NAIF1 is down-regulated in gastric cancer and promotes apoptosis through the caspase-9 pathway in human MKN45 cells

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Abstract. Many key proteins are down-regulated or lose their function during cancer genesis and accelerate the progress of cancer. We found that nuclear apoptosis-inducing factor 1 (NAIF1) was highly expressed in normal gastric tissues but was down-regulated or lost in gastric cancer tissues (P<0.001). NAIF1 expression was higher in well-differentiated (P=0.004) than in moderately- or poorly-differentiated gastric cancer. NAIF1 expression was associated with different T stages (P=0.024). *In vitro*, NAIF1 can inhibit tumor cell proliferation and induce G0/G1 phase cell cycle arrest in the MKN45 cell line. NAIF1 can induce apoptosis through activation of procaspase-9 rather than procaspase-8 followed by activation of the caspase-3 pathway. We designed and constructed two truncation mutants, pEGFP-N1-NLS and pEGFP-N1-GRR, and identified the N-terminal 1-90 amino acid domain of NAIF1, which is a helix-turn-helix motif and which was sufficient for inducing apoptosis. Therefore, these findings suggest that NAIF1 plays an inhibitory role in the initial steps of gastric cancer genesis and may provide new strategies for developing anti-cancer drugs using small molecular polypeptides.

Introduction

Gastric cancer is a highly frequent malignancy worldwide with an estimated incidence of 934000 new cases per year in 2002 (1,2). More than 42% of GC patients are found in China alone (3). Currently, targeted therapies designed to induce apoptosis in cancer cells selectively are the most promising anti-cancer strategies. Many efforts have been made to cure gastric cancer, most of which based on developing effective apoptotic therapeutic approaches such as activation of cell surface death receptors, inhibition of cell survival signaling and others (4-6).

NAIF1 (nuclear apoptosis-inducing factor 1) was found to induce apoptosis when overexpressed in HeLa and HEK293 cells (7). Another study showed NAIF1 was related to transposition because of its trihelix motif (8). However, little information exists on the relationship between NAIF1 and cancer genesis and progress, thus this study investigated the expression and function of NAIF1 in cancer.

We found NAIF1 was highly expressed in normal stomach tissue, but was down-regulated or lost in gastric cancer tissue (P<0.001). This event significantly correlated with the tumor differential T stage. Functional studies indicated NAIF1 can reduce cell viability in human gastric cell line MKN45 by G0/G1 phase cell cycle arrest and induce apoptosis 48 h post-transfection. Western blot analysis indicated NAIF1 triggered procaspase-3 and procaspase-9 activation and PARP cleavage in MKN45. Furthermore, we identified that the 1-90 amino acids domain was the core functional domain of NAIF1 for inducing apoptosis. Thus, our findings provide a potential target for GC apoptotic therapeutic approach.

Materials and methods

Reagents. MTT, PI and DAPI were purchased from Sigma (USA). Monoclonal antibody against β-actin was purchased from Sigma-Aldrich (USA). The antibody against PARP and procaspase-8 were purchased from Beyotime Co. (China), against procaspase-3 and procaspase-9 were purchased from Santa Cruz Biotechnology (USA). Lipofectamine 2000 was purchased from Invitrogen (USA).

Vector construction. The full-length plasmid pEGFP-N1-NAIF1 was kindly provided by Professor Dalong Ma (Peking University). pEGFP-N1-NLS (amino acid residues 1-90) and pEGFP-N1-GRR (amino acid residues 1-117) were prepared by PCR amplification using the pEGFP-N1-NAIF1 plasmid as a template, followed by sub-cloning into the pEGFP-N1

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Abbreviations: PARP, poly (ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; FCM, flow cytomtry; ELISA, enzyme-linked immunosorbent assay; DAB, 3,3'-diaminobenzidine

Key words: nuclear apoptosis-inducing factor 1, gastric cancer, apoptosis, caspase-9

vector to generate a C-terminal EGFP-tagged fusion protein, respectively. The pET-32a-NAIF1 was constructed in the same manner.

Polyclonal anti-NAIF1 antibody preparation. pET-32a-NAIF1 was expressed in *E.coli* BL21(DE3) induced by IPTG. After denaturation, affinity chromatography and renaturation, the purified protein was immunized to rabbits to goat polyclonal antiserum. Rabbit polyclonal antibody was purified using CNBR Sepharose 4B coupled with purified NAIF1 protein. The sensitivity and specificity of the antibody was detected using ELISA, immunofluorescence and Western blot analysis.

Tissue samples and immunohistochemistry. Tissue samples were derived from a total of 129 patients with primary gastric cancer. Normal tissues derived from the same group of patients were also included. All the patients involved in this study underwent surgical treatment at the Cancer Hospital, Chinese Academy of Medical Sciences from 2008 to 2009, and none of them received chemotherapy and/or radiotherapy before surgery. The use of all of the human tissue samples and the experimental procedures for this study were reviewed and approved by the Ethics Committee of the Cancer Institute (Hospital), Peking Union Medical College and Chinese Academy of Medical Sciences. The human multiple cancer/normal tissue microarrays were purchased from Beijing Friendship Hospital. Immunohistochemistry analysis was carried out according to standard procedures. Sections with anti-NAFI1 antibody pre-incubated with the specific NAIF1 peptides at RT for 2 h were also used as preabsorption control. The stain results were read independently by two pathologists. The NAFI1 labeling index (LI) was ranked according to the percentage of positive cell observed. Samples in which >50% of the cell showed positive staining were rated 4, those with 30-49% of cell stained as 3, those with 11-31% of cell stained as 2, those with <10% of nuclei stained as 1, and those with no detectable stained were defined as 0. The positive sample was defined on cut-off point for NAIF1 immunoreactive score at 10% (NAIF1 LI >2).

Cell culture and transfection. The human gastric cancer cell line MKN45 was maintained in RPMI-1640 medium supplemented with 10% FBS in a humidified incubator with 5% CO_2 at 37˚C. DNA transfections were performed using Lipofectamine 2000 according to the manufacturer's instruction.

MTT assay. Cells were placed in 96-well plates at a density of 5000 cells/well in 200 μl medium. At the indicated time-points, 20 μl solution of MTT (5 mg/ml) and 90 μl RPMI-1640 medium was added to the culture medium. After 4 h, DMSO was added to each well to dissolve the formazan. Absorbance was measured at 570 nm on a micro-ELISA reader (Labsystem Multiskan MCC/340, Finland). The data were normalized to solvent treated cultures. All MTT assays were repeated four times.

Analysis of cell cycle and apoptosis by FCM. At 48 h following infection, MKN45 cells were harvested and fixed overnight. Subsequently, the cells were rinsed with PBS, and incubated with 100 µg/ml PI, 0.1 mM EDTA, 0.1% Triton X-100 in PBS, pH 7.4, containing 20 μg/ml RNase A for 30 min. The DNA content of GFP-positive cells was then measured using FCM.

Cell morphology observation stained by DAPI. After transfection, cells were collected and fixed. Then fixed cells were stained with 1 μg/ml DAPI for 10 min in the dark to counterstain nuclei. Stained cell samples were analyzed on fluorescence microscope (Olympus, Japan).

Western blotting. Total protein extracts from cells through RIPA buffer. Then protein was separated on SDS-PAGE and transferred to a PVDF membrane. Antibodies were diluted in TBST (5% milk powder) and incubated at 4˚C overnight, respectively. The appropriate secondary antibody was applied at room temperature for 1 h. Visualization was performed by enhanced chemiluminescence (ECL; Amersham).

Statistical analysis. Data were expressed as the mean ± SD. The variables (e.g., NAIF1 LI of grade, stage, age) were compared by the Kruskal-Wallis non-parametric analysis and Spearman's correlation. For comparing the data of normal and tumor tissue, and other statistical significance of differences observed between groups, the Student's t-test (two-tailed) was used. Values of $P<0.05$ were considered statistically significant.

Results

NAIF1 is highly expressed in normal gastric tissue and down-regulated in gastric cancer. The immunohistochemical staining on the tissue microarrays showed that NAIF1 was highly expressed in liver (both normal and cancer) tissue. NAIF1 was also detected in several other tissues although the stain level was much lower (data not shown). Intriguingly, we found NAIF1 was highly expressed in normal gastric tissue but down-regulated or expression was lost in gastric cancer tissue (Fig. 1). Later, we examined 129 gastric cancer samples and 84 normal gastric samples to further confirm that difference expression of NAIF1. The results of immunohistochemical staining are summarized in Table I. Overall, NAIF1 was highly expressed in all 84 normal tissues, 53 samples (63.1%) scored 3 and 31 samples (36.9%) scored 4 according to the LI scoring standard. On the contrary, in 129 cancer tissue samples, there were 109 samples with negative expression (LI score <1), which account for most of the cancer samples (84.4%). Only 18 samples scored 2 and 2 samples scored 3. The mean value of the NAIF1 LI for the normal and cancer tissue was 3.37±0.49 and 0.53±0.79, respectively. Statistical analysis indicated such expression difference was very significant ($P<0.001$). NAFI1 positive staining was mostly observed in the cytoplasm of gastric cells.

For further investigation, we analyzed the correlation between NAIF1 expression level and clinicopathological parameters. As shown in Table II, the expression level of NAIF1 was much higher in well-differentiated than in moderately- or poorly-differentiated tumor tissues ($P=0.004$). The correlation analysis revealed an association between the NAIF1 LI and different tumor T stage, indicating NAIF1 expression was decreased progressively from T1 to T4 stage. Based on our 129 samples, we did not observe any correlation

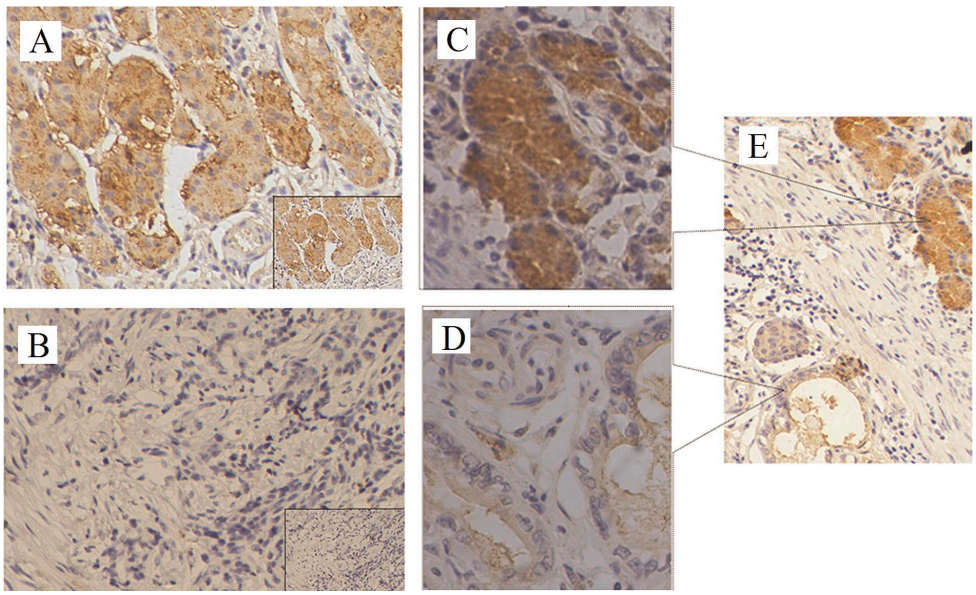

Figure 1. The expression of NAIF1 in gastric normal and cancer tissue. (A) and (C) strong NAIF1 expression was observed in normal gastric tissue, mostly in the cytoplasm (original image x200 and the lower right corner image x100). (B) and (D) in contrast, very weak or no expression of NAIF1 was found in gastric cancer tissue (original image x200 and the lower right corner image x100). (E) A gastric tissue section with both a normal and a cancer part (original x100).

Table I. Immunohistochenimical staining for NAIF1 on the tissue slice of human normal and gastric cancer.

	NAIF1 LI (%)						
Histologic classification	0	1	2	3	4	Mean LI	P-value
Normal tissues	0 (0.0)	0 (0.0)	0 (0.0)	53 (63.1)	31 (36.9)	3.37±0.49	<0.001[a]
Tumor tissues	83 (64.3)	26 (20.1)	18 (14.0)	2 (1.6)	0 (0.0)	0.53±0.79	

[a]Normal tissues vs. tumor tissues (P-value from independent t-test).

between NAIF1 expression and clinical stage, gender, age or other clinicopathological parameters.

NAIF1 reduced cell viability of gastric cancer cell lines. To examine the effect of NAIF1 on the gastric cancer cell proliferation, MTT cell viability assay was performed in the MKN45 cells. As shown in Fig. 2A, the cell proliferation rate decreased gradually in NAIF1 group compared to mock. The significant growth inhibition in NAIF1 transfected MKN45 cells was detected after 5 days of incubation, and that was markedly lower ($P<0.05$) than in pEGFP-N1 transfected MKN45 cells and in control MKN45 cells.

NAIF1 induces cell cycle arrest and apoptosis in gastric cancer cell lines. We transfected the MKN45 cells with pEGFP-N1-NAIF1 to analyze the cell cycle by using FCM. Only GFP-positive cells were analyzed. As illustrated in Fig. 2C, a significant increase in the G0/G1 phase was observed in NAIF1 transfectants (68.9±5.7%) compared to mock (54.4±1.8%). The G2/M phase was significantly decreased in NAIF1 transfectants (5.5±3.5%) compared to mock (11.1±0.2%). The S phase was also decreased after transfection, but less than in G2/M phase (from 34.5±1.7 to 28.5±6.9%). Those results suggested NAIF1 caused a G0/G1 arrest. Moreover, an increased proportion of apoptotic cells in the sub-G1 phase was strongly detected in NAIF1-transfected cells (20.7±5.1%) compared to mock (1.1±0.5%) 48 h post-transfection, indicating NAIF1 can induce apoptosis in MKN45 cells (Fig. 2C).

Based on the above results, nuclear DAPI staining was performed to examine the morphological changes in the nuclei of the cells. Upon transfection with NAIF1, morphological changes were found including the chromatin condensation and the appearance of apoptotic bodies, typical characteristics of apoptosis (Fig. 3).

NAIF1 induces apoptosis through the caspase-9 pathway. Given the distribution of cells in sub-G1 phase was increased and the typical apoptotic morphology changes in the nuclei by NAIF1, we further investigated the apoptosis related proteins by Western blot analysis. As shown in Fig. 4, the PARP and procaspase-3 cleavage is observed in NAIF1-transfected cells, indicating NAIF1 induced apoptosis in MKN45 cells through a caspase pathway.

Table II. Correlations between NAIF1 expression and the clinicopathological variables of the gastric cancers.

Clinicopathological variables	Mean LI	P-value	
		χ^2	r_s
Grade			
Well	1.00±0.91	0.004	0.061
Moderate	0.38±0.70		
Poor	0.48±0.76		
Stage			
I	0.31±0.62	0.186	0.300
II	0.72±0.75		
III	0.59±0.90		
IV	0.58±0.84		
Primary tumor (T) stage			
T1	0.66±0.82	0.083	0.024
T2	0.69±0.93		
T3	0.26±0.56		
T4	0.29±0.67		
Primary tumor (N) stage			
N0	0.62±0.83	0.475	0.135
N1	0.47±0.76		
N2	0.45±0.75		
N3	0.29±0.77		
Primary tumor (M) stage			
M0	0.58±0.81	0.089	0.089
M1	0.00±0.00		
Gender			
Male	0.52±0.81	0.934[a]	
Female	0.53±0.76		
Age			
≥50	0.49±0.77	0.376[b]	
<50	0.63±0.84		

χ^2, P-value from Kruskal-Wallis test. r_s, P-value from Spearman's correlation. [a]Gender, male vs. famale (P-value from ANOVA test). [b]Age, ≥50 vs. <50 (P-value from ANOVA test).

Furthermore, we analyzed caspase-8 for the death receptor-mediated and caspase-9 for the mitochondria-mediated apoptotic pathways. The activation of procaspase-9 was observed in NAIF1-transfected cells. In contrast, proteolytic cleavage of procaspase-8 was not observed under our experimental condition (Fig. 4). These results suggested that NAIF1-induced cell death is functionally linked to activation of caspase-9 and caspase-3.

The N-terminal 1-90 amino acids domain is sufficient for NAIF1 to induce apoptosis. According to the motif scan results, there were two NLS (nuclear location sequence) at the N-terminal and a glycine-rich region (GRR) from amino acids 92-117 in the human NAIF1 protein. Previous study indicated that the GRR motif can promote cell death independently (9). Based on this, we designed two truncated mutation plasmids, pEGFP-N1-NLS(1-90) and pEGFP-N1-GRR(1-117), to investigate the functional domain of NAIF1 (Fig. 2B).

The results of cell cycle analysis showed pEGFP-N1-NLS and pEGFP-N1-GRR also lead to G0/G1 cell cycle arrest (Fig. 2C). Compared to mock, a significant increase in the G0/G1 phase was observed in pEGFP-N1-NLS transfectants (from 65.2±1.0 to 54.4±1.8%) and pEGFP-N1-GRR transfectants (from 64.0±6.4 to 54.4±1.8%). The G2/M phase was decreased from 11.1±0.2 to 2.6±1.4% for pEGFP-N1-NLS transfectants and from 11.1±0.2 to 2.3±2.1% for pEGFP-N1-GRR transfectants. Importantly, the percentage of sub-G1 cells in pEGFP-N1-NLS and pEGFP-N1-GRR transfectants were higher than in pEGFP-N1-NAIF1 transfectants, total of 28.6±6.7 and 33.8±4.1% respectively. The parameter of NAIF1 was 20.7±5.1%.

The results of nuclear DAPI staining analysis showed pEGFP-N1-NLS and pEGFP-N1-GRR also induced apoptosis. The MKN45 cells transfected either of them exhibiting typical morphological changes which indicated they underwent apoptosis (Fig. 3). The following Western blot analysis showed both pEGFP-N1-NLS and pEGFP-N1-GRR can induce cell death through activation of procaspase-9, procaspase-3 and PARP, but no change was observed for procaspase-8. (Fig. 4). All the results indicated, for the induction of apoptosis, that the N-terminal 1-90 amino acids domain of NAIF1 is sufficient to stop this progress. With the sequence GRR, apoptosis can be more efficiently induced in MKN45 cells.

Discussion

It is well known that tumorigenesis is due to the disruption of the balance between cell proliferation and apoptosis, which is maintained by different signaling pathways (10). Many genes playing important roles in apoptosis were down-regulated during tumor progress (11-13). In this study, we show for the first time that NAIF1 is down-regulated or the expression was lost in gastric cancer tissue. Overexpress of NAIF1 can cause G0/G1 arrest inducing apoptosis in gastric cancer cell line MKN45. The first 1-90 amino acids domain of NAIF1 is the minimal functional domain necessary for inducing apoptosis.

By tissue microarrays analysis, NAIF1 has been detected in many tissues. Our result is consistent with previous findings in mRNA level (7), suggesting NAIF1 may have an important role in physiological level. After examining 129 gastric cancer samples and 84 normal gastric samples, we confirmed the difference in expression between normal gastric tissue and gastric cancer tissue (P<0.001). Clinicopathological parameter analysis showed a correlation between tumor pathological T1-4 stage and NAIF1 expression, as well as the expression of NAIF1 at much higher level in well-differentiated tumors compared with in moderately and poorly differentiated tumors (P=0.004). Combining these findings with the negative correlation results of pathological N-stage and M-stage analysis, it shows that the decrease of NAIF1 is an early event in gastric cancer progress. It also hints that NAIF1 plays an

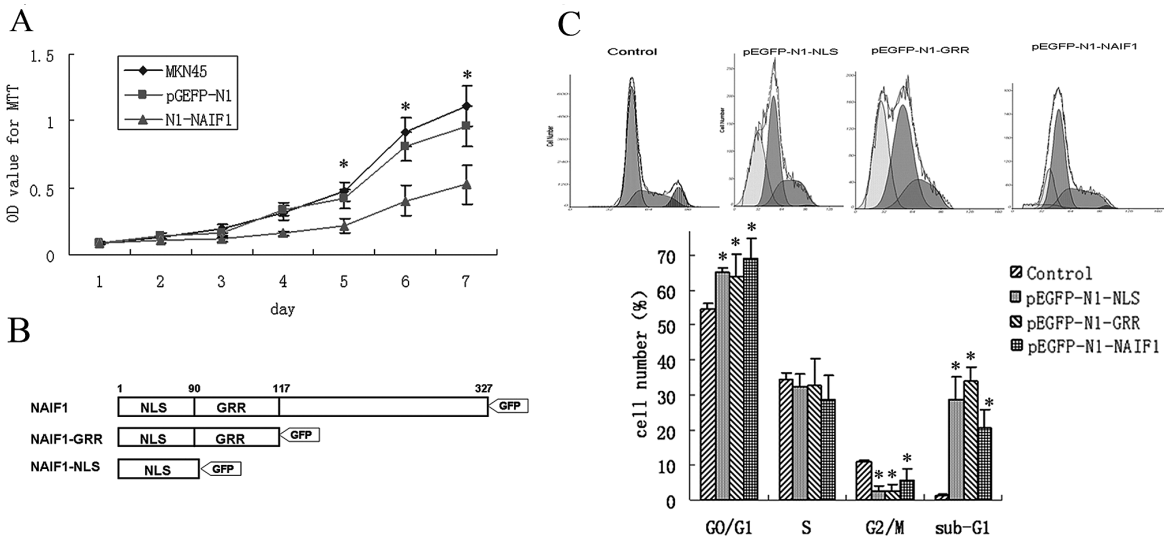

Figure 2. (A) NAIF1 reduced cell viability of gastric cancer cell lines by MTT assay. The significant growth inhibition in NAIF1 transfected MKN45 cells was detected after 5 days incubation, and that was markedly lower (P<0.05) than in pEGFP-N1 transfected MKN45 cells and in control MKN45 cells. *Significant difference (P<0.05) from respective control. (B) The schematic diagram of NAIF1 and its truncated mutations GRR (1-117aa), NLS (1-90aa), all constructed in pEGFP-N1 plasmid. (C) The cell cycle of MKN45 transfected with NAIF1 and the truncated mutations by FCM. Upper, the fitting picture of cell cycle of MKN45 transfected with NAIF1 and the truncated mutation after 48 h by FCM. Only GFP-positive cells were analyzed. Lower, the statistic analysis of cell population in different cell phase. *Significant difference (P<0.05).

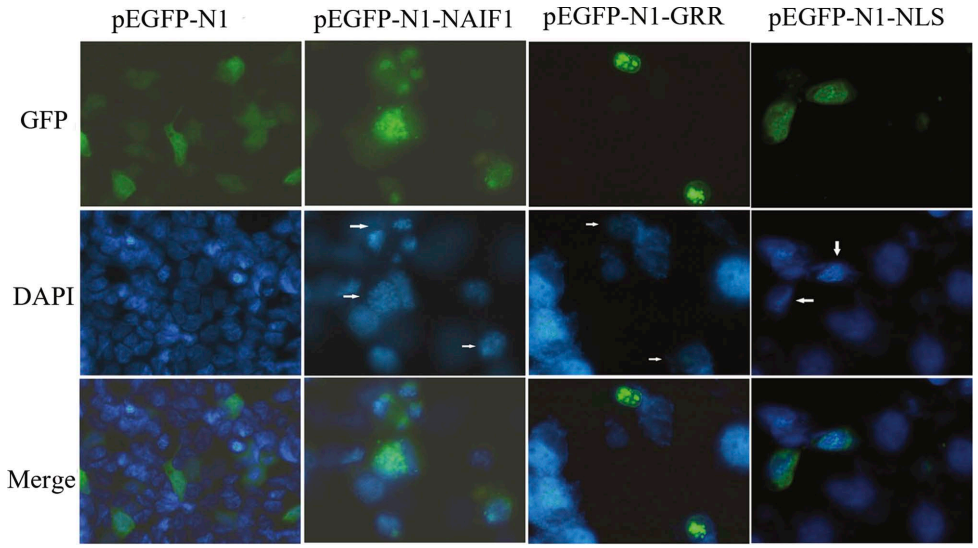

Figure 3. NAIF1 and its truncated mutations induce nuclear morphological changes as indicated by DAPI staining. The upper row shows the GFP-positive cells. Arrows in middle row indicate where the nucleus underwent apoptosis morphological changes. The merge images in lower row indicate cells undergo apoptosis due to NAIF1 and its truncated mutations. Original magnification x200.

inhibitory role in the initial period of gastric cancer genesis. The reason why NAIF1 is down-regulated and what its exact role is during the first period of carcinogenesis is potentially interesting and needs further investigation.

When overexpresing NAIF1 in low background gastric cancer cell line MKN45, we found the growth rate was suppressed markedly, cell arrest and apoptosis was induced by NAIF1. The cell cycle arrest and apoptosis oppose the tumor progress (14,15). Cells can arrest transiently at cell cycle checkpoints to repair the cellular damage. If damage is irreparable, checkpoint signaling might activate pathways that lead to apoptosis. Loss of checkpoint integrity allows the propagation of DNA lesions and results in genomic alterations (16,17). Western blot analysis showed that NAIF1 induces apoptosis through procaspase-9 pathway. Caspase family is stored in most cells as zymogens playing an essential role in the execution of apoptosis. They are divided into apical (-2, -8, -9 and -10) and executioner subsets (-3, -6 and -7) (18,19). Procaspase-9 is activated via a post-mitochondrial route (20). The activation of procaspase-3 and procaspase-9 and PARP cleavage was detected in NAIF1-transfected cells, but the activation of procaspase-8 was not observed, which means NAIF1 carry out its apoptosis-inducing role via intrinsic mitochondria-mediated apoptotic pathways. Whether NAIF1

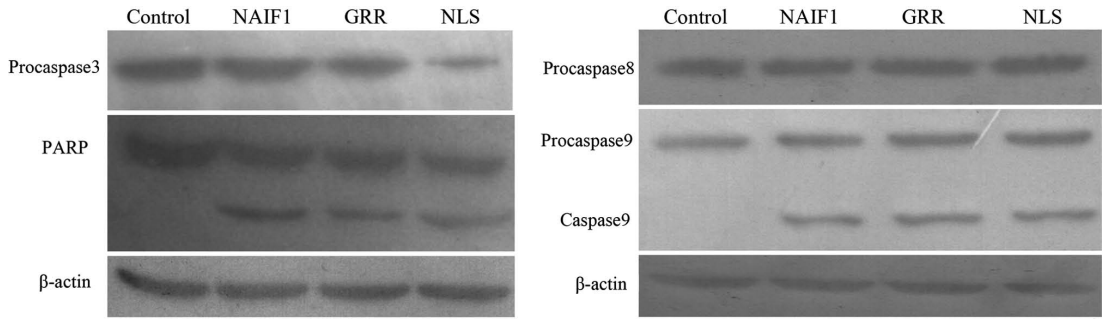

Figure 4. Western blot analysis of apoptosis-related proteins. Western blot analysis of PARP, procaspase-8, -9 and -3 protein expression levels in MKN45 cells 48 h post-transfection. For the full length and truncated NAIF1, all can activate procaspase-3 and its substrate PARP. The activation of procaspase-9 was also observed (35 kDa), but procaspase-8 showed no significant change under our experimental condition. β-actin protein was used as an internal control.

induces apoptosis only via caspase-9 pathway or also through other, like AIF, pathways, needs further investigation.

The motif scan results show there is a glycine-rich region (GRR) from amino acid 92 to 117 in the human NAIF1 protein. GRR has been reported to play important roles in protein-protein interaction, ATP-binding, nucleolar targeting, membrane fusion, ssDNA/RNA-binding activity and inducing apoptosis independently (9,21-25). We were interested whether the apoptosis-inducing function was due to the GRR or not. Therefore, we constructed two truncated mutations, pEGFP-N1-NLS and pEGFP-N1-GRR, for the purpose of identifying the core functional domain of NAIF1. Unexpectedly, the results showed that for inducing apoptosis, the minimum needed is the 1-90 amino acids of NAIF1. With the help of glycine-rich region (91-117aa), it is more efficient to induce apoptosis. The C-terminal of NAIF1 seems to be a slightly negative modulate motif, because the full length NAIF1 induced less sub-G1 populations and weaker activation of the apoptosis-related proteins (Figs. 2C, 3 and 4). We analyzed the secondary structure of NAIF1 protein (http://www.compbio.dundee.ac.uk/www-jpred/). The α-helices structure is predominant in the protein, a helix-turn-helix motif. Recent data have shown helix-turn-helix motif can bind to DNA via the minor groove or interaction with the DNA binding protein, and then regulate the transcription of certain genes (26-28). For example, the LSD1 protein, which forms a compact fold composed of 6 α-helices, binds to the N-terminal tail of histone H3 via the helices motif, repressing the gene expression (29). Thus, we speculate NAIF1 may interact with some DNA binding protein, like histone, to help to change the configuration of DNA and then regulate some gene expression sequentially, inducing apoptosis. The finding that 1-90 amino acids domain of NAIF1 can induce apoptosis provides us the possibility and new strategies for developing anti-cancer drugs of small molecular polypeptides.

NAIF1 is mostly observed in the cytoplasm in normal gastric cells, but NAIF1 and its two truncated mutations were mostly observed in MKN45 nuclei. Based on this, we speculate that NAIF1 plays an important role in control of the expression of some pivotal anti-cancer or apoptotsis-related genes in physiological level. This control may only need a low concentration of NAIF1 in the nuclei. Some mechanisms keep high concentration of NAIF1 in the cytoplasm to guarantee the efficient supplement. When NAIF1 decreased, the expression of some downstream anti-cancer or apoptotic related genes is inhibited, which facilitate tumorigenesis. When the tumor developed to N/M stage, the expression of NAIF1 was dramatically reduced, accompanied with a mechanism which control the high concentration of NAIF1 in the cytoplasm. Thus, when NAIF1 was overexpressed in the MKN45 cancer cells, it gathered in the nuclei and activated the transcription of downstream anti-cancer or apoptotsis-related genes, which then induced apoptosis. The exact mechanism of NAIF1 inducing apoptosis and the physiological role need further exploration.

In summary, our study demonstrated NAIF1 protein is highly expressed in human normal gastric tissue and down-regulated or lost in gastric cancer tissue. When NAIF1 is overexpressed in the gastric cancer cell line MKN45, it causes G0/G1 arrest and induces apoptosis through the procaspase-9 pathway. The N-terminal 1-90 amino acid domain is the core functional domain of NAIF1. Therefore, these findings may provide new strategies for developing anti-cancer drugs of small molecular polypeptides.

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