Chapter 2
Carcinogenesis and Personalization in HPV-Associated Precancer Lesions of the Cervix

Kei Kawana

Abstract Cervical cancer and other HPV-associated cancers are caused by persistent infection with high-risk HPVs, mainly HPV types 16 and 18. Persistent high expression of the *E6* and *E7* oncogenes of high-risk HPV is essential for cell immortalization and transformation into cancer. Therefore, therapeutic agents targeting HPV E6 and E7 have been developed. It is well-known that cervical intraepithelial neoplasia (CIN), a precancerous lesion of cervical cancer, and cervical cancer can be regulated by host immunity. CIN in particular often spontaneously regresses to normal, which is a result of the host's immune response to HPV viral proteins. Therefore, therapeutic vaccines that induce an immune response against HPV have been developed, but have not yet been commercialized. We have been developing therapeutic vaccines against precancerous lesions by applying the mechanism of mucosal immunity. By investigating the antigen expression and immunosuppressive factors involved in the induction of host immunity, we expect to personalize patients who will respond to HPV oncogene-targeting therapy or anti-HPV immunotherapy.

Keywords Cervical cancer · Cervical intraepithelial lesion · Human papillomavirus · Immunotherapy · Mucosal immunology · Carcinogenesis · HPV E6 and E7

2.1 Molecular Biology and Epidemiology of Human Papillomavirus (HPV) Infection

Human papillomavirus (HPV) is a small double-stranded DNA virus that infects only humans. HPV has eight genes: early genes *E1, E2, E4, E5, E6*, and *E7*, and late genes *L1* and *L2*. However, there are more than 200 genotypes of HPV, which are

K. Kawana (✉)
Department of Obstetrics and Gynecology, Nihon University School of Medicine, Tokyo, Japan
e-mail: kawana.kei@nihon-u.ac.jp

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classified according to the homology of the viral genes [1]. HPV infects the stratified squamous epithelium of the skin and mucosal epithelium, and its life cycle is completed only in the epithelium. In the parabasal cells, E6 and E7 are expressed and promote cell proliferation. In the middle layer, E1 and E2 promote replication of the viral genome. Near the surface, structural proteins L1 and L2 are simultaneously expressed to form the viral capsids. The L1/L2 capsids package the viral genome into new viral particles that are released into the vagina, along with a detachment of the stratified squamous epithelium. In latent infection, the viral DNA is bound to the genome of the host cell by a small amount of E2, and is transferred to dividing cells with the viral DNA during mitosis [2]. This mechanism allows HPV to continue to exist in the mucosal basal layer.

There have been many epidemiological studies on high-risk HPV associated with carcinogenesis. HPV testing is a method of detecting HPV DNA (viral genes) by diffusion amplification of DNA extracted from cervical exfoliated epithelial cells. It is possible to identify more than 30 high-risk HPV types. HPV does not cause viremia; therefore, antibody induction is weak and titer is low. The positive rate of HPV antibodies is about 50–70%, although it is difficult to know exactly. Based on the antibody positivity rate and HPV DNA detection rate, it is estimated that 50%–80% of all women are exposed to HPV at least once in their lifetime [3]. In a prospective study, Ho et al. collected vaginal washes from 608 female students at a university in the USA over a 3-year period and used PCR to detect HPV DNA in the suspended cells. Of the group who were positive for HPV DNA (43% of 608 students), 31% became negative within 6 months, 39% became negative within 6–12 months, 11% became negative within 12–18 months, and eventually about 90% of the HPV-DNA-positive group became negative within 2 years [4]. In Japan, the HPV-positive rate among pregnant women in their 20s is reported to be 20–30%, and the HPV-positive rate in Japan is equal to or higher than that in developed and emerging countries overseas. The HPV DNA test positivity rate among Japanese women by age is highest in teenagers, ranging from 30% to 40%. After that, the DNA positivity rate decreases with age to 20–30% in women in their 20s, 10–20% in women in their 30s, and 5–10% in women in their 40s [5].

2.2 Epidemiology of Cervical Cancer

According to the World Health Organization (WHO) announcement at the International Conference on HPV Vaccines held in Geneva in February 1999, the number of HPV carriers worldwide is estimated to increase by 300 million per year. Of these, about 30 million will develop low-grade squamous intraepithelial lesions (LSILs) annually. Lesions more advanced than precancers can be treated; therefore, it is difficult to determine the frequency of cervical cancer in the natural history of the disease, but cases are currently increasing by about 500,000 per year worldwide [6]. The distribution of HPV genotypes in cervical cancer is as follows. In squamous cell carcinoma, HPV16 accounts for about half of the cases, HPV18 is the second

most common, accounting for about 10%, followed by HPV45, 31, and 33. In contrast, in adenocarcinoma and adenosquamous carcinoma, HPV18 accounts for about 40% of cases and is detected to the same extent as HPV16 [3]. HPV18 is detected at a high rate in cervical adenocarcinoma. It has been shown that about 70% of all cervical cancers are caused by HPV16 and 18. However, HPV16 accounts for about 20% of HPV detected in women with no cytological abnormalities, which is a different distribution compared with that in cervical cancer. In Japanese data, the detection frequency of HPV16 and 18 in normal cervical cytology is just over 10%, and HPV52 and 58 are predominant [7]. This indicates that HPV16 and 18 have characteristics that predispose them to invasive cancer. According to the International Agency for Research on Cancer, the odds ratio of developing cervical cancer if any HPV type is detected is 158 times higher than if HPV is not detected. The odds of developing cervical cancer are 434 times higher when HPV16 is detected than when it is undetected, and 248 times higher when HPV18 is detected. This clearly indicates a higher risk of cervical cancer when HPV16 and 18 are detected [3].

2.3 Molecular Biological Mechanisms of HPV Carcinogenesis and the Potential for Therapeutics Discovery Targeting these Mechanisms

The mechanism of HPV-induced carcinogenesis has been intensively studied since the 1980s using molecular biological techniques (Fig. 2.1). The molecular biological changes in HPV-infected cells correlate with the pathological development of cervical intraepithelial neoplasia (CIN). In particular, the fact that squamous

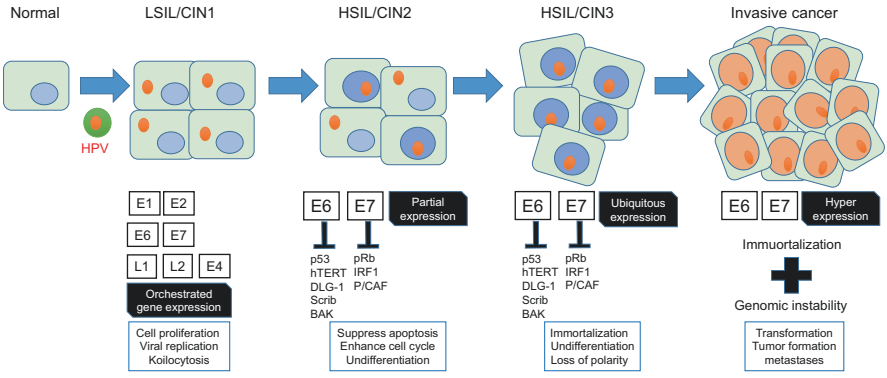

Fig. 2.1 Carcinogenesis of HPV-associated precancer lesions. Squamous neoplastic lesions of the cervix can now be broadly classified pathologically into low- and high-grade squamous intraepithelial lesions (LSILs and HSILs) may be the result of a better understanding of the steps in HPV carcinogenesis. Interestingly, the molecular biological changes in HPV-infected cells correlate with the pathological development of cervical intraepithelial neoplasia (CIN)

neoplastic lesions of the cervix can now be broadly classified pathologically into low- and high-grade squamous intraepithelial lesions (LSILs and HSILs) may be the result of a better understanding of the steps in HPV carcinogenesis. The LSIL→HSIL→squamous cell carcinoma sequence shown in Fig. 2.1 is a common oncogenic process. In contrast, glandular lesions often progress directly from HPV infection to adenocarcinoma in situ and/or invasive adenocarcinoma. In the Bethesda system of cytological diagnosis, the abnormal finding of atypical gland cells (AGC → adenocarcinoma) can be interpreted as rapid progression.

Here, we discuss the general mechanism of HPV carcinogenesis, and the LSIL shows the morphological change of HPV-infected cells with koilocytosis. In LSILs, HPV expresses E6 and E7 proteins to allow infected cells to proliferate near the parabasal layer (lower third of the epithelium) while maintaining squamous differentiation for viral proliferation. E1 and E2 are expressed for replication of the viral genome and L1 and L2 for the synthesis of the viral particle capsid. These viral proteins are expressed in an orderly fashion as the stratified squamous epithelium differentiates. As mentioned above, HPV can always be retained in the basal cells, and the viral genome is always supplied by the basal cells, so HPV does not disappear by shedding [2]. In HISLs, the orderly expression pattern of the virus is lost in some infected cells, and the cancer proteins E6 and E7 are highly expressed. E6 inactivates such enzymes as p53 and hTERT. E7 inactivates Rb protein and keeps the cell cycle going by activating histone deacetylase and cyclin. These functions inhibit apoptosis, enhance the cell cycle, and inhibit squamous cell differentiation. The result is the transformation of infected cells into immortalized cells. CIN2 can be described as a mixture of virus-infected cells and neoplastic cells, and the neoplastic cells can be distinguished from virus-infected cells by their different histopathological features [8]. In CIN3, immortalized neoplastic cells almost completely replace the epithelial lining, and infected cells and cells with koilocytosis are absent from the epithelium. In this state, the squamous epithelium is no longer differentiated, parabasal-like cells with a high N/C ratio proliferate abnormally, cell polarity is disrupted, and histological architecture is lost. These morphological changes have been proven molecularly to be the actions of E6 and E7 oncoproteins [1].

We have studied the agent of antisense RNA encapsulated in polymeric nanomicelles as a molecular targeted therapy to inhibit E6 and E7 expression by nucleic acid therapeutics [9]. This is a therapy in which siRNA specific for HPV E6 and E7 is administered to inhibit transcription of E6/E7 by RNA interference. The target molecule is an exogenous viral protein; therefore, the effect on normal tissues is low, unlike for ordinary molecular targeted therapies. siRNA-based transcriptional repression of viral genes can be observed in cultured cells in vitro, but there is a problem with drug delivery for human application. Therefore, in collaboration with Kazunori Kataoka and Kanjiro Miyata of the University of Tokyo, using nanotechnology, we have produced a therapeutic agent containing siRNA targeting E6/E7 (E6/E7 siRNA) encapsulated in polymeric micelles [10]. E6/E7 siRNA-encapsulated polymeric micelles were prepared for HPV16 and HPV18. To confirm the inhibitory effect of siRNA on E6/E7 expression in vivo, E6/E7 or control siRNA-encapsulated polymeric micelles were intravenously injected into nude mice

transplanted with cervical cancer cells. HPV16 E6/E7 siRNA-encapsulated polymeric micelles suppressed growth of HPV16-positive cervical cancer cell line (SiHa cell) tumors by about 80%, and HPV18 E6/E7 siRNA-encapsulated polymeric micelles suppressed growth of HPV18-positive cervical cancer cell line (HeLa cell) tumors by about 70%, compared with control micelles. The E6/E7 mRNA level in the tumor was significantly decreased in the E6/E7 siRNA polymeric micelle group. Moreover, in the excised tumors, p53 was rescued in a dose-dependent manner by E6/E7 siRNA polymeric micelles [9]. The safety of polymeric nanomicelles in humans has been confirmed for anticancer and granulocyte colony-stimulating factor agents, and the antitumor effect has been confirmed for intravenous administration, which can be used in clinical practice. The manufacturing process for micelles is simple. In the future, there is a possibility that this technology will be developed into a molecular therapy targeting HPV E6/E7.

2.4 HPV-Associated Carcinogenesis from the Viewpoint of Morphology and the Possibility of Drug Discovery Targeting These Mechanisms

High-risk HPV-associated cancers have been reported to include cervical, anal, vulvar, vaginal, and oropharyngeal cancers, of which, cervical cancer is caused by HPV with most highly rate (about 95%). About 70% of cervical cancer and about 90% of anal, vulvar, vaginal, and oropharyngeal cancers are caused by HPV16 or 18. Most HPV-associated carcinogenesis outside the cervix is caused by HPV16 and 18, and HPV16 and 18 are by far the most likely HPV types to cause cancer [3]. In other words, the cervix is the only organ that is uniquely susceptible to cancer caused by high-risk HPVs other than HPV16 or 18. High-risk HPV, in contrast, infects every part of the external genitalia: cervix, vagina, and vulva. Why is cancer more likely to develop in the cervix than in the other external genitalia? These questions can be answered as follows. One of the reasons is the presence of the squamocolumnar junction (SCJ) at the cervix. Tissue stem cells called reserve cells are localized in the SCJ. Reserve cells are referred to as stem cells because they have the pluripotency to differentiate into squamous and glandular epithelium, and have the ability to self-renew. HPV infection and CIN tend to occur in the SCJ or transformation zone, which means that HPV has a propensity to infect self-renewing, pluripotent cells in the SCJ. The monolayer structure of SCJ cells allows for easy entry without the need for deep wounds. SCJ cells also have the ability to differentiate by squamous stratification (squamous metaplasia), which is thought to be induced by HPV [11] (Fig. 2.2a). HPV is able to replicate itself through stratified squamous epithelium, and its dormancy and ability to self-renew allow for repeated latency and viral proliferation. The SCJ is an ideal place for HPV to infect.

When tissue stem cells in the SCJ undergo malignant transformation through HPV carcinogenesis, they have the properties of cancer stem cells (Fig. 2.2b). As a result, they are likely to develop quickly and highly into invasive cancer cells. One of

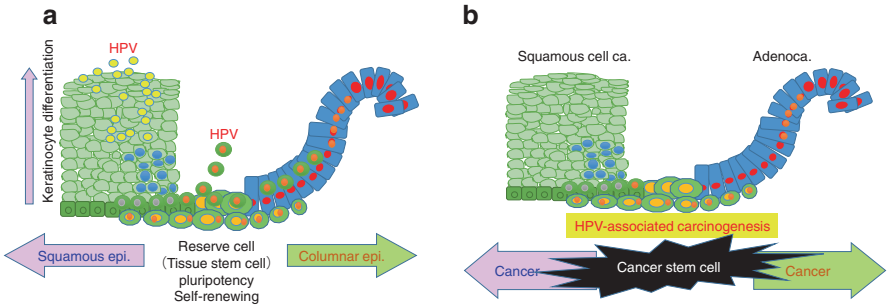

Fig. 2.2 HPV-associated tissue stem cell (reserve cell) and cancer stem cell in the cervix. (**a**) Tissue stem cells called reserve cells are localized in the squamocolumnar junction (SCJ) at the cervix and referred to as stem cells because they have the pluripotency to differentiate into squamous and glandular epithelium, and have the ability to self-renew. SCJ cells also have the ability to differentiate by squamous stratification (squamous metaplasia), which is thought to be induced by HPV. (**b**) When tissue stem cells in the SCJ undergo malignant transformation through HPV carcinogenesis, they have the properties of cancer stem cells. HPV-infected reserve cells are pluripotent, meaning development of squamous cell carcinoma and adenocarcinoma. Stemness of the cancer cells have been identified as a specific mechanism of HPV carcinogenesis in cervical cancer

the reasons why cervical cancer occurs at a young age may be that cervical cancer arises from the SCJ, which has cells that have stem cell features. Furthermore, HPV-infected reserve cells are pluripotent, meaning that a change in the squamous cells results in the development of squamous cell carcinoma, and a change in the glandular cells leads to development of adenocarcinoma. Stem cells and cancer stem cells have been identified as a specific mechanism of HPV carcinogenesis in cervical cancer; therefore, development of therapeutics targeting these stem cell features is expected.

We have succeeded in artificially generating reserve cells as stem cells in the SCJ, and have established a technique to differentiate induced pluripotent stem cells through intermediate mesoderm cells to reserve-cell-like cells called induced reserve cells (iRCs). iRCs are pluripotent cells that can differentiate into both glandular and squamous epithelium in 3D culture and are positive for stem cell markers, Mullerian duct markers, and SCJ markers, making them reserve cell-like [12]. We also have generated iRCs with E6 and E7 oncogenes of HPV16 and 18, and are performing RNA sequencing analysis. By analysis of HPV16 and 18 E6/E7-positive iRCs that maintain stemness, we are exploring new targets based on the gene expression profiles that show characteristics of cancer stem cells.

2.5 Drug Discovery and Development of Immunotherapy Targeting HPV-Associated Cancers

E6 and E7 proteins of HPV are both initiators and promoters of carcinogenesis from HPV infection to cervical cancer (Fig. 2.1). E6 and E7 are ubiquitously expressed in cervical cancer, precancer, and other HPV-associated cancers, and are necessary for maintenance of malignant transformation. E6 and E7 are immunogenic in

humans and are presented as antigens on the surface of cervical cancer and precancer cells, making them so-called cancer antigens. Furthermore, because they are viral proteins, they do not affect normal host cells. HPV E6 and E7 oncoproteins are cancer antigens specific to HPV-associated cancers and are promising target molecules for immunotherapy. Immunotherapies targeting these proteins have been developed over the past two decades; however, none is clinically applicable.

Recently, the expression of immune checkpoints, programmed death (PD)-1/PD ligand (PD-L)1, and MSI-high tumors in cervical cancer have been found, and immunotherapy using immune checkpoint inhibitors (ICIs) has been reconsidered [13]. PD-L1 is upregulated by HPV E5, E6, and E7, and expression of PD-1 and PD-L1 in cervical cancer or CIN is enhanced [14–16]. ICIs, anti-PD-1 and anti-PD-L1, will be combined with HPV E6/E7-targeted cancer immunotherapy and these biomarkers may be used for personalization in HPV-associated cancer or precancer lesions.

2.6 Reason for Promising Immunotherapy for Precancer Lesions CIN2/3

The only treatment available for early cervical cancer and its precancerous lesions, which peak in women in their 20s and 30s, is surgery. At present, there is no treatment available for CIN2/3. Total hysterectomy terminates fertility, and conization is associated with poor perinatal outcomes of subsequent pregnancies. The preterm birth rate increases about threefold in pregnancies after conization, as do the rates of cesarean delivery and low birth weight [17]. The age of patients with CIN3 often coincides with the age at which they become pregnant and give birth; therefore, the increased rate of perinatal complications caused by treatment is a major problem.

In HPV-associated precursor lesions, including precancer, spontaneous regression induced by the host immune response to HPV proteins is often observed because of viral carcinogenesis. Many cohort studies of CIN lesions have demonstrated that within 2 years of follow-up, about 60% of CIN2 and 20% of CIN3 spontaneously regress [18, 19]. This is thought to be a result of the spontaneous induction of host cellular immune responses, mainly against E6 and E7 proteins, in patients with CIN2/3. Research on the application of this natural regression to immunotherapy with therapeutic vaccines has been initiated worldwide since the 1990s.

A large number of clinical trials (Phase I–III) have been conducted on HPV-targeted immunotherapy in CIN2/3. Molecular targets of these studies are mostly the E7 or E6/E7 proteins [20]. In prior clinical trials, vaccine antigen was administered by intramuscular or subcutaneous injection to induce E6/E7-specific cell-mediated immunity in the peripheral blood. However, the immune response does not always correlate with clinical efficacy, and none of the therapeutic vaccines has been commercialized at this time. The most advanced therapeutic vaccine currently in development is VGX-3100 (Inovio Inc.), which is a plasmid DNA vaccine that is injected intramuscularly [21]. In a phase IIb study of 167 patients with HPV16-positive CIN2/3, the rate of histopathological regression and viral clearance was 40% in the VGX-3100 group and 12% in the placebo group in modified intention to treat

analysis. Although there was a significant difference in efficacy, inoculation site adverse events occurred in 98% of patients. In addition, the website reports the results of a phase III study of 201 patients with CIN2/3 as a modified intention to treat population. The primary endpoint was a histologically confirmed LSIL/normal that was negative for HPV16 and/or HPV18 DNA at week 36. The percentage of patients with LSIL/normal and viral clearance was 22.5% with VGX-3100 and 11.1% with placebo, which was not significantly different. Comparison of the primary endpoint results of the phase IIb and III studies showed that the efficacy of VGX-3100 was lower in the phase III trial, although the results in the placebo group were similar. At this point, no promising therapeutic candidates for CIN2/3 have been developed.

2.7 Development of Mucosal Immunotherapy for CIN2/3 Based on Histopathogenesis of CIN2/3

We focused on the fact that CIN2/3 is a mucosal lesion, and developed immunotherapy using the mucosal immune system (called mucosal immunotherapy). In mucosal immunity of the cervical epithelium, gut-associated lymphoid tissue (GALT), including Peyer's patches or mesenteric lymph nodes in the intestinal mucosa, is an organized inductive tissue (Fig. 2.3). Mucosal T cell precursors are primed to produce antigen-specific helper and killer T cells, and are imprinted for

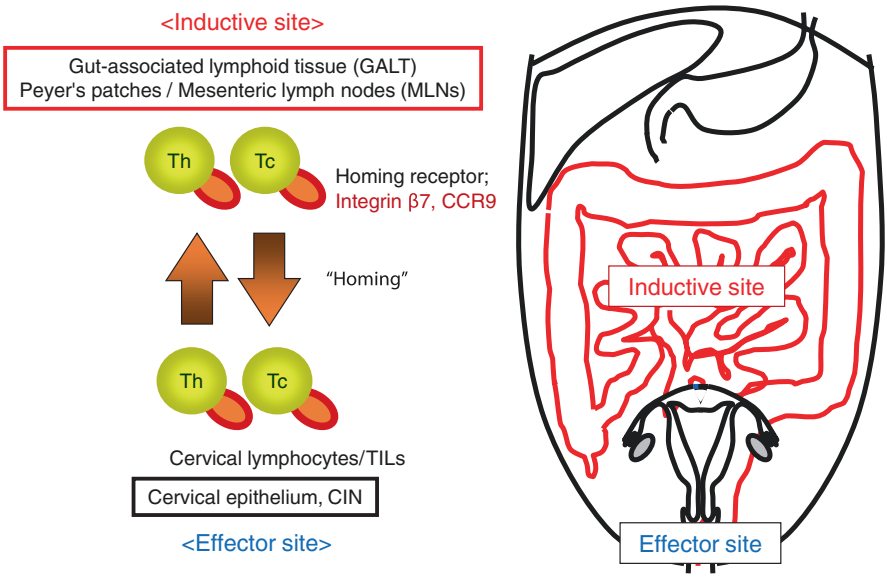

Fig. 2.3 Mucosal immune system in the cervix. In mucosal immunity of the cervix, gut-associated lymphoid tissue (GALT), including Peyer's patches or mesenteric lymph nodes in the intestinal mucosa, is an organized inductive tissue. Mucosal T cell precursors are primed to produce antigen-specific helper and killer T cells, and are imprinted for homing receptors (integrin β7 and CCR9) and T helper (Th)1/Th2 polarization by dendritic cells in GALT. The primed and memory T cells "homing" to the effector sites are activated by antigen stimulation in the mucosal lesions of the cervix

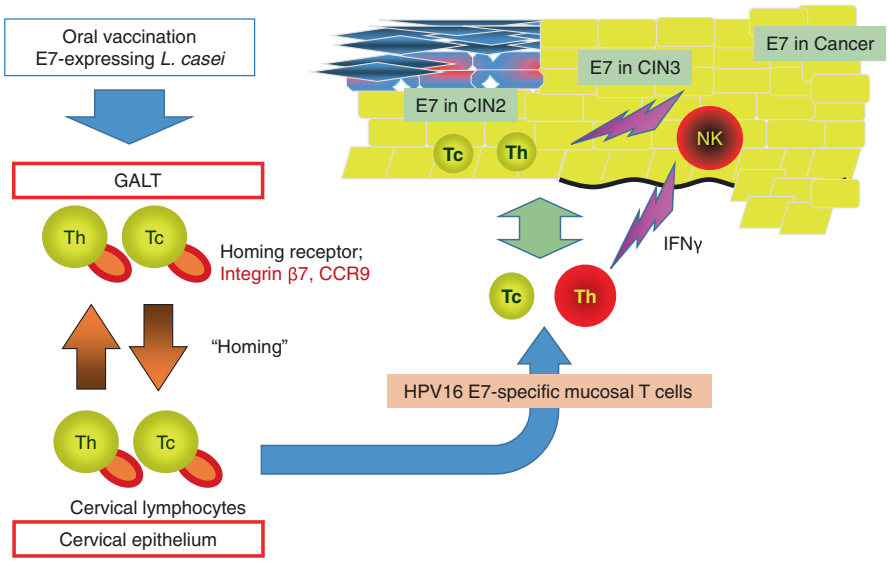

Fig. 2.4 Putative pharmacological effects of oral vaccination with E7-expressing *L. casei*. The orally administered E7-expressing *Lactobacillus*-based therapeutic vaccine are taken up by GALT from the intestinal tract. Mucosal T cells are primed to produce E7-specific immune cells in GALT and home to the cervical mucosa; E7 expressed in CIN2–3 is recognized and TH1 immune responses are elicited, resulting in antitumor effects including NK activity through TH1 cytokines

homing receptors (integrin β7 and CCR9) and T helper (Th)1/Th2 polarization by dendritic cells in GALT. The primed and memory T cells homing to the effector sites are activated by antigen stimulation in the mucosal lesions of the cervix.

Gut-derived mucosal T cells infiltrate CIN as tumor-infiltrating lymphocytes. We found that 20s–40% of the lymphocytes present in the cervical epithelium of patients with CIN were gut-derived integrin $\beta7^+$ and $CCR9^+$ T cells, and CIN was more likely to regress to normal when their content was high [22]. Thus, infiltration of integrin $\beta7^+$ and $CCR9^+$ T cells (especially Th1 T cells) primed and imprinted in the intestinal mucosa contributes to spontaneous regression of CIN.

We hypothesize that by administering cancer antigen HPV E7 to the intestinal mucosa, inducing Th1-type mucosal immunity against E7 by GALT, and homing gut-derived mucosal T cells to the cervical epithelium, E7-specific mucosal Th1 cells recognizing E7-expressing CIN2/3 produce interferon-γ and Th1 responses, including NK cell activity, leading to antitumor activity (Fig. 2.4). We generated *Lactobacillus casei* expressing HPV16 E7 (code name: GLBL101c) as a vaccine antigen for cancer immunotherapy. We confirmed the killer activity of mucosal T cells against HPV16 E7-expressing epithelial (TC-1) cells in preclinical studies in mice [23]. After this, a proof-of-concept clinical study was conducted at the University of Tokyo Hospital under the approval of the Institutional Review Board. The patients had histopathologically confirmed CIN3 that was positive for HPV16 alone, and received GLBL101c orally once a day for 5 days/week for weeks 1, 2, 4,

and 8. For all 17 patients, there were no adverse events of grade 2 or higher, and no grade 1 adverse events were causally related. The clinical efficacy of GLBL101c in the 1.0–1.5 g/day group was 61.5% regression to CIN2 (partial response; PR) at 9 weeks and 38.4% regression to CIN1/normal (complete response; CR) at 12 months from the start of treatment. Since the estimated rate of spontaneous regression of CIN3 is about 10% per year, the regression rate of our study was clearly higher than that of spontaneous regression. In addition, the group with CR/PR had higher induction of E7-specific interferon-γ-producing cells in the cervical epithelium than the nonregressed group had [24].

We conducted a randomized, double-blind, placebo-controlled trial of GLBL101c in CIN2 patients who were positive for HPV16 alone. However, the results showed limited efficacy for CIN2, suggesting higher efficacy may be expected for CIN3, in which E7 is more abundantly expressed than in CIN2 [25]. Compared with the placebo group, there was no difference in adverse events, and safety was confirmed by the second clinical study.

The first-generation, E7-expressing, *Lactobacillus*-based therapeutic vaccine, GLBL101c, used in these two exploratory clinical studies was considered to have limited pharmacological efficacy. Therefore, we developed a next-generation agent (code name: IGMKK16E7) with several times higher E7-specific Th1 immune responses [26]. Then, a phase I/II investigator-initiated clinical trial of IGMKK16E7 was conducted. This was an intergroup, parallel, randomized placebo-controlled trial (mucosal immunotherapy using *Lactobacillus* for treatment of squamous intraepithelial lesion: MILACLE trial) of four groups: placebo, low-dose, medium-dose, and high-dose IGMKK16E7 in HPV16-positive CIN2/3. The target number of enrolled patients was 164 (124 with CIN3 and 40 with CIN2). The primary endpoint was histopathological regression to normal (CR) or CIN1 (PR) at week 16 after the start of treatment [27]. This clinical trial has already finished enrolling patients, and the final analysis is scheduled to be published in Summer 2022.

For immunotherapy of HPV-associated cancer or precancer lesions, expression of viral oncoproteins and/or immunosuppressive biomarkers, including immune checkpoints, could be used to personalize treatment. It is expected that the immune response (pharmacological effect) will differ among patients depending on the expression level of the target molecule of immunotherapy. CIN2/3 lesions with higher E7 expression levels are expected to induce a stronger E7-specific Th1 response, resulting in a higher therapeutic effect. If the expression of immune checkpoints such as PD-L1 is increased by HPV E6/E7, immunosuppression may be enhanced. In this case, a combination of ICIs may be effective. Thus, the indications for immunotherapy and combination with ICIs can be personalized according to the characteristics of each CIN2/3 patient.

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Conflicts of Interest GLOVACC Inc. gifted GLBL101c, IGMKK16E7, and placebo, and partially supported our clinical trial.

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