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Overexpression of nicotinamide *N*-methyltransferase in gastric cancer tissues and its potential post-translational modification

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Abbreviations: 2-DE, 2-dimensional electrophoresis; NNMT, nicotinamide *N*-methyltransferase

Abstract

Gastric cancer is one of the most common cancers worldwide. The purpose of this study was to find out potential markers for gastric cancer. Tumor and normal tissues from 152 gastric cancer cases were analyzed by two-dimensional gel electrophoresis (2-DE). The images of silver stained gels were analyzed and statistical analysis of spot intensities revealed that spot 4262 showed higher expression (5.7-fold increase) in cancer tissues than in normal tissues ($P < 0.001$). It was identified by peptide mass fingerprinting as nicotinamide *N*-methyltransferase (NNMT). A monoclonal antibody with a detection limit down to 10 ng was produced against NNMT in mouse. Using the prepared monoclonal antibody, western blot analysis of NNMT was performed for gastric tissues from 15 gastric cancer patients and two gastric ulcer patients. The results corroborated those of 2-DE experiments. A single spot was detected in gastric ulcer tissues while four to five spots were detected in gastric cancer tissues. In cancer tissues, two additional spots of acidic and basic form were mainly detected on 2-DE gels. This suggests that NNMT receives a post-translational modification in cancer- specific manner.

Keywords: electrophoresis, gel, two-dimensional; nicotinamide *N*-methyltransferase; protein processing, post-translational; proteomics; stomach neoplasms

Introduction

Gastric cancer is the most common cancer in Korea and other Asian countries. Although incidence rates in western world are much lower than in Asia, gastric carcinoma is still a significant worldwide health burden, second only to lung tumors as a leading cause of cancer deaths (Lyman, 1992; Ha *et al*., 2002; Kim *et al*., 2002). To reduce the mortality and improve the effectiveness of therapy, many studies have tried to find key biomarkers. Biomarkers are important molecular signposts of the biologic state of a cell at a specific condition (Tahara *et al*., 1996; Loging *et al*., 2000). Two-dimensional gel electrophoresis (2-DE) is widely used for in investigation of protein complements between diseased and healthy tissue with the purpose of developing diagnostic markers and detecting novel drug targets (Wilkins *et al*., 1996; Gygi *et al*., 2000). Proteomic technologies are providing the tools needed to discover and identify biomarkers associated with diverse diseases and biological processes (Blackstock *et al*., 1999; Hanash, 2003; Choi *et al*., 2004; Eun *et al*., 2004). There have been many studies including analysis of prognostic factors, development of chemotherapeutic agents, and the search for the early detecting molecules, but there are currently very few molecular markers that are clinically in use. Proteomics is one of the technologies that rapidly change our approach to cancer research and can lead to the molecular characterization of cellular events associated with cancer progression, signaling, and developmental stages. 152 cases of gastric cancer and normal tissues were analyzed by two-dimensional electrophoresis, and western blotting. As a result of this study, we suggest that nicotinamide *N*-methyltransferase will be an efficient tumor marker in gastric cancer diagnosis.

Materials and Methods

Materials

IPG strips of pH 3-10, 4-7 were purchased from

Amersham Pharmacia Biotech (APS, Immobiline Dry-Strip, 0.5 × 3 × 180 mm, Uppsala, Sweden) and Bio-Rad (ReadyStrip; 0.5 × 3 × 170 mm, Hercules, CA). Bio-Lyte (pH 3-10) was from Bio-Rad. SDS, acrylamide, methylenebisacrylamide, TEMED, ammonium persulfate, DTT, urea, Tris, glycine, glycerol, and CHAPS were purchased from Bio-Rad or USB (Cleveland, OH). Silver nitrate, coomassie Brilliant Blue G-250, TCA, iodoacetamide, and α-cyano-4-hydroxycinnamic acid were from Sigma (St. Louis, MO). Methanol, ethanol, phosphoric acid, acetic acid and formaldehyde were purchased from Merck (Darmstadt, Germany). Sequencing grade-modified trypsin was obtained from Promega (Madison, WI). Other reagents were obtained from Sigma or Merck.

Stomach tissue samples

Human stomach tissue samples were prepared from resection materials of gastric cancer patients in Gyeongsang National University Hospital. Resections were examined by a pathologist and normal tissue samples were prepared from noncancerous regions.

Preparation of stomach tissue protein samples

Frozen stomach tissue samples (100-200 mg) were homogenized in 2 ml homogenization buffer (50 mM Tris-HCl, pH 7.2) containing a protease inhibitor cocktail [1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, 14 μM E-64] using an utraturrax homogenizer (type T8, IKA Labortechnik, Staufen, Germany) at 25,000 rpm. The mixture was centrifuged at 1,000 *g* for 5 min to remove tissue and cell debris. The supernatant was used as total homogenate. The total homogenate was centrifuged in a Beckman TL-100 table top ultracentrifuge at 100,000 rpm (approx. 430,000 *g*) in a TLA-100.2 rotor for 10 min at 4°C. The supernatant was taken as soluble fraction and the pellet was used for membrane protein preparation. TCA (50% w/v) was added to the supernatant to a final concentration of 10% w/v and the solution was allowed to stand on ice for 30 min. Protein precipitate was collected by spin in a microcentrifuge at 15,000 rpm for 10 min at 4°C, and washed three times in 10% TCA. The precipitate was washed once in diethyl ether and dried under air stream. The dry pellet was dissolved with sonication in the lysis solution [8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 100 mM DTT, 2% w/v Bio-Lyte pH 3-10] and allowed to stand for 1 h at room temperature. After centrifugation at 15,000 *g* for 10 min at 15°C, the supernatant was used as the 2-DE sample for the soluble fraction. The protein samples were stored in aliquots at -70°C until use. For the preparation of the membrane fraction, the ultracentrifugal pellet was washed once in PBS, dissolved in the lysis solution with sonication, centrifuged at 15,000 *g* for 10 min at 15°C, and the supernatant was used as membrane fraction sample. Protein concentration of 2-DE samples was estimated according to using a commercial Bradford reagent (Bio-Rad). BSA was used as standard.

Two-dimensional electrophoresis

IEF was carried out using commercially available, dedicated apparatuses: IPGphor (Amersham Pharmacia Biotech) or Protean IEF Cell (Bio-Rad). IPG strips were used according to the manufacturer's instructions. Samples containing up to 200 μg protein for analytical gels or up to 1 mg for micro-preparative gels, were diluted to 300-350 μl with rehydration solution (8 M urea, 2% CHAPS, 100 mM DTT, 0.5% v/v pH 3-10 IPG buffer, trace bromophenol blue), and applied to strips by overnight rehydration at 50 V. Proteins were focused succeedingly for 1 h at 200 V, 1 h at 500 V, 1 h at 1,000 V, then a gradient was applied from 1,000 to 8,000 V in 30 min, and focusing was continued at 8,000 V for 8.5 h to give a total of 70 kVh on an IPGphor. With Protean IEF Cell, focusing was done initially at 250 V for 15 min, then the voltage was increased to 10,000 V within 3 h, and maintained at 10,000 V for 7 h for a total of 70 kVh. All IEF steps were carried out at 20°C. After the first-dimensional IEF, IPG gel strips were placed in an equilibration solution (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8) containing 1% DTT for 10 min with shaking at 50 rpm on an orbital shaker. The gels were then transferred to the equilibration solution containing 2.5% iodoacetamide and shaken for a further 10 min before placing them on a 7.5-17.5% gradient polyacrylamide gel slab. Separation in the second dimension was carried out using Protean II xi electrophoresis equipment and Tris-glycine buffer (25 mM Tris, 192 mM glycine) containing 0.1% SDS at a current setting of 5 mA/gel for the initial 1 h and 10 mA/gel thereafter. The second-dimensional SDS-PAGE was developed until the bromophenol blue dye marker had reached the bottom of the gel.

Protein visualization and image analysis

For silver staining, following second-dimensional SDS-PAGE, analytical gels were immersed in methanol/acetic acid/water (50:12:38) for 1.5 h, followed by washing twice in 50% ethanol for 20 min. Gels were pretreated for 1 min in a solution of 0.02% $Na_2S_2O_3$. This was followed by three 1 min washes in deionized water. Proteins were stained in a

solution containing 0.2% $AgNO_3$ and 0.075% (v/v) formalin (37% formaldehyde in water) for 20 min, and washed twice in deionized water for 1 min. Subsequently, gels were developed in a solution of 0.06% (v/v) formalin, 2% Na_2CO_3, and 0.0004% $Na_2S_2O_3$. When the desired intensity was attained, the developer was discarded and stopped by 1% acetic acid. For Coomassie blue staining of micro-preparative gels, gels were fixed three times in 30% ethanol containing 2% phosphoric acid for 20 min and rinsed three times in 2% phosphoric acid. Gels were then equilibrated in a solution containing 18% ethanol, 2% phosphoric acid, and 15% ammonium sulfate for 30 min and Coomassie brilliant blue G-250 was added to a final concentration of 1%. Staining was carried out overnight. Protein patterns in the gels were recorded as digitalized images using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, Bio-Rad). Gel image matching was done with PDQuest software (Bio-Rad).

In-gel digestion

In-gel digestion of protein spots on Coomassie- or silver-stained gels was performed essentially as described by Jensen *et al.* (1999). After the completion of staining, the gel slab was washed twice with water for 10 min. The spots of interest were excised with a scalpel, cut into pieces, and put into 1.5 ml microtubes. The particles were washed twice with water for 15 min, and then twice with water/acetonitrile 1:1 (v/v) for 15 min. The solvent volumes were about twice the gel volume. Liquid was removed, acetonitrile was added to the gel particles and the mixture was left for 5 min. Liquid was removed and the particles were rehydrated in 0.1 M NH_4HCO_3 for 5 min. Acetonitrile was added to give a 1:1 v/v mixture of 0.1 M NH_4HCO_3/acetonitrile and the mixture was incubated for 15 min. All liquid was removed and gel particles were dried in a vacuum centrifuge (Heto- Holten, Allerød, Denmark), reswelled in 10 mM DTT/0.1 M NH_4HCO_3, and incubated for 45 min at 56°C to reduce the peptides. After chilling tubes to room temperature and removing the liquid, 55 mM iodoacetamide in 0.1 M NH_4HCO_3 was added, the tubes were incubated for 30 min at room temperature in the dark to *S*-alkylate the peptides. Iodoacetamide solution was removed, the gel particles were washed with 0.1 M NH_4HCO_3 and acetonitrile, dried in a vacuum centrifuge, rehydrated on ice in digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 12.5 ng/μl of trypsin, and incubated for 45 min on ice. Excess liquid was removed and about 20 μl of digestion buffer without trypsin was added. After overnight digestion at 37°C, 25 mM NH_4HCO_3 was added, and the tube was incubated for 15 min. Acetonitrile was added and the tube was incubated for a further 15 min. The supernatant was recovered, and the extraction was repeated twice with 5% formic acid/acetonitrile (1:1, v/v). The three extracts were pooled and dried in a vacuum centrifuge.

MALDI-TOF-MS and data base search

Tryptic peptides were redissolved in a solution containing water, acetonitrile, and trifluoroacetic acid (93:5:2, v/v), and the solution was treated for 5 min in a bath sonicator. Target preparation was carried out by ‘solution-phase nitrocellulose method’. Saturated solution of α-cyano-4-hydroxycinnamic acid (about 40 mg/ml) and nitrocellulose solution (20 mg/ml) were prepared separately in acetone. A mixture of the α-cyano-4-hydroxycinnamic acid solution, nitrocellulose solution, and 2-propanol was prepared at a ratio of 2:1:1. Peptide calibrants (50-200 fmole of each), des-Arg-bradykinin (monoisotopic mass, 904.4681), and neurotensin (1672.9715) were added and the mixture solution was then spotted on the target and dried. Dried samples were

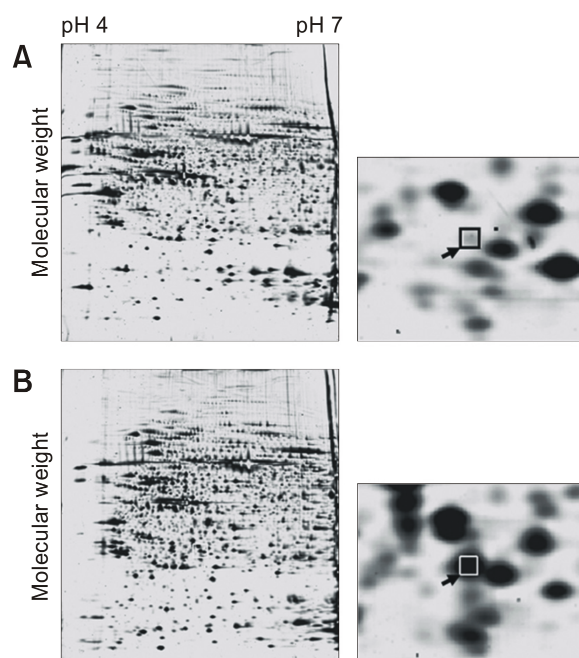

Figure 1. 2-DE protein pattern of normal and cancer stomach tissues. Stomach tissue proteins (50 μg) from the normal (A) and cancer tissues (B) were separated on pH 4-7 IPG strip, in the first dimension and 7.5-17.5% gradient SDS-PAGE gel in the second dimension. Spots were visualized by silver staining. On the right, image crops of the gel region containing the spot 4,262 are shown. The position of the spot indicated by arrows.

washed with 5 μl of 5% formic acid for 10 s, followed by 5 μl of Milli-Q water for 10 s, and then dried spots were analyzed in a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, NA). The spectrometer was run in positive ion mode and in reflector mode with the settings: accelerating voltage, 20 kV; grid voltage, 76%; guide wire voltage, 0.01%; and a delay of 150 ns. The low mass gate was set at 500 *m/z*. Proteins were identified by peptide mass fingerprinting with the search programs MS-FIT (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm) and ProFound (http://129.85.19.192/profound_ bin/WebProFound.exe). The following search parameters were applied: SWISS-PROT and NCBI were used as the protein sequence databases; a mass tolerance of 50 ppm and one incomplete cleavage were allowed; acetylation of the *N*-terminus, alkylation of cysteine by carbamidomethylation, oxidation of methionine, and pyroGlu formation of *N*-terminal Gln were considered as possible modifications.

Generation of monoclonal antibodies

His-tagged fusion protein was expressed in *E. coli* and purified as affinity chromatography method (Ni-NTA agarose, QIAGEN). They were used for immunization of mice and screening of hybridomas secreting Ag-specific monoclonal antibodies, respectively. Each of the 6-week-old female Balb/c mice was given primary injection (intraperitoneal) with 100 μg of the protein emulsified in complete Freund's adjuvant (Sigma-Aldrich Inc.). Two weeks later, each mouse was given one secondary booster injection using the same amount of antigen mixed with incomplete Freund's adjuvant (Sigma-Aldrich Inc.). Booster injections were administered to each mouse at 2-week intervals three more times. Ag-specific antibodies in the serum of immunized mice were tested by Western blot assays prior to hybridoma fusion. The spleen was excised, and hybridoma fusion was performed by standard techniques (Harlow, 1988). mAbs in supernatants of hybridoma cultures were screened in ELISA. Ascites fluid was produced by injecting the hybridoma cells into a pristane-primed Balb/c.

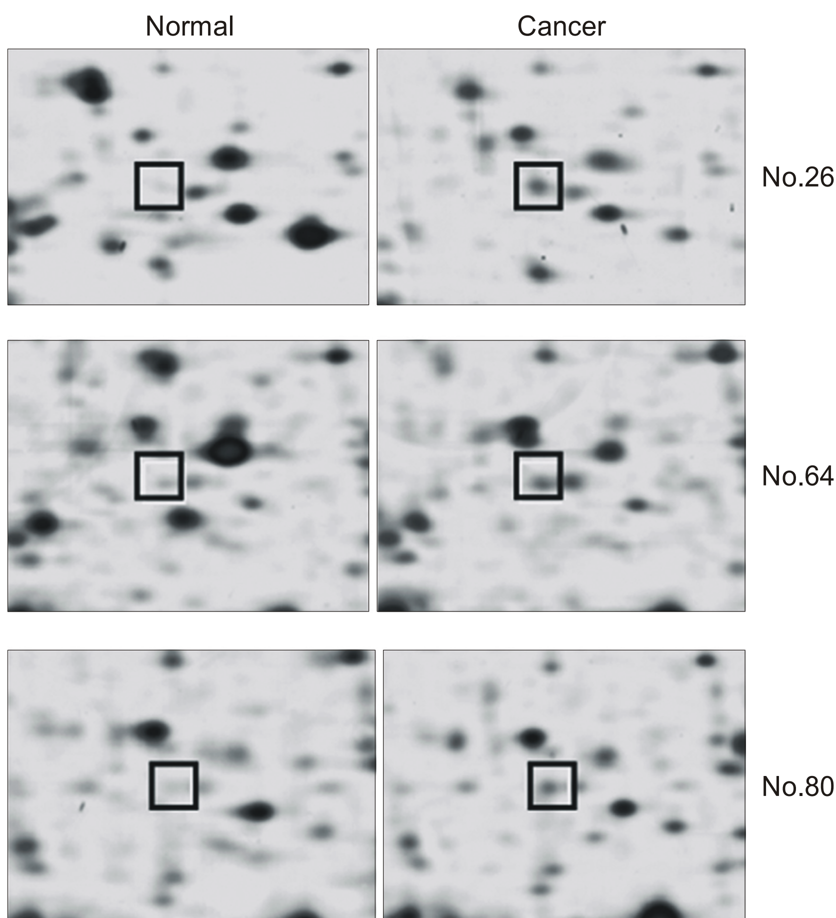

Figure 2. Differentially expressed NNMT in normal and cancer tissues of gastric cancer patients. The positon of NNMT was indicated by squares.

Table 1. Expression level of spot 4262 in normal and cancer tissue of gastric cancer tissues.

Sample No.	N	C	C/N	Sample No.	N	C	C/N
1	347	1064	3.06	45	108	882	8.17
2	246	1630	6.62	46	182	522	2.87
3	257	3156	12.28	47	511	1972	3.86
4	96	515	5.36	48	138	706	5.12
5	0	0	0[a]	49	202	0	–[b]
6	295	829	2.81	50	159	1544	9.71
7	373	1840	4.93	51	866	2686	3.1
8	215	507	2.36	52	0	0	0[a]
9	0	688	+[c]	53	58	1655	28.5
10	0	0	0[a]	54	0	0	0[a]
11	0	0	0[a]	55	234	228	0.97
12	0	0	0[a]	56	235	0	–[b]
13	0	0	0[a]	57	0	0	0[a]
14	0	0	0[a]	58	0	0	0[a]
15	0	0	0[a]	59	218	0	–[b]
16	0	0	0[a]	60	0	0	0[a]
17	0	0	0[a]	61	242	2934	12.1
18	138	873	6.33	62	0	0	0[a]
19	0	0	0[a]	63	1044	165	0.16
20	134	0	–[b]	64	320	419	1.31
21	0	0	0[a]	65	0	0	0[a]
22	0	0	0[a]	66	94	548	5.83
23	0	0	0[a]	67	160	2989	18.7
24	0	0	0[a]	68	108	4298	39.8
25	0	4019	+[c]	69	265	215	0.81
26	92	922	10	70	102	1098	10.8
27	0	0	0[a]	71	178	709	3.98
28	0	0	0[a]	72	474	616	1.3
29	0	0	0[a]	73	132	1089	8.25
30	0	0	0[a]	74	359	602	1.68
31	115	1364	11.9	75	0	1181	+[c]
32	231	3260	14.1	76	178	709	3.98
33	0	0	0[a]	77	621	1622	2.61
34	773	2063	2.67	78	633	1307	2.06
35	209	268	1.28	79	153	1075	7.03
36	195	506	2.59	80	232	750	3.23
37	17	1160	68.2	81	308	4480	14.5
38	116	1694	14.6	82	438	828	1.89
39	518	1438	2.78	83	436	390	0.89
40	405	999	2.47	84	84	2204	26.2
41	160	660	4.13	85	238	2467	10.4
42	214	2664	12.4	86	198	0	–[b]
43	760	1056	1.39	87	706	1150	1.63
44	606	538	0.89	88	411	363	0.88

Table 1. Continued.

Sample No.	N	C	C/N	Sample No.	N	C	C/N
89	200	1230	6.15	122	84	401	4.77
90	451	1147	2.54	123	323	1992	6.17
91	402	0	–[b]	124	105	1030	9.81
92	177	735	4.15	125	211	2067	9.8
93	73	2650	36.3	126	400	1859	4.65
94	0	3190	+[c]	127	918	1899	2.07
95	514	274	0.53	128	187	1138	6.09
96	337	1465	4.35	129	175	2225	12.7
97	0	0	0[a]	130	598	552	0.92
98	63	1218	19.3	131	272	1666	6.13
99	425	1236	2.91	132	23	1077	46.8
100	0	0	0[a]	133	446	1492	3.35
101	168	956	5.69	134	253	3332	13.2
102	0	3235	+[c]	135	403	1591	3.95
103	160	903	5.64	136	482	3196	6.63
104	1092	1424	1.3	137	323	1992	6.17
105	378	1601	4.24	138	718	1684	2.35
106	44	179	2.07	139	272	1942	7.14
107	435	563	1.29	140	551	919	1.67
108	248	1812	7.31	141	105	463	4.41
109	197	419	2.13	142	911	3530	3.87
110	349	2835	8.12	143	232	4045	17.4
111	0	2069	+[c]	144	391	1567	4.01
112	0	389	+[c]	145	643	875	1.36
113	511	0	–[b]	146	620	1259	2.03
114	1202	1297	1.08	147	604	392	0.65
115	0	466	+[c]	148	568	2358	4.15
116	318	3385	10.6	149	670	2149	3.21
117	166	980	5.9	150	683	506	0.74
118	560	485	0.87	151	691	1760	2.55
119	232	1452	6.26	152	1238	1502	1.21
120	374	531	1.42	A.V.E	269	1120	5.7
121	255	1555	6.1	S.D	21	86	

[a]No spot detection. [b]Expressed only in normal tissues. [c]Expressed only in cancer tissues.

Table 2. The results of t-test of spot 4262 expression in gastric cancer tissues.

Average spot volume			Parametric method	
Normal	Cancer	Cancer-Normal	Test statistic	*P* value (< 0.001)
269	1120	851	10.18	7.13×10^{-17}

Western blot analysis

The initial sample for immunoblotting was prepared in the same way as for silver staining. After a run on the 7.5-17.5% gradient SDS-PAGE, the separated proteins were transferred to nitrocellulose membranes. Blocking was performed with a TBST buffer (10 mM Tris-HCl, pH 8.8, 150 mM NaCl, 0.5% Tween-20) containing 5% skim milk (DIFCO). The membranes were then incubated with a mouse monoclonal anti-NNMT antibody (dilution to 1:10,000) for overnight at room temperature. The immunoblots

Parameters Used in Search

Considered modifications: | **Peptide N-terminal Gln to pyroGlu** | **Oxidation of M** | **Protein N-terminus Acetylated** |

Min. # Peptides to Match	Peptide Mass Tolerance (+/-)	Peptide Masses are	Digest Used	Max. # Missed Cleavages	Cysteines Modified by	Peptide N terminus	Peptide C terminus
4	50.000 ppm	monoisotopic	Trypsin	1	carbamidomethylation	Hydrogen (H)	Free Acid (O H)

Result Summary

Rank	MOWSE Score	# (%) Masses Matched	Protein MW (Da)/pI	Species	Accession #	Protein Name
1	9.93e+004	8/30 (26%)	29574.3 / 5.56	HUMAN|280B12748F4488AC	P40261	(NNMT)Nicotinamide N-methyltransferase (EC 2.1.1.1).[Homo sapiens]
2	2.51e+003	4/30 (13%)	28628.2 / 10.97	HUMAN|F1CF6810004F0693	P12980	(LYL1)Lyl-1 protein (Lymphoblastic leukemia derived sequence 1).[Homo sapiens]
3	1.27e+003	4/30 (13%)	85838.8 / 6.28	TUPGB|972C1CA2334C6058	Q95267	(NR3C1..)Glucocorticoid receptor (GR).[Tupaia glis belangeri]
4	1.01e+003	6/30 (20%)	68876.3 / 8.43	YEAST|9ED6EA30BE41B166	P20050	(HOP1..)Meiosis specific protein HOP1.[Saccharomyces cerevisiae]
5	455	5/30 (16%)	24726.6 / 9.45	THEAC|FE2B2B220091017F	Q9HIR5	(RPS3P..)30S ribosomal protein S3P.[Thermoplasma acidophilum]

Detailed Results

1. 8/30 matches (26%). 29574.3 Da. pI = 5.56. Acc. # P40261. HUMAN|280B12748F4488AC. (NNMT)Nicotinamide N-methyltransferase (EC 2.1.1.1).[Homo sapiens].

m/z submitted	MH+ matched	Delta ppm	start	end	Peptide Sequence (Click for Fragment Ions)	Modifications
816.3717	816.4103	-47.3078	124	130	(K)GPEKEEK(L)	
876.4619	876.4943	-36.9963	211	218	(K)FSSLPLGR(E)	
951.4736	951.4974	-24.9748	48	55	(K)IFCLDGVK(G)	
1012.5098	1012.5427	-32.5258	31	39	(R)HSAESQILK(H)	
1205.5491	1205.5512	-1.7830	201	210	(K)SSYYMIGEQK(F)	
1221.5304	1221.5462	-12.9051	201	210	(K)SSYYMIGEQK(F)	1Met-ox
1986.0898	1986.1462	-28.3865	182	200	(R)NLGSLLKPGGFLVIMDALK(S)	
2022.9179	2022.9872	-34.2344	80	96	(K)EIVVTDYSDQNLQELEK(W)	

Click link below to search for another component.
22 unmatched masses:
Click individual mass to do a non-specific cleavage search.
713.4010 804.2702 894.4688 902.4756 1102.6295 1109.4946 1249.6593 1260.6607 1276.6658 1286.7735 1306.6217 1404.8309 1421.6764 1501.7624 1624.7785 1696.7692 1768.8830 1784.7346 1825.7389 1897.7821 1946.8274 2002.0353

The matched peptides cover **29%** (78/264AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 19129

Figure 3. Search results of peptides mass fingerprinting. The spot was identified as nicotinamide *N*-methyltransferase by MALDI-TOF MS, with 8 matching peptides which corresponded to a sequence coverage of 26%.

Table 3. Summary of ELM search results. Total 21 modification sites were reported.

Modification site	Total 21	Cell compartment
CK1 phosphorylation site	6	Nucleus, cytoplasm
CK2 phosphorylation site	2	Nucleus, cytoplasm
Glycosaminoglycan attachment site	4	Extracellular golgi appartus
GSK phosphorylation recognition site	5	Nucleus, cytoplasm
PKA (cell signalling)	1	Cytoplasm, cAMP dependent protein kinase complex
PKA phosphorylation	1	Cytoplasm
MAPK phosphorylation site	1	Nucleus, cytoplasm
SUMO-1 recognition motif	1	Nucleus, PML body

were washed for 30 min, changing the TBST buffer every 10 min, then incubated with a alkaline phosphatase conjugated secondary antibody (dilution to 1:50,000) for 4 h at room temperature. The immunoblots were washed every 10 min for 1 h with changing TBST buffer, and developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP, USB) and *p*-nitro blue tetrazolium chloride (NBT, USB) in alkaline phosphate buffer (100 mM Tris-HCl, pH 9.5, 10 mM NaCl, 5 mM $MgCl_2$).

In vitro phosphorylation

Poly His-tag removed NNMT was mixed with a

kinase reaction buffer comprised of 20 mM Tris HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 100 μM ATP and CK2 (1 unit transfers 1 μmol of phosphate from ATP to casein per min at pH 8.5 and 37°C) in a final volume of 50 μl and incubated for overnight at 30°C. Reactions were easily stopped by treatment with heat, trypsin digestion, or urea. All experiments were repeated at least twice and confirmed by western blotting.

Results

Proteome analysis of gastric cancer tissues reveals overexpression of NNMT

152 cases of gastric cancer and normal tissues were analyzed by two-dimensional electrophoresis. The images of silver stained gels were analyzed with the software PDQust™ (Figure 1, 2). The statistical analysis revealed that the levels of the spot 4,262 in tumor tissues were significantly higher than in normal tissues (Table 1, 2). The spot was identified as nicotinamide *N*-methyltransferase by MALDI-TOF MS, with 8 matching peptides which corresponded to a sequence coverage of 26% (Figure 3). Nicotinamide *N*-methyltransferase (NNMT, E.C.2.1.1.1) is an *S*-adenosyl-L-methionine dependent cytosolic enzyme which catalyses the *N*-methylation of nicotinamide, its predominant route of metabolism.

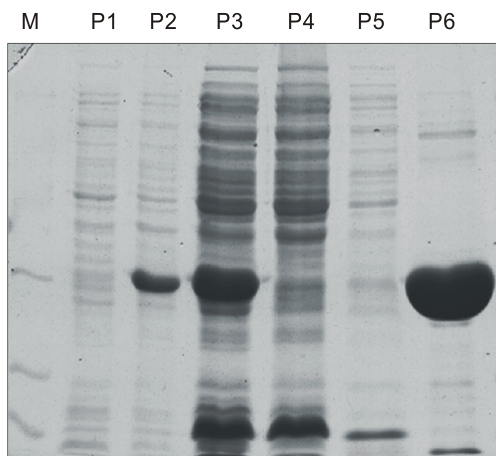

Figure 4. 12% SDS-PAGE of overexpression and purification of NNMT. Cells were induced with 1 mM IPTG and overexpressed protein was purified with Ni-NTA agarose. M, low MW marker; P1, non-induced cells; P2, cells induced with IPTG; P3, cleared lysate; P4, flow-through; P5, wash; P6, eluate.

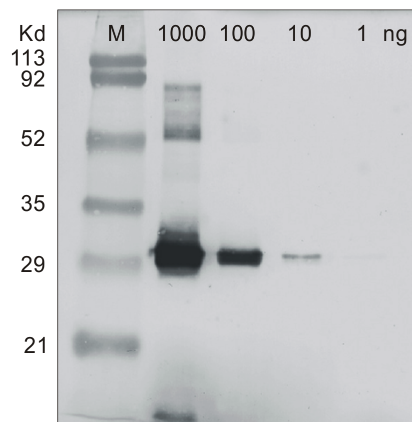

Figure 5. Sensitivity of monoclonal antibody against NNMT. Poly-His tag removed recombinant NNMT was applied to 12% SDS-PAGE and transferred on PVDF membrane. Monoclonal antibody (LP 16-1) against NNMT from ascites fluid was used as a primary anti-body (dilution to 1:10,000).

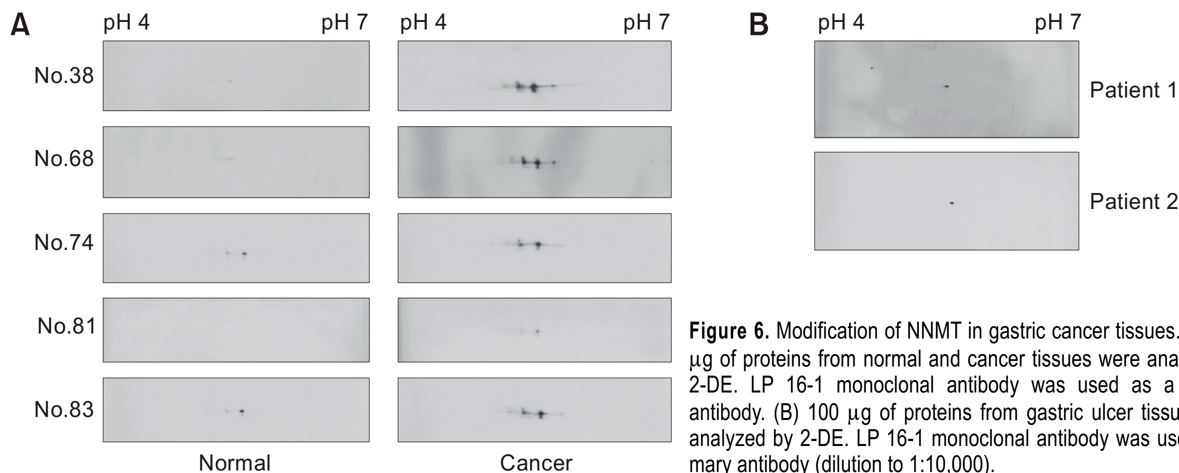

Figure 6. Modification of NNMT in gastric cancer tissues. (A) 100 μg of proteins from normal and cancer tissues were analyzed by 2-DE. LP 16-1 monoclonal antibody was used as a primary antibody. (B) 100 μg of proteins from gastric ulcer tissues were analyzed by 2-DE. LP 16-1 monoclonal antibody was used a primary antibody (dilution to 1:10,000).

Generation of anti-NNMT monoclonal antibody

We produced monoclonal antibody against NNMT to perform further study. Balb/c mice were immunized with purified NNMT, and the spleen cells were fused with myeloma cells. The hybridoma cells producing monoclonal antibody against the NNMT were screened by indirect ELISA, and the monoclonal antibody was obtained from cloning and ascites fluid producing process. The antibody reacted specifically with NNMT protein. The detection limit of the prepared monoclonal antibody was down to about 10 ng (Figure 5).

Western blot analysis of NNMT in gastric cancer tissues and gastric ulcer tissues

Gastric tissues from 15 gastric cancer patients were analyzed by Western blot and the results revealed that NNMT was expressed more highly in cancer tissues than in normal tissues (Figure 6). This result of Western blotting coincided with those of 2-DE experiments. Western blotting revealed that NNMT exists in multiple spots in gastric tissues. In normal gastric tissues, mainly a single spot was predominant in most cases. However, four to five spots were observed in most cancer tissues. We also analyzed gastric tissues from 2 gastric ulcer patients and found only one spot in both cases. The results indicate that the presence of multiple spots of NNMT is highly specific to tumor tissues of stomach. The presence of multiple NNMT spots suggested its post-translarational modification in gastric cancer tissues.

Post-translational modification of NNMT

The pattern of multiple spots with nearly identical MW and distinct pI values suggested that the modification involved a relatively small group with charges, possibly a phosphorylation. By eukaryotic

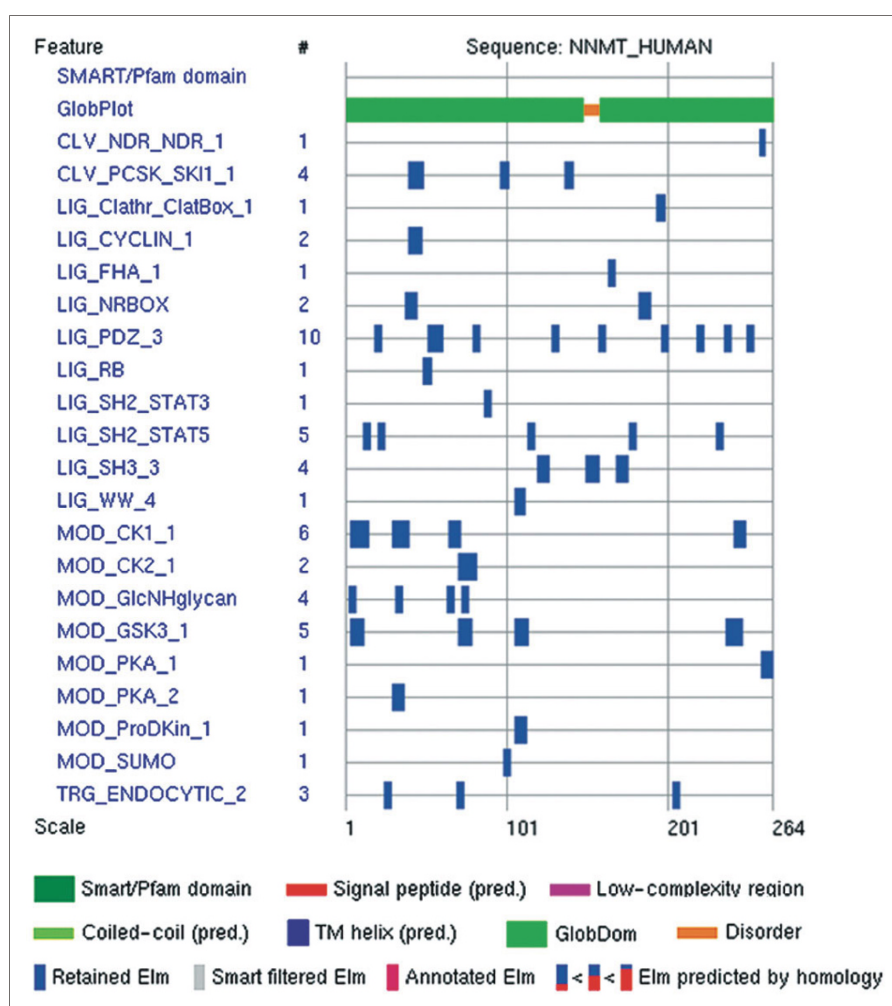

Figure 7. Summary of features reported by ELM search after globular domain filtering and context filtering.

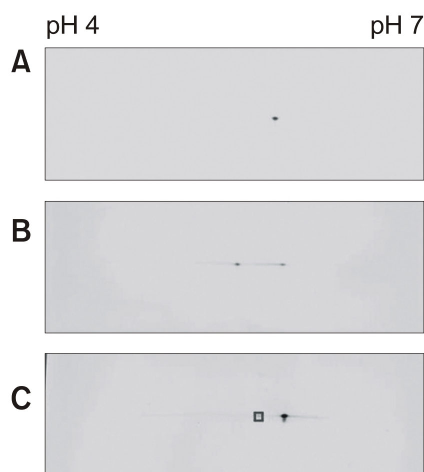

Figure 8. Western blot analysis of NNMT. (A) Recombinant NNMT treated with thrombin. (B) NNMT from gastric ulcer tissues and thrombin-treated recombinant NNMT. (C) NNMT reacted with CK2α. An additional spot was detected on the left of the original spot, as indicated by the square.

linear motif (ELM) search (http://elm.eu.org), NNMT amino acid sequence was found to contain several consensus sequences for modification (Figure 7). Interestingly, casein kinase 2 (CK2) is a ubiquitous eukaryotic Ser/Thr protein kinase and phosphorylates more than 100 substrates and is up-regulated in rapidly dividing cells including most human tumors. So the possibility of NNMT phosphorylation by CK2 was examined. Overexpressed and affinity-purified NNMT was reacted with CK2 *in vitro* and then was analyzed by Western blotting. NNMT treated with CK2 was found in two spots on 2-DE gel, while non-treated control showed a single spot. The additional spot had same molecular weight as the original spot but had a lower pI value (Figure 8). Thus CK2 was capable of phosphorylation of NNMT *in vitro*.

Discussion

Proteomic technologies have been used to identify cancer-specific proteins that are useful for cancer diagnosis, progression, and therapeutic targets (Wilkins *et al*., 1996; Loging *et al*., 2000; Pandey and Mann, 2000). The objective of present study was to identify cancer-associated proteins using a proteomic approach by analyzing protein expression in healthy and cancerous stomach tissues. We found one protein that was expressed at markedly higher levels in cancerous tissue compared with normal stomach tissue. It was identified as nicotinamide *N*-methyltransferase by peptide mass fingerprinting. In a small scale proteomics study of human gastric cancer tissues,a higher expression of NNMT was reported in 8 out of 18 cases examined (Jang *et al*., 2004). However, no statistical analysis of the results was presented and statistical significance of its differential expression between normal and tumor tissues could not be ascertained. We analyzed 152 cases of gastric cancer and normal tissues and statistical analysis showed extremely high significance of the difference ($P < 0.001$). Furthermore, these 2-DE results were verified by Western blotting. Interestingly, Western blotting revealed that NNMT exists in multiple spots in gastric tissues and the presence of multiple NNMT spots is highly specific to tumor tissues of stomach.

Nicotinamide *N*-methyltransferase is an S-adenosyl-L-methionine dependent cytosolic enzyme which catalyses the *N*-methylation of nicotinamide, its predominant route of metabolism (Aksoy *et al*., 1995). Methylation is also an important conjugation reaction in the biotransformation of many drugs and xenobiotics including pyridine and other structurally related compounds (Weinshilboum., 1989). NNMT was first identified by cDNA cloning from the liver and the protein is predicted to be present in the cytosol (Aksoy *et al*., 1995). Several diseases are associated with abnormal nicotinamide metabolism resulting in the production of elevated levels of *N*-methylnicotinamide; these include Parkinson's disease and hepatic cirrhosis (Cuomo *et al*., 1994; Parsons *et al*., 2002). Recently, NNMT was reported as novel serum markers of human colorectal cancer as potential candidates for noninvasive detection of early colorectal neoplasm (Roessler *et al*., 2005).

To our knowledge, NNMT modification has not been reported previously in the human gastric cancer. We performed eukaryotic linear motif search for NNMT to find potential modification sites. NNMT amino acid sequence was found to possess the motifs recognized by CK1 and CK2 for Ser/Thr phosphorylation, glycosaminoglycan attachment sites, GSK phosphorylation sites, PKA recognition sites, a MAPK phosphorylation site and a sumoylation site. Among the various possibilities, we showed that recombinant NNMT was phosphorylated by CK2 *in vitro*.

In the present study, we observed NNMT could be a sensitive and specific tumor marker of gastric tissues. We reported for the first time that NNMT was modified in gastric cancer tissue by Western blot analysis. The mechanism by which NNMT is modified is still unknown and further studies are needed to understand the relationship between gastric cancer and post-translational modification.

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