Identification of the deleted in split hand/split foot 1 protein as a novel biomarker for human cervical cancer

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The morphological transformation of early neoplastic transformation leading to cervical cancer remains problematic. In this work, we have identified deleted in split hand/split foot 1 protein (DSS1) as an early biomarker that is specifically upregulated in premalignant and malignant cervical epithelial cells, but is low or undetectable in non-malignant cells. DSS1 mRNA and protein levels are significantly increased in cultured human cervical carcinoma cell lines originating from primary and metastatic tumors. In fact, >96% of patient tumor tissues were found to have cells with elevated DSS1 when compared with tumor-adjacent normal cells. In histological sections of cervical tissue containing either invasive cervical carcinoma or its precursor lesions, DSS1 was readily detected in the tumor cells. Steady-state DSS1 expression by immunomarked cervical cancer cell lines was found to be necessary for maintenance of their transformed phenotype, since stable shRNA-mediated depletion of DSS1 in HeLa cells inhibited their proliferation and colony-forming activity in monolayer cultures and prevented division of these cells in soft agar. When DSS1 levels are reduced using shRNA, the cells ultimately undergo apoptosis via activation of p53 and the p53 downstream targets, and cleavage of apoptosis-associated proteins including CPP32/caspase-3, poly(ADP-ribose)polymerase and DNA-PKcs. In addition, silencing of DSS1 makes cervical cancer cells sensitive to cell death after treatment with cisplatin. We conclude that the DSS1 protein is critically involved in the maintenance of the transformed phenotype in cervical cancer cells, and that it might be a specific, robust and reliable marker for early detection, diagnosis and treatment.

Introduction

Cervical cancer is a major cause of cancer-related death in women worldwide, in spite of extensive efforts to encourage regular Pap tests.

Abbreviations: AdCs, adenocarcinomas; AdSCCs, adenosquamous cell carcinomas; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; DSS1, deleted in split hand/split foot 1; FBS, fetal bovine serum; HPV, human papillomaviruses; HSIL or LSIL, high- or low-grade squamous intraepithelial lesions; HTBH, Hs68-TEV-Biotinylated region-Hs68; HRP, horseradish peroxidase; IB, immunoblotting; IHC, immunohistochemistry; LVSI, lymphovascular space involvement; MEM, modified Eagle’s medium; PARP, poly(ADP-ribose) polymerase; PIK3CA, phosphoinositide-3-kinase; R313M, RPN3/3-interacting motif; SCCs, squamous cell carcinomas; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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normal cervical tissue. Finally, we report that enforced lowering of DSS1 protein levels in cervical carcinoma cell lines inhibits cell proliferation and neoplastic transformation and causes apoptosis through a p53-dependent CPP32/caspase-3-mediated apoptotic pathway. Taken together, these results suggest that DSS1 may supply the second onco-

genic change, following HPV infection, leading to cervical carcinoma development from HPV-infected cervical epithelial cells.

Materials and methods

Collections of human cervical samples and cell lines

One set of clinical cervical specimens was collected from September 2005 through August 2008 at Chang Gung Memorial Hospital in Taiwan (27). The specimen donors were from patients with high-grade squamous intraepithelial lesions (CIN2 and CIN3), and from patients with early FIGO (The International Federation of Gynecology and Obstetrics) stages of cervical carcinomas receiving radical surgery. Enrollment of specimen donors was performed by a procedure that has been reviewed and approved by the Institutional Review Board (IRB) in Taiwan for the proposed activities. A second set of human specimen donors provided 26 individually matched normal/tumor tissue pairs, obtained from Protein Biotechnologies (San Diego, CA). Recruitment of these cervical tissue donors was under strict IRB-approved protocols, with informed consents, and the utmost attention to issues of patient safety and confidentiality. Finally, normal and malignant human cervical cell lines were obtained from the American Type Culture Collection (ATCC). These cells were cultured in complete medium, as indicated by ATCC, at 37°C in a humidified 95% air/5% CO₂ incubator. The complete medium was supple-

mented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), strepto-
mycin sulfate (100 µg/ml), l-glutamine (2 mM) and sodium pyruvate (1 mM). HeLa, C-33A and M5751 were grown in modified Eagle’s medium (MEM); HeLa S3, Hs68 and HNF in Dulbecco’s MEM; ME-180 and SiHa in McCoy’s 5A; HT-3 and CaSkii in RPMI-1640; C-41 and C-41II in Waymouth’s MB752/1. Human normal primary epidermal keratinocytes (ATCC) isolated from neonatal foreskin were grown in serum-free Dermal Cell Basal Media (ATCC) sup-
plemented with Keratinocyte Growth Kit (ATCC) components. All cell lines were routinely checked for mycoplasma contamination.

Preparation of total RNAs, cDNA synthesis and RT–PCR

Total RNAs were isolated using the TRIZOL reagent according to the manufac-
turer’s manual (Invitrogen, Carlsbad, CA). The single-stranded cDNAs prepared from total RNAs using MMLV SuperScript II reverse transcriptase and oligo-dT primer were employed as a template for PCR. The primers used for PCR were as follows: DSS1 sense, 5′-CACCAGTCATCGAGAGAAGGACGGCTGTA-3′; DSS1 antisense, 5′-CCGGGCTGAAGTGCTCCATCTTAAACCATG-3′; β-actin sense, 5′-CACCAGTCATCGAGAGAAGGACGGCTGTA-3′; β-actin antisense, 5′-CTGAAGAGCTTGCTGCGACGACTAATTC-3′. The primers were synthesized in the DNA Core Facility of the UT Health Science Center (San Antonio). PCR was performed in denaturation (94°C for 45 s), annealing (58°C for 45 s) and extension (72°C for 2 min) cycles, for 30 repetitions, in a Perkin-Elmer 9600 thermal cycler. Two oligos were denatured and re-annealed to form a duplex DNA that contains the small hairpin siRNA target sequence. The insert was then ligated into pGeneClip 3′ vector (Promega, Madison, WI) with the antibiotic marker hygromycin for selection of stable clones in eukaryotic cells.

Selection of the stable shDSS1 clones by RNAi-mediated gene silencing

Transfection of mammalian cells was carried out as described in Amaxa’s pro-
tocol (Lonza, Walkersville, MD). In brief, 2 × 10⁵ cells were electroporated with 4 µg of pGeneClip/NC1 or pGeneClip/shDSS1 in 100 µl of solution R by using program I-013 of the Nucleofector Device of Amaxa (Lonza). Cells were grown in the presence of Hygromycin B (400 µg/ml) to maintain the selection for 2–3 weeks at 37°C in a humidified 95% air/5% CO₂ incubator. DSS1 protein levels in the stable clones knocked down by DSS1 shRNA were compared using IB with a specific DSS1 antibody. Cells were seeded at 2 × 10⁴ or 5 × 10⁴ in 10 cm culture plates. Following selection with Hygromycin B (400 µg/ml) for 2–3 weeks, foci were fixed with 4% of 10% formalin at room temperature. After 2 h fixation, foci were stained with 3 µl of 1% Rhodamine B for 1 h at room temperature. The plates were washed under gentle stream of deionized water and placed upside down on bench to dry. The numbers of foci were counted in triplicate as described previously (22,28,29).

Anchorage-independent assay

Colony formation in soft agar was performed as described previously (22,28,29). In 60 mm culture dishes, cells were re-suspended at 1 × 10⁴ in 0.3% Immunoreactive protein bands were visualized using horseradish peroxidase (HRP)-conjugated mouse or rabbit IgG at 1:3000, and detected by enhanced chemiluminescence (GE Healthcare).

Immunohistochemistry

Adjacent tissue blocks of formalin-fixed, paraffin-embedded cervical tissues were obtained from diagnostic pathology laboratories. All of the sections had been evaluated by a clinical pathologist after hematoxylin and eosin staining (27). The paraffin sections were processed for blotting onto array slide as described previously (31). The array slides contained spot tissues from tumors at distinct progression cervical carcinoma stages, ranging from indi-

cient normal cervix, CIN2 (moderate dysplasia), CIN3 (severe dysplasia/carcinoma in situ) and invasive carcinomas, including SCCs and non-

SCCs (AdCs and AdSCCs). The array slides and full paraffin sections were incubated at 50–60°C for 10 min, and then de-paraffinized and re-hydrated by three successive washes in xylene and graded alcohols (100% → 95% → 70%). Heat-induced antigen retrieval was performed by incubation of the slides 70–75°C for 10 min in 10 mM sodium citrate. The arrays and sections were blocked with a proteinaceous blocking solution and then incubated at 4°C overnight to stop the reaction, counterstained and covered with a mounting medium. The specificity of antibody binding was validated by addition of DSS1 fusion pro-
tein to the antibody, followed by precipitation of antigen–antibody depletion of the immunogenic epitopes pairs and subsequent testing of the antibody for loss of its ability to bind to DSS1 on sections. The micrographs were taken under the light microscope (Leica, Allendale, NJ). The grades of DSS1 staining were evaluated by pathologists using the following categories (1); Score 0 was defined as no staining or dark cytoplasmic/nuclear staining of widely scattered cells (2); Score 1+ was considered as focal moderate staining in ≤50% of cells or pale cytoplasmic/nuclear staining in any proportion of cells not easily seen under a low power field (3); Score 2+ was evaluated as foci darkly staining areas (≥50% of cells) or moderate cytoplasmic/nuclear staining of ≤50% of cells; Score 3+ was graded as dark cytoplasmic/nuclear staining that was easily visible with a low power objective and involves >50% of cells.

Construction for DSS1 small hairpin RNA

Pre-designed silencer select negative controls, NC1 and NC2, and three specific siRNAs (Ambion, Carlsbad, CA), complementary to DSS1 RNA sequence, were validated by qPCR and IB, as described in our earlier stud-

ies (25). Two hairpin oligonucleotides with negative control 1 (NC1) and DSS1-specific siRNA sequences (shDSS1) were synthesized from Sigma–Aldrich, NC1 sense: 5′-TCTCGTAAAGCCGACGACTATCC TTTCTGCTAAATTTAGCTGTCG GCCGTAC-3′; DSS1 antisense: 5′-CTCAGGATAGCACGGCTAAAAATTAGAC GAAAGATTAC GTGCTCGCTTTGCTGTC-3′; shDSS1 sense, 5′-TCTCGGAAATAAGTTGGA ACTTGC-3′; shDSS1 antisense, 5′-CTCGGACCGTTTGGGAGATGTTCTG-3′; shDSS1 sense, 5′-CTCGAGGGAATATTGAGGTATCAGCTGACAGGAGGT CATCATCACCATTAC-3′. Two oligos were denatured and re-annealed to form a duplex DNA that contains the small hairpin siRNA target sequence. The insert was then ligated into pGeneClip 3′ vector (Promega, Madison, WI) with the antibiotic marker hygromycin for selection of stable clones in eukaryotic cells.
Noble Agar (Difco, Franklin Lakes, NJ) in 10% FBS/MEM and layered over 5 ml of 0.5% agar in MEM with 10% FBS. Cells were grown in the presence of Hygromycin B (400 µg/ml) to maintain the selection at 37°C in a humidified 95% air/5% CO2 incubator. Eighteen days after seeding, colonies with >8 cells were counted and photographed under light microscopy (DMI 6000B, Leica).

**DNA ladder assay**

Cellular DNA fragments were isolated as described previously (30). Adherent and non-adherent cells were pooled and lysed with buffer consisting of 50 mM Tris-HCl (pH = 7.5), 20 mM ethylenediaminetetraacetic acid and 1% NP-40. After centrifugation for 1 min at 800 g, the supernatant with DNA fragments was collected. The supernatant was brought to 0.2 µg/µl at 50°C for 1 h, and then reacted with proteinase K (2.5 µg/µl) at 37°C overnight. DNA was extracted using phenol/chloroform, precipitated with 1/10 vol of 3M sodium acetate and 2.5 vol of 100% ethanol and then dissolved in 10 mM Tris-HCl/1 mM ethylenediaminetetraacetic acid (pH = 7.6). The DNA fragments were separated by electrophoresis on 1.2% agarose gels and visualized by ChemiDoc/XRS systems (Bio-Rad).

**Expression vector construction**

Full-length human DSS1 cDNA fragment was generated by RT–PCR from HeLa total RNA using forward (5′-CACCACGATCGGCTGTCGAA AAGAAGACGCC-3′) and reverse primers (5′-CTCGAGACGCGTTGATG TCTCCATCTTGAACGGCTGCTT-3′), and the amplified blunt-end cDNA was directly cloned into the pDNA3.1D/V5-HisTOP6 expression vector (Invitrogen). The pDNA3.1/DSS1-V5-His plasmid was digested sequentially with Mlu I and Sgf I enzymes and the DSS1 insert was ligated into a retroviral vector pQCXIP, harboring a DNA sequence encoding a HTBH tag at its C-terminus (His6-TEV-Biotinylated region-His6) (kindly provided by Dr Lan Huang, Physiology and Biophysics, University of California, Irvine), using a LigaFast™ Rapid DNA Ligation System (Promega). All the construct sequences were verified using an automated Applied Biosystems sequencer and the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Plasmid DNAs were prepared using purification kits from Qiagen (Valencia, CA) and were endotoxin-free when used for transfection into mammalian cells.

**Cell transduction**

The packaging cells RetroPack™ PT-67 (Clontech, Mountain View, CA) were transfected with pQCXIP-HTBH or pQCXIP-DSS1-HTBH retroviral DNA using Lipofectamine PLUS™ reagents (Invitrogen). After 48 h of transfection, the virus-containing medium was collected, filtered and used for targeting into HeLa cells by infection. The recombinant pQCXIP retroviral vector carries HTBH tag or human DSS1-HTBH gene driven by the cytomegalovirus promoter and puromycin gene driven by the long terminal repeat promoter. The virus-infected HeLa stable clones were obtained after at least 2–3 weeks of selection in 10% FBS/MEM medium with 1 µg/ml puromycin (Sigma–Aldrich). Stable clones were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/IB using Streptavidin-HRP at 1:3000 to confirm the protein expression of HTBH or DSS1-HTBH.

**Statistical analysis**

A logistic regression model was employed to examine the effects of strong DSS1 immunohistochemistry (IHC) expression with score of 3+ on each of the pathological risk factors, and the univariate (unadjusted) and multivariate (adjusted) odds ratios in logistic regression were calculated. All statistical analyses were performed by using SPSS 17.0. P < 0.05 was considered statistically significant. Data were also subjected to statistical analysis by one-way analysis of variance (PRISM software version 4.0; GraphPad, San Diego, CA) or Student’s t-test.

**Results**

**Overexpression of DSS1 in human cervical cancer cell lines and in clinical specimens**

Our previous studies have demonstrated that the DSS1 plays a critical role in early skin carcinogenesis (22). Therefore, we hypothesized that...
Fig. 2. DSS1 protein levels in normal cervical cells versus preneoplastic and tumor tissues. (A) Representative photomicrographs showing DSS1 IHC staining in normal cervical epithelium. (B) DSS1 IHC staining in the preneoplastic stage CIN2 and CIN3 tissues, and invasive carcinomas, including SCCs and AdCs. DSS1 protein expression is indicated by solid arrows in brown color. CIN2: 3, 14, 32 and 39; CIN3: 4, 9, 21 and 23; SCCs (low and high): 11, 17, 37 and 69; AdCs: 3, 4, 7 and 19. (C) DSS1 staining percentages in normal tissues, HSIL, including CIN2 and CIN3, and invasive carcinoma (SCCs and non-SCCs). Micrographs, ×10 for low and ×40 for high magnification. Scale bar: 100 µm.
DSS1 may act as a cofactor to promote proliferation and malignant transformation of the cervical epithelium as well, toward the development of cervical cancer. To test this hypothesis, we first compared DSS1 mRNA and protein expression levels by RT–PCR and IB, respectively, in two kinds of immortalized human cervical carcinoma cell lines: one set derived from primary tumors (HeLa, HeLa S3, C-4 I, C-4 II, C-33A and SiHa), and the second set derived from distant metastatic tumors (ME-180/omentum, HT-3/lymph node, MS751/lymph node and CaSkii/small intestine). Figure 1A shows that levels of DSS1 mRNA were significantly upregulated in all of these cervical carcinoma cell lines, compared with levels for two human normal fibroblast lines (HS68 and HNF) and one human normal keratinocytes (HNK), ranging from 6.6- to 13.8-fold (Figure 1A). Similar results were obtained by IB, which disclosed 4.6- to 13.2-fold increased expression of the DSS1 protein in the tumor cells, as compared with the non-malignant fibroblasts and normal keratinocytes (Figure 1B).

We next examined the expression patterns of DSS1 protein in a cohort of specimens from patients with cervical cancer, which were collected as 26 sets of individually matched normal and tumor tissue pairs from patients having various stages and/or grades of cervical cancer. DSS1 protein levels were low or undetectable in cells of normal cervical tissue from each of the specimen pairs (Figure 1C). In contrast, DSS1 protein was highly expressed in >96% of the tumor tissues in these specimen pairs, and higher expression levels of DSS1 protein were readily observed when comparing cells of tumor tissue with cells of the adjacent normal cervix. DSS1 protein levels in the cells of tumor tissues ranged from 1.5- to 371-fold when compared with normal cells (Figure 1C and 1D).

Localisation of DSS1 protein in tissue arrays of invasive cervical carcinoma cells and cells from precursor lesions

To investigate the clinicopathological relevance of upregulated DSS1 protein expression and cervical cancer development in patients, we detected and scored DSS1 protein levels using IHC in 145 cervical specimens blotted in tissue array slides. These 145 tissue specimens from patients with cervical cancer, which were collected as 26 sets of individually matched normal and tumor tissue pairs from patients having various stages and/or grades of cervical cancer. DSS1 protein levels were low or undetectable in cells of normal cervical tissue from each of the specimen pairs (Figure 1C).

Table 1. Logistic regression analysis of clinicopathological factors in relation to strong DSS1 immunohistochemical staining (n = 47)

<table>
<thead>
<tr>
<th>Factors</th>
<th>N</th>
<th>DSS1 staining 3+ (%)</th>
<th>Unadjusted OR*</th>
<th>P-value*</th>
<th>Adjusted OR*</th>
<th>P-value*</th>
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<tr>
<td>SCC</td>
<td>23</td>
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<td>0.256</td>
<td>0.031</td>
<td>0.460</td>
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<td>Non-SCC</td>
<td>24</td>
<td>6 (25.0)</td>
<td>(0.074–0.885)</td>
<td>0.621</td>
<td>(0.114–1.854)</td>
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<td><strong>Grade</strong></td>
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<tr>
<td>Grade 1 and 2</td>
<td>34</td>
<td>13 (38.2)</td>
<td>1.385</td>
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<tr>
<td>Grade 3</td>
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<td>6 (46.2)</td>
<td>(0.381–5.037)</td>
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<td><strong>Tumor size</strong></td>
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<td>&lt;4 cm</td>
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<td>19 (44.2)</td>
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<tr>
<td>≥4 cm</td>
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<td>0 (0)</td>
<td>(0.439–1.204)</td>
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<td>&lt;2/3</td>
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<td>13 (37.1)</td>
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<td>≥2/3</td>
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<td>6 (50.0)</td>
<td>(0.451–6.355)</td>
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<td><strong>Nodal metastasis</strong></td>
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<tr>
<td>Negative</td>
<td>42</td>
<td>16 (38.1)</td>
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<td>3 (60.0)</td>
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<tr>
<td>Positive</td>
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<td>12 (66.7)</td>
<td>(1.717–23.009)</td>
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*OR, odds ratio with 95% confidence interval (CI).
*P < 0.05 is considered significant statistically.

Knockdown of endogenous DSS1 inhibits cellular proliferation and reverses neoplastic transformation

Our findings that DSS1 expression is elevated in cervical tumors suggest that DSS1 plays a pivotal role during the development of human cervical cancer. Therefore, we sought to determine whether the absence of functional DSS1 affects either cellular proliferation or focus-forming activity of cervical epithelial cells in culture, which has long been known to be necessary for cell transformation and clonal expansion of tumor cells (28). As shown in Figure 3A (left), three independent stable clones of HeLa cells (HeLa/shDSS1-C2, -C3 and -C15) were generated which had endogenous DSS1 that was depleted 80–90% when compared with three independent negative control stable HeLa clones (HeLa/Hygro-C4, -C5 and -C20). All three HeLa/shDSS1 stable clones had a decreased cellular proliferation rate by 50–80% when compared with HeLa/Hygro stable clones in which DSS1 was unaltered (Figure 3B; left top). In addition, the HeLa/shDSS1 stable clones exhibited a reduced colony-forming activity by 75% in monolayer cell cultures when compared with HeLa/Hygro cells (Figure 3C and 3D; left). Stable depletion of DSS1 also reduced the ability of transformed cells to proliferate in soft agar assays when comparing HeLa/shDSS1 cells with HeLa/Hygro cell clones (Figure 3E and 3F; left). Notably, however, DSS1 knockdown did not affect proliferation, focus-formation and soft agar cloning efficiency by C-33A and HT-3 cells, neither of which is infected by HPV (both are p53 mutated; Figure 3A–F; right).

Knockdown of endogenous DSS1 causes cell death through p53-dependent pathway

HeLa/shDSS1 cell clones were also compared with negative control HeLa/Hygro cell clones for traits of cellular apoptosis. Figure 4A and 4B shows that cell detachment, cell shrinkage, membrane blebbing, cell pyknosis and margination of nuclear chromatin were readily detectable in HeLa/shDSS1 cells (e.g. C2, C3 and C15), whose DSS1 was knocked down, whereas no such effect was observed in the HeLa/Hygro stable clones whose DSS1 levels were unaltered (e.g. C20). In standard protocols, these clones were infected with HPV. In addition, all three clones were infected with HPV (both are p53 mutated; Figure 3A–F; right).
Fig. 3. Growth inhibitory effects of knockdown of endogenous DSS1 in human cervical carcinoma cells. (A) Selection of the stable DSS1-knockdown clones in HeLa, C-33A and HT-3 cells. The independent pGeneClip/NC1- and shDSS1-transduced stable clones were examined by IB using DSS1-specific antibody (1 µg/ml). Actin was used as an internal loading control. (B) HeLa, but not C-33A or HT-3, DSS1-knockdown stable clones lose growth advantage in monolayer cultures. Growth curves were generated for cells stably expressing NC1 (HeLa/Hygro-C4, -C5 and -C20; C-33A/Hygro-C1; or HT-3/Hygro-C1) or shDSS1 (HeLa/shDSS1-C2, -C3 and -C15; C-33A/shDSS1-C1; or HT-3/shDSS1-C2). Cells were plated at 1 × 10⁴/well onto 6-well plates and grown in MEM (HeLa and C-33A) or RPMI-1640 (HT-3) with 10% FBS in the presence of 400 µg/ml Hygromycin B. The medium was changed every 2–3 days and the cell numbers were counted in triplicate for the following 12 days. Data represent the mean ± SEM of three independent experiments. *HeLa/Hygro-C5 cell proliferation is significantly higher than that of HeLa/shDSS1-C15 cells (P < 0.01). **HeLa/Hygro-C5 is significantly greater than HeLa/shDSS1-C15 (P < 0.001). (C) and (D)
Knockdown of endogenous DSS1 in HeLa cells abrogated focus-forming activity. The DSS1 knockdown cells [HeLa/shDSS1-C2 and -C15 (2 × 10³ each), C-33A/shDSS1-C1 (5 × 10³) and HT-3/shDSS1-C2 (5 × 10³)] and the DSS1-intact cells [HeLa/Hygro-C4 and -C20 (2 × 10³ each), C-33A/Hygro-C1 (5 × 10³) and HT-3/Hygro-C1 (5 × 10³)] were seeded and selected in 400 µg/ml Hygromycin B for 2–3 weeks. Foci were stained with 1% rhodamine B for 1 h at room temperature. The numbers of foci were counted in triplicate. Data represent the mean ± SEM of three independent experiments. *HeLa/Hygro-C4 is significantly greater than HeLa/shDSS1-C2 (P < 0.005). †HeLa/Hygro-C20 is significantly greater than HeLa/shDSS1-C15 (P < 0.005).

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Knockdown of endogenous DSS1 in HeLa cells abrogated focus-forming activity. The DSS1 knockdown cells [HeLa/shDSS1-C2 and -C15 (2 × 10³ each), C-33A/shDSS1-C1 (5 × 10³) and HT-3/shDSS1-C2 (5 × 10³)] and the DSS1-intact cells [HeLa/Hygro-C4 and -C20 (2 × 10³ each), C-33A/Hygro-C1 (5 × 10³) and HT-3/Hygro-C1 (5 × 10³)] were seeded and selected in 400 µg/ml Hygromycin B for 2–3 weeks. Foci were stained with 1% rhodamine B for 1 h at room temperature. The numbers of foci were counted in triplicate. Data represent the mean ± SEM of three independent experiments. *HeLa/Hygro-C4 is significantly greater than HeLa/shDSS1-C2 (P < 0.005). †HeLa/Hygro-C20 is significantly greater than HeLa/shDSS1-C15 (P < 0.005).

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addition, DNA fragmentation, with multiple internucleosomal fragments of 180 bp, were clearly observed in DNA from HeLa/shDSS1 stable clones (C2 and C15) when compared with HeLa/Hygro control cells, whereas no such effects were observed in HeLa/Hygro-C20 control cells. Micrographs, ×10 for low and ×20 and ×40 for high magnification. Scale bar: 100 µm. (C) DNA laddering patterns for DSS1-knockdown versus DSS1-intact cells. DNA fragmentation patterns were clearly observed in DNA preparations from the HeLa DSS1-knockdown clones (HeLa/shDSS1-C2 and -C15), but was not observed in the control clones from HeLa (HeLa/Hygro-C4 and -C20), C-33A (C-33A/Hygro-C1) and HT-3 (HT-3/Hygro-C1), or in the DSS1-knockdown C-33A/shDSS1-C1 and HT-3/shDSS1-C2 cells. (D) Immunoblots of lysates (20 µg) were prepared from DSS1-knockdown clones HeLa/shDSS1-C2 and -C15, DSS1-intact HeLa/Hygro-C4 and -C20 and p53-mutant, DSS1-knockdown C-33A/shDSS1-C1 and HT-3/shDSS1-C2 cells. The blots were incubated with antibodies against DSS1 (1 µg/ml), p53 (1:1000), p21WAF1/CIP1 (1:1000), CPP32/caspase-3 (1:1000), PARP (1:1000), DNA-PKcs (1:1000) and actin (1:2000) (serves as a loading control). (E) Knockdown of p53 in stable clones with a background of downregulated DSS1 rescues cells from death by apoptosis. RNAi transfections were carried out using 100 nM silencer® select validated negative control 1 (NC1) and human p53-specific siRNAs (Ambion; #4390824) (sense: GUAAUACUCUGAGAUCUAC) in DSS1-knockdown cells, HeLa/shDSS1-C2 and -C15. In brief, cells (2 × 10⁶) were electroporated by using program I-013 of the Nucleofector Device (Amaxa). Cells were then cultured and harvested for protein preparation at 48 h after electroporation. The protein levels knocked down by siRNAs were examined by using IB with a specific anti-p53 antibody as indicated. DNA laddering patterns were clearly reduced in DNA preparations from p53-knockdown HeLa/shDSS1-C2 and -C15 stable clones.

**Fig. 4.** Knockdown of endogenous DSS1 causes p53-mediated apoptosis. (A) and (B) shDSS1-induced morphological alterations of HeLa cells observed in monolayer cultures. Characteristics of apoptosis like cell shrinkage and membrane blebbing, indicated by solid arrows, were conspicuous as seen under light microscopy in HeLa/shDSS1-C2, -C3 and -C15 cells, whereas no such effects were observed in HeLa/Hygro-C20 control cells. Micrographs, ×10 for low and ×20 and ×40 for high magnification. Scale bar: 100 µm. (C) DNA laddering patterns for DSS1-knockdown versus DSS1-intact cells. DNA fragmentation patterns were clearly observed in DNA preparations from the HeLa DSS1-knockdown clones (HeLa/shDSS1-C2 and -C15), but was not observed in the control clones from HeLa (HeLa/Hygro-C4 and -C20), C-33A (C-33A/Hygro-C1) and HT-3 (HT-3/Hygro-C1), or in the DSS1-knockdown C-33A/shDSS1-C1 and HT-3/shDSS1-C2 cells. (D) Immunoblots of lysates (20 µg) were prepared from DSS1-knockdown clones HeLa/shDSS1-C2 and -C15, DSS1-intact HeLa/Hygro-C4 and -C20 and p53-mutant, DSS1-knockdown C-33A/shDSS1-C1 and HT-3/shDSS1-C2 cells. The blots were incubated with antibodies against DSS1 (1 µg/ml), p53 (1:1000), p21WAF1/CIP1 (1:1000), CPP32/caspase-3 (1:1000), PARP (1:1000), DNA-PKcs (1:1000) and actin (1:2000) (serves as a loading control). (E) Knockdown of p53 in stable clones with a background of downregulated DSS1 rescues cells from death by apoptosis. RNAi transfections were carried out using 100 nM silencer® select validated negative control 1 (NC1) and human p53-specific siRNAs (Ambion; #4390824) (sense: GUAAUACUCUGAGAUCUAC) in DSS1-knockdown cells, HeLa/shDSS1-C2 and -C15. In brief, cells (2 × 10⁶) were electroporated by using program I-013 of the Nucleofector Device (Amaxa). Cells were then cultured and harvested for protein preparation at 48 h after electroporation. The protein levels knocked down by siRNAs were examined by using IB with a specific anti-p53 antibody as indicated. DNA laddering patterns were clearly reduced in DNA preparations from p53-knockdown HeLa/shDSS1-C2 and -C15 stable clones.
Thus, knocking down 80% of p53 expression in protein level can rescue DSS1-knockdown cells (HeLa/shDSS1-C2 and -C15) from death by apoptosis. Taken together, these data suggest that sustained DSS1 expression suppresses p53 expression and p53-dependent apoptosis in cervical squamous epithelial cells, and that removal of functional DSS1 can reverse the apoptotic defect in cells with wild-type p53 but not mutant p53.

The effects of DSS1 overexpression and knockdown on cisplatin cytotoxicity

To investigate the possible involvement of DSS1 overexpression in the resistance of human cervical epithelial cancer cells to cisplatin, HeLa cells stably expressing HTBH or DSS1-HTBH, were treated with cisplatin at concentrations ranging from 6.25 to 50 µM for 48 h followed by a CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay. As shown in Figure 5A, the protein expression was detected by IB using streptavidin-HRP at a physiological level in HeLa/HTBH (C2 and C16) and HeLa/DSS1-HTBH (C9 and C22) stable clones. In addition, overexpression of DSS1 led to a significant increase in cell viability after treatment with 12.5 µM cisplatin in stable clone HeLa/DSS1-HTBH-C9 compared with the HeLa/HTBH-C2 control cells (*P < 0.001) (Figure 5B). Interestingly, the cisplatin IC50 was significantly higher in HeLa/DSS1-HTBH (C9 and C22) versus HeLa/HTBH (C2 and C16) control cells (Figure 5B). Our results suggest that ectopically enforced constitutive expression of DSS1 can enhance the chemoresistance of human cervical cancer cells to cisplatin. On the contrary, to determine whether downregulation of DSS1 expression can sensitize human cervical cancer cells to cisplatin and cause cell death. To address this, DSS1-knockdown (HeLa/shDSS1-C2 and -C15) and DSS1-intact (HeLa/Hygro-C4 and -C20) cells were treated with various concentrations of cisplatin for 48 h, followed by incubation with MTS reagents for 4 h. Cell viability was measured by MTS assay, as absorbance at 490 nm using an ELISA plate reader to assess sensitivity. Data represent the mean ± SEM of three independent experiments.

Discussion

The goal of this study was to better understand the role of DSS1 in the molecular pathogenesis of human cervical cancer by using well-defined clinical specimens and in vitro cell culture model systems. Here, we have demonstrated five principal findings: (i) DSS1 mRNA and/or protein are overexpressed in human cervical carcinoma cell lines and in tumors from patients with high grades and/or stages of
cervical cancer (Figure 1); (ii) in cervical tissue, DSS1 protein is specifically expressed and localized in the highly proliferative squamous or glandular regions, and is elevated during early high-grade squamous intraepithelial lesions (HSIL) (Figure 2); (iii) knockdown of endogenous DSS1 inhibits proliferation, membrane activity, and prevents the proliferation of cancerous cervical epithelial cells in soft agar (Figure 3); (iv) functional knockdown of DSS1 results in apoptosis mediated through a functional p53-dependent signaling pathway (Figure 4); and (v) silencing of DSS1 confers sensitivity to the DNA-damaging agent cisplatin in human cervical cancer cells (Figure 5). We conclude that DSS1 protein is a novel biomarker for early detection, diagnosis and treatment of human cervical carcinomas.

A previous study from our laboratory showed that constitutive expression of DSS1 in JB6 Cl 41-5a preneoplastic epidermal cells was positively correlated with increased cell growth rate, elevated focus-forming ability and enhanced cellular transformation, suggesting that DSS1 has oncogenic properties in early skin carcinogenesis (22). DSS1 mRNA was highly expressed and localized to the rapidly proliferative squamous regions of TPA-induced papillomas and malignancies, with some expression in the adjacent epidermis and hair follicles (22). DSS1 protein was also markedly increased in TPA-induced skin hyperplasia, papillomas and malignant tumors in mice (22), and was also significantly upregulated in UV-induced SCCs (32). In agreement with these previous results, we have shown here that DSS1 is highly expressed in metaplastic squamous or glandular tissues, and that it is elevated even more in HSIL, including CIN2 and CIN3/CIS (Figure 2B and 2C). These data suggest to us that upregulation of DSS1 protein is a critical event during the progression of precancerous lesions into more invasive cervical carcinomas. It will be interesting to determine whether DSS1 upregulation is a universal event for epithelial cell carcinogenesis or whether it is limited to specific epithelial cell types.

Notably, cervical cancer patients with LVSI had tumors whose cells exhibited a significant increase in DSS1 protein expression levels (strong staining with score of 3+) when compared with tumors from patients without LVSI (Table 1 and Supplementary Table 1, available at Carcinogenesis Online). Several studies have recently identified LVSI as an independent surgical-pathological predictor of recurrence, exclusively for bulky stage IB cervical cancer, and LVSI is used as a significant prognostic factor for shortened disease-free survival, despite an early stage cervical cancer diagnosis (33,34). Although LVSI as an independent risk factor is controversial, it has been shown to be one of the independent predicting factors for pelvic node metastases in numerous peer-reviewed studies (35,36). This nodal status is considered to be the major prognostic factor affecting survival among women with early stage cervical cancer (37,38). Currently, LVSI is one of three intermediate-risk factors predictive of early invasive cervical carcinoma after radical surgery, and all three may be useful in decision making for the use of post-operative adjuvant radiation therapy. Our results strongly suggest that upregulation of DSS1 protein expression is potentially positively correlated with the risk of tumor recurrence and negatively associated with disease-free survival in early stage cervical cancer patients with no apparent lymph node metastasis. Therefore, it will be important to assess clinical relevance of DSS1 expression for the cases of advanced-stage cervical cancer with concurrent cisplatin-based chemoradiation, which is a standard therapy at present, to determine whether tumors whose cells have strong DSS1 expression (+), either in the cytoplasm and/or nucleus, predict tumor recurrence and a shorter survival time.

Cervical cancer patients are almost always infected by high-risk types of HPV such as 16, 18, 31, 33 and 45 (27). However, only 10% of HPV-infected patients will develop a series of precancerous lesions, including LSIL and HSIL, and only about 0.8% of cases will progress to the more invasive cervical cancers like SCCs and AdCs (1,4). In a mouse model for cervical cancer, where the HPV16 oncogenes, E6 and E7, have been transduced into the normal cervix, tumor promoters like TPA or mutagens such as N-methyl-N-nitro-N-nitrosoguanidine promote tumor development in a dose-dependent fashion (39). This work indicates that HPV acts as an initiator to induce intraepithelial dysplastic changes, although oncoprotein expression in infected cells is not sufficient to trigger tumor formation. Thus, a cofactor is required to promote cervical neoplastic progression. Here, we have provided data that support our central hypothesis that DSS1 plays a pivotal role in the molecular pathogenesis of human cervical cancer. It follows that identifying agents that elevate levels of DSS1 may provide the second event, or cofactor, necessary for full transformation of oncogenic HPV-infected cervical cells. Although we did not detect the expression level of DSS1 protein in patient 17, it could be due to association of any other pathways like PI3KCA, which is frequently involved in cellular proliferation (immortality) and neoplastic (malignant) transformation during the development of cervical cancer (9).

Previous studies have shown that treatment of human cervical carcinoma cells with the proteasome inhibitor, bortezomib, restores normal expression levels and functions of p53, which in turn triggers the death of the treated cells through the CPP32/caspase-3-dependent apoptotic pathway (40). It is interesting in this regard that HPV-positive cervical cancer cells are more sensitive to bortezomib or to another proteasome inhibitor, MG-132, than are HPV-negative cervical cancer cells or primary human keratinocytes (40,41). Additionally, downregulation of HPV16 or HPV18 E6/E7 by specific E6 siRNA also leads to growth inhibition of HPV16 or HPV18-positive cervical cancer cells, and eventually causes cell death via CPP32/caspase-3-mediated p53-dependent apoptosis (42,43). More recent work has shown that targeting E6 or XIAP in combination with cisplatin can efficiently potentiate rhTRAIL-induced CPP32/caspase-8/-3-mediated apoptosis in HPV-positive cervical cancer cells (44). In this study, we have shown that DSS1 expression is upregulated in cervical tumors, and that enforced downregulation of DSS1 in tumor cells makes them susceptible to apoptosis in a p53-dependent manner, suggesting that the proteasome, HPV oncoproteins and DSS1 function in a linear pathway to disturb regulatory mechanisms that govern cervical epithelial cell proliferation and apoptosis. It will be important now to determine how oncogenic HPV infection affects DSS1 expression immediately after infection, because a cause and effect relationship has not yet been established.

In summary, this research is innovative in identifying the DSS1 protein as a novel potential biomarker for early detection, diagnosis and treatment of human cervical cancer. First, we have demonstrated that DSS1 is specifically expressed and localized in the highly proliferative regions of the human cervical carcinoma. Secondly, tumors from patients with higher grades and/or stages of cervical cancer contain cells with significantly higher levels of DSS1 expression, compared with cells from tumor-adjacent normal cervical tissue. Thirdly, we have more fully elucidated the mechanisms by which knockdown of DSS1 expression causes cell death and reverses neoplastic transformation of cervical cancer cells in vitro. Lastly, our results point to a novel therapeutic strategy that may reverse tumor cell drug resistance (e.g. cisplatin) through inactivation of DSS1 expression in the cells.

Supplementary material
Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/.

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