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The antiapoptotic protein, FLIP, is regulated by heterogeneous nuclear ribonucleoprotein K and correlates with poor overall survival of nasopharyngeal carcinoma patients

L-C Chen[1,6], I-C Chung[2,6], C Hsueh[1,3], N-M Tsang[4], L-M Chi[1], Y Liang[1], C-C Chen[1], L-J Wang[5] and Y-S Chang*[,1,5]

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) mediates antiapoptotic activity in part by inducing downstream antiapoptotic genes. To systematically identify hnRNP K targets in nasopharyngeal carcinoma (NPC), affymetrix chips were used to identify genes that were both overexpressed in primary NPC and downregulated by hnRNP K knockdown in NPC-TW02 cells. The resulting gene set included the antiapoptotic gene, *FLIP*, which was selected for further study. In cells treated with hnRNP K siRNA, TRAIL-induced apoptosis was enhanced and the FLIP protein level was reduced. Promoter, DNA pull-down and chromatin-immunoprecipitation assays revealed that hnRNP K directly interacts with the poly(C) element on the *FLIP* promoter, resulting in transcriptional activation. Through iTRAQ-mass spectrometric identification of proteins differentially associated with the poly(C) element or its mutant, nucleolin was determined to be a cofactor of hnRNP K for *FLIP* activation. Furthermore, FLIP was highly expressed in tumor cells, and this high-level expression was significantly correlated with high-level hnRNP K expression ($P = 0.002$) and poor overall survival ($P = 0.015$) as examined in 67 NPC tissues. A multivariate analysis confirmed that FLIP was an independent prognostic factor for NPC. Taken together, these findings indicate that FLIP expression is transcriptionally regulated by hnRNP K and nucleolin, and may be a potential prognostic and therapeutic marker for NPC.
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Heterogeneous nuclear ribonucleoprotein K (hnRNP K) belongs to the hnRNP family of proteins. The members of this family interact directly with DNA and RNA through their K-homology domains and regulate gene expression at multiple levels, including transcription, RNA splicing, RNA stability and translation.[1–2] The expression of hnRNP K has been shown to be aberrantly increased in numerous cancers,[3–6] and we recently reported that high-level hnRNP K expression is correlated with poorer overall survival (OS) and decreased metastasis-free survival among nasopharyngeal carcinoma (NPC) patients.[5] Our findings were consistent with those from clinical correlation studies in oral squamous cell carcinoma[4] and prostate cancer.[3]

HnRNP K is a nucleocytoplasmic shuttling protein that is primarily located in the nucleus for transcriptional regulation.[1] However, cytoplasmic accumulation of hnRNP K through ERK-mediated phosphorylation of hnRNP K serines-284 and -353 has been reported in cervical carcinoma HeLa,[7] chronic myelogenous leukemia[8] and NPC[9] cells. The tumorigenic activity of hnRNP K appears to be conferred through its ability to increase proliferation,[10] antiapoptotic effects,[9] clonogenic potential[8] and metastasis.[11] These functions may be due, at least in part, to the ability of hnRNP K to upregulate the *c-myc*,[8,12] *thymidine phosphorylase* (*TP*)[9] and *eIF4E*[10] genes through transcriptional or post-transcriptional regulation. However, the full spectrum of targets regulated by hnRNP K has not as yet been systematically examined.

The acquisition of resistance to apoptosis is considered a hallmark of cancer, and impairment of apoptotic pathways is one of the underlying mechanisms of chemoresistance in cancer cells. The TRAIL ligand is known to be involved in apoptotic activity; TRAIL interacts with the DR4 and DR5 death receptors, thereby regulating the extrinsic apoptotic pathway through sequential recruitment of the Fas-associated death domain and inactive procaspase 8 (FLICE) to form the death-inducing signaling complex (DISC).[13] At the DISC, activation of procaspase 8 triggers the activation of effector caspases 3 and -7, resulting in apoptosis. TRAIL has been shown to induce apoptosis in a broad range of tumor cells and in numerous preclinical trials, without evidence of systemic toxicity.[13] TRAIL triggers apoptosis in cancer cells regardless of the p53 status, making it a particularly attractive agent for cancer cells that have become resistant to chemo- or radiotherapy through inactivation of p53.[13] The combined

[1]Chang Gung Molecular Medicine Research Center, Chang Gung University, Taoyuan, Taiwan; [2]Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan; [3]Department of Pathology, Chang Gung Memorial Hospital at Lin-Kou, Taoyuan, Taiwan; [4]Department of Radiation Oncology, Chang Gung Memorial Hospital at Lin-Kou, Taoyuan, Taiwan and [5]Graduate Institute of Basic Medical Sciences, Chang Gung University, Taoyuan, Taiwan
*Corresponding author: Y-S Chang, Graduate Institute of Basic Medical Sciences, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-shan, Taoyuan, Taiwan 333, Taiwan. Tel: 886 3 2118800 x5131; Fax: 886 3 2118683; E-mail: ysc@mail.cgu.edu.tw
[6]These authors contributed equally to this work.
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Abbreviations: hnRNP K, heterogeneous nuclear ribonucleoprotein K; NPC, nasopharyngeal carcinoma; FLICE, inactive procaspase 8; DISC, death-inducing signaling complex
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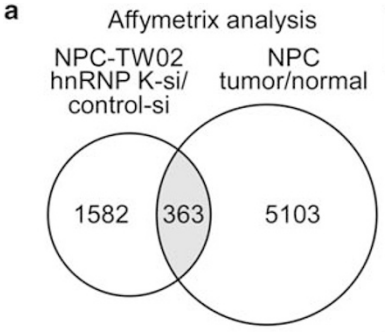
use of TRAIL plus traditional chemotherapeutic agents or radiotherapy has been shown to enhance cancer-killing efficacies and restore the TRAIL sensitivity in previously TRAIL-resistant cells. Phase I and II clinical trials with rhTRAIL and agonist antibodies are ongoing.[13] One important inhibitor of TRAIL-induced apoptosis is the FLICE-inhibitory protein (FLIP; CFLAR), which is predominantly expressed as long ($FLIP_L$) and short ($FLIP_S$) splice forms, and functions to inhibit caspase 8.[14] Both isoforms of FLIP are recruited to the DISC, where they prevent the full activation of caspase 8 and inhibit apoptosis.[14] FLIP has been found to be overexpressed in various cancers,[15–16] and FLIP overexpression has been shown to determine tumoral resistance to treatments with death ligands such as FasL and TRAIL.[14] Conversely, downregulation of FLIP sensitizes previously resistant tumor cells to these ligands.[14]

NPC is relatively rare in Caucasians, but the disease is relatively common in the Southeastern region of China and in Taiwan. NPC is sensitive to radiation therapy, and more advanced cases may be treated with a combination of radio- and chemotherapy. Although the survival rate of NPC is ~92% at 1 year and ~50% at 5 years, 20–25% of patients display distant metastases after treatment.[17] Thus, it is essential that we develop more sensitive and effective therapeutic strategies against NPC. In this study, we identified *FLIP* as a target gene of hnRNP K in NPC cells, and found that *FLIP* expression is regulated by the hnRNP K/nucleolin complex through direct interaction with a poly(C) sequence in the *FLIP* promoter. Finally, we found that FLIP overexpression was significantly correlated with hnRNP K overexpression and poor OS in NPC patients.

Results

Affymetrix analysis of hnRNP K targets. HnRNP K-mediated upregulation of TP can prevent hypoxia-induced apoptosis[9] and has been associated with poor prognosis in NPC patients,[5] suggesting that hnRNP K may function as an upstream regulator protein of cancer. To identify additional genes positively regulated by hnRNP K, we performed systematic microarray expression profiling using the Affymetrix HG U133 plus 2.0 chip (Affymetrix, Santa Clara, CA, USA) on samples representing NPC tumor tissues, adjacent normal tissues, control NPC-TW02 cells and hnRNP K-knockdown NPC-TWO2 cells. As shown in Figure 1a, 1945 genes were reduced in NPC-TW02 cells transiently transfected with hnRNP K siRNA *versus* those transfected with the negative control siRNA (cutoff value, 1.5-fold), whereas 5466 genes were elevated 1.5-fold or more in NPC tissues *versus* adjacent normal tissues. Comparison of these data sets revealed that 363 genes were elevated in NPC tissues and downregulated in hnRNP K-knockdown cells; these were considered potential targets for hnRNP K (Figure 1a). Ontological analysis of these 363 genes



Genes	NPC-TW02 Fold reduction	NPC Fold increase
RANTES	0.06	1.64
FLIP	0.16	2.26
IL7R	0.18	4.94
MMP13	0.23	4.29
ITGB6	0.30	5.92
DcR3	0.35	2.97
Rad9B	0.51	18.09
TP	0.61	3.46
Rap2A	0.65	3.30

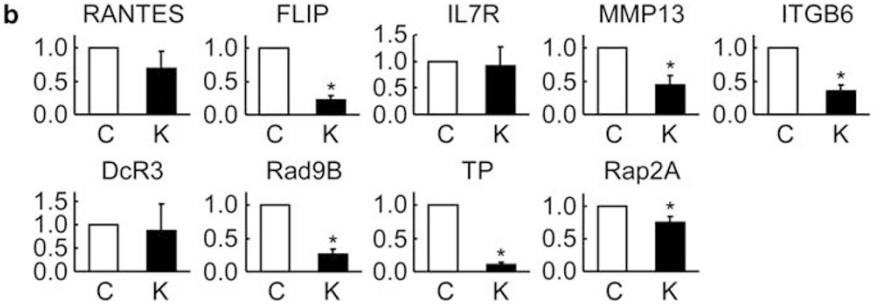


Figure 1 *FLIP*, *MMP13*, *ITGB6*, *Rad9B* and *Rap2A* are potential targets of hnRNP K in NPC cells. (**a**) Systematic identification of genes upregulated by hnRNP K. The open circles represent genes whose expressions were reduced by at least 1.5-fold in hnRNP K-knockdown NPC-TW02 cells *versus* controls and increased by at least 1.5-fold in nine NPC tissues compared with pooled adjacent normal tissues, as assessed using an Affymetrix HG U133 plus 2.0 microarray set. The gray ellipses represent the genes that were both reduced by hnRNP K knockdown and increased in NPC tissues. The bottom panel shows the fold changes of the nine genes shown in the gray ellipses. (**b**) Changes in mRNA expression were assessed in cells treated with hnRNP K siRNA (K) or control siRNA (C). Forty-eight hours after siRNA transfection, cells were harvested for RNA preparation. Relative transcript amounts were measured by quantitative RT-PCR, and the results were normalized with regard to *GAPDH* expression. All data are presented as the mean ± S.D. from at least three experiments. $^*P < 0.05$

revealed that 20 and 26 of the genes reportedly contribute to cancer development-related antiapoptosis and migration, respectively (Supplementary Tables S1 and S2). Consistent with our previous findings,[5,9] TP was overexpressed in NPC tissues and downregulated by hnRNP K knockdown in NPC cells (Figure 1a).

To validate our results, we used quantitative RT-PCR to examine the mRNA expression levels of the apoptosis-related genes *FLIP*,[14] *DcR3*,[18] *Rad9B*[19] and *IL7R*,[20] and the migration-associated genes *RANTES*,[21] *MMP13*,[22] *ITGB6*[23] and *Rap2A*[24] (Figure 1b). The mRNA levels of *FLIP*, *MMP13*, *ITGB6*, *Rad9B* and *Rap2A* were significantly reduced (to 0.2-, 0.4-, 0.4-, 0.3- and 0.8-fold, respectively) in hnRNP K-knockdown cells compared with control siRNA-transfected NPC-TW02 cells. As *FLIP* gene expression appeared to be reduced the most, we further investigated the regulation and antiapoptotic role of hnRNP K-mediated *FLIP*.

HnRNP K-mediated FLIP expression contributes to resistance against TRAIL-induced apoptosis in NPC cells. FLIP is an important inhibitor of TRAIL-induced apoptosis, and FLIP overexpression has been correlated with poor prognosis in colorectal cancer[15] and bladder urothelial carcinoma.[16] TRAIL is a ligand of the DR4 and DR5 death receptors, and these interactions can induce apoptosis in cancer cells.[13] However, the role of hnRNP K in TRAIL-induced apoptosis has not as yet been established. To gain insight into the potential role of hnRNP K in TRAIL-induced apoptosis, we tested FLIP expression in hnRNP K-knockdown and control cells in the presence and absence of TRAIL in three NPC cell lines, NPC-TW01, -TW02 and -TW04. As shown in Figure 2a, the level of FLIP protein was reduced significantly in hnRNP K siRNA-treated NPC cells compared with cells treated with control siRNA regardless of TRAIL treatment. As typical apoptotic morphologies and cellular shrinkage were observed in TRAIL-treated hnRNP K-knockdown NPC-TW02 cells (Figure 2b, left panel), we further measured the effect of hnRNP K knockdown on TRAIL-induced apoptosis using Annexin V staining. As shown in Figure 2b (the bottom part of the left panel), TRAIL dose-dependently induced apoptosis in both hnRNP K-knockdown and control NPC-TW02 cells, but TRAIL-induced apoptosis was higher in hnRNP K-knockdown cells *versus* controls (34.2 *versus* 15.2% in 100 ng/ml-treated cells; $P<0.05$). Similar results were found in both NPC-TW01 and -TW04 cells (Figure 2b, right panel).

We next examined whether FLIP is involved in the enhancement of TRAIL-induced apoptosis in hnRNP K-knockdown cells. As shown in Figure 2c, TRAIL treatment significantly increased the proportion of apoptotic cells from 17.9% in control cells to 34.1% in hnRNP K-knockdown cells. However, the apoptotic effect of TRAIL was obviously abolished in cells transfected with a FLAG-tagged FLIP expression vector, with the percentage of apoptotic cells decreasing from 34.1 to 21.6%. As TRAIL has been shown to mediate apoptosis by binding to DR4 and DR5, we examined the expression of these death receptors in hnRNP K-knockdown and control NPC-TW02 cells. We found that the knockdown of hnRNP K did not affect the expression of DR4 or DR5 (Figure 2d). Taken together, our findings reveal that hnRNP K knockdown decreases the expression of FLIP but not DR4 and DR5, and enhances the sensitivity of NPC cells to TRAIL-induced apoptosis.

HnRNP K regulates the *FLIP* promoter directly through its poly(C) element. We next sought to determine the mechanism(s) underlying hnRNP K knockdown-mediated FLIP downregulation. Analysis of the *FLIP* promoter sequence revealed four potential hnRNP K-binding poly(C) or C-C-C-C elements, R1 to R4 (Figure 3a).[1] To distinguish between transcriptional activation and post-transcriptional regulation, we examined the effect of hnRNP K knockdown on *FLIP* promoter activity and mRNA stability. We transfected a *FLIP* promoter reporter construct (-1179 to $+281$) into hnRNP K-knockdown NPC-TW02 cells and examined reporter activity 24 h later. HnRNP K knockdown significantly inhibited *FLIP* promoter activity by 54.3% compared with that in control cells ($P<0.001$) (Figure 3b). In contrast, the half-life of the *FLIP* mRNA was similar in control (8.4 ± 1.8 h) and hnRNP K-knockdown (7.8 ± 1.5 h) cells, as determined by the actinomycin D blockade of *de novo* RNA synthesis followed by the quantitative RT-PCR of *FLIP* mRNA levels at 1, 2, 4 and 8 h post-treatment (Figure 3c). These results suggest that the hnRNP K-mediated changes in *FLIP* gene expression are due to promoter inhibition, not mRNA destabilization.

To verify the ability of hnRNP K to bind to the predicted poly(C) elements, we performed DNA pull-down assays with R1, -2, -3 and -4 DNA-specific probes (Figure 3a). Our results showed that hnRNP K specifically bound to probe R4, but not to the other three probes (Figure 3d). Mutation of the poly(C) element in R4 (R4mut) abolished this binding, indicating that the interaction between hnRNP K and R4 is specific (Figure 3d). To further support our contention that hnRNP K can interact with the endogenous *FLIP* promoter, we performed a chromatin-immunoprecipitation analysis. As shown in Figure 3e, hnRNP K specifically immunoprecipitated with the R4 region of the *FLIP* promoter, but not with R1, R2 or R3.

To better delineate the function of the poly(C) element (-26 to -22) in R4 for *FLIP* promoter activity, we generated *FLIP* mini-promoter-reporter constructs (-37 to $+281$) with and without a mutation in the poly(C) element (-37R4mut), and transfected these constructs into hnRNP K-knockdown and control NPC-TW02 cells. As shown in Figure 3f, the wild-type promoter activity was significantly reduced (by 64.5%) in hnRNP K-knockdown *versus* control cells. In contrast, the poly(C) mutant promoter showed substantially reduced basal activity (to 48.6%) compared with the wild-type promoter, but was resistant to hnRNP K knockdown. Taken together, these results indicate that the poly(C) element (-26 to -22) is critical for hnRNP K-mediated *FLIP* promoter activity.

Identification of *FLIP* promoter poly(C) element-binding proteins. To identify other proteins involved in *FLIP* promoter regulation, proteins capable of binding to the mutant and wild-type poly(C) element (-26 to -22) were profiled using iTRAQ mass spectrometry (LTQ-Orbitrap, Thermo Electron, Bremen, Germany). Compared with the proteins that bound to the wild-type probe, 367 proteins showed 1.3-fold or larger reductions in the amount of

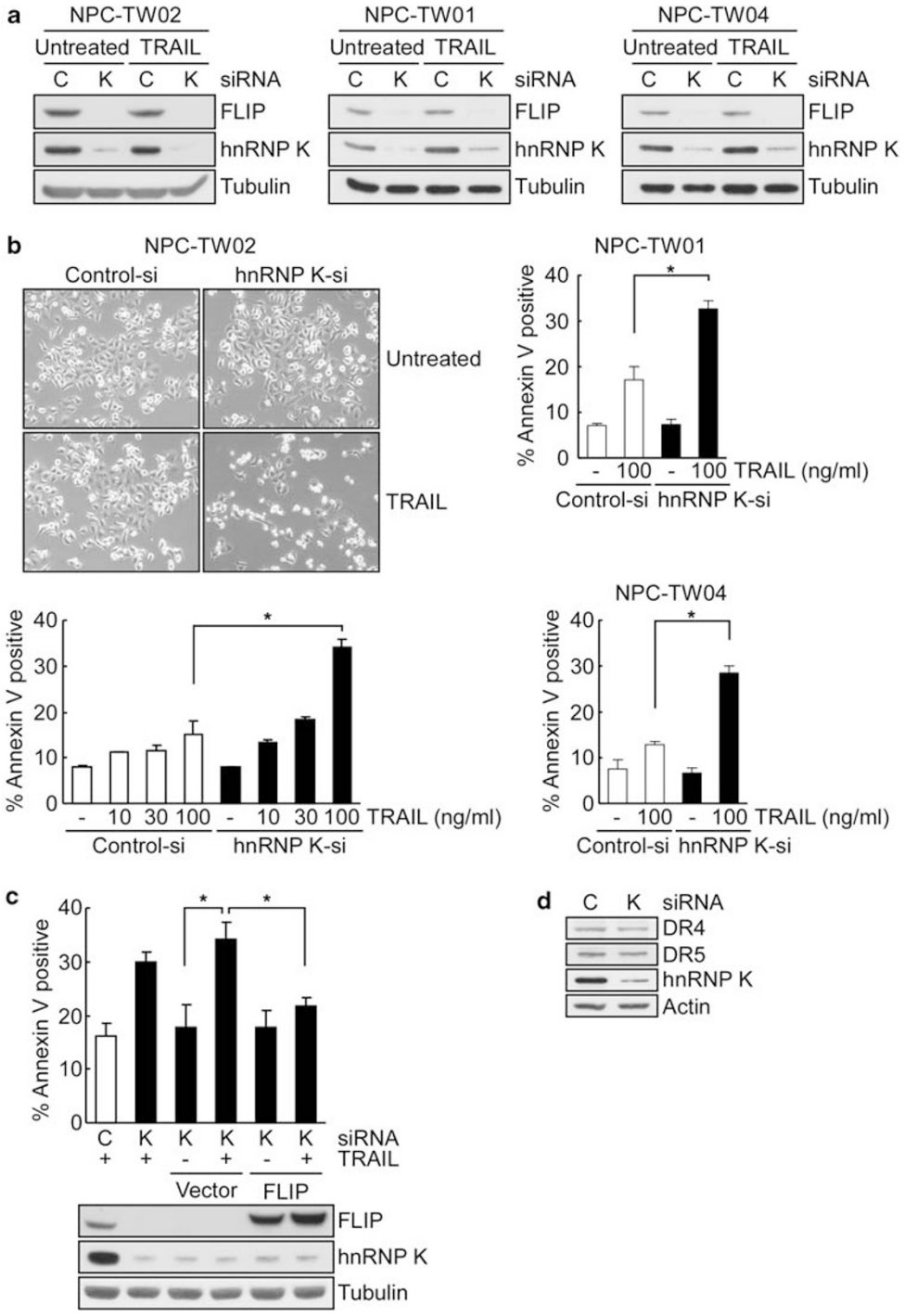


Figure 2 Prevention of TRAIL-induced apoptosis by hnRNP K through FLIP. (**a**) Effect of hnRNP K knockdown on FLIP expression in three NPC cell lines, NPC-TW01, -TW02 and -TW04. NPC cells transfected with hnRNP K (K) or control (C) siRNA were treated with or without 100 ng/ml TRAIL for 48 h. The levels of FLIP, hnRNP K and tubulin (loading control) protein were determined by western blotting. (**b**) Enhancement of TRAIL-induced apoptosis by hnRNP K knockdown. Morphology of NPC-TW02 cells transfected with hnRNP K or control siRNA, followed by treatment with or without 100 ng/ml TRAIL for 48 h. The bottom and right panels show the percentage of apoptotic cells in three NPC cell lines, NPC-TW01, -TW02 and -TW04, respectively, as assessed using Annexin V staining. All data are presented as the mean ± S.D. from at least three experiments. *$P<0.05$. (**c**) Effect of ectopic FLIP on TRAIL-induced apoptosis in hnRNP K-knockdown cells. NPC-TW02 cells transfected with hnRNP K (K) or control (C) siRNA were transfected with constructs encoding FLAG-tagged FLIP or empty vector, and further treated with or without 100 ng/ml TRAIL for 48 h. The levels of FLIP, hnRNP K and tubulin (loading control) were determined by western blotting. All data are presented as the mean ± S.D. from at least three experiments. *$P<0.01$. (**d**) The expression levels of the TRAIL receptors, DR4 and DR5, in hnRNP K-knockdown cells. NPC-TW02 cells transfected with hnRNP K (K) or control (C) siRNA were incubated for 48 h. The levels of DR4, DR5, hnRNP K and actin (loading control) were determined by western blotting

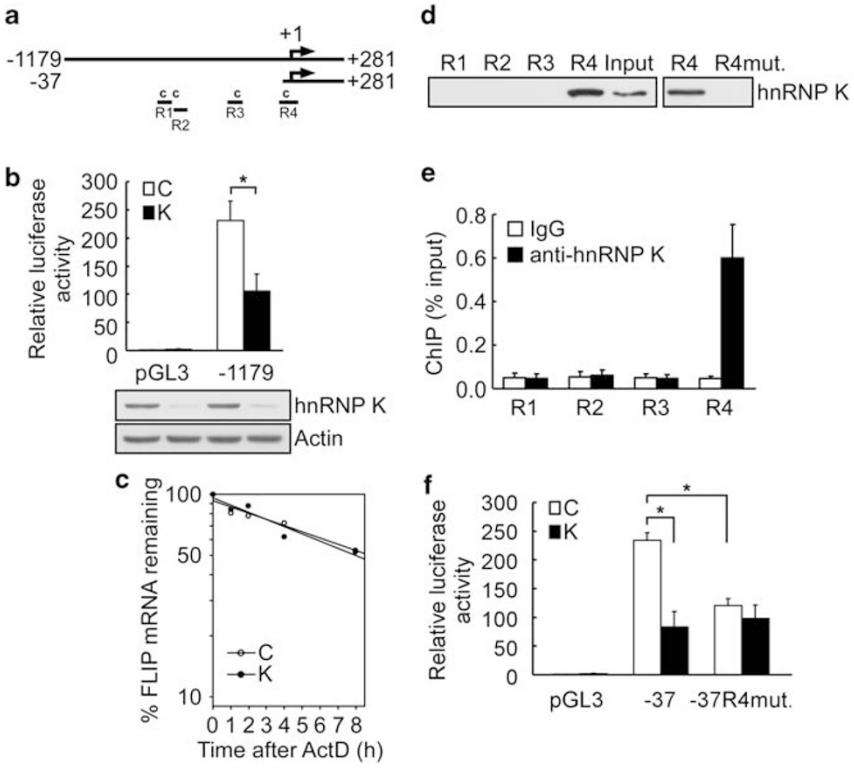


Figure 3 The poly(C) element in the *FLIP* promoter is directly bound by hnRNP K. (**a**) Schematic of the *FLIP* promoter (−1179 to +281) containing four poly(C) sequences (C, −667 to −664, −600 to −597, −280 to −277 and −26 to −22). The numbers 1–4 indicate sequences R1–R4, as used for the DNA pull-down and ChIP assays. The arrow indicates the transcription start site. (**b**) Inhibition of *FLIP* promoter activity by hnRNP K knockdown. The pGL3-basic vector (pGL3, promoterless luciferase vector) and a *FLIP* promoter construct (−1179, encompassing nucleotides −1179 to +281) were transfected into NPC-TW02 cells 48 h after the cells had been transfected with control siRNA (C) or hnRNP K siRNA (K). Firefly and Renilla luciferase activities were determined 24 h later. The levels of hnRNP K were determined by western blotting; actin was measured as a loading control. *$P<0.01$ (**c**) The half-life of *FLIP* mRNA in hnRNP K-knockdown cells. The levels of *FLIP* mRNA in NPC-TW02 cells transfected with control siRNA (C) or hnRNP K siRNA (K) for 48 h were measured following treatment with actinomycin D for 1, 2, 4 and 8 h. (**d**) Binding of hnRNP K to a poly(C) element of the *FLIP* promoter *in vitro*. Probes encompassing the region shown in panel **a** were generated by PCR amplification using 5′ biotin-conjugated primers and templates from wild-type (for R1, R2, R3 and R4) and mutant (for R4mut.) *FLIP* promoters. DNA pull-down assays were performed with nuclear extracts isolated from NPC-TW02 cells. The hnRNP K levels of the immunoprecipitates and 2% inputs were determined by western blotting. (**e**) HnRNP K binds to the *FLIP* promoter sequence *in vivo*. Chromatin immunoprecipitation was performed using nuclear extracts from NPC-TW02 cells and an antibody against hnRNP K, followed by quantitative PCR of the sequence within the *FLIP* promoter region shown in panel **c**. Mouse IgG immunoprecipitation was performed as a negative control. (**f**) The poly(C) sequence is a responsive element for hnRNP K-mediated promoter activity. NPC-TW02 cells were pretreated with control siRNA (C) and hnRNP K siRNA (K) for 48 h and then transfected with pGL3-basic (pGL3), or *FLIP* promoter constructs −1179 (−1179 to +281), −37 (−37 to +281) or −37R4mut (−37 to +281) containing the mutant poly(C) sequence. Firefly and Renilla luciferase activities were determined at 24 h post-transfection. *$P<0.01$

Table 1 hnRNP K/FLIP promoter-associated proteins identified by iTRAQ analysis

Protein name	Accession number	Gene name	Unique peptides[a]	Ratio (MU/WT)
Transcription factor AP-2α[b]	P05549	TFAP2A	7	0.198
Heterogeneous nuclear ribonucleoprotein K	P61978	HNRNPK	16	0.257
Heterogeneous nuclear ribonucleoprotein F[c]	P52597	HNRNPF	7	0.533
Nucleolin[c]	P19338	NCL	23	0.606
Histone acetyltransferase p300[b]	Q09472	EP300	7	0.718
Nuclease-sensitive element-binding protein-1[c]	P67809	YBX1	2	0.745
Transcriptional repressor protein YY1[b]	P25490	YY1	6	1.163

[a]The number of peptides used for identification of the proteins. [b]The transcription factors predicted using Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). [c]The hnRNP K interacting proteins.

DNA–protein complexes pulled down by the poly(C)-mutated R4 probe. The results of proteins that were previously known as hnRNP K-associated proteins or the factors predicted for having binding sites on *FLIP* promoter are shown in Table 1. Three of the differentially interacting proteins – nucleolin,[2] hnRNP F[25] and YB-1[26] – were previously reported to associate with hnRNP K. Two others, AP-2α and p300, were predicted to bind to R4 of the *FLIP* promoter based on a publicly available website for predicting transcription factor-binding elements (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

YY1 were also predicted to bind to elements within R4, but did not show differential binding with the wild-type *versus* mutated probes.

To further examine these candidate proteins, we used western blotting to analyze their protein levels in pulled-down DNA–protein complexes, and found that the bindings of AP-2α and nucleolin to probe R4 were markedly reduced to 0.2- and 0.5-fold, respectively, by the poly(C) mutation (Figure 4a). We then tested whether AP-2α and nucleolin formed a protein complex with hnRNP K. As shown in Figure 4b, nucleolin and hnRNP K, but not AP-2α, were present in immunoprecipitates separately generated using hnRNP K- and nucleolin-specific antibodies. When we used an anti-AP-2α antibody, neither nucleolin nor hnRNP K were detected in the immunoprecipitates. These results suggest that hnRNP K formed a protein complex with nucleolin, but not with AP-2α. These results collectively suggest that nucleolin and AP-2α may bind to the *FLIP* promoter in an hnRNP K-dependent and -independent manner, respectively.

Nucleolin and AP-2α are responsible for *FLIP* gene regulation. Next, we investigated whether hnRNP K, nucleolin and AP-2α have direct effects on *FLIP* promoter activity, hnRNP K-, nucleolin- and AP-2α-knockdown. NPC-TW02 cells were separately transfected with reporter constructs corresponding to the *FLIP* mini promoter, and reporter activity was examined after 24 h (Figure 4c). Our results showed that hnRNP K, nucleolin and AP-2α knockdown dramatically inhibited *FLIP* promoter activity by 62.1 ($P<0.01$), 73.8 ($P<0.01$) and 51.1% ($P<0.01$), respectively. To further test whether FLIP expression could be physiologically regulated by these three proteins, FLIP protein and mRNA levels were examined in NPC-TW02 cells transfected with siRNA directed against hnRNP K, nucleolin and AP-2α. As shown in Figure 4d, *FLIP* mRNA levels were significantly reduced to 0.36-, 0.61- and 0.78-fold in cells transfected with siRNA against hnRNP K, nucleolin and AP-2α, respectively, compared with cells transfected with the control siRNA. Similarly, FLIP protein levels were significantly reduced to 0.1-, 0.3- and 0.8-fold in cells transfected with siRNA against hnRNP K, nucleolin and AP-2α, respectively (Figure 4d). We further assessed the impact of nucleolin and AP-2α knockdown on TRAIL-induced apoptosis in NPC-TW02 cells. As shown in Figure 4e, nucleolin knockdown significantly enhanced TRAIL-induced apoptosis (28.4%) compared with the cells transfected with control siRNA (15.8%). On the other hand, AP-2α knockdown had lesser effect (21.2%) compared with hnRNP K or nucleolin knockdown. Our results suggest that hnRNP K, nucleolin and AP-2α may be involved in the transcriptional activation of the *FLIP* promoter. hnRNP K and nucleolin knockdown significantly downregulate FLIP expression and subsequently enhance TRAIL-induced apoptosis.

Elevated FLIP correlates with hnRNP K in NPC tissues. No previous study has examined FLIP expression in NPC tissues. Here, we examined 67 NPC biopsies by immunohistochemical staining with an anti-FLIP antibody. As shown in Figures 5a and b, FLIP was highly expressed in tumor cells, whereas only relatively weak staining was seen in the adjacent normal nasopharyngeal epithelium. Forty of the tumor specimens (60%; Figure 5g) showed highly intense FLIP staining and were designated 'FLIP high' (Figure 5c), whereas the remaining 27 specimens (40%) showed weaker staining intensities (Figure 5e) and were designated 'FLIP low.' Representative specimens are shown at lower magnification ($\times 200$) in Supplementary Figure S1.

To examine the physiological role of hnRNP K in FLIP expression in primary NPC samples, we further analyzed the correlation between the levels of FLIP expression and the levels of hnRNP K expression in NPC tissues. Consecutive tissue slides of the same set of specimens were subjected to immunohistochemistry (IHC) using an anti-hnRNP K antibody. As shown in Figure 5, 30 specimens (45%) showed high levels of hnRNP K (Figure 5d), whereas 37 specimens (55%) showed low levels of hnRNP K (Figure 5f). Statistical analysis using the Pearson χ^2-test showed that high-level hnRNP K was positively correlated with high-level FLIP expression (Figure 5g; $P=0.002$). These results strongly suggest that FLIP expression is positively regulated by hnRNP K in NPC tumor cells.

Association of FLIP with OS. To evaluate the prognostic significance of FLIP under current therapeutic protocols, we subjected the same retrospective cohort of 67 NPC patients to a clinical outcome assessment study. No significant correlations were found between high-level FLIP expression and clinicopathological features, including age, gender, tumor stage, node stage, AJCC stage and chemotherapy (Supplementary Table S3). A Kaplan–Meier survival analysis was then carried out to determine the OS for patients as a function of FLIP expression. As shown in Figure 5h, there was a significant difference in OS between patients with high and low levels of FLIP expression ($P=0.015$). We next conducted a multivariate analysis of FLIP with age, gender, tumor stage, node stage and chemotherapy, and found that high FLIP expression ($P=0.005$) was an independent prognostic predictor of poor OS (Table 2).

Discussion

Overexpression of hnRNP K is found in various cancers and has been correlated with poor prognosis.[3–6] In this study, we showed a new function for hnRNP K in FLIP regulation and resistance to TRAIL-induced apoptosis. The poly(C) sequence at -26 to -22 bp upstream of the *FLIP* transcription start site is bound by the hnRNP K/nucleolin complex, which is responsible for transcriptional activation. Moreover, induction of FLIP by hnRNP K increases the resistance of NPC cells to TRAIL-induced apoptosis. Importantly, high-level FLIP expression was found to be correlated with poor prognosis and increased expression of hnRNP K in NPC patients. Our findings collectively show that hnRNP K and nucleolin form a complex on the *FLIP* promoter, thereby inducing FLIP expression through transcriptional activation. This provides a mechanistic explanation for the correlation between FLIP and hnRNP K in NPC. In addition, FLIP may serve as an independent prognostic and therapeutic marker for NPC.

HnRNP K is overexpressed in various cancers, and this overexpression has been correlated with poor prognosis[3–6]

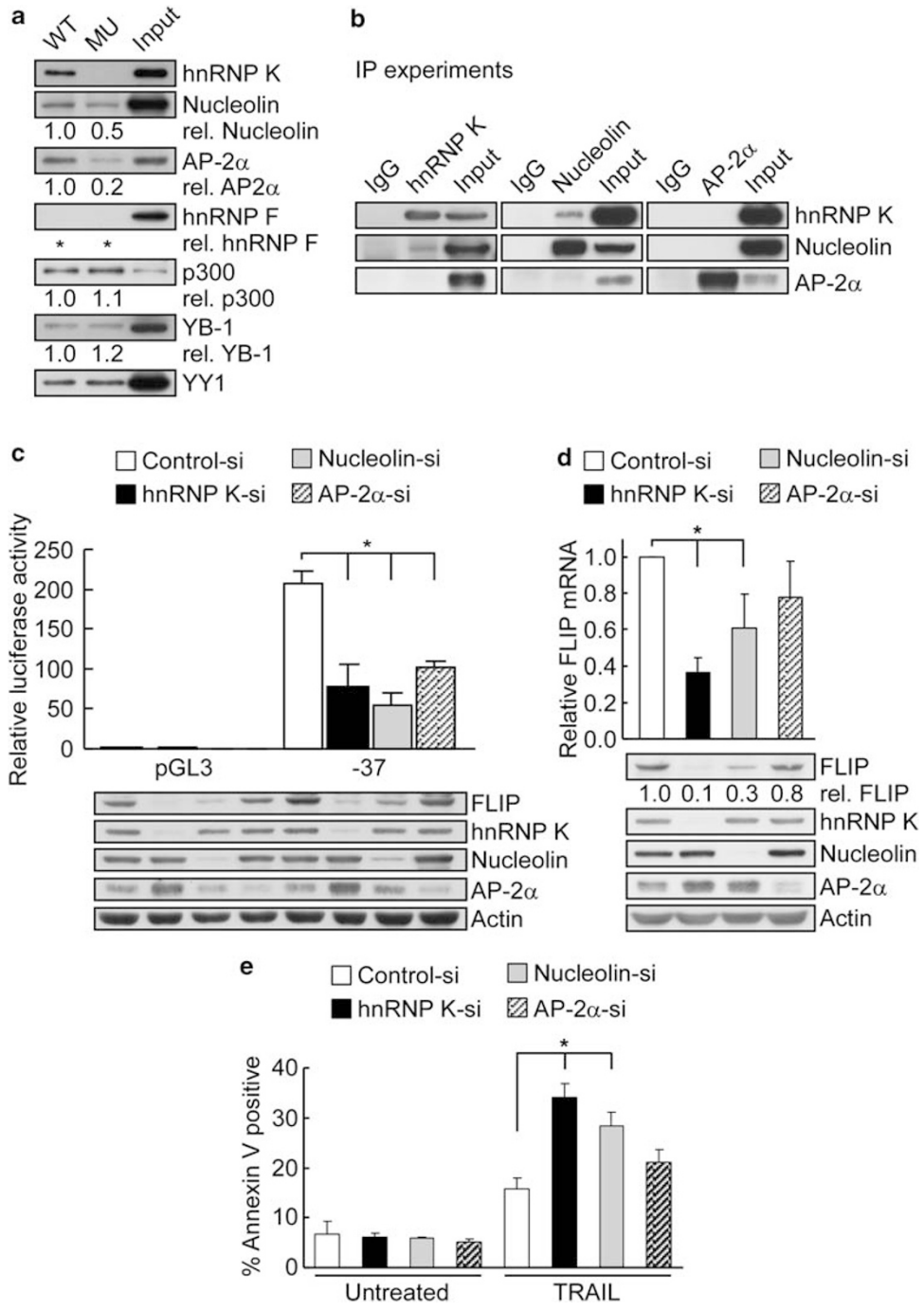


Figure 4 Validation of the *FLIP* promoter poly(C) element-binding proteins identified by iTRAQ. (**a**) Binding of AP-2α and nucleolin to the *FLIP* promoter poly(C) element. Proteins enriched from NPC-TW02 nuclear extracts by DNA pull-down with wild-type (WT) and poly(C)-mutated (MU) R4 probes were analyzed by western blotting. YY1 was detected as an internal control. The level of MU relative to WT is indicated. * Below the level of detection. (**b**) HnRNP K and nucleolin interacting with each other, but not with AP-2α. HnRNP K, nucleolin and AP-2α, were immunoprecipitated from NPC-TW02 nuclear extracts and detected by western blotting using specific antibodies. (**c**) Inhibition of *FLIP* promoter activity by nucleolin and AP-2α knockdown. The pGL3-basic vector and *FLIP* promoter constructs (encompassing nucleotides -1179 or -37 to $+281$) were transfected into NPC-TW02 cells 48 h after the cells had been transfected with control siRNA or siRNA against hnRNP K, nucleolin or AP-2α. Firefly and Renilla luciferase activities were determined at 24 h post-transfection. The levels of FLIP, hnRNP K, nucleolin, AP-2α and actin (loading control) protein were determined by western blotting. (**d**) Effect of nucleolin and AP-2α knockdown on FLIP expression. Forty-eight hours after siRNA transfection, the levels of FLIP proteins and mRNA were determined by western blotting and quantitative RT-PCR, respectively. The amounts of FLIP protein and mRNA were normalized with respect to the levels of actin and *GAPDH*, respectively, and the resulting values from the control siRNA treatment were set as 1.0. *$P<0.01$. (**e**) Effect of nucleolin and AP-2α knockdown on TRAIL-induced apoptosis. The percentage of apoptotic cells of NPC-TW02 cells transfected with hnRNP K, nucleolin, AP-2α or control siRNA, followed by treatment with or without 100 ng/ml TRAIL for 48 h, as assessed using Annexin V staining. All data are presented as the mean $\pm$ S.D. from at least three experiments. *$P<0.05$

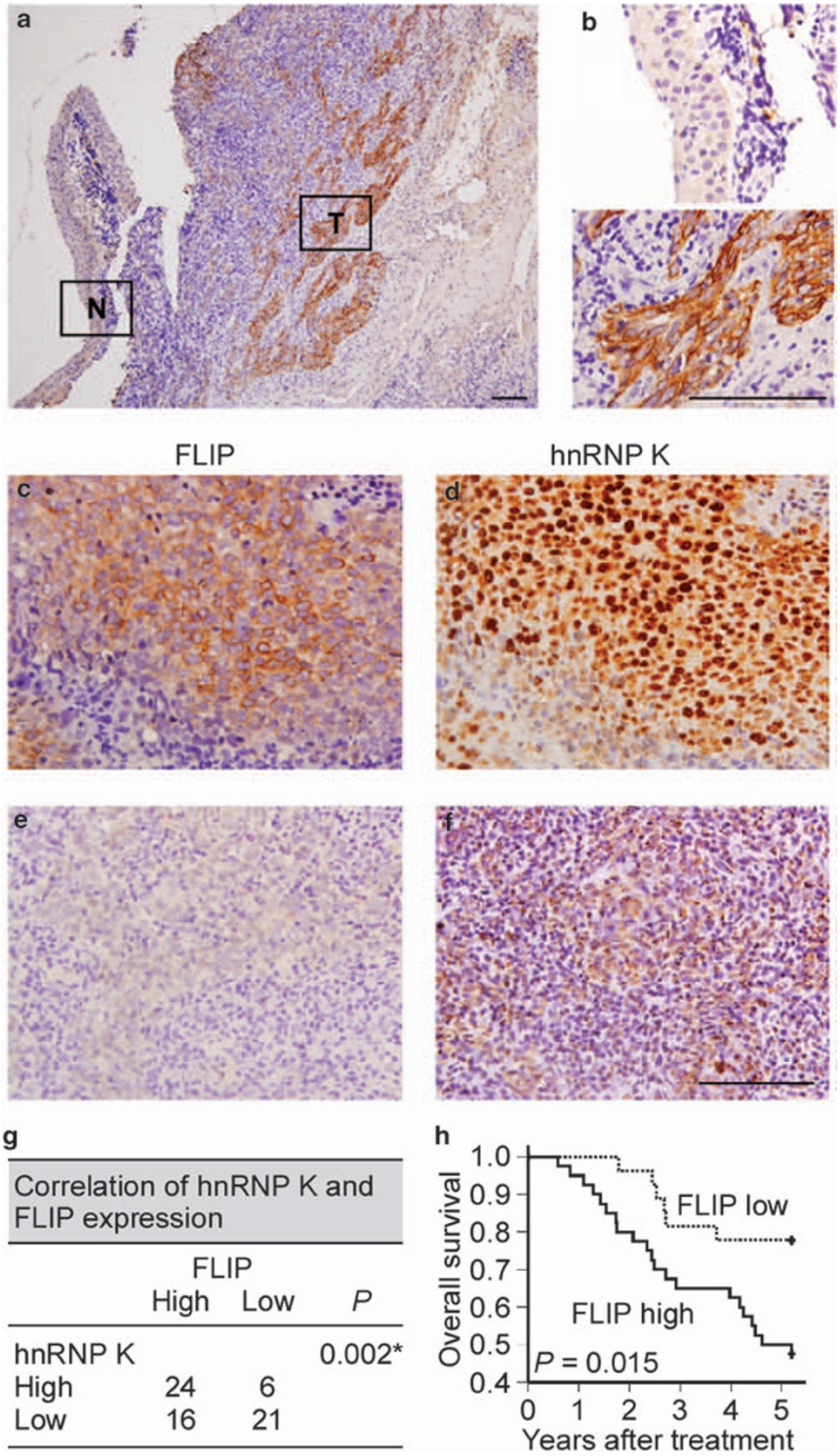


Figure 5 Correlation of FLIP with hnRNP K and overall survival in NPC patients. (**a**) Immunohistochemical staining of FLIP in NPC specimens. Representative images of NPC specimens containing tumor (T) and adjacent nontumor (N) cells stained with a specific anti-FLIP antibody are shown at $\times$ 100 magnification. The T and N areas are shown at $\times$ 400 magnification in (**b**), in the upper and lower panels, respectively. Consecutive NPC tissue sections were stained using anti-hnRNP K and anti-FLIP antibodies, and were evaluated immunohistochemically. (**c**) and (**d**) Tumor cells with high-level FLIP and hnRNP K expression are shown. (**e**) and (**f**) Tumor cells with relatively weak expression of FLIP and hnRNP K are shown at $\times$ 400 magnification. Scale bar, 100 μm. (**g**) Correlation of FLIP and hnRNP K expression. * Statistically significant by χ^2-test. (**h**) Kaplan–Meier survival analysis of OS as a function of elevated FLIP expression in NPC patients. FLIP high, $n = 40$ and FLIP low, $n = 27$

Table 2 Cox multivariate regression analysis of the association between FLIP and OS of NPC patients

Characteristics	Patients ($n=67$)		
	Hazards ratio	95% Confidence interval	*P*
Age[a]			0.055
>Median	1.00	Reference	
⩽Median	0.41	0.162–1.019	
Gender			0.125
Male	1.00	Reference	
Female	2.09	0.814–5.388	
Tumor stage			
1	1.00	Reference	
2	1.46	0.396–5.381	0.570
3	4.45	1.289–15.359	0.018[b]
4	5.53	1.527–20.041	0.009[b]
Node stage			
0	1.00	Reference	
1	1.82	0.579–5.714	0.306
2	2.55	0.665–9.786	0.172
3	5.17	1.519–17.597	0.009[b]
Chemotherapy			0.042[b]
No	1.00	Reference	
Yes	0.32	0.104–0.958	
FLIP			0.005[b]
Low	1.00	Reference	
High	4.67	1.604–13.588	

[a]Median age is 46 years. [b]With statistic significance.

suggesting that hnRNP K contributes to tumor progression and malignancy. Recent work has shown that hnRNP K regulates antiapoptosis and cell migration, two biological functions that are critically related to cancer development.[9,11] In this study, we identified 46 genes involved in apoptosis and migration as being overexpressed in NPC and upregulated by hnRNP K. Our differentially expressed gene set included *TP*[9] and *ITGB6*,[27] along with 44 other genes that had not been previously reported as hnRNP K targets. Among these differentially expressed genes, *FLIP*,[15–16] *FGF2*,[28] *TP*,[5] *SULF2*[29] and *MMP13*[30] are known to be overexpressed in various cancers and have been associated with poor prognosis. Thus, our findings suggest that several genes downstream of hnRNP K are overexpressed in cancer and may promote tumor development.

FLIP is an antiapoptotic molecule critical to TRAIL-induced apoptosis.[14] Consistent with this antiapoptotic function of FLIP, we found that hnRNP K-mediated FLIP expression is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. Although FLIP is known to be regulated at the translational and post-translational levels[14,31] we herein show that hnRNP K can directly bind to the poly(C) element of the *FLIP* promoter, transcriptionally activating *FLIP* and consequently preventing apoptosis. Although transcription of the *FLIP* gene is also regulated by NF-κB,[32] c-Myc,[33] nuclear factor of activated T cells,[34] androgen receptor response element[35] and c-Fos,[36] we found that hnRNP K is indispensable for the transcriptional activation of *FLIP*. Previous studies have shown that hnRNP K can regulate the expression of TP[9] and gastrin[2] post-transcriptionally. However, we did not find evidence that hnRNP K affects the stability of *FLIP* mRNA (Figure 3c), although *FLIP* mRNA could be regulated by IRF8 through mRNA stabilization.[37] Instead, similar to the hnRNP K-mediated induction of *c-myc*, *Src* and *eIF4E* gene expression through binding of poly(C) elements in their gene promoters,[1] we herein show that the poly(C) element of the *FLIP* promoter is important for its recognition and activation by both hnRNP K and nucleolin.

NF-κB has a major role in the transcriptional activation of multiple antiapoptotic genes during cancer progression. The prototypical NF-κB complex is a p65/p50 heterodimer that is important for NF-κB-mediated antiapoptotic effects.[38] In the absence of appropriate stimuli, NF-κB is sequestered in the cytoplasm by IκBα protein. On stimulation by factors such as TNFα, IκBα protein is degraded by proteasome, leading to NF-κB nuclear translocation and transcriptional activation.[39] Although hnRNP K knockdown-enhanced TRAIL-induced apoptosis is correlated with the loss of FLIP expression, we assess the impact of hnRNP K knockdown on NF-κB activation. As shown in Supplementary Figure S2a and S2b, TNFα stimulation induced nuclear translocation of p65 and p50, as well as downregulation of IκBα, which were not affected by hnRNP K knockdown. In addition, TNFα treatment had no effect on the survival of NPC-TW02 cells regardless of hnRNP K knockdown (Supplementary Figure S2c). These results together suggest that hnRNP K knockdown-enhanced TRAIL-induced apoptosis resulted from the loss of FLIP expression, rather than from the alteration of NF-κB activation.

Nucleolin is a multifunctional protein involved in ribosome biogenesis,[40] transcriptional regulation[40] and mRNA stability.[2] Nucleolin is an abundant protein, but its overexpression has been correlated with poor prognosis in various cancers.[40] Currently, a guanine-rich aptamer of nucleolin, AS1411, is in phase II clinical trials as an anticancer drug for acute myeloid leukemia and renal cell carcinoma.[40] Recent work has shown that nucleolin can bind to a guanine-rich DNA sequence upstream of the *c-myc* promoter (nt -142 to -115) and that it can function as a transcriptional repressor in MCF10A cells.[41] Other studies have revealed that a cytosine-rich DNA sequence corresponding to the same region of the *c-myc* promoter can also be bound by hnRNP K, leading to transcriptional activation in HeLa cells.[12] Both nucleolin and hnRNP K favor the binding of single-strand DNA over double-strand (ds)DNA.[41–42] We suggest that these previous findings seem to indicate that hnRNP K and nucleolin may recognize the same promoter site, where they appear to function as cofactors for transcriptional regulation. Consistent with this hypothesis, nucleolin and hnRNP K can form a cytoplasmic protein complex that acts to stabilize *gastrin* mRNA.[2] Here, we show for the first time that hnRNP K and nucleolin can function as cofactors for promoter activation.

TRAIL-based systems, many of which are currently undergoing preclinical trials, are thought to have high therapeutic potential because TRAIL induces apoptosis in a broad range of tumor cells, spares normal cells, and does not appear to be associated with systemic toxicity.[13] However, various cancer cells can resist TRAIL-induced apoptosis by overexpressing FLIP. It is hoped that the development of agents capable of inhibiting FLIP expression could sensitize cancer cells

resistant to TRAIL, thereby recovering the efficacy of TRAIL-based cancer therapies in resistant tumors.[14] This study, which provides insight into the induction of FLIP by hnRNP K and nucleolin, may aid in the development of new agents capable of inhibiting FLIP expression. In particular, we suggest that the nucleolin-targeting aptamer, AS1411, could prove useful for sensitizing cells to TRAIL-induced apoptosis through inhibition of FLIP expression.

In conclusion, we show herein that hnRNP K and nucleolin exert an antiapoptotic function by inducing the antiapoptotic protein, FLIP, through binding to the poly(C) element of the *FLIP* promoter and inducing transcriptional activation. FLIP is overexpressed in NPC and is associated with poor prognosis. We believe that high-level expression of hnRNP K, nucleolin and FLIP could be prognostic for TRAIL resistance in NPC patients. Finally, we propose that hnRNP K, nucleolin and FLIP should all be considered potential targets for developing new anticancer agents.

Materials and Methods

Quantitative RT-PCR. RNA samples from NPC tissues and NPC-TW02 cells were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of RNA (1 μg) was performed using ImProm-II (Promega, Madison, WI, USA) and oligo(dT)$_{15}$ primers (Promega). The primers used to amplify the cDNA corresponding to *FLIP*, *RANTES*, *IL7R*, *MMP13*, *ITGB6*, *DcR3*, *Rad9B*, *TP*, *Rap2A* and *GAPDH* are presented in Supplementary Table S4. Quantitative RT-PCR was performed on a Light-Cycler (Roche Diagnostics), according to the manufacturer's instructions, using the FastStart DNA Master SYBR Green I reagent (Roche Diagnostics, Mannheim, Germany). The gene expression results were normalized with regard to the expression of *GADPH*. For mRNA half-life assessment, actinomycin D (5 μg/ml) was added 48 h after transfection with control or hnRNP K-targeting siRNA (see below), and RNA was prepared at the indicated times.

RNA interference. *SMART*pool reagents, including four 21-bp RNA duplexes targeting hnRNP K, AP-2α and nucleolin, respectively, were purchased from Dharmacon (Lafayette, CO, USA), and the negative control siRNA was synthesized by Research Biolabs Ayer Rajah Industrial Estate (Research Biolabs, Singapore, Singapore). The oligonucleotide sequences are presented in Supplementary Table S5. NPC cells were transfected with 50 nmol/l dsRNA duplexes and 50 μg dsRNA transfection reagents (*Trans*IT-TKO; Mirus Bio Corporation, Madison, WI, USA) according to the manufacturer's protocol. At 6 h post-transfection, the siRNA-containing medium was replaced with fresh complete medium. After a further 48 h of culture, cells were harvested, and cell extracts were prepared and subjected to western blotting to confirm target gene knockdown.

DNA pull-down. Probes corresponding to the four potential binding elements (R1-R4) were generated by PCR using appropriate biotinylated primers and the *FLIP* promoter construct (-1179 to $+281$) as a template. The specificity and requirement of the poly(C) nucleotides within the four probes for hnRNP K binding were further determined using a poly(C) mutant probe that was PCR-generated from a template that had been mutated by site-directed mutagenesis. The biotinylated probes were conjugated with M-280 Streptavidin Dynabeads (Invitrogen) in binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 1 mM Na_3VO_4, 5 mM DTT, 5% glycerol and 0.3% NP-40) for 40 min at room temperature. NPC-TW02 cells were extracted using the Compartmental Protein Extraction reagent (Millipore, Billerica, MA, USA), and nuclear fractions (50 μg) were incubated with unconjugated Dynabeads (Invitrogen) in the presence of 25 μg/ml poly (dI:dC) for 20 min at room temperature. The unbound fraction was incubated with 250 μg of Dynabeads bound to 50 pmol of immobilized wild-type or mutant *FLIP* promoter for 1 h at room temperature. The Dynabead-bound complexes were collected using a Dynal MPC-S magnetic particle concentrator (Dynal, Lake Success, NY, USA) and washed with binding buffer. The DNA-bound proteins were eluted in SDS sample buffer and assayed by western blotting.

Chromatin immunoprecipitation. NPC-TW02 cells were crosslinked by treatment with 1% formaldehyde for 10 min at room temperature, and the reactions were quenched with glycine (0.125 M) for 10 min at room temperature. Cell pellets were incubated with RSB buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and protease inhibitors) for 10 min at 4 °C, an equal volume of RSB buffer containing 0.4% NP-40 was added, and samples were incubated at 4 °C for an additional 10 min. The samples were then centrifuged at 500 g for 5 min at 4 °C, the supernatants were removed, and the nuclear pellets were washed with RSB buffer and SNSB buffer (10 mM Tris-HCl (pH 7.4), 1 M NaCl, 1 mM EDTA, 0.1% NP-40 and protease inhibitors). The nuclei were incubated with RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1% NP-40 and protease inhibitors). The resulting nuclear fractions were sonicated on ice and diluted 10-fold in WCE buffer (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2 M NaCl, 0.5% Triton X-100, 10% glycerol and protease inhibitors). The diluted samples were centrifuged at 13 000 r.p.m. for 15 min at 4 °C. Approximately 500 μg of the resulting chromatin solution was used for immunoprecipitation. Samples were precleared with 30 μl of 50/50 salmon sperm DNA/protein A agarose slurry (Millipore, cat. no. 16–157C) for 1 h at 4 °C. The precleared samples were incubated overnight at 4 °C with 5 μg of an anti-hnRNP K antibody (Invitrogen). Mouse IgG1 was used as a control antibody. The immunocomplexes were collected with 30 μl of 50/50 salmon sperm DNA/protein A agarose slurry for 1 h at 4 °C. The beads were washed with a 9 : 1 WCE/RIPA buffer, washed with Tris-EDTA, and then resuspended in 100 μl Tris-EDTA. An equal volume of 2 $\times$ PK buffer (200 mM NaCl, 1% SDS and 600 μg/ml proteinase K) was added to the immunoprecipitates and the input samples, and all samples were incubated for 3 h at 56 °C. The samples were then de-crosslinked by overnight incubation at 65 °C. DNA fragments were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and quantitative PCR was performed using the appropriate primers against the four potential hnRNP K-binding elements.

Patients, clinical staging, treatment and assessment of clinical outcome. The retrospective cohort comprised 67 NPC patients who had been admitted to Chang Gung Memorial Hospital (CGMH; Lin-Kou) from 1990 to 1999. Clinical stage was defined according to the 2002 cancer staging system revised by the American Joint Committee on Cancer. Histological typing was done according to the World Health Organization (WHO) classification criteria, as previously described.[5] This study was reviewed and approved by the institutional review board and ethics committee of CGMH. Informed consent was obtained from all patients. All enrolled patients had been treated with definitive radiotherapy (cumulative dose of external beam radiotherapy $>$R64.8 Gy). Among them, 16 patients had received additional chemotherapy from the Department of Medical Oncology at CGMH. Patients diagnosed with distant metastatic disease at presentation (M1 stage) and/or those who had undergone previous treatment at another institute were excluded from this study. For all enrolled patients, pathology reports were obtained from pathologic databases and medical records, and reviewed for confirmation of the NPC diagnosis (reviewed by C Hsueh). Information on stage, treatment and follow-up were collected from hospital tumor registries and medical files, as was limited information on family history. Patient characteristics and clinical features are summarized in Supplementary Table S3. The median age at diagnosis was 46 years (range, 25.5–76.9), and the male-to-female ratio was $\sim$3.8 : 1. The clinicopathological features were comparable between patient subgroups classified according to FLIP status. The primary end point was OS, which was calculated from the date of diagnosis to the date of death or the last follow-up.

Immunohistochemical staining. Immunohistochemical analyses were performed using an automatic IHC-staining device, according to the manufacturer's instructions (Bond-max Automated Immunostainer; Vision Biosystems, Melbourne, Australia). Tissue sections were retrieved using Bond Epitope Retrieval Solution 1 (Vision BioSystems) and stained with antibodies against hnRNP K (mouse monoclonal antibody, 1 : 300 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and FLIP (rabbit polyclonal antibody, 1 : 100 dilution; Santa Cruz Biotechnology). A polymer detection system (Bond Polymer Refine; Vision BioSystems) was used to reduce nonspecific staining. Tissue sections were treated with liquid DAB reagent using 3′-diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin as the counterstaining reagent. For analysis of hnRNP K expression, specimens in which $>$50% of the tumor cells displayed strong staining were defined as having a 'high-level' hnRNP K expression, and those where $\leqslant$50% of tumor cells stained strongly were defined as having 'low-level' hnRNP K expression. For analysis of FLIP expression, specimens in which $>$50% of tumor cells displayed

positive staining were defined as having 'high-level' FLIP expression, and those in which $\leqslant$50% tumor cells displayed positive staining were defined as having 'low-level' FLIP expression. FLIP- and hnRNP K-positive tumor cells in representative microscopic fields were scored independently by two experienced pathologists.

Statistical analysis. All statistical analyses were performed using the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). The data were analyzed using Student's *t*-test. Differences were considered significant at a level of $P<0.05$.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)