EXPRESSION OF INHIBITOR OF APOPTOSIS FAMILY PROTEINS IN HUMAN ORAL SQUAMOUS CELL CARCINOGENESIS

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Accepted 9 July 2010
Published online 21 October 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/hed.21582

Abstract: *Background.* The purpose of this study was to determine inhibitor of apoptosis (IAP) expression, its relationship with *p53*, and epigenetic change in oral carcinogenesis that remain to be elucidated.

Methods. We measured IAP and *p53* expression in 44 oral potentially malignant disorders and their corresponding malignant-transformed oral squamous cell carcinomas (OSCCs), and in 44 other non-transformed oral potentially malignant disorders. IAP and *p53* expression in 10 fresh OSCCs, together with epigenetic change of their mutation, were also determined.

Results. Normal mucosa did not express IAP/mutated *p53*. Oral potentially malignant disorders that underwent transformation exhibited high IAPs (>90%) and less-consistent mutated-*p53* (34%) expression, whereas transformed OSCCs exhibited high IAP and mutated-*p53* expression. Fresh OSCCs exhibited 80% to 100% IAP mRNA expression and 50% protein, mRNA, and *p53* mutation expression. Normal tissues revealed DNA methylation of IAP, whereas cancerous tissues overexpressing IAP exhibited hypomethylation.

Conclusion. This study showed that IAP expression is an early event in oral carcinogenesis and that epigenetic and genetic pathways are associated with IAP expression in OSCC.
 Head Neck **33:** 985–998, 2011

Keywords: inhibitors of apoptosis; oral carcinoma; oral potentially malignant disorder; *p53*; epigenetic; hypomethylation

Apoptosis, or programmed cell death, is a controlled process of cellular disassembly that occurs in response to internal or external apoptotic signals. It is an intricate and greatly synchronized process essential for normal development and has miscellaneous functional roles such as in embryogenesis, tissue homeostasis, and tumorigenesis in multicellular organisms.[1] Apoptosis is regulated by a cascade of cysteine proteases called caspases, which are formed in cells as inactive zymogens and transform to active proteases after proteolysis.[2] Organisms have to firmly modulate and tightly control the caspase cascade, which begins with the activation of an initiator caspase (such as caspases 8 and 9) followed by the activation of an effector caspase (such as caspases 3, 6, and 7).[3]

The inhibitors of apoptosis (IAP) are a family of proteins that act as intrinsic negative regulators of the above-mentioned caspase cascade and are the only identified endogenous proteins that interfere with the activity of both initiator and effector caspases.[4] To date, to our knowledge, 8 human IAP family members have been reported: 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, apollon, livin (ML-IAP, KIAP), and IAP-like protein 2.[4] These IAPs are characterized by the presence of one or more 70 to 80 amino acid N-terminal domains, designated as the baculovirus IAP repeat; some bind and suppress activated caspases, including effector caspases 3 and 7 and initiator caspase 9.[5]

The *p53* tumor-suppressor gene participates in cell cycle checkpoint mechanisms, suppressing cell-cycle progression, and executing apoptosis in response to DNA damage.[6] Regarding the 2 common animal models for chemically induced oral cancer, the 7,12-dimethylbenz[a]anthracene-induced hamster buccal-pouch cancer model (HBPCM) and the 4-nitroquinoline-1-oxide-induced rat oral cavity cancer model,[7] an association between p53 expression and IAPs has been found only in the former,[8] and not in the rat oral cavity cancer model, although IHC expression of survivin has been noted recently during 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis.[9] Therefore, the relationship between the contributions

Correspondence to: L.-M. Lin
Contract grant sponsor: This research was supported by a grant from the National Science Council, ROC; contract grant number: NSC 95-2314-B-037-071.
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of the IAP family and p53 in human oral squamous cell carcinogenesis remains an attractive area for study.

The study of epigenetics has exerted a large impact on our understanding of oral cancer biology. Epigenetics is defined as the stable heritable changes in gene expression without alteration in DNA sequences.[10] One form of epigenetic modification is known as DNA methylation, in which hypomethylation leading to oncogene activation occurs, whereas hypermethylation causes tumor suppressor gene silencing.[11] Hypomethylation of IAP genes in HBPCM has recently been demonstrated,[12,13] but, nevertheless, the regulation of IAP family members by an epigenetic mechanism is still a promising area for investigation in human oral squamous cell carcinogenesis.

As the expression of the IAP family, and its relationship with *p53* and epigenetic change, in human oral carcinogenesis remains to be elucidated, this study intended to examine the protein and mRNA expression of IAPs and *p53* in oral potentially malignant disorders and squamous cell carcinomas (SCCs) of the human oral mucosa. The association of expression of the IAP family members with epigenetic change and mutant *p53* in human oral SCCs (OSCCs) was also investigated. Due to the relative paucity of commercial antibodies and published cDNA sequences for baculoviral IAP repeat-containing ubiquitin-conjugating enzyme, livin, and IAP-like protein 2, only 5 of 8 IAP family members (namely: survivin, XIAP, cIAP1, cIAP2, and NAIP) were included in this study.

MATERIALS AND METHODS

Source of Tissues. The tissue specimens used in this study were obtained from patients who had visited our institution between 1991 and 2001. This study was approved by the institutional review board of the Ethics Committee for Scientific Research on Human Beings at our institution. Categories of oral potentially malignant disorders used for the present study was based on the classification of the World Health Organization in 2005.[14] All cases of OSCCs in this study were classified according to the primary site as described in the International Classification of Diseases (ICD 140–145) for Oncology (World Health Organization, 1988).[15]

Forty-four cases (3.02%) within a cohort of 1458 patients (average age, 47.5 years; range, 17–86 years) of various oral potentially malignant disorders of the human oral mucosa with malignant transformation to OSCCs were identified, with a mean follow-up time of 42.64 months. Strict selection criteria were followed as described in a previous study.[16] In brief, 2 criteria must be fulfilled to diagnose progression to oral cancer: the first is that the potential oral cancer must develop at the same site as the initial lesion; the second is that the time taken for this progression must exceed 6 months. Histologic diagnoses of these 44 human oral potentially malignant disorders included epithelial hyperplasia/hyperkeratosis ($n = 15$); submucous fibrosis ($n = 8$); epithelial dysplasia with submucous fibrosis ($n = 2$); epithelial dysplasia with hyperkeratosis/epithelial hyperplasia ($n = 6$); verrucous hyperplasia ($n = 10$), and lichen planus ($n = 3$). In addition, 44 other age-matched (average age, 46.5 years; range, 19–80 years) cases of oral potentially malignant disorders that had not undergone malignant transformation during the same observation period were also included. All patients, with the exception of those with lichen planus, had been exposed to risk factors such as betel-quid chewing, cigarette smoking, or alcohol consumption. There were no signs of dysplasia in the lichen planus lesions. Five samples of paraffin-embedded human normal oral mucosa tissue from healthy individuals who did not chew betel-quid, drink alcohol, or smoke cigarettes were used as controls. Serial 4-μm-thick sections of the 44 potentially malignant disorder samples and the corresponding malignantly transformed SCC specimens, plus sections of the 44 cases of oral potentially malignant disorders that had not undergone malignant transformation, and those of the control patients, were prepared for immunohistochemical (IHC) analysis.

Owing to restrictions specified by the institutional review board, no fresh tissues of oral potentially malignant disorders could be included in this study. Ten other specimens of OSCC (4 buccal; 5 tongue; 1 gingiva) were obtained from fresh tissue samples of surgically resected tumors from 9 male patients and 1 female patient aged between 22 and 70 years (mean, 48.8 years old). None of the patients had received any preoperative radiotherapy or chemotherapy, and all 10 patients had been exposed to risk factors such as betel-quid chewing, cigarette smoking, and alcohol consumption. Most identified malignancies were well differentiated, with the exception of one that was deemed to be moderately differentiated. Disease stage was defined as stage I to IV (stage IV, 6 patients; stages II and III, 2 patients each) in accordance with the TNM classification.[17] A portion of the surgically resected oral tissue was immediately frozen in liquid nitrogen for subsequent DNA/RNA extraction and investigation by reverse transcription-polymerase chain reaction (RT-PCR), mutation analysis for *p53* gene, and methylation assay. Another portion was fixed in 10% neutral buffered formalin solution for histologic diagnosis and subsequent IHC analysis.

Five fresh samples of human normal oral mucosa tissue taken from sex-matched and age-matched healthy individuals aged 18 to 82 years (mean, 30.4 years old) were used as controls; none of these control patients had ever chewed betel-quid, drank alcohol, or smoked cigarettes. Portions of normal oral tissue samples were immediately frozen in liquid nitrogen for subsequent DNA/RNA extraction, mutation analysis for the *p53* gene, RT-PCR investigation and

methylation assay; another portion of each sample was fixed in 10% neutral buffered formalin solution for IHC analysis.

Semiquantitative Immunohistochemistry. After tissue sectioning, staining was performed using a standard avidin-biotin-peroxidase complex method.[18] Antibodies for the detection of IAP proteins were obtained from Abcam Corporation (Cambridge, United Kingdom). Rabbit polyclonal antibodies against human, rat, and mouse survivin (catalog number ab469), XIAP (catalog number ab21278), cIAP1 (catalog number ab2399), cIAP2 (catalog number ab23423), NAIP (catalog number ab25968), and wild-type p53 (catalog number ab4060) were used, the specificities of which have been established in previous studies.[4,19–22] Monoclonal antibody NCLp53-D07 (mAb-DO7; Novocastra, Newcastle, United Kingdom) was used for the identification of p53 protein. The mAb-DO7 antibody detects both wild-type and mutant forms of p53.[23]

Tissue sections were mounted on gelatin-chrome alum-coated slides. After 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 3 times (5 minutes 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 minutes. Before IHC staining, a 10% solution of normal rabbit serum was applied for 60 minutes to tissue sections to inhibit nonspecific staining. The sections were subsequently incubated with antibodies against *survivin*, XIAP, cIAP1, cIAP2, NAIP, and wild-type p53 (catalog number ab4060; all antibodies were used at a dilution of 1:100) 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 using mAb-DO7. The sections were then treated with mAb-DO7 at a dilution of 1:200 for 2 hours at room temperature. After subsequent rinsing with TBS (3 times, 10 minutes each), tissue sections were stained for survivin, XIAP, cIAP1, cIAP2, NAIP, and wild-type p53 (catalog number ab4060) were then incubated for 60 minutes at room temperature with biotin-conjugated goat anti-rabbit immunoglobulin G (Vector Labs, Burlingame, CA; 1:100). In contrast, the sections stained for p53 using mAb-DO7 were treated with biotinylated anti-mouse immunoglobulin G antibody (Vector Labs; 1:100) for 30 minutes. After these procedures, all sections were again washed with TBS (3 times, 10 minutes each) and then incubated with an avidin-biotin-peroxidase complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA) for a further 60 minutes. After washing with TBS (3 times, 10 minutes each), peroxidase binding was visualized as brown reaction products resulting from a benzidine reaction. The sections were then counterstained with Mayer's hematoxylin. Each set of experiments included specimens known to express survivin, XIAP, cIAP1, cIAP2, NAIP, and p53 (catalog number ab4060, mAb-DO7), which served as positive controls and ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

The immunostained sections were compared with the corresponding hematoxylin-eosin stained sections to establish a topographic relationship between positive-stained areas and histopathologic diagnoses. The percentage of positive-stained tumor cells was determined semiquantitatively by assessing the entire tumor section, and each sample was then assigned 1 of the following 6 staining scores: 0 (0%), 1 (less than 10%), 2 (11% to 25%), 3 (26% to 50%), 4 (51% to 75%), 5 (76% to 90%), or 6 (91% to 100%). Immunohistochemical staining was classified as negative if the staining was apparent in 10% of the cells or less (scores 0–1) and positive when over 10% (scores 2–6) were stained. The IHC staining of each section was evaluated by 2 experienced oral and maxillofacial pathologists (the first 2 authors of this article), who independently evaluated the staining scores. When disagreement existed between the 2 observers, a consensus was reached by discussion. Interobserver agreement was evaluated using kappa statistics[24]: a kappa value of less than 0.40 was considered to show poor agreement; a value of 0.40 to 0.59, fair agreement; a value of 0.60 to 0.74, good agreement; and a value of 0.75 to 1.00, excellent agreement.

DNA/RNA Isolation. Genomic DNA was extracted from each specimen by proteinase K digestion and phenol-chloroform extraction, as described elsewhere.[25] Total RNA was extracted by homogenizing the tissue specimens in guanidium isothiocyanate, followed by ultracentrifugation in cesium, as described previously in another article.[26]

Reverse Transcription-Polymerase Chain Reaction. Isolated total RNA (1 μg) was reverse transcribed to cDNA in a reaction mixture (of a final volume of 20 μL) containing $MgCl_2$ (4 μL; 5 μM]), 10× reverse transcription buffer (2 μL; 10 m*M* Tris-HCl, [pH 9.0], 50 m*M* KCl, 0.1% Triton X-100), dNTP mixture (2 μL; 1 m*M* each), recombinant RNasin ribnonuclease inhibitor (0.5 μL; 1U/μL), avian-myeloblastosis-virus reverse transcriptase (15 units; High Conc.; 15 units/μg), and oligo (dT)15 primer (0.5 μg; catalog number A3500, Promega, Madison, WI) and incubated for 15 minutes at 42°C. The avian-myeloblastosis-virus reverse transcriptase was then inactivated by heating for 5 minutes at 99°C and incubating at 0 to 5°C for a further 5 minutes.

Oligonucleotide primers were obtained from Genset Corp (La Jolla, CA). The primer pairs were chosen

Table 1. Oligoprimers from GenBank used for RT–PCR.

Primer	Sequences	Exon no.	Product (bp)	Accession no.
cIAP1	Forward: 5′–CAG CCT GAG CAG CTT GCA A–3′ Reverse: 5′–GCC CAT TTC CAA GGC AGA T–3′	3	354	BC016174
cIAP2	Forward: 5′–TCC GTC AAG TTC AAG CCA GTT–3′ Reverse: 5′–TCT TTT TCC TCA GTT GCT CTT TCT CT–3′	4–6	328	BC037420
NAIP	Forward: 5′–GCT TCA CAG CGC ATC GAA–3′ Reverse: 5′–ATG AGA GAC CCA AAA TCC GAA A–3′	12–14	446	U80017
XIAP	Forward: 5′–AGT GGT AGT CCT GTT TCA GCA TCA–3′ Reverse: 5′–GTT CCT CGG GTA TAT GGT GTC TGA–3′	1	360	U45880
Survivin	Forward: 5′–AGA ACT GGC CCT TCT TG GA–3′ Reverse: 5′–AAG GAA AGC GCA ACC GGA CG–3′	1–3	200	BC016174
p53	Forward: 5′–CTG AGG TTG GCT CTG ACT GTA CCA CCA TCC–3′ Reverse: 5′–CTC ATT CAG CTC TCG GAA ACA TCT CGA AGC G–3′	6–10	370	NM000546
β-actin	Forward: 5′–AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT–3′ Reverse: 5′–AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC–3′	3–4	350	X00351

Abbreviations: RT–PCR, reverse transcription–polymerase chain reaction; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein; cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2 , cellular inhibitors of apoptosis protein 2; No., number; bp, base pair.

from the published cDNA sequences of *survivin*, *XIAP*, *cIAP1*, *cIAP2*, *NAIP*, *p53,* and β-*actin* from GenBank (Table 1). All selected primers detect mRNA sequences of human tissues; the *p53* primer is able to detect both wild-type and mutant sequences.[27] 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 (of 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 m*M*), 10× reverse transcription buffer (8 μL; 10 m*M* Tris-HCl, pH 9.0, 50 m*M* KCl, 0.1% Triton X-100), upstream primer (50 pmol), downstream primer (50 pmol), and *Taq* DNA polymerase (2.5 units; Promega, catalog number M7660).

PCR reactions were carried out on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). The thermocycling conditions included denaturing at 94°C for 1 minute (1 cycle), then denaturing at 94°C (1 minute), annealing and extending at 72°C (1 minute) for 30 cycles, then a final extension at 72°C for 7 minutes for 1 cycle. The annealing conditions used were: 1 minute at 55°C for *survivin* and *p53*, 59°C for *XIAP*, 50°C for *cIAP1* and *cIAP2*, 56°C for *NAIP*, and 62°C for β-*actin*. The β-*actin* primers were used as positive controls; reactions were also performed without RNA and reverse transcriptase as negative controls. Amplification products were analyzed by electrophoresis in 2% agarose gel along with the relevant DNA molecular-weight marker (Boehringer, Mannheim, Germany) and stained with ethidium bromide. Photographs were obtained with a Polaroid DS-300 camera, and the PCR products were visualized as bands with a UV transilluminator.

Mutation Analysis of the *p53* Gene. Single-stranded conformation polymorphism analysis was used to analyze the tissue samples of 10 fresh OSCCs and 5 normal fresh oral tissues for mutations within exons 6 to 10 of the *p53* gene, which is the region most frequently affected by mutations in human tumors. Samples showing altered electrophoretic mobility were re-amplified in another reaction and analyzed by direct sequencing.

PCR was performed with 1 μg of genomic DNA, 200 ng of each oligoprimer (Table 1), 200 μM dNTPs, 1× PCR reaction buffer, and 2.5 units *Taq* polymerase. Aliquots of PCR-amplified mixture were diluted with 2 mL of distilled water and spun in a Midro 120 micro-concentrator (Hettich, Alpharetta, GA) to remove excess oligoprimers and dNTPs. DNA was then resuspended in 50 μL of 10 m*M* Tris (pH 8.0) and 1 m*M* EDTA. Direct sequencing of the amplified products of both strands was performed as per the instructions included in the Promega fmol DNA sequencing system technical manual to confirm and characterize the nature of the mutation; the sequences obtained were then compared with the known sequence of *p53* obtained from GenBank (accession No. NM000546).

Polymerase Chain Reaction–Based Methylation Assay. A PCR-based methylation assay was used to analyze DNA samples obtained from the 10 fresh OSCC samples and 5 fresh normal tissue samples. With reference to the known sequences in GenBank, oligoprimers for methylation assay of the 5 IAP members (*cIAP1*, *cIAP2*, *NAIP*, *survivin*, and *XIAP*) were deliberately designed so as not to contain any cytosine nucleotides, but each PCR product would include at least one site of CpG islands that could be digested by both *Msp* I and *Hind* III (Table 2). Therefore, only 1 set of oligoprimers was required for the methylation assay.[28]

Nondigested genomic DNA was modified by bisulfite treatment, converting unmethylated cytosine to

Table 2. Oligoprimers from GenBank used for methylation-specific PCR.

Primer	Sequences	Nucleotide no.	Product (bp)	Accession no.
cIAP1	Forward: 5′–TTA TTT GTG GAT AAG AAT ATG AAG–3′ Reverse: 5′–AAA AAT CCT TAT TTT AAA ACA CAA–3′	1643–2293	674	BC016174
cIAP2	Forward: 5′–AGA AGA TGA AAT AAG GGA AGA GGA G–3′ Reverse: 5′–TTT TAC TTC ACT TAC AAT TTC AAT AAT–3′	1405–2210	832	BC037420
NAIP	Forward: 5′–AAA AAG AAA AGA AAA GAA AAG AAA AAT–3′ Reverse: 5′–TCT ACT AAT TCT TTT TTT TCT TTT TT–3′	2303–2927	650	U80017
XIAP	Forward: 5′–TTT AGG TGA AGG TGA TAA AGT AAA GTG–3′ Reverse: 5′–TTC ACA TCA CAC ATT CAA TCA–3′	906–1769	884	U45880
Survivin	Forward: 5′–AAT AAG AAG AAA GAA TTT GAG GAA A–3′ Reverse: 5′–AAA AAA ACA CAA CAA CAA AAA AAC T–3′	554–1113	584	NM_001012271

Abbreviations: PCR, polymerase chain reaction; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein; cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2 , cellular inhibitors of apoptosis protein 2; No., number; bp, base pair.

thymidine and leaving methylated cytosine unchanged. Approximately 10 μg of DNA treated with bisulfite and 10 μg untreated DNA were digested with *Hind* III (1 μg/unit; Toyobo, Tokyo, Japan) at 37°C for 12 hours and the digested DNA then precipitated with ethanol dissolved in distilled water. The DNA was further digested with mCpG-sensitive *Msp* I (1 μg/unit; Toyobo, Tokyo, Japan) at 37°C for 24 hours. Then, the nondigested bisulfite-treated and untreated DNA samples and the digested treated and untreated DNA samples were subsequently amplified in a final volume of 25 μL containing 1 μL of nondigested/digested DNA, 2.5 pmol of specific oligoprimers for *survivin*, *XIAP*, *cIAP1*, *cIAP2*, and *NAIP* (Table 2), 50 μM of dNTPs, 10 m*M* Tris-HCL buffer (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 0.5 unit of AmpliTaq Gold (Applied Biosytstems, Foster City, CA) on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94°C for 1 minute (1 cycle), then denaturing at 94°C (1 minute), annealing and extending at 72°C (1 minute) for 40 cycles, then a final extension at 72°C for 7 minutes for 1 cycle. The annealing conditions used were 58°C (1 minute) for all 5 IAPs. Negative control experiments without primers were also performed. After purification using a PCR Product Purification Kit (Qiagen, Valencia, CA), the amplified PCR products were separated in 2% agarose gel and visualized by ethidium bromide staining.

Statistical Analyses. Statistical analysis was performed using Jump 7.0 software (SAS Institute, Cary, NC). Any p values $< .05$ were considered significant.

RESULTS

Semiquantitative Immunochemistry

Inhibitors of Apoptosis and p53. The interobserver agreement for the assessment of immunoscores among the 2 observers was excellent, with a kappa value of 0.91. The scores of IHC expression (based on the percentage of positive-stained cells, not staining intensity) for the 5 IAPs (*survivin*, XIAP, cIAP1, cIAP2, and NAIP) and p53 proteins in human oral potentially malignant disorders and their malignantly transformed SCCs, and in human oral potentially malignant disorders that had not undergone transformation and the 10 fresh OSCCs are summarized in Table 3.

Oral potentially malignant disorders with malignant transformation showed high expression of IAPs (cIAP1, survivin: 100% each, cIAP2: 97.7%, NAIP: 95.5%, and XIAP: 93.2%) and less consistent expression of nuclear staining of p53 (mAb-DO7; 34%), whereas the malignantly transformed SCCs showed higher expression of IAPs (cIAP1, cIAP2, NAIP, survivin: 100% each, and XIAP: 93.2%) and a slightly higher (45.5%) percentage of p53 expression (mAb-DO7). Oral potentially malignant disorders without malignant transformation also showed high expression of IAPs (cIAP1, cIAP2: 95.5% each, survivin: 93.2%, NAIP, and XIAP: 90.9% each) and a lower (25.0%) percentage of p53 expression (mAb-DO7). Similarly, the 10 fresh OSCCs demonstrated high expression of IAPs (cIAP1, NAIP, XIAP, survivin: 100% each, *cIAP2*: 80.0%) and a lower (50.0%) percentage of p53 expression (mAb-DO7). In contrast, a complete absence of staining for wild-type p53 was observed in all sections of human oral potentially malignant disorders with transformation and the malignantly transformed SCCs, and in all sections of human oral potentially malignant disorders that had not undergone transformation, and in the 10 fresh OSCCs. All sections of human normal oral mucosa showed negative staining for the 5 IAPs and p53 (mAb-DO7). Representative IHC stainings of the 5 members of the IAP family and p53 (mAb-DO7) for human oral potentially malignant disorders and the corresponding malignantly transformed OSCCs are shown in Figure 1, whereas those for oral potentially malignant disorders that had not undergone transformation to OSCCs are shown in Figure 2.

A representative negative IHC staining of p53 (wild-type) antibody for OSCC and a positive staining for positive control tissue of a sample of oral lymphoma are shown in Figures 3A and 3B. For the 5 fresh normal tissue samples, negative IHC staining for the 5 IAPs and p53 was noted in all cases except

Table 3. Scores for immunohistochemical expression of the five inhibitors of apoptosis family and p53 proteins for the 44 human oral potentially malignant disorders (PMDs) and the corresponding malignantly transformed oral squamous cell carcinomas (SCCs), and 44 PMDs without malignant transformation and 10 fresh OSCCs.

		Staining score							
		Negative staining		Positive staining					
	Lesions	[1]0	1	2	3	4	5	6	*p* value
cIAP1	[2]PMD transforming	0	0	3	1	9	29	2	[8].2290
	[3]PMD non-transforming	0	2	5	3	12	22	0	
	[4]SCC, paraffin	0	0	0	1	4	29	10	[9].0013*
	[5]SCC, fresh	0	0	0	1	2	4	3	[10].3532
cIAP2	PMD transforming	0	1	8	6	6	20	3	.3972
	PMD non-transforming	0	2	10	8	9	15	0	
	SCC, paraffin	0	0	1	0	5	26	12	<.0001*
	SCC, fresh	0	2	0	0	3	5	0	.1701
NAIP	PMD transforming	0	2	2	15	7	18	0	.1764
	PMD non-transforming	0	4	5	18	9	8	0	
	SCC, paraffin	0	0	0	0	6	32	6	<.0001*
	SCC, fresh	0	0	0	0	1	7	2	.8546
XIAP	PMD transforming	0	3	3	7	12	15	4	.1395
	PMD non-transforming	0	4	8	9	14	9	0	
	SCC, paraffin	0	4	1	5	5	16	13	.1119
	SCC, fresh	0	0	1	1	0	4	4	.8893
Survivin	PMD transforming	0	0	3	9	5	23	4	.0564
	PMD non-transforming	0	3	5	12	9	15	0	
	SCC, paraffin	0	0	1	0	4	24	15	.0002*
	SCC, fresh	0	0	0	0	1	6	3	.9564
[6]p53	PMD transforming	0	29	9	5	0	1	0	.0913
	PMD non-transforming	0	33	11	0	0	0	0	
	SCC, paraffin	0	24	3	8	4	5	0	.0036*
	SCC, fresh	0	5	2	1	1	1	0	.9732
[7]p53	PMD transforming	44	0	0	0	0	0	0	1.0000
	PMD non-transforming	44	0	0	0	0	0	0	
	SCC, paraffin	44	0	0	0	0	0	0	1.0000
	SCC, fresh	10	0	0	0	0	0	0	1.0000

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

Note: 1: Staining scores: 0 (0%); 1 (1% to 10%); 2 (11% to 25%); 3 (26% to 50%); 4 (51% to 75%); 5 (76% to 90%); 6 (91% to 100%) of positive-stained cells. 2: Number of cases of potentially malignant disorder with malignant transformation. 3: Number of cases of PMD without malignant transformation. 4: Number of cases of malignantly transformed OSCC. 5: Number of cases of fresh oral squamous cell carcinoma (OSCC). 6: Detected by mAb–DO7 antibody. 7: Detected by wild-type antibody (catalog number ab4060). 8: p value of compared immunoscores of the 5 inhibitors of apoptosis family and p53 proteins for the 44 PMDs with malignant transformation and the other 44 PMDs without transformation (chi-square test). 9: p value of compared immunoscores of the 5 inhibitors of apoptosis family and p53 proteins for the 44 PMDs and the corresponding malignantly transformed OSCCs (Wilcoxon signed-rank test). 10: p-value of compared immunoscores of the 5 inhibitors of apoptosis family and p53 proteins for the 44 malignantly transformed OSCCs and 10 fresh OSCCs (chi-square test).

**Statistically significant ($p < .05$).*

case 4, which showed positive staining for *p53* (wild-type) antibody (Figure 3C). On the other hand, representative IHC staining of p53 (mAb-DO7) in the fresh OSCCs is shown in Figure 3D.

Statistical Analysis. With the exception of XIAP, the scores for the 4 other IAPs and p53 were significantly elevated in the corresponding SCCs as compared with the oral potentially malignant disorders ($p < .05$, Wilcoxon signed-rank test; Table 3). On the other hand, there were no statistical differences between oral potentially malignant disorders that had undergone malignant transformation and those that had not ($p > .05$; chi-square test; Table 3), or between malignantly transformed OSCCs and fresh OSCCs ($p > .05$; chi-square test; Table 3) for expression of the 5 IAPs and p53.

Significant correlations among the IHC staining scores for the 5 IAPs in the 44 oral potentially malignant disorders with malignant transformation were noted, with the exception of *survivin* and cIAP1, survivin and XIAP, and NAIP and XIAP ($p < .05$; chi-square test; Table 4). In addition, significant correlations were observed between the IHC staining scores for survivin and cIAP1, and *survivin* and cIAP2, in the 44 malignantly transformed SCCs, but not among the other IAPs ($p < .05$, Nominal logistic fit test; Table 4). In contrast, we could only find a significant correlation between the IHC staining scores for p53 and cIAP2 in the oral potentially malignant disorders with malignant transformation, and no correlation was observed among the other IAPs (Table 4); furthermore, no significant correlation of the IHC staining scores for p53 with any of the 5 IAPs in the 44 malignantly transformed OSCCs was noted (Table 4).

No statistical significance was observed in the comparison of the IHC scores of true dysplastic lesions and non-dysplastic lesions (lichen planus and hyperkeratosis/epithelial hyperplasia; $p > .05$, 1-way

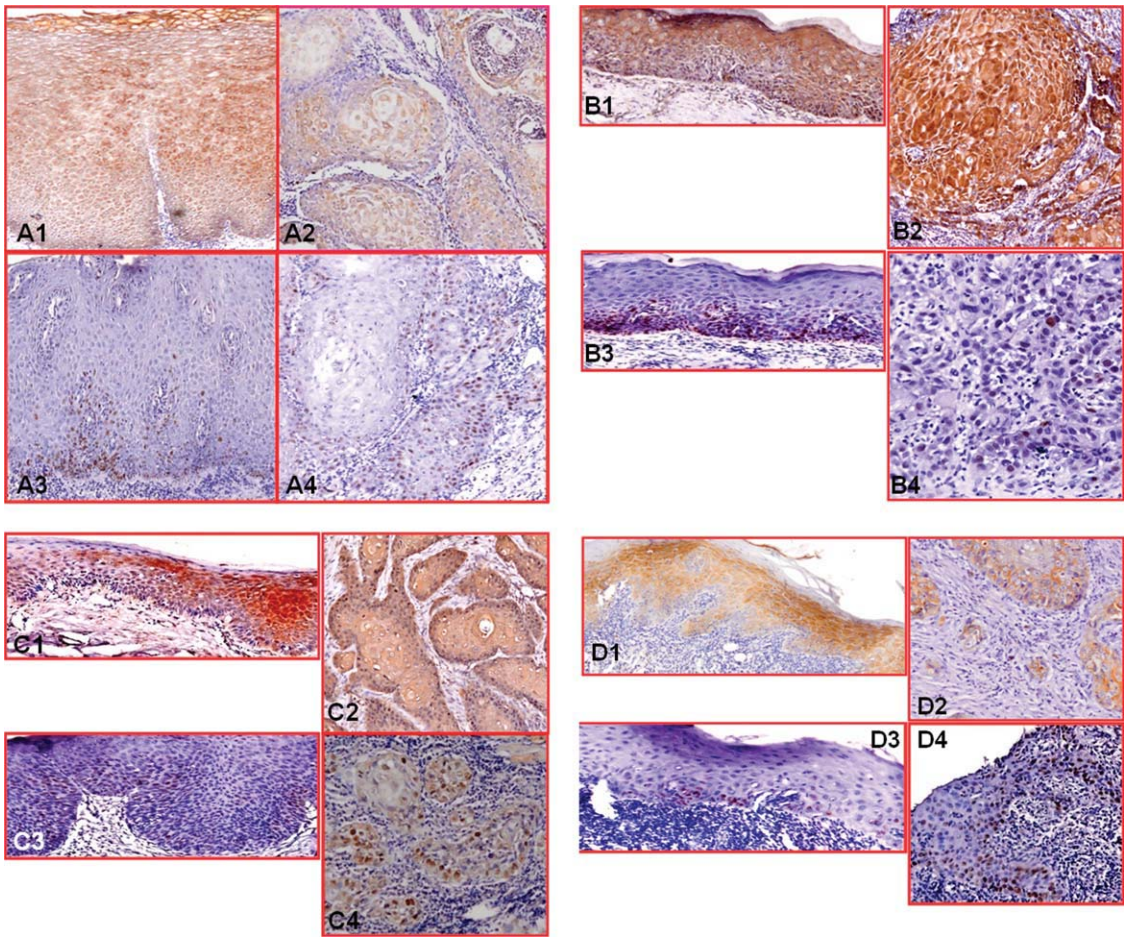

FIGURE 1. Representative immunohistochemical staining of cIAP2 and p53 (mAb-DO7) for human oral potential malignant disorders and the corresponding malignantly transformed squamous cell carcinomas (SCCs; avidin-biotin-peroxidase complex staining, ×100). A1: epithelial hyperplasia (EH; cIAP2, score 6). A2: malignantly transformed SCC from EH (cIAP2, score 5). A3: EH (p53, score 1). A4: malignantly transformed SCC from EH (p53, score 2). B1: submucous fibrosis (SF) (cIAP2, score 5). B2: malignantly transformed SCC from SF (cIAP2, score 6). B3: SF (p53, score 2). B4: malignantly transformed SCC from SF (p53, score 1). C1: epithelial dysplasia (ED) with SF (cIAP2, score 4). C2: malignantly transformed SCC from ED with SF (cIAP2, score 6). C3: ED with SF (p53, score 2). C4: malignantly transformed SCC from ED with SF (p53, score 5). D1: lichen planus (LP) (cIAP2, score 5). D2: malignantly transformed SCC from LP (cIAP2, score 5). D3: LP (p53, score 1, ×40). D4: malignantly transformed SCC from LP (p53, score 3). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

analysis of variance). There were significant correlations of the IHC expression of cIAP1 ($p = .0133$, linear regression analysis) and XIAP ($p = .0354$, linear regression analysis) of the 10 fresh OSCCs with p53 (mAb-DO7) but not of the other 3 IAPs with p53 ($p > .05$, linear regression analysis).

The survival rate of the 44 malignantly transformed OSCC cases was estimated to be 54.4% (survival plot); however, there was no significant correlation between the IHC staining scores for all OSCCs and survival rate, nor was any correlation observed for other clinical parameters (data not shown).

Reverse Transcription-Polymerase Chain Reaction

Inhibitors of Apoptosis and p53. After RT-PCR, *survivin, XIAP, NAIP,* and *cIAP1,* mRNA was observed to be present in all of the 10 fresh tissue specimens of OSCC, evidenced as bands corresponding to 200 base pair (bp), 360 bp, 446 bp, and 328 bp PCR products, respectively (Figure 4), whereas *cIAP2* mRNA was evident as a band corresponding to a 328 bp PCR product in 8 of 10 OSCC tissue samples. No *survivin, XIAP, NAIP, cIAP1*, or *cIAP2* mRNAs were detected in any of the samples of normal oral mucosa or the negative control without primers (Figure 4).

The *p53* mRNA was detected in half of the 10 samples of OSCCs, observed as a band corresponding to a 370 bp product (Figure 4). No *p53* mRNA was detected in any of the samples of negative controls without primers, but *p53* mRNA (370 bp) was detected in 1 of 5 normal mucosa tissue samples (Figure 4). All samples, with the exception of the negative controls without primers, revealed bands corresponding to β-*actin* (350 bp;

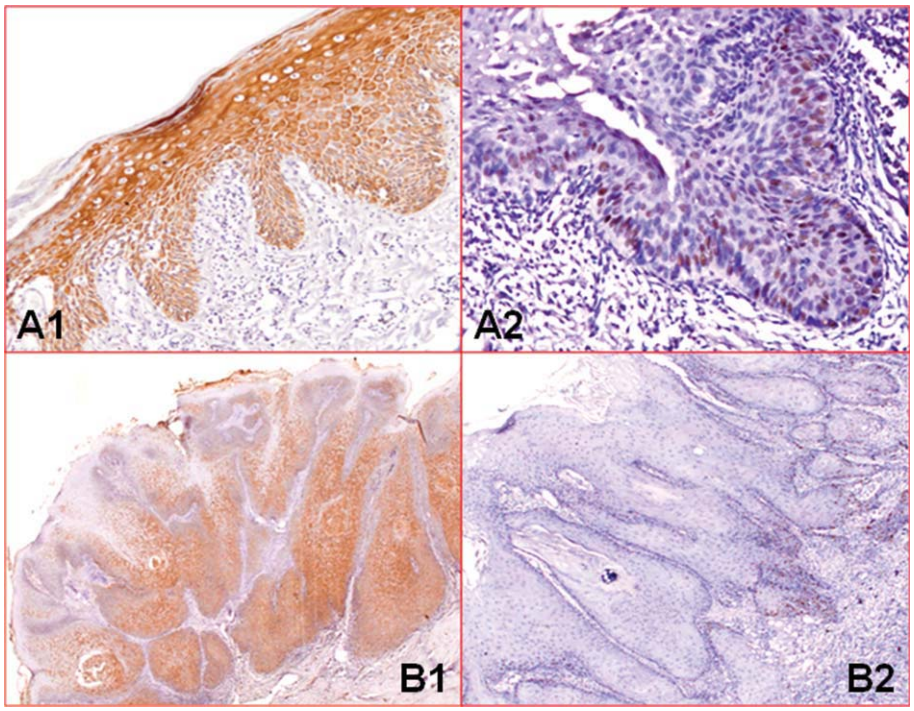

FIGURE 2. Representative immunohistochemical staining of cIAP2 & p53 (mAb-DO7) for human oral potential malignant disorders without malignant transformation (avidin-biotin-peroxidase complex staining). A1: epithelial dysplasia (ED) with epithelial hyperplasia (EH; cIAP2, score 6, ×100). A2: ED with EH (p53, score 3, ×200). B1: verrucous hyperplasia (VH) (cIAP2, score 5, ×40). B2: VH (p53, score 1, ×40). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

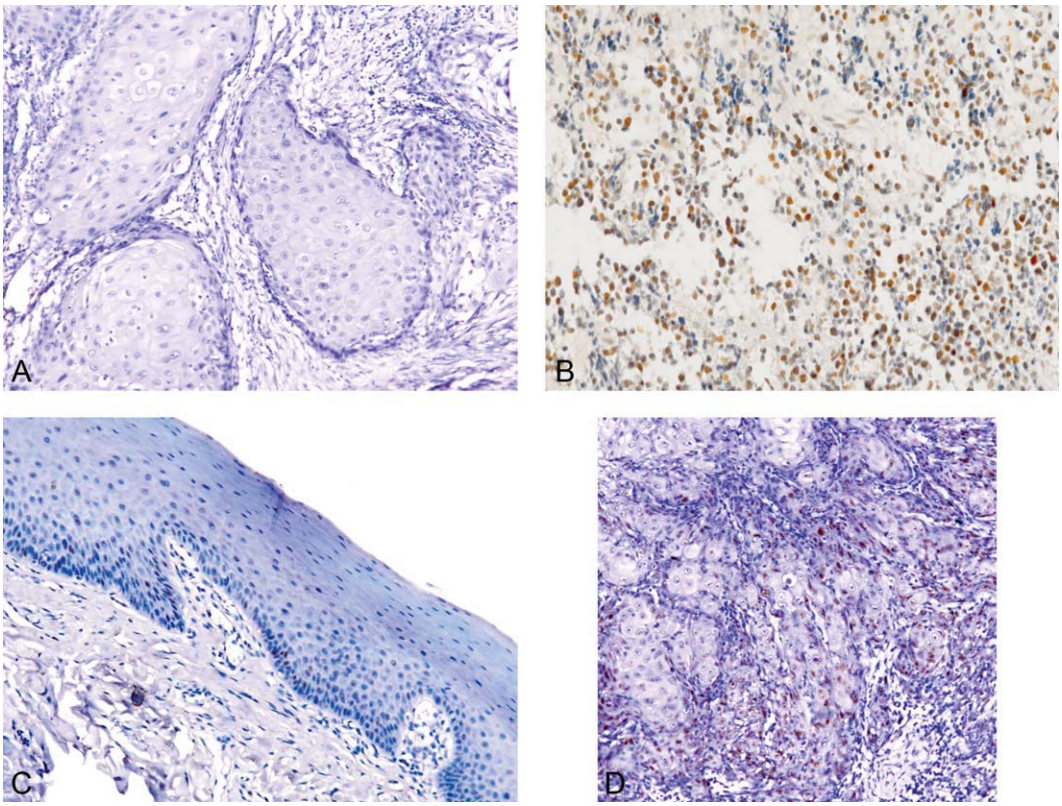

FIGURE 3. **(A)** Representative negative immunohistochemical staining of wild-type p53 for oral squamous cell carcinoma (SCC) (×200). **(B)** Positive staining for positive control tissue of a case of oral lymphoma (×100). **(C)** Positive staining for wild-type p53 of case 4 of normal tissue (×100). **(D)** Positive staining of p53 (mAb-DO7) for a representative sample of fresh OSCC (score 3, ×200; avidin-biotin-peroxidase complex staining). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 4. Correlation of scores for immunohistochemical expression among the 5 inhibitors of apoptosis family and p53 proteins of the 44 human oral potential malignant disorders and the corresponding malignantly transformed oral squamous cell carcinomas.

	cIAP1	cIAP2	NAIP	XIAP	Survivin	p53
cIAP1		0.0998*	0.3460	0.1706	0.0167‡	0.8759
		0.0253†,§	0.0016§	0.0166§	0.0860	0.6917
cIAP2	0.0998		0.5077	0.1831	0.0462‡	0.6005
	0.0253§		0.0205§	0.0067§	0.0008§	<0.0001§
NAIP	0.3460	0.5077		0.4758	0.8611	0.3783
	0.0016§	0.0205§		0.5185	0.0129§	0.5814
XIAP	0.1706	0.1831	0.4758		0.2309	0.4253
	0.0166§	0.0067§	0.5185			0.1610
survivin	0.0167‡	0.0462‡	0.8611	0.2309		0.7930
	0.0860	0.0008§	0.0129§	0.1279		0.6376
p53	0.8759	0.6005	0.3783	0.4253	0.7930	
	0.6917	<0.0001§	0.5814	0.1610	0.6376	

Abbreviations: NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein; cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2 , cellular inhibitors of apoptosis protein 2; No., number; bp, base pair.
**Malignantly transformed oral squamous cell carcinoma.*
†Oral potentially malignant disorder.
‡Statistically significant ($p < .05$, Nominal logistic fit test).
§Statistically significant ($p < .05$, chi-square test).

Figure 4). Additionally, the results regarding the mRNA level of the 5 members of the IAP family and *p53* obtained using RT-PCR were consistent with the protein level findings for IHC staining for both the OSCCs and normal tissues.

Statistical Analyses. As compared with normal tissues, the frequency of expression of the 5 *IAP*s and *p53* mRNA in OSCCs was significantly elevated ($p < .05$, chi-square test; Table 5).

Mutation Analysis of the *p53* Gene. Upon direct sequencing of the PCR products for genomic *p53* DNA from the 10 oral fresh SCC tissue specimens, 2 cases were observed to have a point mutation (C→ G at nucleotide 1131 for exon 8 and G→C at nucleotide 1261 for

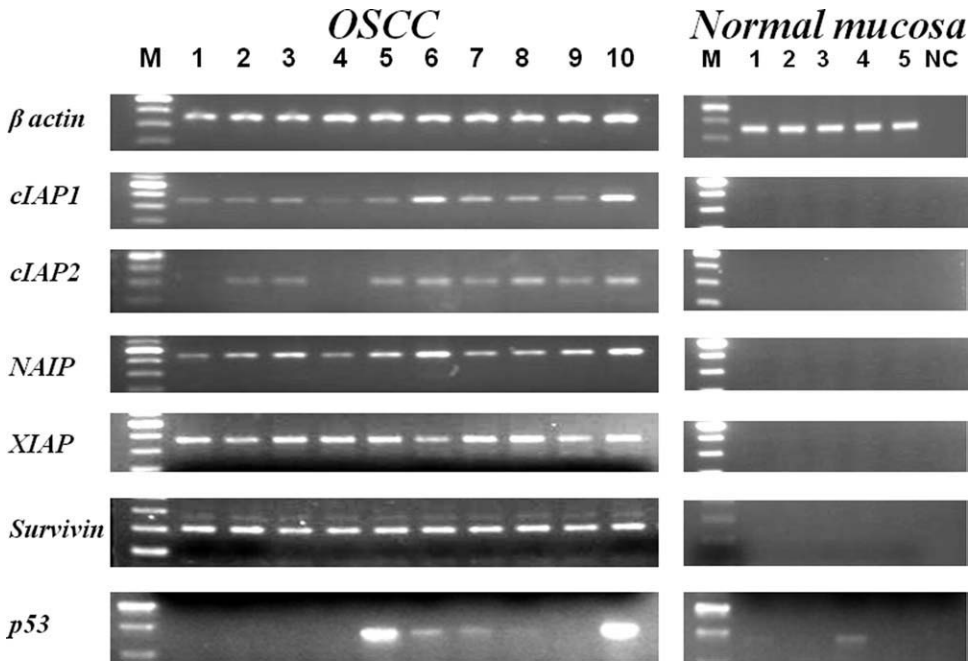

FIGURE 4. After reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, bands corresponding to mRNA of cellular inhibitors of apoptosis protein 1 (*cIAP1;* 354 bp), cellular inhibitors of apoptosis protein 2 (*cIAP2;* 328 bp), neuronal apoptosis inhibitory protein (*NAIP;* 446 bp), X-linked inhibitors of apoptosis protein (*XIAP;* 360 bp), *survivin* (200 bp), and *p53* (370 bp) were evident in all of the 10 specimens of human oral squamous cell carcinomas (OSCCs; lanes 1–10). No bands for the 5 IAPs were detected in any of the 5 samples of normal mucosa (lanes 1–5), and *p53* was evident in only 1 (lane 4) of 5 samples. All samples (lanes 1–10 for OSCCs; lanes 1–5 for normal mucosa) revealed bands of β-*actin* (350 bp), with the exception of the negative control without primers (lane N); lane M: molecular-weight markers.

Table 5. Comparison of mRNA expression (frequency) of oral squamous cell carcinoma with that of normal oral mucosa.

	Survivin	XIAP	cIAP1	cIAP2	NAIP	p53 (mutant)
Oral squamous cell carcinoma	10/10*	10/10	10/10	8/10	10/10	5/10
Normal oral mucosa	0/5	0/5	0/5	0/5	0/5	0/5
	$p = .0001^{\dagger}$	$p < .0001^{\dagger}$	$p = .0001^{\dagger}$	$p = .0001^{\dagger}$	$p = .0001^{\dagger}$	$p = .0001^{\dagger}$

Abbreviations: NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitors of apoptosis protein; cIAP1, cellular inhibitors of apoptosis protein 1; cIAP2 , cellular inhibitors of apoptosis protein 2.
**Frequency of mRNA expression.*
†Statistically significant ($p < .05$).

exon 10; Figure 5A); 1 case had 3 point mutations (G→C at nucleotide 1088 and nucleotide 1096 for exon 8; C→G at nucleotide 1102 for exon 8); 1 case had mutations at nucleotides 1023 to 1076 for exons 7 to 8; and in 1 case, a frame-shift mutation for exons 6 to 10 was observed. Additionally, the results for the 5 cases of fresh OSCCs showing *p53* mutations were consistent with those for the protein levels of *p53* of the same 5 positive cases (mAb-DO7) with IHC staining. On the other hand, direct sequencing of the PCR product of genomic *p53* DNA for the 5 normal fresh tissue specimens showed no alteration from the known sequence of *p53* from GenBank (accession No. NM000546), indicating no occurrence of mutation (Figure 5B).

Polymerase Chain Reaction-Based Methylation Assay. For normal oral mucosa tissue, PCR products of 584 bp (*survivin*), 884 bp (*XIAP*), 650 bp (*NAIP*), 674 bp (*cIAP1*), and 832 bp (*cIAP2*) were observed in nondigested DNA samples treated with bisulfite and untreated nondigested samples, whereas no PCR products were observed in digested DNA samples.

For all tissues of OSCCs, PCR products of 584 bp (*survivin*), 884 bp (*XIAP*), 650 bp (*NAIP*), 674 bp (*cIAP1*), and 832 bp (*cIAP2*) were observed in nondigested DNA samples not treated with bisulfite and digested DNA samples both treated with bisulfite and untreated, whereas no PCR products were observed in nondigested DNA samples not subjected to bisulfite treatment. Representative results of the PCR-based methylation assay are shown in Figure 6.

DISCUSSION

One of the purposes of this study was to evaluate whether IAP expression of oral potentially malignant disorders is related to malignant transformation. However, we failed to establish a significant relationship between IAP expression and oral potentially malignant disorders malignant transformation. Indeed, malignant transformation is a complex issue contributing to a multi-factorial aspect of oral potentially malignant disorders, and the results obtained,

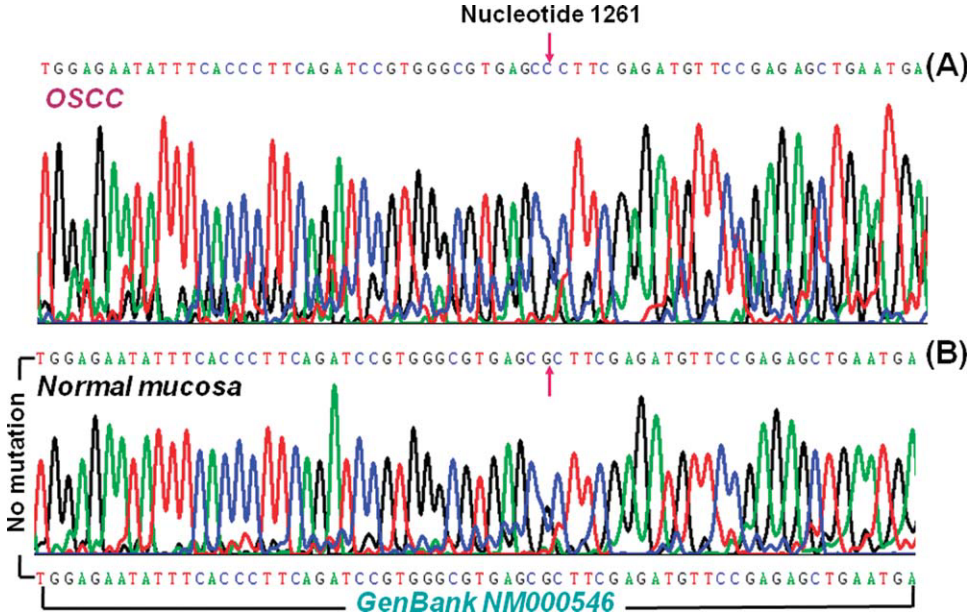

FIGURE 5. Representative sample of direct sequencing of the polymerase chain reaction (PCR) products for genomic *p53* DNA in human oral squamous cell carcinoma (OSCC) tissue specimens showing a point mutation of G to C at nucleotide 1261 (red arrow) **(A)**, whereas no alteration in the sequence of *p53* in normal mucosa was found **(B)**. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

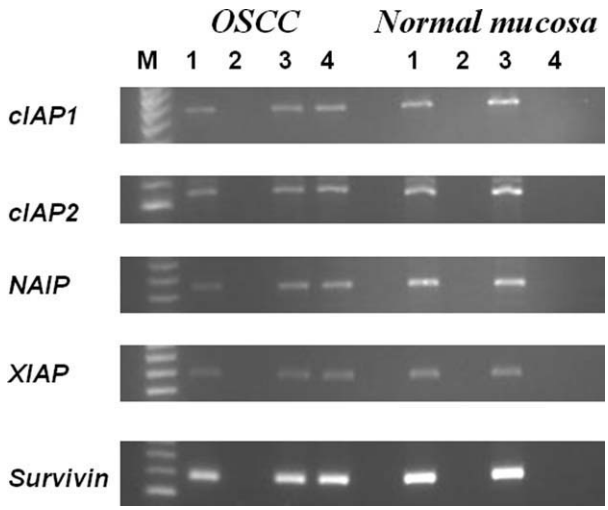

FIGURE 6. Representative results of polymerase chain reaction (PCR)-based methylation assay of cellular inhibitors of apoptosis protein 1 (*cIAP1*), cellular inhibitors of apoptosis protein 2 (*cIAP2*), neuronal apoptosis inhibitory protein (*NAIP*), X-linked inhibitors of apoptosis protein (*XIAP*), and *survivin* genes in human oral squamous cell carcinomas (OSCCs) and normal mucosa (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).

therefore, may be because there were not enough samples of individual oral potentially malignant disorders with malignant transformations collected in this study. In addition, ours was a wide-ranging cohort of patients with oral potentially malignant disorders consisting of cases ranging from low-risk to high-risk malignancies, which may have different pathogeneses.[14] Nevertheless, significant elevation of 4 IAPs (*survivin*, *cIAP1*, *cIAP2*, and *NAIP*, and not *XIAP*) was observed in malignantly transformed OSCCs as compared with the corresponding oral potentially malignant disorders (Table 3). Hence, according to our results, IAP expression may indeed be 1 of the contributing factors to malignant transformation of oral potentially malignant disorders, but whether or not it is a determining factor remains to be elucidated in future studies. Because it is not easy to accumulate a large sample size for each category of oral potentially malignant disorders with overt malignant transformation, using standardized criteria for malignant transformation of oral potentially malignant disorders[17] and the study design of this study, a multi-institutional or multinational study could be instigated with a large sample size of individual oral potentially malignant disorders, which may provide more insight into this issue.

In normal tissue, including oral mucosa, homeostasis is acquired by balancing the rate of cell proliferation with an equal rate of cell death,[29] and impaired balance between cell proliferation and apoptosis is crucial to the development of malignant neoplasms. IAP family members are important regulators of apoptosis that serve as endogenous inhibitors of caspase family cell death proteases.[30] A role of IAP-mediated inhibition of apoptosis in the development of cancers has been hypothesized,[31] and a relationship of some IAP family members with human OSCCs has also been reported. However, only survivin has been extensively studied, and an upregulation of survivin expression is regarded as an important early event in human oral carcinogenesis.[32–34] However, to our knowledge, the implication of a role for members of the IAP family other than survivin in human oral carcinogenesis has seldom been studied. Hence, the assessment of an association of IAP with early-stage human oral carcinogenesis was another purpose of this study. We observed significant protein overexpression of the 5 IAP family members (survivin, XIAP, cIAP1, cIAP2, and NAIP) in oral potentially malignant disorders as compared to normal oral mucosa, indicating that IAP expression is an early event in human oral carcinogenesis. This finding is also compatible with our results from a previous animal study using an HBPCM.[13]

The epigenetic pathway of IAP overexpression in oral cancerous tissues is yet to be completely understood. Amplification of the *survivin* locus on chromosome 17 and DNA demethylation of its promoter region have been suggested as potential mechanisms of *survivin* overexpression in certain cancers.[35] In this study, we demonstrated that normal tissue contains a *survivin*-methylated, *cIAP1*-methylated, *cIAP2*-methylated, *XIAP*-methylated, and *NAIP*-methylated allele, whereas no evidence of methylation was observed in cancerous tissues. Although incomplete restriction digestion or oversensitivity in detecting hypermethylation is possible in a pure PCR-based methylation assay, the overexpression of IAP family members in human OSCCs noted in this study may be interpreted as suggestive of regulation via epigenetic dysregulation of DNA hypomethylation. Most studies on the epigenetics of head and neck SCC have primarily focused on promoter hypermethylation, which is considered a mechanism of inactivation of many tumor suppressor genes such as *p16*, *E-cadherin*, *DAP-kinase,* and many others.[11,36] To the best of our knowledge, this study is the first to have demonstrated DNA hypomethylation of the 5 studied members of the IAP family in human oral cancerous tissues, suggesting a modality of activation of oncogenes of IAPs. However, further study using a method involving the sequencing of several CpG islands should be performed to further confirm the correctness of our PCR results. Furthermore, a designed methylated DNA and a known unmethylated DNA should be used as positive and negative control samples, respectively, in the same assay to check for

methylation and non-methylation of the *IAP* genes in the samples.

Wild-type *p53* has been reported to inhibit the promoter activity of *survivin*, leading to the downregulation of *survivin* expression and hence suggesting the existence of a negative feedback loop between *survivin* expression and wild-type *p53*. Loss or mutation of *p53* might result in the overexpression of *survivin*[37,38]; however, the association between *p53* and other IAP family members has not yet been studied. Therefore, examination of the potential regulation of the overexpressed IAP by mutant *p53* in OSCC was also an objective of this study. Two different p53 antibodies were used in this study. The antibody (catalog number ab4060) that recognized the wild-type p53 protein revealed a complete absence of staining for all the OSCCs examined, including the 44 malignantly transformed and 10 fresh OSCCs. The possibility of denaturing p53 protein using paraffin-embedded samples is limited because efficient detection of wild p53 status of the positive control has been attained in this study. Another antibody (mAb-DO7) recognized both wild-type and mutant p53 proteins, which yielded approximately 46% and 50% for the 44 malignantly transformed OSCCs and 10 fresh OSCCs, respectively, in this study. Immunostaining cannot be used as a surrogate tool for the detection of *p53* gene mutation. Nevertheless, given that there was a high incidence of *p53* mutation (approximately 49%) in OSCC samples from patients with betel-quid chewing and cigarette smoking habits,[39] we believe that the *p53*-DO7 antibody should have picked up a certain amount of mutant *p53* protein in the tissues of OSCCs examined in this study.

On the other hand, direct sequencing of the PCR product of genomic *p53* DNA obtained from the normal oral tissue specimen showed the exact same sequence as the known sequence of *p53* obtained from GenBank (accession No. NM000546), indicating that the DNA sequencing in this study was of acceptable quality. Significantly, mutations of exons 6 to 10 of the *p53* gene were identified in 5 of 10 fresh OSCC samples examined in this study. Mutation of the *p53* gene has also previously been reported in a HBPCM[40] in vitro, in a human oral cancer cell line,[41] and in human OSCC in vivo.[42] The frequency of *p53* mutations in our cohort of 10 fresh OSCCs may have a potential bias because only *p53* mutations within exons 6 to 10 were analyzed. Soussi and Béroud[43] analyzed the entire *p53* gene and noted that approximately 14% of mutations were located outside exons 5 to 8, with a significant number of mutations in exons 4, 10, and, to a lesser extent, exon 9. However, this does not detract from the significance of our results, which showed *p53* mutations compatible with the findings regarding mRNA and protein expression, because all 5 cases of fresh OSCCs exhibited *p53* mutations consistent with the same 5 positive case results for protein levels of p53 (mAb-DO7) with IHC staining. In addition, a significant correlation of IHC expression of 2 of 5 IAPs was observed in the 10 fresh OSCCs with p53 (mAb-DO7). Hence, the *p53* mutation status of OSCCs may at least partially have some influence on the expression of IAP proteins.

Taken together, the results of this study indicate that overexpression of IAP family members in human OSCCs might be related by both genetic (partially by mutant *p53*) and epigenetic mechanisms. Upon subjection to the accumulated harmful effects of risk factors such as betel-quid chewing, cigarette smoking, alcohol drinking, or others, the induced mutant *p53* in oral mucosa keratinocytes could cause breakdown of the negative feedback loop originally existing between IAP and wild-type *p53*. Hence, expression of the IAP family would be deregulated and upregulation would occur in oral mucosa keratinocytes, developing into carcinoma. However, 1 point of caution should be mentioned in analyzing the results of the present study, the protein and mRNA expressions of the 5 members of the IAP family were higher than those of *p53*, indicating that IAP expression may not be solely caused by *p53* abnormalities. Although it is possible that *p53* contributes to IAP deregulation in a subset of oral cancer, and other as yet unknown genetic dysregulations, may also contribute to the upregulation of IAP in human oral carcinogenesis. Given that genetics and epigenetics are complementary in cancer etiology,[12] further study on the potential interaction between genetic and epigenetic changes would enable clarification of the mechanism of upregulation in the IAP family. If a correct regulatory pathway for the IAP family is discovered, it would be a valuable early diagnostic cancer marker.

The concept of "field cancerization" is well recognized for patients who frequently come into contact with mutagens, suggesting that the entire aerodigestive tract mucosa is exposed to carcinogens, and multiple foci of transformed tissue clones are expected in these patients at high-risk of malignancy.[44] The enhanced expression of IAPs and mutant *p53* observed in this study would then be indicative of the manifestation of field carcinogenesis in those patients heavily exposed to carcinogens (except for the lichen planus group). On the other hand, all of the lichen planus patients in this study were at low risk of malignancy because they were histologically found to be free of epithelial dysplasia and had no oral risk factors for malignancy. Is it perhaps indicated, therefore, that enhanced IAPs and mutant *p53* expression plays a potential role in oral tumorigenesis by inhibiting apoptosis in the category of patients with lichen planus?

In this study, no significant statistical differences were noted in a comparison of the clinical characteristics of the patients with OSCC in which expression of the 5 IAP members was observed, although some IAPs, such as *cIAP1* and particularly *survivin*, have been found to be related to clinical outcomes in previous studies.[32–34,45] On the other hand, the *cIAP1* and *cIAP2* genes are located on human chromosome 11q, whereas the *NAIP*, *survivin*, and *XIAP* genes are

situated on human chromosomes 5q, 17q, and Xq, respectively,[13] all of which are regions of high incidence of chromosomal abnormalities associated with carcinomas of the head and neck, including OSCC.[46–49]

As all 5 IAP family members (both protein and mRNA) were found to be overexpressed in OSCCs in this study, a potential synergy among IAP family members, and hence a putative common regulatory mechanism, might also be hypothesized. Moreover, we found in this study that the protein expression of these 5 IAP family members in both human oral potentially malignant disorders and SCC were largely statistically correlated with each other (Table 4). To date, little accuracy is known regarding the pathways of interaction among IAP family members. A potential interaction between *survivin* and *XIAP* has been reported: *survivin* was found to bind to second mitochondria-derived activator of caspase (Smac), releasing its suppression of *XIAP* and resulting in *XIAP* activation.[50]

Finally, one point concerning protein and mRNA expression of *survivin* is worthwhile to be noted. Lo Muzio et al[51] reported low positivity for survivin protein expression in normal oral mucosa despite that it is limited to few basal cells; however, we found complete negative survivin staining for all normal oral mucosa in the current study. This minor difference of staining is perhaps due to the different antibodies with different sensitivities being used. On the other hand, *survivin* mRNA has not been detected for all normal oral mucosa in our study but low expression of *survivin* mRNA have been reported in 2 most recent studies.[52,53] Again, this disparity of mRNA expression may be attributed to the more sensitive real-time RT-PCR that was used in these 2 recent studies, whereas a less sensitive traditional RT-PCR is applied for the current study. Accordingly, human normal mucosa shows the absence or significant downregulation of *survivin* expression compared to cancerous tissues and can be regarded as *survivin*-negative; however, it is not entirely negative.

In conclusion, the results of this study suggest that the IAP family may be a contributing factor in human oral squamous cell carcinogenesis, and it can be inferred that the expression of IAP family members may be associated with genetic (at least partly by mutant *p53*) and epigenetic regulatory changes.

Acknowledgments. We acknowledged the assistant of Dr. B.-C. Wu, Department of Oral & Maxillofacial Surgery, Chung-Shan Medical University Hospital, Taichung, and also all the teaching staff of the Department of Oral & Maxillofacial Surgery, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, for this study.

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