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ORIGINAL ARTICLE

Expression of inhibitors of apoptosis family protein in 7,12-dimethylbenz[a]anthracene-induced hamster buccal-pouch squamous-cell carcinogenesis is associated with mutant p53 accumulation and epigenetic changes

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Summary

Fifty outbred Syrian golden hamsters were equally divided into three experimental groups and two control groups. The pouches of the experimental groups were painted bilaterally with a 0.5% 7,12-dimethylbenz[a]anthracene (DMBA) solution thrice a week for 3, 7 and 14 weeks. One of the control groups was applied with mineral oil while another control group remained untreated throughout the experiment. Neither survivin nor cIAP2 could be detected in any of the control tissues, whereas survivin and cIAP2 were found to be significantly increased in 3-, 7- and 14-week DMBA-treated pouches compared with the control pouches. Expression of XIAP, cIAP1 and NAIP were noted for both the control and 3-, 7- and 14-week DMBA-treated pouches, but levels were found to be significantly elevated in the experimental groups compared with the control pouches. p53 was not detected in any control tissues, but was significantly increased in 3-, 7- and 14-week DMBA-treated pouches. Direct sequencing revealed a point mutation (C → G) of *p53* for pouch tissues treated with DMBA for 3 and 7 weeks, and there was a wide variation in the *p53* sequence of the 14-week DMBA-treated pouch tissues, as compared with the control tissues. The control tissues had a *survivin*- and *cIAP2*-methylated allele, whereas the DMBA-treated tissues showed no evidence of *survivin*- and *cIAP2*-methylation. Neither the control nor DMBA-treated pouches showed evidence of *XIAP*-, *cIAP1*- or *NAIP*-methylation. Our results suggest that the expression of inhibitors of apoptosis family in DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis may be modulated by both genetic (mutant *p53*) and epigenetic mechanisms.

Keywords
cIAP1, cIAP2, DMBA carcinogenesis, hamster, IAP, NAIP, survivin, XIAP

Apoptosis, also termed programmed cell death, is a complex and highly regulated process, important for normal development and the functioning of diverse roles such as embryogenesis and tissue homeostasis, as well as tumourigenesis in multicellular organisms (Gupta 2002). Apoptosis is executed by a family of cysteine proteases known as caspases, which are produced in cells as inactive zymogens and become active proteases subsequent to proteolysis (Nicholson 1999). This caspase family of cysteine proteases performs an essential role in orchestrating the sequential breakdown of cells. The organism must tightly modulate the caspase cascade, commencing with the activation of an upstream or initiator caspase (such as caspases 8 and 9) and leading to the activation of a downstream or effector caspase (such as caspases 3, 6 and 7) (Cohen 1997).

The inhibitors of apoptosis (IAP) are a family of proteins functioning as intrinsic negative regulators of the aforementioned caspase cascade (Liston *et al.* 2003) and are the only known endogenous proteins that interfere with the activity of both initiator and effector caspases (Liston *et al.* 2003). Eight human IAP family members have been recognized so far: neuronal apoptosis inhibitory protein (NAIP), X-linked inhibitors of apoptosis protein (XIAP), cellular inhibitors of apoptosis protein 1 (cIAP1), cellular inhibitors of apoptosis protein 2 (cIAP2), survivin, baculoviral IAP repeat-containing ubiquitin-conjugating enzyme (BRUCE) (apollon), livin (ML-IAP, KIAP) and IAP-like protein 2 (ILP-2) (Liston *et al.* 2003). They are characterized by the presence of one or more 70–80 amino acids N-terminal domain called the *baculovirus* IAP repeat; some bind and potently inhibit activated caspases, including the effector caspases 3 and 7 and the initiator caspase 9 (Deveraux & Reed 1999). In addition, several IAPs also contain a carboxyl terminal really interesting new gene (RING) zinc-finger domain, which binds ubiquitin-conjugating enzymes that promote degradation of IAP caspase complexes (Yang *et al.* 2000). Moreover, a caspase recruitment domain (CARD), a conserved domain, has been found in cIAP1 and cIAP2, but the function of this domain in these two members is currently unknown (Salvesen & Duckett 2002). Some characteristics, as well as the number and distribution of these three kinds of domain amongst the IAP family members, are summarized in Table 1.

Hamster buccal-pouch mucosa provides one of the most widely accepted experimental models for oral carcinogenesis (Gimenez-Conti & Slaga 1993). Despite anatomical and histological differences between (hamster) pouch mucosa and human buccal tissue, experimental carcinogenesis protocols for the former induce premalignant changes and carcinomas that are similar to the development of premalignancy and malignancy in human oral mucosa (Morris 1961).

The *p53* tumour-suppressor gene is implicated in cell cycle checkpoint mechanisms, inhibiting cell cycle progression and inducing apoptosis in reaction to DNA damage (Gottlieb & Oren 1998). Given that an association of p53 expression and survivin, as well as XIAP, has been previously noted in hamster buccal-pouch carcinogenesis (Hsue *et al.* 2007), the relationship of other members of the IAP family and p53 is still an interesting area for study. On the other hand, epigenetic mechanisms such as DNA methylation have been implicated in cell proliferation, differentiation and genomic integrity. Although an association of survivin expression in hamster buccal-pouch carcinomas with epigenetic alteration has been reported in our laboratory recently (Chen *et al.* 2005a), the regulation of IAP family members other than survivin by an epigenetic mechanism is still a promising area in the investigation of oral carcinogenesis.

Consequently, as the expression of the IAP family, as well as its association with p53 and epigenetic alterations, in experimental oral carcinogenesis is not completely understood, this study was designed to investigate the protein and mRNA expression of five IAP family members (survivin, XIAP, cIAP1, cIAP2 and NAIP) in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch squamous-cell carcinogenesis. The relationship between the expression of the IAP family and p53 status, as well as epigenetic alterations in DMBA-induced hamster pouch squamous-cell carcinogenesis, was also examined.

Materials and methods

Animals and treatments

Outbred, young (6-week-old), male Syrian golden hamsters (*Mesocricatus auratus*; 50 animals, purchased from the National Science Council Animal Breeding Center, Taipei, China), weighing approximately 100 g at the beginning of the experiment, were randomly divided into three experimental and two control groups (10 animals per group). The animals were housed under constant conditions (22 °C, 12-h light/dark cycle) and supplied with tap water and standard Purina laboratory chow *ad libitum*. Appropriate animal care and an approved experimental protocol ensured humane treatment, and all procedures were conducted in accordance with the NIH *Guide for the Care and Use of Animals*. After allowing the animals 1 week of acclimatization to their new surroundings, both pouches from all animals in the experimental groups were painted with a 0.5% DMBA solution at 9 a.m. on Monday, Wednesday and Friday of each week, using a No. 4 sable-hair brush. Bilateral pouches from each animal of one control group were similarly treated with

mineral oil. Approximately 0.2 ml of the appropriate solution was applied topically to the medial walls of both pouches at each painting. The second control group of animals remained untreated throughout the experiment.

At the end of 3 weeks (3 days after the last treatment), all animals from one of the experimental groups were simultaneously killed by administration of a lethal dose of diethyl ether, at 9 a.m., to avoid any influence of diurnal variation (Lin & Chen 1997). Their pouches were exposed by dissection and examined grossly; both pouches were then excised and placed on cardboard to prevent distortion of the pouch tissues. After 7 weeks of treatment, the animals from one of the two remaining experimental groups were killed in a similar manner. Then, at 14 weeks, the animals from the last experimental group and those from the two control groups were killed, using the same procedure.

A portion of the pouch tissue was immediately frozen in liquid nitrogen for subsequent DNA/RNA extraction, RT-PCR reaction and methylation assay investigation, whilst another portion was routinely processed for light microscopy by being fixed in 10% neutral-buffered formalin solution for about 24 h, dehydrated in a series of ascending-concentration alcohol solutions, cleared in xylene, and embedded in paraffin for immunohistochemical staining.

Immunohistochemistry

Following tissue sectioning, staining was performed using a standard avidin-biotin peroxidase complex (ABC) method (Hsu *et al.* 1981). Antibodies for IAP proteins were obtained from Abchem Corporation, Cambridge, UK. Rabbit polyclonal antibodies against human, rat and mouse survivin (Cat. No. ab469), XIAP (Cat. No. ab21278), cIAP1 (Cat. No. ab2399), cIAP2 (Cat. No. ab23423) and NAIP (Cat. No. ab25968) were used and their specificity has been established in previous studies (Liston *et al.* 2001; Barnes *et al.* 2006; Hsue *et al.* 2008). Monoclonal antibody NCLp53-D07 (mAb DO7; Novocastra, Newcastle, UK) was used for the identification of p53 protein. The mAb DO7 antibody detects both wild-type and mutant forms of p53 (Vojtesek *et al.* 1992).

Tissue sections were mounted on gelatin–chrome alum-coated slides. Following deparaffinization in xylene (twice) and rehydration in a decreasing-concentration ethanol series (absolute, 95%, 70% and 30% ethanol, and then water), tissue sections were microwave-treated thrice (5 min each time) in a citrate buffer (10 mM; pH 6.0) to retrieve antigenicity. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 60 min. Prior to immunohistochemical staining, a 10% solution of normal rabbit serum was applied for 60 min to tissue sections to inhibit non-specific staining. These sections were subsequently incubated with antibodies against survivin, XIAP, cIAP1, cIAP2 and NAIP (1:100 each) overnight at 4 °C. A blocking solution of 2% low-fat milk powder in Tris-buffered saline (TBS, with 0.02% sodium azide) was applied to those sections to be stained for p53 protein. These sections were then treated with mAb DO7 at a dilution of 1:200 for 2 h at room temperature. Following subsequent rinsing with TBS (thrice, 10 min each), tissue sections stained for survivin, XIAP, cIAP1, cIAP2 and NAIP were then incubated for 60 min at room temperature with biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA, USA; 1:100). In contrast, the sections stained for p53 were treated with biotinylated anti-mouse IgG antibody (Vector; 1:100) for 30 min. Following this, all sections were again washed with TBS (thrice, 10 min each) and then incubated with an avidin–biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA, USA) for a further 60 min. After washing with PBS (thrice, 10 min each), peroxidase binding was visualized as brown reaction products via a benzidine reaction. The sections were then counterstained with Mayer's haematoxylin. Each set of experiments included a human buccal squamous-cell carcinoma specimen known to express survivin, XIAP, cIAP1, cIAP2, NAIP and p53, which served as a positive control and ensured the reproducibility of the staining process. The scores for positive staining were classified as: 0, <10%; 1, 10–24%; 2, 25–49%; 3, 50–74%; 4, 75–89%; 5, 90–100%. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

DNA/RNA isolation

Genomic DNA was extracted from each pouch specimen by proteinase K digestion and the phenol–chloroform extraction procedure, as described elsewhere (Sambrook & Russell 2001). Total RNA was extracted by homogenizing the pouch tissue specimens in guanidium isothiocyanate, followed by ultracentrifugation in caesium, as described previously (Chomczynski & Sacchi 1987).

Semi-quantitative reverse transcription-polymerase chain reaction

Isolated total RNA (1 μg) was reverse-transcribed to cDNA in a reaction mixture (with a final volume of 20 μl) containing $MgCl_2$ (4 μl; 5 mM), 10× reverse transcription buffer (2 μl; 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100), dNTP mixture (2 μl; 1 mM each), recombinant

RNasin® ribnonuclease inhibitor (0.5 μl; 1 μ/μl), avian myeloblastosis virus (AMV) reverse transcriptase (15 units; High Conc.; 15 μ/μg), and oligo(dT)15 primer (0.5 μg; catalogue no. A3500; Promega, Madison, WI, USA). The reaction mixture was incubated at 42 °C for 15 min. The AMV reverse transcriptase was inactivated by heating at 99 °C for 5 min and then incubating at 0–5 °C for a further 5 min.

Oligonucleotide primers were purchased from Genset Corp. (La Jolla, CA, USA). The primer pairs were chosen from the published cDNA sequences of survivin, XIAP, cIAP1, cIAP2, NAIP, p53 and β-actin (Table 2). All these chosen primers were applicable to rodent tissues; therefore, they were justified to be used to detect hamster sequences. The 20 μl first-strand cDNA synthesis reaction product obtained from the reverse transcriptase reaction was diluted to 100 μl with nuclease-free water. The PCR amplification reaction mixture (with a final volume of 100 μl) contained diluted, first-strand cDNA reaction product (20 μl; <10 ng/μl), cDNA reaction dNTPs (2 μl; 200 μM each), $MgCl_2$ (4 μl; 2 mM), 10× reverse transcription buffer (8 μl; 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100), upstream primer (50 pmol), downstream primer (50 pmol) and Taq DNA polymerase (2.5 units; Promega, Cat. No. M7660).

The PCR steps were carried out on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94 °C for 1 min (one cycle), then denaturing at 94 °C (1 min), annealing at 55 °C (1 min) for survivin and p53, at 59 °C (1 min) for XIAP, at 50 °C (1 min) for cIAP1 and cIAP2, at 56 °C (1 min) for NAIP, or at 60 °C (1 min) for β-actin, and extending at 72 °C (1 min) for 30 cycles, then a final extension at 72 °C for 7 min. The β-actin primers were utilized as positive controls. Negative controls, i.e. those conducted in the absence of RNA and reverse transcriptase, were also performed. Amplification products were analysed by electrophoresis in a 2% agarose gel along with the relevant DNA molecular-weight marker (Boehringer, Mannheim, Germany) and stained with ethidium bromide. Photographs were taken with a Polaroid DS-300 camera. The PCR products were visualized as bands with a UV transilluminator. Densitometric analysis of the PCR products was measured as the ratio of the expression of the five members of the IAP family and p53 with respect to the expression of β-actin, using the ONE-DSCAN I-D GEL ANALYSIS Software (Scanalytics, Inc., Rockville, MD, USA). The PCR products were then sequenced to confirm their identities using a T7 Sequenase kit (version 2.0; Amersham International, Little Chalfont, UK).

Polymerase chain reaction-based methylation assay

To investigate whether methylation of a CpG island of survivin, XIAP, cIAP1, cIAP2 and NAIP could be implicated in mRNA expression, a polymerase chain reaction-based methylation assay was used to analyse DNA samples obtained from the samples of hamster pouch mucosa. Genomic DNA was modified by bisulphite treatment, converting unmethylated cytosine to thymidine and leaving methylated cytosine unchanged (Herman *et al.* 1996). Approximately 10 μg of DNA with and without bisulphite treatment was digested with *Hin*dIII (1 μg/unit; Toyobo, Tokyo, Japan) at 37 °C for 12 h. The digested DNA was then precipitated with ethanol dissolved in distilled water. The DNA was further digested with mCpG-sensitive *Hpa* II (1 μg/unit; Toyobo) at 37 °C for 24 h. The non-digested DNA samples with and without bisulphite treatment, as well as the digested DNA samples with and without bisulphite treatment, were subsequently amplified with specific primers for survivin, XIAP, cIAP1, cIAP2 and NAIP (Table 2). The polymerase chain reactions were performed in a final volume of 25 μl, containing 1 μl of non-digested/digested DNA, 2.5 pmol of each specific primer, 50 μM of dNTPs, 10 mM Tris–HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.5 unit of AmpliTaq Gold (Applied Biosytstems, Foster City, CA, USA). Negative controls were also performed. The amplified PCR products were separated in a 3% agarose gel and visualized by ethidium bromide.

Results

Gross observation and histopathology

The gross and histopathological changes in the untreated, mineral oil-treated and DMBA-treated pouches were similar to those described in our previous study (Chen *et al.* 2005b). Upon gross examination, no apparent changes for any of the mineral oil-treated or untreated pouches were observed. Thickened mucosa, with a rough surface and of whitish granular appearance, were noted in the 3 and 7 weeks DMBA-treated pouches, with 100% tumour incidence in all samples after 14 weeks of DMBA treatment. No significant histological changes were observed for any of the mineral oil-treated or untreated pouches. However, hyperkeratosis was noted in the 3 weeks DMBA-treated pouches and areas of epithelial hyperplasia were observed in the 7 weeks DMBA-treated pouches. Furthermore, infiltrative squamous-cell carcinomas were detected in the 14-week DMBA-treated mucosa.

Table 1 Some characteristics of the eight human inhibitors of apoptosis (IAP) family members

IAP family members	Size (kDa)	Chromosome positions	Number of domains	Caspase specificity
XIAP (ILP1)	57	Xq25	BIR: 3 RING: 1	Caspase 3, 7, 9
cIAP1	70	11q22-q23	BIR: 3 RING: 1 CARD: 1	Caspase 3, 7, 9
cIAP2	68	11q22-q23	BIR: 3 RING: 1 CARD: 1	Caspase 3, 7, 9
LIVIN (ML-IAP)	31	20q13.3	BIR: 1 RING: 1	Caspase 3, 7, 9
ILP2#	?	19q 13.3-q13.4	BIR: 1 RING: 1	Caspase 9
NAIP*	156	5q13.1	BIR: 3 RING: 0	Caspase 3, 7
Survivin	16.5	17q25	BIR: 1 RING: 0	Caspase 3, 7
BRUCE	528	2p21-p22	BIR: 1 RING: 0	Caspase 3, 7

*The first characterized mammalian IAP.
#The most recently identified IAP (a tissue-specific homologue of XIAP).
BIR, baculovirus IAP repeat; RING, really interesting new gene; CARD, caspase recruitment domain; XIAP, X-linked inhibitors of apoptosis protein; cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2, cellular inhibitors of apoptosis protein 2; ILP2, IAP-like protein 2; NAIP, neuronal apoptosis inhibitory protein; . BRUCE, BIR repeat-containing ubiquitin-conjugating enzyme.

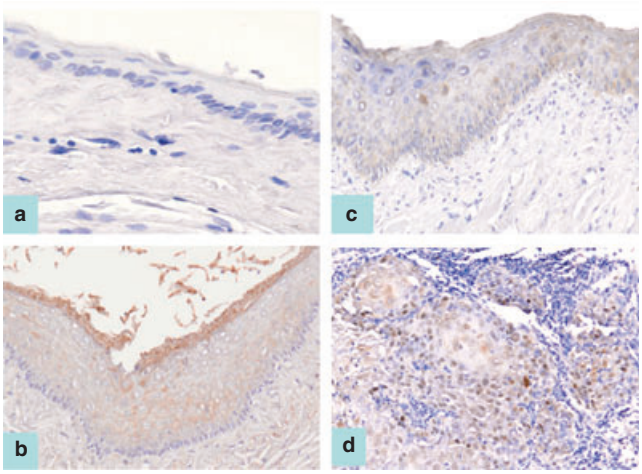

Figure 1 cIAP2 staining was not detected in untreated hamster pouch tissue specimen (a, ABC stain ×100). Cytoplasmic staining of cIAP2 proteins was detected in the whole epithelial layer (except the basal layer) of a representative 3-week 7,12-dimethylbenz[a]anthracene (DMBA)-treated pouch tissue sample (b, ABC stain ×200). Patchy cytoplasmic staining was detected in the whole layer of a representative specimen of 7-week DMBA-treated pouch tissue (c, ABC stain ×100). Cytoplasmic cIAP2 staining was shown in a representative specimen of hamster pouch tissue treated with DMBA for 14 weeks (d, ABC stain ×100). A similar staining pattern is also observed for survivin immunostaining.

Table 2 Oligoprimers 1 used for RT-PCR and oligoprimers 2 used for PCR-based methylation assay

Primers	Sequences	Product (bp)	Accession no.
cIAP1	Forward 1: 5-CAG CCT GAG CAG CTT GCA A-3′	354	BC016174
	Reverse 1: 5-GCC CAT TTC CAA GGC AGA T -3′	674	
	Forward 2: 5′ TTA TTT GTG GAT AAG AAT ATG AAG 3′		
	Reverse 2: 5′ AAA AAT CCT TAT TTT AAA ACA CAA 3′		
cIAP2	Forward 1: 5-TCC GTC AAG TTC AAG CCA GTT-3′	328	BC037420
	Reverse 1: 5-TCT TTT TCC TCA GTT GCT CTT TCT CT-3′	832	
	Forward 2: 5′ AGA AGA TGA AAT AAG GGA AGA GGA G 3′		
	Reverse 2: 5′ TTT TAC TTC ACT TAC AAT TTC AAT AAT 3′		
NAIP	Forward 1: 5-GCT TCA CAG CGC ATC GAA-3′	446	U80017
	Reverse 1: 5-ATG AGA GAC CCA AAA TCC GAA A -3′	650	
	Forward 2: 5′ AAA AAG AAA AGA AAA GAA AAG AAA AAT 3′		
	Reverse 2: 5′ TCT ACT AAT TCT TTT TTT TCT TTT TT 3′		
XIAP	Forward 1: 5-AGT GGT AGT CCT GTT TCA GCA TCA-3′	360	U45880
	Reverse 1: 5′-GTT CCT CGG GTA TAT GGT GTC TGA-3′	884	
	Forward 2: 5′ TTT AGG TGA AGG TGA TAA AGT AAA GTG 3′		
	Reverse 2: 5′ TTC ACA TCA CAC ATT CAA TCA 3′		
Survivin	Forward 1: 5-AGA ACT GGC CCT TCT TG GA-3′	200	BC016174
	Reverse 1: 5-AAG GAA AGC GCA ACC GGA CG-3′	584	
	Forward 2: 5′ AAT AAG AAG AAA GAA TTT GAG GAA A 3′		
	Reverse 2: 5′ AAA AAA ACA CAA CAA CAA AAA AAC T 3′		
β-actin	Forward 1: 5-AGA ACT GGC CCT TCT TGG A-3′	350	X-00351
	Reverse 1: 5-AAG GAA AGC GCA ACC GGA CG-3′		

cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2, cellular inhibitors of apoptosis protein 2; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein.

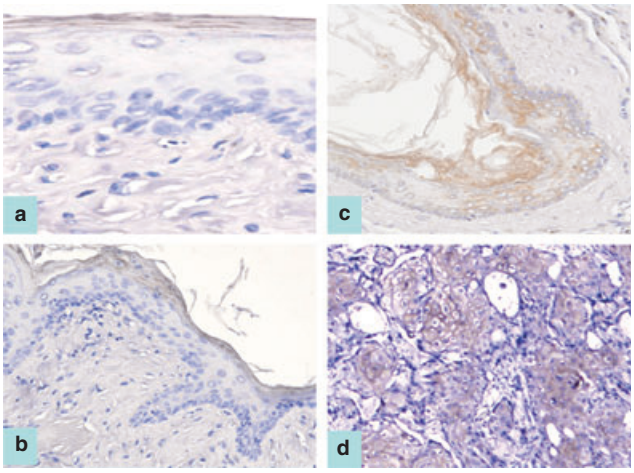

Figure 2 cIAP1 staining was located in the outermost keratinized layer of an untreated pouch tissue specimen (a, ABC stain ×200), as well as a representative 3-week 7,12-dimethylbenz[a]anthracene (DMBA)-treated pouch tissue (b, ABC stain ×100). Patchy cytoplasmic staining was detected in the whole layer of a representative specimen of 7-week DMBA-treated pouch tissue (c, ABC stain ×100). A representative specimen of pouch tissue treated with DMBA for 14 weeks demonstrated cytoplasmic cIAP1 staining (d) (ABC stain ×100). A similar immunostaining pattern is also observed for XIAP and NAIP proteins.

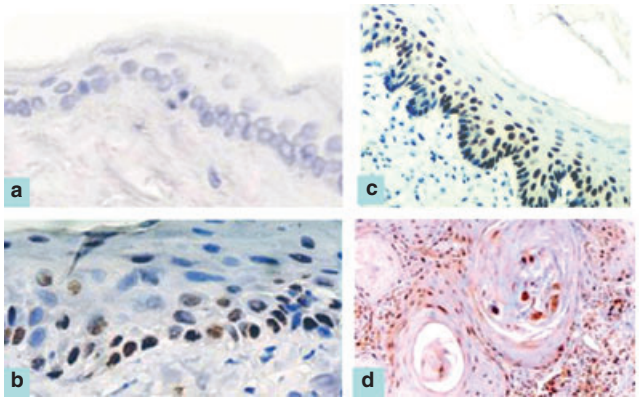

Figure 3 Negative p53 staining was observed for an untreated pouch tissue specimen (a, ABC stain ×100). Nuclear staining of p53 was noted in representative samples from 3-week (b, ABC stain ×200), 7-week (c, ABC stain ×100) and 14-week (d, ABC stain ×100) 7,12-dimethylbenz[a]anthracene-treated pouch tissue.

Table 3 Scores for immunohistochemical expression of the five members of the inhibitors of apoptosis family and p53 proteins for 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch squamous-cell carcinogenesis

	cIAP1	cIAP2	NAIP	XIAP	Survivin	p53
Untreated	1	0	1	1	0	0
Mineral oil-treated	1	0	1	1	0	0
3-week DMBA-treated	*2	*4	*3	*4	*3	*3
7-week DMBA-treated	*4	*4	*3	*4	*4	*3
14-week DMBA-treated	*4	*4	*3	*5	*4	*4

Staining scores: 0, <10%; 1, 10–25%; 2, 25–50%; 3, 50–75%; 4, 75–90%; 5, 90–100%.
*Statistical significance as compared with untreated or mineral oil-treated tissues ($P < 0.0001$, chi-square test).
cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2, cellular inhibitors of apoptosis protein 2; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein.

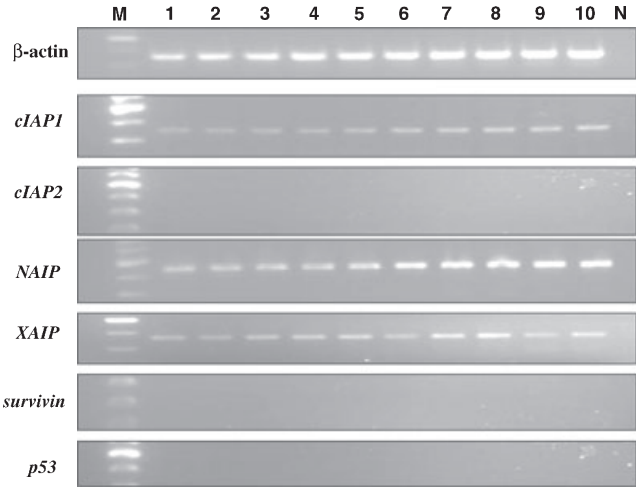

Figure 4 Upon RT-PCR, bands corresponding to mRNA of *cIAP1* (354 bp), *cIAP2* (328 bp), *NAIP* (446 bp), *XIAP* (360 bp), *survivin* (200 bp) and *p53* (370 bp) were evident in all of the hamster buccal-pouch tissue specimens treated with 7,12-dimethylbenz[a]anthracene (DMBA) for 14 weeks (lanes 1–10). All samples (lanes 1–10), except the negative control samples (lane N), revealed bands of β-actin (350 bp). Similar results were observed for hamster pouch tissue treated with DMBA for 3 and 7 weeks. Lane M: molecular weight markers.

Immunohistochemistry

The pattern of immunohistochemical staining for the five members of the IAP family in our study can be classified into two categories: (1) survivin and cIAP2; (2) XIAP, cIAP1 and NAIP.

Survivin and cIAP2

Neither survivin nor cIAP2 staining could be found in any of the untreated or mineral oil-treated hamster pouch tissue

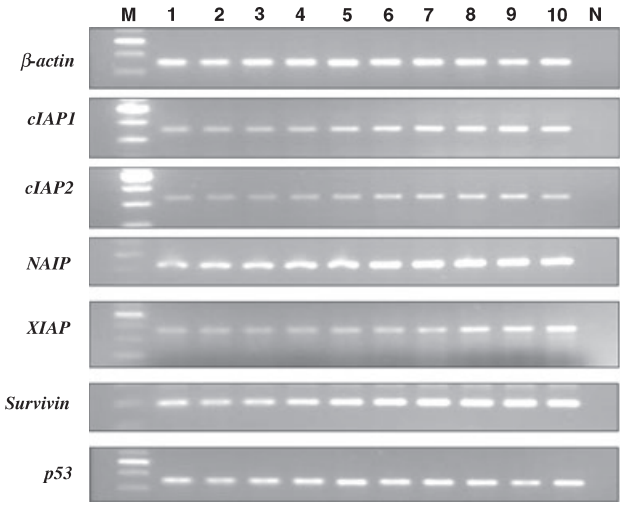

Figure 5 For all of the untreated samples (lanes 1–10), upon RT-PCR, bands corresponding to mRNA of *cIAP1* (354 bp), *NAIP* (446 bp) and *XIAP* (360 bp) were detectable, whereas no bands for *cIAP2*, *survivin* and *p53* were detected. All samples (lanes 1–10), except the negative control samples (lane N), revealed bands of β-actin (350 bp). Similar results were observed for mineral oil-treated pouch tissues. Lane M: molecular weight markers.

specimens (Figure 1a). Cytoplasmic staining of survivin or cIAP2 proteins was detected in the entire epithelial layer (except the basal layer) in all hamster pouch tissues treated with DMBA for 3 weeks (Figure 1b). In addition, cytoplasmic staining of survivin or cIAP2 protein was observed in all specimens of hamster buccal-pouch tissue treated with DMBA for 7 weeks (patchy staining; Figure 1c) and 14 weeks (Figure 1d). No obvious variation in the staining intensity was found for all hamster pouch tissues treated with DMBA for 3, 7 and 14 weeks respectively.

XIAP, cIAP2 and NAIP

XIAP, cIAP1 or NAIP staining was located in the outermost keratinized layer of the untreated, mineral oil-treated and 3-week DMBA-treated pouch tissues (Figure 2a,b); however, the possibility of 'edge-effect' artefact should also be alerted. Cytoplasmic staining of XIAP, cIAP1 or NAIP proteins was found to extend downwards and was detected in the whole epithelial layer in all specimens of hamster buccal-pouch tissue treated with DMBA for 7 weeks (patchy staining; Figure 2c). Similar cytoplasmic staining of XIAP, cIAP1 or NAIP proteins was observed for all specimens of pouch tissue treated with DMBA for 14 weeks (Figure 2d). Significant deviation in the staining intensity was not being noted for all the control and experimental tissues.

p53

No p53 staining could be detected in any of the untreated or mineral oil-treated pouch tissue specimens (Figure 3a), whereas nuclear staining of p53 was noted for all pouch tissues treated with DMBA for 3 (Figure 3b), 7 (Figure 3c) and 14 weeks (Figure 3d). Apparent difference in the

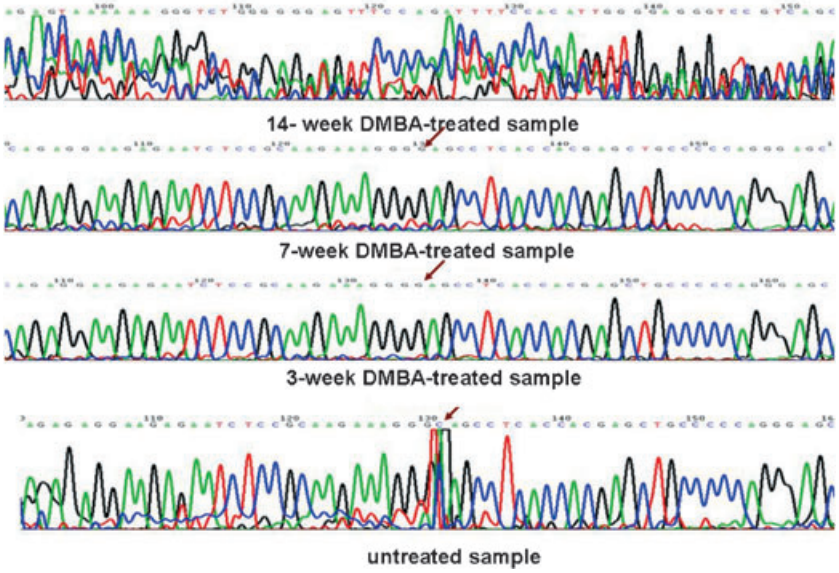

Figure 6 Upon direct sequencing of the PCR product of *p53* (370 bp), a point mutation of C to G was noted for the hamster buccal-pouch tissue specimens treated with 7,12-dimethylbenz[a]anthracene (DMBA) for 3 and 7 weeks, as compared with the untreated tissue specimens (arrow). A great variation of the sequence of *p53* for hamster buccal-pouch tissue specimens treated with DMBA for 14 weeks as compared with the untreated pouch tissue specimens was found.

Table 4 Mean mRNA expression of the five inhibitors of apoptosis family members and p53 for 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch squamous-cell carcinogenesis

	Untreated	Mineral oil-treated	3-week DMBA-treated*	7-week DMBA-treated*	14-week DMBA-treated*
cIAP1	0.1108 ± 0.0365	0.2008 ± 0.0640	0.1518 ± 0.0602	0.3590 ± 0.0951	0.6313 ± 0.1166
cIAP2	0	0	0.1293 ± 0.0325	0.2124 ± 0.0670	0.2759 ± 0.0887
NAIP	0.4264 ± 0.2456	0.4935 ± 0.0599	0.7286 ± 0.2263	0.9867 ± 0.2868	1.9495 ± 0.4451
XIAP	0.1294 ± 0.0376	0.3042 ± 0.1266	4.3607 ± 0.5956	4.6138 ± 0.5818	5.3556 ± 0.9270
Survivin	0	0	1.3858 ± 0.5409	2.3059 ± 0.3641	1.1561 ± 0.2815
p53	0	0	0.1227 ± 0.0382	1.0079 ± 0.2870	1.9559 ± 0.3381

*Statistical significance as compared with untreated or mineral oil-treated tissues with the exception for comparison of the average mRNA expression in mineral oil-treated tissues for cIAP1 with that for the 3 weeks DMBA-treated tissues ($P < 0.0001$, one-way ANOVA).
cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2, cellular inhibitors of apoptosis protein 2; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein.

Table 5 Correlation of the mean mRNA expression of the five inhibitors of apoptosis family members for 7,12-dimethylbenz[a]anthracene-induced hamster buccal-pouch squamous-cell carcinogenesis

Variables	By variables	Mean ± standard deviation	Correlation coefficients (r)*
cIAP1		0.2871 ± 0.2004	
	NAIP	0.9168 ± 0.6198	0.8339
	cIAP2	0.1235 ± 0.1227	0.7918
	XIAP	2.9527 ± 2.3436	0.6596
	survivin	0.9696 ± 0.9398	0.4399

*$P < 0.0001$, linear regression analysis.
cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2, cellular inhibitors of apoptosis protein 2; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein.

Table 6 Correlation of mean mRNA expression of the five inhibitors of apoptosis family members with p53 for 7,12-dimethylbenz[a]anthracene-induced hamster buccal-pouch squamous-cell carcinogenesis (linear regression analysis)

Variable	By variables	Mean ± standard deviation	Correlation coefficients (r)*
p53		0.6173 ± 0.7992	
	NAIP	0.9168 ± 0.6198	0.9310
	cIAP1	0.2871 ± 0.2004	0.8949
	cIAP2	0.1235 ± 0.1227	0.8402
	XIAP	2.9527 ± 2.3436	0.7183
	survivin	0.9696 ± 0.9398	0.4847

cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2, cellular inhibitors of apoptosis protein 2; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein.
*$P < 0.0001$, linear regression analysis.

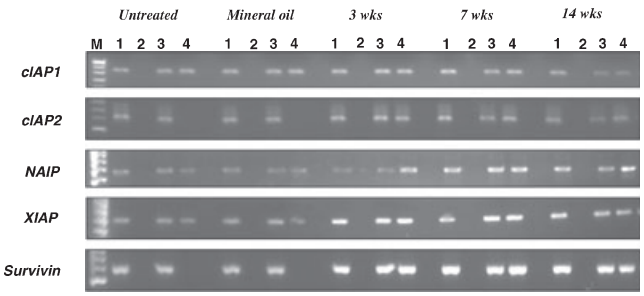

Figure 7 Representative results of RT-PCR-based methylation assay of *cIAP1*, *cIAP2*, *NAIP*, *XIAP* and *survivin* genes for 3-, 7- and 14-week 7,12-dimethylbenz[a]anthracene-treated, untreated and mineral-oil treated hamster buccal-pouch tissues. Lane 1: PCR products for non-digested DNA samples without C → T conversion; lane 2: PCR products for digested DNA samples without C → T conversion; lane 3: PCR products for non-digested DNA samples with C → T conversion; lane 4: PCR products for digested DNA samples with C → T conversion; lane M: molecular weight markers.

staining intensity was not found for all pouch tissues treated with DMBA for 3, 7 and 14 weeks.

Statistical analyses

The scores based on percentage of positive stained cells (not staining intensity) of the five IAP family members and p53 for DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis are summarized in Table 3. Upon statistical analysis, the expression of these five IAP family members and p53 for 3-, 7- and 14-week DMBA-treated hamster pouch tissues was significantly increased compared with the expression of untreated and mineral oil-treated pouch tissues ($P < 0.0001$, chi-square test, Table 3).

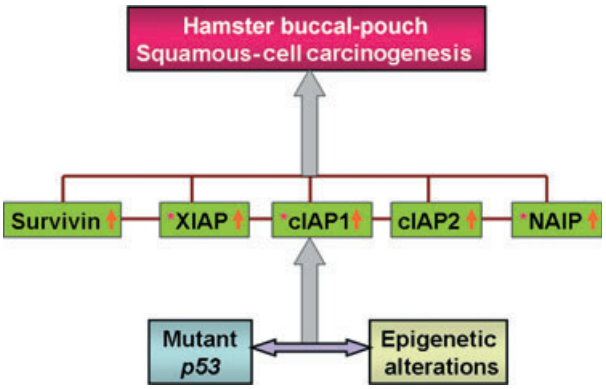

Figure 8 Hypothesis of the genetic and epigenetic regulation of inhibitors of apoptosis (IAP) family members for 7,12-dimethylbenz[a]anthracene-induced hamster buccal-pouch squamous-cell carcinogenesis (brown line: interactions amongst the IAP family members remain unclear; purple double-head horizontal arrow: exact interrelationship between mutant p53 mechanism and epigenetic alteration is still uncertain; grey single-head vertical arrow: induction; orange single-head vertical arrow: over-expression of IAP members; brown asterisk: possibly partially regulated by epigenetic changes).

Semi-quantitative reverse transcription-polymerase chain reaction

Survivin and cIAP2. Upon RT-PCR, *survivin* mRNA and *cIAP2* mRNA were evident as a band corresponding to a 200 and 328 bp PCR product, respectively, in all of the hamster buccal-pouch tissue specimens treated with DMBA for 3, 7 and 14 weeks (Figure 4), whereas no *survivin* mRNA and *cIAP2* mRNA was detected in the samples of the untreated, the mineral oil-treated or the negative control animal tissue (Figure 5). All samples, except the negative control samples, revealed bands of β-actin (350 bp) (Figures 4 and 5).

XIAP, cIAP1 and NAIP. Upon RT-PCR, mRNA for *XIAP*, *cIAP1* and *NAIP* was detectable as a band corresponding to a 360, 354 and 446 bp PCR product, respectively, in all of the 3-, 7- and 14-week DMBA-treated hamster buccal-pouch tissue specimens (Figure 4). Messenger RNA for *XIAP*, *cIAP1* and *NAIP* was also detected in the tissue derived from the untreated, mineral oil-treated tissues and negative control animals (Figure 5). All samples, except the negative control samples, revealed bands of β-actin (350 bp) (Figures 4 and 5).

p53. Messenger RNA for *p53* corresponding to a band of 370 bp was observed for all the hamster buccal-pouch tissue specimens treated with DMBA for 3, 7 and 14 weeks (Figure 4). No such bands were noticed for the tissue samples of untreated, mineral oil-treated tissues and the negative-control animals (Figure 5). All samples, except the negative control samples, revealed bands of β-actin (350 bp) (Figures 4 and 5).

On the other hand, upon direct sequencing of this 370 bp band, a point mutation of C to G was noted for the hamster buccal-pouch tissue specimens treated with DMBA for 3 and 7 weeks, as compared with the untreated tissue specimens (Figure 6). Furthermore, there was a great variation for the sequence of the hamster buccal-pouch tissue specimens treated with DMBA for 14 weeks as compared with the untreated tissue specimens (Figure 6).

Statistical analyses. The mean RNA expression of these five members of the IAP family and p53 for DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis is summarized in Table 4. It was noted that the average mRNA expression in mineral oil-treated control tissues for cIAP1 is higher than that for the 3-week DMBA-treated experimental tissues. Also, the mean mRNA expression of survivin for the 14-week DMBA-treated tissues was lower than that for both the 3- and 7-week DMBA-treated tissues. It might be due to the fact that a wide range of variation was noted within these data categories (cIAP1, 3-week DMBA treatment; survivin, 14-week DMBA treatment). Upon statistical analysis, the mean RNA expression of these five members of the IAP family and p53 was significantly elevated for 3-, 7- and 14-week DMBA-treated pouch tissues compared with the untreated and mineral oil-treated tissues ($P < 0.0001$, one-way ANOVA, Table 4) with the exception for comparison of the average mRNA expression in mineral oil-treated tissues for cIAP1 with that for the 3-week DMBA-treated tissues.

On the other hand, the mean RNA expression of these five members of the IAP family for DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis was correlated to each other (linear regression analysis, Table 5); furthermore, it was significantly correlated to that of p53, with a decreasing order of correlation of NAIP, XIAP, cIAP2, cIAP1 and survivin (linear regression analysis, Table 6).

Polymerase chain reaction-based methylation assay

Survivin and cIAP2. The methylation status of the *survivin* and *cIAP2* genes in all of the 3-, 7- and 14-week DMBA-treated, untreated and mineral oil-treated hamster buccal-pouch tissues was investigated. For the untreated and mineral oil-treated tissues, non-digested DNA samples with and without bisulphite conversion showed PCR products of 584 bp (*survivin*) and 832 bp (*cIAP2*), respectively, whereas

digested DNA samples with and without bisulphite treatment did not show any PCR products.

On the other hand, for the 3-, 7- and 14-week DMBA-treated hamster buccal-pouch tissues, non-digested DNA samples without bisulphite treatment, as well as the digested DNA samples with and without bisulphite treatment, showed a PCR product of 584 bp (*survivin*) and 832 bp (*cIAP2*), respectively, while non-digested DNA samples with bisulphite treatment did not show a PCR product. The representative results of the RT-PCR-based methylation assay are shown in Figure 7.

XIAP, cIAP1 and NAIP. The methylation status of *XIAP, cIAP1* and *NAIP* genes in all of the 3-, 7- and 14-week DMBA-treated, untreated and mineral oil-treated hamster buccal-pouch tissues was investigated. For the untreated and mineral oil-treated tissues, non-digested DNA samples with and without bisulphite treatment, as well as digested DNA samples with bisulphite treatment, showed a PCR product for *XIAP* (884 bp), *cIAP1* (674 bp) and *NAIP* (650 bp), respectively, whereas non-digested DNA samples without bisulphite treatment showed no evidence of a PCR product. Similar results were obtained for all the 3-, 7- and 14-week DMBA-treated hamster buccal-pouch tissues. The representative results of the RT-PCR-based methylation assay are shown in Figure 7.

Discussion

The implication of IAP-mediated inhibition of apoptosis in some cancers has been hypothesized (LaCasse *et al.* 1998), and the association of IAP family members, chiefly survivin, with human oral squamous-cell carcinomas has also been studied (Tanimotoa *et al.* 2005; Jane *et al.* 2006). However, the relationship of the IAP family with chemically induced oral experimental carcinogenesis has rarely been studied. Only two IAP family members, namely survivin and XIAP proteins, have been reported to be overexpressed in DMBA-induced hamster buccal-pouch carcinogenesis recently in our laboratory (Chen *et al.* 2005a; Hsue *et al.* 2008). In the present study, besides further analysing the mRNA expression of survivin and XIAP, we also demonstrate the protein and mRNA expression of three other IAP family members (cIAP1, cIAP2 and NAIP) for DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis. This is, to the best of our knowledge, the first study to document the sequential expression (both protein and mRNA) of these five IAP family members (survivin, XIAP, cIAP1, cIAP2 and NAIP) in chemically induced oral experimental carcinogenesis. Our results indicate that expression of these five IAP family members (both protein and mRNA) is an early event in DMBA-induced pouch carcinogenesis. These findings are consistent with the results for human oral carcinogenesis (Tanimotoa *et al.* 2005; Jane *et al.* 2006). Thus, the present study further indicates that the IAP family may participate in oral carcinogenesis.

The mechanisms of IAP overexpression in cancerous tissues are largely uncertain. As all five IAP family members (both protein and mRNA) have been found to be significantly overexpressed in the present study, a synergistic effect amongst IAP family members, and therefore a probable common regulatory mechanism, might be supposed. Amplification of the *survivin* locus on chromosome 17 and DNA demethylation of its promoter region have been regarded as potential mechanisms of survivin overexpression in some cancers (Altieri 2001), including human oral cancer and chemically induced hamster buccal-pouch carcinoma (Tanaka *et al.* 2003; Chen *et al.* 2005a). In the present study, we have demonstrated that the control tissues (untreated and mineral oil-treated) had a *survivin*- and *cIAP2*-methylated allele, whilst the 3-, 7- and 14-week DMBA-treated tissues showed no evidence of *survivin*- and *cIAP2*-methylation. Also, neither the control nor the DMBA-treated pouches showed evidence of *XIAP*-, *cIAP1*- and *NAIP*-methylation. Therefore, these results indicate that overexpression of survivin and cIAP2 in DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis may be regulated by an epigenetic mechanism whereas overexpression of XIAP, cIAP1 and NAIP may also (possibly partially) modulated by the same mechanism.

On the other hand, p53 loss or mutation might result in the upregulation of survivin expression (Hoffman *et al.* 2002; Mirza *et al.* 2002), but the relevance of p53 regulation to other IAP family members has not yet been elucidated. In the present study, we demonstrate that the overexpression of these five IAP family members (both protein and mRNA) is associated with *p53* mutation for DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis.

Mirza *et al.* (2002) and Hoffman *et al.* (2002) indicated that accumulation of wild-type p53 suppresses p53 promoter activity, resulting in the downregulation of survivin expression, and therefore hypothesize a negative feedback loop between survivin expression and wild-type p53. Based on the findings of the present study, overexpression of the IAP family in DMBA-induced hamster pouch squamous-cell carcinogenesis might be regulated by both genetic (mutant *p53*) and epigenetic mechanisms. Following gradual increasing concentration of DMBA, the induced mutant p53 in pouch keratinocytes could produce failure of the negative feedback loop originally present between IAP and wild-type p53.

Consequently, the IAP family expression would become uncontrolled and hence become upregulated, a state for developing carcinoma tissues. Given that genetics and epigenetics are complementary in the field of cancer aetiology (Feinberg 2004), in the future, further study on the interaction between *p53* gene expression and epigenetic alterations should be performed to evaluate the interrelationship of *p53* gene mutation and epigenetic alteration in the IAP family. If an exact mechanism for the IAP family is understood, it would be a useful diagnostic marker of cancer and a potential target for the treatment of oral cancer.

In the present study, we found that the mRNA expression of these five IAP family members is correlated to each other; however, to date, the exact pathway of interaction amongst IAP family members is not yet been completely understood. A possible interaction between IAP family members has been reported; survivin was demonstrated to bind second mitochondria-derived activator of caspase (Smac), relieving its inhibition of XIAP, and then allowing XIAP to function (Song *et al.* 2003). Further exploration of the interaction pathways amongst IAP family members for DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis is essential in order to elucidate the association between coexpression of IAP family members and oral experimental carcinogenesis. Moreover, the finding of overexpression of multiple IAP family members in our study raises the possibility that effective strategies will require IAP antagonists that are able to suppress multiple members of the IAP family.

In conclusion, our findings suggest that the IAP family could play a pivotal role in DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis and what is, to the best of our knowledge, the first demonstration that their expression may be regulated by both genetic (mutant *p53*) and epigenetic mechanisms in this experimental model system for oral carcinogenesis, although their interrelationship and the exact interactions amongst IAP family members remain to be elucidated (Figure 8).

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