Mice xenograft experiments

Immortalised endometriotic epithelial cells were resuspended in growth media (10^8 cells per ml) and were subcutaneously injected (0.1 ml) into the base of the bilateral flank of female BALB/c nu/nu mice (age range 7–9 weeks, SLC, Hamamatsu, Japan). Tumour growth was monitored weekly until confirmed tumours were visualised or at least for 2 months unless tumour formation was detected. All the experiments have been carried out with the ethical committee approval and meet the standards required by the UKCCCR guidelines (Workman *et al*, 2010).

RESULTS

Generation of immortalised epithelial cells from ovarian endometrioma

Endometriotic tissues were collected from the surface epithelia of ovarian endometrioma of two patients, a 27-year-old (patient 1) and a 44-year-old (patient 2) patient who underwent laparoscopic ovarian cystectomy. These tissues were minced and digested in a collagenase solution. Endometriotic glands were then roughly isolated by serial filtration from the stromal cells, followed by direct pick-up, one by one, using microscopic manipulation (Figure 1A). Approximately 100 glands were individually seeded on the wells of plastic dishes and were infected with various combinations of lentiviral vectors for expression of *cyclinD1*, *cdk4*, *dominant negative p53* and *hTERT*. For comparative purposes, various combinations of retroviral vectors for expression of HPV16 *E6*, *E7* and *hTERT* (Kyo *et al*, 2003) were also introduced (Figure 1). Combinatorial transfection of at least three out of these genes successfully generated a total of five independent cell populations from the two patients that achieved >40 population doubling (PD). Two of these populations were transfectants harbouring the *cyclinD1*, *cdk4* and *hTERT* genes, two harboured the *E6*, *E7* and *hTERT* genes and the other population harboured *cyclinD1*, *cdk4*, dominant negative *p53* (*DN-p53*) and *hTERT* genes. Morphologically, all of these cells exhibited a small round shape that was compatible with an epithelial origin and formed a mesh-like structure on plastic dishes (Figure 1B). Introduction of the *hTERT* gene alone, or together with *cdk4*, generated cells from both patients that passed through 10 PD, but finally led to growth arrest at PD between 15–40, during which they exhibited morphological change to a large and flat shape. This phenomenon was determined to be senescence because these cells stained positive for the senescence-associated β-gal (Figure 1C). The cells derived from patient 1 and 2 that gained an extended life span, following the introduction of *cyclinD1*, *cdk4* and *hTERT* genes (and *DN-p53*), were named as EMosis-CC/TERT1 (and EMosis-CC/TERT/DNp53-1) and EMosis-CC/TERT2, respectively, and the cells into which HPV *E6/E7/TERT* were introduced were named as EMosis-E6/E7/TERT1 and EMosis-E6/E7/TERT2, respectively. These cells continued to grow for over 100 PD (Figure 1E), without any morphological change or senescence-associated β-gal staining (Figure 1D). To date, these cells have grown for over 200 PD and continue to grow. We have therefore concluded that these cells have gained immortal phenotypes. These findings indicate that co-expression of *cyclinD1* and *cdk4* are required in order to overcome the premature senescence of endometriotic epithelial cells and that these genes, combined with the expression of *hTERT*, are sufficient for their immortalisation, whereas the additional inactivation of *p53* is not necessarily required for immortalisation.

Expression of epithelial markers and sex steroid receptors

To confirm the origin of the immortalised cells, we next examined the expression of various epithelial and stromal cell markers using RT–PCR analysis. All isolated cells that had an extended life span expressed cytokeratin 8 mRNA, whereas mRNA expression of the stromal marker FSP1 was not observed (Figure 2). The mRNA expression of CD10, a marker that is characteristic of endometrial and endometriotic stromal cells (Sumathi and McCluggage, 2002;

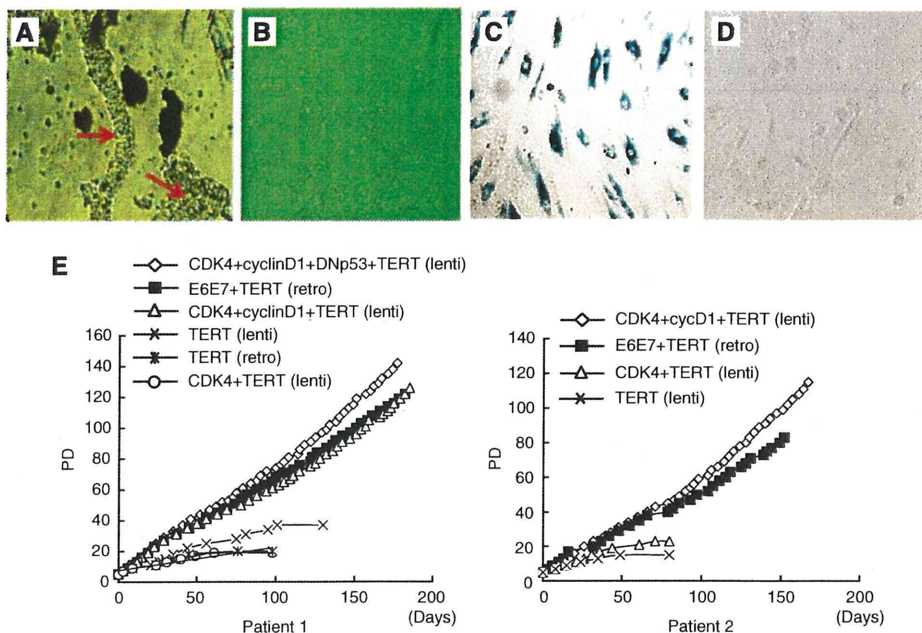

Figure 1 Morphological characteristics and proliferative life span of epithelial cells from ovarian endometrioma transfected with various genetic factors. (**A**) Phase contrast image of glandular clusters isolated from ovarian endometrioma tissues. Individual clusters were directly picked up, one by one, using microscopic manipulation and were transfected with various genetic factors. Glandular clusters are shown in arrows. (**B**) Phase contrast image of representative isolated clones (EMosis-CC /TERT-1 cells) cultured on plastic dishes are shown. (**C**) β-gal staining of cells from patient 1 transfected with *hTERT* alone (population doubling (PD): 20). (**D**) β-gal staining of EMosis-CC /TERT-1 cells (PD: 100). (**E**) The growth characteristics of transfected cells are represented as a growth curve. The genetic factors introduced are shown. Abbreviations: lenti = lentiviral vectors; retro = retroviral vectors.

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Toki *et al*, 2002), was not detected in EMOsis-CC/TERT1 or EMOsis-E6/E7/TERT1 cells but was detected in EMOsis-CC/TERT2 and EMOsis-E6/E7/TERT2 cells, whereas CD10 mRNA was not detected in primary endometriotic glands isolated from patients 1 and 2 (Figure 2). We also verified the epithelial origin of these clones using immunocytochemistry. As shown in Figure 3, all of these cells stained positive for pan-cytokeratin. However, although EMOsis-CC/TERT1 cells were negative for CD10, EMOsis-CC/TERT2 cells exhibited apparent CD10 staining.

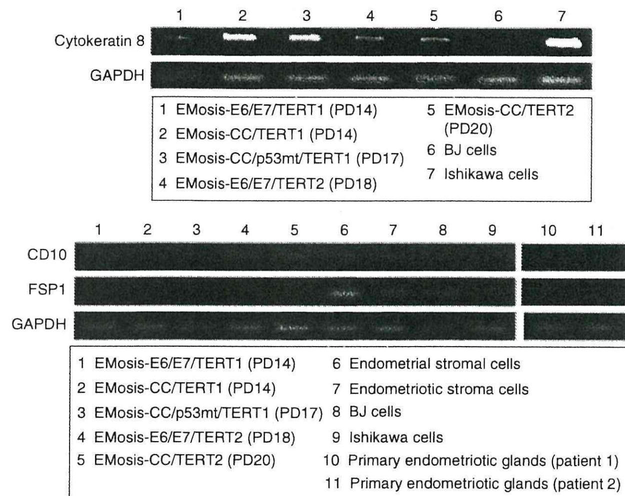

Figure 2 RT–PCR analysis of the expression of epithelial and stromal markers in immortalised epithelial cells from ovarian endometrioma. The expression of cytokeratin 8, CD10 and FSP1 in endometriotic epithelial cells immortalised by various genetic factors was examined using RT–PCR. Ishikawa and BJ cells were used as controls for epithelial and fibroblast cells, respectively. Primary endometriotic glandular cells without transfection, isolated from the ovarian endometrioma of patient 1 or 2, were used as negative controls for CD10 or FSP1 expression. GAPDH expression was assayed as a loading control.

We further investigated steroid-receptor expression in these cells using RT–PCR. ERα and progesterone receptor B (PRB) were expressed in all cell types that had an extended life span, except for EMOsis-E6/E7/TERT1 that lacked ERα expression (Figure 4A). Because expression of the PR isoform PRA, which has an 164 amino-acid deletion of PRB (Kastner *et al*, 1990), can not be discriminated from that of PRB using RT–PCR because of their identical gene sequences, we performed western blot analysis to distinguish the protein expression of these two PR isoforms. There was no detectable protein expression of PRA or even of PRB in any cell type except for EMOsis-CC/TERT1 that had detectable PRB protein expression (Figure 4B). The expression of ERα was not detected in these immortalised cells by western blot analysis (Figure 4C). These results were summarised in Supplementary Table 2. Aromatase expression is another factor that needs to be considered in relation to steroid-receptor expression. A tritiated water assay revealed that there was no detectable aromatase expression in any of the immortalised cells using assay conditions under which control primary endometriotic stromal cells exhibited significant aromatase activity (Figure 4D).

Lack of transformed phenotypes in immortalised epithelial cells from ovarian endometrioma

We next sought to determine whether these immortal cells had acquired a transformed phenotype. First, their growth properties were examined using a soft-agar colony formation assay. A total of 2×10^5 cells were seeded on soft agar on 6-cm dishes and colonies with diameters >0.2 mm were counted after incubation for 2 weeks. Ishikawa or BJ cells were simultaneously examined as positive or negative controls, respectively. Although Ishikawa cells formed distinct colonies, neither the immortal epithelial cells nor the BJ cells formed colonies (Figure 5A). Tumourigenicity of these cells was also examined using nude mice. Control Ishikawa cells

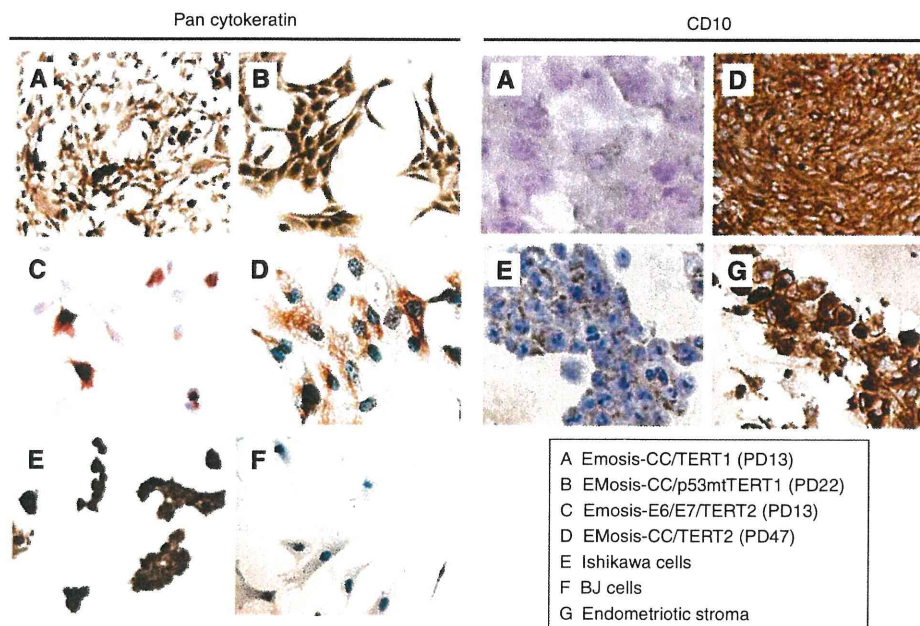

Figure 3 Immunocytochemical analyses of cytokeratin and CD10 expression in immortalised epithelial cells from ovarian endometrioma. The expression of cytokeratin and CD10 in endometriotic epithelial cells that were immortalised by various genetic factors and cultured on LAB TEK chamber slides was examined using immunocytochemistry. Ishikawa and BJ cells were used as controls for epithelial and fibroblast cells, respectively. Primary stromal cells without transfection, isolated from the ovarian endometrioma of another patient, were used as a positive control for CD10.

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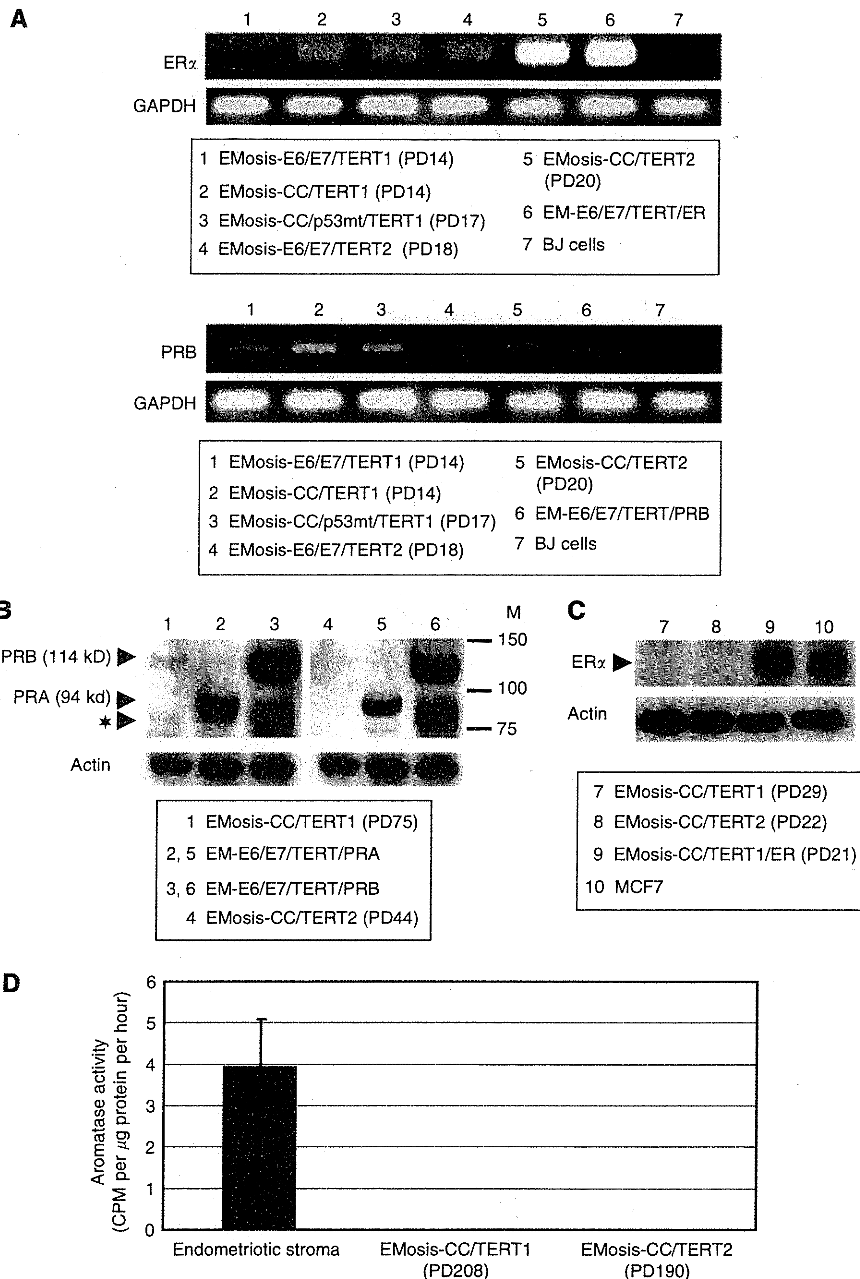

Figure 4 Sex steroid-receptor expression in, and aromatase activity of, immortalised epithelial cells from ovarian endometrioma. (**A**) RT–PCR analysis of expression of the oestrogen receptor α (ERα) or the progesterone receptor B (PRB). EM-E6/E7/TERT/ER cells are immortalised endometrial epithelial cells in which ERα cDNAs were stably transfected and were used as a positive control for ERα. EM-E6/E7/TERT/PRB cells are immortalised endometrial epithelial cells in which PRB cDNAs were stably transfected. Because our primer sets for PRB were designed to amplify the sequences containing *PRB* gene promoter in order to distinguish from PRA transcript, they can detect only intrinsic PRB mRNA but not extrinsic, overexpressed PRB mRNA that lacks promoter sequences. The weak PRB band in EM-E6/E7/TERT/PRB cells is therefore derived from intrinsic PRB. BJ cells were used as a negative control for ERα and PRB expression. GAPDH was used as a loading control. (**B**) Western blot analysis of expression of the progesterone receptor. EM-E6/E7/TERT/PRA or EM-E6/E7/TERT/PRB cells are immortalised endometrial epithelial cells in which PRA or PRB cDNAs were stably transfected and were used as a positive control for PRA or PRB expressions, respectively. Although EM-E6/E7/TERT/PRA cells showed a clear PRA band by western blotting (94 kDa), EM-E6/E7/TERT/PRB cells displayed two bands; one band was of the expected size of intact PRB (114 kDa); the other band was located just below the PRA band (identified by the symbol: ★) and was not a PRA band but a degraded PRB band, which was confirmed by another western blot analysis using a PRB-specific antibody (data not shown). EMosis-CC/TERT1 cells exhibited a weak, but distinct, PRB band but not a PRA band. M: protein weight marker. (**C**) Western blot analysis of expression of the ER. There was no detectable protein expression of ERα in EMosis-CC/TERT1 or EMosis-CC/TERT2 cells. EMosis-CC/TERT1/ER cells, generated by the introduction of ERα cDNA into EMosis-CC/TERT1 cells, were confirmed to have significant ERα expression. MCF7 cells were used as a positive control of ERα expression. (**D**) Analysis of aromatase activity using a tritiated water assay. Primary endometriotic stromal cells isolated from the ovarian endometrioma of another patient were used as a positive control of aromatase activity. Both EMosis-CC/TERT1 and EMosis-CC/TERT2 cells lacked aromatase activity.

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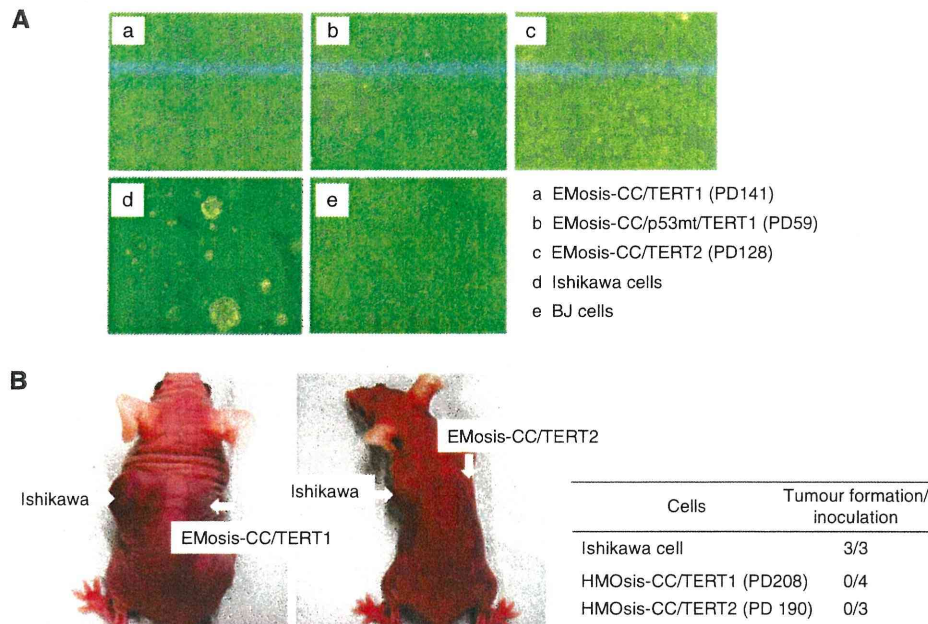

Cells	Tumour formation/ inoculation
Ishikawa cell	3/3
HMOsis-CC/TERT1 (PD208)	0/4
HMOsis-CC/TERT2 (PD 190)	0/3

Figure 5 Analysis of the transformed phenotypes of immortalised epithelial cells from ovarian endometrioma. (**A**) Anchorage-independent growth was examined using a soft-agar colony formation assay. A total of 2×10^5 Ishikawa cells or immortalised cells were seeded onto soft agar and colonies >0.2 mm were counted after incubation for 2 weeks. Ishikawa and BJ cells were used as a positive and negative control for colony formation, respectively. (**B**) *In vivo* growth was examined using a tumour formation assay in nude mouse. Immortalised epithelial cells from ovarian endometrioma were resuspended in growth media (10^8 cells per ml) and were subcutaneously injected (0.1 ml) into the base of the bilateral flank of female BALB/c nu/nu mice (age range 7–9 weeks, SLC). Tumour growth was monitored weekly until confirmed tumours were visualised or at least for 2 months unless tumour formation was detected.

formed a subcutaneous tumour in mice 6 weeks after inoculation, but immortal epithelial cells were not able to form any tumour even 2 months after inoculation (Figure 5B).

Responsiveness of immortalised epithelial cells from ovarian endometrioma to progestin and oestrogen

We next examined the responsiveness of the immortalised epithelial cells to progestin. EMOsis-CC/TERT1 and EMOsis-CC/TERT2 cells were treated with MPA, dienogest or progesterone at a concentration of 1 or 100 nM for different time periods. Cell growth was then examined by counting cell numbers. Treatment with MPA or dienogest at a concentration of 10 or 100 nM significantly inhibited the growth of both cell types at 72 h (Figures 6A and B). Treatment with progesterone at 10 or 100 nM significantly inhibited the growth of EMOsis-CC/TERT1 at 72 h but only had a marginal effect on EMOsis-CC/TERT2 cells (Figure 6C). We performed these inhibitory experiments in growth media containing serum, considering clinical situations in which progestin is administrated *in vivo*. However, we also confirmed that progestin inhibited the growth of these cells in phenol red-free media containing charcoal-treated serum, although the extent of inhibition was less than that in normal growth media (data not shown), probably because of the cytostatic conditions of such media. These findings suggest that the immortalised epithelial cells preserved cell responsiveness to progestin.

We further examined the responsiveness of the immortalised epithelial cells to oestrogen. EMOsis-CC/TERT1 and EMOsis-CC/TERT2 cells were treated with 10 or 100 nM of 17β estradiol (E2) for different time periods. We failed to find any effect of E2 on the growth of either cell type (data not shown). This result was likely to be due to the low levels of ERα expression, which could only be faintly detected using RT–PCR. We therefore sought to over-express ERα in EMOsis-CC/TERT1 cells via lentiviral introduction of ERα cDNA, and obtained ERα-overexpressing EMOsis-CC/TERT1 cells (EMOsis-CC/TERT1/ER). Sufficient expression of ERα in these cells was confirmed by western blot analysis (Figure 4C). The growth of EMOsis-CC/TERT1/ER cells was significantly activated by treatment with 17β-estradiol (E2) at a concentration of 100 nM (Figure 6D). Thus, we had successfully generated immortalised epithelial cells from ovarian endometrioma that still had the property of oestrogen or progestin responsiveness.

DISCUSSION

Although stromal cells in endometriotic tissues are easily isolated and grown under usual culture conditions, epithelial cells are hard to purify and propagate *in vitro*. This difficulty is mainly because of the rarity of epithelial cells in endometriotic tissues as well as to their shorter life span due to two barriers against their *in vitro* growth. To overcome these barriers, we first purified glandular fragments from endometriotic tissues that were treated with collagenase-based reagents via microscopic manipulation. We succeeded in immortalising endometriotic glandular cells through combinatorial introduction of the two genetic factors (*cyclin D1/cdk4*) that inhibit Rb functions together with *hTERT*. Special attention must be paid to contamination of the purified glandular fragments by stromal cells. This is because small amounts of stromal cells might possibly attach to the epithelial clusters providing a limitation to the purification of epithelial cells in glandular clusters. We therefore carefully judged whether the immortalised cells that we obtained were of epithelial origin. RT–PCR and immunocytochemical analyses confirmed the expression of epithelial markers in these cells. The introduction of *hTERT* alone, or the combination of *CDK4* and *hTERT*, failed to immortalise these cells. This result might provide further evidence of the epithelial origin of these cells, because stromal cells are usually immortalised by the introduction of *hTERT* alone (Kiyono *et al*, 1998; Morales *et al*, 1999). Indeed, Krikun *et al* (2004)

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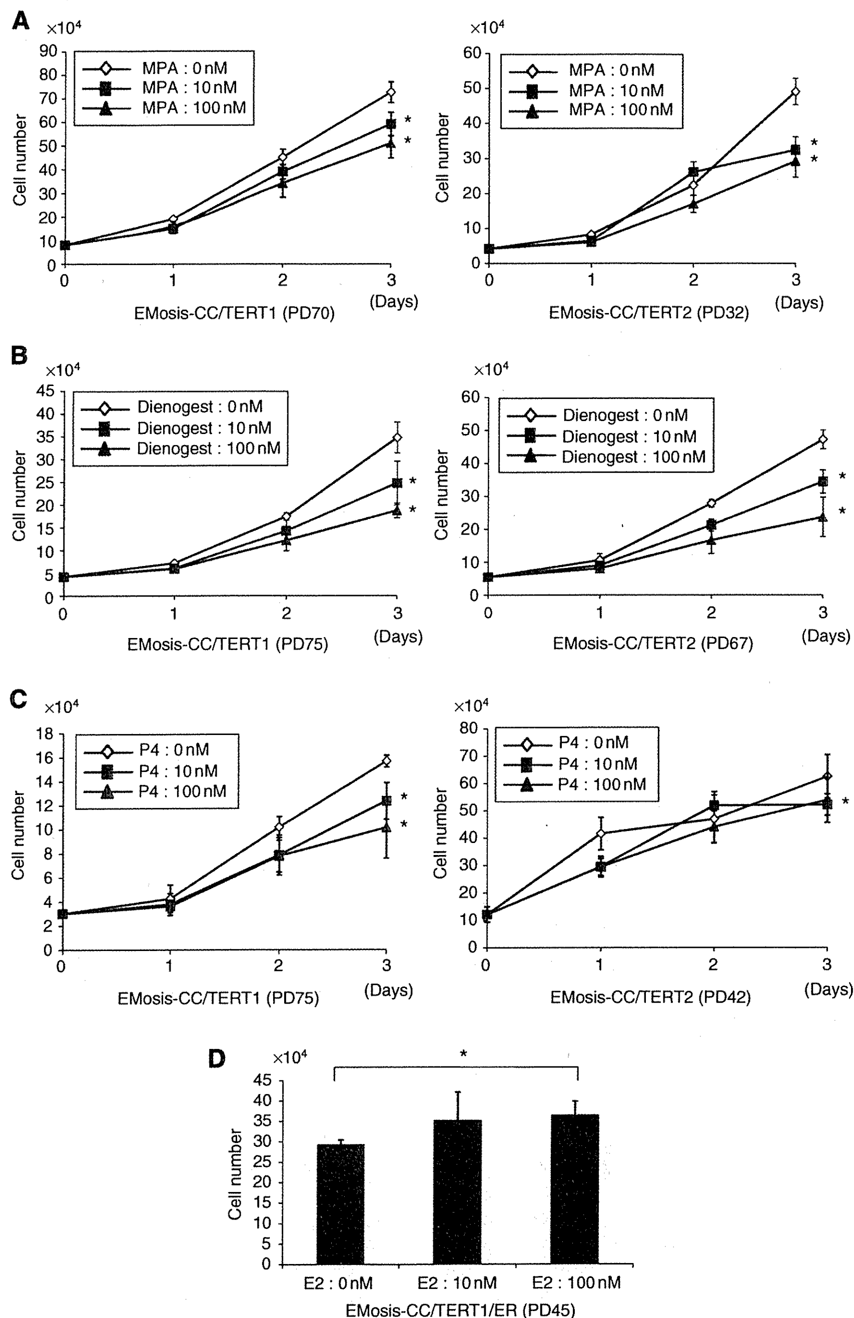

Figure 6 Effect of progestin and oestrogen on the growth of immortalised epithelial cells from ovarian endometrioma. EMosis-CC/TERT1 or EMosis-CC/TERT2 cells were seeded on six-well dishes and were treated with or without MPA (**A**), dienogest (**B**) or progesterone (P4) (**C**) at a concentration of 10 or 100 nM for the indicated number of days. Cell growth was monitored by counting cell numbers. Data are presented as means ± s.d. of three independent experiments. $^*P<0.05$. (**D**) EMosis-CC/TERT1/ER cells were generated by the introduction of ERα cDNA into EMosis-CC/TERT1 cells and confirmed to have significant ERα expression (Figure 4C). EMosis-CC/TERT1/ER cells were seeded on six-well dishes and were treated with or without MPA estradiol (E2) at a concentration of 10 or 100 nM for the indicated number of days. Cell growth was monitored by counting cell numbers on day 5 after treatment. Data are presented as means ± s.d. of three independent experiments. $^*P<0.05$.

confirmed immortalisation of endometriotic stromal cells by the introduction of *hTERT* alone. The requirement of two genetic factors that inhibit Rb function in addition to *hTERT* for cell immortalisation is consistent with observations in other epithelial cell types (Kiyono *et al*, 1998). Even the additional introduction of *DN-p53* failed to immortalise these cells, suggesting that they do not have high malignant potential, despite their invasive behaviour *in vivo*, which is reminiscent of cancer.

Although some studies showed that endometriotic tissues expressed PRB (Shen *et al*, 2008), others demonstrated that PRA was predominantly expressed and that PRB expression was low or absent (Attia *et al*, 2000; Wu *et al*, 2006). In the present study, one strain of the immortalised cells (EMosis-CC/TERT1) expressed PRB that was detectable using western blot analysis, whereas the other strain (EMOsis-CC/TERT2) did not. The reason why PRA was not detected in our western analysis remains unclear, but the

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expression might weaken or diminish during *in vitro* culture and/or the subsequent immortalisation step. Both EMosis-CC/TERT1 and EMosis-CC/TERT2 cells responded well to progestin, exhibiting significant growth retardation. It is of particular interest that, even though EMosis-CC/TERT2 cells only weakly expressed PRB, which was only detectable using RT-PCR, they were responsive to progestin, suggesting that such a low level of PRB expression was sufficient for a progestin effect. To our knowledge, this is the first demonstration of cultured epithelial cells from ovarian endometrioma that have stable progestin responsiveness. These cells are therefore a valuable tool for the study of progestin action in endometriosis. Progestin resistance is one of the characteristics of this disease (Vercellini *et al*, 2003; Bulun *et al*, 2006). However, some patients (approximately 50-70%) respond well to progestin-related agents, whereas others do not (Vercellini *et al*, 2003; Momoeda *et al*, 2009). Although the molecular mechanisms of this diversity among patients are not fully understood, some studies have indicated that the absence of, or decrease in, PR expression, possibly via promoter hypermethylation, has a key role in progestin resistance (Wu *et al*, 2006; Burney *et al*, 2007). We recently reported that fork head protein O1 (FOXO1) is a direct target of progestin for inhibiting endometrial epithelial growth (Kyo *et al*, 2011). Phosphorylated Akt has a critical role in this pathway by inhibiting FOXO1 activity, and the status of Akt is a predictor of progestin responsiveness in this cell type. It is therefore of interest to know whether a similar scenario of FOXO1 regulation by progestin exists in endometriotic epithelial cells, and this possibility is under investigation.

CD10 is a characteristic marker of both endometrial and endometriotic stromal cells (Toki *et al*, 2002, Sumathi and McCluggage, 2002). Although the endometriotic epithelial cells were isolated from patient 2 lacked CD10 expression before transfection, the EMosis-CC/TERT2 cells did express CD10 and, in addition, retained CK8 expression as demonstrated using RT-PCR (Figure 3). One possible explanation of these inconsistent results is that the contaminated stromal cells might have had a growth advantage during the immortalisation steps and therefore became the predominant population in the immortal cell culture. However, this possibility is not likely because these immortalised cells continued to express cytokeratin, which was confirmed using both RT-PCR and immunocytochemistry. Alternatively, the process of epithelial mesenchymal transition might be involved in this inconsistent expression of CD10, and this possibility is also under investigation. Recently, several novel mechanisms have been proposed to explain endometrial and endometriotic regeneration. One study showed that epithelial cells in the endometrium might have originated from stromal cells via cellular transdifferentiation (Garry *et al*, 2010). Another study suggested that both epithelial and stromal cells in eutopic/ectopic endometrium might arise from a common cell type with stem-like properties (endometrial stem/progenitor cells) (Maruyama *et al*, 2010). Both studies thus proposed a common origin of epithelial and stromal cells in eutopic/ectopic endometrium. The expression of the stromal marker (CD10) in endometriotic epithelial cells that was observed in the present study may be consistent with a hypothesis. We consider that this phenomenon is interesting and that it will be worthwhile to carry out further extensive analysis to uncover the origin of endometriotic cells.

Aromatase p450 is expressed in a number of tissues such as ovarian granulose cells, adipose tissue, skin fibroblasts and brain (Simpson *et al*, 1994). Aromatase catalyses the conversion of androstenedione to estrone, which is further converted to the potent oestrogen E2 by the enzyme 17β-hydroxysteroid dehydrogenase type 1. It is known that aromatase is absent in normal endometrium, whereas it is expressed in eutopic/ectopic endometrium in patients with endometriosis (Bulun *et al*, 1993; Noble *et al*, 1997; Zeitoun *et al*, 1999), where it increases local oestrogen production and thereby contributes to the development of this disease. Although it is well established that aromatase activity exists in stromal cells in endometriotic tissues, and this activity is well characterised, the presence and the role of aromatase in epithelial cells are largely unknown. Although a few immunohistochemical studies have shown aromatase expression in endometriotic epithelial cells (Kitawaki *et al*, 1997; Bulun *et al*, 2001), no study has confirmed this finding in *in vitro* cultures, probably because of the difficulty of *in vitro* culture of these cells. Our cell system gave us the opportunity to explore this point and demonstrated that the immortalised epithelial cells from ovarian endometrioma completely lacked aromatase activity, as determined using a tritiated water assay. Therefore, our result strongly supports the absence of aromatase activity in endometriotic epithelial cells.

Although ovarian endometrioma is a benign tumour, some endometriomas have been known to develop into malignant tumours with clear cell or endometrioid-type histology (Wölfler *et al*, 2005; Mandai *et al*, 2009). Although some genetic factors, including *PTEN* mutation, are known to be associated with ovarian cancers arising from endometrioma (Anglesio *et al*, 2010; Jones *et al*, 2010; Wiegand *et al*, 2010), the molecular mechanisms of carcinogenesis are largely unknown. The immortalised cells we established were found to lack transformed phenotypes. These cells might therefore be an ideal model for the study of carcinogenesis, in which candidate genetic factors can be introduced or knocked down, enabling the identification of genetic factors required for transformation. In particular, the ERα-expressing EMosis-CC/TERT1/ER cells appear to be suitable for such purposes. We are currently testing *in vivo* propagation of these cells in NOG mice together with endometriotic stromal cells to reconstitute endometriotic tissues with glandular structures, or hopefully, cancerous tissues after various genetic manipulations. Thus, these cells will be essential for the complete understanding of the multistep carcinogenesis of ovarian endometrioma and hopefully may be useful for identification of novel molecular therapeutic targets.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

REFERENCES

Anglesio MS, Carey MS, Köbel M, Mackay H, Huntsman DG, Vancouver Ovarian Clear Cell Symposium Speakers (2010) Clear cell carcinoma of the ovary: A report from the first Ovarian Clear Cell Symposiumh. *Gynecol Oncol* **121**: 407-415

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Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, Bulun SE (2000) Progesterone receptor isoform A but not B is expressed in endometriosis. *J Clin Endocrinol Metab* **85:** 2897 - 2902

Bulun SE, Mahendroo MS, Simpson ER (1993) Polymerase chain reaction amplification fails to detect aromatase cytochrome P450 transcripts in normal human endometrium or decidua. *J Clin Endocrinol Metab* **76:** 1458 - 1463

Bulun SE, Yang S, Fang Z, Gurates B, Tamura M, Zhou J Sebastian S (2001) Role of aromatase in endometrial disease. *J Steroid Biochem Mol Biol* **79:** 19 - 25

Bulun SE, Cheng YH, Yin P, Imir G, Utsunomiya H, Attar E, Innes J, Julie, Kim J (2006) Progesterone resistance in endometriosis: link to failure to metabolize estradiol. *Mol Cell Endocrinol* **248:** 94 - 103

Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, Nezhat CR, Lessey BA, Giudice LC (2007) Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology* **148:** 3814 - 3826

Garry R, Hart R, Karthigasu KA, Burke C (2010) Structural changes in endometrial basal glands during menstruation. *BJOG* **117:** 1175 - 1185

Gurates B, Sebastian S, Yang S, Zhou J, Tamura M, Fang Z, Suzuki T, Sasano H, Bulun SE (2002) WT1 and DAX-1 inhibit aromatase P450 expression in human endometrial and endometriotic stromal cells. *J Clin Endocrinol Metab* **87:** 4369 - 4377

Jones S, Wang TL, Shih Ie M, Mao TL, Nakayama K, Roden R, Glas R, Slamon D, Diaz Jr LA, Vogelstein B, Kinzler KW, Velculescu VE, Papadopoulos N (2010) Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science* **330:** 228 - 231

Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P (1990) Two distinct estrogen-regulated promoters generatetranscripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* **9:** 1603 - 1614

Kitawaki J, Noguchi T, Amatsu T, Maeda K, Tsukamoto K, Yamamoto T, Fushiki S, Osawa Y, Honjo H (1997) Expression of aromatase cytochrome P450 protein and messenger ribonucleic acid in human endometriotic and adenomyotic tissues but not in normal endometrium. *Biol Reprod* **57:** 514 - 519

Kiyono T, Hiraiwa A, Ishii S, Takahashi T, Ishibashi M (1994) p53-mediated transactivation by E6 of type 1, but not type 5, 8, or 47, human papillomavirus of cutaneous origin. *J Virol* **68:** 4656 - 4661

Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* **396:** 84 - 88

Krikun G, Mor G, Alvero A, Guller S, Schatz F, Sapi E, Rahman M, Caze R, Qumsiyeh M, Lockwood CJ (2004) A novel immortalized human endometrial stromal cell line with normal progestational response. *Endocrinology* **145:** 2291 - 2296

Kurman RJ, Craig JM (1972) Endometrioid and clear cell carcinoma of the ovary. *Cancer* **29:** 1653 - 1664

Kyo S, Nakamura M, Kiyono T, Maida Y, Kanaya T, Tanaka M, Yatabe N, Inoue M (2003) Successful immortalization of endometrial glandular cells with normal structural and functional characteristics. *Am J Pathol* **163:** 2259 - 2269

Kyo S, Sakaguchi J, Kiyono T, Shimizu Y, Maida Y, Mizumoto Y, Mori N, Nakamura M, Takakura M, Miyake K, Sakamoto M, Inoue M (2011) Forkhead transcription factor FOXO1 is a direct target of progestin to inhibit endometrial epithelial cell growth. *Clin Cancer Res* **17:** 525 - 537

Maruyama T, Masuda H, Ono M, Kajitani T, Yoshimura Y (2010) Human uterine stem/progenitor cells: their possible role in uterine physiology and pathology. *Reproduction* **140:** 11 - 22

Mandai M, Yamaguchi K, Matsumura N, Baba T, Konishi I (2009) Ovarian cancer in endometriosis: molecular biology, pathology, and clinical management. *Int J Clin Oncol* **14:** 383 - 391

McMeekin DS, Burger RA, Manetta A, DiSaia P, Berman ML (1995) Endometrioid adenocarcinoma of the ovary and its relationship to endometriosis. *Gynecol Oncol* **59:** 81 - 86

Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM (1998) Development of a self-inactivating lentivirus vector. *J Virol* **72:** 8150 - 8157

Momoeda M, Harada T, Terakawa N, Aso T, Fukunaga M, Hagino H, Taketani Y (2009) Long-term use of dienogest for the treatment of endometriosis. *J Obstet Gynaecol Res* **35:** 1069 - 1076

Morales CP, Holt SE, Ouellette M, Kaur KJ, Yan Y, Wilson KS, White MA, Wright WE, Shay JW (1999) Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet* **21:** 115 - 118

Ness RB (2003) Endometriosis and ovarian cancer: thoughts on shared pathophysiology. *Am J Obstet Gynecol* **189:** 280 - 294

Noble LS, Takayama K, Zeitoun KM, Putman JM, Johns DA, Hinshelwood MM, Agarwal VR, Zhao Y, Carr BR, Bulun SE (1997) Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. *J Clin Endocrinol Metab* **82:** 600 - 606

Olive DL, Schwartz LB (1993) Endometriosis. *N Engl J Med* **328:** 1759 - 1769

Oral E, Ilvan S, Tustas E, Korbeyli B, Bese T, Demirkiran F, Arvas M, Kosebay D (2003) Prevalence of endometriosis in malignant epithelial ovary tumours. *Eur J Obstet Gynecol Reprod Biol* **109:** 97 - 101

Ramirez RD, Sheridan S, Girard L, Sato M, Kim Y, Pollack J (2004) Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer Res* **64:** 9027 - 9034

Sasaki R, Narisawa-Saito M, Yugawa T, Fujita M, Tashiro H, Katabuchi H, Kiyono T (2009) Oncogenic transformation of human ovarian surface epithelial cells with defined cellular oncogenes. *Carcinogenesis* **30:** 423 - 431

Schreiber E, Matthias P, Muller MM, Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17:** 6419

Shen F, Wang Y, Lu Y, Yuan L, Liu X, Guo SW (2008) Immunoreactivity of progesterone receptor isoform B and nuclear factor kappa-B as biomarkers for recurrence of ovarian endometriomas. *Am J Obstet Gynecol* **199:** 486. e1 - 486.e10

Shozu M, Zhao Y, Simpson E.R (1997) Estrogen Biosynthesis in THP1 Cells Is Regulated by Promoter Switching of the Aromatase (CYP19) Gene. *Endocrinology* **138:** 5125 - 5135

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr Rev* **15:** 342 - 355

Sumathi VP, McCluggage WG (2002) CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming a diagnosis of endometriosis. *J Clin Pathol* **55:** 391 - 392

Toki T, Shimizu M, Takagi Y, Ashida T, Konishi I (2002) CD10 is a marker for normal and neoplastic endometrial stromal cells. *Int J Gynecol Pathol* **21:** 41 - 47

Vercellini P, Frontino G, De Giorgi O, Pietropaolo G, Pasin R, Crosignani PG (2003) Continuous use of an oral contraceptive for endometriosis-associated recurrent dysmenorrhea that does not respond to a cyclic pill regimen. *Fertil Steril* **80:** 560 - 563

Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, McConechy MK, Anglesio MS, Kalloger SE, Yang W, Heravi-Moussavi A, Giuliany R, Chow C, Fee J, Zayed A, Prentice L, Melnyk N, Turashvili G, Delaney AD, Madore J, Yip S, McPherson AW, Ha G, Bell L, Fereday S, Tam A, Galletta L, Tonin PN, Provencher D, Miller D, Jones SJ, Moore RA, Morin GB, Oloumi A, Boyd N, Aparicio SA, Shih IeM, Mes-Masson AM, Bowtell DD, Hirst M, Gilks B, Marra MA, Huntsman DG (2010) ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med* **363:** 1532 - 1543

Wölfel T, Hauer M, Schneider J, Serrano M, Wölfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Büschenfelde KH, Beach D (1995) A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* **269:** 1281 - 1284

Wölfler MM, Nagele F, Kolbus A, Seidl S, Schneider B, Huber JC, Tschugguel W (2005) A predictive model for endometriosis. *Hum Reprod* **20:** 1702 - 1708

Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham D, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA, An ad hoc committee of the National Cancer Research Institute (2010) Guidelines for the welfare and use of animals in cancer research. *Br J Cancer* **102:** 1555 - 1577

Wu Y, Strawn E, Basir Z, Halverson G, Guo SW (2006) Promoter hypermethylation of progesterone receptor isoform B (PR-B) in endometriosis. *Epigenetics* **1:** 106 - 111

Zeitoun KM, Takayama K, Michael MD, Bulun SE (1999) Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of SF-1 and COUP-TF to the same cis-acting element. *Mol Endocrinol* **13:** 239 - 253

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ORIGINAL ARTICLE

Characterization of Gut-Derived Intraepithelial Lymphocyte (IEL) Residing in Human Papillomavirus (HPV)-Infected Intraepithelial Neoplastic Lesions

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C-C chemokine receptor type 9, cervical intraepithelial neoplasia, genital tract, integrin $\alpha E\beta 7$, intraepithelial lymphocyte, mucosal immunity

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Problem
Mucosal T cells are the most likely direct effectors in host anti-human papillomavirus adaptive immunity and regression of cervical intraepithelial neoplasia (CIN) lesions. There are no studies addressing intraepithelial lymphocytes (IELs) in CIN lesions.

Method of study
Cervical lymphocytes were collected using cytobrushes from patients with CIN and analyzed by FACS analysis. Comparisons were made between populations of cervical T cells in CIN regressors and non-regressors.

Results
A median of 74% of cervical lymphocytes were $CD3^+$ T cells. Populations of integrin $\alpha E\beta 7^+$ IEL in CIN lesions varied markedly among patients (6–57%). Approximately half of integrin $\beta 7^+$ T cells were CD45RA-negative memory T cells. The number of integrin $\alpha E\beta 7^+$ cells among cervical T cells was significantly higher in CIN regressors when compared to non-regressors.

Conclusion
Higher cervical IEL numbers are associated with spontaneous regression of CIN. Accumulation of cervical integrin $\alpha E\beta 7^+$ IEL may be necessary for local adaptive effector functions.

Introduction

Lymphocytes involved in the mucosal immune system are found in the inductive sites of organized mucosa-associated lymphoid tissues (MALT) and in a variety of effector sites such as the mucosa of the intestine, respiratory tract, and genital tract.[1] The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin $\alpha 4\beta 7$ and C-C chemokine receptor type 9 (CCR9). Lymphocyte-expressed integrin $\alpha 4\beta 7$ and CCR9 bind to their natural ligands, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25 (TECK), respectively, which are expressed on the cell surface of

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endothelial cells in submucosal post-capillary venules.[1] In the intestine, mucosal dendritic cells (DCs) in gut-associated lymphoid tissues (GALT) regulate the expression of integrin α4β7 on activated effector and regulatory lymphocytes in a retinoic acid–dependent manner.[1–3] Mucosal T cells expressing integrin $\alpha4\beta7^+$ are known to circulate in peripheral blood from inductive sites and to home to the lamina propria (LP) at effector sites via α4β7-MAdCAM-1 and CCR9-CCL25 interactions.[4] Integrin $\alpha4\beta7^+$ T cells can differentiate into $\alpha E\beta7^+$ T cells upon exposure to TGF-β,[5] and the expression of integrin αEβ7 facilitates the retention of lymphocytes in the epithelium via interactions with E-cadherin.[4] Integrin αEβ7 is a specific marker of intraepithelial lymphocytes (IELs) residing in mucosal epithelia, and those cells expressing this antigen on their surface were initially educated in the gut.

The cervical mucosa is a very common site for pathogen invasion and is the primary transmission site for human papillomavirus (HPV), *Chlamydia trachomatis,* and human immune deficiency virus type 1 (HIV-1). A well-organized mucosal defense system in the cervical mucosa is critical to human health. Mucosal epithelial cells in the human cervix are active participants in such immunological protection.[6] However, the lymphocytes populating the cervical mucosal tissues, especially cervical IELs, have been poorly studied. Mucosal T cells in the murine genital tract express a large amount of integrin α4β7 on their cell surface,[7] and MAdCAM-1 is expressed on endothelial cells in the submucosa of murine fallopian tubes infected with *C. trachomatis.*[8] Several studies have demonstrated that human genital mucosa expresses MAdCAM-1 endogenously[9] and that GALT-derived integrin $\alpha4/E\beta7^+$ T cells home to the genital mucosa.[10,11] This T-cell homing and the expression of integrin αE increase in the presence of cervicitis and vaginitis.[10,11] Although integrin $\beta7^+$ mucosal T cells have been found in the cervical mucosa, a local inductive site (i.e., MALT) has never been demonstrated histologically.[11] We hypothesized that GALT may act as the inductive site for cervical IELs.

Human papillomavirus infection is a major cause of cervical cancer, and its precursor lesion, cervical intraepithelial neoplasia (CIN), develops in the epithelium. Natural history studies of CIN[12,13] show that most infections and CIN lesions resolve spontaneously but some persist and progress to cervical cancer. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions[14,15] suggest that cell-mediated immune response against HPV antigens is important in the control of HPV infection and progression to CIN. More controversial are the relative roles of systemic and local mucosal immune responses in the HPV pathogenesis. Trimble et al.[16] reported that naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN 2/3 lesions, but Nakagawa et al.[17] demonstrated a positive association between systemic cell-mediated immune responses to HPV E6 and HPV/CIN regression.

We studied the local mucosal cell–mediated immune response to HPV antigens by characterizing cervical mucosal immune cells collected non-invasively, using only a cytobrush. We confirmed that the collected $CD3^+$ cervical T cells were intraepithelial in origin (integrin $\alpha E\beta7^+$ IELs). Approximately half of the integrin $\beta7^+$ T cells were memory T cells. Finally, integrin $\beta7^+$ intraepithelial T cells increased significantly in the patients whose CIN lesions regressed spontaneously regardless of HPV genotype.

Materials and methods

Study Population

Cervical cell samples were collected using a cytobrush from 86 patients under observation after being diagnosed with CIN by colposcopically directed biopsy. All women gave written informed consent, and the Research Ethics Committee of the University of Tokyo approved all aspects of the study. Patients with known, symptomatic, or macroscopically visible vaginal inflammation or sexually transmitted infections were excluded from our study. Samples for HPV genotyping were collected at the first follow-up examination after diagnosis. Cervical lymphocytes were collected from non-menstruating patients at their latest follow-up visit. To study the potential association between cervical IEL characteristics and CIN progression, CIN patients with the regression of cervical cytology (cases) were matched with control patients who did not exhibit cytologic regression over the same time period (measured from initial detection of abnormal cytology). In this study, cytological regression was defined as normal cytology at two or more consecutive evaluations conducted at 3 to 4-month intervals. Thirteen patients were enrolled in the regression group, and the median

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follow-up duration was 27 (12–38) months. Thirteen pairs of follow-up time-matched patients with persistent cytological abnormalities were enrolled in the non-regression group, and the median follow-up time was 24 (12–40.5) months.

HPV Genotyping

DNA was extracted from cervical smear samples using the DNeasy Blood Mini Kit (Qiagen, Crawley, UK). HPV genotyping was performed using the PGMY-CHUV assay method.[18] Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and human leukocyte antigen-DQ (HLA-DQ) primer sets. Reverse blotting hybridization was performed. Heat-denatured PCR amplicons were hybridized to specific probes for 32 HPV genotypes and HLA-DQ reference samples. The virological background (HPV genotyping) of 86 patients in our study was shown in Table I. Here, HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs according to International Agency for Research on Cancer multicenter study.[19]

Table I Human Papillomavirus (HPV) Genotype Distribution

HPV type	Total numbers (%)
16	19 (18.4)
18	7 (6.8)
31	2 (1.9)
33	1 (1.0)
35	1 (1.0)
39	1 (1.0)
45	1 (1.0)
51	7 (6.8)
52	20 (19.4)
53	4 (3.9)
56	3 (2.9)
58	12 (11.7)
59	3 (2.9)
68	3 (2.9)
82	1 (1.0)
6	2 (1.9)
54	1 (1.0)
55	1 (1.0)
66	4 (3.9)
69	1 (1.0)
70	3 (2.9)
83	3 (2.9)
84	2 (1.9)
Total	103 (100)

Patients infected with multiple HPV types were included. Of 86 patients, 32 (37%) were infected with multiple types. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs.[19]

Collection and Processing of Cervical Specimens

Cervical cells were collected using a Digene cytobrush as described previously.[20] The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was placed in a 15-mL tube containing R10 media [RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B] and an anticoagulant (0.1 IU/mL of heparin and 8 nM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37°C for 15 min with shaking, the cytobrush was removed. The tube was centrifuged at 330 × *g* for 4 min. The pellet was resuspended in 10 mL of 40% Percoll, layered onto 70% Percoll, and centrifuged at 480 × *g* for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was >95%, as confirmed by trypan blue exclusion test, and fresh samples were immediately used for further analysis.

Immunolabeling and Flow Cytometry

Cervical immune cell preparations were immunolabeled, incubated on ice for 30 min, washed twice with FACS buffer (10% FCS, 1 mM EDTA, and 10 mM NaN_3) and fixed by adding paraformaldehyde in PBS to a final concentration of 1%.

The following fluorochrome-conjugated mouse monoclonal antibodies specific for human leukocyte surface antigens were used: a fluorescein isothiocyanate (FITC)-conjugated pan leukocyte marker (FITC-anti CD45), a B lymphocyte marker (FITC-anti CD19), a cytotoxic T-cell marker (FITC-anti CD8), a helper T-cell marker (FITC-anti CD4), an integrin β7 marker (FITC-anti integrin β7), a phycoerythrin (PE)-conjugated integrin α4 marker (PE-anti integrin α4), an integrin αE marker (PE-anti integrin αE), a C-C chemokine receptor type 9 marker (PE-anti CCR9), a marker for naïve cells (PE-anti CD45RA), a phycoerythrin cyanine 5 (PC5)-conjugated pan T lymphocyte marker (PC5-anti CD3), a natural killer cell marker (PC5-anti CD56), and an allophycocyanin (APC)-conjugated pan T lymphocyte marker (APC-anti CD3). Cell preparations were labeled in parallel with appropriate isotype control

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antibodies. Antibodies were purchased from eBioscience (San Diego, CA, USA) and Beckman Coulter (Brea, CA, USA). Data were acquired using three-color flow cytometry on FACSCalibar (Becton-Dickinson, Texarkana, TX, USA). The positions of lymphocytes and monocytes were determined on the forward scatter versus side scatter (SSC) profile. The positions of pan-lymphocytes and T lymphocytes were determined by CD45 and CD3 gating, respectively. As the percentage of B cells among cervical lymphocytes is known to be low (less than a few percentage) when compared to the 20% level seen in peripheral blood,[20] the presence of elevated CD19^{+} B cells in cervical specimens would indicate contamination with peripheral blood. For our investigations, cervical samples with more than 3% B cells were excluded from analysis.

Statistical Analysis

Statistical analyses, including calculation of medians and interquartile ranges (IQRs), were performed using the commercial statistical software package JMP® (SAS, Cary, NC, USA). Wilcoxon rank sum test or Fisher's exact test was applied for matched paired comparisons. *P*-values ≤0.05 were considered significant.

Results

Purification of Cervical Leukocytes Collected from CIN Lesions

To characterize mucosal cellular immune responses in HPV-infected lesions, cervical samples, including exfoliated epithelial cells and cervical lymphocytes, were collected from CIN lesions positive for any HPV genotype using a cytobrush. Cervical samples were fractionated over a discontinuous Percoll density gradient to remove cervical epithelial cells, and the layer between Percoll and culture medium was collected. Cervical lymphocytes were identified among isolated cells using standard SSC and CD45 gating (Fig. 1). Approximately 10^4–10^5 CD45^{+} cells were isolated from patients' cervices. CD45^{+} cells primarily consisted of lymphocytes (Fig. 1, circle) and granulocytes (Fig. 1, square). A minority of the cells included in the square in Fig. 1 were monocytes (data not shown). Two representative cases are provided in Fig. 1: the upper panel represents a patient with numerous granulocytes and a relatively small population of CD45^{+} lymphocytes (10%), whereas the lower panel represents a patient with few granulocytes and a high number of lymphocytes (30%).

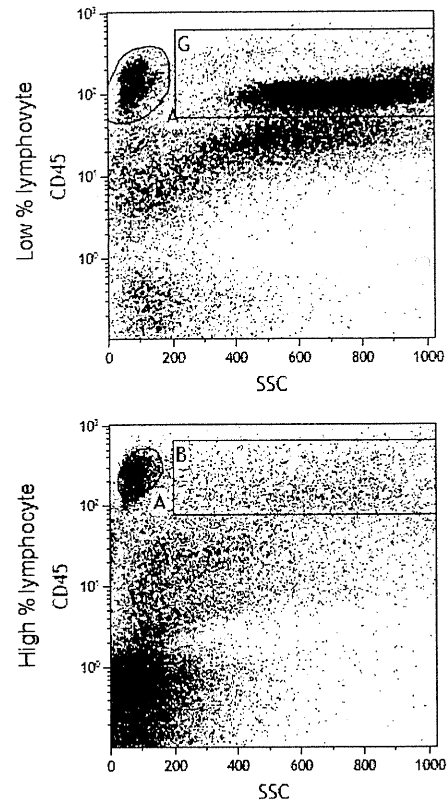

Fig. 1 Flow cytometric analysis of cervical mucosal cells using CD45/SSC gating. Processed cervical specimens were analyzed by flow cytometry and CD45/SSC gating. CD45^{+} cervical leukocytes are comprised of lymphocytes (circle) and granulocytes/monocytes (square). Upper and lower panels were representative of patients with low (about 10%) and high (about 30%) numbers of lymphocytes among their CD45^{+} cervical leukocytes, respectively. The absolute number of isolated cervical lymphocytes remained relatively constant among study subjects.

Characterization of Cervical T Cells in CIN Lesions

The majority of cervical lymphocytes isolated from CIN lesions were CD3^{+} T cells [median 74% (IQR: 59–82)]. CD19^{+} B cells were rarely found [median 0.45% (IQR: 0.04–1.40)]. In Fig. 2, CD3-gated cervical T cells were characterized by flow cytometry, and each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively. A median of 54%

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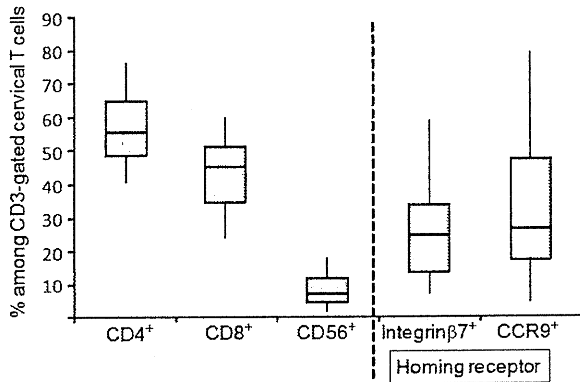

Fig. 2 Characterization of the cervical CD3+ lymphocytes. CD3-gated cervical T cells consisted of CD3+ CD4+ T cells [median 54% (IQR: 49–65), $n = 28$], CD3+ CD8+ T cells [median 46% (IQR: 35–51), $n = 28$], and CD3+ CD56+ natural killer T cells [median 5.6% (IQR: 4.5–12), $n = 17$]. Twenty-four percentage (IQR: 13–34, $n = 43$) and 27% (IQR: 17–47, $n = 27$) of cervical T cells were integrin β7+ and CCR9+, respectively. Each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively.

(IQR: 49–65) of cervical T cells were CD3+ and CD4+, while a median of 46% (IQR: 35–51) expressed CD3 and CD8, demonstrating that CD8+ T cells are relatively abundant among cervical T cells. The CD4/CD8 ratio of 1.15 in the cervix was clearly lower than the value of 2.0 found in peripheral blood. Among CD3+ cells, a median of 5.6% (IQR: 4.5–12) were CD56+ natural killer T (NKT) cells.

Those cervical CD3+ T cells that were originally derived in the gut were defined by expression of the gut mucosa-specific cell-surface antigens integrin β7+ and CCR9+. A median of 24% (IQR: 13–34) of cervical T cells expressed integrin β7 and 27% (IQR: 17–47) expressed CCR9 (Fig. 2).

Notably, more than 90% (median: 99.1, IQR: 95.3–100) of the integrin β7+ cells co-expressed the αE subunit (integrin αEβ7+ cells; Fig. 3a). Integrin α4+ cells were rarely present among the integrin β7+ cells (Fig. 3b). Approximately 40% (median: 40.1, IQR: 33.2–44.2) of cervical integrin β7+ cells were integrin αEβ7+ CCR9+ double positive (Fig. 3c).

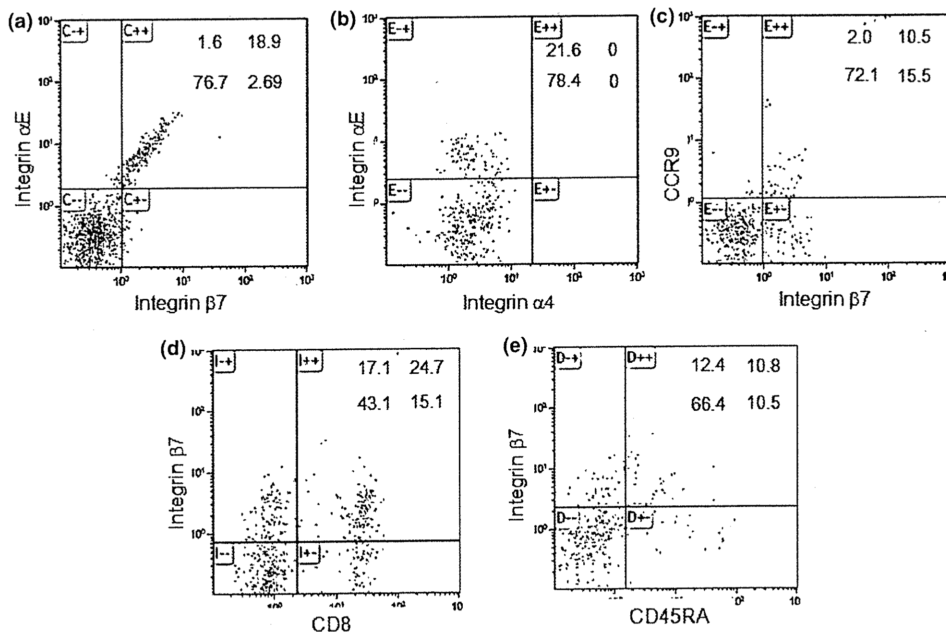

Fig. 3 Characterization of CD8, CD45RA, and homing receptors specific for gut-derived mucosal T cells among CD3+ cervical T cells. Representative flow cytometry analyses of CD3-gated cervical T cells. (a) More than 90% of integrin β7+ cervical T cells were integrin αE+ intraepithelial lymphocyte. (b) Integrin α4+ LPL were negligible in our cervical samples. (c) Among integrin β7+ cells, approximately 40% were CCR9+. (d) Forty-two percentage of total cervical T cells and 53% of integrin β7+ T cells were CD8+. (e) About half of the integrin β7+ T cells were CD45RA-negative memory cells.

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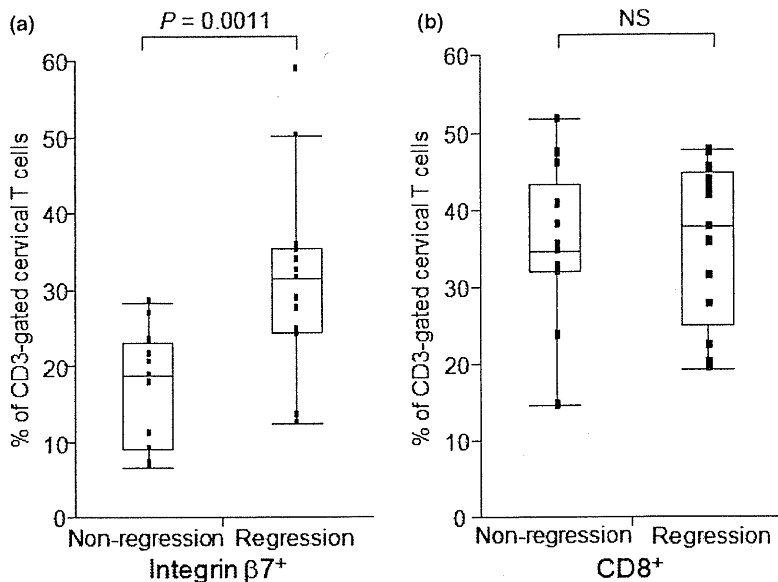

Fig. 4 Association between gut-derived cervical intraepithelial lymphocyte and cervical intraepithelial neoplasia (CIN) regression. Populations of integrin $\beta7^+$ (a) and $CD8^+$ (b) cells among $CD3^+$ cervical T cells were compared between CIN regression (n = 13) and non-regression (n = 13) groups, paired according to follow-up duration. A P-value ≤0.05 was considered significant using Wilcoxon rank test comparisons.

$CD8^+$ memory T cells are essential for adaptive cytotoxic immune responses to CIN.[21,22] Among our patients with CIN, the proportion of integrin $\beta7^+$ cervical T cells that expressed CD8 [median 53% (IQR: 28–69)] was greater than that for total cervical T cells (Fig. 3d). Approximately half [median 43% (IQR: 31–57)] of integrin $\beta7^+$ T cells were CD45RO memory T cells, while the other half were CD45RA effector T cells (Fig. 3e).

Association of Gut-derived Cervical IEL with CIN Course

Integrin β7 is a more ubiquitous homing receptor in mucosal lymphocytes rather than integrin αE or α4. To determine whether there was an association between the presence of gut-derived cervical IEL and spontaneous regression of CIN, comparisons were made between populations of integrin $\beta7^+$ $CD3^+$ and $CD8^+$ $CD3^+$ cervical T cells in CIN regressors (n = 13) and non-regressors (n = 13), paired according to their duration of follow-up. No significant differences were seen in the detection rates of high-risk HPV (69 versus 77%, P = 0.50), the squamous intraepithelial lesion (SIL) grade (high-grade SIL: 54 versus 54%, P = 0.65), and the median ages (34 years old versus 35) of patients in the regression and non-regression groups. The percentage of integrin $\beta7^+$ cervical T cells varied from 6 to 57% among the 26 study subjects. Among regressors, integrin $\beta7^+$ cervical T cells comprised a median of 31.6% (IQR: 24.5–35.5) of $CD3^+$ cervical T cells; the rate among non-regressors was 18.8% (IQR: 9.2–23.3), P = 0.0011 (Fig. 4a). In contrast, there was no difference in populations of $CD8^+$ $CD3^+$ cervical T cells between CIN regressors and non-regressors (Fig. 4b). The proportion of $CCR9^+$ and $CD45RA^+$ $CD3^+$ cervical T cells was likewise similar in the two groups (data not shown).

Discussion

Human papillomavirus preferentially infects, and CIN develops in the human cervical epithelium. It is clear that HPV antigens are recognized by the systemic cell-mediated immune system, but remains unclear whether systemic cellular immune responses predict the regression of CIN.[16,17] Local mucosal immune responses in the cervix are likely to be important in immunological clearance of CIN lesions. Integrin α4β7 is essential for recruiting activated mucosal lymphocytes from the circulation into local LP in a manner entirely dependent on interaction between lymphocyte integrin α4β7 and the MAdCAM-1 that is constitutively expressed on LP post-capillary venules.[23] In contrast, integrin αE (CD103) β7 is expressed by only 2% of circulating blood lymphocytes, but more than 90% of IEL and a minority of lamina propria lymphocyte (LPL); its ligand is E-cadherin expressed on the epithelial cells.[24] Activated integrin $\alpha4\beta7^+$ T cells differentiate within the

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LP into integrin αEβ7$^+$ T cells upon exposure to TGF-β locally secreted by epithelial cells.[5] Binding of integrin αEβ7 to E-cadherin provokes retention of the activated IEL within the epithelium. Recognition of target epithelial cells by IELs is important in the initiation of cytolytic effector function by activated IELs and modulation of adaptive immune responses to control potentially destructive epithelial immunity. Adhesion of integrin αEβ7$^+$ IEL to epithelial E-cadherin is promoted by CCL25–CCR9 interactions.[4] This suggests that, when compared to integrin α4β7$^+$ LPL, integrin αEβ7$^+$ IELs may be more directly linked to essential adaptive immune responses to target epithelial cells at local effector sites.

Several studies have reported that integrin α4β7 is expressed on gut-derived mucosal lymphocytes within the cervix.[9,11] However, our data indicate that more than 90% of integrin β7$^+$ T cells were positive for integrin αE and few express α4. Pudney et al.[10] have shown using immunohistochemistry that integrin αEβ7$^+$ lymphocytes are primarily located in the epithelium of the ectocervix and often occur as focal accumulations in the LP of the transformation zone. Our brushing methodology enables us to preferentially collect cervical mucosal lymphocytes from the epithelium and occasionally from the LP. Others who have recently reported that nearly all cervical tissue T cells are integrin α4β7$^+$[9] used cervical tissue specimens and equally valuable methodologies that would be expected to isolate cells from deeper within the cervical tissue, possibly favoring isolation of LPL over cells tightly adhered to the epithelium.

Our cervical samples were contaminated by numerous granulocytes, a finding supported by several previous studies using cervical mucosa unlike peripheral blood samples.[10,11] Granulocyte contamination variability was likely the result of differing levels of cervical inflammation among patients. Although the number of lymphocytes among CD45$^+$ cervical leukocytes varied from 10 to 30%, the absolute number of cervical lymphocytes present in a sample appeared to be relatively constant and independent of patient source. The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin β7 and CCR9. We showed that integrin β7 and CCR9 did not always co-express. This agrees with reports showing that expression of the mucosal homing receptors, integrin β7 and CCR9, is not always linked, but instead depends on lymphocyte differentiation and the location of the effector sites infiltrated by these cells.[25,26]

Expression of MAdCAM-1 is essential for trafficking of integrin α4β7$^+$ lymphocytes into the LP, while the expression of E-cadherin on the epithelium is essential for the retention of integrin αEβ7$^+$ lymphocytes. Inflammation of the mucosa enhances MAdCAM-1 expression on the endothelial cells of post-capillary venules in the genital tract,[8] and inflammatory changes are often observed in CIN when compared with normal cervical mucosa.[27,28] Trimble et al.[9] reported that MAdCAM-1 expression correlates with non-specific CD8$^+$ LPL infiltration of the LP and CIN regression. In our sampled IELs, there was no association between CD8$^+$ cells and CIN regression. Studies have also demonstrated that oncoproteins from high-risk HPV subtypes downregulate E-cadherin expression in CIN lesions and that this downregulation is closely associated with disease progression.[29–31] E-cadherin plays an important role in the maintenance of normal adhesion in epithelial sites and its loss is associated with poor prognosis for many tumors other than CIN.[32] The downregulation of E-cadherin may interfere with the retention of integrin αEβ7$^+$ T cells in CIN lesions, and our results suggest that IEL retention varies among patients with CIN. We have shown that populations of integrin αEβ7$^+$ IEL in CIN lesions vary markedly among patients and that higher IEL numbers are associated with spontaneous regression of CIN. Although HPV-specific cytotoxic T lymphocyte activity was not investigated here, the accumulation of integrin αEβ7$^+$ IEL in CIN lesions and their association with CIN regression suggests these cells, rather than non-specific CD8$^+$ T cells, may have important local effector functions in the cervical epithelium. In the present study, the adaptive immune system was focused, but the innate immune responses play equally important roles in controlling HPV infection. Daud et al.[33] has recently reported the mechanism of interference with innate immune system by HPV16, dampened toll-like receptor expression, which results in the viral persistence. The interaction of innate with adaptive immunity at the local mucosa should be investigated.

In summary, our report is the first to specifically phenotype cervical IEL in CIN lesions. Our results indicate that the presence of elevated numbers of gut-derived integrin αEβ7$^+$ IELs in specimens gathered from patients with CIN using a cervical cytobrush may represent a possible biomarker for CIN regression. Sampling of cervical IEL using this methodology is relatively non-invasive and techni-

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cally easier than the isolation of cervical LPL from tissue biopsies. Future investigations using our sampling methods will focus on HPV-specific cell-mediated immune responses by cervical IELs isolated from patients with CIN. These and related investigations should improve our understanding of cervical mucosal immunity and hasten the development of a therapeutic HPV vaccine.

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References

1 Gorfu G, Nieves JR, Ley K: Role of beta7 integrins in intestinal lymphocyte homing and retention. *Curr Mol Med* 2009; 9:836–850.

2 Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY: Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 2004; 21:527–538.

3 Edelee F, Molenaar R, Gutle D, Dudda JC, Jakob T, Homey B, Mebius R, Hornef M, Martin SF: Cutting edge: instructive role of peripheral tissue cells in the imprinting of T cell homing receptor patterns. *J Immunnol* 2008; 181:2745–2749.

4 Ericsson A, Svensson M, Arya A, Agace WW: CCL25/CCR9 promotes the induction and function of CD103 on intestinal intraepithelial lymphocytes. *Eur J Immunol* 2004; 34:2720–2729.

5 El-Asady R, Yuan R, Liu K, Wang D, Gress RE, Lucas PJ, Drachenberg CB, Hadley GA: TGF-b-dependent CD103 expression by $CD8^+$ T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *J Exp Med* 2005; 201:1647–1657.

6 Kawana K, Matsumoto J, Miura S, Kawana Y, Nagamatsu T, Yasugi T, Fujii T, Yang H, Quayle AJ, Taketani Y, Schust DJ: Expression of CD1d and ligand-induced cytokine production are tissue-specific in mucosal epithelia of the human lower reproductive tract. *Infect Immun* 2008; 76:3011–3018.

7 Hawkins RA, Rank RG, Kelly KA: Expression of mucosal homing receptor α4β7 is associated with enhanced migration to the Chlamydia-infected murine genital mucosa in vivo. *Infect Immun* 2000; 68:5587–5594.

8 Kelly KA, Rank RG: Identification of homing receptors that mediate the recruitment of CD4 T cells to the genital tract following intravaginal infection with *Chlamydia trachomatis*. *Infect Immun* 1997; 65:5198–5208.

9 Trimble CL, Clark RA, Thoburn C, Hanson NC, Tassello J, Frosina D, Kos F, Teague J, Jiang Y, Barat NC, Jungbluth AA: Human papillomavirus 16-associated cervical intraepithelial neoplasia in humans excludes CD8 T cells from dysplastic epithelium. *J Immunol* 2010; 185:7107–7114.

10 Pudney J, Quayle AJ, Anderson DJ: Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol Reprod* 2005; 73:1253–1263.

11 Kelly KA, Wiley D, Wiesmeier E, Briskin M, Butch A, Darville T: The combination of the gastrointestinal integrin (α4β7) and selectin ligand enhances T-cell migration to the reproductive tract during infection with *Chlamydia trachomatis*. *Am J Reprod Immunol* 2009; 61:446–452.

12 Holowaty P, Miller AB, Rohan T, To T: Natural history of dysplasia of the uterine cervix. *J Natl Cancer Inst* 1999; 91:252–258.

13 Moscicki AB, Schiffman M, Kjaer S, Villa LL: Chapter 5: updating the natural history of HPV and anogenital cancer. *Vaccine* 2006; 24:42–51.

14 Ellerbrock TV, Chiasson MA, Bush TJ, Sun XW, Sawo D, Brudney K, Wright TC Jr.: Incidence of cervical squamous intraepithelial lesions in HIV-infected women. *JAMA* 2000; 283:1031–1037.

15 Ognenovski VM, Marder W, Somers EC, Johnston CM, Farrehi JG, Selvaggi SM, McCune WJ: Increased incidence of cervical intraepithelial neoplasia in women with systemic lupus erythematosus treated with intravenous cyclophosphamide. *J Rheumatol* 2004; 31:1763–1767.

16 Trimble CL, Peng S, Thoburn C, Kos F, Wu TC: Naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN2/3. *Cancer Immunol Immunother* 2010; 59:799–803.

17 Nakagawa M, Gupta SK, Coleman HN, Sellers MA, Banken JA, Greenfield WW: A favorable clinical trend is associated with CD8 T-cell immune responses to the human papillomavirus type 16 E6 antigens in women being studied for abnormal pap smear results. *J Low Genit Tract Dis* 2010; 14:124–129.

18 Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ: Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000; 38:357–361.

19 Bosch FX, Sanjose S: Human papillomavirus and cervical cancer – burden and assessment of causality. *J Natl Cancer Inst Monogr* 2003; 31:3–13.

20 Prakash M, Patterson S, Kapembwa MS: Evaluation of the cervical cytobrush sampling technique for the preparation of $CD45^+$ mononuclear cells from the human cervix. *J Immunol Methods* 2001; 258:37–46.

21 Passmore JA, Milner M, Denny L, Sampson C, Marais DJ, Allan B, Gumbi PP, Hitzeroth II, Rybicki EP, Williamson AL: Comparison of cervical and blood T-cell responses to human papillomavirus-16 in women with human papillomavirus-associated cervical intraepithelial neoplasia. *Immunology* 2006; 119:507–514.

22 Monnier-Benoit S, Mauny F, Riethmuller D, Guerrini JS, Capilna M, Felix S, Seilles E, Mougin C, Pretet JL: Immunohisto-chemical analysis of $CD4^+$ and $CD8^+$ T-cell subsets in high risk human papillomavirus-associated pre-malignant lesions of the uterine cervix. *Gynecol Oncol* 2006; 102:22–31.

23 Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Bucher EC: Role of alpha 4-integrins lymphocyte homing to mucosal tissues in vivo. *J Immunol* 1994; 152:3282–3293.

24 Cepek KL, Parker CM, Madara JL, Brenner MB: Integrin alpha E beta 7 mediates adhesion of T lymphocytes to epithelial cells. *J Immunol* 1993; 150:3459–3470.

25 Mora JR, Andrian UHV: T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol* 2006; 27:235–243.

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26 Carramolino L, Zaballos A, Kremer L, Villares R, Martin P, Ardavin C, Martinez AC, Marquez G: Expression of CCR9 beta-chemokine receptor is modulated in thymocyte differentiation and is selectively maintained in CD8+ T cells from secondary lymphoid organs. *Blood* 2001; 97:851–857.
27 Kobayashi A, Greenblatt RM, Anastos K, Minkoff H, Massad LS, Young M, Levine AM, Darragh TM, Weinberg V, Smith-McCune KK: Functional attributes of mucosal immunity in cervical intraepithelial neoplasia and effects of HIV infection. *Cancer Res* 2004; 64:6766–6774.
28 Kobayashi A, Weinberg V, Darragh T, Smith-McCune K: Evolving immunosuppressive microenvironment during human cervical carcinogenesis. *Mucosal Immunol* 2008; 1:412–420.
29 Branca M, Giorgi C, Ciotti M, Santini D, Di Bonito L, Costa S, Benedetto A, Bonifacio D, Di Bonito P, Paba P, Accardi L, Mariani L, Syrjanen S, Favalli C, Syrjanen K: Down-regulation of E-cadherin is closely associated with progression of cervical intraepithelial neoplasia (CIN), but not with high-risk human papillomavirus (HPV) or disease outcome in cervical cancer. *Eur J Gynecol Oncol* 2006; 27:215–223.
30 Matthes K, Leong CM, Baxter L, Inglis E, Yun K, Backstrom BT, Doorbar J, Hibma M: Depletion of langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down regulation of E-cadherin. *J Virol* 2003; 77:8378–8385.
31 Welles A, Yates C, Shepard CR: E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* 2008; 25:621–628.
32 Boulenouar S, Weyn C, Noppen MV, Moussa AM, Favre M, Delvenne PO, Bex F, Noel A, Englert Y, Fontaine V: Effects of HPV-16 E5, E6 and E7 proteins on survival, adhesion, migration and invasion of trophoblastic cells. *Carcinogenesis* 2010; 31:473–480.
33 Daud II, Scott ME, Ma Y, Shiboski S, Farhat S, Moscicki AB: Association between toll-like receptor expression and human papillomavirus type 16 persistence. *Int J Cancer* 2011; 123:879–886.

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Introducing HPV vaccine and scaling up screening procedures to prevent deaths from cervical cancer in Japan: a cost-effectiveness analysis

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Objective To assess the cost-effectiveness of universal vaccination of 11-year-old girls against human papillomavirus (HPV) infection and increased screening coverage to prevent cervical cancer in Japan where the coverage of Papanicolaou smears is very low.

Design A cost–utility analysis from a societal perspective.

Setting Japan, 2010.

Population The female Japanese population aged 11 years or older.

Methods A Markov model of the natural history of cervical cancer was constructed to compare six strategies: i.e. a screening coverage rate of 20, 50 and 80% with and without routine vaccination at age 11.

Main outcome measures Cervical cancer incidence, quality-adjusted life years (QALYs), costs and incremental cost-effectiveness ratios.

Results Expanding the coverage of Papanicolaou smears from the current level of 20–50 and 80% yields a 45.5 and 63.1% reduction in cervical cancer incidence, respectively. Impact of combined strategies increases with coverage. Coverages of 20, 50 and 80% showed a 66.1, 80.9 and 86.8% reduction in disease, respectively. The costs of strategies with vaccination are four times higher than the cost of strategies without vaccination. Vaccinating all 11-year-old girls with bivalent vaccines with a Papanicolaou smear coverage rate of 50% is likely to be the most cost-effective option among the six strategies.

Conclusions The introduction of HPV vaccination in Japan is cost-effective as in other countries. It is more cost-effective to increase the coverage of the Papanicolaou smear along with the universal administration of HPV vaccine.

Keywords Cost-effectiveness analysis, economics, human papillomavirus, vaccines.

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Introduction

Cervical cancer is the fifth leading cause of female cancer death in the world.[1] The overall frequency of cervical cancer in Japan, including carcinoma *in situ*, was reported as 17 000 per year.[2] In Japan, it is the third leading cause of cancer death among women <40 years of age.[2] The age-adjusted mortality rate of cervical cancer in Japan has remained at almost the same level for the past two decades, although it has declined in the USA and UK.[2–4]

Screening with cervical cytology [i.e. Papanicolaou (Pap) smear] has been the key national strategy for early detection and treatment of cervical cancer to reduce its burden.[5] However, the coverage of Pap smear screening in Japan remains between 10 and 20%,[6] much lower than in other countries such as the UK (81%), France (54%) and the USA (>82%).[7]

Persistent human papillomavirus (HPV) infection, particularly with oncogenic types 16, 18, 52 and 58, is associated with a higher risk of incident cervical cancer precursor lesions.[8] A prophylactic vaccine to prevent infection from HPV16 and 18 to reduce the burden of cervical cancer has been developed and implemented in some countries.[9] The idea of introducing HPV vaccine in the Japanese population has evoked public debate and become a huge political

issue. The bivalent vaccine was officially approved for use in Japan by the end of 2009, but there has been an on-going debate on whether the use of HPV vaccine should be underpinned by tax-payers' money and if so, how much the government should spend.[9]

Despite its proven cost-effectiveness in other settings[10–12] a simple extrapolation of the costs and effectiveness of HPV vaccine in countries other than the Japanese setting is not appropriate because of the differences in cervical cancer epidemiology and health systems. The prevalence of HPV types differs between geographic regions. In the case of squamous cell carcinoma, HPV16 was the predominant type (46–63%) followed by HPV18 (10–14%), 45 (2–8%), 31 (2–7%) and 33 (3–5%) in all regions except Asia, where HPV types 58 (6%) and 52 (4%) were more frequently identified.[13] In Japan, HPV52 and HPV58 are most frequently found in squamous intraepithelial lesion following HPV16.[14] A relatively lower prevalence of HPV16 and HPV18 in Japan has cast doubt on the effectiveness of the current HPV vaccine when compared with other countries.[15]

Only one study has evaluated the cost-effectiveness of HPV vaccination in the Japanese setting.[16] However, the study did not compare strategies with a variable screening rate. Nor did it consider the effect of HPV type prevalence by age in Japan. Therefore, a cost-effectiveness analysis of screening coverage and vaccination, taking into account the age-specific prevalence by HPV type in the Japanese setting is urgently needed to inform and support policy decisions. Healthcare resources are limited; resources dedicated to screening and vaccination are no longer available for alternative healthcare uses and therefore the chosen strategy should represent a cost-effective use of scarce resources. The major objective of the present study is to assess the cost-effectiveness of universal vaccination against HPV in Japan from a societal perspective where the coverage of Pap smears is low and HPV oncogenic types are different from in other settings.

Methods

Natural history model of HPV infection

We developed a state-transition Markov model that simulates the natural history of HPV infection and carcinogenesis, in which transitions take place from one state to another at 1-month intervals (Figure 1). The model has 25 Markov states. The entry point into the model is girls aged 11 years with no previous exposure to HPV. We assumed that when girls/women enter the model, they start sexual activities, so acquiring a risk for HPV with the currently observed probabilities. In each cycle, they proceed to one of the four states: HPV16 and 18 DNA-positive group (HPV16 and 18), the other high-risk HPV DNA-positive group (other HR), the low-risk HPV DNA-positive group (LR), and the non-infected group (Normal) using monthly transition probabilities based on the systematic review of published literature.[17]

Each group follows a natural history unless they are screened. When cervical intraepithelial neoplasia (CIN) 2, 3 or invasive cancer is identified by the screening, a treatment intervention conditional on cancer stage is implemented. After the treatment for an invasive cancer, a certain proportion of patients die whereas others survive according to the survival probabilities compiled from the cancer registry data.[18,19] Age-dependent transition rates of disease progression from the susceptible to those infected with either HPV16/18, other HR (excluding HPV16 and 18) or LR were estimated from recent Japanese data.[20] All individuals are followed up for 50 years until they reach 60 years of age, which is the average retirement age in Japan, unless they die earlier.

Vaccine efficacy

The vaccine efficacy was evaluated in eight randomised controlled trials.[21,22] There was a substantial variation in follow-up periods and outcome measures among the studies. The World Health Organization adopted CIN2/3 as surrogate endpoints for cervical cancer in trials assessing vaccine efficacy.[21] In our model we used the relative risk of HPV16 and 18 persistent infection risk, as the vaccine immunises against the contraction of HPV. The hypothesis that persistent infection with one of the 15 carcinogenic HPV types is the fundamental cause of cervical cancer is clearly supported by scientific evidence.[23] We assumed relative risks of 0.12 (95% CI 0.03–0.48) for persistent HPV16 and 18 infection and 0.5 (range 0.3–0.7) for persistent HPV high-risk type excluding 16, 18.[21,22,24,25] Additionally, we assumed 100% lifetime protection against HPV16 and 18 once fully vaccinated.

Intervention strategies

The bivalent vaccine was approved for use in women and girls who are over 10 years old. The Japan Society of Obstetrics and Gynaecology recommended administering HPV vaccine among girls from 11 to 14 years of age as a priority, partly because they are old enough to understand the meaning of the vaccination and partly because the vaccination in this age group is efficient and ensures early protection against HPV with high immunogenicity. Therefore, for strategies which include vaccination, all 11-year-old girls are vaccinated at the entry point into the model. We assumed that there is no exposure to any HPV types before the entry to the model. At 20 years of age, they start receiving screening every 2 years according to the current Japanese recommendations.[5] Our reference strategy is the screening programme only with the current level of

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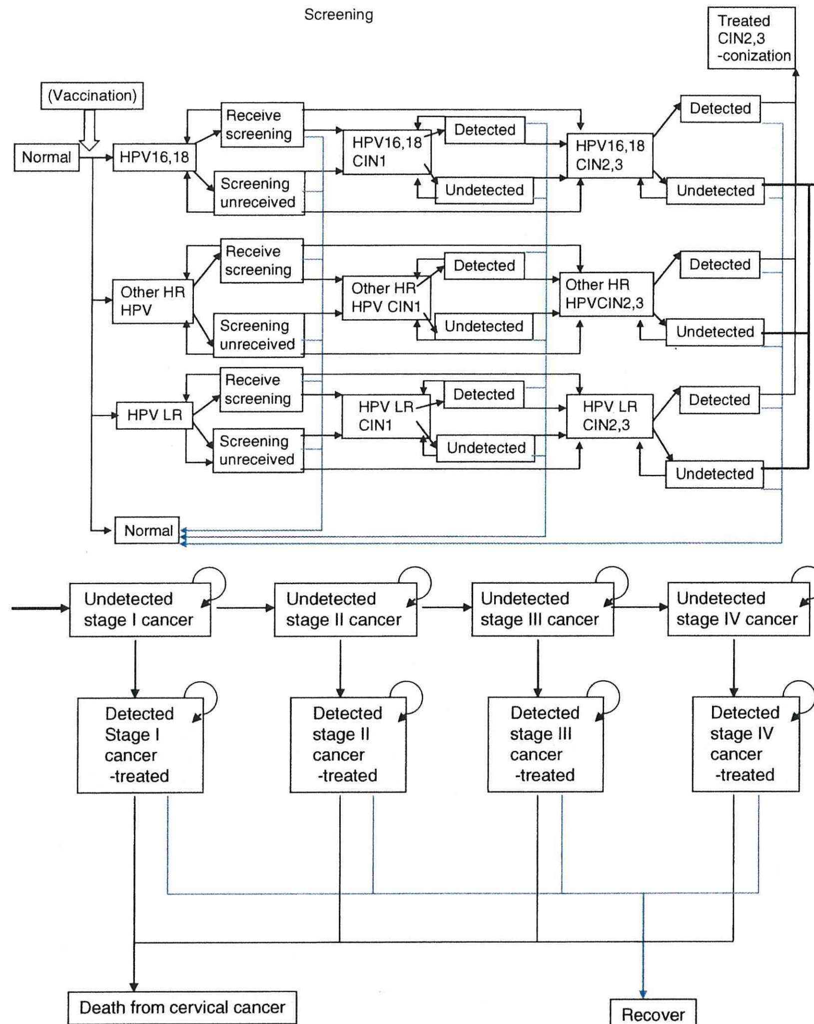

*The probabilities to die from other causes are included at all each states.

Figure 1. HPV natural history model.

Table 1. Strategies

1. 20% coverage rate of screening with no vaccination
2. 50% coverage rate of screening with no vaccination
3. 80% coverage rate of screening with no vaccination
4. 20% coverage rate of screening with vaccination for all 11-year-old girls
5. 50% coverage rate of screening with vaccination for all 11-year-old girls
6. 80% coverage rate of screening with vaccination for all 11-year-old girls

coverage (i.e. 20%).[6,7] Table 1 summarises six strategies that were analysed in the present study. The sensitivity of the Pap smear was assumed to be 94.7% as previously reported.[26] The specificity (reported to be 98.9%) is not included in the model because screening will be repeated in false positives as determined by the cytology results.

Survival rates of women with cervical cancer

We used the data from life tables of Japanese vital registration to estimate the population-based mortality rates by age from cervical cancer and other competing risks.[27] Cumulative nationwide survival rates by cancer stages of FIGO classification were not available in Japan. We adopted the data from the US SEER programme (Surveillance Epidemiology and End Results),[18,19] which were calibrated using data from an existing Japanese regional cancer registry.[28]

Transition probabilities

Several natural history models of HPV have been developed and used in policy evaluations.[29,30] Whereas a particular

parameter has been common to several natural history models, there is a huge variation in the structure and parameters used in the previous models.[29] We used age-dependent type-specific HPV prevalence data from Japanese women[20] to derive transition probabilities from the susceptible to those infected with HPV16/18, other HR types and LR types.

Other model parameters were estimated from systematic literature reviews and then calibrated to the Japanese setting (Table 2).[17] We simulated the model by using the transition rates of CIN2, 3 to the undetected stage I cancer of HPV16/18 and other HR depending on their age groups in Japan. Then we adjusted them by using the data of age-dependent incident rates of cervical cancer. We validated the model by goodness-of-fit statistics using age-dependent mortality rates of cervical cancer.

Table 2. Transition rates

Variable	Baseline values	Range
Progression		
HPV DNA to CIN1		
Low-risk HPV	0.0264	0.0245–0.0284
High-risk 16, 18 HPV	0.0150	0.0026–0.0274
High-risk other HPV	0.0376	0.0271–0.0480
HPV DNA to CIN2, 3		
Low-risk HPV	0.00003	0.000003–0.00006
High-risk 16, 18 HPV	0.0012	0.000014–0.0024
High-risk other HPV	0.000025	0.000002–0.00005
CIN1 to CIN2, 3		
Low risk HPV	0.0003	0.00002–0.0005
High-risk 16, 18 HPV	0.0042	0.0001–0.0082
High-risk other HPV	0.0015	0.0001–0.0028
CIN2, 3 to undetected stage 1 cancer		
High-risk 16, 18 HPV	0.0049*	0.00001–0.0098
High-risk other HPV	0.0088*	0.00004–0.0176
Progression rates in unscreened women with cancer		
Stage I to stage II	0.0188	
Stage II to stage III	0.0250	
Stage III to stage IV	0.0375	
Regression		
HPV DNA to Normal		
Low-risk HPV	0.1951	
High-risk 16, 18 HPV	0.1951	
High-risk other HPV	0.1951	
CIN1 to Normal		
Low-risk HPV	0.0854	
High-risk 16, 18 HPV	0.1406	0.1316–0.1497
High-risk other HPV	0.0430	
CIN2, 3 to Normal (70% of women)		
Low-risk HPV	0.0145	0.0052–0.0238
High-risk 16, 18 HPV	0.0045	0.0010–0.0080
High-risk other HPV	0.0082	0.0029–0.0134
CIN2, 3 to HPV DNA or to CIN1 (15% of women each)		
Low-risk HPV	0.0031	0.0011–0.0051
High-risk 16, 18 HPV	0.0010	0.0002–0.0017
High-risk other HPV	0.0018	0.0006–0.0029

*Multiplied by age-dependent rate derived from calibration.

Cost estimation

A societal perspective was adopted for this cost analysis. Cost estimates are presented in Table 3 that include programme costs and time costs. We approximated the programme costs by using the current national tariff used by the national health insurance scheme.[31] These data were cross-validated by the cost of treatments and care for gynaecological patients at the University of Tokyo Hospital between August 2007 and November 2009. Both variable costs and doctor's fees are included in the programme costs according to the fee schedule set by the national tariff.[31] We estimated patients' time cost by using the national average hourly wage of part-time workers from a national survey.[32]

Cost-effectiveness analysis

We calculated quality-adjusted life-years (QALYs) from the model outputs on incidence, duration and mortality. The quality-of-life weights for different health states were based on those used in previous studies (Table 4).[33–35] All costs and benefits were discounted at 3%, a frequently used rate for cost-effective analysis done in Japanese settings.[36]

In line with a standard health economic evaluation, strategies are ranked in order of effectiveness after excluding dominated strategies.[37] Incremental cost-effectiveness ratios (ICERs) are then calculated for each strategy relative to the next best alternative. The preferred strategy is the most effective strategy with an ICER within the willingness to pay threshold of 4.5 million yen. A commonly applied threshold for acceptable cost-effectiveness in the USA is $50,000;[38] it is often used as a basis of cost-effective analysis in a Japanese setting.

Table 3. Cost data

Costs involving patient's time costs	Yen
Screening visit (Pap-test) per event	7460
CIN1 detected patient per month	4228
CIN2, 3 detection per event	28,360
Conisation cost per case	310,900
Treatment cost for stage I cancer case	664,300
Treatment cost for stage II cancer per case	2,869,900
Treatment cost for stage III cancer per case	3,066,500
Treatment cost for stage IV cancer per case	2,940,200
Average monthly wage for a Japanese case	226,100
Vaccination cost (for three doses/visits)	58,000

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