Gain of function mutation of *NOTCH1* was detected in many cases of T-cell acute lymphoblastic leukemia,[13] and this finding gave rise to the concept of treatment by inhibiting the formation of Notch transactivation complex.[14] In cervical cancer, the upregulation of NOTCH1[15,16] and NOTCH2,[16] and an increase in nuclear localization of NICD[17] were documented. On the basis of these observations, activation of Notch signaling is thought to be associated with the development of cervical cancer.[18] However, promotion of carcinogenesis by Notch signal activation seems contradictory to the phenotypes observed in the *Notch1* knockout mouse, which exhibits an increase in the incidence of papilloma and chemical-induced skin cancer,[8] and the transgenic mouse of the pan-Notch inhibitor, dominant-negative *Mastermind-like 1*, which shows dysplasia and SCC of skin.[19] In fact, expression of NOTCH1 is decreased in skin cancer,[20,21] suggesting that *NOTCH1* acts as a tumor suppressor. To solve the discrepancy of the proposed role of Notch, accurate knowledge of its expression is crucial. To assess its contribution in cancer, the Notch expression should be evaluated in comparison with the normal cells from which the cancer cells originated. However, it appears that the expression of Notch in human cancers has not been properly evaluated along this line. Even the localization of Notch proteins in normal adult tissue is unclear, partially, because of the difficulty of detection.

To address this issue, we have engaged in an examination of Notch expression in adult human tissues. In this study, we first demonstrate that NOTCH1 is predominantly expressed in the basal cells of normal squamous epithelium. Then we expand the examination to neoplasms that have originated from squamous epithelium and show that Notch1 expression is downregulated in these lesions. Cell culture experiments indicate that reduction of NOTCH1 expression associates with abnormal differentiation represented by alteration of keratin subtype expression. Our data suggest that aberrant epithelial differentiation in squamous neoplasms is caused by the reduction of NOTCH1 expression, which in turn unveils its essential function in the maintenance of normal epithelial integrity.

MATERIALS AND METHODS

Clinical Specimens

A total of 56 specimens of oral lesions (epithelial dysplasia and squamous cell carcinoma) were collected at the Dental Hospital of Tokyo Medical and Dental University. Pharyngeal cancers were excluded; the case summaries are shown in Supplementary Material 1. A total of 20 specimens of esophageal lesions (squamous cell carcinoma) and normal tissues were collected at Medical Hospital of Tokyo Medical and Dental University; the case summaries are shown in Supplementary Material 2. A total of 57 specimens of uterine cervical lesions (intraepithelial neoplasm and squamous cell carcinoma) were collected at Keio University Hospital; the case summaries are shown in Supplementary Material 3. The tissues were fixed in formalin and embedded in paraffin according to the routine laboratory protocol. HPV genotyping was performed as previously described[22] from the conventional cytology specimens. All experiments were approved by the ethics committees of both universities.

cDNA Microarray Analysis

Oral squamous cell carcinoma (OSCC) cells were taken from surgically excised specimens by laser capture microdissection and were subjected to cDNA microarray analysis, as previously described.[23]

Immunostaining and *In Situ* Hybridization

Immunohistochemical staining was performed using the Sequenza (Thermo Fisher Scientific, MA, USA). For antigen retrieval, the sections were autoclaved in alkaline buffer (10 mM Tris (pH = 9.0) and 1 mM EDTA) at 120 °C for 20 min. The primary antibodies used in this study were anti-Notch1 (EP1238Y, Epitomics, CA, USA); cleaved Notch1 (Val1744; D3B8, Cell Signaling, MA, USA) Notch2 (D67C8, Cell Signaling); Notch3 (D11B8, Cell Signaling); Hes1 (EPR4226, Epitomics); Hey1 (polyclonal, Millipore, MA, USA); keratin 5 (K5; EPR1600Y, Epitomics); K13 (EPR3671, Epitomics); K13 (alternative antibody, KS-1A3, Leica Microsystems, Wetzlar, Germany); K15 (EPR1614Y, Epitomics); K17 (D73C7, Cell Signaling); K17 (alternative antibody, E3, Dako, Glostrup, Denmark); K18 (DC10, Dako); pan-Keratin (AE1/AE3, Dako); Vimentin (SP20, Epitomics); E-cadherin (36/E-Cadherin, BD transduction laboratories, CA, USA); Desmoglein3 (3G133, Santa Cruz, CA, USA); p63 (4A4, Dako); β-tubulin (9F3, Cell Signaling) and β-actin (C-2, Santa Cruz). EnVision Dual Link (Dako) was used as the secondary antibody. Coloration was done in DAB substrate. For immunofluorostaining, Alexa Fluor 488 anti-rabbit IgG (Invitrogen, CA, USA) and DAPI were used. *In situ* hybridization to mouse E18.5 embryos was conducted as previously described.[24] Antibody adsorption test was performed as follows. HEK293 cells were transfected with *Notch1* or *NOTCH3* and were fixed 48 h after transfection. The anti-NOTCH1 or anti-NOTCH3 antibody (diluted 1/500) was applied to the fixed cells, respectively, and incubated for 1 h. The adsorbed supernatants were collected and used for immunohistochemical staining. The immunostaining results were compared with that using the antibody adsorbed to mock-transfected cells.

Protein Extraction from Formalin-Fixed Paraffin-Embedded Specimens

Formalin-fixed paraffin-embedded specimens were sectioned at 10 μm thick and deparaffinized. Tissues were manually dissected under a microscope. The collected tissues were heated at 95 °C for 1 h and then at 60 °C for 4 h in the protein extraction buffer (50 mM Tris (pH = 8.0), 5 mM EDTA, 2% SDS).

Genes

Mouse *Notch1* was provided by Dr J. Nye and constitutive active *Notch1* were previously described.[25] Notch extracellular domain (NECD) was created by ligating the PCR amplified *Notch1* extracellular and transmembrane regions (1M to 1755G) into *pAcGFP1-C2* (Clontech). Human *Notch3* was provided by Dr A. Joutel. *Rbpj* cDNA was provided by the RIKEN BioResource Center (Ibaraki, Japan) courtesy of Dr T. Honjo and cloned into *pCMV-Tag4* (Stratagene, CA, USA), and dominant-negative *Rbpj (R218H)* was created by PCR mutagenesis. Dominant-negative *Dll1* (chick) was previously described.[26] Human *TP63α(TA)* was purchased from Invitrogen. *TP63α(ΔN)* was created as follows. A plasmid (*pBK-CMV-dN*) was made, which contained the sequence corresponding to the *TP63α(ΔN)*-specific N-terminal region by ligating annealed oligoniculeotides into *pBK-CMV* (Stratagene). As the N-terminal *TP63α(TA)*-specific region incidentally coincides to 5′ of MSC1 site, the N-terminal region was deleted from *TP63α(TA)* by MSC1 digestion, and the fragment was ligated into *pBK-CMV-dN*. The resulting plasmid was confirmed by DNA sequencing to carry *TP63α(ΔN)* identical to the wild type. Human *KRT13* and *KRT15* promoters were cloned by PCR from the BAC clone RP11-156A24, and human *KRT17* promoter was cloned from the BAC clone RP13-415G19, both of which were provided by the BACPAC Resources Center (CA, USA). The sequences of the PCR primers used in the cloning procedures will be provided on request. Stealth RNAs for human *NOTCH1* were purchased from Invitrogen. The sequence of the plus strand of the dsRNA is 5′-UCGCAUUGACCAUUCAAACUGGUGG-3′.

Cell Culture Experiments

GE-1, Ca9-22, HeLa and CaSki cells were provided by the RIKEN BioResource Center. HSC-3 and 293 cells were provided by the Japanese Collection of Research Bioresources (Osaka, Japan). Human foreskin (HFS) cells were purchased from Kurabo (Osaka, Japan). Transfections were performed using Lipofectamine 2000 (Invitrogen) or by calcium phosphate method. Cell proliferation was evaluated using a Cell Counting Kit (Dojindo, Kumamoto, Japan). Cells were lysed in RIPA buffer and the concentration of the protein was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). Western blot analysis and RT-PCR were performed according to standard protocols.[27] Three-dimensional culture was conducted using Millicell Culture Inserts (Millipore). Real-time RT-PCR was performed using a Lightcycler (Roche, Basel, Switzerland). The PCR primer sequences are as follows: hN1-5865F, 5′-CAACATCCAGGACAACATGG-3′; hN1-6093R, 5′-GGACTTGCCCAGGTCATCTA-3′; hN2-7187F, 5′-ATGCTTCCTCAAATGCTGCT-3′; hN2-7513R, 5′-TCATTTCTCTCCCGGATGAC-3′; hN3-7275F, 5′-GTCTGGGACCTCCTTCTTCC-3′; hN3-7628R, 5′-CCAAGGGTGCCTACTTGGTA-3′; hN4-6444F, 5′-TGCAGGCATATGGGATGTAA-3′; hN4-6665R, 5′-CATCCCCACAGTGGAGTTCT-3′; HES1-468F, 5′-GCGGACATTCTGGAATGACA-3′; HES1-594R, 5′-CGTTCATGCACTCGCTGAAG-3′; HEY1-485F, 5′-GATGACCGTGGATCACCTGAA-3′; HEY1-584R, 5′-CCGAAATCCCAAACTCCGATAG-3′; GAPDH-275F, 5′-GCACCGTCAAGGCTGAGAAC-3′; GAPDH-417R, 5′-ATGGTGGTGAAGACGCCAGT-3′.

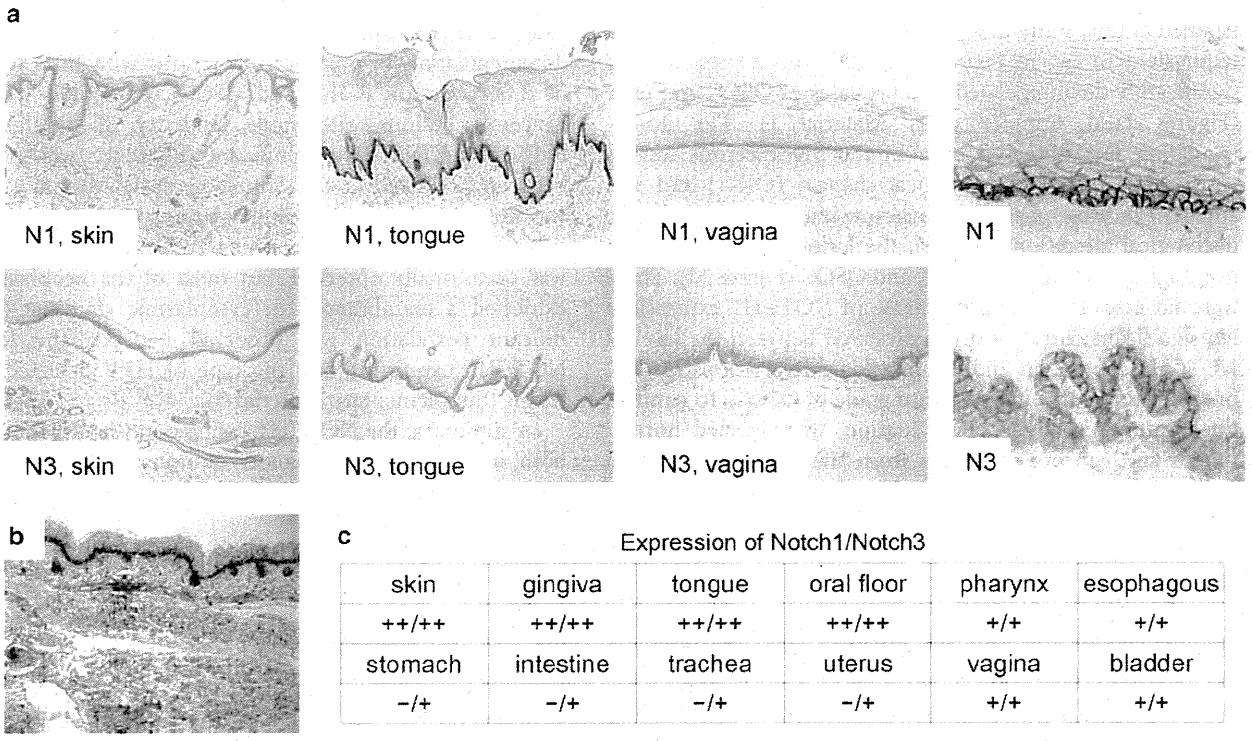
Luciferase Activity Assay

KRT13, *KRT15* and *KRT17* promoter fragments were cloned as described in the Genes section, and ligated into *pGL4.10.* (Promega, WI, USA). The promoter construct of 0.1 μg, 0.1 μg of the *Notch1* construct and 0.01 μg of the *Renilla* luciferase standardization plasmid were transfected into GE-1 cells on 48-well plates and the luciferase activity was measured 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega). All transfections were done in triplicate and the experiments were repeated at least twice.

RESULTS

Expression of NOTCH1 in Basal Cells of Squamous Epithelium

In the pilot study, we screened for optimal antibodies that clearly detect an endogenous level of Notch in western blot analysis. The selected antibodies revealed that NOTCH1, NOTCH2 and NOTCH3 were expressed in primary HFS cells and HEK293 cells, whereas NOTCH4 was not detected (data not shown). This result was confirmed by RT-PCR analysis, which revealed expression of *NOTCH1*, *NOTCH2* and *NOTCH3* but not *NOTCH4* (data not shown). These (anti-NOTCH1 and anti-NOTCH3) antibodies reacted with recombinant Notch1 (mouse) and NOTCH3 (human), respectively, which were overexpressed in HEK293 cells as well as with the endogenous proteins, as revealed both by western blot analysis and immunofluorescent staining, validating their specific reactions (Supplementary Material 4A, B). We then examined their immunohistochemical expression in various human adult tissues. Distinct expression of NOTCH1 was observed in the basal cells of squamous epithelium (Figure 1a). This was consistent with the result of *in situ* hybridization, which exhibited basal-cell expression of *Notch1* in an embryonic mouse skin (Figure 1b). The NOTCH1 protein was detected mainly on the plasma membrane, which is consistent with its function as a transmembrane receptor (Figure 1a). Nuclear staining was rarely observed. Antibody adsorption test showed significantly reduced staining when the antibody was absorbed to *Notch1*-transfected cells, validating the usability in immunohistochemical detection (Supplementary Material 4C). Both cornified- and non-cornified squamous epithelia expressed NOTCH1. The expression in the esophagus and vagina appeared weaker than that in the skin and oral mucosa (Figure 1c), suggesting that the epithelium of ectodermal origin (skin, oral mucosa) expresses NOTCH1 more than that of endodermal origin (esophagus, vagina). Basal (myoepithelial) cells in the secretory glands were weakly



Expression of Notch1/Notch3

skin	gingiva	tongue	oral floor	pharynx	esophagous
++/++	++/++	++/++	++/++	+/+	+/+
stomach	intestine	trachea	uterus	vagina	bladder
−/+	−/+	−/+	−/+	+/+	+/+

Figure 1 Expression of NOTCH1 and NOTCH3 in various tissues. (**a**) Immunohistochemical staining of adult human skin, tongue and vagina shows distinct expression in the basal cells. Membrane staining pattern is obvious (right). (**b**) *In situ* hybridization on the skin of mouse E18.5 embryo, using *Notch1* RNA probe. (**c**) Simplified summary of the expression in the epithelium of each tissue.

positive for NOTCH1 (data not shown). In the uterus, the columnar epithelium, including subcolumnar reserve cells, was almost negative for NOTCH1. In the digestive tract, NOTCH1 was detected faintly only in the basal crypts (data not shown). In the subepithelial tissue, NOTCH1 was detected weakly in the vascular endothelial cells (data not shown). NOTCH3 was also expressed in the basal cells of squamous epithelium, showing membranous localization (Figure 1a). The staining pattern of NOTCH3 was a little different from that of NOTCH1, showing weak ubiquitous cytoplasmic staining also in glandular cells (data not shown). This staining in glandular cells seemed nonspecific because it did not decrease when the adsorbed antibody was used, whereas the staining in the basal cells weakened (data not shown). The NOTCH2 antibody was not applicable to immunohistochemical detection, showing no staining in any tissues (data not shown). In summary, NOTCH1 and NOTCH3 were expressed mainly in the basal cells of squamous epithelium.

NOTCH1 Expression Is Downregulated in Squamous Neoplasms

As the anti-NOTCH3 antibody yielded some nonspecific staining that would hamper correct evaluation, we determined to focus on NOTCH1 in this study and expanded our investigations to neoplasms. Because the squamous epithelia were dominant sites of NOTCH1 expression, we chose squamous neoplasms that develop at these sites and investigated whether the expression is upregulated, downregulated or unchanged. For this purpose, we collected surgical specimens of squamous cell carcinoma (cancer). To assess the contribution of NOTCH1 in cancer progression, surgical specimens of intraepithelial neoplasm (precancer) were also collected. To compensate for the organ-specific differences, specimens were collected from three different tissue sources—oral mucosa, esophagus and uterine cervix.

First we examined the NOTCH1 expression in 56 cases of oral epithelial neoplasm, including OSCC and oral intraepithelial neoplasm (OIN). We randomly collected specimens that contained normal epithelium, and the expression was evaluated by the staining intensity in individual tumor cells in comparison with that in normal basal cells within the same specimen. The distinct staining pattern with abrupt changes at the interface between normal epithelium and neoplasm enabled us to adopt this methodology. The expression was scored as being at one of five levels: level 4, upregulated expression compared with the normal basal cells; level 3, expression level similar to the normal basal cells (equivalent to '+ +' in Figure 1c); level 2, expression level less than the normal basal cells; level 1, stained only faintly

(stained at least more than fibroblasts); or level 0, undetected (equivalent to '–' in Figure 1b). NOTCH1 expression was significantly downregulated in most cases of OSCC and OIN (Figures 2a–d, Supplementary Material 1). The downregulation was observed even in the precancerous lesions with minimum histopathological changes (OIN1) and was apparent in most of the precancerous lesions with moderate histological alterations (OIN2), the lesions with prominent histological alterations (OIN3), and OSCC (Figure 2d). There were no cases showing an increase of NOTCH1 expression. No significant correlation was observed between the level of NOTCH1 expression and the histological variations of OIN or the histological differentiation grade of OSCC. To confirm the immunohistochemical evaluation, we dissected normal tissues and cancers separately from the sections, extracted proteins and conducted western blot analysis. In all, 4 out of 10 cases were informative, all of which showed reduced NOTCH1 expression in cancer, normalized to keratin 5 (Figure 2e). This result is consistent with the immunohistochemical observations. Proteins of sufficient amount and quality were not obtained from the other six specimens, probably due to the fixation period. The downregulation of *NOTCH1* in OSCC was also confirmed by cDNA microarray analysis of 41 OSCC *vs* 7 normal oral epithelia ($P<0.001$), with the average expression in OSCC reduced to 0.43-fold compared with the normal control (Figure 2f). The cDNA microarray showed that *NOTCH2* and *NOTCH3* were also downregulated in OSCC to 0.78-fold and 0.83-fold, respectively (data not shown).

Next, we examined the immunohistochemical expression of NOTCH1 in squamous cell carcinoma of esophagus (ESCC; Supplementary Material 2 and 5). Downregulation was evident in 17 out of 20 cases while weak expression remained. The remaining three cases retained a considerable expression, but there were no cases with NOTCH1 upregulation.

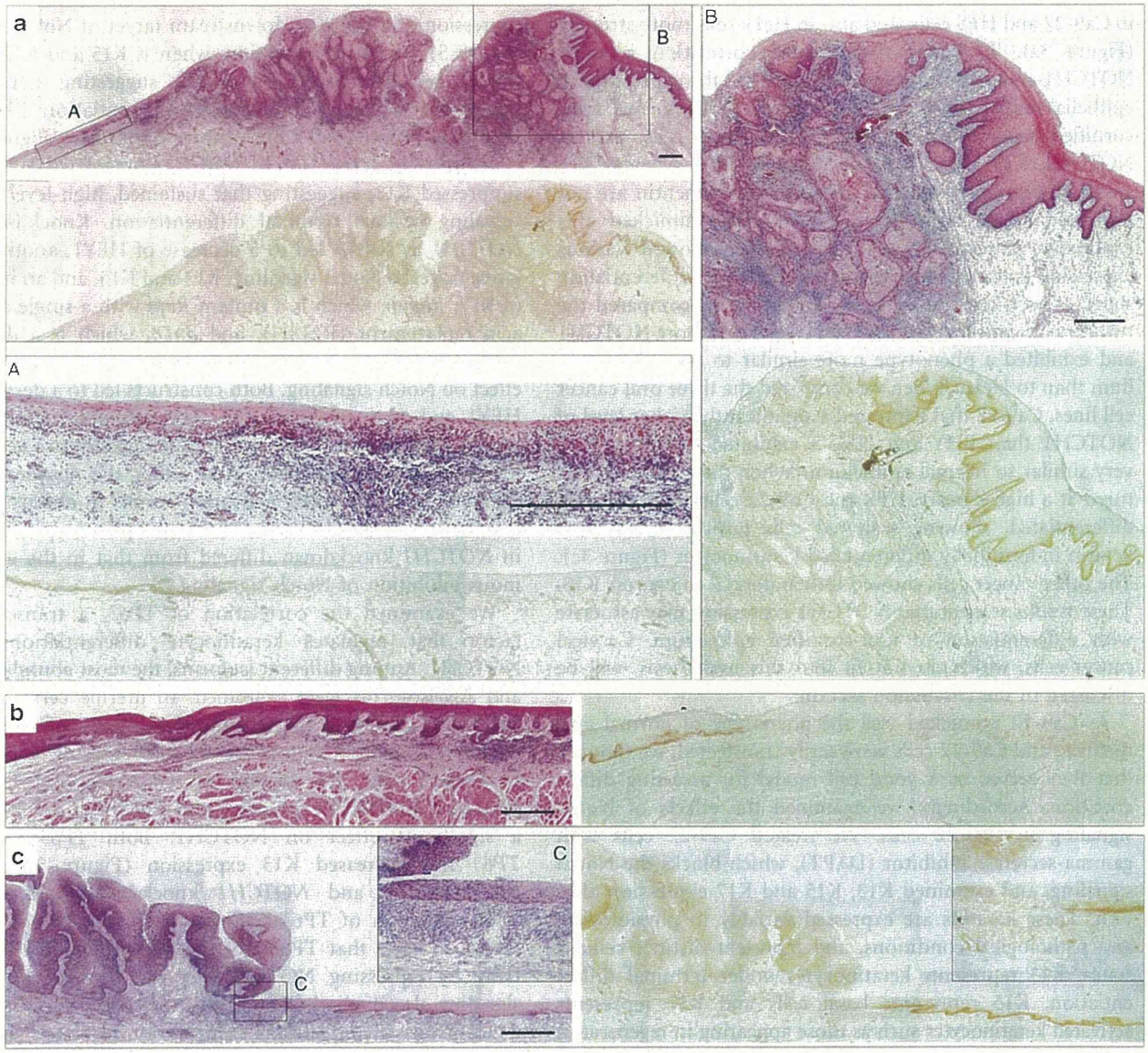
Next, we examined the immunohistochemical expression of NOTCH1 in uterine cervical lesions (Supplementary Material 3, 6). NOTCH1 expression tended to be downregulated in cervical intraepithelial neoplasm (CIN) and cervical squamous cell carcinoma (CSCC). However, more than 50% of the cases retained considerable expression (Supplementary Material 6). More cases showed NOTCH1 downregulation in precancerous lesions with little tendency of differentiation (CIN3 and CSCC), compared with precancerous lesions with some tendency of differentiation (CIN1 or CIN2). No significant difference was observed between CIN3 and CSCC (Supplementary Material 6). There was not a single case showing apparent NOTCH1 upregulation in the individual cancer cells. Indistinct nuclear staining was occasionally observed, but most of the neoplastic cells exhibited a membranous or cytoplasmic staining. No significant correlation was observed between the level of NOTCH1 expression and the type of HPV detected in the lesion (Supplementary Material 3).

In summary, the NOTCH1 expression was downregulated both in precancer and cancer of non-cornified squamous epithelium.

NOTCH1 Regulates the Differentiation of Squamous Epithelium

The fact that NOTCH1 was downregulated not only in cancer but also in precancer suggests that aberrant NOTCH1 expression associates with changes of epithelial properties observed both in precancers and cancers. The essential properties that discriminate cancer from precancer are invasion and resultant metastasis, and our data suggest that the downregulation of NOTCH1 may not contribute to these cancer-specific events. In contrast, dysregulation of differentiation is a common feature observed both in cancer and precancer, appearing as abnormality of cell alignment, stratification and keratinization. Considering that Notch governs cell-to-cell signaling, we hypothesized that reduction of Notch signaling might affect the cell-to-cell-based regulation of epithelial differentiation. To test this hypothesis, we conducted cell culture experiments. First, we examined Notch expression in cell lines derived from cervical cancers (HeLa, CaSki), oral cancers (BHY, Ca9-22, HSC-3) and primary HFS cells. Western blot analysis revealed that both NOTCH1 and NOTCH3 were expressed most abundantly in the normal cells, and variably, but at much lower levels, in the cancer cell lines (Figure 3a), which is a finding consistent with the observation that NOTCH1 expression was downregulated in squamous neoplasms. NOTCH2 was considerably expressed

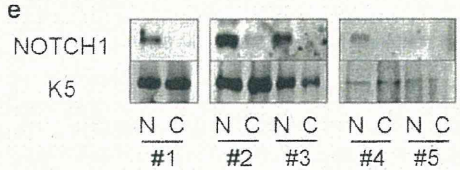
Figure 2 Expression of NOTCH1 in oral squamous cell carcinoma (OSCC, cancer) and oral intraepithelial neoplasm (OIN, precancer). (**a**) OSCC associated with OIN. Low-magnification view and the highlighted borders of the lesion (A, B). NOTCH1 expression is significantly reduced in OIN (A) and OSCC (B). Clear demarcations of NOTCH1 expression are seen, which coincides with the border of the lesion. Scale bar, 0.5 mm. (**b**) OIN1 (mild epithelial dysplasia). NOTCH1 is significantly downregulated even in this lesion with minimum histological change, and the border of NOTCH1 expression coincides with the border of the lesion. Scale bar, 0.5 mm. (**c**) Papillary-type OSCC. Low-magnification view with the highlighted border of the lesion (C). Downregulation of NOTCH1 is evident. Scale bar, 0.5 mm. (**d**) Numbers of cases (and percentage) with reduction of NOTCH1 expression. G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated OSCCs. (**e**) Western blot analysis using surgical specimens. Formalin-fixed paraffin-embedded tissues were separately dissected from normal (N) and cancer (C) tissues, and the proteins were extracted. A sufficient amount of protein was obtained from 4 out of 10 cases, in which NOTCH1 expression was reduced in cancer (Case #1 to #4). Keratin 5 (K5) was used for standardization. Case #5 is shown as an example of a non-informative case. (**f**) cDNA microarray analysis of 41 OSCC and 7 normal control epithelia. Vertical axis corresponds to globally normalized signal intensity of *NOTCH1* mRNA expression. *NOTCH1* is significantly downregulated in OSCC compared with normal epithelium ($^*P<0.001$). Short bars, expression in each case; long bars, mean; error bars, s.e.



d

OIN		
OIN1	OIN2	OIN3
8/10 (80%)	12/13 (92%)	10/10 (100%)
OSCC		
G1	G2	G3
14/16 (88%)	8/8 (100%)	2/3 (67%)

e



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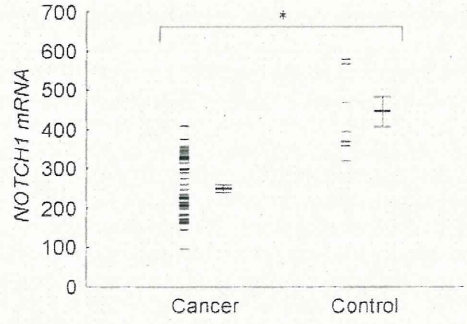


in Ca9-22 and HFS cells, and also in HeLa cells more strongly (Figure 3a). To assess the possible correlation between NOTCH1 and differentiation, we examined the expression of epithelial markers in these cells (Figure 3b). In normal non-cornified squamous epithelium, the basal cells express NOTCH1, K15, K19, CDH1, DSG3 and TP63, whereas K16 and K17 expression is weak, and K18 and vimentin are not expressed (data not shown). HFS cells mimicked this expression pattern. The cancer cell lines showed various expression patterns, reflecting the diversities in differentiation states of each cell line (Figure 3b). When we compared the two cervical cancer cell lines, CaSki expressed more NOTCH1 and exhibited a phenotype more similar to normal epithelium than to HeLa. When we compared the three oral cancer cell lines, Ca9-22 that expressed a significantly higher level of NOTCH1 than BHY and HSC-3 exhibited the phenotype very similar to normal epithelium. When the cells were cultured at a high density, HFS and Ca9-22 cells spontaneously differentiated, showing scattered cells positive for K13, a keratin in terminally differentiated keratinocytes (Figure 3c). The other cancer cells showed little potential to express K13. These results suggest that NOTCH1 expression may associate with differentiation of non-cornified epithelium. Cervical cancer cells, which do not fit into this hypothesis, will be discussed in the Discussion section.

As Ca9-22 mimicked well the phenotype of normal epithelium, and Ca9-22 cells were easily transfected, we thought that they served as a good cell model for assessing differentiation. Accordingly, we examined the effects of Notch signaling in Ca9-22 cells. We treated Ca9-22 cells with gamma-secretase inhibitor (DAPT), which blocks the Notch signaling, and examined K13, K15 and K17 expression after 72 h. These keratins are expressed variably in physiological and pathological conditions, and represent distinct cellular states; K13 represents keratinocytes under terminal differentiation, K15 represents basal cells and K17 represents activated keratinocytes such as those appearing in regenerative epithelium and cancer. DAPT treatment led to reduced expression of a putative downstream target of Notch signaling, HES1, and K13 expression, whereas K15 and K17 levels were largely unchanged (Figure 3d), suggesting that Notch signaling is necessary for terminal differentiation. Next the cells were transfected with various constructs (Figure 3e). Constitutively, active Notch1 (NICD) upregulated HES1 and suppressed K13, suggesting that sustained, high-level Notch signaling inhibits terminal differentiation. Knockdown of *NOTCH1* by siRNA led to a decrease of HEY1, another putative target of Notch signaling, K13 and K15, and an increase of K17. *Rbpjm*, which is a mutant *Rbpj* with a single amino-acid replacement (R218H), and *dnDl*, which is a deletion mutant of *Dll1*, are both known to pose a dominant-negative effect on Notch signaling. Both constructs led to a decrease of HEY1 and an increase of K13. These results indicate that proper expression of NOTCH1 and its signaling are necessary for terminal differentiation, and either the absence of its signaling or its untimely activation results in abnormal differentiation. It should be noted that the phenotype observed in *NOTCH1* knockdown differed from that in the autonomous inhibition of Notch signaling.

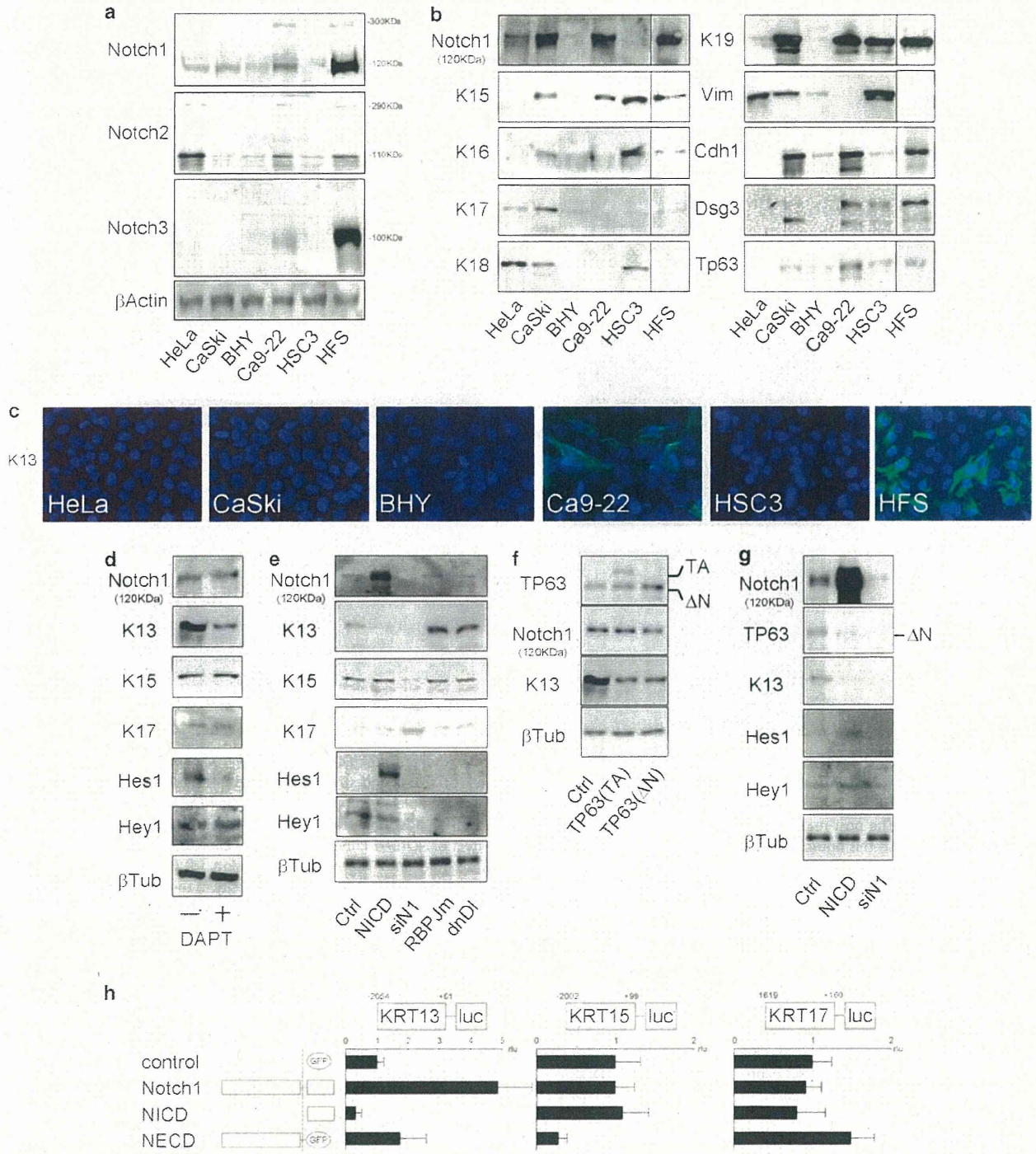
We examined the correlation of TP63, a transcription factor that regulates keratinocyte differentiation, with NOTCH1. Among different isoforms, the most abundant TA- and ΔN-isoforms were examined. In uterine cervical cells, TP63(ΔN) acts as a transcriptional repressor of *NOTCH1*, which results in maintenance of self-renewing capacity.[28] In Ca9-22 cells, transfection of *TP63(ΔN)* only slightly decreased NOTCH1 expression and *TP63(TA)* did not have a significant effect on NOTCH1. Both *TP63(TA)* and *TP63(ΔN)* decreased K13 expression (Figure 3f). *NICD* overexpression and *NOTCH1* knockdown both led to downregulation of TP63(ΔN) and K13 (Figure 3g). These results indicate that TP63(ΔN) inhibits differentiation partially by repressing NOTCH1 expression, whereas Notch signaling also mediates TP63(ΔN) expression.

To further investigate the contribution of Notch signaling in regulation of keratin subtype expression, we conducted

Figure 3 NOTCH1 regulates keratin expression. (**a**) Expression of NOTCH1, NOTCH2 and NOTCH3 in cervical cancer cell lines (HeLa, CaSki), oral cancer cell lines (BHY, Ca9-22, HSC-3) and primary human foreskin (HFS) cells, as revealed by western blot. All the antibodies recognize an epitope in the intracellular domain. Most proteins were detected as a furin-cleaved form containing the transmembrane and intracellular domains. A small amount of full-length protein was also detected. Gamma-secretase-cleaved C-terminal fragment (NICD) was not observed in NOTCH1 and NOTCH3 blots. (**b**) Expression of various keratins (K), vimentin (Vim), E-cadherin (Cdh1), desmoglein 3 (Dsg3) and TP63 in various cells, as revealed by western blot. As the full-length NOTCH1 was observed at a much weaker intensity and in proportion to the intensity of the 120 kDa protein, only the 120 kDa protein of NOTCH1 is shown. (**c**) K13 expression in Ca9-22 and HFS cells cultured at a high density. Immunofluorostaining was conducted using the secondary antibody labeled by Alexafluor 488. The nuclei were counterstained by DAPI. K13-expressing cells appear in a scattered manner. HeLa, CaSki, BHY and HSC-3 showed no K13 expression. (**d**) Ca9-22 cells were treated with only DMSO or 2.5 μM DAPT at 80% confluency and were cultured for 72 h. An equal amount of protein was subjected to western blot analysis for keratins (K), HES1, HEY1 and β-tublin (βTub). (**e**) Ca9-22 cells were transfected at 30% confluency with an empty plasmid (Ctrl), NICD, dominant-negative RBPJ (R218H, RBPJm), dominant-negative Dll1 (dnDl) or siRNA for *NOTCH1* (*siN1*) and were cultured for 72 h. (**f**) Ca9-22 cells were transfected with *TP63(TA)* or *TP63(ΔN)* and were incubated for 72 h. Western blot analysis revealed the endogenous expression of TP63 (ΔN), but not TP63 (TA). NOTCH1 was only slightly downregulated by TP63 (ΔN). K13 was downregulated both by TP63 (TA) and TP63 (ΔN). (**g**) Ca9-22 cells were transfected with *NICD* or siRNA for *NOTCH1* and were incubated for 72 h. Both *NICD* and *siN1* downregulated TP63 and K13. (**h**) Luciferase reporter assay for human *KRT13*, *KRT15* and *KRT17* promoters. The promoter construct plus of 0.1 μg and 0.1 μg of the Notch1 construct and 0.01 μg of the *Renilla* luciferase standardization plasmid (*pEF-RL*). Transfections were done into GE-1 cells on 48-well plates and the luciferase activity was measured 48 h after transfection using Dual-Luciferase Reporter Assay System (Promega). The error bars denote standard errors. rlu; relative luciferase unit.

promoter analysis of the keratin genes using GE-1 cells that were established from normal mouse gingival epithelium. We co-transfected the cells with *Notch1*, *NICD* or *NECD*, and the luciferase reporter constructs for *KRT13*, *KRT15* or *KRT17* promoters. NECD is a membrane-tethered Notch1 extracellular domain whose intracellular domain was replaced by AcGFP. Notch1 increased the *KRT13* promoter activity while NICD decreased it. NECD decreased the *KRT15* promoter activity and slightly increased the *KRT17* promoter activity (Figure 3h). These results suggest that NOTCH1-expressing cells have more potential to differentiate, but Notch signaling itself autonomously inhibits differentiation and directs the cell to maintain basal-cell phenotype.

Next we examined the effect of *NOTCH1* knockdown in normal epithelial cells using the primary HFS cells. The knockdown efficiency was more than 90% as revealed by



western blot analysis (Figure 4a). *NOTCH1* knockdown significantly suppressed the expression of *HEY1*, whereas *HES1* showed only slight (20%) reduction, as revealed by real-time PCR (Figure 4b, real-time PCR data are not shown). Cell proliferation was not altered by *NOTCH1* knockdown (data not shown). The total amount of keratin protein was unchanged, and vimentin was not induced by *NOTCH1* knockdown (Figure 4c). *NOTCH1* knockdown led to downregulation of K13 and K15, and upregulation of K17. Immunocytostaining using the anti-K13 antibody revealed that *NOTCH1* knockdown resulted in a decrease both of the staining intensity in individual cells and of the number of stained cells (Figure 4d). The expression of CDH1 (E-cadherin), DSG3 (desmoglein 3) and TP63 was not significantly

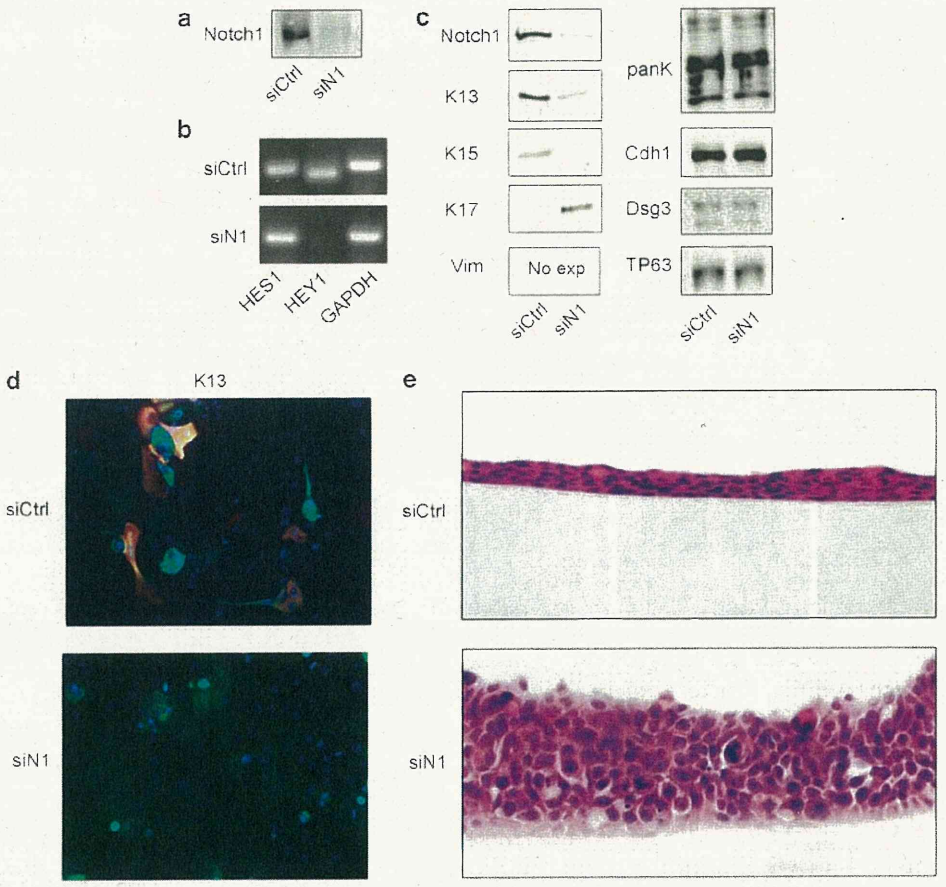
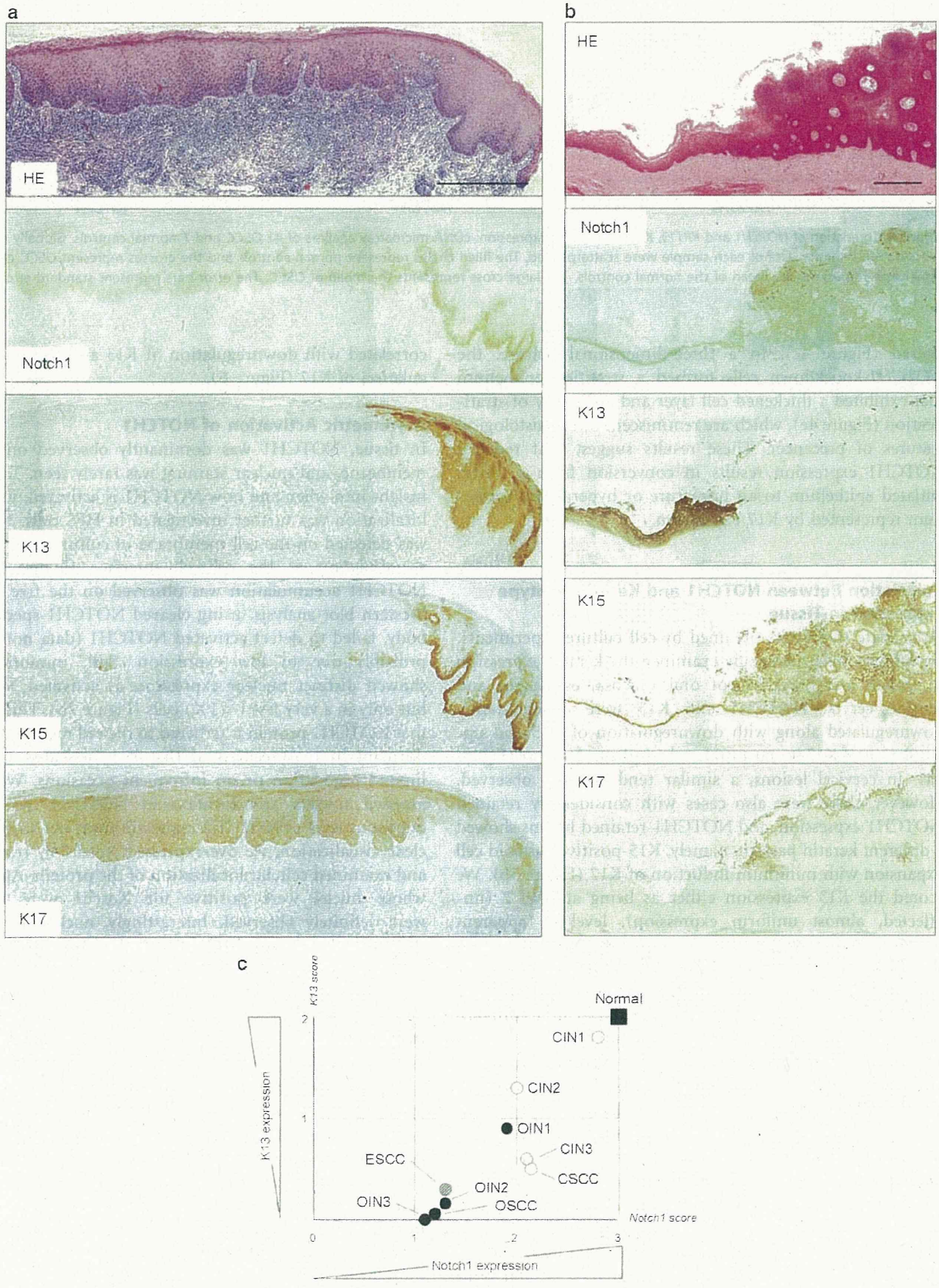


Figure 4 Notch1 knockdown in primary foreskin (HFS) cells results in immature epithelium. (**a**) Efficiency of *NOTCH1* knockdown. HFS cells were transfected with negative control siRNA (*siCtrl*) or siRNA for *NOTCH1* (*siN1*) were incubated for 7 days and subjected to western blot analysis. (**b**) Effect of *NOTCH1* knockdown on the target genes. Real-time RT-PCR revealed that *HEY1* was almost completely suppressed, whereas *HES1* was downregulated only by 20% (data not shown). The post-real-time PCR samples were diluted and amplified for an additional two cycles in order to make them visible on gel electrophoresis. Thus this figure actually shows conventional RT-PCR. (**c**) The effect of *NOTCH1* knockdown on the expression of keratinocyte differentiation markers. K, keratin; Vim, vimentin; panK, pan-keratin; Cdh1, E-cadherin; Dsg3, desmoglein 3; No exp, no expression. (**d**) Expression of K13 in HFS cells transfected with *siCtrl* or *siN1* 7 days after transfection, revealed by immunofluorostaining. The fluorescence intensity in the individual cells and the number of positively stained cells decreased in the siN1-transfected cells. (**e**) Vertical sections of three-dimensionally cultured HFS cell layers, stained with hematoxylin and eosin. Cells were transfected and seeded in Millicell culture inserts and cultured for 10 days.

Figure 5 Immunohistochemical expression of NOTCH1, K13, K15 and K17 in squamous neoplasms. (**a**) In this OIN case, the lesion shows concomitant downregulation of NOTCH1, K13 and K15, with complimentarily induced expression of K17. Scale bar, 0.5 mm. (**b**) In this CIN case showing condylomatous proliferation, the level of NOTCH1 expression in the individual cells is retained, and basaloid cells expressing NOTCH1 and K15 expand in the whole layer. K17 induction is faint. Scale bar, 0.5 mm. (**c**) Schematic summary of the immunohistochemical expression of NOTCH1 and K13 in various squamous neoplasms. Each group of the lesions is plotted by the average scores of NOTCH1 and K13 expression. OIN, oral intraepithelial neoplasm; OSCC, squamous cell carcinoma of the oral cavity; ESCC, squamous cell carcinoma of the esophagus; CIN, cervical intraepithelial neoplasm; CSCC, squamous cell carcinoma of the cervix.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Normal
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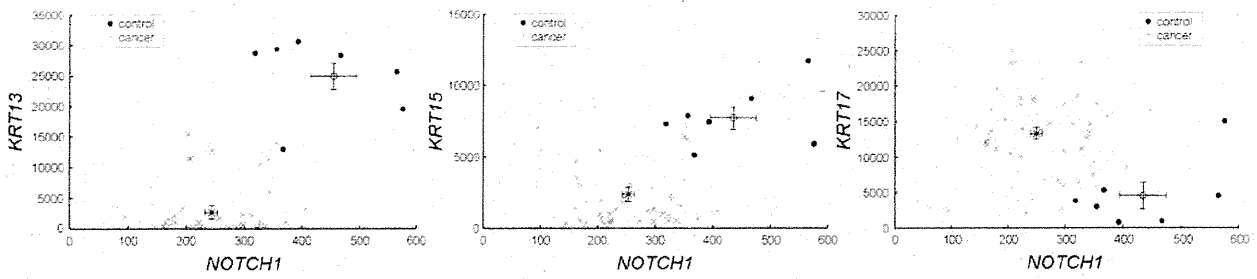

Figure 6 Correlation of *NOTCH1* and *KRT13*, *KRT15* or *KRT17* expression. cDNA microarray analysis of 41 OSCC and 7 normal controls. Globally normalized hybridization intensity data of each sample were scatterplotted. The filled circles represent normal controls and the crosses represent OSCC cases. The blank circle represents the mean of the normal controls. The large cross represents the mean of OSCC. The error bars represent standard errors.

altered (Figure 4c). In a three-dimensional culture, the *NOTCH1*-knockdown cells formed a stratified epithelium that exhibited a thickened cell layer and a disarray of stratification (Figure 4e), which are reminiscent of the histological features of precancer. These results suggest that reduced NOTCH1 expression results in conversion from a differentiated epithelium to an immature or hyperplastic epithelium represented by K17 expression.

Correlation Between NOTCH1 and Keratin Subtype Expression in Tissue

To validate the results obtained by cell culture experiments, we immunohistochemically examined the keratin expression in cancer and precancer of oral mucosa, esophagus and uterine cervix. NOTCH1 and K13 were concomitantly downregulated along with downregulation of K15 and upregulation of K17 in most oral and esophageal lesions (Figure 5a). In cervical lesions, a similar tendency was observed. However, there were also cases with considerably retained NOTCH1 expression, and NOTCH1-retained lesions showed a different keratin pattern, namely, K15-positive basaloid cell expansion with minimum induction of K17 (Figure 5b). We scored the K13 expression either as being at level 2 (unaffected, almost uniform expression), level 1 (apparent downregulation but patchy expression remaining) or level 0 (almost complete loss of expression), and analyzed the correlation of these three expression levels with the NOTCH1 scores. Loss of K13 expression was observed in accordance with NOTCH1 downregulation (Figure 5c). The average scores of K13 and NOTCH1 decreased in accordance with the grades of OIN and CIN, although the high-grade lesions (OIN2, OIN3, CIN3) showed scores similar to those of OSCC or CSCC (Figure 5c), indicating that these precancers and cancers in each site are essentially the same lesions in the context of NOTCH1 and K13 expression. The cervical lesions, especially low-grade CIN, tended to show more NOTCH1 and K13 expression. Correlation between NOTCH1 expression and keratin expression was further assessed in oral cancers using microarray data. Scatter plots revealed that the downregulation of NOTCH1 in cancer correlated with downregulation of K13 and K15, and upregulation of K17 (Figure 6).

Asymmetric Activation of NOTCH1

In tissue, NOTCH1 was dominantly observed on the cell membrane, and nuclear staining was rarely seen. To gain an insight into when and how NOTCH1 is activated, its cellular localization was further investigated in HFS cells. NOTCH1 was detected on the cell membrane of culture cells, showing accumulation at the cell-cell interface (Figure 7a). No NOTCH1 accumulation was observed on the free surfaces. Western blot analysis, using cleaved NOTCH1-specific antibody, failed to detect activated NOTCH1 (data not shown), probably due to low expression. Still, immunostaining showed distinct nuclear expression of activated NOTCH1, but only in a very few ($<1\%$) cells (Figure 7b). This suggests that NOTCH1 protein is tethered to the cell membrane and is dormant in most cells, and that it is activated only in a limited population or on infrequent occasions. We further assessed nuclear translocation of NOTCH1. As the endogenous level of NOTCH1 expression was not sufficient for clear visualization, we overexpressed *Notch1* by transfection and examined cellular localization of the protein. Again, cells whose nuclei were positive for Notch1 were rare but were definitely observed. Interestingly, nuclear localization of Notch1 was occasionally observed in one of two neighboring cells, which appeared as postmitotic daughter cells (Figure 7c).

DISCUSSION

We demonstrated that NOTCH1 is expressed predominantly in the basal cells of normal squamous epithelium. Contrary to our findings, previous reports had documented the expression in both basal and suprabasal layers of the skin[4,21] and in the uterine cervix,[15,16] as well as in the suprabasal layer of the cornea,[29] but we believe the present study correctly demonstrates the basal-cell dominant NOTCH1 expression in human squamous epithelium and its neoplasms. The most prominent feature that supports the validity of our immunohistochemical examination is the sharp demarcation of NOTCH1 expression that matched to the border between

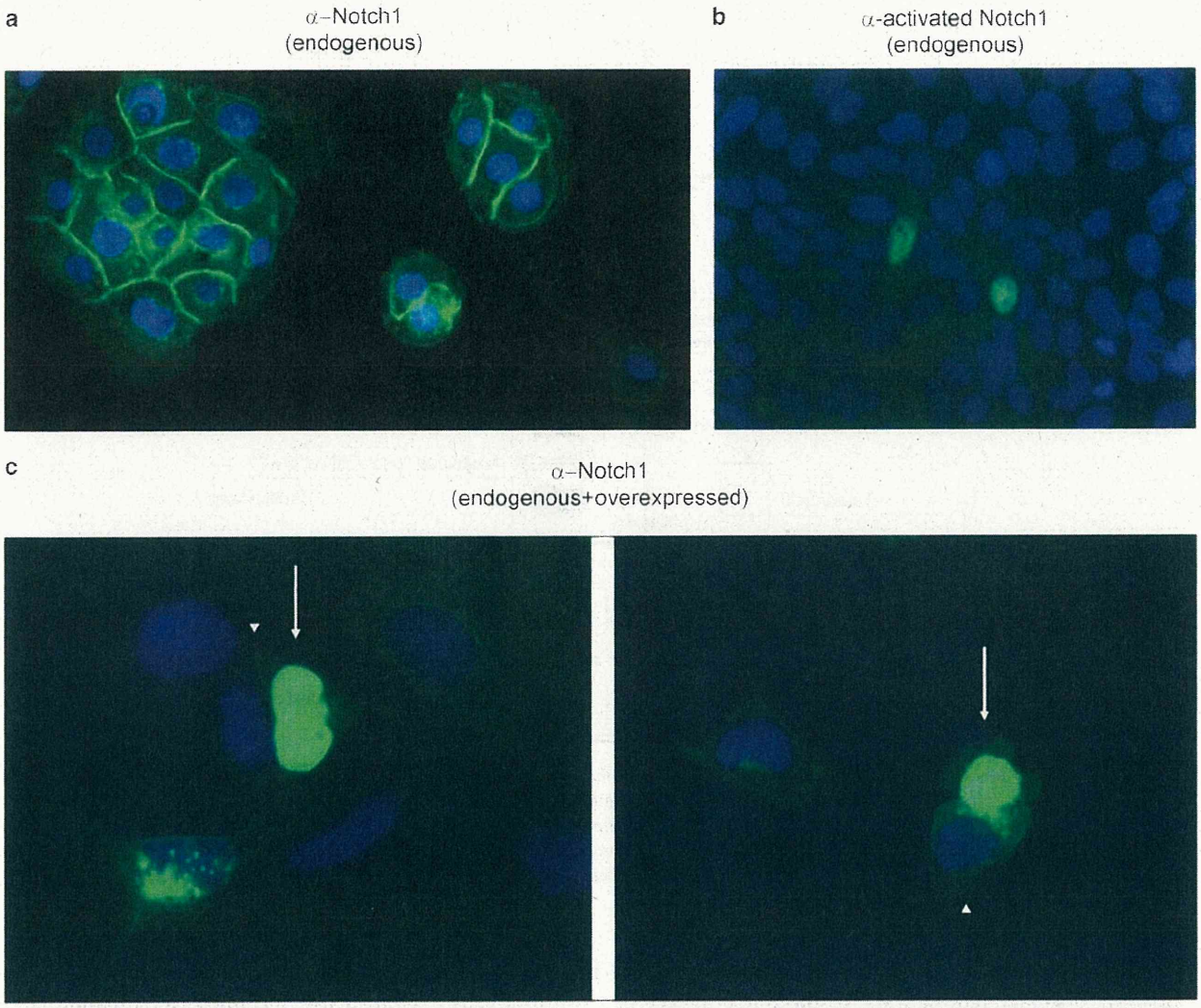

Figure 7 Nuclear translocation of NOTCH1 is rare, and it occasionally occurs asymmetrically in cultured keratinocytes. (**a**) Immunofluorostaining of HFS cells using the anti-NOTCH1 antibody. NOTCH1 protein accumulates at the cell–cell interface. Little protein was detected on the free surface or in the nuclei. (**b**) Immunofluorostaining of HFS cells using the anti-cleaved NOTCH1(Val1744) antibody. This activated form of intracellular domain of NOTCH1 is detected in the nuclei, but only in a very few cells—far <1% of the total population. (**c**) Immunofluorostaining of Ca9-22 cells transfected with wild-type mouse Notch1 using the anti-NOTCH1 antibody. The antibody crossreacts with mouse Notch1. Endogenous expression of NOTCH1 is observed as fine membranous or cytoplasmic staining. The transfected cells exhibit much stronger expression, facilitating detection. Nuclear localization of the NOTCH1 is observed in one of two neighboring cells, which appear as postmitotic daughter cells. Arrow: postmitotic cell with nuclear NOTCH1; arrowhead: postmitotic cell without nuclear NOTCH1.

normal and neoplastic epithelia, which would never be observed in nonspecific staining.

Although elevated Notch signaling has been suggested in tumor development,[13,30–32] neither an increase of NOTCH1 expression nor its nuclear translocation was observed in the cases we examined. The cDNA microarray of OSCC showed that neither *NOTCH2* nor *NOTCH3* was upregulated in OSCC, suggesting that quantitative compensation is unlikely. These results indicate that upregulation of Notch-dependent signaling may not make a major contribution to the development and progression of squamous cell carcinoma. Conversely, consistent reduction of NOTCH1 expression in squamous neoplasms was evident. Also, *NOTCH1* has recently been shown to be mutated in 11–15% of head and neck cancer, and about 40% of the mutations were predicted to generate truncated NOTCH1 proteins, whereas no apparent activating mutation was found.[33,34] These results suggest that NOTCH1 may function as a tumor suppressor gene rather than as an oncogene in squamous neoplasms.

We demonstrated that impaired Notch1 signaling led to abnormal differentiation represented by alterations of keratin subtype expression, which was commonly observed not only in cancers but also in precancers. The role of Notch in regulation of squamous epithelium differentiation has also been suggested by studies using cultured cervical[28] and esophageal keratinocytes.[35] Collectively, these results indicate that

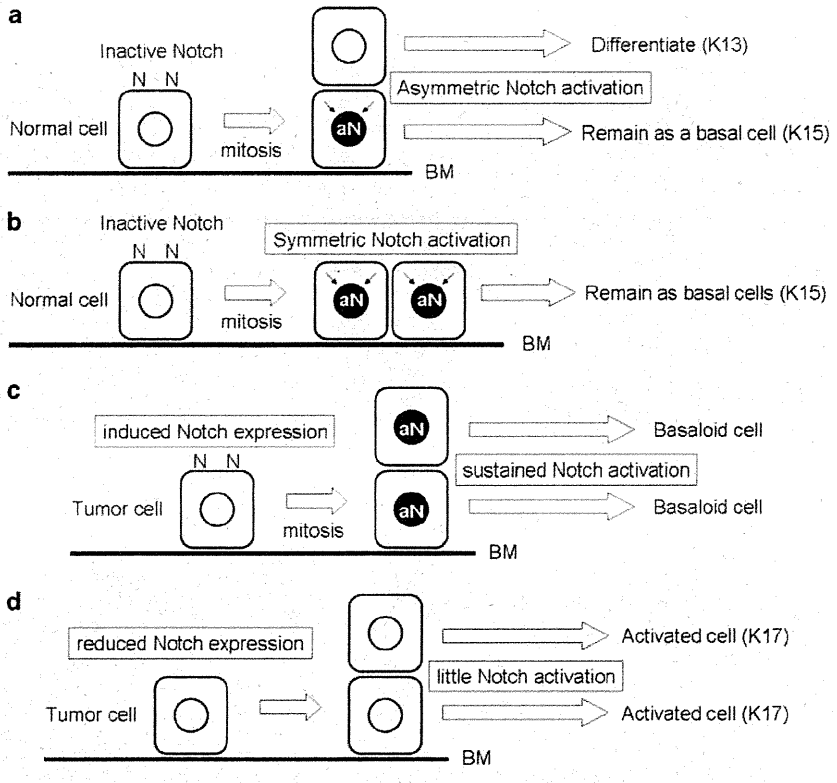

Figure 8 Proposed model of Notch-mediated mechanism of non-cornified squamous epithelium differentiation. (**a**) NOTCH1 is selectively activated in the basal cell (K15 +) when the sister cell divides basoapically, and directs it to remain as a basal cell (K15 +), whereas the apical daughter cell without Notch signal input differentiates (K13 +). (**b**) When the sister cells divide symmetrically, Notch is activated in both cells, which remain as basal cells (K15 +). (**c**) If the asymmetric Notch signaling is impaired and both basal and apical daughter cells undergo Notch activation, both remain as basaloid cells (K15 +). (**d**) The cells with reduced NOTCH1 expression convert to activated cells (K17 +) that lead to a hyperplastic phenotype.

reduced NOTCH1 expression affects the terminal differentiation, thus highlighting the essential role of NOTCH1 in maintaining normal epithelial integrity. In addition, we examined five cases of traumatic ulcer and found that NOTCH1 was downregulated in the regenerative epithelium, accompanied with loss of K13 expression and robust induction of K17 (K.S., unpublished observations). This suggests that the downregulation of NOTCH1 expression level is an inherent mechanism for switching the epithelium from a normal and mature state to an activated and immature state.

Cervical cancers tended to retain NOTCH1 expression compared with oral and esophageal cancers. We evaluated the expression in the cervical cancer cells in comparison with the neighboring vaginal squamous epithelium. However, this evaluation method might be misleading because cervical cancers arise from the reserve cells beneath the columnar epithelium of endocervix,[36] and the vaginal epithelium is not the origin of cervical cancers. As the reserve cells are almost negative for NOTCH1, it can also be said that NOTCH1 expression is increased in cervical cancer compared with its original cell type.

We hypothesized that strong NOTCH1 expression may correlate with a tendency for differentiation toward squamous epithelium. To check this hypothesis, we additionally examined five specimens of squamous metaplasia caused by obstruction of the minor salivary gland duct. As expected, NOTCH1 was significantly induced in ducts, which show ectopic K13 induction and squamous metaplasia (Supplementary Material 7). HPV infection to metaplastic epithelium in the transformation zone initiates progression to CIN or CSCC,[37] and *NOTCH1* is upregulated by HPV E6 and E7 oncoproteins, which are almost uniformly expressed in cervical cancer.[38] Virally induced NOTCH1 expression would tend to be maintained in CIN and CSCC, but would be no more upregulated. In this context, upregulation of NOTCH1 may have an essential role only in generation of metaplastic epithelium.

HPV has been detected only in a minority of OSCC cases, excluding pharyngeal cancer[39] and ESCC.[40] This is probably attributable to the difference in the NOTCH1 expression pattern between oro-esophageal and cervical cancers. The NOTCH1 expression patterns seem to underlie their histopathological differences. Expansion of a basaloid cell population is usually observed in CIN, whereas this finding is exceptional in OIN, whose basaloid cells are usually limited to the lower part of the epithelium. Remaining NOTCH1

expression appears to autonomously direct the cell to maintain the basaloid phenotype.

In our series of cell culture experiments, Notch signaling exhibited seemingly diverse effects. For example, both activation of Notch signaling and downregulation of NOTCH1 expression inhibited differentiation. To better understand such diverse effects, we have developed the following model, which is consistent with the experimental and histopathological findings, and also with the self-organizing nature of stratified epithelium. NOTCH1 is selectively activated in the basal cell when the sister cell divides basoapically, and acts to direct the basal cell to remain as a basal cell, whereas the apical daughter cell without Notch signal input is directed to differentiate (Figure 8a). When the sister cell divides laterally, Notch is activated in both cells, which directs them both to be basal cells (Figure 8b). If asymmetric Notch signaling is impaired, and both basal and apical daughter cells undergo Notch activation, both would remain as basaloid cells (Figure 8c), causing expansion of the basal-cell layer. Cells with reduced NOTCH1 expression convert to activated cells that lead to a hyperplastic phenotype (Figure 8d). In either case, impaired Notch signaling causes an immature epithelium. Although future research is required to confirm this model, it is consistent with the results of genetically engineered mouse experiments.[8,35]

Besides its role as a receptor, Notch is considered to have a function as a modulator of cell adhesion.[7] The NOTCH1 accumulation on the plasma membranes between neighboring cells and the rare observation of the activated form support this notion. Reduced Notch expression may facilitate the cell dissociation and movement that are required for regenerative epithelium and cancer invasion.

Altogether, we assume that NOTCH1 functions in two ways: it mediates the balance between populations of basal cells and differentiated cells in normal epithelium by symmetric and asymmetric activation; and in pathological conditions, such as wound healing, precancer and cancer, its expression is reduced, which converts the cells into an activated and immature state.

Impaired asymmetric cell division affects epidermal Notch signaling and results in defects in stratification and differentiation, suggesting that Notch is an effector of asymmetric cell division.[41] The mechanism of asymmetric Notch activation is unclear. JAG1, one of the canonical ligands, was expressed in the suprabasal layers (K.S., unpublished observations), which suggests that the signal is directionally transmitted from an apical cell to a basal cell because of these localizations of ligand- and receptor-expressing cells. However, JAG1 was also expressed in the basal cells, and the significance of the co-expression of receptors and ligand in the basal cells is yet to be elucidated. Accumulating evidence indicates that the canonical Notch ligands also act as a cell-autonomous repressor of Notch signaling,[26,42–46] suggesting that JAG1 expression in the basal cells may inhibit the signaling. Another possible mechanism of the asymmetric activation is the suppression of Notch signaling by protein degradation mediated by Numb, which is distributed differentially in the daughter cells and governs asymmetric cell division.[47,48]

The transcription of *NOTCH1* gene is suppressed by TP63(ΔN) in cervical keratinocytes,[28] and TP63(ΔN) inhibits differentiation in the oropharyngeal SCC cell line.[49] Our findings using Ca9-22 cells are consistent with these results, suggesting that the interplay between NOTCH1 and TP63(ΔN) in differentiation is common in non-cornified epithelia of various sites. In addition, we found that TP63(ΔN) expression was affected by Notch signaling, suggesting a feedback relationship between these factors. It should be noted that this hypothetical NOTCH1-TP63 interplay does not resemble the expression patterns of these factors in cancer tissues, in which TP63 is uniformly expressed in both basal and suprabasal layers of normal epithelium and cancer (data not shown). Thus, the NOTCH1-TP63 interplay is merely one of many mechanisms that govern the epithelial cell behaviors.

In summary, NOTCH1 is expressed predominantly in the basal cells of squamous epithelium, and it is generally downregulated in squamous neoplasms, even at early stages. Reduction of NOTCH1 expression directs the basal cells to cease terminal differentiation, resulting in an immature epithelium, which may have an essential role in the histopathogenesis of dysplastic features commonly observed in precancerous epithelium. These findings suggest that normal epithelial integrity is autonomously maintained by this evolutionarily conserved cell-to-cell signaling system.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGEMENT

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Expression of basal cell keratin 15 and keratin 19 in oral squamous neoplasms represents diverse pathophysiologies

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Histology and Histopathology (in press)

Abstract

Human epithelium contains keratin, which is expressed during differentiation. Depending on the target cell type, different types of keratin are expressed, and their alterations seem to represent changes in cell properties. The basal cells of oral epithelium express keratin 5 (K5), K14, K15 and K19, but their alterations in tumors are unclear. To address this issue and to seek possible diagnostic application, we examined the expression of these keratins in oral squamous cell carcinoma (OSCC) and squamous intraepithelial neoplasm (SIN). cDNA microarray analysis of 43 OSCC revealed slight upregulation of *KRT14*, downregulation of *KRT15* and *KRT19*, and unaltered *KRT5* expression. There were great variations in *KRT15* and *KRT19* expression across each cancer. Well-differentiated OSCC tended to express more *KRT15* and less *KRT19* compared to moderately- or poorly-differentiated OSCC. *KRT15* was positively correlated with differentiation-related keratin, *KRT13*. These observations were further investigated by immunohistochemical examination. K5 and K14 were ubiquitously expressed in all 50 OSCC and 50 SIN examined. K15 and K19 were generally downregulated, but were considerably retained in about half of the cases and showed diverse expression patterns. K15-positive cancers tended to show a well-differentiated phenotype, and K19-positive cancers tended to show more invasive tumor fronts. Most K19-positive cancers appeared to develop with little associating SIN. K19 was consistently downregulated in SIN, while K15 was downregulated mainly in high grade SIN. In summary, K15 and K19, unlike K5 or K14, are expressed variably in both SIN and OSCC, which reflects the differences in their pathogenesis and biological behaviors, suggesting their prospective applications as markers for subclassifying OSCC and SIN.

Introduction

Intermediate filaments are one of three types of cytoskeletal elements that are essential for cell shape, motility and structural integrity (Moll *et al.*, 2008). Intermediate filaments belong to a large and diverse gene family, and they are expressed selectively in specific cell types. For example, vimentin, desmin and glial acidic protein are expressed mainly in mesenchymal, muscle and glial cells, respectively (Coulombe *et al.*, 2001). Keratins are epithelial-specific intermediate filament proteins that comprise the largest numbers of subtypes (Moll *et al.*, 2008). Classified as acidic or basic, they are arranged in heterotypic pairs and integrated into multimeric filaments (Moll *et al.*, 2008). These subtypes seemingly have evolved from the simple epithelial keratin pairs, keratin 8 (K8) and K18, through a series of tandem gene duplications (Blumenberg, 1988). These keratins are expressed depending on cell type and differentiation (Moll *et al.*, 1982; Fuchs, 1995; Moll, 1998; Moll *et al.*, 2008). Although the mechanism of selective expression is unclear, it facilitates cell typing, and its alteration represents certain changes in cell property. Furthermore, accumulating evidence suggests that keratin expression itself elicits alterations in cell property (Hutton *et al.*, 1998; Wawersik and Coulombe, 2000; Kim *et al.*, 2006; Depianto *et al.*, 2010). In a stratified epithelium, basal and suprabasal layers express different sets of keratins. In the non-cornified squamous epithelium of oral mucosa, the basal cells express one basic keratin, K5, and three acidic keratins, K14, K15 and K19 (Sakamoto *et al.*, 2011). In the suprabasal layer, these basal cell keratins disappear, and K4 and K13 become a dominant pair. Our previous study showed that this strict control of differentiation and stratification is invariably affected in oral epithelial neoplasms, and the expression of K4 and K13 is consistently downregulated in oral squamous cell carcinoma (OSCC) and squamous intraepithelial neoplasm (SIN, oral epithelial dysplasia and carcinoma in situ). This indicates that aberrant terminal differentiation is a common feature in these lesions, which is represented by loss of K4 and K13 expression (Sakamoto *et al.*, 2011). OSCC and SIN show great variations in their histologies and biological behavior. We asked whether the keratin patterns reflect those differences across tumors. For example, whether ectopic induction of K1 and K10 in the suprabasal layer correlates with orthokeratotic lesion (Sakamoto *et al.*, 2011). Here, we focused on the keratins expressed in the basal cells. We thought that these keratins are more essential than the keratins expressed in the suprabasal layer because the basal cell properties determine the epithelial differentiation fate and the interaction with stroma, which influence tumor behavior. The expression of these keratins in OSCC has not been systematically investigated, except that changes of K14 and K19 expression in OSCC have been

documented in several studies, in which K14 was either downregulated (Vaidya *et al.*, 1996; Farrar *et al.*, 2004) or upregulated (Su *et al.*, 1996; Ohkura *et al.*, 2005), and K19 was also either downregulated (Su *et al.*, 1996; Crowe *et al.*, 1999; Kobayashi *et al.*, 2009) or upregulated (Lindberg and Rheinwald, 1989; Xu *et al.*, 1995; Fillies *et al.*, 2007; Zhong *et al.*, 2007; Safadi *et al.*, 2010). The results reported to date are confusing, and the roles of the altered keratin expression in the pathogenesis are still unknown.

In this study, we examine the expression of K5, K14, K15 and K19 in OSCC and SIN. We show that their expression is altered in different manners. K5 is largely unchanged, whereas K14 is upregulated. K15 and K19 are downregulated, but unlike K14, they show great variations depending on the case, which correlate to histopathological features of each OSCC. The significance of these keratin expressions in oral epithelial neoplasms is discussed, and possible applications to pathology practice are shown.

Materials and methods

cDNA microarray analysis

Cancer cells were collected from surgically-excised specimens from 43 patients by laser capture microdissection of frozen sections and were subjected to cDNA microarray analysis, as previously described (Tomioka *et al.*, 2006). Histological grade of differentiation was evaluated by oral pathologists, and the 43 cancers consisted of 17 WHO grade I, 20 grade II and 6 grade III OSCC. Epithelial portions free of cancer, dysplasia or any histopathological alterations were collected from 7 specimens and microdissected, and they were used as normal controls.

Tissue specimens

Fifty formalin-fixed paraffin-embedded specimens of OSCC (32 OSCC with SIN and 18 OSCC without SIN) plus 18 SIN specimens and 5 epulis specimens were collected from the archives of the dental hospital at Tokyo Medical and Dental University. As a normal control, paraffin-embedded archival specimens of tongue, pharynx, skin and submandibular gland taken by autopsy were used. The experimental procedures were approved by the Tokyo Medical and Dental University Ethics Committee. The OSCC patients were 29 males and 21 females, aged 32–84 years (mean 61.7), and the primary sites of cancer were the tongue (34), gingiva (5), buccal mucosa (6), oral floor (4) and palate (1). The SIN patients were 11 males and 7 females, aged 40-76 (mean 64.7), and the sites of SIN were the tongue (12), gingiva (4) and buccal mucosa (2). Histological grading of OSCC and SIN was done according to WHO criteria (Barnes *et al.*, 2005).

The pattern of invasion was evaluated according to the criteria used in the malignancy grading system developed by Anneroth (Anneroth *et al.*, 1987).

Immunohistochemical staining

Immunohistochemical staining was conducted as previously described (Sakamoto *et al.*, 2011). In brief, sections were heat-treated in alkaline buffer (10mM Tris (pH 9.0) and 1mM EDTA) at 120°C for 20 minutes. The primary antibodies used in this study were anti-K5 (EPR1600Y, Epitomics); K14 (LL002, Abcam); K15 (EPR1614Y, Epitomics); K19 (EP1580Y, Epitomics); Ki-67 (MIB-1, Dako) and CD44 (EPR1013Y, Epitomics) antibodies. They were diluted 1/500 in 50 mM Tris (pH 7.4) and 0.1% Tween 20 and used for overnight incubation at 4 °C. As a negative control, the primary antibodies were replaced by normal rabbit or mouse IgG. After washing with TBST (10 mM Tris (pH 7.4), 50 mM NaCl, 0.1% Tween 20), the sections were incubated with EnVision Dual Link (Dako) at room temperature for 1 hr. Coloration was done in DAB substrate. For immunofluorescent double staining, two antibodies (K15 and Ki-67 or K19 and Ki-67) were mixed for primary antibody reaction, and Alexa Fluor 488 anti-rabbit IgG (Invitrogen) and Alexa Fluor 594 anti-mouse IgG (Invitrogen) were mixed for secondary antibody reaction. Fluorescence images were digitally obtained from three representative areas of each specimen by Axiocam (Zeiss). For evaluation of the Ki-67 labeling indices, numbers of K15+ cells and K15+Ki-67+ cells, or numbers of K19+ cells and K19+Ki-67+ cells were counted on the computer screen, and the ratios of K15+Ki-67+ / K15+ and K19+Ki-67+ / K19+ were calculated.

Statistical analyses

Microarray data was analyzed by Pearson's product moment correlational analysis or Student's t-test. G factor, D factor and S factor were analyzed by Pearson's chi-square test. I factor was analyzed using the Cochran-Armitage test. A p-value less than 0.05 was considered statistically significant. A p-value less than 0.1 was considered to indicate a tendency.

Results

cDNA microarray analysis of basal cell keratins in OSCC

cDNA microarray analysis of OSCC collected by laser-capture microdissection revealed complex alterations in the expression of keratin subtypes. In our previous study, we evaluated the average values of the expression, and performed further studies on *keratin 4* (*KRT4*) and *KRT13* that showed the most significant changes (Sakamoto *et al.*, 2011).

In this study, we focused on the keratins expressed in the basal layer; *KRT5*, *KRT14*, *KRT15* and *KRT19*. To clarify the expression in each case, we plotted the globally-normalized microarray signals of each sample on dual logarithmic axis charts (Fig.1A, B). *KRT5* expression in OSCC (11500±558; mean±s.e.) showed little diversity, as revealed by a small dispersion along the horizontal axis, and the expression did not differ ($p=0.40$) from that in normal epithelium (11233±777) (Fig.1A). *KRT14* expression in OSCC (25783±992) showed a 1.6-fold upregulation over normal epithelium (16107±2165) ($p=0.003$). Normal epithelia co-expressed *KRT15* and *KRT19*, as revealed by the localization at the right upper corner in Fig.1B, wherein the expression of *KRT15* (8295±605) was 5-fold higher than that of *KRT19* (1752±285). In OSCC, *KRT15* (2735±545) was reduced to 0.31-fold ($p<0.001$), and *KRT19* (639±180) reduced to 0.44-fold ($p=0.007$) compared to normal epithelium. When the expression in each OSCC was individually evaluated, the reduction rates of *KRT15* and *KRT19* were diverse, as revealed by a great dispersion of the scatter plot (Fig.1B). Thirty-three percent of OSCC showed more than 10-fold reduction of *KRT15* (represented by a left shift of one gauge in Fig.1B). Sixty-three percent showed more than 10-fold reduction of *KRT19* (represented by a downshift of one gauge in Fig.1B), and 42% showed more than 100-fold reduction of *KRT19* (represented by a down shift of two gauges in Fig.1B). In contrast, there were OSCCs that considerably retained these keratin expressions: 21% of OSCC showed more than half (a left shift of approximately 0.3 gauge) of the normal *KRT15* ; and 23% showed more than half (a downshift of approximately 0.3 gauge) of the normal *KRT19*. Great variations in *KRT15* and *KRT19* expression across different cancers contrasted well with unvarying changes of *KRT5* and *KRT14*. We hypothesized that the expression levels of these keratins may reflect diverse biological properties of each cancer. With this in mind, we compared the expression of *KRT15* and *KRT19* with clinicopathological parameters, including age, sex, site, size of tumor and histological grade. Only the histological grade showed correlation with *KRT15* and *KRT19*. Well-differentiated OSCC showed higher expression of *KRT15* compared to moderately- or poorly-differentiated OSCC, and moderately-differentiated OSCC tended to express more *KRT15* than did poorly-differentiated OSCC (Fig. 1C). *KRT19* expression was significantly higher in moderately- or poorly-differentiated OSCC compared to well-differentiated OSCC (Fig. 1D). To further evaluate the correlation between keratin expression and differentiation, correlation coefficients between *KRT15* or *KRT19* and the other major basic keratins were calculated in 43 OSCC data (Table 1). Positive correlation was indicated between *KRT15* and *KRT13*(Table 1, Fig. 1E). Since K13 is a differentiation-related keratin, this