Review

3D Modelling of HPV-Related Tumors and Their Microenvironment

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Review

3D Modelling of HPV-Related Tumors and Their Microenvironment

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Abstract: Human Papillomavirus (HPV) related cancers account for a large percentage of cancers. The tumor microenvironment (TME) is a complex structure and contributes significantly to tumor development, progression, and invasion. The TME is particularly pronounced in HPV-related cancers and HPV is known to influence the TME. In cancer research, there is an urgent need for new models to deepen our understanding of the pathophysiology of the disease and to facilitate the development of innovative therapies. In this context, 3D cell culture has emerged as a promising method in cancer research, that offers significant potential compared to conventional approaches. 3D modelling can produce results that are more consistent with clinical outcomes without the ethical concerns and problems of animal models. In this review, our aim is to discuss the tumor microenvironment in HPV-related cancers and demonstrate the importance of advancing 3D cell culture models for this purpose.

Keywords: 3D-cell culture; tumor microenvironment; HPV-induced cancers

1. Introduction

Cancer is an urgent public health issue globally, and cancer types particularly associated with Human Papillomavirus (HPV) are gaining increasing importance. HPVs are small circular viruses with double-stranded DNA [1]. About 5% of all cancers are related to HPV infections [2]. HPV is classified into low and high-risk types based on oncogenic risk. Low-risk types (Types 6 and 11) are associated with skin and anogenital warts, whereas high-risk types (Types 16, 18, 31, and 45) are predisposed agents for anogenital cancers [3–5]. Understanding the interaction of HPV infection on carcinogenesis and cancer progression plays a vital role in the development of prevention, early detection, and treatment strategies. HPVs are the primary cause of nearly all cervical cancers (CVC) and a significant portion of other cancers in the mucosal lining of the anogenital tract, as well as in the head-neck, and even lung region [6]. HPV presents as a critical public health problem because of its ability to infect different anatomical regions and induce malignant transformations. Understanding the effects of HPV on the genetic material of the host cell is necessary to elucidate the mechanisms of cancer development in HPV-associated cancers [7–10].

In in vitro 3D cancer models, cancer cells are co-cultured with diverse stromal cell varieties, replicating tumor characteristics in a laboratory setting. This approach reveals increased invasiveness and resistance to anti-cancer therapies compared to traditional 2D cell culture, highlighting its promise for drug screening applications [11]. Modeling cancers represents a groundbreaking step in cancer research. These studies aim to explore further the effects of cancer in vitro environments, thereby working to develop more effective treatments and preventive measures for these types of cancers. The models provide a window to understand critical processes such as cancer development,

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metastasis, and progression, while also revealing the role of other cells and the extracellular matrix (ECM) in these processes, offering a more comprehensive view of cancer biology. Such research forms an important foundation for developing new strategies in cancer management. This review aims to explore the microenvironment in HPV-associated cancers and understand the current status of 3D models of HPV-associated cancers in the existing literature.

2. Overview of Human Papillomavirus (HPV) and Its Implications in Cancer Development

High risk HPV is a tumorigenic DNA virus that causes epithelial proliferation on skin and mucosal surfaces and is the most common sexually transmitted infection [12,13]. The most lethal type of cancer known in women is CVC, and its primary cause are HPV types 16, 18, 45 and 56 [14,15]. According to the National Cancer Institute (2019) data for 2023, there were 13,960 new CVC diagnoses with an estimated death count of 4,310. Although HPV is generally recognized as the predominant etiological factor in CVC, it has also been reported to play a role in the pathogenesis of other malignancies such as oral squamous cell carcinoma [16,17]. Squamous cell carcinomas of the head and neck, the seventh most diagnosed cancers worldwide, also account for 5% of all cancer cases [18,19]. Similarly, about 85% of anal cancers caused by HPV are reported to be squamous cell carcinomas [20].

In a study showing the strong association between HPV infection and colorectal cancer, HPV antigen was detected in 23% of normal colon samples, 60% of adenomas, and 97% of carcinomas [21]. Detection of HPV DNA in invasive penile cancer is relatively rare, and the incidence of penile cancer is lower compared to CVC [22–24]. The best-known skin cancers are melanoma and non-melanoma cancers, both of which are influenced by HPV in association with UV exposure [25,26].

HPV mediated carcinogenesis is mainly related with E6 and E7 proteins of HPV. They are intranuclear proteins and even though they have different properties, they act cooperatively in promotion of carcinogenesis [27]. E7 binds to retinoblastoma protein (Rb) and retinoblastoma pocket proteins which are p107 and p130. This binding causes inhibition or proteasomal degradation of Rb which results with the increased shift to S phase of cell cycle. This shift leads to an increase in cell proliferation and therefore, increase in viral gene transcription. E7 also contributes to G1-S phase entry by binding to p21 and p27. As a response to increased expression of proliferative proteins, p53 expressions normally increase to counter excessive proliferative activation [28]. Since p53 expression disrupts viral gene replication, HPV developed a counter mechanism against p53 using E6 protein. E6 can bind to p53 to prevent its tumor-suppressive effects [29]. Also, E6 causes degradation of p53 via the ubiquitin pathway [30]. Even though E6 mainly shows its activity by p53 regulation, there are p53-independent effects of E6 as well, such as mitogenic effects, inhibition of apoptosis and interfering with the organization of the tissue [31]. E5, another oncoprotein of HPV, plays an immunosuppressive role by negatively regulating anti-viral IFN response pathways, antigen processing and antigen presentation [32]. E5 hinders the transportation of HLA-1 molecule to cell surface for immune recognition and keep it inside the Golgi apparatus. This incident happens due to E5's interaction with HLA-1's heavy chain via E5's first hydrophobic domain [33]. HPV 16 E5 is shown to diminish recognition by CD8+ T cells [34]. Figure 1 summarizes the effect of HPV on the cell through the E5, E6, and E7 proteins.

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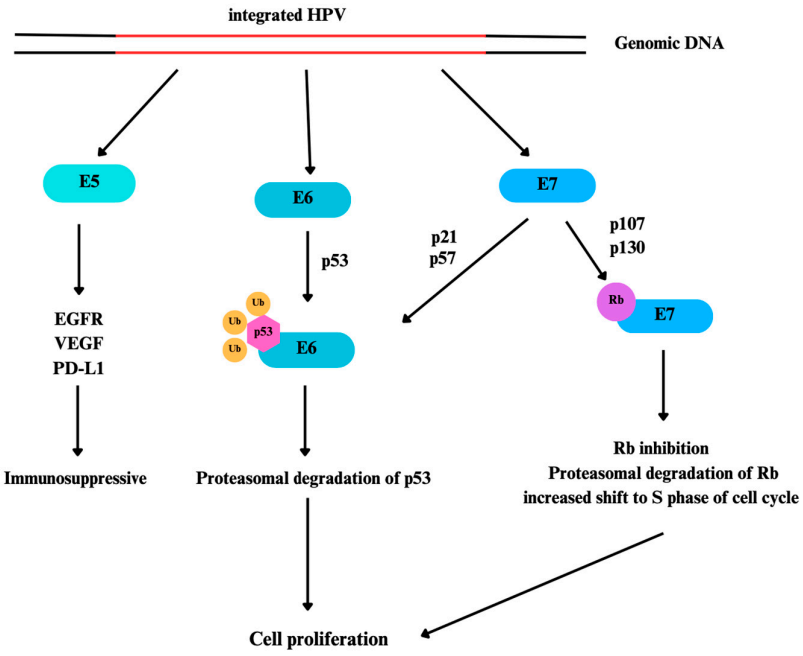

Figure 1. Illustration of HPV E5/E6/E7-mediated cellular transformation.

3. The role of HPV on the Tumor Microenvironment

The mechanisms by which HPV leads to cancer are quite complex and involve numerous molecular interactions. The infection itself, even with the virus's integration into the host cell's genome, is not sufficient for carcinogenesis [35,36]. However, it results in a series of events, such as genetic rearrangements, which lead to genomic instability and increase the risk of neoplastic transformation. HPV integration leads to the production of inflammatory mediators that promote cancer progression [37].

Active molecules in seminal fluid can induce inflammation in the cervical mucosa by attracting and activating leukocytes, thereby increasing inflammation [38,39]. Interestingly, it has been observed that IL-6 levels are regulated differently in HPV-positive squamous cell carcinoma of the head and neck than in HPV-negative cases. IL-6 has been found to increase in HPV-16 and/or HPV-18 positive cells, which is associated with a poor clinical prognosis of patients [40].

Regulation of the immunosuppressive cytokines IL-10 and TGF-β has been observed in HPV-positive keratinocytes [41,42]. The simultaneous expression of these cytokines is associated with the activation and polarization of immunosuppressive regulatory T cells [43].

Tumors are not only made up of cancer cells, but have a complex structure consisting of immune cells and various molecules that remodel the environment. This inner structure of tumors other than cancer cells, is called the tumor microenvironment (TME). The TME actively contributes to tumorigenesis, tumor progression, invasion and metastasis [44].

Lymphocytes are leukocytes that are identical in appearance but differ in their functions. There are three types of lymphocytes according to their functions: T lymphocytes, B lymphocytes and natural killer (NK) cells [45]. T lymphocytes develop in the thymus, hence the name. They are able to recognize antigens that are presented to them and react appropriately depending on their subgroup [46]. There are many subsets of T cells. The most important subsets that actively contribute to TME are CD4+ T cells (also called helper T cells, Th) and CD8+ T cells (also called cytotoxic T cells). CD4+ T cells are the mediators of antitumor immunity and show their effect by directly killing cancer cells, stimulating immune cells that play a role in innate immunity and preventing angiogenesis [47]. Regulatory T cells (Treg) are a remarkable subset of CD4+ T cells. They have a highly immunosuppressive profile and their abundance in TME is associated with unfavorable outcomes

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[48]. CD8+ T cells are the strongest effectors of the antitumor response and are considered the basis of immunotherapy. Despite the presence of CD8+ T cells, tumor growth is not prevented. This phenomenon is also known as the "Hellstrom paradox", which states that tumor-reactive CD8+ T cells lose their function during tumor development [49]. NK cells are cytotoxic lymphocytes that, unlike CD8+ T cells, do not require prior antigen exposure to exert their antitumor effect [50]. Despite their different antitumor mechanisms, cancer cells have developed different strategies against the activity of NK cells and their infiltration into the TME [51,52].

Macrophages are one of the most important mediators of innate and adaptive inflammatory responses. There are two main types of macrophages. M1 macrophages, which have a proinflammatory function, exhibit antitumor effects through mechanisms such as efficient regulation of Th responses, promotion of T cell proliferation, IFNγ secretion, secretion of IL12, which has proinflammatory potent antitumor effects, and reduction in secretion of factors that contribute to cancer progression, such as VEGF, MMPs and CCL18 [53]. In contrast, M2 macrophages, which have anti-inflammatory effects, show tumor-supportive effects through various mechanisms such as immunosuppression, secretion of tumor-promoting growth factors such as PDGF, TGFβ1, HGF, promotion of angiogenesis, and facilitating the transformation of fibroblasts into cancer-associated fibroblasts [54]. Macrophages that contribute to TME are referred to as tumor-associated macrophages (TAMs). TAMs are in a constant state of flux between types M1 and M2 [55]. They play an important role in angiogenesis, migration, invasion, epithelial-to-mesenchymal transition, interaction with cancer stem cells, and immunosuppression. TAMs are associated with poor overall survival in CVC and other HPV-induced cancers [56]. TAMs remain poorly understood in HPV-positive tumors. However, the increasing three-dimensional (3D) models will enable more accurate research aimed at investigating the interaction of TAMs with TME [57].

Neutrophils are the most abundant type of leukocyte in the blood and act as the first line of defense under physiological conditions. There are three recognized mechanisms for neutrophils against pathogens: Phagocytosis, degranulation, and the formation of extracellular traps for neutrophils through the release of nuclear material. Beyond these functions, however, research describes many other mechanisms, including active involvement in cancer [58]. As in infectious and inflammatory diseases, an increase in peripheral neutrophil counts is also seen in cancer patients. Several studies have shown that an increased ratio of neutrophils to lymphocytes indicates a poor prognosis [59]. Neutrophils that engage in TME undergo phenotypic changes and are referred to as tumor-associated neutrophils (TANs). Similar to macrophages, there are two main phenotypes of TANs: antitumor (TAN1) and pro-tumor (TAN2) [60]. TAN1 cells target cancer cells directly by releasing their granule content, which contains elastase and myeloperoxidase, or indirectly by forming extracellular traps and secreting cytokines such as IFN-γ and IL-12. TAN2 cells promote tumorigenesis and progression, but their mechanisms are not fully understood [61].

Eosinophils are leukocytes that are mainly involved in parasitic infections. They also have important functions in various diseases such as asthma, eosinophilic gastrointestinal diseases, and systemic hypereosinophilic diseases [62]. In addition to their functions in physiological and pathological conditions, eosinophils regulate numerous immune cells such as lymphocytes, macrophages, and neutrophils [63]. Typically, eosinophils are not regarded as major contributors of TME. However, eosinophils have several roles in many stages of cancer. They may appear as anti-tumorigenic, pro-tumorigenic, or silent bystanders depending on cancer type [64]. Eosinophils are known to infiltrate the TME and this occurrence has been termed tumor-associated tissue eosinophilia (TATE). TATE predicts a better prognosis for patients with solid tumors [65]. Because of eosinophils' involvement in the TME and their potential role in treatment, they are worthy of cancer research. The importance of TME in viral-induced carcinogenesis, especially in HPV-induced mucosal cancers, is paramount [66,67]. In addition, HIV-positive patients with HPV-related tumors have particular problems, such as earlier onset and resistance to standard treatments, highlighting the intricate interplay between HPV infection and the immune system within the TME [68].

Understanding these interactions is of central importance for the development of effective therapeutic strategies against HPV-induced carcinogenesis.

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4. Characterization of the Tumor Microenvironment in HPV-Associated Cancers

The rise in global oral cancer cases has been confined to a 2.3% yearly increase specifically in diagnoses of tongue, tonsil, and oropharyngeal cancers linked to HPV [69]. In HPV-related oropharyngeal squamous cell carcinoma (OPSCC) patients, T cell infiltration is to a greater extent, and higher IFNγ, lower IL-4, and low transforming TGF-β expressions are seen which indicates a good prognosis [70]. A noteworthy increase in the percentage of effector T cells to naïve T cells is seen in HPV-positive OPSCC compared to HPV-negative OPSCC [71]. A notable increase in CD8+ T cells in circulation and TME has been found and this elevation significantly correlates with a better prognosis [72]. Literature has controversial results regarding the role of Tregs in OPSCCs and this may be related to inconsistent techniques that are used for detection of Tregs [73]. Elevated levels of M1/M2 and M2 macrophage infiltration are seen in tumor stroma of HPV-positive OPSCC patients compared to HPV-negative patients. Interestingly, a greater infiltration of CD163+ macrophages has been found in HPV-negative patients which indicates shorter metastasis-free survival and overall survival [74]. Similarly, another study proclaims that HPV positivity in OPSCC patients significantly indicates a better overall survival up to twenty years [75]. Al-Sahaf et al. have observed a significant increase in neutrophil levels in HPV-negative OPSCC patients compared to HPV-positive patients which suggests a better prognosis for HPV-positive patients [76]. Eosinophils were found to be a positive indicator of prognosis in HPV-positive patients and disparately a negative indicator in HPV-negative patients. Same study also revealed that total peripheral eosinophil count is higher in HPV-negative patients compared to HPV-positive patients [77].

Untreated HPV infections are the cause of 95% of all CVCs [78]. There is an unanimity that CVC is an immunogenic tumor since it's caused by persistent infection with HPV [79]. CVC patients either lack or have severely diminished HPV-16 specific CD4+ T-cell response [80]. Inhibition of CD4+ and CD8+ T cell responses to HPV are associated with the CVC development [81]. Also, CD4+/CD8+ ratio is found to be significantly lower in CVC tumor than in peripheral blood [82]. Decreased CD4+ T cell ratio is strongly correlated with rapid tumor growth and lymph node metastasis in CVC [83]. Treg infiltration correlates with CVC progression and decreased Treg levels show improved outcomes [84]. NK cell ratio is greater in stage II CVC in comparison to stage I CVC patients. Also, patients with NK cell population greater than 15% have significantly higher clinical stage and invasion depth compared to those with lesser than 15% [85]. Elevated levels of CD56high NK cells in tumor tissue compared to peripheral blood in CVC patients have been demonstrated. Conversely, CD56low NK cells are present in peripheral blood to a greater extent [86]. Macrophages in the TME of CVC are mainly polarized towards the M2 phenotype and this polarization also correlates with poor response to chemoradiation [87]. Increased TAM density is markedly associated with poor survival in CVC [88]. Also, there is a modulation of TAMs by CVC cells via lactate secretion [89]. In terms of distinguishing precancerous lesions from CVC, increased neutrophil-to-lymphocyte ratio is predictive for CVC [90]. A recent study has established that high peripheral neutrophil to lymphocyte ratio is correlated with poor prognosis in early CVC [91]. Increased eosinophil levels and eosinophil-to-lymphocyte ratio indicate a favorable overall survival in CVC patients [92]. Microenvironment of CVC can be summarized as shown in Figure 2.

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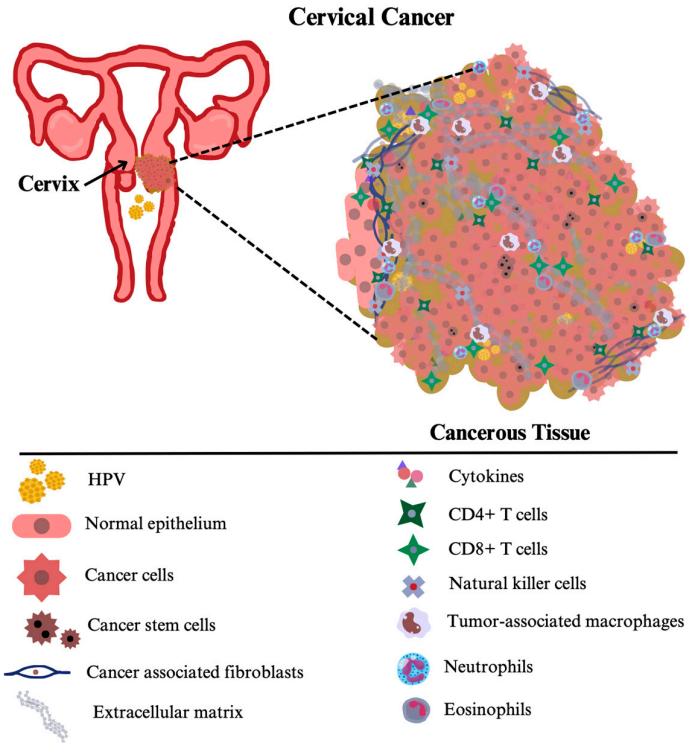

Figure 2. Schematic illustration of TME of CVC. Many immune cells and CAFs contribute to TME. Also, extracellular matrix plays an important role in TME. Taken together, TME has vital roles in carcinogenesis, cancer progression, invasion, and metastasis in CVC.

Penile cancer is a rare condition in industrialized nations; however, they have high incidences in developing regions [93]. Limited availability and access to medical healthcare in these regions may benefit greatly from advances in treatment. Detection of HPV in penile cancers is highly inconsistent which may explain the heterogeneity of literature considering the presence of HPV in penile cancers [94]. There is an increase of cytotoxic T cells in HPV-positive patients compared to HPV-negative patients. This increase is associated with poor overall survival [95]. Increased CD8+ T cell infiltration rate is related with decreased lymph node metastasis in penile cancer patients [96]. Literature investigating Tregs in penile cancers is scant [97]. NK cell activity has shown a noteworthy decrease in penile carcinoma [98]. CD68+ macrophages are found to be in high densities, and they are associated with a more favorable overall survival and decreased risk of regional recurrence [95]. Stromal CD68+ macrophages are inversely associated with lymph node metastasis [99]. Increased intratumoral CD163+ M2 macrophages are significantly associated with higher lymph node metastasis [96]. Higher neutrophil-to-lymphocyte ratio is associated with worse prognosis and lymph node metastasis in penile cancer patients [100]. Eosinophil infiltration has been demonstrated in penile cancers and seems to improve the 5-year survival, however researchers point out the small sample group and suggest broader research in this subject [101].

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. Although HPV is not considered a major risk factor for CRC, the presence of HPV infection is associated with a higher risk of CRC [102]. In addition, the overall prevalence of HPV in CRC patients is 31.9%, further supporting the association between HPV and CRC [103]. In HPV-positive CRC patients, there is an increase in T cells compared to HPV-negative patients [104]. An increase in Tregs was also observed [105]. M1 macrophage infiltration is increased in HPV-positive CRC. The same study also showed that the expression of MHC-II by M1 macrophages is increased and that these findings together directly increase the antitumor response [104]. The literature investigating the HPV-

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associated CRC microenvironment is insufficient and new studies may shed light on our understanding and thus improve treatment options.

Anal cancer is one of the rare cancers however its incidence increases every year by 2.7%. Anal squamous cell cancer (ASCC) is the most common form of anal cancer [106]. Approximately 90% of anal cancers are associated with HPV infection [107]. High amounts of tumor-infiltrating lymphocytes are related to better prognosis in ASCC patients [108]. As a result, IFNγ secretion and increased PD-L1 expression are seen within TME [109]. Studies regarding Tregs in ASCC are limited and have controversial results. The roles of TAMs in ASCC remain mostly unresolved [110]. Neutrophilia strongly predicts overall survival, progression, and failure-free survival in APSCC patients [111]. Increased neutrophil-to-lymphocyte ratio is associated with poor overall survival and cancer-specific survival [112]. In HPV-positive ASCC patients, low eosinophil levels correlate with better disease-free survival [77].

Normally, the supporting structure, the so-called stroma, has been regarded merely as a scaffold on which the epithelial cells "sit". However, it is known that the communication between stromal cells and HPV-infected epithelial cells plays a crucial role in carcinogenesis. Stromal fibroblasts, which are the most important cellular component of connective tissue, provide signals that significantly influence the development and progression of cancer. Cancer-associated fibroblasts (CAFs) are fibroblasts that are activated by paracrine mediators produced by cancer cells. They can promote HPV-mediated carcinogenesis through various mechanisms via stromal-epithelial interactions [113]. The ability of CAFs to promote immunosuppression in HPV-associated cancer, as well as their ability to enhance cell growth and metastasis, has been reported several times [114–116].

It has been indicated that bidirectional communication between epithelial cells and the TME affects a range of processes from tumor initiation to neoplastic progression, and even to metastasis and drug resistance. Researchers believe that in response to this communication, the TME alters various stromal-epithelial signaling activities crucial for HPV-positive epithelial cell growth, disease initiation, and progression. Therefore, there is a clear need for 3D in vitro model systems to better understand the interactions between HPV-infected tissue and the surrounding TME. These systems are crucial for comprehending the fundamental mechanisms of cancer and developing new treatment strategies.

5. Utilization of 3D In Vitro Models in HPV-Related Cancer Research

For about a century, cancer research has progressed by focusing on the cellular structures of tumors [117]. Cell lines derived from various cancer types have been developed. These cell lines can be easily multiplied in culture and reflect the characteristics of specific cancer types. They have become a significant tool in cancer research [118]. However, commercially available tumor-derived cell lines often have genetic characteristics that differ from the original tumor cells [117]. Furthermore, cell lines undergoing extended cultures may exhibit heightened susceptibility to genetic alterations, varied morphological characteristics, and increased vulnerability to contamination [119,120]. Although extensive evidence supports the superiority of 3D cell models, engineered with materials optimized for cellular growth in the laboratory over traditional two-dimensional (2D) cultures [121], preclinical research predominantly utilizes 2D cultures due to their simpler cultivation process [122].

Culturing commercial cell lines which are modified for easier replication and using materials that don't harm these cells in cell culture is practical in terms of in vitro cancer studies. However, these factors often remain inadequate and unreliable to mimic an organism. The significant gap between 2D cell cultures studied in the laboratory and clinical studies is attempted to be filled with animal model experiments; however, both the inconsistency between human and animal responses and substantial time and cost expenditures are incurred as a result [123]. In 2D cell cultures, the cells grow on polystyrene plates and form individual layers. Therefore, the culture lacks the complex interactions between cells and the surrounding ECM as well as the interactions between neighboring cells that are present in 3D cell culture models [124]. Anticancer drugs used in 2D cell cultures usually reach the cells without any physical barriers. However, the drugs have to overcome many factors in the organism that prevent their spread throughout the tumor [125]. 3D organization of the tumor

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mass and associated stroma fundamentally alters the tumor spread profile via cell-cell contacts and cell-matrix interactions [126].

In the literature, mice have been considered a good model for mimicking human cancers, particularly for animal model approaches. However, the significant differences among tumors and ethical concerns have emphasized the need for a new model [127,128]. Therefore, in the literature, studies are attempting to mimic the ECM by using different biomaterials on the lung [129], breast [130], prostate [131], and kidney [132] to investigate such cancer development.

While different forms of biomaterials affect cell culture efficiency, 3D culture methods contribute to the advancement of many applications, particularly in the fields of cancer and stem cell research, as well as drug and toxicity screening [133]. Cells in 2D are exposed to a homogeneous environment with sufficient oxygen and nutrients, whereas cells in solid tumors are exposed to gradients of critical chemical and biological signals, which can have both stimulating and inhibitory effects on tumor progression [134]. When comparing 3D and 2D gene expressions, the crucial difference emerges in culture systems rather than cell lines [135]. Schematic illustration of the comparison between 2D and 3D culture models Figure 3.

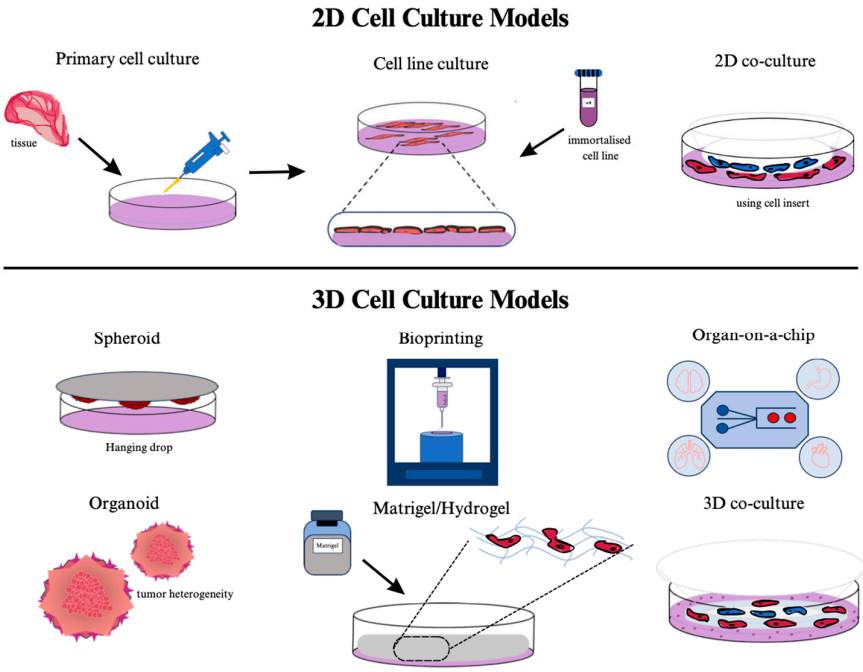

Figure 3. 2D cell culture systems grow cells in a flat, single-layer environment, which restricts them to only cell-cell interactions. In contrast, 3D cell culture models better mimic the tumor microenvironment by allowing cells to interact with each other and the surrounding matrix in a three-dimensional space. This 3D approach can support multiple cell types within one environment, reflecting the complexity and diversity of actual tumors.

In this field of research, there is considerable amount of interest in identifying 3D models that best reflect the specific characteristics of the TME. With the increase in studies in this area over the past decade, interest in 3D modeling has grown exponentially. Approximately 18,000 publications that have been found in the search for "3D cancer models" in 2024 can be seen as a precursor to the emergence of new and valuable techniques for studying tumor and TME interactions.

6. 3D Modelling of HPV-Induced Cancers

According to the National Cancer Institute (NCI), major HPV-related cancers are anal cancer, cervical cancer, oropharyngeal cancer, penile cancer, vaginal cancer, and vulvar cancer. Also, other

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cancers such as colorectal cancer, skin cancer, and lung cancer may be related to HPV infection. 3D models of HPV-related cancer models are summarized in Table 1.

Cervical Cancers

When examining 3D printing articles mainly related to CVC, studies are aimed at analyzing cell behavior and simulations designed to visualize CVC. The second type of 3D CVC cancer model provides visual interaction between doctors and patients, facilitating surgical simulation for medical personnel [136,137]. However, in this review, we will focus on the studies of the first type. Zhao et al.'s (2014) study aimed to create cervical tumor models using HeLa cells and gelatin/alginate/fibrinogen hydrogels with 3D printing technique and reported the comparison of these models with traditional 2D cultures. The results indicated that printed 3D models offered successfully simulated tumor properties such as higher proliferation rate, cellular spheroid formation, elevated MMP protein expression, and chemoresistance, suggesting a broad application potential of the novel 3D cell printing technology in tumor biology and tumor heterogeneity studies [138]. Muniandy et al. (2021) successfully established 3D spheroids of CVC cell lines using the liquid overlay method, revealing distinct behaviors in different cell types: HeLa and CaSki formed compact spheroids while SiHa formed flat ones. HeLa and CaSki spheroids showed aggressive invasion, while SiHa spheroids displayed incremental growth and invasion [139]. Zuk and colleagues created 3D artificial tissues in de-epidermized dermal scaffolds using normal and cancerous cervical cell lines in their study in 2017. While the normal tissue model exhibited distinct epithelial layers and specific cytokeratin expressions, the cancer model demonstrated dysplastic changes and different cytokeratin expressions [140]. Additionally, De Gregorio et al. developed a novel organotypic model for cervical tumors and identified key molecules involved in the epithelial-mesenchymal transition process. The systematic characterization of stromal factors highlighted the significance of potential therapeutic targets and emphasized the importance of treatments targeting not only cancer cells but also the stroma [141]. Thippabhotla et al. (2019) compared extracellular vehicles derived from 2D and 3D cell cultures and found that 3D culture-derived extracellular vesicles (EVs) more closely resembled in vivo circulating EVs from CVC patients, suggesting a specific sorting process [64]. In the study conducted by Lin and colleagues (2024), it is demonstrated that C3 PLP2+ Tumor Epithelial Progenitor Cells play a significant role in cervical carcinoma in the generated 3D model, and the PLP2+ Tumor EPC score is shown to be an independent prognostic factor [142]. Another study was conducted by Jackson et al. (2020), their method involved the successful creation of a 3D culture by growing primary human epithelial cells on a dermal equivalent using collagen fibers, derived from both HPV-negative and HPV-positive oral and cervical tissues [143]. The studies conducted by Mao et al., on human CVC, including research on cell proliferation, epithelial-mesenchymal transition, and drug resistance, demonstrated that 3D culture models yield much more effective results compared to 2D culture systems [144].

Oropharyngeal Cancers

Miserocchi et al. (2021), who created a 3D model with a collagen-based scaffold using HPV-positive and HPV-negative oropharyngeal squamous cell carcinoma cells (OSCC), emphasized the in vitro model's ability to represent the different pathophysiological nature and drug sensitivity of the two tumor types [145]. Colley et al. (2011) investigated where three different in vitro models demonstrating the transition from dysplasia to in situ carcinoma histopathologically were utilized for the tissue engineering of oral mucosa. These models were further examined through in vivo comparisons. In this context, they demonstrated that when natural dermis was used, it was possible to culture dysplasia and tumor cell invasion *in vitro*, mimicking the morphological appearance and histological features observed in vivo [146]. In another study, Almela et al. (2018) printed a scaffold with suitable pore sizes in 3D using an injectable β-tricalcium phosphate paste. Human primary oral squamous cell carcinoma cells were seeded onto this scaffold, successfully establishing a short-term in vitro model, although not long-lasting [147]. In another study, Noi and colleagues (2018) used a commercially purchased 3D culture system. They suggest that signaling pathways are significantly

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altered only through this method, contributing to invadopodium formation and the development of oral cancer in experiments conducted within the 3D culture system [148]. In another study, Vincent-Chong and Seshadri (2020) emphasized the importance of strategies aiming to successfully identify the response of the subtype of oral cancer and sensitize tumors to radiation, stating that the 3D organoid platform is a valuable tool for screening radiosensitizers and investigating the efficacy of novel immunotherapeutic strategies [149]. Lei et al. developed a perfusion 3D cell culture microfluidic chip for real-time and non-invasive monitoring of cell proliferation and chemosensitivity. They demonstrated that oral cancer cells proliferation and chemosensitivity in 3D cell culture format can be monitored by impedance measurement [150]. In another investigation anticipating the prospective importance of personalized medicine, Igawa et al. (2023) have established a 3D oral cancer model incorporating oral cancer cells and normal oral fibroblasts [151].

In their study, Tanaka et al. (2018) established three well-characterized primary head and neck squamous cell carcinoma HPV-positive organoid lines, from which 2D cell lines can also be easily obtained. They demonstrated that by conducting drug resistance studies on cells derived from organoids, they could obtain the best possible response similar to the original tumor [152]. In another study, Sawant et al. (2016) created a 3D culture by embedding primary keratinocytes in a collagen matrix with fibroblasts and, for comparison, seeding them in a flat collagen matrix without growth factors, for coculture with fibroblasts. While the 3D coculture containing fibroblasts yielded results closer to in vivo conditions, they noted some limitations such as limited sample quantity due to being obtained from humans, contamination based on poor hygiene, and inter-donor variability [153].

Table 1. 3D models to mimic tumor and TME in HPV related cancers.

HPV-related cancer types	Culturing Model	Used Cells	ECM Matrix	References
CVC	Hydrogel	Hela cells	Gelatin/alginate/fibrinogen	[138]
CVC	Spheroid System	HeLa (HPV18), CaSki (HPV16), SiHa (HPV16), C33A (non-HPV), HT3 (non-HPV)	Bovine collagen I matrix	[139]
CVC	Scaffold	human keratinocyte cell line (NTERT), cervical cancer cell line (C33A)	Human skin (Euro Skin Bank, Netherlands)	[140]
CVC	Scaffold	SiHa (ATCC® HTB-35), Cervical CAF isolated from squamous intraepithelial lesion, and invasive cervical carcinoma biopsies	Fibronectin solution human fibroblasts	[141]
CVC	Hydrogel	Hela cells (ATCC® CCL-2™)	Peptide-based	[64]
CVC	Hydrogel	SiHa and Hela cells	Matrigel matrix (BD Biosciences, USA)	[142]
CVC	Scaffold	primary human cervical carcinoma	Collagen, glycosaminoglycan, and elastin	[144]
CVC and OSCC	Organotypic Raft	primary epithelial cells (human tonsillar keratinocytes or human cervical keratinocytes), J2 fibroblasts, Human fibroblasts hTERT	Collagen-fibroblast mixture	[143]
OSCC	Scaffold	UPCI:SCC090 (CRL-3239), UM-SCC-6 Squamous Carcinoma Cell Line	Type I collagen	[145]
OSCC	Scaffold	Normal human oral keratinocytes and fibroblasts were isolated from oral mucosa biopsies,	Collagen mixture and fibrin adhesive sealant	[147]

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		Primary alveolar human osteoblasts were isolated from aspirated waste bone chips		
OSCC	Spheroid System	D20, FaDu, DOK, Cal27 (CRL-2095) and, Normal oral keratinocytes and fibroblasts were isolated from biopsies	Human cadaver skin (Euroskin, Beverwijk, Holland)	[146]
HNSCC	Organoid	Individual patient's tumors	-	[152]
Human tongue squamous cell carcinoma	Organotypic Culture	Individual patient's tumors (keratinocytes and fibroblasts)	Collagen Matrix	[153]
Human tongue cancer tissue	Silica fibre sheet	HSC-3 and HSC-4	-	[148]
Human oral cancer	Microfluidic chip	OEC-M1	-	[150]
Human oral cancer	Hydrogel	Human primary normal oral fibroblasts human oral squamous carcinoma cell lines	Collagen Matrix	[151]

In addition to the major cancers affected by HPV, the 3D models of the cancer types that are minorly affected by HPV are as follows:

Colorectal Cancer

The organoid model of the mouse intestine developed by Sato et al. in 2009 has provided important progress in CRC organoid models. Sato et al. also improved their model in 2011 to adapt it for human small intestine and colon [154]. McGuckin et al. have achieved to produce the first long-term 3D CRC model. Abbas et al. (2023) found significant differences in cell proliferation, death phase profiles, tumor-related gene expression, and drug sensitivity in 3D colorectal cancer cultures compared to 2D cultures [155]. Another study on drug resistance was conducted by Koch et al. in 2021, which found that 3D colorectal cancer cells were more resistant to radiation and certain chemotherapy drugs compared to 2D cell cultures [156]. Magdeldin et al. (2014) developed and established a physiologically relevant model for colorectal cancer using HT29 and HCT116 cell lines, focusing on 3D in vitro model, gene expression profiles, oxygen and nutrient gradients, and pharmacokinetics [157].

Skin Cancer

Skin cancer can be categorized into two main types, nonmelanoma and melanoma, based on the histological characteristics of the malignant cells [158]. Berning et al. (2015) constructed a complex dermal equivalent using normal human dermal fibroblasts, enabling long-term epidermal regeneration. Furthermore, by incorporating cells from various stages of malignancy using inserts, they developed a human skin cancer model reflecting tumor-specific growth and invasion profiles [159]. In another study highlighting significant differences in treatment outcomes between 2D and 3D cultures, Vörsmann et al. (2013) developed an organotypic human skin-melanoma model [160]. In another study, they proposed a collagen-based membrane for early invasion investigations of human melanoma and various drug trials [161].

7. Limitations and Future Directions in 3D Model-Based Cancer Research

3D culture systems offer a significant opportunity for investigating cancer immunology; however, they also come with some limitations. The increased complexity of these systems can create challenges in replicating and interpreting results across experiments and within them. Additionally,

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compared to 2D culture systems, 3D culture systems can be more costly and less accessible. Researchers may struggle to capture images of 3D cultures by some microscopy techniques due to their depth and limited transparency. When it comes to studying immunological environment of cancer using 3D culture systems, it can be challenging to culture primary immune cells long-term in both 2D and 3D environments. Additionally, existing 3D models of the tumor microenvironment might not fully capture the complexity of human tumors, which often involve various immune and stromal cell populations. Accurately replicating this complexity and ensuring that immunotherapeutic agents can effectively penetrate the ECM are critical considerations [162]. The movement of immune cells is vital to their function and activity in the tumor microenvironment, so it must be a primary consideration in 3D cell culture assays [163]. Moreover, along with the multi-step interactions between various cell types, the extraordinary complexity of the immune system may limit the applicability of simple heterotypic or multicellular culture methods. Before starting 3D culture, a tumor model must be sufficiently characterized compared to the original tumor tissue to ensure accurate interpretation of results. Each experiment should include 3D models representing different regions of the tumor to mimic tumor heterogeneity. The availability of sufficient tissue material and ethical issues related to tumors taken from patients are among the difficulties of 3D models. A significant disadvantage is the inability to replicate ex vivo results from individual tumors in an in vivo environment. Therefore, large patient samples, as in clinical trials, are required to obtain reliable results.

The in vitro utilization of 3D models highlights the ability to provide a realistic simulation of the tumor microenvironment by replicating cellular heterogeneity, gene expression patterns, cell differentiation, hypoxia induction, activation of cell signaling pathways, and cell-cell/cell-ECM interactions. These models enable the in vitro assessment of various aspects of tumor biology such as chemoresistance, migration, and invasion, making them a valuable tool in cancer research [164]. Interestingly, tumor cells in some cancer patients exhibit intrinsic resistance to a wide spectrum of chemotherapeutic drugs without prior exposure to these cytotoxic agents [165]. This intrinsic drug resistance has been assigned to the increased expression of multidrug resistance (MDR) proteins by cancer cells [166,167]. Hypoxia [168], low nutrient availability, and low pH which are characteristics of tumor tissue [169] have been suggested to increase the expression of MDR proteins through specific cellular signaling pathways. While an MDR-permissive environment can be partially recreated in 2D cultures, the absence of 3D architectural context hinders the recapitulation and conservation of MDR behaviors [170].

Upon reviewing the literature, it is notable that particularly HPV-infected 3D cell cultures have not been extensively developed. Furthermore, studies on the microenvironment in many HPV-induced cancer types remain scarce. Looking ahead, we envision that the penetration of various drugs through 3D models, drug absorption according to ECM and TME, and assessment of potential drug candidates can be more accurately evaluated in HPV-induced tumors.

8. Conclusions

In recent years, cancer researchers have been emphasizing the importance of the 3D TME that influences tumor cell behavior. However, much of cancer research, especially in the field of drug screening, remains confined to the 2D cell culture paradigm. This review, as evidenced by various publications, has robustly demonstrated the superiority of 3D growth in modeling tumor cell behavior and drug response. Additionally, many studies indicate the need for more accurate modeling of tumor stroma, as the ECM serves as a regulator of tumor cell behavior and is one of the new cancer treatment targets. 3D in vitro culture systems successfully mimicking cancer cell behavior in vivo have become important tools for drug screening, angiogenesis, immunotherapy, and hypoxic conditions. 3D environments developed with high precision and accuracy, utilizing hydrogel materials containing various growth stimulants or inhibitors, may play a significant role in future cancer treatments.

On the other hand, despite gaining deeper insights into HPV-mediated cancer formation, many questions regarding virus-host interactions remain unanswered. Particularly, uncertainties persist

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regarding how HPV affects antitumoral immunity in the context of radiotherapy and immune checkpoint blockade. In this review, we have examined significant progress made in creating in vitro models that better reflect HPV-associated cancer types. We have focused on 3D models expected to more closely resemble the in vitro environment than the standard 2D settings used in many new models. We believe that in future cancers or non-cancerous diseases of all kinds, the penetration of various drugs through 3D models, the absorption of drugs according to ECM and TME, and potential drug candidates can be more accurately evaluated.

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