvement of EVs in the pathogenesis of HPV tumorigenesis dates back to 2009, when the presence of extracellular Survivin in HPV-18 positive cells HeLa was suggested [63]. Cell medium containing Survivin shows anti-apoptotic, proliferative, and pro-metastatic potential with respect to inactive T34A mutant [63]. After 2 years, the same researchers showed that extracellular Survivin was integrated in Exos and that proton irradiation caused synthesis and release of these Exos [64]. These Survivin-positive Exos were then investigated for their protein cargo content by examining the stress-induced proteins. Therefore, the presence of other inhibitor of apoptosis proteins (IAPs) as XIAP, c-IAP1, c_IAP2, and Livin/ML-IAP was shown [65, 66]. The presence of IAPs relied upon HPV oncoproteins, since Exos obtained from E6- and E7-silenced HeLa cells showed a decrease in the expression of these inhibitors; surprisingly, E6-/E7-silenced HeLa secreted more Exos than control cells [66]. The cargo content of HeLa-derived, Survivin-positive Exos was additionally described at the level of miRNAs [67]. The researchers demonstrated that 52 miRNAs were deregulated and the expression of 23 out of these was influenced by E6/E7 silencing. Most of the upregulated miRNAs play a pro-proliferative, anti-apoptotic, and anti-senescent role. The downregulated ones play instead opposite functions. Very important is also the fact that miRNA content of Exos showed a comparative but not superimposable profile of expression, since 11 out of 46 miRNAs found in Exos are not deregulated

References		Type of EVs	EV source	Purification method	Cargo type	Cargo hallmark
Aromseree et al.	[77]	Exos	EBV-infected LCLs	Differential centrifugation	Viral mRNAs	EBER1 EBER2
Chiantore et al.	[68]	Exos	E6-/ E7-transduced primary human keratinocytes	Differential centrifugation	miRNAs	miR-222
Carolis et al.	[82]	EVs	Sera	Differential centrifugation	DNA	Circular HPV DNA
Gaiffe et al.	[75]	ABs	HeLa CaSki	—	DNA	E6 and E7 DNA
Gezer et al.	[70]	Exos	HeLa	Differential centrifugation plus filtration	lncRNAs	lincRNAp21 CCND1-ncRNA HOTAIR, TUG1 GAS5, MALAT1
Harden et al.	[69]	Exo-EVs	Primary human keratinocytes	Total exosome isolation reagent	miRNAs	miRNAs connected to apoptosis, necrosis, and cell viability
Hermetet et al.	[76]	ABs	HeLa CaSki	—	—	—
Honegger et al.	[66]	MVs	HeLa	Differential centrifugation	Proteins	Survivin, XIAP, c-IAP1, Livin
Honegger et al.	[67]	Exos	HeLa	Differential centrifugation	miRNAs	Several miRNAs deregulated
Kannan et al.	[81]	Exos	Sera UM-SCC-104	Commercial kits	Proteins	HPV16-E7 MUC16 SIRPA
Khan et al.	[63]	Exos	HeLa S	Differential centrifugation	Proteins	Survivin
Liu et al.	[79]	Exos	Cervicovaginal lavages	Differential centrifugation	miRNAs	miR-21 miR-146a
Rana et al.	[72]	EVs	Primary human keratinocytes	Differential centrifugation	Proteins	IL-36γ
Valenzuela et al.	[65]	Exos	HeLa	ExoQuick Kit	Proteins	Survivin, c-IAP1, c-IAP2, XIAP
Zhang et al.	[80]	Exos	Cervicovaginal lavages	Differential centrifugation	lncRNAs	HOTAIR, MALAT1, MEG3

Table 1.
EV type and cargo in HPV+ cells/specimens.

in cells, proposing the presence of specific mechanisms for incorporation of these miRNAs into Exos. The analysis was also performed in the HPV-16-positive cell line SiHa with superimposable results, proposing that HPV deregulation of miRNA

expression is not genotype-specific [67]. The study of miRNAs performed in Exos obtained from primary keratinocytes transduced with E6 and E7 from HPV-16 or HPV-38 confirmed the results obtained in the cell lines by Honegger et al. and showed in these vesicles the presence of mRNA coding for E6 and E7 and the capacity of Exos to transfer these mRNAs to non-transduced keratinocytes [68]. Focusing on a panel of some tumor-related miRNAs, Harden and Muller acquired comparable expression profiles between cell- and Exos-related miRNAs [69]. Other researchers demonstrated the presence of long noncoding RNAs (lncRNAs) into HeLa-derived Exos checking the presence of lincRNA-p21, CCNDA1-ncRNA, HOTAIR, TUG1, and GAS5 [70]. Significantly, lincRNA-p21, the most overexpressed lncRNA in Exos compared to parental cells, is a repressor of p53-dependent transcriptional responses [71] suggesting that his horizontal transfer may influence gene expression in acceptor cells.

EVs derived from HPV-positive cells are likewise able to horizontally transfer cytokines and mRNA thereof, consequently assuming an immunomodulatory role in the cancer microenvironment. Rana et al. showed the presence of proinflammatory cytokine IL-36γ into EVs isolated from Poly(I:C)-treated keratinocytes [72]. Since it has been reported that HPV-16 suppressed the Poly(I:C)-induced expression of several proinflammatory genes [73], it is conceivable to think that IL-36γ expression in EVs is suppressed by HPVs. According to this anti-inflammatory function, the expression of many proinflammatory cyto- and chemokine mRNAs is deregulated in E6-/E7-transduced keratinocytes, and as in the case of miRNAs, this profile is sufficiently conserved in EVs with a statistically significant reduction of IL-1α, IL-1β, CCL27, CXCL1, CXCL3, and angiogenin mRNAs [74].

Different classes of EVs have been also related to HPV-induced tumorigenesis. It has been shown that ABs transfer viral DNA to nonprofessional phagocytic cells [75]. Specifically, these researchers showed that human primary fibroblasts could internalized ABs derived from apoptotic Hela or CaSki (HPV16+) cells and following this internalization showed a transformed phenotype (i.e., development in soft agar, aneuploidy, diminished expression of p53 and p21). This internalization of apoptotic cells, made by both professional and non-professional phagocytic cells and called efferocytosis, happens in a time- and stage-dependent manner since only late apoptotic fragments were able to be taken up by normal fibroblasts; on the contrary, professional phagocytic cells internalize with high effectiveness and degrade both early and late ABs without indications of transformation [76].

EVs, and especially Exos, may be involved in the viral crosstalk, as in the case of coinfection with HPV and Epstein–Barr virus (EBV). It has been reported that Exos from EBV-positive lymphoblastoid cell lines and containing EBV small noncoding RNAs, called EBERs, are delivered to HPV-positive keratinocytes [77]. In EBV-positive cells, EBERs can affect innate immunity and cell proliferation [78], but in the case of their horizontal transfer to HPV-positive keratinocytes, their role remains elusive [77].

The delivery potentiality of EVs, in terms of cargo content and possibility to recover them from virtually all the bodily fluids, makes them an ideal diagnostic and prognostic tool to monitor tumor onset and progression as well as therapy effectiveness while avoiding invasive procedures. The liquid biopsy approach has been recently applied also in HPV-positive carcinomas. The first attempt to study EVs in specimens collected from HPV-positive cancer dates back to 2014 [79] when it was demonstrated that exosomal miR-21 is overexpressed in HPV-positive normal specimens and even more in HPV-positive cervical cancer specimens compared to normal HPV-negative ones. In addition, Exos from cervicovaginal lavages of both HPV-positive normal and cancer patients contain more HOTAIR, MALAT1, and MEG3 lncRNAs [80]. Moreover, in HPV-16-associated oropharyngeal cancer

(HPVOPC), serum collected from patients contains Exos expressing key altered proteins, namely, mucin-16 (MUC-16) and signal regulatory protein α (SIRPA) as well as E7 oncoprotein [81]. Similarly, Exos derived from a HPVOPC cell line displayed the ability to induce epithelial-mesenchymal transition (EMT) and invasiveness of two human mammary epithelial cell lines [81]. Finally, the presence of EVs containing HPV DNA was analyzed in the serum of patients with breast cancer. The authors found two out of eight HPV DNA-positive specimens among patients with ductal carcinoma in situ with a complete correspondence between tissue and serum-derived EV specimens and one out of fourteen HPV DNA-positive specimens among benign breast disease-affected women [82].

4. Engineered exosomes in immunotherapy of HPV-related tumors

Exosomes, in virtue of the properties of stability, biocompatibility, and low immunogenicity and of the possibility to be loaded with a cargo, are increasingly explored as therapeutic delivery agents for drugs and small molecules in a number of pathological conditions. Importantly, exosomes hold good stability while preserving the activity of the molecules shipped, which are fundamental characteristics for delivery, while the outstanding hallmark concerns safety, being a cell-free and very controllable system. In the recent past, exosomes have been investigated for the delivery of siRNAs, miRNAs, shRNAs, and anti-inflammatory/anticancer agents as curcumin and doxorubicin and paclitaxel.

Working either through the parental cells or the environmental milieu, exosomes can be tailored to target definite diseases, e.g., by expressing specific surface ligands and receptors and loading them with therapeutic agents. In alternative to the manipulation of exosomes through the biogenesis in the parental cell line, exosomes can be purified and then modified for incorporating therapeutic molecules. Exosomes delivered by systemic route preferentially accumulate in the liver, spleen, and kidneys, but by scaling the dosage and changing the route of administration, it is possible to affect biodistribution. As the exosome tropism is affected by the donor cell type which determines the membrane composition, attention must be paid to the fact that tumor exosome content is enriched in molecules that can promote tumorigenesis.

A number of applications of therapeutic exosomes in pathologic conditions such as diabetes, cancer, and cardiovascular and neurological diseases are under investigation, with some of them in phase I and one (advanced non-small lung cancer) in phase II clinical trial. For their part, HPV tumors represent *per se* targetable lesions because of their confined localization and lend themselves to being attacked by an exosome-based immunotherapeutic approach that can be either active or passive.

The first studies utilizing exosomes in immunotherapy are dated 20 years ago and used B-cell-derived exosomes to induce MHC-restricted T-cell responses [4]. In the next studies, exosomes were proposed as tumor vaccines for a number of cancers such as melanoma, glioma, hepatocellular carcinoma, and renal cell carcinoma [83–89], due to their capability to deliver antigens from professional APCs such as DCs to other APCs.

Cervical cancer immunotherapy using dendritic cells pulsed with exosomes derived from HeLa cells was recently proposed. Monocyte-derived dendritic cells from cervical cancer patients were matured in vitro to DCs and pulsed with exosomes purified from HeLa culture medium with an opportune protocol. These HeLa-exo pulsed DCs in co-culture experiments with autologous lymphocytes showed the ability to stimulate a strong T-cell activation and a CTL-specific cytotoxic response [90]. In a mouse animal model, HPV-specific cytotoxic activity useful in cervical cancer immunotherapy was also demonstrated using exosomes [91].

Proteins localized into the membrane compartment generating exosomes can be used as carriers for delivering other proteins into the exosomes: this is the case of the HIV-1 Nef protein. A Nef mutant (Nef^{mut}) was generated from a natural defective HIV-1 expressing a Nef variant with the property to accumulate in abnormal quantities in the raft membranes and then in exosomes, compared to the wild-type protein [92]. This 27 kDa Nef^{mut}, both myristylated and palmitoylated, was appropriately engineered to work as a carrier for other proteins long up to 630 amino acids, once fused to its COOH terminus. It has been demonstrated that the fusion products can be incorporated not only into genome-free viral particles of HIV-1, working as a vaccine [93], but also into exosomes of cells transfected with the corresponding DNA constructs.

To develop a therapeutic vaccine for the cure of HPV16-related tumors, the HPV16-E7 and HPV16-E6 tumor-specific antigens were fused to Nef^{mut} and expressed in HEK 293 T cells into the exosomes, anchored to the membrane by Nef^{mut}. Mice harboring subcutaneous HPV16-specific tumors, when immunized with recombinant Nef^{mut}-E7 or Nef^{mut}-E6 exosomes, developed a strong specific cell-mediated immune response, which were able to block the growth and reduce the burden of the tumors generated by injection of TC-1 tumor cells [94, 95]. The production of recombinant exosomes and their purification for therapeutic vaccine development are nevertheless multistep procedures difficult to scale-up. For this reason, the Nef^{mut} expression in exosomes was also pursued and obtained from Nef^{mut}-E7 or Nef^{mut}E6 DNA plasmids in vitro and in vivo. The results allowed to develop an upgraded HPV16 therapeutic vaccine based on the DNA inoculation of Nef^{mut}-E7 and Nef^{mut}-E6 plasmids [96].

The peculiarity of the Nef-fused antigens is to be cross presented to the T cells once they reach the dendritic cells in form of exosomes, forming the platform for the genesis of CTL vaccines [97].

Nef^{mut} is also being investigated for the delivery of therapeutic intracellular antibodies against the HPV-E6 and HPV-E7 oncoproteins. Anti-HPV16-E6 and anti-HPV16-E7 antibodies in single-chain format were previously shown to exert anti-proliferative activity in HPV-positive cells in vitro and therapeutic efficacy against HPV-positive tumors in animal models [98]. Preliminary experiments show that exosomes purified from cells transfected with plasmids expressing the Nef^{mut}-anti-16E7 scFv fusion protein contain an antibody in an active conformation able to bind to the target E7 (Accardi et al., unpublished), exactly as what occurs for the scFv delivered as DNA or protein [98, 99]. In view of the translational application of these antibodies in the clinic for the treatment of preneoplastic HPV-related lesions localized in the anogenital area or even in the oropharynx, exosomes can represent a safe and easy delivery tool. These results represent an incentive to continue the studies, albeit with the awareness of the limits of this strategy, mainly consisting of the lack of reliable standardized methods for the control of exosome contents and purification and of the limited number of exosomes that can be produced.

5. Conclusions

Our knowledge of the EV biology and role, mainly with respect to virus infection, is still in its infancy. Studies have demonstrated that the delivery of viral and cell factors by EVs empowers the control of neighboring unaffected cells. Communication by MVs enables the virus to interact with, and control, cell microenvironment. Various reports proposed that virus infections use the cell vesiculation pathways for virus maturation, immune evasion, and intercellular correspondence. In addition, there is expanding proof of tumorigenic properties

of Exos and MVs derived from cancer cells, depending on either a direct effect of oncogenes transferred to beneficiary cells or an indirect effect of modification of the TME. Importantly, in virus-induced cancer cells, the Exos cargo is different from that of the non-infected cells. These EVs seem to be enriched with viral or cell oncogenic factors as oncoproteins and oncomiRs. Thus, viral-modified Exos may function to horizontally transfer oncogenic molecules among neighboring cells, but they are also armed to manipulate the TME by favoring angiogenesis and inflammation or exerting immunosuppressive effects.

6. Executive summary

- Extracellular vesicles are mediators of intercellular and intra- and intertissue signals that transfer discrete cargo composed by nucleic acids, proteins, and lipids.
- Extracellular vesicles are involved in both physiological and pathological processes.
- Tumors exploit extracellular vesicles to reshape the microenvironment, reprogram normal neighboring cells, evade cell-mediated immune responses, and prepare pre-metastatic niches.
- Human papillomavirus infection has a deep impact on extracellular vesicle cargo in terms of protein expression, mRNA, lncRNAs, and miRNA content as well as viral DNA transfer via apoptotic bodies.
- Mediators transferred by extracellular vesicles released from HPV-positive cells are involved in proliferative, anti-apoptotic, and anti-senescence pathways.
- Extracellular vesicles released from HPV-positive cells contain mRNAs for cytokines and chemokines suggesting a role of these mediators in the reshape of tumor microenvironment.
- Due to their stability and low immunogenicity, extracellular vesicles represent an ideal tool for the delivery of chemotherapeutic drugs to tumor cells as well as for the stimulation of antitumoral immune responses.
- In the next few years, tumor-specific and tumor-associated extracellular vesicles will represent a versatile diagnostic and/or prognostic marker to be detected in blood samples in the liquid biopsy.

7. Future perspective

EVs may represent an innovative tool for the diagnosis, the prognosis, and the follow-up of malignancies as well as for the delivery of anticancer drugs. The ability to vehiculate and protect their cargo from lytic enzymes as well as immune effector mechanisms makes EVs an ideal support for the so-called liquid biopsy and for the next-generation cancer therapy. Indeed, valuable markers as cancer-associated DNAs, mRNAs, miRNAs, and other lncRNAs are protected from the activity of nucleases present in the plasma. Tumor-specific or tumor-associated antigens could be more efficiently presented to immune cells or transferred to antigen-presenting

cells using EVs rather than artificially synthesized liposomes. These scenarios are predictable for all the malignancies, especially those associated with oncoviruses where tumor-specific antigens are well expressed (i.e., E6 and E7 in the case of HR-HPV). Nevertheless, little is known about the biogenesis and release as well as the cargo content of EVs especially in terms of expression of specific tumor- or tissue-associated EV antigens. In the next few years, "benchtop" research will be focused on these aspects of EV biology, thereby leading to "bedside" diagnostic and clinical application of EVs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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