Involvement of *N*-acetyltransferase human in the cytotoxic activity of 5-fluorouracil

Kazuko Takubo[a,b], Hiroyuki Tsuchiya[a], Akihiro Kurimasa[a], Thomas Arnesen[c], Kazuo Ryoke[b] and Goshi Shiota[a]

_N_-acetyltransferase human (NATH) participates in a posttranslational modification of the proteins and has been reported to play a role in apoptosis. In this study, the involvement of NATH in the cytotoxic action of 5-fluorouracil (5-FU) in human squamous cell carcinoma HEp-2 cells was examined. We found that 5-FU decreased NATH expression in a dose-dependent and time-dependent manner. No change was observed after treatment with bleomycin, nedaplatin, mitomycin C, or methotrexate. Interestingly, knockdown of NATH by small interfering RNA resulted in the downregulation of thymidylate synthase mRNA expression and induced apoptosis. Conversely, NATH overexpression facilitated cell proliferation independent of the presence of 5-FU. The effect of NATH knockdown on the expression of proteins in HEp-2 cells was examined using two-dimensional gel electrophoresis and mass spectrometry. Profilin 1, CutA, ras-related nuclear protein, annexin A5, enolase 1, and elongation factor 1 alpha 1 were found to be upregulated and 14-3-3η, tublin, nuclear auto antigenic sperm protein, heat shock protein 70, and heat shock protein 90 were downregulated by knockdown of NATH. The results of this study suggest that NATH plays an important role in the cytotoxic activity of 5-FU. *Anti-Cancer Drugs* 20:668–675 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2009, **20**:668–675

Keyword: apoptosis, 5-fluorouracil, *N*-acetyltransferase human, proteomic analysis, squamous cell carcinoma

[a]Division of Molecular and Genetic Medicine, Department of Genetic Medicine and Regenerative Therapeutics, Graduate School of Medicine, [b]Division of Oral and Maxillofacial Biopathological Surgery, Department of Medicine of Sensory and Motor Organs, Tottori University, Yonago, Japan and [c]Department of Molecular Biology, University of Bergen, Bergen, Norway

Correspondence to Dr Kazuko Takubo, DDS, Division of Molecular and Genetic Medicine, Department of Genetic Medicine and Regenerative Therapeutics, Graduate School of Medicine, Tottori University, Nishimachi 36-1, Yonago 683-8504, Japan
Tel: +81 859 38 6435; fax: +81 859 38 6430; e-mail: kazuko@med.tottori-u.ac.jp

Received 16 December 2008 **Revised form accepted** 24 April 2009

Introduction

Acetylation is one of many posttranslational modifications of proteins and is conserved among diverse organisms. Histone *N*-ε-acetylation and *N*-ε-deacetylation are the fundamental processes required for transcriptional regulation, in which histone acetyltransferases and histone deacetylases (HDACs) play central roles. For instance, HDAC inhibitors, such as trichostatine A, have been reported to induce growth arrest, differentiation, and apoptosis in various tumor cells [1–3]. Thus, HDAC inhibitors are expected to form a new class of chemotherapeutic agents. In contrast, it has been suggested that as many as 70% of soluble proteins (cytoplasmic and nucleoplasmic) in eukaryotes are subjected to *N*-α-acetyl modification [4,5], in which the α-amino groups of glycine, alanine, serine, threonine, and methionine residues are subjected to acetylation by *N*-α-acetyltransferases [6].

N-acetyltransferase human (NATH) is a homolog of yeast *N*-acetyltransferase 1 protein and mouse *N*-acetyltransferase 1 protein [7–9]. NATH mRNA is ubiquitously expressed at a low level in human adult tissues, especially in the brain, heart, and bone marrow [9]. It has been reported that NATH forms an *N*-α-acetyltransferase complex with human arrest defective 1 (hARD) and human *N*-acetyltransferase 5 (hNAT5) on the ribosomal subunit, implying a cotranslational acetyltransferase function of the complex [10–12]. Interestingly, the dissociation of NATH and hARD1, which results in decreased acetyltransferase activity, was observed during apoptosis [10]. Although the biological function of NATH and the *N*-α-acetyltransferase complex in normal and malignant cells has not yet been clarified, NATH mRNA expression has been reported be upregulated in papillary thyroid carcinomas [9,13]. Moreover, small interfering RNA (siRNA)-mediated knockdown of NATH and hARD1 induced apoptosis, and sensitized cells to daunorubicin-induced apoptosis [14]. In this study, we examined whether NATH is involved in the cytotoxic activity of 5-fluorouracil (5-FU) in squamous cell carcinoma HEp-2 cells. We found that 5-FU treatment resulted in the downregulation of NATH and the knockdown of NATH by short interfering RNA (siRNA) downregulated thymidylate synthase (TS) mRNA expression and induced apoptosis of the cells. Conversely, NATH overexpression facilitated cell proliferation independent of the presence of 5-FU. These data suggest

0959-4973 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32832d5159

that NATH plays an important role in the cytotoxic activity of 5-FU.

Materials and methods

Chemicals

The chemotherapeutic agents used in this study were 5-FU (a gift from Kyowa Hakko Co. Ltd., Tokyo, Japan), bleomycin (Wako Pure Chemical Industries, Osaka, Japan), nedaplatin (a gift of Shionogi & Co. Ltd., Osaka, Japan), mitomycin C (MMC; Wako Pure Chemical Industries), and methotrexate (MTX; Nacalai Tesque, Kyoto, Japan). Hoechst 33258 was purchased from Sigma (St. Louis, Missouri, USA).

Cell culture

HEp-2, Ca9-22, HSC-3, and HSC-4 human head and neck squamous cell carcinoma lines, KYSE70 human esophagus cancer cell line, PANC-1 human pancreas cancer cell line, HuH-7 human liver cancer cell line, and DLD-1 human colon cancer cell line were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2.

Plasmid DNA construction

Human NATH cDNA, obtained from total RNA of HEp-2 cells, was amplified by polymerase chain reaction (PCR) using KOD Plus (TOYOBO, Osaka, Japan) with primers (5′-GAG CTC GGA TCC GAA ACG ATG CCG GCC GTG AGC CTC CCG-3′ and 5′-GAC TCG AGC GGC CGC CCA ATT TCA TTG GCC AGT TCT TC-3′) containing *Bam*HI and *Not*I restriction sites. The amplified cDNA was digested by the restriction enzymes and ligated into *Bam*HI/*Not*I-digested pcDNA3 (pcDNA3/NATH; Invitrogen, Carlsbad, California, USA). To introduce a V5 epitope tag on the C-terminus of NATH, the *Bam*HI-*Not*I fragment of pcDNA3/NATH was inserted into the same restriction sites of pcDNA6/V5-His A (pcDNA6/NATH; Invitrogen).

Transfection

Plasmid DNA was introduced into HEp-2 cells by electroporation (Celljject Pro; ThermoHybaid, Ashford, UK). NATH-specific siRNAs (si4 and si6; Invitrogen) and negative control siRNA (Negative Control Low GC Duplex; Invitrogen) were transfected into the cells using Lipofectamine2000 (Invitrogen), according to the manufacturer's instruction. The sequences of si4 and si6 were 5′-GGG AUA AAG ACA AUC UUC AAA UCU U-3′ and 5′-GGU UAU GCU AUU GCU UAC CAU UUA U-3′, respectively.

WST assay

The Water-Soluble Tetrazolium Salt assay was performed with TetraColor ONE (Seikagaku Co., Tokyo, Japan). Growth inhibition by the chemotherapeutic agents and siRNAs was determined by measurement of the optical density at 450 and 600 (as a reference) nm with a microplate reader (X/Fluor4; Tecan Japan Co. Ltd., Kanagawa, Japan).

RNA isolation and real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen). cDNA was prepared from the RNA using SuperScript First-Strand Synthesis System (Invitrogen). Real-time reverse transcription PCR (RT-PCR) was carried out using the LightCycler system (Roche Diagnostics, Osaka, Japan). Relative expression levels of each transcript were normalized to that of β-actin mRNA. The following primers were used for detection of specific genes: NATH-forward, 5′-AGG AAT TCT GAT TCA TTG CCA CAC-3′; NATH-reverse, 5′-TAT AGC TCG CTT CTG ACT AGA AGG ATC TAA-3′; hARD-forward, 5′-GCA CCT CTA TTC CAA CAC CC-3′; hARD-reverse, 5′-CTT CAT GGC ATA GGC GTC C-3′; hNAT5-forward, 5′-CAC TCT TCC AGC CTT CCT TCC-3′; hNAT5-reverse, 5′-CGT ACA GGT CTT TGC GGA TGT C-3′; TS-forward, 5′-GCT TCC AGT GGA GGC ATT T-3′; TS-reverse, 5′-CAA TCA CTC TTT GCA GTT GGT C-3′; β-actin-forward, 5′-CAC TCT TCC AGC CTT CCT TCC-3′; and β-actin-reverse, 5′-CGT ACA GGT CTT TGC GGA TGT C-3′.

Flow cytometry analysis

After trypsin treatment, cells were fixed in 70% ethanol-PBS at −20°C overnight. Cells were washed once and then incubated in 100 µl PBS containing 0.25 mg/ml RNase (Nippon gene, Osaka, Japan) at 37°C for 1 h. Subsequently, the cells were incubated with propidium iodide (50 µg/ml in PBS) in the dark for 30 min at room temperature. DNA contents of the cells were analyzed by flow cytometry (EPICS ALTRA; Beckman Coulter Inc., Fullerton, California, USA) and the proportion of cells in the subG1 phase was determined by EXPO32 Analysis software (Applied Cytometry Systems, Sheffield, UK).

Western blot analysis

Cells were homogenized with ice-cold radio immunoprecipitation assay buffer [50 mmol/l Tris–HCl (pH 7.9), 150 mmol/l NaCl, 1% nonyl phenoxylpolyethoxylethanol-40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease inhibitors (Complete; Roche Applied Science, Mannheim, Germany). The protein concentration was determined using the Bio-Rad protein assay (BioRad Laboratories, Hercules, California, USA). Western blot analysis was performed according to the standard protocol, using antibodies against poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, California, USA), caspase-8 (Santa Cruz Biotechnology), caspase-9 (Cell Signaling Technology), and actin (Santa Cruz Biotechnology). The antibody against NATH was previously reported [14].

Proteomic analysis

NATH-specific siRNA (si4) and negative control siRNA were transfected into HEp-2 cells using Lipofectamine2000. After 48 h incubation, SDS sample buffer (1% SDS, 5 mol/l Tris–HCl pH 6.8, 1% dithiothreitol) was added to each culture dish and protein was collected. Isoelectic focusing was performed using Immunobiline dry strip pH 3–10 (GE Healthcare, Buckinghamshire, England) or Ampholine pH 3.5–10.0 for IEF (GE Healthcare). SDS-polyacrylamide gel electrophoresis was performed on a 9–18% gradient gel. Protein spots were visualized by SYPRO Ruby (Invitrogen), and analyzed using ImageMaster 2D 5.0 Platinum (GE Healthcare). Among protein spots that differed by more than 1.5 between si4 and negative control siRNA, those with intensities by which sufficient amount of protein can be estimated were selected for mass spectrometry (MS) analysis. MS and MS/MS spectra were obtained using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics K.K., Osaka, Japan). Identification of proteins was carried out using MASCOT software (Matrix Science K.K., Tokyo, Japan) with the NCBInr database.

Statistical analysis

Student's *t*-test was used to examine the significant intergroup differences and *P* value of less than 0.05 was considered to be significant.

Results

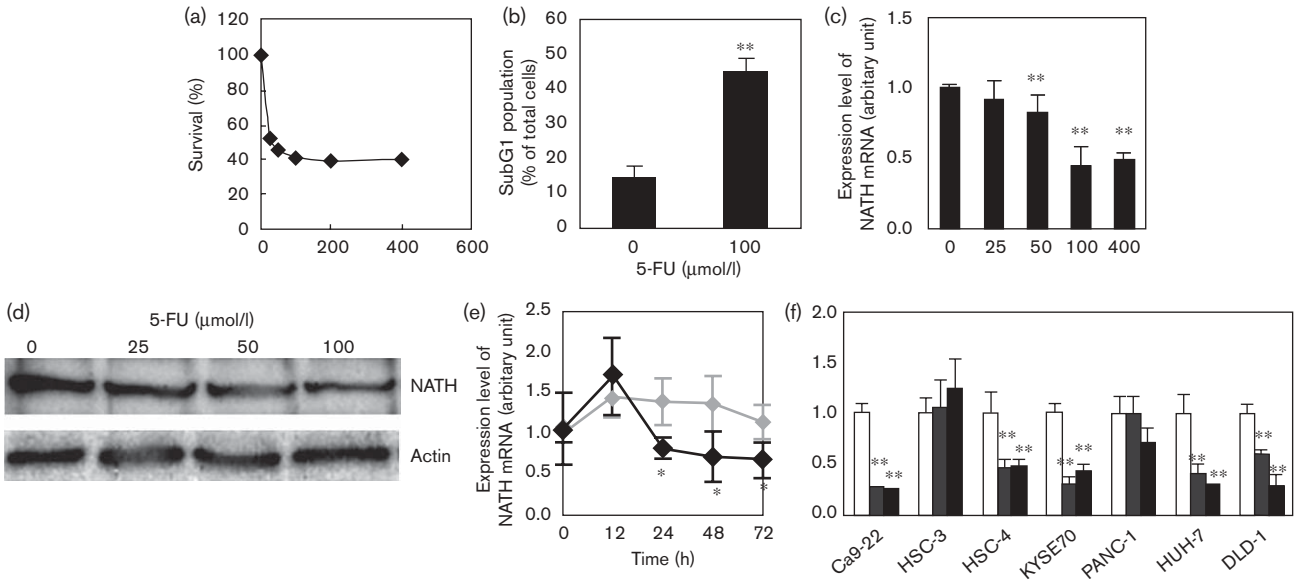
5-fluorouracil-induced *N*-acetyltransferase human downregulation

The effect of 5-FU on the viability of HEp-2 cells was examined by the WST assay. The cell viability decreased in a dose-dependent manner up to 100 μmol/l 5-FU, and maintained a plateau at higher concentrations (Fig. 1a). As judged by Hoechst33258 staining, 5-FU caused apoptosis of HEp-2 cells (data not shown). In addition, the subG1 population of HEp-2 cells treated with 100 μmol/l 5-FU was significantly increased (Fig. 1b). Concomitantly, NATH mRNA and protein levels were apparently downregulated in a dose-dependent manner (Fig. 1c and d). The NATH mRNA expression level was decreased in a time-dependent manner after treatment with 5-FU (Fig. 1e). Although hARD and hNAT5 have been reported to form a complex with NATH [12], both mRNA expressions were significantly upregulated in the presence of 5-FU (data not shown). In other cell lines that are also sensitive to 5-FU, NATH mRNA expression level was significantly downregulated by 5-FU treatment, except in HSC-3 and PANC-1 cell lines (Fig. 1f).

Induction of apoptosis by *N*-acetyltransferase human knockdown

The effect of siRNA-mediated knockdown of NATH expression on cell viability was examined. Two siRNAs

Fig. 1



5-fluorouracil (5-FU)-induced *N*-acetyltransferase human (NATH) downregulation. (a) Cell viability of HEp-2 cells at the indicated concentrations of 5-FU for 48 h. (b) SubG1 population of HEp-2 cells at 48 h after treatment with 0 and 100 μmol/l 5-FU. (c) NATH mRNA expression in HEp-2 cells treated with the indicated concentrations of 5-FU for 48 h. (d) NATH protein expression in HEp-2 cells, after treatment of the indicated concentrations of 5-FU for 48 h. Actin was used as an internal control. (e) NATH mRNA expression in HEp-2 cells treated with the 0 μmol/l (gray line) and 100 μmol/l (black line) 5-FU for the indicated times. (f) NATH mRNA expression in Ca9-22, HSC-3, HSC-4, KYSE70, PANC-1, HuH-7, and DLD-1 cells treated with 0 μmol/l (blank bars), 50 μmol/l (gray bars) and 100 μmol/l (black bars) 5-FU for 48 h. (a, b, c, e) Data are shown as means ± SD ($n=3$). *$P<0.05$, **$P<0.01$ versus 5-FU 0 μmol/l or 0 h.

(si4 and si6) with different target sites in the NATH mRNA were used. Both siRNAs effectively knocked down NATH mRNA expression at 48 h posttransfection in HEp-2 cells (Fig. 2a). In addition, the viability of HEp-2 cells transfected with si4 and si6 was significantly decreased, as compared with those transfected with a negative control siRNA (Fig. 2b). Although siRNA transfection provided a greater reduction in NATH mRNA expression as compared with 5-FU treatment (Figs 1c and 2a), 5-FU showed more potent cytotoxic activity compared with the NATH-specific siRNA (Figs 1a and 2b). These results indicate that 5-FU has diverse mechanisms of action, one of which is involved in the downregulation of NATH (also see the discussion).

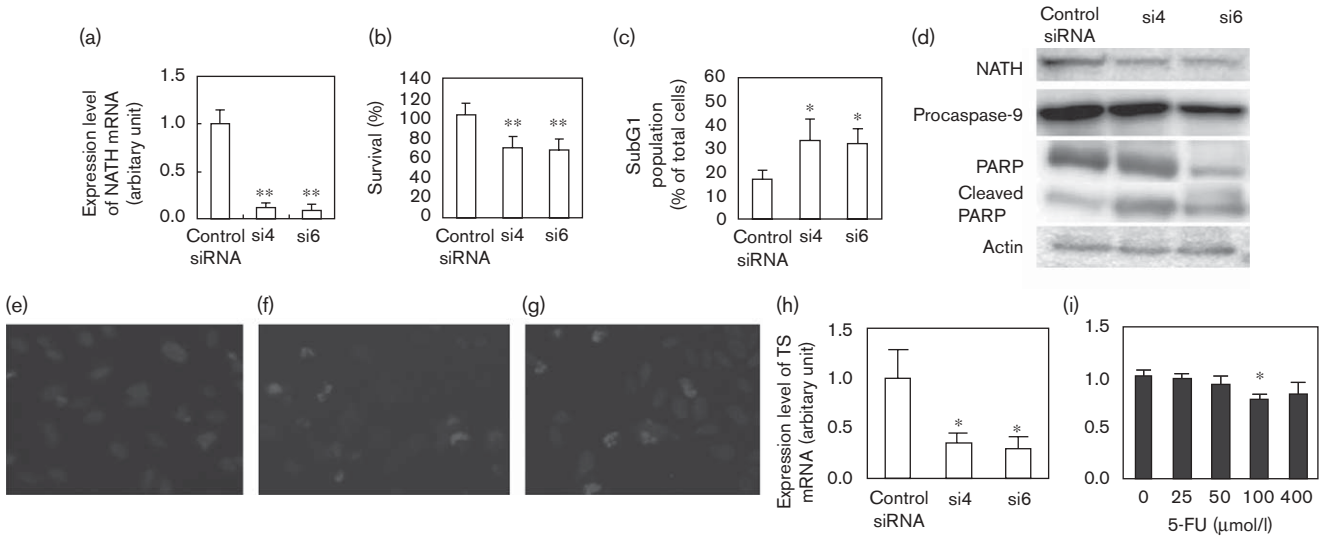
To address whether siRNA-mediated NATH knockdown induces apoptosis in HEp-2 cells, we quantified a subG1 population by flow cytometry. HEp-2 cells transfected with si4 and si6 showed a two-fold increase in the subG1 population, indicating that NATH knockdown induced apoptosis in the cells (Fig. 2c). Moreover, western blot analysis showed decreases in NATH, procaspase-9, and PARP proteins in the cells transfected with si4 and si6. A notable increase in cleaved PARP was also observed, indicating caspase-3 activation (Fig. 2d). Despite the robust reduction in NATH mRNA expression, NATH protein level was decreased by the siRNAs to a similar magnitude to that by 5-FU treatment (Fig. 1d). A notable increase in cleaved PARP was also observed, indicating caspase-3 activation (Fig. 2d). Consistent with these observations, Hoechst33258 staining revealed that si4 and si6 increased the number of cells with apoptotic nuclear morphology (Fig. 2f and g), compared with those transfected with negative control siRNA (Fig. 2e). These data suggest that caspase-3 and caspase-9 are involved in the apoptosis of HEp-2 cells induced by NATH knockdown. However, alteration in caspase-8 expression was not observed (data not shown), suggesting that this apoptosis is not attributable to the death receptor pathway.

It has been well established that the cytotoxic action of 5-FU is mediated through the inhibition of TS activity. Therefore, real-time RT-PCR was carried out to investigate the effect of NATH on TS expression. TS mRNA expression dramatically decreased in HEp-2 cells transfected with NATH siRNA (Fig. 2h). Interestingly, a moderate but significant decrease in TS expression was observed when HEp-2 cells were treated with 5-FU (Fig. 2i). This result suggests that NATH downregulation plays a role in one of the mechanisms by which 5-FU inhibits TS.

Increased cell viability by *N*-acetyltransferase human overexpression

To explore the biological function of NATH, we examined whether overexpression of NATH affects cell

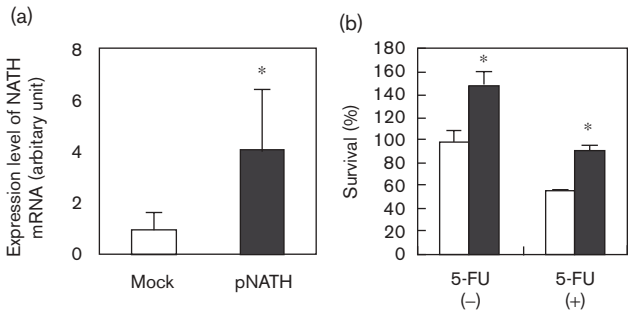
Fig. 2



Induction of apoptosis by knockdown of *N*-acetyltransferase human (NATH). (a) NATH mRNA expression levels at 48 h after transfection of control small interfering RNA (siRNA) and NATH-specific siRNAs (si4 and si6) in HEp-2 cells. (b) Cell viability of HEp-2 cells at 48 h after transfection of control siRNA, si4 and si6. (c) SubG1 population of HEp-2 at 48 h after cells transfection of control siRNA and NATH-specific siRNAs. (d) Expression of NATH, procaspase-9, and poly (ADP-ribose) polymerase (PARP) at 48 h after transfection of control siRNA and NATH-specific siRNAs. Actin was used as an internal control. (e) Hoechst33258 staining of HEp-2 cells transfected with negative control siRNA. At 48 h after transfection, the cells were stained, incubated with 10 μmol/l Hoechst33258 for 1 h. (f) Hoechst33258 staining of HEp-2 cells transfected with si4. (g) Hoechst33258 staining of HEp-2 cells transfected with si6. (h) Thymidylate synthase (TS) mRNA expression levels at 48 h after transfection of control siRNA and NATH-specific siRNAs (si4 and si6) in HEp-2 cells. (i) TS mRNA expression in HEp-2 cells at the indicated concentration of 5-fluorouracil (5-FU) for 48 h. (a, b, c, h, i) Data are shown as means ± SD ($n=3$). *$P<0.05$, **$P<0.01$ versus negative control siRNA or 5-FU 0 μmol/l.

viability in the presence and absence of 5-FU. In HEp-2 cells transfected with pcDNA6/NATH, real-time RT-PCR showed an approximately four-fold increase in NATH mRNA expression (Fig. 3a). 5-FU treatment resulted in a similar magnitude of a decrease in cell viability irrespective of NATH overexpression (Fig. 3b). This result suggests that the 5-FU-induced cytotoxicity involves various mechanisms, which cannot be explained solely by NATH downregulation. More importantly, it was suggested that NATH overexpression directly stimulates cell proliferation.

Fig. 3



Association of cytotoxic action of 5-fluorouracil (5-FU) with *N*-acetyltransferase human (NATH) mRNA level. (a) NATH mRNA expression levels at 24 h after transfection with pcDNA6/V5-HisA (mock; blank bar) and pcDNA6/NATH (pNATH; dark gray bar). (b) Effect of NATH expression on cytotoxity induced by 5-FU. Cell viability was determined at 36 h after treatment of 5-FU in the cells that had been transfected with pcDNA6/V5-HisA (blank bar) and pcDNA6/NATH (dark gray bar) for 12 h. (a, b) Data are shown as means ± SD ($n=3$). *$P<0.05$ versus mock or 5-FU 0 μmol/l.

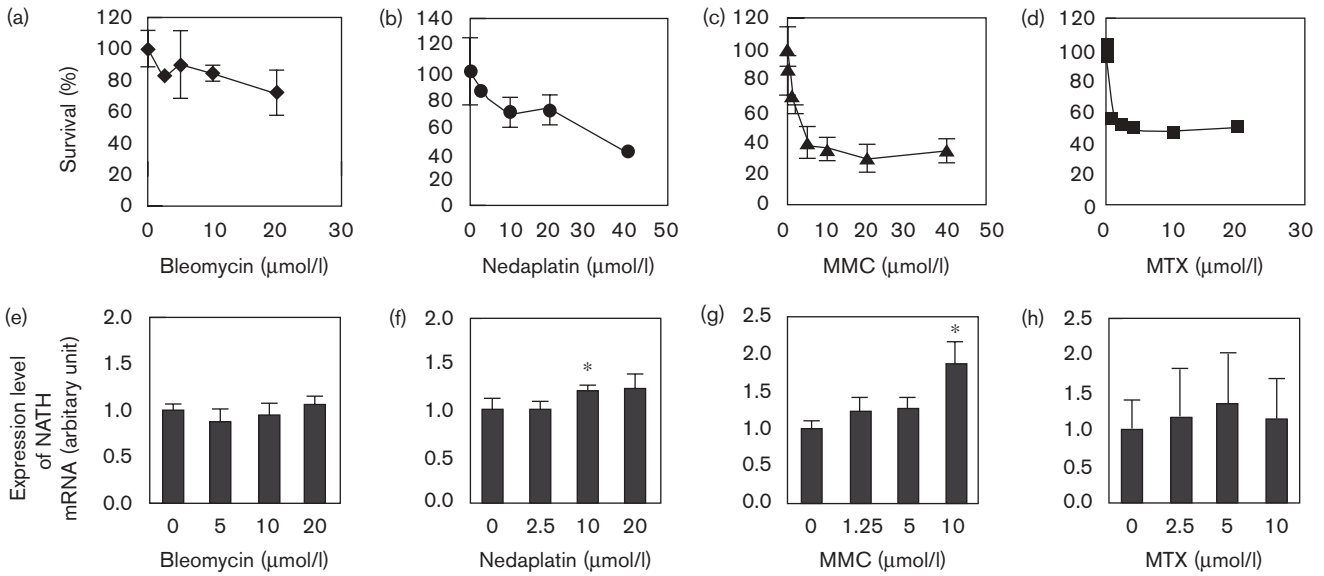
Effect of other chemotherapeutic agents on *N*-acetyltransferase human expression

The WST assay showed that bleomycin, nedaplatin, MMC, and MTX exerted cytotoxic effect on HEp-2 cells in a dose-dependent manner although the sensitivity varied among the agents (Fig. 4a–d, respectively). To examine whether these agents had any effect on NATH mRNA expression, NATH mRNA was measured. NATH mRNA expression in HEp-2 cells was not altered by treatment with bleomycin (Fig. 4e). However, NATH mRNA was increased at 10 μmol/l nedaplatin and 10 μmol/l MMC (Fig. 4f and g, respectively). These results suggest that the cytotoxic action of these agents is different from that of 5-FU. Interestingly, MTX did not affect the NATH expression although folate metabolism is the target of MTX as well as 5-FU (a TS inhibitor) (Fig. 4h). These results suggest that the cytotoxic actions of bleomycin, nedaplatin, MMC, and MTX are related to another mechanisms than NATH downregulation.

Identification of differentially expressed proteins in *N*-acetyltransferase human-knocked-down HEp-2 cells

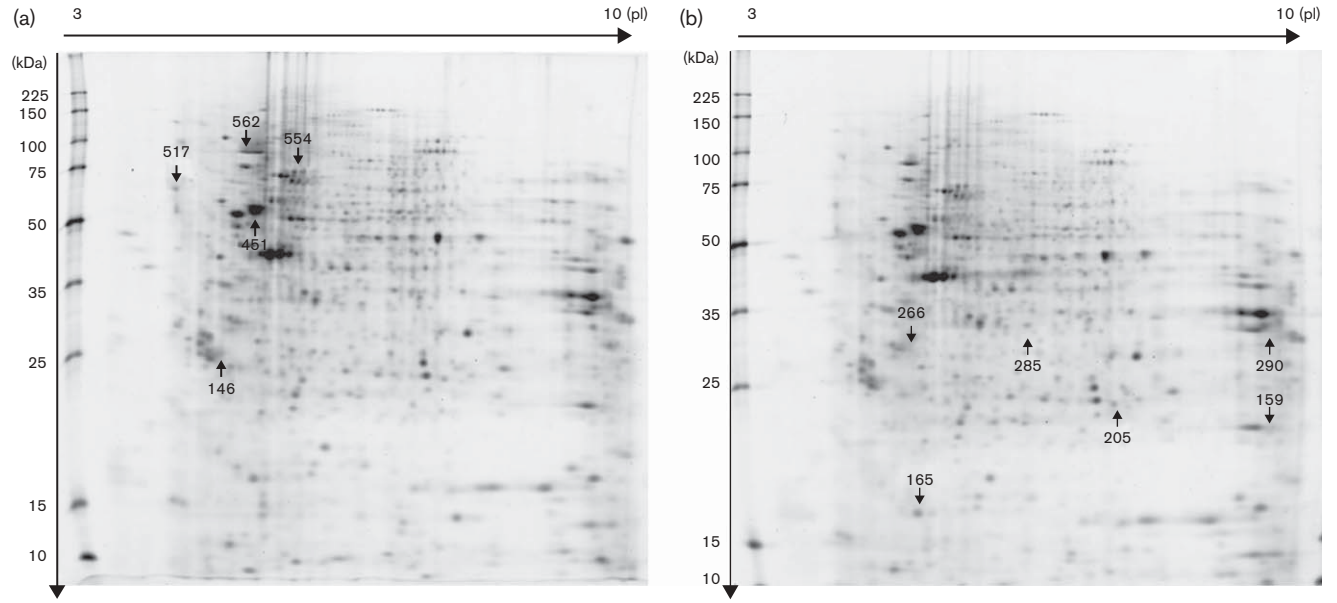
Using image analysis following two-dimensional gel electrophoresis, 34 spots were picked up, in which 18 were upregulated and 16 were downregulated, respectively,

Fig. 4



Effect of other chemotherapeutic agents on *N*-acetyltransferase human (NATH) mRNA expression. (a–d) Cell viability of the HEp-2 cells treated for 48 h with the indicated concentrations of bleomycin, nedaplatin, mitomycin C (MMC), and methotrexate (MTX), respectively. (e–h) NATH mRNA expression in the HEp-2 cells treated for 48 h by bleomycin, nedaplatin, MMC, and MTX, respectively. (a–h) Data are shown as means ± SD ($n=3$). *$P<0.05$ versus 0 μmol/l each chemotherapeutic agent.

Fig. 5



Two-dimensional gel analysis of differentially expressed proteins between (a) negative control siRNA and (b) NATH-specific siRNA. Proteins were solubilized in SDS sample buffer according to the procedure detailed in 'Material and methods' and separated by first-dimension pH 3–10 immunobilized pH gradient strips followed by second-dimensional separation by SDS-polyacrylamide gel electrophoresis. Differentially expressed protein spots marked with arrows were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Table 1 Proteins identified by MALDI-TOF MS

Spot no.[a]	Accession no.[b]	Protein name	Mass weight (Da)	pI	%Vol si4	%Vol negative	Ratio si4/negative	Function
Upregulated proteins								
159	4826898	Profilin 1	15 216	8.44	0.85	0.30	2.85	Actin-binding protein
165	7341255	CutA	14 769	4.80	0.44	0.20	2.17	Unknown
205	4092054	RAN	24 668	7.00	0.24	0.14	1.68	GTPase
266	809185	Annexin A5	35 840	4.94	0.36	0.22	1.61	Ca^{2+}-dependent membrane binding protein
285	4503571	ENO1	47 481	7.01	0.40	0.18	2.19	Glycolytic protein
290	927065	EEF1A1	43 139	8.54	0.77	0.36	2.13	Elongation factor protein
Downregulated proteins								
146	437363	14-3-3η	28 343	4.76	0.16	0.25	0.63	Neuronal protein
451	37492	Tublin	50 746	5.02	0.45	0.71	0.63	Cytoskeletal protein
517	184433	NASP	85 424	4.27	0.19	0.31	0.63	Histone chaperone
554	12653415	HSP70	73 967	6.03	0.19	0.31	0.62	Molecular chaperone
562	306891	HSP90	83 584	4.97	0.87	1.70	0.51	Molecular chaperone

EEF1A1, elongation factor 1 alpha 1; ENO1, enolase 1; HSP70, heat shock protein 70; HSP90, heat shock protein 90; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NASP, nuclear auto antigenic sperm protein; pI, isoelectric point; RAN, ras-related nuclear protein.
[a]Spot numbers refer to Fig. 5a and b.
[b]Description corresponds to the protein name given by National Center for Biotechnology Information database.

in HEp-2 cells transfected with NATH-specific siRNAs (Fig. 5a and b). By a MASCOT search, six upregulated proteins were identified as profilin 1, CutA, ras-related nuclear protein (RAN), annexin A5, enolase 1 (ENO1), and elongation factor 1 alpha 1, and five downregulated proteins were identified as 14-3-3η, tublin, nuclear auto antigenic sperm protein, heat shock protein 70 (HSP70), and heat shock protein 90 (Table 1).

Discussion

Since 1957 when 5-FU was discovered as a promising chemotherapeutic agent [15–17], this pyrimidine analog

has been widely used in cancer chemotherapy, for example, in cervical carcinoma, head and neck cancer, colorectal cancer, and hepatocellular carcinoma [18–21]. It has been reported that 5-FU induces cell cycle arrest in S phase in a colon cancer cell line and HeLa cells [22,23] by either inhibition of TS activity and DNA synthesis or incorporation into RNA and disruption of RNA and protein synthesis [24]. However, the molecules that are involved in the mechanism of 5-FU action have not yet been fully elucidated.

It was reported that NATH forms an *N*-α-acetyltransferase complex with hARD and hNAT5 on the ribosome [12], suggesting that NATH plays a role in translation-coupled *N*-α-acetylation of newly synthesized polypeptides. Moreover, it has been reported that the knockdown of NATH, or other *N*-α-acetyltransferase subunits, induces growth arrest and apoptosis in HeLa cells, and sensitized HeLaS3 cells to daunorubicin-induced apoptosis [14]. In addition, our preliminary study identified the NATH gene, whose expression was dramatically altered by 5-FU, by differential display screening (data not shown). Hence, we examined whether NATH is involved in the cytotoxic action of 5-FU.

As mentioned above, TS is one of the important factors whose activity influences the cytotoxic effect of 5-FU [24]. Our study, using NATH-specific siRNA, showed that NATH knockdown resulted in a significant decrease in the TS mRNA expression. However, although NATH expression was markedly downregulated in the presence of 5-FU, the TS mRNA expression level was significantly, but only slightly lowered in HEp-2 cells. Evans *et al.* [25] reported that HEp-2 cells were relatively resistant against 5-FU treatment among tumor cell lines. The report also suggests that the cytotoxic action of 5-FU in HEp-2 cells is largely dependent on its incorporation into RNA, but not on TS inhibition. Thus, our observation implies that HEp-2 cells may have acquired a survival advantage against 5-FU, which counteracts the decrease in the TS mRNA expression induced by NATH downregulation. Clarification of the mechanism of this counteraction will allow us to achieve further improvement in the clinical efficacy of 5-FU, especially in patients with refractory tumor.

In contrast, it was shown that NATH overexpression facilitated cell proliferation independent of the presence of 5-FU. This result indicates that although the effect on the acquisition of resistant phenotype is still uncertain, the dysregulation of NATH expression may be involved in the development of tumor cells [9,13]. Further study must be performed to clarify how NATH regulates TS expression and cell proliferation in detail.

The mechanisms by which 5-FU downregulates NATH expression and upregulates hARD and hNAT5 expressions remain to be clarified. Interestingly, siRNA-mediated knockdown of NATH did not affect the expression levels of hARD and hNAT5 (data not shown). Given that the *N*-α-acetyltransferase complex consists of three subunits, NATH knockdown might be sufficient to disturb the function of the complex. Alternatively, 5-FU-mediated transcriptional regulation of NATH and the other two genes might involve independent mechanisms. Further study is required to investigate the interaction of NATH with hARD and hNAT5, the activity of the *N*-α-acetyltransferase complex, and the target proteins for the *N*-α-acetyltransferase complex.

In this study, the proteomics analysis revealed that NATH knockdown caused upregulation of proteins including profilin 1, CutA, RAN, annexin A5, ENO1, and elongation factor 1 alpha 1, and caused downregulation of proteins including 14-3-3η, tubulin, nuclear auto antigenic sperm protein, HSP70, and heat shock protein 90. Interestingly, downregulation of HSP70 and ENO1, and upregulation of RAN by 5-FU treatment in a colorectal cancer cell line SW480 have been reported [26]. It is of great interest to clarify how siRNA-induced knockdown of NATH leads to the downregulation of these proteins. Although the downregulation of TS mRNA level by siRNA-mediated NATH knockdown was observed, no spot identified as TS was obtained. This may be because of our selection criteria for MS analysis (i.e. that the spots with intensity by which sufficient amount of protein can be estimated were selected).

In this study, we found that 5-FU downregulates NATH expression not only in HEp-2 cells, but also in other cancer cell lines, and the downregulation of NATH plays an important role in 5-FU-induced cytotoxicity.

Conclusion

We demonstrated that 5-FU decreased the mRNA level of NATH, which is involved in apoptosis and sensitivity to chemotherapeutic agents. Our study sheds light on the importance of NATH for cancer chemotherapy as well as cancer development.

Acknowledgement

Conflicts of interest: none.

References

1 Yoshida M, Huremia R, Nishiyama M, Komatsu Y, Nishino N, Horinouchi S. Histone deacetylase as a new target for cancer chemotherapy. *Cancer Chemother Pharmacol* 2001; **48**:S20–S26.
2 Gennaro ED, Bruzzese F, Caraglia M, Abruzzese A, Budillon A. Acetylation of proteins as novel target for antitumor therapy: review article. *Amino Acids* 2004; **26**:435–441.
3 Monneret C. Histone deacetylase inhibitors. *Eur J Med Chem* 2005; **40**:1–13.
4 Bradshaw RA, Brickey WW, Walker KW. N-terminal processing: the methionine aminopeptidase and N alpha-acetyl transferase families. *Trends Biochem Sci* 1998; **23**:263–267.

5 Brown JL. The N-terminal region of soluble proteins from procaryotes and eukaryotes. *Biochem Biophys Acta* 1970; **221**:480–488.
6 Polevoda B, Norbeck J, Takakura H, Blomberg A, Sherman F. Identification and specificities of N-terminal acetyltransferases from Saccharomyces cerevisiae. *EMBO J* 1999; **18**:6155–6168.
7 Mullen JR, Kayne PS, Moerschell RP, Tsunasawa S, Gribskov M, Shepanski MC, *et al*. Identification and characterization of genes and mutants for N-terminal acetyltransferase from yeast. *EMBO J* 1989; **8**:2067–2075.
8 Sugiura N, Adams SM, Corriveau RA. An evolutionarily conserved N-terminal acetyltransferase complex associated with neuronasal development. *J Biol Chem* 2003; **278**:40113–40120.
9 Fluge Ø, Bruland O, Akslen LA, Varhaug JE, Lillehaug JR. NATH, a novel gene overexpressed in papillary thyroid carcinomas. *Oncogene* 2002; **21**:5056–5068.
10 Arnesen T, Anderson D, Baldersheim C, Lanotte M, Varhaug JE, Lillehaug JR. Identification and characterization of the human ARD1-NATH protein acetyltransferase complex. *Biochem J* 2005; **386**:433–443.
11 Gautschi M, Just S, Mun A, Ross S, Rücknagel P, Dubaquie Y, *et al*. The yeast N alpha-acetyltransferase NatA is quantitatively anchored to the ribosome and interacts with nascent polypeptides. *Mol Cell Biol* 2003; **23**:7403–7414.
12 Arnesen T, Anderson D, Torsvik J, Halseth HB, Varhaug JE, Lillehaug JR. Cloning and chracterization of hNAT5/hSAN: an evolutionarily conserved component of the NatA protein N-α-acetyltransferase complex. *GENE* 2006; **371**:291–295.
13 Arnesen T, Gromyko D, Horvli O, Fluge Ø, Lillehaug J, Varhaug JE. Expression of N-acetyl transferase human and human arrest defective 1 proteins in thyroid neoplasms. *Thyroid* 2005; **15**:1131–1136.
14 Arnesen T, Gromyko D, Pendino F, Ryningen A, Varhaug JE, Lillehaug JR. Induction of apoptosis in human cells by RNAi-mediated knockdown of hARD1 and NATH, components of the protein N-α-acetyltransferase complex. *Oncogene* 2006; **25**:4350–4360.
15 Heiderberger C, Chaudhuri NK, Danneberg P, Mooren D, Griesbach L, Duschinsky R, *et al*. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature* 1957; **30**:663–666.
16 Spiegelman S, Sawyer R, Nayak R, Ritzi E, Stolfi R, Martin D. Improving the anti-tumor activity of 5-fluorouracil by increasing its incorporation into RNA via metabolic modulatuion. *Proc Natl Acad Sci U S A* 1980; **77**:4966–4970.
17 Parker WB, Cheng YC. Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol Ther* 1990; **48**:381–395.
18 Aoki Y, Sato T, Watanabe M, Sasaki M, Tsuneki I, Tanaka K. Neoadjuvant chemotherapy using low-dose consecutive intraarterial infusions of cisplatin combined with 5-fluorouracil for locally advanced cervical adenocarcinoma. *Gynecol Oncol* 2001; **81**:496–499.
19 Christiansen H, Hermann RM, Hille A, Weiss E, Nitsche M, Martin A, *et al*. Concomitant radiochemotherapy in primary inoperable advanced head and neck cancer with 5-fluorouracil and mitomycin-c. *Head Neck* 2004; **26**:845–853.
20 Ducreux M, Bouche O, Pignon JP, Mousseau M, Raoul JL, Cassan P, *et al*. Randomised trial comparing three different schedules of infusional 5FU and raltitrexed alone as first-line therapy in metastatic colorectal cancer. *Oncology* 2006; **70**:222–230.
21 Uka K, Akita H, Takaki S, Miki D, Kawaoka T, Jeong SC, *et al*. Pretreatment predictor of response, time to progression, and survival to intraarterial 5-fluorouracil/interferon combination therapy in patients with advanced hepatocellular carcinoma. *J Gastroenterol* 2007; **42**:845–853.
22 Inaba M, Mitsuhashi J. Flow cytometric analysis of cell-killing of 5-fluorouracil in human colorectal cancer cells. *Oncol Res* 1994; **6**:303–309.
23 Xiao Z, Xue J, Sowin TJ, Rosenberg SH, Zhang H. A novel mechanism of checkpoint abrogation conferred by Chk1 downregulation. *Oncogene* 2005; **24**:1403–1411.
24 Van Tris B, Pinedo HM, Giaccone G, Peters GJ. Downstream molecular determinants of response to 5-fluorouracil and antifolate thymidylate synthase inhibitors. *Ann Oncol* 2000; **11**:385–391.
25 Evans RM, Laskin JD, Hakala MT. Assessment of growth-limiting events caused by 5-fluorouracil in mouse cells and in human cells. *Cancer Res* 1980; **40**:4113–4122.
26 Wong CS, Wong VW, Chan CM, Ma BB, Hui EP, Wong MC, *et al*. Identification of 5-fluorouracil response proteins in colorectal carcinoma cell line SW480 by two-dimensional electrophoresis and MALDI-TOF mass spectrometry. *Oncol Rep* 2008; **20**:89–98.