The Tumor Suppressor UCHL1 Forms a Complex with p53/MDM2/ARF to Promote p53 Signaling and Is Frequently Silenced in Nasopharyngeal Carcinoma

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Abstract

Purpose: Nasopharyngeal carcinoma is prevalent in southern China and Southeast Asia, with distinct geographic and ethnic distribution. One candidate susceptibility locus has been identified at 4p11-14, with the associated candidate gene(s) not identified yet. This study investigated the role of ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) in nasopharyngeal carcinoma pathogenesis.

Experimental Design: UCHL1 expression and methylation were examined in nasopharyngeal carcinoma. Furthermore, the mechanism of its tumor-suppressive function was elucidated in nasopharyngeal carcinoma cells.

Results: Through genomewide expression profiling, we identified UCHL1, a 4p14 gene normally expressed in normal upper respiratory tract tissues, being silenced in all nasopharyngeal carcinoma cell lines. Its silencing is mediated by CpG methylation because UCHL1 promoter methylation was detected in all silenced cell lines, and pharmacologic demethylation reactivated UCHL1 expression along with concomitant promoter demethylation. UCHL1 methylation was also frequently detected in primary tumors but only weakly detected in few normal nasopharyngeal tissues, indicating that the methylation-mediated silencing of UCHL1 is important in nasopharyngeal carcinoma pathogenesis. Ectopic UCHL1 expression dramatically inhibited the growth of nasopharyngeal carcinoma cells through promoting tumor cell apoptosis. We further found that UCHL1 formed a complex with p53/p14ARF/Mdm2 p53 binding protein homolog (mouse), MDM2 and activated the p53 signaling pathway. UCHL1 expression extended p53 and p14ARF protein half-life and shortened MDM2 protein half-life.

Conclusions: These results indicate that UCHL1 could deubiquitinate p53 and p14ARF and ubiquitinate MDM2 for p53 stabilization to promote p53 signaling, thus involved in nasopharyngeal carcinoma pathogenesis, whereas it is frequently silenced in this tumor.

Nasopharyngeal carcinoma is rare in most part of the world but prevalent in southern China, Southeast Asia, and some north African areas (1, 2). Its incidence is ~100-fold higher in southern Chinese of Cantonese origin than in Caucasians in Western countries. Southern Chinese who immigrated to other Western countries also have a higher risk for nasopharyngeal carcinoma as compared with local population (3). Familial clustering of nasopharyngeal carcinoma has been well documented in not only southern Chinese population but also non-Chinese, low-risk populations (3). These unique features strongly indicate the critical role of genetic susceptibility in its development. In a recent genomewide linkage analysis of nasopharyngeal carcinoma pedigrees from Cantonese-speaking Chinese, one susceptibility locus was identified at 4p11-p14 (4, 5), indicating the presence of cancer genes, including tumor suppressor genes, critical for nasopharyngeal carcinoma development at this locus.

Inactivation of tumor suppressor genes is important for tumor development (6), through either genetic or epigenetic alterations or a combination of both (7). Epigenetic silencing of tumor suppressor genes, including promoter CpG island methylation and histone modifications, leads to the disruption of tumor suppressor gene functions and involves in tumor development (8). A growing number of
tumor suppressor genes, such as p16, RASSF1A, BLU, CHFR, TSLC1, DLC1, PCDH10, and WIFI, have been found silenced by promoter methylation in nasopharyngeal carcinoma (2). Located at 4p14, ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) is a member of the ubiquitin carboxy terminal hydrolase family targeting ubiquitin-dependent protein degradation pathway, with both ubiquitin hydrolase and dimerization-dependent ubiquitin ligase activities (9), playing important roles in multiple cellular processes such as proliferation, cell cycle, apoptosis, and intracellular signaling.

The role of UCHL1 in tumorigenesis has been complex, from tumor suppressive to oncogenic, depending on the tumor type (10–14). UCHL1 methylation has been reported in multiple tumors, including esophageal (15), gastric (16), renal (17), prostate (18), head and neck squamous cell carcinoma (19), hepatocellular (12), ovarian (20), and colorectal cancers (12, 21), supporting a critical role of UCHL1 in tumor suppression. UCHL1 methylation could be used as a biomarker for the diagnosis and prognosis prediction of certain tumors (10, 15, 21, 22). However, the role of UCHL1 in nasopharyngeal carcinoma pathogenesis remains unclear. In addition, the association of UCHL1 with human diseases is puzzled by its dual enzymatic activities, with little known about its two opposing enzymatic activity in cancer pathogenesis.

Through expression subtraction, suppression subtractive hybridization, we identified UCHL1 as a downregulated gene in nasopharyngeal carcinoma, making it a candidate tumor suppressor gene for this tumor. Here, we fully characterized its epigenetic alteration and functions in nasopharyngeal carcinoma cells. We found that UCHL1 was frequently silenced by promoter CpG methylation in nasopharyngeal carcinoma; it acts as a functional tumor suppressor gene for nasopharyngeal carcinoma through stabilizing p53 through deubiquitinating p53 and p14ARF alternative reading frame (ARF) and ubiquitinating MDM2, which is mediated by its hydrolase and ligase activities, further resulting in the induction of tumor cell apoptosis.

**Materials and Methods**

**Cell lines and samples**

Nasopharyngeal carcinoma cell lines (C666-1, CNE1, HK1, HONE1, and HNE1) and one immortalized but non-transformed nasopharyngeal epithelial cell line (NP69) were used (23). All nasopharyngeal carcinoma cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. NP69 was cultured as described (24). For pharmacologic demethylation, cells were freshly seeded at a density of 1 × 10⁶ cells/mL. After overnight culture, cells were treated with 5 or 10 μmol/L of DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (Sigma) for 3 days. Primary tumor and normal nasopharyngeal tissue samples have been described previously (25–29). Genomic DNA and total RNA were extracted with TRI Reagent (Molecular Research Center, Inc.). Normal adult tissue RNA samples were purchased commercially (26).

**Antibodies**

Antibodies used were UCHL1 (ab10404; Abcam), cleaved caspase-3 (9661), cleaved poly(ADP-ribose) polymerase (PARP; 9541; Cell Signaling), p53 (M7001), anti-mouse IgG–horseradish peroxidase (P0161), anti-rabbit IgG–horseradish peroxidase (P0448; Dako), p21(OP64; Calbiochem), p14(sc-8340), MDM2 (sc-813), His (sc-804), hemagglutinin (sc-7392; Santa Cruz), green fluorescent protein (GFP; Roche), α-tubulin (Lab Vision Corporation).

**Construction of the UCHL1-expressing vector**

The full-length open reading frame of UCHL1 was obtained by reverse transcriptase-PCR (RT-PCR) amplification of human normal trachea RNA (Clontech) using the high-fidelity Accuprimmer Taq polymerase (Invitrogen). The PCR product was then cloned into the pCR4-TOPO vector (Invitrogen). After sequence verification, the insert was subcloned into neomycin-resistant mammalian expression vector pcDNA3.1 (-) using the XbaI and KpnI sites. The plasmids for transfection were prepared with endotoxin-free QIAGEN plasmid preparation midi kit (QIAGEN).

**Semiquantitative RT-PCR**

Total RNA was reverse-transcribed using MuLV reverse transcriptase (Applied Biosystems) and random hexamer. PCR was done using Go-Taq (Promega) as previously described (12). The UCHL1-specific primers are 5′-AGCT-CAAGCCGATGGAGATC (forward) and 5′-CCCTTCAG-CTCTTCAATCTG (reverse). PCR was done for 32 cycles with the annealing temperature of 55°C. GAPDH was used as a control to assess the overall cDNA content.

**Methylation-specific PCR**

Genomic DNA was chemically modified with sodium metabisulfite (30, 31). The bisulfite-modified DNA was...
amplified using primer pairs that specifically amplify either methylated or unmethylated sequences of the UCHL1 promoter. The primers specific for methylated sequence are 5′-TTTATTTGGTCGCGATCGTTC and 5′-AAACTA-CATCTTCGCGAAACG. The primers specific for unmethylated sequence are 5′-GGGTTTGTATTTATTTGGTTGT and 5′-CTTAAACTACATCTTCACAAAACA. All methylation-specific PCR primers have been tested to be specific, without amplifying any not-bisulfited genomic DNA sample. Methylation-specific PCR was done for 38 cycles with the annealing temperature of either 62°C (methylated) or 58°C (unmethylated).

**Bisulfite genomic sequencing**

Bisulfite genomic sequencing was done to confirm the methylation-specific PCR results (30, 31). Briefly, bisulfite-treated genomic DNA was amplified with bisulfite genomic sequencing primers (containing no CpG site), specific for a fragment of the UCHL1 CpG island containing 49 CpG sites and spanning the sites analyzed by methylation-specific PCR (Fig. 1A). PCR was done for 40 cycles with the annealing temperature of 55°C. The primers used are 5′-GGTTTTATATATTGGAATATTT and 5′-CTTAAACTACATCTTCACAAAACA. The PCR products were cloned into the pCR4-TOPO vector (Invitrogen). At least five colonies were randomly chosen...
for sequencing, with the M13 reverse primer and the ABI Prism Dye Terminator Cycle Sequencing Kit using an ABI Prism 3100 DNA Sequencer (Applied Biosystems).

**Colony formation assay**

Colony formation assay was carried out as previously described (26). Briefly, cells were cultured overnight in a 12-well plate (1.0 × 10⁵ per well) and transfected with empty vector or the UCHL1 expressing vector using FuGENE 6 (Roche). Forty-eight hours later, the transfectants were replated in triplicate and cultured for 10 to 15 days in complete medium containing G418 (400 μg/mL). Surviving colonies were stained with gentian violet after methanol fixation and visible colonies (≥50 cells) were counted.

**Immunoprecipitation and Western blot**

CNE1 or HONE1 cells were transfected with UCHL1-expressing plasmid. Forty-eight hours after the transfection, cellular lysates were prepared by incubating the cell pellets in lysis buffer (50 mmol/L Tris-HCl, pH 8.0; 150 mmol/L NaCl; 0.5% NP40) for 30 minutes on ice, followed by centrifugation at 14,000 × g for 15 minutes at 4°C. For immunoprecipitation, 200 μg of protein was incubated with specific antibodies (1-2 μg) overnight at 4°C with constant rotation; then 20 μL of protein A or G agarose beads was added and incubated for an additional 2 hours. Beads were then washed five times using the lysis buffer. Between washes, the beads were collected by centrifugation at 3,000 × g for 30 seconds at 4°C. The precipitated proteins were eluted from the beads by resuspending the beads in 2× SDS-PAGE loading buffer and boiling for 5 minutes. Immunoprecipitation proteins or cell lysates were resolved using SDS-PAGE gels and transferred onto nitrocellulose membranes. For Western blot, membranes were incubated with appropriate antibodies for 1 hour at room temperature or overnight at 4°C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using Western blot Luminol reagent (GE Healthcare Bio-Sciences) according to the manufacturer’s protocol.

**Half-life analysis**

CNE1 or HONE1 cells cultured in 24-well plates were transiently transfected with UCHL1 expression plasmid and cultured for 40 hours. The cells were then incubated with cycloheximide (20 μg/mL; Sigma) to inhibit further protein synthesis. Following incubation for 0, 5, 10, 20, 40, or 60 minutes, cells were harvested. Western blot was done as described above. The relative p53, MDM2, and p14ARF levels were quantified by densitometry analysis using the ImageJ1.410 image processing software.
Ubiquitination assay

CNE1 or HONE1 cells were cotransfected with His-tagged ubiquitin and UCHL1-expressing plasmids. Cells were subjected to lysis as described above and analyzed by immunoprecipitation with p53, MDM2, and p14ARF antibodies, followed by immunoblot with His antibody to detect ubiquitinated p53, MDM2, and p14ARF proteins. HEK293T cells were transfected with UCHL1 (0.5 or 1 μg), GFP-p53, HA-MDM2, p14ARF, or His-tagged single-lysine 48 ubiquitin plasmids, respectively. After treatment as described above, ubiquitinated p53, MDM2, or p14ARF was immunoprecipitated from the cell extracts.

Fig. 3. Re-expression of UCHL1 inhibits nasopharyngeal carcinoma cell growth and induces apoptosis through activating the p14ARF-p53 tumor suppressive pathway. A, the effect of ectopic UCHL1 expression on nasopharyngeal carcinoma cell growth was assessed by monolayer colony formation. Quantitative analyses of colony numbers are shown in the right as values of mean ± SD. Ps were calculated using the Student’s t test. *, P < 0.01. B, Western blot showed cleaved caspase-3 and PARP in UCHL1-transfected CNE1 and HONE1 cells. C, Western blot analysis was done using p53, p14ARF, MDM2, and p21 antibodies. α-Tubulin expression level was used as a control.
with the relevant antibody, followed by immunoblot with His antibody to detect ubiquitinated p53, MDM2, or p14ARF proteins.

Results

Identification of UCHL1 as a downregulated gene in nasopharyngeal carcinoma

To screen for deregulated genes in nasopharyngeal carcinoma, we did suppression subtractive hybridization using nasopharyngeal carcinoma cell lines and the immortalized but nontransformed nasopharyngeal epithelial cell line NP69 (32). Among a few target genes identified, one is the UCHL1 (Fig. 1A). Meanwhile, independent experiments comparing the whole-genome expression profiles between nasopharyngeal carcinoma cell lines and NP69 with Affymetrix cDNA microarrays also identified this gene as a downregulated gene in nasopharyngeal carcinoma.6 We thus examined UCHL1 expression by semiquantitative RT-PCR in nasopharyngeal carcinoma cell lines. UCHL1 is highly expressed in normal trachea and larynx tissues, as well as NP69, but downregulated or totally silenced in nasopharyngeal carcinoma cell lines (Fig. 1B).

Promoter CpG methylation of UCHL1 in nasopharyngeal carcinoma cell lines

We further evaluate whether methylation is involved in UCHL1 silencing in nasopharyngeal carcinoma. A typical CpG island spanning the UCHL1 exon 1 was identified by CpG island Searcher (http://ccnt.hsc.usc.edu/cpgislands2; Fig. 1A), suggesting that UCHL1 is subject to methylation-mediated silencing. We thus examined its methylation using methylation-specific PCR. UCHL1 was methylated in all silenced cell lines but only partially methylated in NP69, with no methylation detected in normal trachea and larynx tissues (Fig. 1B). We also confirmed UCHL1 methylation by high-resolution bisulfite genomic sequencing analysis of 49 CpG sites within the CpG island, including CpG sites analyzed by methylation-specific PCR. Nearly all CpG sites examined were methylated in nasopharyngeal carcinoma cell lines (Fig. 1C).

Demethylation restored UCHL1 expression and frequent UCHL1 methylation in primary tumors

To assess whether CpG methylation is responsible for the transcriptional silencing of UCHL1, we treated nasopharyngeal carcinoma cell lines with 5-aza-2-deoxycytidine (32, 33). UCHL1 expression was dramatically reactivated in all cell lines after drug treatment, along with the evidence of promoter demethylation (Fig. 2A), showing that CpG methylation of the UCHL1 promoter silences its expression in tumor cells.

Fig. 4. UCHL1 regulate p53, MDM2, and p14ARF stability. HONE cells (A) and CNE1 cells (B) were transfected with UCHL1. Forty hours later, cells were treated with 20 μg/mL cycloheximide for the indicated periods, followed by Western blot using antibody against endogenous p53, MDM2, p14ARF, or UCHL1, with α-tubulin as the loading control (left). The relative p53, MDM2, and p14ARF levels were quantified by densitometry analysis (right).

We next investigated UCHL1 methylation in primary tumors. UCHL1 methylation was detected in 82.9% (34 of 41) of primary tumors but only weakly seen in some normal nasopharyngeal tissues (3 of 9 cases; Fig. 2B-D).

**Ectopic expression of UCHL1 inhibited nasopharyngeal carcinoma cell growth and induced apoptosis**

We compared the growth characteristics of two cell lines with silenced UCHL1 before and after ectopic expression of UCHL1 by monolayer colony formation assay. The number of colonies formed by UCHL1 re-expressed cells was significantly less than that with empty vector (P < 0.01; Fig. 3A). Furthermore, UCHL1-induced apoptosis was determined by measuring caspase-3 activity. Western blot showed obvious increase of cleaved caspase-3 in UCHL1-transfected cells. Meanwhile, increased cleaved PARP was also observed in UCHL1-transfected cells, consistent with the activation of apoptosis (Fig. 3B).

**UCHL1 activated the p53 signaling pathway**

To investigate the molecular mechanism of the tumor suppressive functions of UCHL1, we evaluated the effect of UCHL1 on p53 expression, which is a key molecule regulating apoptosis and cell cycle. Results showed that p53 increased at the protein level in UCHL1-transfected cells; simultaneously, p21, a direct downstream target of p53 signaling, also increased, indicating that UCHL1 could promote the accumulation and activation of p53 (Fig. 3C).

To determine whether p14ARF-promoted MDM2 degradation is involved in the stabilization of p53 in the presence of UCHL1, the expression of p14ARF and MDM2 was evaluated. Transfection of UCHL1 significantly increased the p14ARF protein level and reduced the MDM2 protein level (Fig. 3C), suggesting that UCHL1 promotes the accumulation of p53 through activating p14ARF while inactivating MDM2 signaling.

Furthermore, the effects of UCHL1 overexpression on the half-life of endogenous p53, MDM2, and p14ARF in the presence of cycloheximide were examined. The data showed that UCHL1 overexpression significantly extended the half-life of p53 and p14ARF, on the contrary, shortened the half-life of MDM2 (Fig. 4A and B), suggesting that UCHL1 probably acts as an ubiquitin ligase for p53, MDM2, and p14ARF, and leads to the activation of p53 signaling pathway.

![Fig. 5. UCHL1 formed a complex with p14ARF-MDM2-p53. A, overexpressed UCHL1 in HONE1 and CNE1 cells, followed by immunoprecipitation with p53 or MDM2 antibodies and immunoblot with antibodies against MDM2, p53, p14ARF, and UCHL1. B, interaction of UCHL1 with p14ARF-MDM2-p53 in 293T cells determined by coimmunoprecipitation analysis. C, the model of complex formation of UCHL1-p14ARF-MDM2-p53. D, proposed mechanism of UCHL1 acting as a tumor suppressor gene in carcinogenesis UCHL1 promotes the stabilization and activation of p53 signaling through deubiquitinating p14ARF, p53, and ubiquitinate MDM2, further leading to apoptosis, autophagy, and cell cycle arrest.](image-url)
UCHL1 stabilized p53 in the ubiquitin-proteasome pathway

UCHL1 seems to have two opposing enzyme activities and modulates cellular protein stability/degradation through the ubiquitin proteasome system. To test whether UCHL1 mediates p53 stabilization by regulating the p14ARF-MDM2-p53 pathway through ubiquitin-proteasome system, ubiquitination assay was done. We firstly evaluated the possible interaction of UCHL1 with p14ARF, MDM2, and p53 by overexpressing UCHL1 in CNE1 and HONE1 cells. Results revealed that UCHL1 formed a complex with p14ARF, MDM2, and p53 (Fig. 5A and C). Endogenous association of UCHL1 and p53/MDM2/p14ARF was also confirmed in HEK293T cells (Fig. 5B and C).

Furthermore, we found that ectopic UCHL1 overexpression in CNE1 and HONE1 cells could decrease the ubiquitination of endogenous p53 and p14ARF while increasing the ubiquitination of MDM2 (Fig. 6A). Exogenous expression of MDM2, ubk48, p53, and UCHL1 in HEK293T cells further confirmed the role of UCHL1 on p53, MDM2, and p14ARF in the ubiquitin-proteasome pathway (Fig. 6B). These results indicate that UCHL1 could deubiquitinate p53 and p14ARF and also ubiquitinate MDM2, which further resulted in the stabilization of p53 and tumor cell apoptosis (Fig. 5D).

Discussion

The predilection of nasopharyngeal carcinoma to Cantonese and the familial clustering of some cases suggest that genetic susceptibility plays an important role in nasopharyngeal carcinoma etiology. A susceptibility locus was recently identified at 4p11-p14 by genomewide linkage analysis of familial nasopharyngeal carcinoma (4, 5). Here, we identified a 4p14 gene, UCHL1, being downregulated in nasopharyngeal carcinoma cell lines, as a candidate tumor suppressor gene. We found that UCHL1 was silenced by promoter CpG methylation in nasopharyngeal carcinoma cell lines, whereas pharmacologic demethylation...
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Reactivated its expression. Methylation was also frequently detected in primary tumors but seldom in normal nasopharyngeal tissues, suggesting that epigenetic silencing of UCHL1 is important for nasopharyngeal carcinoma pathogenesis. Functionally, ectopic expression of UCHL1 in silenced tumor cells significantly suppressed their colony formation and induced cell apoptosis through caspase activation, indicating that UCHL1 is a functional tumor suppressor gene for nasopharyngeal carcinoma.

The ubiquitin-proteasome system is frequently disturbed in cancers (34). Compared with other ubiquitin enzymes, UCHL1 possesses two opposite activities in regulating ubiquitin pathway (9, 35): hydrolase, removing and recycling ubiquitin molecules from degraded proteins, which is important to sustain protein stabilization; and ligase, linking ubiquitin molecules together for target protein degradation. Thus, one possible mechanism by which UCHL1 functions as tumor suppressor gene is through ubiquitinizing oncoproteins and deubiquitinating tumor suppressor genes. Previous studies have shown that UCHL1 promotes the degradation of cell cycle inhibitor p27Kip1 (36, 37) and stabilizes NF-κB (38) and p53 (12, 19), but the specific substrates of UCHL1 in cancers are largely unclear.

p53 is one of two major tumor suppressive pathways in the development of human cancers. The signaling pathways regulating p53 abundance have yet to be fully elucidated (39). p14ARF-MDM2-p53 is an important tumor suppression pathway regulating p53 stabilization, which results in two critical tumor suppressive events, cell cycle arrest and apoptosis (40–42). p14ARF can form a stable ternary complex with MDM2 and p53, further stabilizing p53 protein by promoting the degradation of MDM2 (40, 43). p14ARF (44), MDM2 (45), and p53 (46) are all regulated through the ubiquitin-proteasome pathway, whereas MDM2 is known as a ubiquitin E3 ligase for p53 (47). We found that ectopic expression of UCHL1 could activate the p53 pathway through increasing the protein levels of p14ARF and p53 and downregulating the protein level of MDM2. Furthermore, half-life analysis showed that the half-life of p53 or p14ARF protein was significantly extended, whereas the half-life of MDM2 significantly shortened under UCHL1 overexpression, indicating that UCHL1 mediates the activation of p53 by protein degradation pathway.

Accumulation of p53 protein is common in nasopharyngeal carcinoma tumors, whereas p53 mutations could be detected in nasopharyngeal carcinoma cell lines but only infrequent in nasopharyngeal carcinoma tumors (48, 49). In our study, the complex of UCHL1-p14ARF-MDM2-p53 was further confirmed by immunoprecipitation, suggesting that UCHL1 could stabilize p53 through regulating the stabilities of p14ARF, MDM2, and p53. Ubiquitination analysis indeed showed that UCHL1 promoted the deubiquitination of p14ARF and p53 and, meanwhile, the ubiquitination of MDM2, thus elucidating the molecular mechanism of the activation of p53 signaling by UCHL1. However, whether UCHL1 mediate these effects through its two opposite enzymatic activities (50), E3 ligase and hydrolase activity, needs to be explored further. Our results also revealed another mechanism of UCHL1 acting as a tumor suppressor gene, that is, through the induction of apoptosis.

In summary, we showed that UCHL1 is frequently inactivated by promoter methylation in nasopharyngeal carcinoma cell lines and tumors. Restoration of UCHL1 could activate the p14ARF-p53 signaling pathway with its two opposing enzyme activities in the ubiquitin pathway, further resulting in tumor cell apoptosis (Fig. 5D). Thus, our study extends the current knowledge of the tumor suppressor gene functions of UCHL1 in carcinogenesis and also provides us clue to further develop UCHL1 methylation as a molecular marker for nasopharyngeal carcinoma detection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Jing Tan for constructing the UCHL1-expressing plasmid and Prof. George Tsao, Dolly Huang, Guiyuan Li, and Kaitai Yao for some cell lines.

Grant Support

Hong Kong RGC grant (CUHK4443/05M), a Chinese University of Hong Kong direct grant (Q. Tao), and the National Natural Science Foundation of China (30801337).

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Received 12/04/2009; revised 03/24/2010; accepted 04/02/2010; published OnlineFirst 04/15/2010.

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Clinical Cancer Research.
2958 Clin Cancer Res; 16(11) June 1, 2010

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