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SHORT COMMUNICATION

Survivin expression is regulated by an epigenetic mechanism for DMBA-induced hamster buccal-pouch squamous-cell carcinomas

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Summary Apoptosis, also known as programmed cell death, is regulated by a number of inhibitory or stimulatory factors. In addition to the pro- and anti-apoptotic Bcl-2 family proteins, there is also a family of inhibitors of apoptosis protein (IAP). *Survivin*, a member of this IAP family, is selectively upregulated in most tumours. The objective of the present study was, therefore, to investigate the protein and mRNA expression of *survivin*, as well as the methylation status of the CpG sites in exon 1 of the *survivin* gene for 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch squamous-cell carcinomas. Immunohistochemical analysis for protein expression, RT-PCR for mRNA expression, and a PCR-based methylation assay were performed on 26 samples of hamster buccal pouches. The total study population was assigned into either one experimental group (15-week DMBA treatment; $n = 13$) or two control groups (untreated: $n = 6$; mineral-oil treated $n = 7$). Cytoplasmic staining of *survivin* protein and mRNA were detected in all of the hamster buccal-pouch tissue specimens treated with DMBA, whereas neither survivin protein nor *survivin* mRNA were noted for all of the untreated and mineral oil-treated hamster buccal-pouch tissue specimens. Furthermore, all the untreated and mineral-oil treated samples had a *survivin*-methylated allele, whereas the DMBA-treated cancerous tissues showed no evidence of *survivin* methylation. The results suggest that *survivin* may play an important role in DMBA-induced hamster buccal-pouch carcinomas, and that the gene expression may be modulated by an epigenetic mechanism.

Apoptosis, also termed programmed or physiological cell death, plays diverse roles in embryogenesis and normal homeostasis, as well as in tumorigenesis.[1–3] Apoptotic processes are regulated by a number of factors, which have inhibitory or stimulatory effects.[2–4] Besides the pro- and anti-apoptotic Bcl-2 family proteins,[5,6] there is another family of inhibitors of apoptosis protein (IAP). IAP, first described as baculovirus gene products, are characterized by a novel domain of approximately 70 amino acids, known as the baculoviral IAP repeat (BIR). This domain is able to inhibit caspases and,

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thus, suppress apoptosis.[7–9] Proteins containing BIR domains have been found in a wide range of eukaryotic species, and eight IAP members have been identified in humans.[9] *Survivin* is a member of the IAP family. Its gene, which is situated on chromosome 17q25, is a unique bifunctional protein that inhibits apoptosis by suppressing caspase-3 and caspase-7, and modulating the G2/M phase of the cell cycle by associating with the mitotic spindle microtubules.[10] *Survivin* expression is absent from normal adult tissues, but can be recognized in developing fetal tissues and different types of neoplasms.[11–13]

Hamster buccal-pouch mucosa provides one of the most widely accepted experimental models for oral carcinogenesis.[14] 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 in terms of the development of both premalignancy and malignancy in human oral mucosa.[15] Genetic alterations such as *p53*, *p63*, *p73* and *iNOS* in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinogenesis have previously been studied in our laboratory.[16–19] To date, however, little is known with respect to the role of epigenetic alterations in hamster buccal-pouch model. The purpose of this study was, therefore, to investigate the protein and mRNA expression of *survivin* for DMBA-induced hamster buccal-pouch squamous-cell carcinomas. Additionally whether *survivin* expression was regulated by an epigenetic mechanism, methylation status of the CpG sites in exon 1 of the *survivin* gene was also evaluated in DNA samples from DMBA-induced hamster buccal-pouch carcinomas.

Outbred, young (6 weeks of age), male, Syrian golden hamsters (*Mesocricatus auratus*; $n = 26$) were purchased from the National Science Council Animal Breeding Center, Taipei, ROC. They weighed approximately 100 g each at the commencement of the experiment. The animals were randomly divided into one experimental ($n = 13$) and two control groups consisting of untreated ($n = 6$) and mineral-oil treated subgroups ($n = 7$). The animals were housed under constant conditions (22 °C and a 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 guidelines promulgated by the NIH Guide for the Care and Use of Animals. After allowing the animals one week of acclimatization to their new surroundings, both pouches from all of the animals from the experimental group 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 from the mineral-oil 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 untreated group of six animals remained untreated throughout the experiment.

At the end of 15 weeks (3 days subsequent to the last treatment), the experimental group of 13 animals was omitted from the painting schedule and, in order to avoid the influence of diurnal variation, simultaneously and humanely sacrificed at 9 a.m. by the administration of a lethal dose of diethyl ether.[20] The animals' pouches were exposed by dissection and examined grossly. Both pouches were then excised. A portion of the pouch tissue was immediately frozen in liquid nitrogen for subsequent DNA/RNA extraction, RT-PCR reaction, and methylation assay, whilst another portion was 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 study. Finally, all of the animals in the two control groups were sacrificed at the end of the experiment.

Genomic DNA was extracted from each pouch specimen by proteinase *K* digestion and the phenol–chloroform extraction procedure, as described elsewhere.[21] Total RNA was extracted by homogenizing the pouch-tissue specimens in guanidium isothiocyanate followed by ultracentrifugation in caesium chloride, as described previously.[22]

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 concentration: 15 μ/μg), and oligo(dT)15 primer (0.5 μg; Promega, Catalogue No. A3500, WI, USA). The reaction mixture was incubated for 15 min at 42 °C. The AMV reverse transcriptase was inactivated by heating for five minutes at 99 °C 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 for *survivin* (GenBank Accession No. AB-028869) and β-actin (GenBank Accession No. X-00351). Primers used were as follows: 5′-AGAACTGGCCCTTCTTGGA-3′ (forward) and 5′-AAGGAAAGCGCAACCGGAC-

G-3′ (reverse) for *survivin*, yielding a 200-bp product; 5′-AACCGCGAGAAGATGACCCAGATCATGTTT-3′ (forward) and 5′-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3′ (reverse) for β-actin, yielding a 350-bp product. 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, Catalogue No. M7660).

The PCR steps were performed using a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94 °C for 1 min (1 cycle), then denaturing at 94 °C (1 min), annealing at 55 °C (1 min) for *survivin* or at 60 °C (1 min) for β-actin, and extending at 72 °C (1 min) for 30 cycles, with a final extension at 72 °C (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 included. Amplification products were analyzed by electrophoresis in a 2% agarose gel along with the relevant DNA molecular-weight marker (Boehringer, Mannheim, Germany) and stained with ethidium bromide. The PCR products were visualized as bands with a UV transilluminator. Photographs were taken with a Polaroid DS-300 camera.

To investigate whether methylation of CpG islands of the *survivin* exon 1 could implicate *survivin* mRNA expression, a PCR-based methylation assay was used to analyze DNA samples obtained from samples of hamster-pouch mucosa. Approximately 10 μg of DNA was digested with *Hin*dIII (1 μg/unit; Toyobo) at 37 °C for 12 h. The digested DNA was then precipitated with ethanol, and dissolved in distilled water. The DNA was further digested with mCpG-sensitive *Hpa*II (1 μg/unit; Toyobo) at 37 °C for 12 h. The *Hpa*II-digested DNA was subsequently amplified with specific primers for *survivin* exon 1 (forward: 5′-GACTACAACTCCCGGCACACCCCGC-3′; reverse: 5′-CCCAGGAGGCCGGGCAGTCTCACCC-3′). The polymerase chain reactions were performed in a final volume of 25 μl containing: 1 μl of digested DNA; 2.5 pmol of each specific primer; 50 μM of dNTPs; 10 mM of Tris—HCL buffer (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.5 units of AmpliTaq Gold (Applied Biosystems; Foster City, CA, USA). Negative controls were also included. The amplified PCR products were separated in a 3% agarose gel and visualized by ethidium bromide. PCR amplification of the *survivin* gene or cDNA was tested in a preliminary experiment by direct product sequencing.

Following tissue section, staining was performed using a standard avidin—biotin peroxidase complex (ABC) method.[23] Tissue sections were mounted on gelatin-chrome alum-coated slides. Subsequent to deparaffinization in xylene (twice) and rehydration in a descending-concentration ethanol series (absolute, 95%, 70% and 30% ethanol, and then water), tissue sections were microwave treated three times (5 min each) in citrate buffer (10 mM; pH = 6.0) in order to retrieve antigenicity. The tissue sections were then treated in H_2O_2—methanol (0.3%), and normal goat serum (10%; Dako, Santa Barbara, CA, USA). All sections were subsequently incubated with the goat anti-rat *survivin* polyclonal antibody (1:100; Santa Crutz Biotechnology Inc., Santa Crutz, CA, USA), at room temperature for 60 min. These sections were then incubated for a further 30 min at room temperature in the presence of biotinylated goat anti-rabbit IgG, and then for a final 30 min with ABC (Dako). The peroxidase-binding sites were visualized as brown reaction products of the benzidine reaction. The sections were subsequently counterstained with haematoxylin. Positive and negative controls were used for each experiment. Immunohistochemical staining was classified as negative if staining was apparent for l0% of the cells or less, and positive where this was over 10%.

Gross and histopathological changes in the 15-week DMBA-treated pouches were similar to those described in our previous study.[16—19] Invasive squamous-cell carcinomas with 100% tumour incidence were apparent for all of the DMBA-treated pouches. The mineral oil-treated and untreated pouches revealed no obvious changes associated with such treatment.

Upon RT-PCR, *survivin* mRNA was detectable as a band corresponding to a 200-bp PCR product in all of the 13 hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks, whereas no *survivin* mRNA was detected in the tissue deriving from the untreated animals, or the mineral oil-treated tissues or negative-control samples.

Methylation status of the *survivin* gene was investigated for all of the three groups of tissues. All untreated and mineral-oil treated samples had a PCR product of 297 bp, indicating a *survivin*-methylated allele, whereas no evidence of *survivin* methylation was detected in the DMBA-treated cancerous tissues. Representative results from RT-PCR and methylation assay are depicted in Fig. 1A and B.

From immunohistochemistry, no *survivin* staining could be found for any of the untreated and mineral-oil treated hamster pouch-tissue specimens (Fig. 1C), whereas cytoplasmic staining of *survivin*

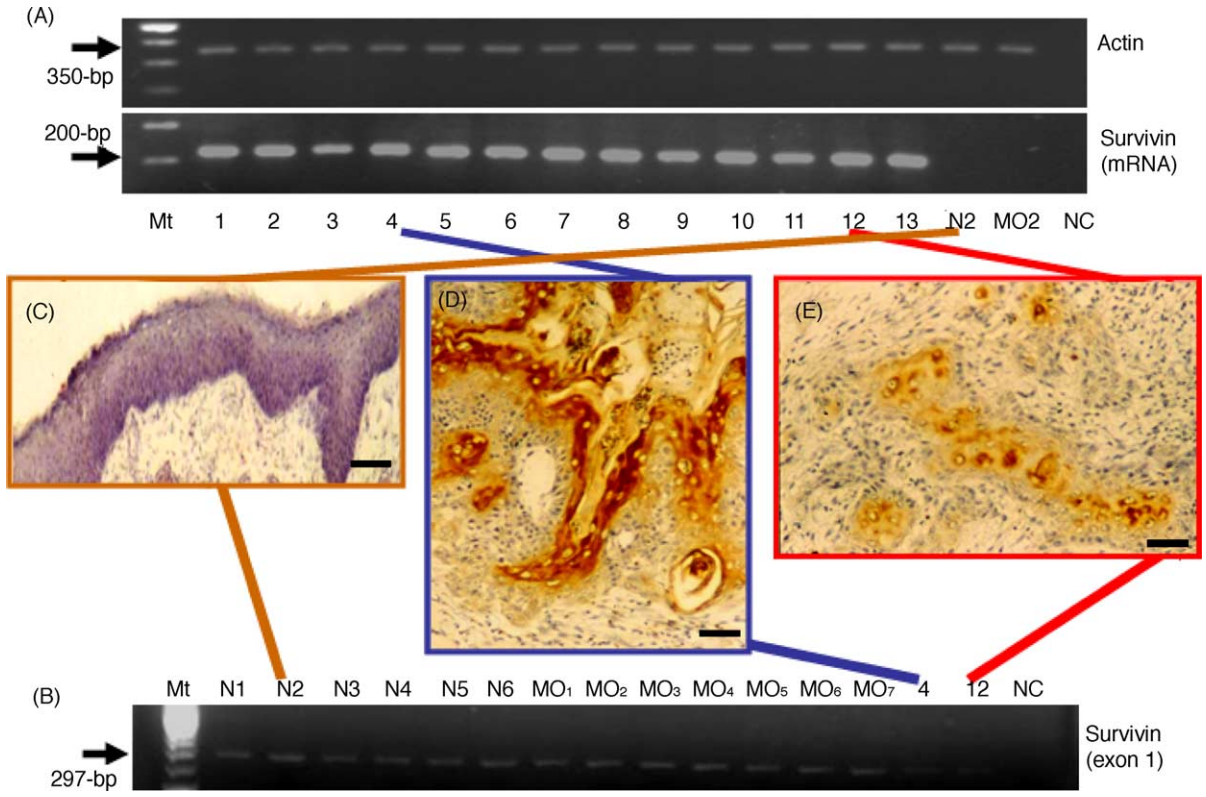

Fig. 1 Representative results for (A) RT-PCR, (B) methylation assay, and (C—E) immunohistochemistry in the DMBA-induced hamster buccal-pouch carcinoma. (A) *Survivin* mRNA was detectable as a band corresponding to a 200-bp PCR product in all 13 hamster buccal-pouch specimens treated with DMBA for 15 weeks (lanes 1—13). No *survivin* mRNA was noted in the representative samples for the untreated (lane N_2: animal 2 in the untreated group) or the mineral-oil treated tissues (lane MO_2: animal 2 in the mineral-oil treated group), or the negative controls (NC); Mt: molecular-weight marker. (B) All the untreated (lanes N_1—N_6) and mineral-oil treated samples (lanes MO_1—MO_7) expressed a 297-bp PCR product indicating a *survivin*-methylated allele. There was no evidence of *survivin*-methylation in the DMBA-treated cancerous tissue (lanes 4 and 12: animals 4 and 12, respectively, in the experimental group). Correspondingly, no survivin protein was present in animal 2 in the untreated group (C) (ABC stain × 100, bar = 20 μm), while the protein was present in the animals 4 (D) (ABC stain × 100, bar = 20 μm) and 12 (E) in the experimental group (ABC stain × 100, bar = 20 μm).

proteins was detected in the tumour cells for all specimens of hamster buccal-pouch tissue treated with DMBA for 15 weeks (Fig. 1D and E).

The present study is the first time to demonstrate that DMBA-induced hamster buccal-pouch carcinomas express *survivin* (protein and mRNA). The expressions of protein and mRNA are consistent. Furthermore, this expression is highly cancer-specific because it was positive for 100% of the pouch carcinoma tissues, whereas there was no *survivin* expression for either the untreated or mineral-oil treated pouch tissues. This implies that *survivin* may play an important role in susceptibility to DMBA-induced squamous-cell carcinomas. However, whether the expression may also be an early event remains to be established in the DMBA-induced hamster buccal-pouch model. Subsequent study using two-stage carcinogenesis using this animal model would help to clarify this proposition. In addition, the results of the current study are compatible with those of a number of reports of human oral squamous-cell carcinomas,[24—27] however, different positive rates have also been reported.[24—27] A 100%-positive rate of expression was found in this animal study, compared to analogous reported rates ranging from 47.8% to 100% in previous human studies.[24—27] This contradictory result may be a reflection of the fact that the human studies utilized heterogeneous populations while the present study utilized a homogenous well-controlled animal sample.

Significantly, methylation of the *survivin* gene was noted for all our untreated and mineral-oil treated samples, whereas no methylation was determined for all of the DMBA-treated cancerous tissues. These findings indicate that *survivin* gene expression may be regulated by an epigenetic mechanism. The role of carcinogen in causing epigenetic changes has been well-documented.[28—30] Some environmental carcinogens such as cadmium,

arsenic and nickel have been implicated in epigenetic carcinogenesis.[28–30] Cadmium inhibits the DNMT1 DNA methyltransferase gene causing hypomethylation.[28] Arsenic has been found to induce *ras* hypomethylation[29] whereas nickel is implicated in altered DNA methylation and histone acetylation.[30] Therefore, in keeping with the aforementioned findings of these environmental carcinogens, it is reasonable to suggest that DMBA (a poetent chemical carcinogen) is contributed to the promoter hypomethylation of *survivin* in chemically induced hamster buccal-pouch carcinoma. Our results have also been consistent with those of a previous in vivo study of human oral squamous-cell carcinoma[25] and an in vitro investigation of an ovarian cancer cell line.[31]

As outlined above, highly selective cancer expression was determined for *survivin* in the DMBA-induced hamster buccal-pouch carcinomas in this report, suggesting that it could be a potential tumour marker in the hamster buccal-pouch model. In addition, previous studies have shown that *survivin* targeting using *survivin* antisense cDNA[32] or a mutant *survivin* adenovirus[33] may be a useful approach for selective cancer gene therapy. Taken together, our results indicate that the DMBA-induced hamster buccal-pouch mucosa may serve as a suitable experimental model for investigation of the potential of these targeting strategies as novel therapeutic tools for oral squamous-cell carcinomas.[34]

A significant correlation between *p53* and *iNOS* as well as an overexpression of both *p53* and *iNOS* have been reported in DMBA-induced hamster buccal-pouch carcinogenesis.[16–18] Furthermore, both *p63* and *p73* being two close relatives to p53 have also been found to be overexpressed in DMBA-induced hamster buccal-pouch carcinomas.[18,19] Recent evidence suggests that both survivin and p53 may be coupled to the apoptotic pathway through Apaf-1/caspase-9.[5] In addition to converging on the same components of the apoptotic pathway, there may be a direct relationship between *survivin* and *p53*, given the recent evident that wild-type *p53* binds to the *survivin* promoter in vivo and suppresses its gene expression.[35] Hence, further study to investigate the coexpression of *survivin* and *p53*, *p63*, *p73* and *iNOS* in hamster buccal-pouch model is highlighted in order to evaluate whether the epigenetic and genetic changes caused by DMBA may interact in that epigenetic alterations may determine the effect of subsequent genetic insults in chemically induced hamster buccal-pouch carcinogenesis, and the epigenetic and genetic changes may play a complementary role in experimentally induced oral carcinogenesis. A significant association between survivin expression and accumulation of mutant p53 has been demonstrated in gastric cancer.[36]

Finally, the wild-type adenomatous polyposis coli protein can regulate *survivin* expression in colon cancer.[37] It seems reasonable to suggest, therefore, that an investigation of the relationship between *survivin* expression and presence of these tumour suppressors in DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis is warranted.

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