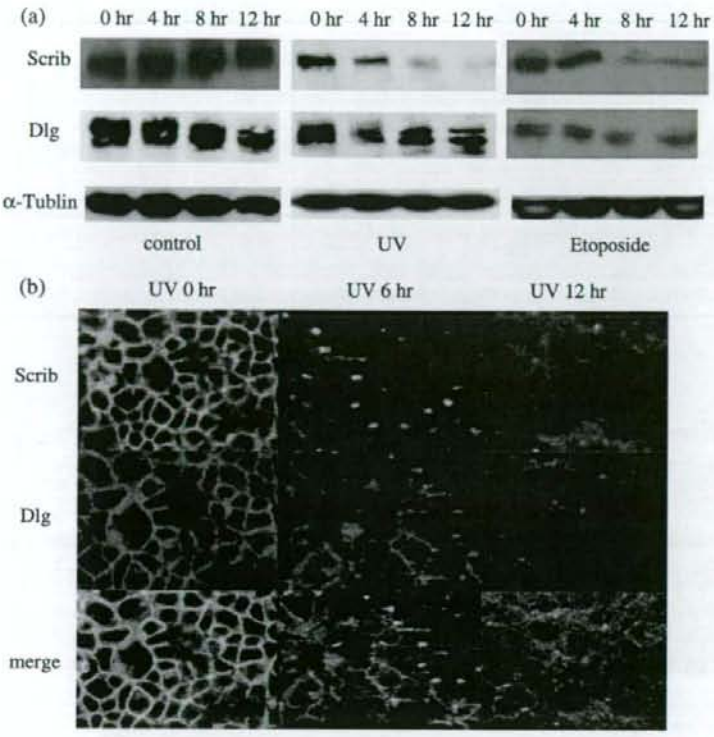


Figure 5 Loss of hScrib expression is earlier event in progressive apoptosis than that of hDlg. Expression of human homologues of *Drosophila* neoplastic tumor suppressor proteins, hScrib and hDlg, was analyzed by the Western blotting (a) and immunofluorescence staining (b) with anti-hScrib and anti-hDlg antibodies during progression of apoptosis. Both of assays indicate that hScrib is targeted by proteolysis earlier than hDlg in the progression of apoptosis induced by UV irradiation.

expression vector was lower than that in cells transfected with control vector, which is consistent with the previous report (Nakagawa & Huibregtse 2000). After induction of apoptosis, caspase-dependent cleavage of hScrib was more evident in cells transfected with control vector comparing with that in cells transfected with E6 expression vector (Fig. 11). The p220 hScrib was not observed in cells transfected with control vector and in those transfected with E6 expression vector after 24 h of UV irradiation, suggesting the possibility that E6 protein expression partially inhibit caspase-dependent cleavage of hScrib.

Discussion

Tissue homeostasis is kept in normal epithelial cells under the surveillance of programmed cell death mechanism (Igney & Krammer 2002). Over-proliferated or over-damaged cells are eliminated by the self-destruction mechanism called apoptosis (Steller 1995; Thompson 1995; Song & Steller 1999). Disruption of intrinsic cell–cell contact is a critical step in the process of apoptosis (Rosenblatt *et al.* 2001). A proteolytic cascade mediated by the caspases family of cysteine proteinases, which specifically cleave target proteins after aspartate residues, has a central role in cell death machinery (Brancolini *et al.* 1997). A number of proteins localized at the adherens and tight junctions have been reported to be targeted by caspases, including E-cadherin, β-catenin, FAK, PAK2, fodrin, plakoglobin, hDlg, focal adhesion kinase, ZO-1, ZO-2, occludin, MAGI-1 and MAGI-2 (Rudel & Bokoch 1997; Wen *et al.* 1997; Janicke *et al.* 1998; Levkau *et al.* 1998; Steinhusen *et al.* 2000, 2001; Bojarski *et al.* 2004; Gregorc *et al.* 2005, 2007; Ivanova *et al.* 2007). We have identified that hScrib is targeted for cleavage by executioner caspase activated by death ligands TNF-α and FAS ligands and UV irradiation. These data indicate that caspase-dependent cleavage of hScrib is a general event in apoptosis.

hScrib is human homologue of *Drosophila* tumor suppressor protein Scribble (Nakagawa & Huibregtse 2000). In *Drosophila*, three tumor suppressor genes (TSGs) *lgl*, *dlg* and *scrib* are categorized as neoplastic TSGs, in which mutation causes loss of apico-basolateral cellular polarity and tissue architecture and simultaneously induces extensive over proliferation in epithelia and neuroblasts (Bilder *et al.*

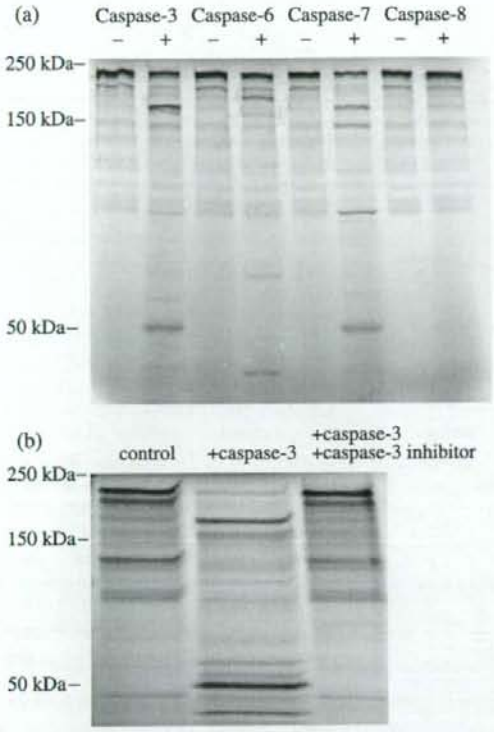


Figure 6 *In vitro* cleavage of hScrib with the executioner caspases. (a) *In vitro* translated [^{35}S] methione-labeled hScrib was incubated with recombinant caspase-3, caspase-6, caspase-7 and caspase-8 as described in the Materials and methods. Cleavage of hScrib by the executioner caspases was confirmed by the SDS electrophoresis and autoradiography. (b) The *in vitro* cleavage of hScrib by caspase-3 was completely repressed by the presence of caspase-3 inhibitor.

2000; Bilder 2003, 2004; Humbert *et al.* 2003; Hariharan & Bilder 2006). The *scrib* mutant clones proliferate, but these excess cells are eliminated by JNK-dependent apoptosis (Brumby & Richardson 2003; Pagliarini & Xu 2003; Tapon 2003). Loss of *scrib* mutation in activated Ras-expressing cells disrupted the epithelial structure of the eye imaginal disc and led to progressive invasion into neighboring structure (Pagliarini & Xu 2003). These data suggest the possibility that disruption of tissue polarity by loss of hScrib is involved in human carcinogenesis in concert with activated expression of oncogenic Ras. hScrib has been shown to be a functional homologue of the *Drosophila* Scribble (Dow *et al.* 2003). hScrib can rescue loss of polarity and inhibit tumorous overgrowth of *scrib* mutant *Drosophila* (Dow *et al.* 2003). Mammalian Scribble was shown to have crucial role in promotion of cell polarity in migrating astrocyte and epithelial cells (Osmani *et al.* 2006; Dow *et al.* 2007).

hScrib localizes at the adherens junction in normal epithelial cells and its expression is down-regulated in the precursor lesions and invasive cancers in the uterine cervix and colon (Nakagawa *et al.* 2004; Gardiol *et al.* 2006). Loss of hScrib expression was observed at the early stage of apoptosis identified by the positive TUNEL signal. hDlg is human homologue of *Drosophila* neoplastic tumor suppressor protein Discs large and is targeted for ubiquitin-mediated degradation by the high-risk HPV E6 protein (Gardiol *et al.* 1999). We analyzed loss of expression of these human homologues of *Drosophila* neoplastic tumor suppressor proteins during apoptosis and found that loss of hScrib expression is earlier event than that of hDlg. These data indicate that proper expression of hScrib is essential for construction of adherens junction and elimination of hScrib expression is also crucial for the disruption of junctional protein complex in damaged cells during apoptosis.

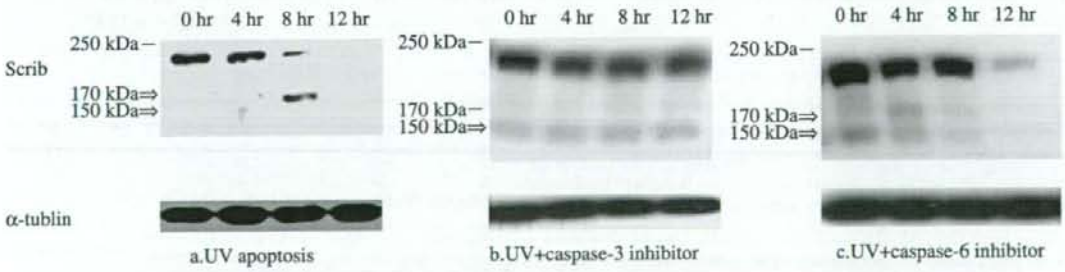


Figure 7 *In vivo* cleavage of hScrib by caspase-3 and caspase-6. The hScrib expression was lost by the irradiation of UV. The lost of hScrib expression after the apoptosis induction was inhibited by the presence of the inhibitor of executioner caspases, especially by the caspase-3 inhibitor. Note that generation of p170 hScrib was repressed by the caspase-3 inhibitor, but not by the caspase-6 inhibitor.

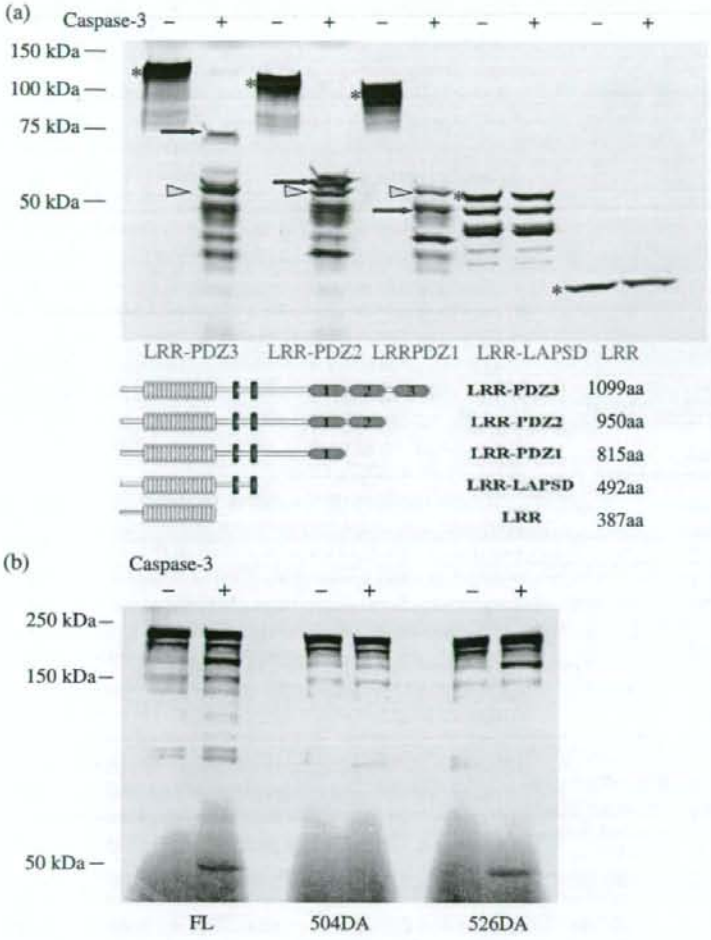
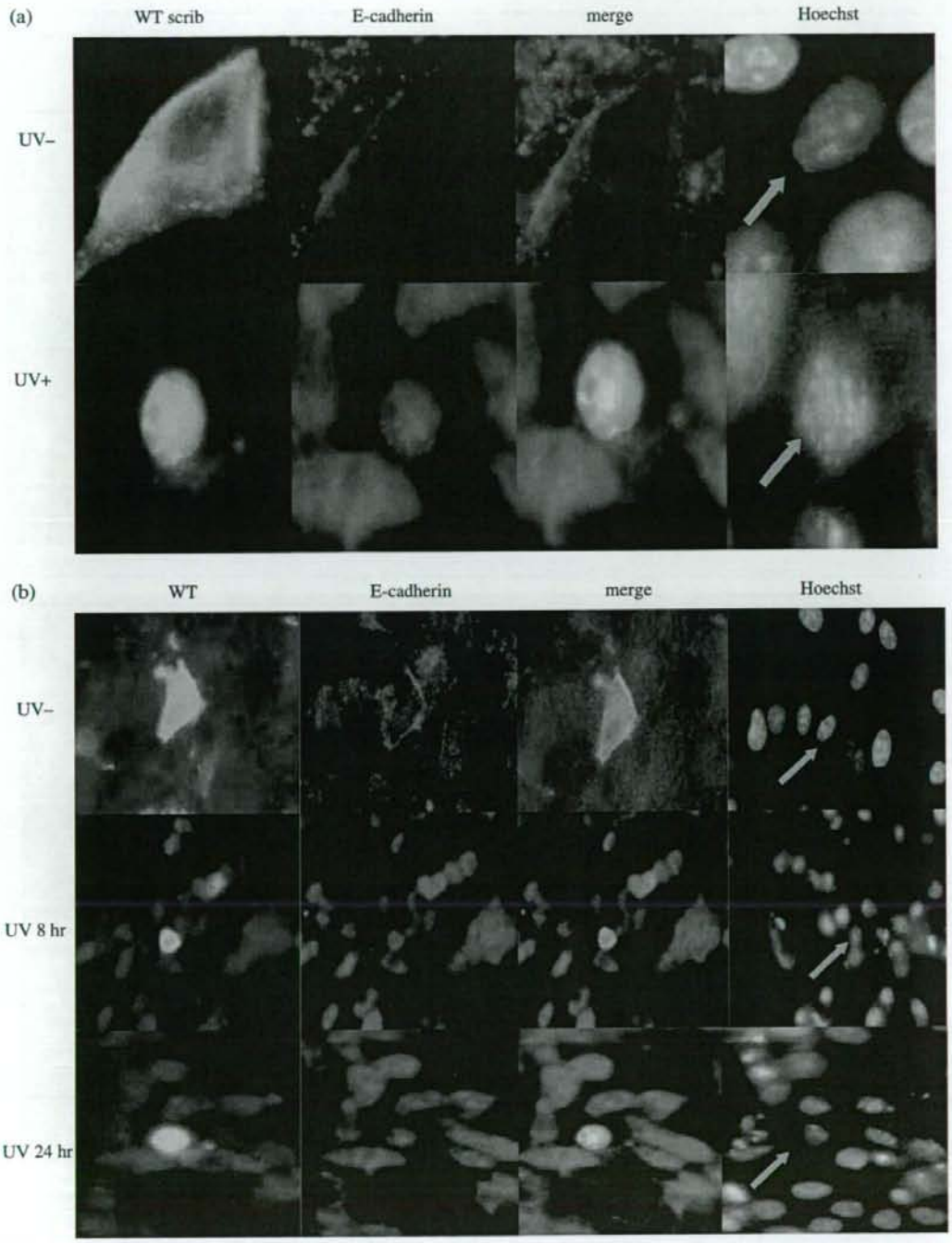


Figure 8 Analysis of cleavage site of hScrib by the caspase-3. (a) *In vitro* translated [^{35}S]-methione-labeled hScrib deletion mutants were incubated in the presence of recombinant caspases-3. The Scheme of hScrib C-terminal deletion mutants is shown in Fig. 8a. Human Scrib mutants, LRR–PDZ3, LRR–PDZ2 and LRR–PDZ1 were cleaved by recombinant caspase-3, whereas LRR–LAPSD and LRR were not susceptible for cleavage by caspase-3. Note that the protein band with same molecular weight (marked by the blue arrow head), which is considered to be the N-terminal part of proteins cleaved by caspase-3, is seen in cleaved LRR–PDZ3, LRR–PDZ2 and LRR–PDZ1 (Fig. 8a). The full-length translated hScrib mutants were indicated by the asterisk. Several fragments with much smaller sizes are seen for translated hScrib deletion mutants, especially for LRR–LAPSD. The protein bands, which are considered to be the C-terminal part of LRR–PDZ3, LRR–PDZ2 and LRR–PDZ1 hScrib mutants cleaved by caspase-3 are indicated by the red arrows. (b) Wild-type hScrib and Alanine substitution hScrib mutants of Asp 504 (D504A) and Asp526 (D526A) were tested for *in vitro* cleavage in the presence of recombinant caspases-3. Human Scrib D504A mutant is resistant to caspase-3 dependent cleavage.

We screened hScrib amino acids sequence for the potential cleavage site by caspase and found two D-X-X-D sequences (D_{1068}-V-R-D_{1071} and D_{1131}-P-T-D_{1134}), which are typical caspase-3 recognition sequences (Talanian *et al.* 1997). None of single amino acid substitution of these four Asp residues rendered hScrib resistant for caspase-dependent cleavage (K. S. and S. N. unpublished data). hScrib is a member of LAP (LRRs and PDZ domains) proteins. It has 16 canonical LRRs at the N-terminal region and four copies of the PDZ domain in its C-terminus (Santoni *et al.* 2002). Between these structures lies a 38-amino acid LRR-like domain called LAPSD-a. A second conserved sequence specific to LAP proteins and unrelated to LRR motifs between LRRs and PDZ domains resides at the downstream of LAPSD-a and is named as LAPSD-b. We investigated the region responsible for caspase-dependent cleavage by using deletion mutants of hScrib and found that PDZ domains are not targeted for cleavage by caspase-3 and that the

Figure 9 GFP-fused wild-type hScrib and hScrib D504 mutant were transfected in to MDCK cells. Apoptosis was induced with UV irradiation 48 h post-transfection. Cells were analyzed for the immunofluorescence staining of E-cadherin. Hoechst staining was carried out for the analysis of nuclear fragmentation. The expression of hScrib and E-cadherin was lost in the wild-type hScrib transfected cells (arrow), which show the fragmented nucleus, after induction of apoptosis. Note that expression of E-cadherin is intact as a control in cells transfected with hScrib D504A mutant (arrow), which is resistant for caspase-dependent cleavage, whereas nucleus shows typically apoptotic signal with condensed fragmentation.



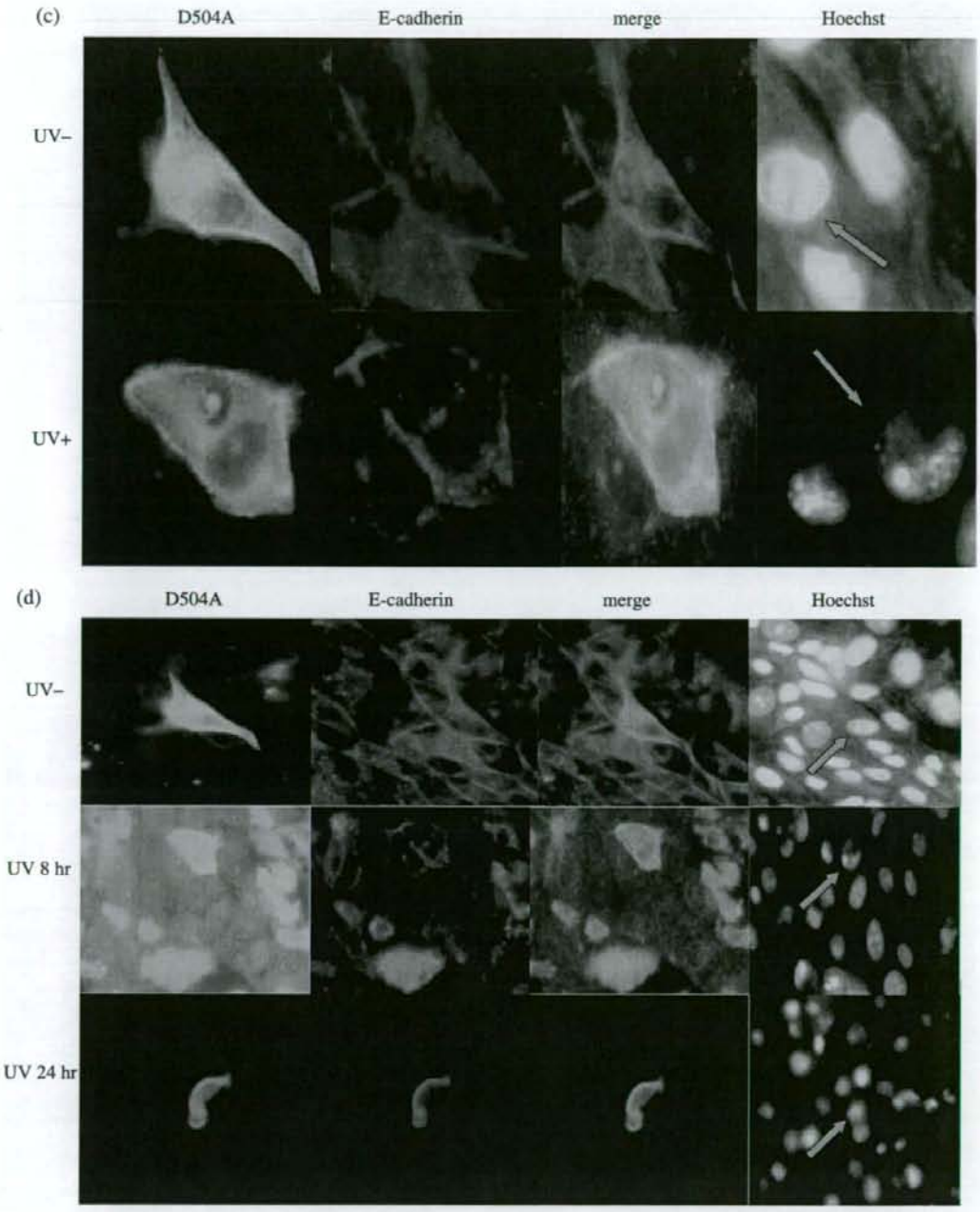


Figure 9 *Continued*

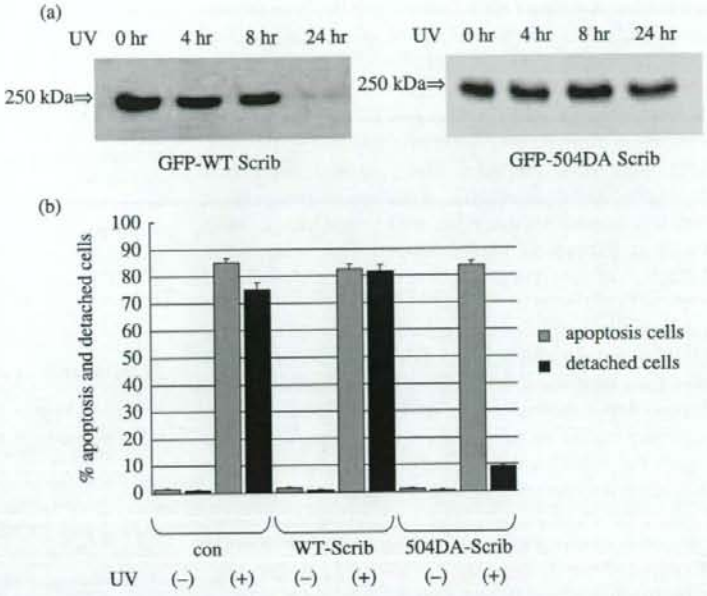


Figure 10 Human Scrib D504A mutant is resistant to caspase-dependent cleavage induced by apoptosis. (a) Immunoblotting analysis confirmed that over-expressed GFP–hScrib D504A mutant is resistant to caspase-dependent cleavage activated by apoptosis induction with UV irradiation. (b) To quantitate the effect of WT and Asp504Ala hScrib mutant on apoptosis induction and cellular detachment, 300 MDCK cells transfected with control vector, GFP–WT hScrib vector, or GFP–hScrib D504A mutant vector were analyzed for apoptosis signals and cellular detachment by Hoechst staining and immunofluorescence analysis of E-cadherin, respectively. The ratio of apoptosis positive cells and detached cells as evidenced by loss of E-cadherin in 300 cells transfected with each vector was analyzed for absence and presence of UV irradiation and shown as histograms.

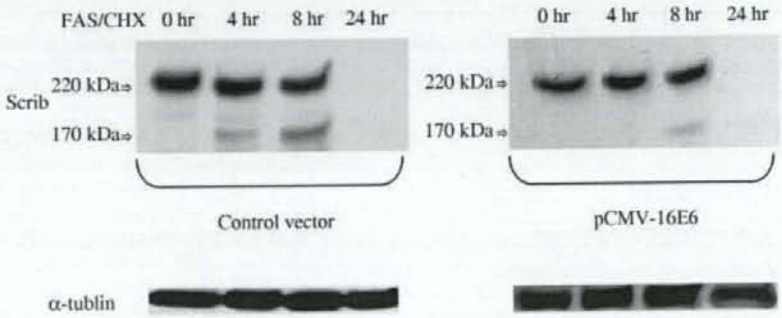


Figure 11 The effect of HPV E6 on the caspase-dependent cleavage of hScrib. Apoptosis was induced by UV irradiation at 48 h post-transfection of control vector or E6 expression vector. Prior the apoptosis induction, hScrib expression level was lower in cells transfected with E6 expression vector. After induction of apoptosis, caspase-dependent cleavage of hScrib was more obvious in cells transfected with control vector as evidenced by the generation of p170 at 4 h post-UV irradiation comparing with that in cells transfected with E6 expression vector. Note that p170 was not observed in cells transfected with E6 vector after 4 h of UV irradiation.

amino acids sequence between LRRs and PDZ domain 1 is potential caspase-dependent cleavage site. The site-specific mapping of a critical amino acid for the caspase-dependent cleavage of hScrib with mutagenesis showed that first ASP residue (amino acid 504) in the region between LAPSD-b and PDZ domain 1 is targeted for cleavage by caspase-3. hScrib N-terminal region containing LRRs, LAPSD-a and LAPSD-b ($hScrib_{1-724}$ and $hScrib_{1-518}$) is reported to localize at the basolateral epithelial membrane (Navarro *et al.* 2005). Our previous study showed that $hScrib_{1-495}$ localizes in the cytoplasm, not at the membrane (Nagasaka *et al.* 2006). It is possible that the cleaved hScrib at amino acid 504 by caspase-3 ($hScrib_{1-504}$) does not target the basolateral membrane. For *Drosophila* Scribble, multi-step localization through LRRs and PDZ domains are necessary for establishment of cortical polarity (Albertson *et al.* 2004; Zeitler *et al.* 2004). hScrib has been shown to be involved in polarity

control in migrating cells by interacting βPIX exchange factor and APC (Audebert *et al.* 2004; Takizawa *et al.* 2006; Dow *et al.* 2007). hScrib have been reported to interact with ZO-2 and zyxin-related proteins, Lipoma Preferred Partner (LPP) protein and TRIP6, at epithelial cellular junctions through its PDZ domains (Metais *et al.* 2005; Petit *et al.* 2005a,b). The caspase-3 dependent cleavage of hScrib at amino acid 504 might disrupt these protein complexes formations at the epithelial cellular junctions through its PDZ domains. The resistance to distraction of adherens junction in the apoptosis-induced epithelial cells transfected with hScrib D504A mutant indicates that caspase-3 dependent cleavage of hScrib is a critical step for elimination of dying cell from normal cells. Our analysis of the effect of E6 expression on the caspase-dependent cleavage of hScrib indicated the possibility that E6 partially inhibit the cleavage. These data suggest the possibility that E6 render some cellular fractions of hScrib resistant to the caspase-dependent cleavage. Further investigations would be required to show comprehensive mechanisms underlying the partial inhibition of caspase-dependent cleavage of hScrib by E6 protein.

In summary, we found that hScrib, which has a fundamental role in tissue polarity architecture, is a novel death substrate targeted by caspase-3. The caspase-dependent cleavage of human homologues of *Drosophila* neoplastic tumor suppressors, hScrib and hDlg, is considered to be an essential step in the elimination of apoptotic cells from the surrounding healthy cells.

Experimental procedures

Tissue culture and apoptosis induction

Human HaCat, CaCo-2 cells and Hela cells were grown in DMEM supplemented with 10% fetal bovine serum. Before induction of apoptosis, cells were plated onto 10-cm dishes and allowed to reach to the confluency. Apoptosis was induced by irradiating UV light (0.24 J) or adding 200 nM etoposide (Sigma, St Louis, MO), 500 ng/mL anti-Fas (MBL, Nagoya, Japan), 100 μg/mL Cycloheximide (CHX) (Sigma) and/or 2000 U/mL TNF (Relia Tech GmbH, Braunschweig, Germany) into the medium. In an additional experiment using caspase inhibitors, 50 μM Z-DEVD-FMK (R&D systems, Minneapolis, MN or Ac-VEID-CHO (Biomol, Pennsylvania, PA) was added into the medium and apoptosis was induced as described above.

Western blotting

Following apoptosis induction, cells were harvested at the indicated hours after induction of apoptosis. The protein concentration of the samples was equalized and samples were analyzed by electrophoresis on 6% SDS PAGE. Levels of hScrib and hDlg protein were determined by Western blotting using ECL advance Western blotting Detection Kit (GE Healthcare Bio-science, Piscataway, NJ) according to the manufacture's instructions. The expression of hScrib was detected using the anti-hScrib goat monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or the anti-hScrib polyclonal antibody raised in rabbit against its PDZ domains as an antigen. The expression of hDlg was detected using the anti-hDlg mouse monoclonal antibody. (Santa Cruz Biotechnology). The expression of Lamin B1 was detected using the anti-Lamin B1 mouse monoclonal antibody. (Santa Cruz Biotechnology). The expression of procaspase-3 was detected using the anti-caspase-3 mouse monoclonal antibody. (Santa Cruz Biotechnology). The expression of GFP–Scrib was detected using the anti-GFP mouse monoclonal antibody (Zymed, San Francisco, CA).

Fluorescence microscopy

HaCaT and CaCo-2 cells were grown overnight on cover slips before induction of apoptosis. Cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS for 30 min at the times indicated, followed by permeabilization with 0.2%(v/v) Triton X-100 in PBS for 5 min. After extensive washing with 1% BSA–PBS, the cells were incubated with anti-hScrib antibody diluted 1 : 400, and anti-hDlg diluted 1 : 100 in PBS for 60 min. Following an additional round of wash with PBS containing 1% BSA, cells were incubated with donkey anti-goat and rabbit anti-mouse Allexa488 and 568 conjugated antibodies (Invitrogen, Eugene, OR) for 60 min. Expression of protein was investigated under the confocal fluorescence microscopy.

To analyze apoptosis signal, cells were incubated with Hoeshst33342 (Sigma) for 7 min, washed in PBS with 1% BSA, and then mounted on slides.

MDCK cells were transfected with GFP-tagged human scribble constructs, using the PolyFect Transfection Reagent (Qiagen, Hiden, Germany) or Effectene Transfection Reagent (Qiagen) according to manufacturer's instructions. To see the effect of HPV E6 on the caspase-dependent cleavage of hScrib during apoptosis, 293T cells were transfected with HPV E6 expression plasmid (Nakagawa & Huibregtse 2000).

Apoptosis is induced 48 h post-transfection with UV irradiation. At the indicated hours, cells were collected and treated as described above or stained with anti-E-cadherin antibody (BD Transduction Laboratories, Franklin Lakes, NJ) and Alexa568 conjugated anti-mouse antibodies (Molecular Probes, Eugene, OR). In addition, Hoeshst33342 was used to stain the nuclei. Morphological changes of cells induced of apoptosis were monitored using confocal fluorescence microscopy. To quantify the effect of WT hScrib or hScrib mutant D504A on cellular detachment during apoptosis, number of cells showing apoptosis (fragmentation of nucleus, and shrinkage of cytoplasm) and cellular detachment (loss of E-cadherin) were analyzed in 300 MDCK cells transfected with control vector, GFP–WT hScrib, or GFP–hScrib D504A mutant.

In vitro translation of proteins

Proteins were expressed using the Promega TNT coupled transcription-translation Rabbit-Reticulocyte lysate system (Promega,

Madison, WI) according to the manufacture's instructions and radio-labeled with [^{35}S]-methionine (PerkinElmer, Waltham, MA).

Caspase cleavage assays

For *in vitro* caspase cleavage assay, *in vitro* translated hScrib labeled with [^{35}S] methione was incubated in the presence of recombinant caspase-3 (Chemicon, Temecula, CA), caspase-6 (Alexis, Lausen, Switzerland), caspase-7 (Chemicon) or Caspase-8 (BioVision, San Francisco, CA) at 37 °C for 1 h. The reaction was terminated by the addition of SDS loading buffer and boiling. The reaction mixtures were analyzed by SDS-PAGE and autoradiography.

Plasmids

For *in vitro* expression, the cDNA for Scrib was subcloned into the BamHI/NotI sites of pCDNA3. The Scrib Ala substitution mutants of Asp were constructed using overlap polymerase chain reaction (PCR) with Scrib cDNA as a template using the following primers:

5′-CCTTGCCAGCCA**GCC**TCTGGGTCGCCC-3′ (Asp504Ala)

5′-GGCCTGAGTGAA**GCC**TCTCGCCCATCTGCC-3′ (Asp526Ala)

5′-GTGAACGGGCAA**GCC**GTGCGGGATGCC-3′ (Asp_{1068}Ala)

5′-CAAGACGTGCGG**GCT**GCCACGCACCAAG-3′ (Asp_{1071}Ala)

5′-GGCAACCCCCGC**GCC**CCCACAGACGAG-3′ (Asp_{1131}Ala)

5′-CGCGACCCCACA**GCC**GAGGGCATCTTC-3′ (Asp_{1134}Ala)

To generate the deletion mutants of hScrib, the following cDNA sequences were amplified with polymerase chain reaction (PCR) and subcloned into pCDNA3: LRR + PDZ 1–3 (amino acids 1–1096); LRR + PDZ 1–2 (amino acids 1–953); LRR + PDZ 1a (amino acids 1–819); LRR + PDZ 1b LRR + LAPSDb (amino acids 1–495).

For GFP fusions wild type and mutant *human scribble* cDNA were cloned into the HindIII/EcoRI sites of pEGFP–C1 vector.

TUNEL assay

Human HaCaT cells were grown overnight on cover slips before induction of apoptosis.

After induction of apoptosis, cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 30 min, followed by permeabilization with 0.2% (v/v) Triton X-100 in PBS for 5 min.

The TUNEL assay was carried out using Promega Dead-END™ Fluorometric TUNEL System (Promega) according to the manufacture's instructions.

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Oral pilocarpine (5 mg t.i.d.) used for xerostomia causes adverse effects in Japanese

Naoki Nakamura [a,*], Nakashi Sasano [a], Hideomi Yamashita [a], Hiroshi Igaki [a], Kenshiro Shiraishi [a], Atsuro Terahara [a], Takahiro Asakage [b], Kazunari Nakao [b], Yasuhiro Ebihara [b], Kuni Ohtomo [a], Keiichi Nakagawa [a]

[a] *Department of Radiology, Tokyo University Hospital, Tokyo, Japan*
[b] *Department of Otolaryngology, Tokyo University Hospital, Tokyo, Japan*

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Abstract

Objective: To evaluate Japanese tolerability to pilocarpine of 5 mg t.i.d.
Methods: From January 2006 to July 2006, 39 patients with xerostomia received 5 mg t.i.d. pilocarpine for at least for 12 weeks unless they had experienced unacceptable adverse effects. All patients received radiotherapy that included the parotid glands in the radiation field >50 Gy. The body weights of the patients ranged from 42 to 73 kg (median 60 kg).
Results: Thirty-six of the 39 patients were evaluable. The tolerated rate was only 47%. Of the 25 patients whose body weights were less than 65 kg, the tolerated rate was 36%, whereas the rate of the 11 patients whose body weights were 65 kg or above was 72% ($p = 0.050$). The most common adverse effect was sweating with an incidence of 64%. Response rate, which was defined as the total number of patients with an increase of at least 25 mm from the baseline in the VAS score divided by the number of maintaining patients among those who started pilocarpine after more than 4 months from the start of radiotherapy, was 40% at 12 weeks ($n = 15$).
Conclusion: For Japanese, 5 mg t.i.d. pilocarpine caused a high incidence of unacceptable adverse effects. A lower dose of pilocarpine needs to be considered.

Keywords: Pilocarpine; Xerostomia; Japanese; Tolerability; Sweating; Radiotherapy

1. Introduction

Lifelong xerostomia associated with salivary dysfunction is a most unpleasant adverse effect resulting from high dose irradiation delivered to the head and neck region [1–5]. Patients with xerostomia experience significant oral discomfort and difficulties in speaking, swallowing, and sleeping [6–9]. These conditions can lead to severe oral disease, nutritional deficiencies and marked decline in quality of life [10].

Treatment of xerostomia is difficult, and previous treatments have included saliva substitutes, hard candy, antimicrobial rinses, and fluoride treatments, all of which have generally been inadequate. Pilocarpine hydrochloride, however, has been approved for effective treatment of radiation-induced xerostomia in many countries. It is a naturally occurring alkaloid that has a broad range of pharmacologic effects, including increasing secretion from the exocrine glands (sweat, salivary, lacrimal, gastric, pancreatic, and intestinal glands). The clinical efficacy of 5–10 mg pilocarpine three times per day (t.i.d.) daily to reduce the symptoms of xerostomia has been studied in several trials in the Western countries [8–18].

On the other hand, pilocarpine is known to cause various kinds of adverse effects expected for a cholinergic agonist (e.g., sweating, rhinitis, nausea, urinary frequency)

* Corresponding author at: Department of Radiology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 5880 8667; fax: +81 3 5880 8935.
E-mail address: nnakamur-tky@umin.ac.jp (N. Nakamura).

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[8,11,12]. The severity of these adverse effects is thought to be dose related. According to Rieke et al. pilocarpine's adverse effects were considered acceptable by patients taking 5 mg t.i.d. [11].

Pilocarpine has been available for clinical use from October 2005 in Japan. However, a study has not yet been conducted to determine if 5 mg t.i.d. is the best dose of pilocarpine for Japanese who probably have a different body mass index than Westerners. The purpose of this study was to evaluate the tolerability of Japanese to 5 mg oral pilocarpine t.i.d. daily during or after radiotherapy in head and neck cancer.

2. Materials and methods

2.1. Patients

Thirty-nine patients, who had been suffering from radiation-induced xerostomia, received pilocarpine during or after radiotherapy in our institute. They started to receive pilocarpine between January 2006 and July 2006. All of the patients had head and neck carcinomas and received >50 Gy radiotherapy that included the parotid glands in the radiation field.

The patients' characteristics are summarized in Table 1. Thirty-nine patients consisted of 35 males and 4 females with a median age 61 years. The body weights of the patients ranged from 42 to 73 kg (median 60 kg). Twenty-eight (72%) patients had pharyngeal cancer. Five (13%) patients had laryngeal cancer, three (8%) had oral cancer, two (5%) had unknown primary cancer, and one (3%) had paranasal cavity cancer.

Twenty-three (59%) patients received definitive radiotherapy, while 16 (41%) patients received post-operative radiotherapy. Twenty (51%) patients received concurrent chemotherapy. The prescribed irradiation dose ranged from 60 to 72 Gy (median 70 Gy). The mean dose to both salivary glands ranged from 19.9 to 57.3 Gy (median 41.1 Gy).

Table 1
Patients' characteristics

Characteristics	$n = 39$
Age, years (median)	39–84 (61)
Gender (%)	
Female	10
Male	90
Body weights (%)	
<65 kg	67
>65 kg or =65 kg	33
Tumor site (%)	
Pharynx	72
Epipharynx	15
Mesopharynx	15
Hypopharynx	31
Larynx	13
Oral cavity	8
Primary unknown	5
Paranasal cavity	3
Intent of radiotherapy (%)	
Definitive radiotherapy	59
Post-operative radiotherapy	41
Prescription dose, Gy (median)	60–72 (70)
Mean dose of both salivary glands, Gy (median)	19.9–57.3 (41.1)
Concurrent chemotherapy (%)	
Yes	51
No	49
Duration from the beginning of radiotherapy to the beginning of pilocarpine (%)	
<120 or =120 days	15
>120 days	85

n = number of patients.

2.2. Pilocarpine

All patients received a daily dose of pilocarpine of 5 mg t.i.d. They were seen prior to initiation of treatment and at 2-week intervals thereafter, and continued to receive pilocarpine for at least 12 weeks whether effective or not unless they had experienced unacceptable adverse effects.

The duration from the beginning of radiotherapy to the start of pilocarpine ranged from 2 weeks to 72 months (median 8 months). Thirty-three (85%) patients started pilocarpine 4 months or later from the start of radiotherapy.

2.3. Study outcomes

The primary outcomes were determined by the rate that pilocarpine was maintained for 12 weeks, with or without any adverse effects (defined as tolerated rate). Secondary outcomes included the incidence of adverse effects, and the subjective symptoms of xerostomia.

Adverse effects were reported by telephone as they occurred, or at the bi-weekly appointments throughout the study.

The subjective assessment of efficacy was undertaken through the use of visual analog scales (VAS) every 4 weeks. The 100 mm visual analog scale (VAS) was used to record the response. Patients were asked to rate their condition of the dryness of the mouth on a scale from 0 to 100. This questionnaire was completed before starting pilocarpine for a period of 12 weeks. A patient with an increase of at least 25 mm from the baseline in the VAS score was defined as a "Responder." Response rate was defined as the total number of "Responder" divided by the number of maintaining patients. Response rate, in addition, was calculated among the 30 patients who started pilocarpine after more than 4 months from the start of radiotherapy and were considered to have fixed symptoms of xerostomia.

3. Results

Three of the 39 patients were excluded from the analysis because they stopped taking pilocarpine within 12 weeks for

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Table 2
Tolerability of pilocarpine with 5 mg t.i.d.

Status	n	%
Tolerable without any adverse effects	6	17
Tolerable with some adverse effects	11	30
Unacceptable adverse effects	19	53

n = number of patients.

reasons other than adverse effects. (Two of them refused to continue because of insufficient efficacy, and one stopped because of beginning other medication which should not be used concurrently with pilocarpine.).

3.1. Tolerability

Of the remaining 36 patients, the tolerated rate was as low as 47%. Only 17 of the 36 patients were able to continue pilocarpine for 12 weeks with or without any adverse effects. Nineteen (53%) stopped taking pilocarpine within 12 weeks because of unacceptable adverse effects (Table 2).

The duration from the beginning to stopping pilocarpine due to adverse effects ranged from 3 to 42 days (median 7 days).

When we divide patients in less or more than 65 kg, the tolerated rates between two groups showed a significantly difference. Of the 25 patients whose body weights were less than 65 kg, the tolerated rate was 36%, whereas the tolerated rate of the 11 patients whose body weights were 65 kg or more was 72% ($p = 0.050$, calculated by a χ^2-test) (Table 3).

3.2. Incidence of adverse effects

The most common adverse effect was sweating, and its incidence was 64%. Other adverse effects reported included nausea, rhinitis, headache, cervical pain, fatigue, dazzling, oversalivation, and paresthesia of the tongue (Table 4).

Table 3
Tolerated rate according to the patients' body weights

Body weights	n	Tolerated rate (%)
<65 kg	25	36
>65 kg or =65 kg	11	72

n = number of patients; $p = 0.050$.

Table 4
Incidence of adverse effects with a probable relationship to pilocarpine

Adverse effects	% (n = 36)
Sweating	64
Nausea	8
Rhinitis	6
Headache	3
Cervical pain	3
Fatigue	3
Dazzling	3
Oversalivation	3

n = number of patients.

Table 5
Response rate

	Pretreatment	4 weeks	8 weeks	12 weeks
n	30	20	15	15
Response rate (%)	–	10	27	40

n = number of patients.

With the exception of paresthesia of the tongue, all of the other adverse effects which caused patients to quit taking pilocarpine disappeared within 1 week of stopping and were probably related to pilocarpine.

3.3. Subjective symptoms of xerostomia

Response rates at 4, 8, and 12 weeks were 10, 27, and 40%, respectively (Table 5).

4. Discussion

The most common adverse effect of pilocarpine is sweating, and its incidence is thought to be dose related. In the review of two prospective randomized trials that included 369 patients, Rieke et al. reported that the incidence of sweating with pilocarpine was 29% with 5 mg t.i.d, while it was 68% with 10 mg t.i.d. [11] They concluded that the adverse effects were considered acceptable by patients taking 5 mg t.i.d. They also reported that an improvement in dryness was obtained in 51% of patients receiving 5 mg t.i.d. pilocarpine, which was equally effective as 10 mg t.i.d, but 2.5 mg t.i.d was judged to be an ineffective dose.

Our investigation indicated that the incidence of sweating was 62%, and the tolerability was very low in spite of using 5 mg t.i.d. Conceivably, the physical difference between the Japanese and Westerners may explain the discrepancy between our results and those reported by Rieke et al. [11].

In our study, the tolerated rate in patients whose body weights were less than 65 kg was much lower than that in patients whose weights were 65 kg or above ($p = 0.050$). For Japanese patients, especially for those weighting 65 kg or less, 5 mg t.i.d. of pilocarpine appears to be an over dose. The proper dose of pilocarpine may be a little lower than 5 mg t.i.d. for the average Japanese, and perhaps the tolerability can be raised without decreasing efficacy when using a proper dose.

In conclusion, an oral pilocarpine dose of 5 mg t.i.d. caused a high incidence of unacceptable adverse effects for Japanese. A lower dose of pilocarpine needs to be considered in conjunction with body weights to find a proper dose.

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Umami taste dysfunction in patients receiving radiotherapy for head and neck cancer

Hideomi Yamashita [a,*], Keiichi Nakagawa [a], Yoshio Hosoi [b], Azusa Kurokawa [c], Yusuke Fukuda [c], Ichiro Matsumoto [c], Takumi Misaka [c], Keiko Abe [c]

[a] *Department of Radiology, University of Tokyo Hospital, 7-3-1, Hongo, Bunkyou-ku, Tokyo 113-8655, Japan*
[b] *Section of Radiation Biology, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan*
[c] *Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan*

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KEYWORDS
Umami taste;
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Whole-mouth taste method

Summary Taste loss is a major cause of morbidity in patients undergoing head and neck irradiation. Previous studies have reported the alteration of the four basic tastes in patients with head and neck cancer during radiotherapy. However, only a few studies have been conducted on the effects of irradiation on the function of *umami* taste, a novel and basic taste recently recognized. In a prospective study, 52 patients undergoing radical head and neck irradiation were assessed for taste loss. Taste ability was measured by the taste threshold for *umami* quality using the whole-mouth taste method in patients before, during, and immediately after radiotherapy. *Umami* taste declined of the 3rd week after the start of radiotherapy and improved of the 8th week.

Introduction

Taste dysfunction is one of the most frequent complaints of patients undergoing radiation therapy (RT) for head and neck cancer. Complaints of taste disorders have been reported in 75% of patients with head and neck cancer undergoing radiation, and 93% of these patients complain of long-term xerostomia.[1] Many patients undergoing dose-intensive radiation experience reduced taste (ageusia) or altered taste (dysgeusia), which may have a significant impact on quality of life (QOL). Patients with taste disturbance experienced greater weight loss than those who did not report a change in taste.[2] On the other hand, patients with taste loss had a worse outcome than those did not lose their sense of taste and were able to maintain their food intake and nutritional support.[3] To design a diet that maximizes

* Corresponding author. Tel.: +81 3 5800 8667; fax: +81 3 5800 8935.
E-mail address: yamashitah-rad@h.u-tokyo.ac.jp (H. Yamashita).

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on the remaining taste abilities might result in the most palatable diet to the patients with taste loss and thus, better outcome and QOL might be expected. This would require individual diet management and especially, depend on a well understanding of the changes of the five basic tastes.

In addition to sweet, salty, bitter, and sour, a novel taste that is referred to by the Japanese word *umami* has come to be recognized as a "fifth taste".[4–7] *Umami* taste is found in a diversity of foods like fish, meat, milk, tomato, and some vegetables,[5] and considered to have an important role in the determination of food palatability as well as the intake of food.[8] In Japan, palatable and flavor enhancing taste is given a descriptor word *umami*, which means delicious. In 1908 Ikeda[9] extracted the glutamic acid from seafood and firstly put forward the conception of independent *umami* taste. Unfortunately, *umami* was not internationally accepted as a basic taste because it was supposed that *umami* could be duplicated with appropriate combinations of the other four basic tastes. However, Ikeda's pioneering opinion can be much supported by recent researches. According to the excellent review of taste that was recently published,[10] *umami* is considered to be one of the five basic tastes.

The relationship between changes in the taste recognition threshold for the new taste *umami* and the timing of radiation were analyzed.

Materials and methods

The subjects were 52 patients who underwent RT for their head and neck cancers at the Tokyo University Hospital from April 2002 to August 2007. None of the patients was treated with surgery prior to RT. The malignancies were distributed among the 52 patients as follows: nasopharyngeal cancer, 5; oropharyngeal and hypopharyngeal cancer, 1; oropharyngeal cancer, 17; hypopharyngeal cancer, 20; and the other head and neck cancers, 9. The mean age was 64 years (range, 29–89 years). There were 46 men and six women. Most patients were in good general condition {the 90% rate of Karnofsky performance status was 69% (36/52)}. In most patients (48/52), the RT was administered as a dose of 2 Gy once a day, five times each week. The total RT period ranged from 38 to 62 days (median: 47 days). Conventional radiation technique was used in this study. Only photon energy was used. Off-cord reductions were performed at 40 Gy in 20 fractions. The anterior oral tongue was deflected from the radiation volume after off-cord reduction. Concurrent chemotherapy was allowed in this study. Thirty-three subjects (63%) underwent chemotherapy combined with RT.

The cancers were limited to the head and neck area. Patients who had only a part of tongue within the radiation field were excluded from the study.

LINAC (6 MV in most cases) was used as a radiation source. In most cases, from the start to 40 Gy in 20 fractions, the radiation method was in three fields (their gantry angles were 0, 80, and 280° and beam weight was 1:1:1) in order to include the bilateral whole neck lymph nodes within radiation field. The radiation treatment of the nasopharyngeal and hypopharyngeal cancer also included the oral tongue within the volume of tissue radiated. That is why all patients received radiation dose of at least 11.4 Gy to the anterior tongue. After that, up to 60 Gy in 30 fractions, two shrinking and right and left opposing fields were used. In addition, the radiation field to the tumor bed was reduced. Most patients received a total radiation dose of 72 Gy in 36 fractions (mean: 68.4 Gy, range of dose: 36–72 Gy). The determination of the radiation fields was confirmed with linacgraphy. The planning was based on a three-dimension CT in all patients.

No tumor ablative procedures, or alteration of altering salivary beds, were performed in this study. No patients were taking Salagen or amifostine. None of the enrolled subjects had total or partial glossectomies.

All subjects gave written informed consent before entry into the study. The subjects had no intercurrent illnesses that affected salivary function (i.e., Sjögren's syndrome, human immunodeficiency virus [HIV]). No concurrent medicines altering the taste of the subjects were administered.

The taste recognition threshold for *umami* was measured using the whole-mouth taste method. Test solutions of monosodium glutamate (MSG; 25, 50, 75, or 100 mM) were prepared, and the subjects were tested with 10 mL of each concentration for a recognition threshold. First, the subject was asked to rinse mouth with distilled water and perceive the *umami* taste of the distilled water. Then, using a polyethylene pipette, 10 mL solution of the lowest concentration of one taste was circularly dropped into the mouth of the subject. The subject was instructed to identify the taste and then spat out the solution. When a wrong response was made, the next higher concentration would be applied. The lowest concentration that the subject continuously recognized the stimuli for two times was defined as the recognition threshold.

These taste recognition threshold measurements were performed once before RT and weekly thereafter from the first week to 10–12 weeks after the start of RT. At the same time, the subjects were questioned about xerostomia and mucositis by the radiation oncologists weekly.

Xerostomia or mouth dryness was classified into grade 0, normal; grade 1, mild and slight dryness of mouth, or symptomatic (dry or thick saliva) without significant dietary alteration; grade 2, moderate dryness of mouth, or symptomatic and significant oral intake alteration (e.g., copious water, other lubricants, diet limited to purees and/or soft, moist foods); and grade 3, complete dryness of mouth, or symptoms leading to inability to adequately aliment orally; IV fluids, tube feedings, or TPN indicated. Stomatitis due to radiation was classified into grade 1, erythema of the mucosa or minimal symptoms, normal diet; grade 2, patchy ulcerations or symptomatic but can eat and swallow modified diet; grade 3, confluent ulcerations or bleeding with minor trauma or symptomatic and unable to adequately aliment or hydrate orally; and grade 4, tissue necrosis, significant spontaneous bleeding, or symptoms associated with life-threatening consequences.

Results

Patients

The mean and median total doses of RT for tip of the tongue were 13.5 Gy and 13.3 Gy (range, 11.4–14.8 Gy) and for the

posterior part of the tongue were 68.4 Gy and 70 Gy (range, 36–72 Gy). The median dose to the affected side of salivary glands was 64 Gy.

Salivary function of the subjects was normal before treatment, but most subjects complained of xerostomia and/or stomatitis from the third week after the start of RT. Grade 3 of xerostomia and/or stomatitis occurred in approximately half of patients (29/52).

Taste recognition

In twenty-five patients (48%), the taste recognition threshold for *umami* did not fall and retained the state of the pre-RT. In the other patients (52%), the threshold deteriorated at the 2nd–5th weeks (median: the 3rd). Figure 1 shows changes in the taste recognition threshold for *umami* every week during and after RT. The sensitivity of taste declined significantly between the start of testing and the 3rd week after beginning RT (at 30 Gy) when compared with the state immediately before the start of RT ($p = 0.0027$). The paired *t*-test was used in calculating these values. The significance level was set at 0.05. On the 8th week after the start of RT, the sensitivity of taste improved significantly.

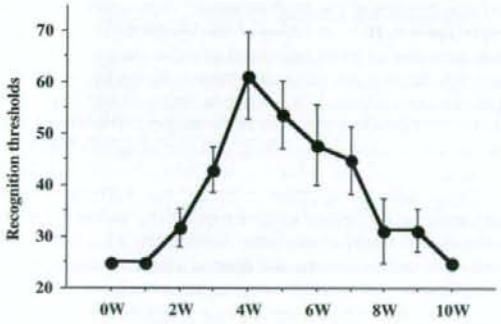

Figure 1 Weekly recognition thresholds of *umami* taste during and after radiation therapy, shown by mean ± SD of the concentration numbers.

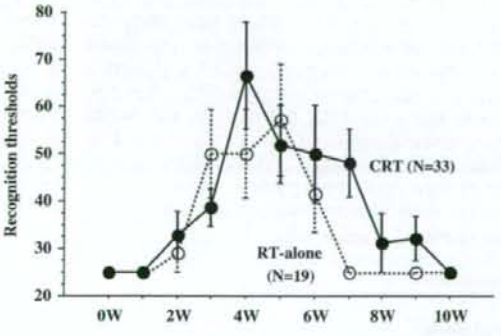

Figure 2 Weekly recognition thresholds of *umami* taste during and after radiation therapy, shown by mean ± SD of the concentration numbers divided by with or without chemotherapy.

In 15 of 34 patients given concurrent chemotherapy (44%), the threshold did not fall. On the other hand, the threshold deteriorated in eight of 18 patients without chemotherapy (44%). Consequently, there was no difference in the effect with and without chemotherapy (Fig. 2).

Discussion

The findings of a prospective study are provided that examines altered taste in patients who are receiving RT with or without chemotherapy for head and neck cancers. This entity of altered taste appears to bother patients but is very much understudied with only a relatively small number of publications occurring over the last few years. Moreover, the *umami* taste quality is only recently recognized and, to my knowledge, there is only one previous report studying taste loss of the *umami* taste during and after RT.[11] Thus, the present study is thought to represent interesting work and new information on the subject.

As shown in our previous reports,[12,13] there was a significant impairment of the threshold of all four basic tastes (sweet, sour, salt, and bitter) at 3 weeks after starting RT, and this impairment continued for 8 weeks. In the present study, the thresholds of *umami* taste increased significantly after irradiation at 3 weeks and recovered at 8 weeks. The impairment pattern was similar to that of 4 basic tastes. This result came up to our expectations. No difference is expected between the impacts of RT on for example sour taste of *umami* taste since there is no different physiology involved, different anatomy.

The reason why only *umami* taste was measured and the 4 other tastes were not evaluated is that the taste disc method on the 4 basic tastes is covered by health insurance in Japan. On the contrary, since there is no taste disc on *umami* taste, we used the whole-mouth method. This is why we presented the result of only *umami* taste in the present study.

In this study, some patients (actually 48%) showed no decline in *umami* taste. There was no link with irradiated dose or other medications. The reason was supposed that the taste recognition threshold for *umami* was measured using the whole-mouth taste method. The whole-mouth method cannot detect the subtle difference in taste threshold compared with the taste disc method and so on.

Treatment of mucositis did not impact the measurement. Pain medications such as non-steroidal anti-inflammatory drugs or oral morphine drugs as the treatment of mucositis have been used. Xerostomia may be an important contributor to *umami* taste.

Antineoplastic drugs that have been associated with taste changes include cisplatin, carboplatin, cyclophosphamide, doxorubicin, 5-fluorouracil, levamisole, and methotrexate.[14] But in this study, as shown in Figure 2, antineoplastic drugs had no or little effect on these patients. Xerostomia, which can be due to RT, may be responsible for taste changes. Damage to salivary glands may reduce the flow of saliva to such an extent that taste substances are not diluted and do not reach the receptor, which may result in food that is tasteless.[14] Changes in the oral flora, with overgrowth of fungi, some bacterial species, and increased dental caries may also lead to altered taste.[15]

Table 1 Wilcoxon signed-rank test comparing with 0 weeks

	0 W	1 W	2 W	3 W	4 W	5 W	6 W	7 W	8 W	9 W
p-value		–	0.0831	0.0027	0.0006	0.0009	0.0172	0.0104	0.3434	0.1679
Mean	25	25	32	43	61	54	48	45	31	31
SD	0	0	18	19	39	30	27	25	13	12

A similar study has previously been published. Shi et al. in Kyushu University[11] first observed the alteration of *umami* taste in patients following head and neck irradiation. In their study, the thresholds of *umami* taste increased after irradiation at 15 Gy. Then, unlike the classic four basic tastes, *umami* taste showed a significant impairment at 30 Gy and reached the peak of mean threshold at 45 Gy. Among the five basic tastes, *umami* taste showed a distinctive pattern of impairment. On the contrary, the distinctive pattern of impairment comparing with the other four basic tastes cannot be found. Our previous study[13] suggested that the other four basic tastes also showed the same pattern of impairment and recovery as *umami*.

In the present study, the recognition thresholds are nearly totally recovered at week eight after the start of RT. Since the treatment took seven weeks, the acute side effects including radiation mucositis or xerostomia were expected to be maximal at week eight. However, the taste thresholds were already largely recovered. It may be because the anterior tip of the tongue was no longer in the radiation fields from 40 Gy (week four) (see Table 1).

According to our previous report,[12] the taste loss is likely to be caused by damage to the taste cells but not by an impairment of the taste nerve fibers. Shatzman and Mossman[16] studied the effects of irradiation on preparations of enriched bovine taste bud membranes by using differential and sucrose gradient centrifugation. They found that a radiation dose of 70 Gy reduced the protein content in the membrane-enriched fraction. However, radiation seemed to have no effect on the amount of cyclic adenosine monophosphate (AMP), which is bound to the membrane and acts as a second messenger. These results suggest that radiation may cause a structural change in the membranes of the taste buds, but the membranes remain normal with respect to function, which is consistent with the suprathreshold taste performance results in this study. If the taste cell membranes and nerve fibers function normally after irradiation, the function of taste intensity–concentration curves should not change significantly.

Umami taste is now recognized as the fifth basic taste category in mammals. It has been suggested that this taste category evolved to enhance detection of amino acids (e.g. glutamate and aspartate) and oligopeptides in foods.[17–19] Monosodium glutamate (MSG) is a prototypic *umami* substance that is widely used as a research tool and flavor enhancer.[5,8,20,21] Preclinical studies have indicated that MSG solutions may evoke *umami* taste through interactions with G-protein-coupled taste receptor (T1R1/T1R3)[21] and/or a ligand activated ion channel[22–24] expressed in taste receptor cells. The taste response to MSG is not observed in T1R1/T1R3 knockout mice.[25] Therefore, this may suggest that irradiation damages the function of taste receptor expression cells.

Taste thresholds are influenced by the quality and quantity of the saliva, which especially by the use of parotid sparing techniques recovers in the year after RT. So it would be very interesting to repeat the test two months, six months and/or one year after RT. This is the subject of a following study. It has been shown several other authors that taste recovers slowly up to one year after treatment.[26]

Comparison with our own previous study with another patients and another method (i.e. disc method)[12], during head and neck irradiation the clinical impairment of *umami* taste is not different from that of the other four basic tastes. Shi et al.[11] concluded the opposite. They did find a different impairment pattern of the *umami* taste compared to the other taste qualities.[11] This reason might be that Shi et al.[11] examined only up to the 60 Gy and additionally only at the time point of pre-RT, 15, 30, 45, and 60 Gy and, on the other hand, we performed taste test every week from pre-RT to 9 weeks. Other authors[27,28] have found that the bitter and/or salt taste quality were affected most as compared to sweet and sour.

With the implementation of new radiation techniques, such as conformal and intensity-modulated RT (IMRT) in head and neck irradiation, the late-radiation effects can probably be reduced since these new techniques become more and more standard and since these techniques reduce the radiation dose to the salivary gland tissue and to the oral tongue. As shown in our previous report,[13] the importance of the irradiated tongue volume in relation to taste changes. In the group including most of the tongue within the radiation fields, there was a significant impairment and improvement of the threshold of all four basic tastes. However, this was not seen in the group not including the tip of the tongue within the radiation fields. According to Fang et al.,[29] the exception was that patients treated by IMRT had a both statistically and clinically significant improvement in global QOL, fatigue, taste/smell, dry mouth, and feeling ill at the time point of 3 months after RT. These modified techniques may result in a reduced number of taste buds irradiated and thus, might be helpful to preserve taste function against radiation damage. This QOL study show that taste loss is a significant chronic complication of head and neck therapy. The present study is a short-term assessment of taste impairment and recovery only by the end of RT or immediately after RT. We are planning to continue this taste test in patients who are followed up after completing RT.

Conflict of interest statement

None declared.

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診療

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転移性脊椎腫瘍のIMRTによる再照射の初期経験

原田英幸*1 西村哲夫*1 永田 晋*1 古谷和久*1 朝倉浩文*1 橋本孝之*1
水本斉志*1 高橋 満*2 片桐浩久*2 高木辰哉*2 村田秀樹*2

❶ 背 景

癌の脊椎転移の患者では，痛みが患者の生活の質を下げるだけでなく，硬膜外腔への腫瘍進展による脊髄の圧迫や脊髄の虚血などにより脊髄横断症状を呈し，下肢対麻痺となることもある。放射線治療は，こうした脊椎転移による症状を緩和する，あるいは歩行機能を維持するために有効である[1]。しかしながら，2.5～13%の症例では照射した脊椎に再増悪を認める[2)3)]。この場合に再照射を安全に行うことができれば有望な治療法といえるが，正常臓器の耐容線量を超えることによる有害事象への危惧から一般にはあまり行われていない。

近年，高精度放射線治療の進歩にはめざましいものがあり，従来頭蓋内病変へのみ適応されていた定位放射線治療が体幹部に適応され，早期肺癌の治療成績は著明に向上した。また強度変調放射線治療（Intensity Modulated Radiotherapy：IMRT）が脳腫瘍，頭頸部癌，前立腺癌において保険適応となり，正常臓器への線量を軽減しつつ腫瘍への高線量処方が可能となり，臨床の場での適用が広がっている。本稿では，当院において既照射の脊椎転移再増悪で，再照射が不可避となり，通常の方法では照射が困難と考えられた症例に対するIMRTの初期経験を報告する。

❷ 対象と方法

脊椎転移の再増悪に対するIMRTの適応基準として，以下の条件を満たす症例を対象とした。1）転移性脊椎腫瘍に対して，すでに放射線治療を施行している病変，2）臨床的あるいは画像的に再増悪が認められていること，3）手術や他の治療の適応が少ないと判断されていること，4）治療体位が保持できること，5）書面による同意が得られていること。しかし，原病の進行により全身状態が悪化している場合や極めて生命予後が不良であることが想定される場合，その他担当医が適応でないと判断される場合は対象としなかった。

以上の条件を満たした症例に対して2006年12月に治療を開始し，2007年8月までに治療を施行した8症例について検討した。

❸ 治療計画・検証

当院では，リニアックを用いた定位放射線照射を頭蓋内および体幹部に対して実施している。CT（computed tomography）は東芝社製Asteion，リニアックはシーメンス社製Primusであり，CTとリニアックは同一寝台となっている。頭蓋内病変に対する定位放射線照射では，アイソセンターにおいて3mm

*1 H. Harada, T. Nishimura, S. Nagata, K. Furutani, H. Asakura, T. Hashimoto, M. Mizumoto 静岡県立静岡がんセンター 放射線治療科 *2 M. Takahashi, H. Katagiri, T. Takagi, H. Murata 同 整形外科
〔索引用語：転移性脊椎腫瘍，再照射，強度変調放射線治療〕