Roles of the PDZ-binding motif of HPV 16 E6 protein in oncogenic transformation of human cervical keratinocytes

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Key words
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Infection with specific human papillomaviruses (HPVs), such as HPV-16 and -18, is a major risk factor for human cancer of uterine cervix. From the fact that E6 and E7 genes are almost exclusively expressed in cervical cancer cells and that they can inactivate tumor suppressors, p53 and pRB, respectively, they are believed to play key roles in cervical carcinogenesis. In addition to inducing p53 degradation, numerous studies have indicated that the E6 protein has many other targets. The C-terminal PDZ-binding motif is specifically conserved among E6 proteins of high risk HPVs, and is essential to bind and enhance degradation of several PDZ domain-containing proteins, including DLG1, DLG4, SCRIB, MAGI1 and PTPN13.1–6 Accumulating lines of evidence suggest that the PDZ domain-binding motif is particularly important for transformation and tumorigenesis in cultured cells, transformation of primary human keratinocytes, and hyperplasia and carcinogenesis in E6-transgenic mice.1,7 and some of the PDZ proteins are known to have tumor suppressor functions.8–12 Interestingly, the E7 protein but not the E6 protein of Rhesus papillomavirus type 1 (RhPV1), which is closely related to HPV-1613 and causes anogenital malignancy in their host,14 has the PDZ-binding motif at the C-terminus, and interact with the cell polarity regulator PAR3,15 belonging to the same pathway of regulation as SCRIB and DLG1. Other viral oncoproteins such as HTLV1 Tax and Adenovirus E4ORF1 also conserve the C-terminal PDZ-binding motif, suggesting a role in their viral life cycle and oncogenic potential of these viruses.2

However, little is known about which PDZ domain-containing target(s) are important for HPV-induced cervical carcinogenesis. We previously demonstrated that transduction of oncogenic HRAS (HRASG12V) and MYC together with HPV16 E6E7 is sufficient for highly tumorigenic transformation of primary human cervical keratinocytes (HCKs).16 More recently, we found HRASG12V and HPV16 E6E7 is sufficient for tumorigenic transformation of HCKs.17 Having taken advantage of this system, we found the PDZ domain-binding motif plays an important role in tumorigenic transformation of...
primary HCKs, and further investigated which PDZ domain-containing target(s) are critical for HPV-induced cervical carcinogenesis.

Materials and Methods

Cell culture and cell lines. Normal human cervical keratinocytes were obtained with written consent from patients who underwent abdominal surgery for a gynecological disease other than cervical cancer. HCK1, HCK4 and HCK12 were used in this study. These cells were established by transduction of human TERT into HCK1, HCK4 and HCK12 cells, and maintained in low-calcium serum-free keratinocyte-growth medium (KGM) (Epilife-KG2, KURABO Industries, Ltd, Osaka, Japan). Cervical cancer cell lines, HeLa, CaSkii, SiHa, C33A, OMCD4 and Yumoto were maintained in Dulbecco’s modified Eagle medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). All cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C.

Vector construction and retroviral infection. Construction of the retroviral expression vectors, pCLXSH-hTERT, pCLXSN-16E6, pCLXSN-16E7, pCLXSN-16E6, and its mutant has been described previously. (Table S1) Similarly, pCLXSN-6E6, -11E6, -18E6, -26E6, 30E6, -31E6, -33E, -35E6, -39E6, -43E6, -45E6, -52bE6, 54E6, -58E6, -59E6 were prepared by using the Gateway system so that each construct contains Kozak consensus sequence and E6 open reading frame. The retroviral expression vectors has been described previously.(20) The construction of the puromycin resistant gene in pCMSCVpuro with a segment containing blasticidin-S resistant gene. The construction of the Gateway system so that each construct contains Kozak consensus sequence and E6 open reading frame. Human HRASG12V cDNA in pBabe-puro-HrasG12V (a gift from Dr. Hahn WC) was cloned and recombined into retroviral expression vectors to generate pCMSCVbsdHRASG12V. pCMSCVbsd was produced by replacing the puromycin resistant gene in pCMSCVpuro with a segment containing blasticidicin-S resistant gene. The construction of the destination vector pDEST-SI-CMCSVpuro, the entry vector pENTR-HIR-stuffer and the short-hairpin RNA (shRNA) retroviral expression vectors has been described previously.(20) The targeted sequences for MAGI1, SCRIB, DLG1, DLG4, PAR3 and firefly luciferase were listed in Table S2. The production of recombinant viruses and selection of infected HCKs were detailed earlier.(16)

Western analysis. Whole-cell proteins were extracted in lysis buffer (0.5% NP40, 1 mM DTT, 50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.02% NaN₃) supplemented with 5% (v/v) protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan). Gels were loaded with 20 μg of total cell lysate per lane as described previously. (19) A monoclonal antibody to HPV16E6 (clone 47A4) was generated by using the N-terminal 16 amino acids peptide of HPV16E6 as an antigen. All other antibodies were purchased as follows: monoclonal antibodies against HPV16E6 (clone 8C9; Invitrogen, Carlsbad, CA, USA), DLG4 (PSD95) (Upstate, K28/43), DLG1 (Santa Cruz, Dallas, TX, USA) and firefly luciferase were listed in Table S2. The production of recombinant viruses and selection of infected HCKs were detailed earlier.(16)

Whole-cell proteins were extracted in lysis buffer (0.5% NP40, 1 mM DTT, 50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.02% NaN₃) supplemented with 5% (v/v) protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan). Gels were loaded with 20 μg of total cell lysate per lane as described previously. (19) A monoclonal antibody to HPV16E6 (clone 47A4) was generated by using the N-terminal 16 amino acids peptide of HPV16E6 as an antigen. All other antibodies were purchased as follows: monoclonal antibodies against HPV16E6 (clone 8C9; Invitrogen, Carlsbad, CA, USA), DLG4 (PSD95) (Upstate, K28/43), DLG1 (Santa Cruz, Dallas, TX, USA) and firefly luciferase were listed in Table S2. The production of recombinant viruses and selection of infected HCKs were detailed earlier.(16) Western analysis. Whole-cell proteins were extracted in lysis buffer (0.5% NP40, 1 mM DTT, 50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.02% NaN₃) supplemented with 5% (v/v) protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan). Gels were loaded with 20 μg of total cell lysate per lane as described previously. (19) A monoclonal antibody to HPV16E6 (clone 47A4) was generated by using the N-terminal 16 amino acids peptide of HPV16E6 as an antigen. All other antibodies were purchased as follows: monoclonal antibodies against HPV16E6 (clone 8C9; Invitrogen, Carlsbad, CA, USA), DLG4 (PSD95) (Upstate, K28/43), DLG1 (Santa Cruz, Dallas, TX, USA) and firefly luciferase were listed in Table S2. The production of recombinant viruses and selection of infected HCKs were detailed earlier.(16)
CO₂ environment at 37°C. After 2 weeks, colonies were fixed, stained and counted as previously described.\(^{(20)}\)

**Colony formation in soft agar medium.** Cells were seeded at 5 × 10⁴ cells per 6-well plate in an appropriate medium. Colonies were counted after 3 weeks as previously described.\(^{(16)}\)

**Tumorigenesis in nude mice.** All surgical procedures and care administered to the animals were in accordance with institutional guidelines. A 100 µL volume of cells in a 1:1 mixture of Matrigel (BD Biosciences, San Jose, CA, USA) was subcutaneously injected into female BALB/c nude mice (Clea Japan Inc., Tokyo, Japan). The expression of human involucrin in all tumors was determined by Western blots with antibodies against human involucrin (clone SY5; Sigma) that do not react with mouse epidermis to confirm that the tumors were derived from implanted HCKs (data not shown).

**Results**

The C-terminal PDZ domain-binding motif of HPV16 E6 is critical for inducing enhanced proliferation, anchorage-independent growth and tumorigenic potential of HCK cells expressing E7 and HRAS\(^{G12V}\). Recently, we reported that introduction of HPV16 E6 and E7 (E6E7) and HRAS\(^{G12V}\) was sufficient for tumorigenic transformation of HCK1T cells, which are normal cervical keratinocytes transduced with hTERT, and primary normal cervical keratinocytes.\(^{(16)}\) In order to examine biological functions of E6 in HPV-induced cervical carcinogenesis, HCK1T cells expressing HPV16 E7 and HRAS\(^{G12V}\) were first established and then each of wild type and three mutants of HPV16 E6 were transduced into the cells. 16E6Δ151 is defective in binding to PDZ domains, 16E6SAT is defective in degradation of p53 and 16E6SATΔ151 is defective in both activities (Table S1). As expected, p53 and the downstream effector p21 were downregulated by 16E6 and 16E6Δ151 but not by 16E6SAT containing R85/P9A/R10T substitutions,\(^{(21)}\) and 16E6SATΔ151. On the other hand, DLG1, MAGI1, SCRIB and PTPN13 were downregulated by 16E6 and 16E6SAT but not by 16E6Δ151 and 16E6SATΔ151 (Fig. 1a,c). Interestingly, PAR3 was also decreased in parallel with downregulation of these reported E6 targets. Since the anti-16E6 monoclonal antibody (clone 47A4) raised against the N-terminal 16 amino acids is unable to detect 16E6SAT, which has mutations in the epitope, expression of these mutants were confirmed by RT-PCR (Fig. 1b, upper panel). These cell lines were characterized by proliferation in ordinary culture, clonogenic assay, anchorage-independent growth and tumorigenic potential in nude mice (Fig. 2). Wild type 16E6 induced enhanced proliferation of the cells, and the two mutants defective in p53 degradation showed profoundly reduced activity, whereas 16E6Δ151 showed intermediate activity (Fig. 2a). Similar tendency was observed in clonogenic assay (Fig. 2b,c). However, anchorage-independent growth capacity and tumorigenic potential were strongly reduced in the E6 mutants defective in binding to PDZ domains, whereas E6 SAT mutant clearly enhanced tumorigenic potential of the cells though not to the levels of...
wild type 16E6 (Fig. 2d–f). The results were confirmed by two independently established HCKs, HCK4T and HCK12T (Fig. S1a–c for HCK4T and d–f for HCK12T). Since the expression of cytokeratin 7, which is recently reported as a marker of cells-of-origin of cervical cancer,(22,23) was detected in HCK1T cells but not in HCK4T and HCK12T cells by western blotting (data not shown) and the tumorigenicity was severely impaired in HCK1T-E7-HRASG12V16E6A151 mutant, we considered HCK1T to be more relevant to analyze cervical carcinogenesis, and used in the following analyses.

Depletion of single PDZ domain-containing proteins partially restored the reduced anchorage-independent growth and tumorigenicity by 16E6A151 mutant. In order to examine the effect of knockdown of PDZ domain containing proteins on the tumor promoting ability of 16E6, we designed shRNAs specifically knock down each PDZ domain-containing proteins. These include DLG1, DLG4, MAGI1, SCRIB, PTPN13 and PAR3. Among a few different shRNAs to each target tested, the most potent shRNAs were chosen for transduction into HCK1T cells expressing E7, HRASG12V and 16E6A151 (Fig. 3a). Knockdown of SCRIB, MAGI1 and PAR3 significantly enhanced anchorage-independent growth, while that of DLG1, DLG4 and PTPN13 showed only marginal effects. However, effect of any single shRNA did not completely restored the reduced activity of 16E6A151 (Fig. 3b). Consistent with these results, tumorigenicity of the cells was also significantly restored by depletion of SCRIB, MAGI1 or PAR3, while weakly by depletion of DLG1, DLG4 or PTPN13 (Fig. 3c). However, the restoration of tumorigenicity of HCK1T-E7-HRASG12V-16E6A151 by single knockdown of SCRIB, MAGI1 or PAR3 was partial, indicating that reduction of single PDZ domain containing protein by E6 is not sufficient for its ability to promote tumor growth.

Depletion of MAGI1, SCRIB and PAR3 cooperatively enhanced anchorage-independent growth and tumorigenicity of HCKs expressing E7, HRASG12V and 16E6A151. Since the wild-type E6
protein inactivates multiple PDZ-domain containing proteins simultaneously, we tried to examine the effect of concurrent depletion of multiple target proteins. To this end, we transduced representative combinations of shRNAs doubly or triply at the multiplicity of infection of 5. Depletion of the target proteins was confirmed by Western blot (data not shown). Every pair of MAGI1- SCRIB- and PAR3-shRNA examined cooperatively enhanced anchorage-independent growth of the cells, and combination of these three shRNAs further enhanced the anchorage-independent growth to the level that wild type 16E6 could induce (Fig. 3b). In addition, the combination of SCRIB-, DLG1- and DLG4-shRNA resulted in similar level as the wild type E6 did. The tumorigenicity of these cells were then examined in nude mice. The combination of MAGI1- and SCRIB-shRNA cooperatively enhanced the tumor growth more than any single shRNA did, however not to the level as the wild type 16E6 (open square and closed circle, respectively in Fig. 3d). Similarly, triple knockdown of DLG1, DLG4 and SCRIB shRNA enhanced the tumor growth more than any single shRNA did but did not fully restore the wild type level. Notably, triple knock-down of MAGI1, SCRIB and PAR3 surpassed the tumor growth induced by wild type 16E6 (closed triangle in Fig. 3d).

PAR3 downregulation by E6 is not dependent on E6AP. Since we found that HPV16 E6 reduced PAR3 level in the PDZ-binding motif-dependent manner (Fig. 1) and depletion of PAR3 had the strongest effect to enhance tumorigenicity of HCKIT cells expressing HRASG12V and 16E6A151 (Fig. 3), we examined whether PAR3 is downregulated in cervical cancer cell lines. Interestingly, in HPV-positive cervical cancer cells, PAR3 were generally reduced in compared to normal keratinocytes such as HCKIT and HaCaT cells and HPV negative cervical cancer cells including C33a, OMC4 and Yumoto cells, similar to DLG4, MAGI1 and p53 (Fig. 4a). Several reports suggested that degradation of not only p53 but also some of PDZ-domain containing proteins by HPV16E6 was mediated by E6AP(4,24,25). Therefore, we examined whether the reduction of PAR3 in HPV positive cervical cancer cells is dependent on the ubiquitin ligase, E6AP or not. Upon depletion of E6AP, levels of p53 but not PAR3 were increased in HeLa and SiHa cells (Fig. 4b). These results indicate that PAR3 is a direct target of HPV16E6. In this study, the anchorage-independent growth to the level that wild type E6 did. The tumorigenicity of these cells were then examined in nude mice. The combination of MAGI1- and SCRIB-shRNA cooperatively enhanced the tumor growth more than any single shRNA did, however not to the level as the wild type 16E6 (open square and closed circle, respectively in Fig. 3d). Similarly, triple knockdown of DLG1, DLG4 and SCRIB shRNA enhanced the tumor growth more than any single shRNA did but did not fully restore the wild type level. Notably, triple knock-down of MAGI1, SCRIB and PAR3 surpassed the tumor growth induced by wild type 16E6 (closed triangle in Fig. 3d).

Discussion

We first confirmed downregulation of the PDZ-domain containing proteins, which were reported to be targets of HPV16 E6, in normal cervical keratinocytes, HCKIT cells, by transduction of HPV16 E6 in the C-terminal PDZ-binding motif-dependent manner. Thereby we found that PAR3 was also downregulated by transduction of HPV16 E6. In this study, the C-terminal deletion mutant of E6, E6aΔ151, showed markedly reduced ability to induce anchorage-independent growth and tumorigenicity compared with wild-type E6 in corporation with E7 and HRASG12V. Surprisingly, another mutant of E6, E6SAT, which specifically lacks a function to target p53 for degradation, showed only marginally reduced transforming abilities compared to wild-type E6. The results clearly indicate the importance of the C-terminal PDZ-binding motif of E6 in transformation of cervical keratinocytes.

Then we found that single knockdown of SCRIB, MAGI1 or PAR3 significantly increased the anchorage-independent growth of HPV such as HPV43 and 54. Surprisingly, PAR3 was most strongly reduced by E6 protein of HPV54, but not at all by that of HPV6 and 11 (Fig. 4c).
and tumorigenic ability of HCK1T cells expressing HRAS<sup>G12V</sup>, HPV16 E7 and HPV16 E6Δ151, and the combinatorial knockdown of SCRIB and MAGI1 markedly restored the tumorigenic ability. Additional knockdown of PAR3 further enhanced both anchorage-independent growth and tumorigenicity which is rather higher than that induced by wild type HPV16 E6. Though RhPV1 E7 but not E6 conserves PDZ-binding motif at the C-terminus and targets PAR3 for degradation<sup>12</sup> and HPV18 E6 can bind and induce mislocalization of PAR3 protein in a PDZ-dependent manner,<sup>26</sup> PAR3 has not been reported as a degradation target of E6 proteins of high-risk HPVs. We found levels of PAR3 protein were decreased in HCK1T cells by HPV16 E6 in the C-terminal PDZ-binding motif dependent manner. Furthermore, the E6 proteins of many other HPVs with the exception of those of HPV6, 11, 45 also induced reduction of PAR3 in HCK1T cells.

E6 proteins of HPV16 and 18 target similar but different sets of cellular proteins with different specificities.<sup>27</sup> Not only a group of HPVs including high-risk mucosal HPVs, but also some other HPVs such as HPV40 can target a similar set of PDZ proteins and can disturb epithelial polarity.<sup>28</sup> Interestingly, CRPV E6 can also associate with DLG1 through the C-terminal PDZ-binding motif which is required for transformation of NIH3T3 cells.<sup>29</sup> Thus, papillomaviruses in different strategies, indicating evolutional importance of this cradle has evolved to target similar set of proteins with different strategies, indicating evolutional importance of this function in the viral life cycle. These target proteins have an important role in epithelial polarity which is maintained by interdependent control of three complexes, PATJ complex, SCRIB complex and PAR3 complex.<sup>30</sup>

In transformation of mouse primary tongue epithelial cells, inactivation of PTPN13 has almost equivalent function to HPV16 E6 in cooperation with Ra<sup>G12V</sup> (<sup>L</sup>PTPN13 (FAP1) is suggested as a potential E6 target when DLG1 was identified as the first example of the E6 target.<sup>1</sup> In colorectal cancers, mutations of many protein tyrosine phosphatases including PTPN13 were identified.<sup>31</sup> However, knockdown of PTPN13 had only marginal effects on anchorage-independent growth as well as tumor growth (Fig. 3b,c). Thus, relevant target(s) of HPV16 E6 might depend on cell types. HPV16 is a causative agent for development of not only cervical cancer but also oral squamous cell carcinomas including tongue cancer. Thus it will be intriguing to examine whether PTPN13 functions as a tumor suppressor in human tongue keratinocytes.

We found downregulation of PAR3 by E6 proteins of HPV16. HPV18 E6 was reported to induce mislocalization of PAR3 and to inhibit tight junction formation without reducing the protein levels in 293T and HaCaT cells.<sup>26</sup> In our study, PAR3 levels were clearly decreased by HPV16 E6 at least partly in the PDZ-binding motif-dependent manner. Since depletion of E6AP did not recover the expression of PAR3 and proteasome inhibitors restored levels of PAR3 in HCK1T-E7-HRAS<sup>G12V</sup> cells expressing wild type E6 or E6Δ151 and SiHa cells, E6 may facilitate proteasomal degradation of PAR3 in an E6AP independent manner (Figs. 4a,b and S2). Some of the E6 target proteins including PATJ are suggested to be E6AP-independent.<sup>32–34</sup> We initially considered that degradation of other PDZ-domain-containing proteins might indirectly decrease the stability of the PAR3 complex. However, surprisingly, the E6 protein of low-risk HPV54, which does not conserve the C-terminal PDZ-domain binding motif, had the strongest ability to downregulate PAR3 protein in HCK1T cells among 17 types of HPVs examined. These results imply that E6 is able to enhance proteasomal degradation of PAR3 with multiple mechanisms. Further study is required to elucidate the precise mechanisms how E6 proteins of these HPVs can downregulate PAR3.

Clearly, our study indicates that the C-terminal PDZ-binding motif of E6 protein plays important roles in the development of cervical carcinogenesis. Thus it is conceivable to consider that difference in oncogenic potential among high-risk HPVs at least partly depends on the difference in the function of the C-terminal motif of the E6 proteins. Targeting this function of E6 as well as the well-known function of E6 to inactivate p53 could be a therapeutic option in HPV-positive cancers.

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Disclosure Statement

Y. I. is an employee of Promega corporation and S-I. O. is an employee of BML, Inc.

References

1 Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T, Ishibashi M. Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci USA 1997; 94: 11612–6.

2 Lee SS, Weiss RS, Javier RT. Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci USA 1997; 94: 6670–5.

3 Nakagawa S, Huibregtse JM, Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. Mol Cell Biol 2000; 20: 8244–53.

4 Handa K, Yugawa T, Narisawa-Saito M, Ohno S, Fujita M, Kiyono T. E6AP-dependent degradation of DLG4/PSD95 by high-risk human papillomavirus type 16 E6 protein. J Virol 2007; 81: 1379–89.

5 Glaunsinger BA, Lee SS, Thomas M, Banks L, Javier R. Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. Oncogene 2000; 19: 5270–80.

6 Sapanos WC, Hoover A, Harris GF et al. The PDZ binding motif of human papillomavirus type 16 E6 induces PTPN13 loss, which allows anchorage-independent growth and synergizes with ras for invasive growth. J Virol 2008; 82: 2493–500.

7 Nguyen ML, Nguyen MM, Lee D, Griep AE, Lambert PF. The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6’s induction of epithelial hyperplasia in vivo. J Virol 2003; 77: 6957–64.

8 Hu Y, Li Z, Guo L et al. MAGI-2 Inhibits cell migration and proliferation via PTEN in human hepatocarcinoma cells. Arch Biochem Biophys 2007; 467: 1–9.

9 Wu X, Hepner K, Castelino-Prabhu S et al. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. Proc Natl Acad Sci USA 2000; 97: 4233–8.

10 Zhu H, Rosenberg A, Bergami KC et al. Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. Cell 2008; 135: 865–78.

11 Watson RA, Thomas M, Banks L, Roberts S. Activity of the human papillomavirus E6 PDZ-binding motif correlates with an enhanced morphological transformation of immortalized human keratinocytes. J Cell Sci 2003; 116: 4925–34.
Table S2.
Sequences of the targeted sites.

Table S1
The effects of proteosomal inhibitors, MG132 or Epoximicin on PAR3 level was examined in indicated cells.

Fig. S2.
HCKs, HCK4T (a, b, c) and HCK12T (d, e, f).

Fig. S1.
Additional Supporting Information may be found online in the supporting information tab for this article:

12 Lee C, Laimins LA. Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. J Virol 2004; 78: 12366–71.
13 Ostrow RS, McGlennen RC, Shaver MK, Kloster BE, Houser D, Faras AJ. A rhasas monkey model for sexual transmission of a papillomavirus isolated from a squamous cell carcinoma. Proc Natl Acad Sci USA 1990; 87: 8170–4.
14 Wood CE, Chen Z, Cline JM, Miller BE, Burk RD. Characterization and experimental transmission of an oncogenic papillomavirus in female macaques. J Virol 2007; 81: 6339–45.
15 Tomaic V, Gardiol D, Massimi P, Ozbun M, Myers M, Banks L. Human and primate tumour viruses use PDZ binding as an evolutionarily conserved mechanism of targeting cell polarity regulators. Oncogene 2009; 28: 1–8.
16 Narisawa-Saito M, Yoshimatsu Y, Ohno S et al. An in vitro multistep carcinogenesis model for human cervical cancer. Cancer Res 2008; 68: 5699–705.
17 Narisawa-Saito M, Inagawa Y, Yoshimatsu Y et al. A critical role of MYC for transformation of human cells by HPV16 E6E7 and oncogenic HRAS. Carcinogenesis 2012; 33: 910–7.
18 Okamoto T, Aoyama T, Nakayama T et al. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. Biochem Biophys Res Commun 2002; 295: 354–61.
19 Narisawa-Saito M, Handa K, Yagawa T, Ohno S, Fujita M, Kiyono T. HPV16 E6-mediated stabilization of ErbB2 in neoplastic transformation of human cervical keratinocytes. Oncogene 2007; 26: 2988–96.
20 Yagawa T, Handa K, Narisawa-Saito M, Ohno S, Fujita M, Kiyono T. Regulation of Notch1 gene expression by p53 in epithelial cells. Mol Cell Biol 2007; 27: 3732–42.
21 Kiyono T, Foster SA, Koop JL, McDougall JK, Galloway DA, Klingelhutz AJ. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 1998; 396: 84–8.
22 Chopjitt P, Pientong C, Sunthamala N et al. E6D25E, HPV16 Asian variant shows specific proteomic pattern correlating in cells transformation and suppressive innate immune response. Biochem Biophys Res Commun 2016; 478: 417–23.
23 Herfs M, Yamamoto Y, Laury A et al. A discrete population of squamo-columnar junction cells implicated in the pathogenesis of cervical cancer. Proc Natl Acad Sci USA 2012; 109: 10516–21.
24 Kuballa P, Matentzoglu K, Scheffner M. The role of the ubiquitin ligase E6-AP in human papillomavirus E6-mediated degradation of PDZ domain-containing proteins. J Biol Chem 2007; 282: 65–71.
25 Jing M, Bohl J, Brimer N, Kinter M, Vande Pol SB. Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. J Virol 2007; 81: 2231–9.
26 Facciusi F, Bugnon Valdano M, Marziali F et al. Human papillomavirus (HPV)-18 E6 oncoprotein interferes with the epithelial cell polarity Par3 protein. Mol Oncol 2014; 8: 533–43.
27 Kranjec C, Banks L. A systematic analysis of human papillomavirus (HPV) E6 PDZ substrates identifies MAGI-1 as a major target of HPV type 16 (HPV-16) and HPV-18 whose loss accompanies disruption of tight junctions. J Virol 2011; 85: 1757–64.
28 Van Doorslaer K, DeSalle R, Einstein MH, Burk RD. Degradation of human PDZ-proteins by human alphapapillomaviruses represents an evolutionary adaptation to a novel cellular niche. PLoS Pathog 2015; 11: e1004980.
29 Du M, Fan X, Hanada T et al. Association of cottontail rabbit papillomavirus E6 oncoproteins with the hDlg/SAP97 tumor suppressor. J Cell Biochem 2005; 94: 1038–45.
30 Feigin ME, Muthuswamy SK. Polarity proteins regulate mammalian cell-cell junctions and cancer pathogenesis. Curr Opin Cell Biol 2009; 21: 694–700.
31 Wang Z, Shen D, Parsons DW et al. Mutational analysis of the tyrosine phosphatase in colorectal cancers. Science 2004; 304: 1164–6.
32 Pim D, Thomas M, Javier R, Gardiol D, Banks L. HPV E6 targeted degradation of the discs large protein: evidence for the involvement of a novel ubiquitin ligase. Oncogene 2000; 19: 719–25.
33 Storr CH, Silverstein SJ. PATJ, a tight junction-associated PDZ protein, is a novel degradation target of high-risk human papillomavirus E6 and the alternatively spliced isoform 18 E6. J Virol 2007; 81: 4080–90.
34 Grm HS, Banks L. Degradation of hDlg and MAGIs by human papillomavirus E6 is E6-AP-independent. J Gen Virol 2004; 85: 2815–9.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Tumorigenic potentials of expression of E7-HRASG12V with the wild type or E6 Δ151 were examined in two independently established HCKs, HCK4T (a, b, c) and HCK12T (d, e, f).

Fig. S2. The effects of proteosomal inhibitors, MG132 or Epoximicin on PAR3 level was examined in indicated cells.

Table S1 Biological activities of HPV16E6 and its mutants used in this study.

Table S2. Sequences of the targeted sites.