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［原著］Proteomic Changes in a Squamous Cell Carcinoma Cell Line Induced by an Effector Kinase of a Small G Protein Rap 2

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Proteomic Changes in a Squamous Cell Carcinoma Cell Line Induced by an Effector Kinase of a Small G Protein Rap2

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ABSTRACT

The Traf2- and Nck-interacting kinase (TNIK) is a downstream effector of a Ras-like small G protein Rap2 identified by us. Very little is known about TNIK's roles in physiological or pathological cellular processes. To examine the effect of activated TNIK in cancer cells, over-expression of TNIK was induced by the use of TetOff system in a squamous cell carcinoma cell line. Global protein expression profiling was carried out by Ettan™ two-dimensional fluorescence difference gel electrophoresis (2-D DIGE). Proteins were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. By comparing the cells with or without over-expressed TNIK, 2-D DIGE revealed up-regulation of three proteins and down-regulation of six proteins by TNIK. Peptide mass fingerprinting with MALDI-TOF mass spectrometry identified five out of these nine proteins: isovaleryl coenzyme-A dehydrogenase (up-regulated 2.46-fold, $p<0.05$); Rho GDP dissociation inhibitor alpha (down-regulated 2.35-fold, $p<0.05$); stomatin-like protein 2 (down-regulated 1.57-fold, $p<0.05$); ribosomal protein, mitochondrial, S22 (down-regulated 1.78-fold, $p<0.05$); ornithine aminotransferase (down-regulated 1.34-fold, $p<0.05$). Three down-regulated proteins, Rho GDP dissociation inhibitor alpha, stomatin-like protein 2, and ornithine aminotransferase were candidates for cancer prognostic marker or target proteins for chemotheraputic drug development. *Ryukyu Med. J., 26 (3,4) 135～145, 2007*

Key words: Proteomics, Rap2, MAP4K, squamous cell carcinoma

INTRODUCTION

Rap2 is a small G protein homologous to Ras, a prototypical oncogene product[1]. Members of Ras family play crucial roles in regulation of various cellular processes such as cell growth, differentiation and death. The family consists of subfamilies such as Ras, Rap, Ral, etc. The Rap subfamily is the closest relative of the Ras subfamily. Rap subfamily contains Rap1 and Rap2, which are about 60% identical to each other. Rap1's function is often analogous to that of Ras, but it sometimes counteracts Ras function, because they share identical amino acid sequence in their effector-binding region. For instance, Rap1 interacts with Raf-1, a downstream effector of Ras. However, Rap1 cannot activate Raf-1 for complex reasons, and as a result, leads to suppression of Ras-mediated activation of extracellular signal-activated kinase (ERK), the growth-related mitogen-activated protein kinase (MAPK). On the other hand, Rap1 interacts with and activates another Ras effector, B-raf, more efficiently than Ras and plays a major role in regulation of ERK in some cell types.

In contrast to Ras/Rap1, little had been known about Rap2's role in cellular signaling. The effector-binding region of Rap2 differs from those in Ras and Rap1 by a single amino acid: Rap2 has Phe at

amino acid position 39, but Ras/Rap1 have Ser. This difference might suggest that Rap2 has its specific downstream effectors. In our previous study, therefore, Rap2-specific interacting proteins were searched from rat brain by a focused proteomic approach: the combination of Rap2-affinity column chromatography and protein identification by mass spectrometry. A protein of 155 kDa was isolated and identified as Traf2- and Nck-interacting kinase (TNIK)[2]. TNIK belongs to the Ste20 group of kinases that act upstream of c-Jun N-terminal kinase (JNK), a stress-related MAPK[3]. In addition, we have isolated another Ste20 group kinase, HPK/GCK-llike kinase (HGK), also known as MAPK kinase kinase kinase 4 (MAP4K4), as another Rap2-specific interacting protein by the yeast two-hybrid screening[4]. Thus, it turned out that Ras/Rap1 and Rap2, with different effector-binding region at position 39, act upstream of different types of MAPKs, namely, ERK and JNK, respectively.

Ste20 group members share similar kinase domains to that of STE20, a yeast MAP4K[3]. The group consists of two families. The first family, p21-activated kinase (PAK) family contains C-terminal catalytic and N-terminal regulatory domains, while the second family, the germinal center kinase (GCK) family contains N-terminal kinase and C-terminal regulatory domains. TNIK and HGK/MAP4K4 belong to the latter, and their C-terminal regulatory domain is the binding site for Rap2. TNIK and HGK/MAP4K4 are about 90% identical in their kinase and regulatory domains, but identity drops to about 50% in intermediate domain between the kinase and the regulatory domains. One reason for this drop is the presence of three alternatively spliced modules (amino acids 447-475, 537-591 and 795-802 of human TNIK), and the largest human TNIK isoform consists of 1360 amino acids. TNIK is named after its ability to interact with adaptor proteins Traf2 and Nck at this intermediate domain[5]. However, no evidence has been reported so far that Traf2 or Nck activates TNIK, thus Rap2 is the only known upstream activator of TNIK.

Regarding JNK activation by TNIK, some controversy exists as to whether GCK family members should be termed "MAP4K" or not, since they appear to activate MAP3Ks without phosphorylating them. Rather, the members help formation of MAP3K oligomers: the C-terminal regulatory domains of the GCK family members have an ability to oligomerize and at the same time serve as binding sites for MAP3Ks. Thus, formation of GCK family member oligomers through the C-terminal regulatory domains in turn leads to formation of MAP3K oligomers, within which trans-phorphorylation and activation of MAP3K take place: kinase domain of the family members are not necessary[3]. Consistent with this, simple artificial over-expression of the GCK family members can activate JNK[3]: this is also the case for TNIK, and over-expression of a kinase-deficient mutant TNIK can lead to activation of JNK[5].

Very little is known about TNIK's roles in physiological or pathological cellular processes. One obvious approach to address this issue, which is not in the scope of this study, is to subject cells with activated or inactivated TNIK to a variety of cell biological assays. However, this kind of hypothesis-driven approach could overlook something that is com pletely unexpected. One non-hypothesis-driven approach is the proteomic approach[6]. Recently, the strategy of combining Ettan™ two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) system (GE Healthcare) with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has become one of standard strategies for proteomic analysis[7]. The 2-D DIGE system has circumvented the limitations of previous stain-based two-dimensional electrophoresis system. In this system, proteins in the control and test samples are labeled with different fluorescent CyDyes™ such as Cy3 and Cy5. This allows two-dimensional separation of the control and test sample pair on the same gel, enabling perfect protein spot matching. Also, linear dynamic ranges for fluorescent dyes are five orders of magnitude, allowing very accurate quantitative comparisons. Protein spots showing quantitative differences are examined by MALDI-TOF mass spectrometry, and proteins contained in the spots are identified.

In the present study, we have attempted to examine TNIK's role in cancer cells by the above proteomic approach: we have examined the effect of over-expressed TNIK on global protein expression profile in a cancer cell line *in vitro*. We have chosen a mouse squamous cell carcinoma cell line Pam212, which forms tumors in mice after simple subcutaneous implantation[9], allowing *in vivo* test of *in vitro* findings.

MATERIALS AND METHODS

Generation of Pam212 cell clones for induced expression of TNIK

Unless otherwise indicated, Pam212 cells were maintained in a standard growth medium, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with penicillin and streptomycin. To establish cells that can be induced to over-express TNIK, we used the Rev-Tet-Off System (BD Clontech). According to the manufacturer's protocol, two DNA constructs were transduced into Pam212 cells using retroviruses: [1] a construct for expression of a transcriptional activator protein, tTA, that allows induction of a gene of interest only in the absence of tetracycline (i.e., tetracycline shuts off the induction); [2] a construct that expresses a cDNA for the longest isoform of human TNIK[2] fused to the HA-epitope tag (HA-TNIK) under the control of tTA. The neomycin- and hygromycin-resistant genes contained in the tTA and HA-TNIK constructs, respectively, allowed selection of doubly transduced cells. The selected cells over-expressed HA-TNIK only when cultured in the absence of tetracycline derivative, doxycycline (Dox). For cloning of highly over-expressing cells using 96-well culture plates, selected cells were diluted to the point of one cell per 200 μl of medium, and 100 μl were added to each well. Cloned cells that formed single colonies were transferred to 60-mm dishes, propagated, and subjected to Tet-regulated induction of HA-TNIK.

Tet-regulated induction of HA-TNIK

Until the time of induction, cloned cells were propagated in the standard growth medium containing 1 μg/ml of Dox to suppress expression of HA-TNIK. For induction, cells were trypsinized, and trypsin was inactivated with DMEM supplemented with 10% "Tet System-Approved FBS" (Clontech) to avoid any contamination of tetracycline derivatives from ordinary FBS. The cells were fed with the same Dox-free medium next day and further incubated for two more days before experiments (72 h induction, "TNIK cells"). On the other hand, to prepare cells that are not induced, cells were grown for 3 days in the presence of Dox. Induction of HA-TNIK was examined with Western immunoblotting with a mouse monoclonal anti-HA antibody as described previously[2]. Two clones (lines A and B) that exhibited strong induction were selected and maintained. The same 72 h induction protocol was used throughout the study.

Protein extraction and fluorescent dye labeling

Protein extraction from Pam212 cells and fluorescent dye labeling were carried out similarly as described previously for ocular tissues except that cells were harvested by scraping, collected by centrifugation, and homogenized by sonication only[10, 11]. Briefly, confluent control cells and TNIK cells in 60-mm dishes were washed in ice-cold phosphate-buffered saline, and collected in 150 ml of homogenization buffer (2 M thiourea, 7 M urea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propane- sulfonate (CHAPS), 30 mM Tris-HCl, pH 8.5), supplemented with Complete EDTA-free Protease Inhibitors (Roche). After homogenization with a brief sonication, the crude homogenate was centrifuged at 16,000 g for 30 min at 4oC. Proteins in the supernatant were purified with the 2-D Clean Up Kit (GE) and re-dissolved in the homogenization buffer. Protein concentration was determined using the 2-D Quant Kit (GE) and adjusted to 5 mg/ml, before storing at -80oC. After thawing on ice and centrifugation at 16,000 g for 10 min at 4oC, proteins were labeled with CyDye DIGE fluorescent dyes according to manufacturer's instructions ("minimal labeling" protocol, GE). Control and TNIK cell samples were differentially labeled with Cy3 and Cy5, respectively, or vice versa.

Two-dimensional separation of labeled proteins and image analysis

For the first dimension of isoelectric focusing (IEF), an immobilized pH gradient (IPG) dry strip was used (pH 4-7, 18-cm linear, Immobiline Dry Strips, GE). A pair of labeled control and TNIK cell samples (50 mg proteins each) were mixed (total 24 ml), combined with 320 ml of rehydration buffer [8 M urea, 2% CHAPS, 0.002% w/v bromophenol blue (BPB), 0.5% v/v IPG buffer (GE) and 1.2% v/v Destreak Reagent (GE)] and applied to the dry strip. After overnight "rehydration-loading" (also called "in-gel rehydration") of sample proteins, the strip was subjected to IEF with LKB2117 Multiphor II (GE) and a programmed voltage gradient (0 to 500 V ramp for 1 min, 500 to 3,500 V ramp for 1.5 h, and 3,500 V for 6 h). For the second dimension of sodium dodecyl sulfate-polyacrylamide gel electroporesis

(SDS-PAGE), the strip after IEF was equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% BPB and 1% w/v dithiothreitol). The strip was then mounted on top of a 12.5% gel (1-mm thick, 24 x 24 cm) that was cast using low fluorescence glass plates (Nihon EIDO, Japan) and fixed in place with a shark-tooth comb. SDS-PAGE was carried out at a constant temperature (20oC) and constant power (60 mA) until the BPB dye front was approximately 1 mm from the bottom of the gel. The gel was scanned using the Typhoon Variable Mode Imager (GE) to acquire Cy3 and Cy5 images. Protein spots from each image were outlined and quantified using Image Quant Tool and Image Master Platinum software programs (GE). The quantity of an individual spot in each image was expressed as % volume (%V), relative to the sum of all detected spots from each image[12].

Screening and identification of differentially expressed proteins

Only protein spots that exhibited similar TNIK-induced alteration (up- or down-regulation) in all 2-D DIGE analyses were selected. Using the %V value, a ratio of TNIK-induced alteration (%V in TNIK cells/%V in control cells) was calculated for each analysis. Subsequently, the average ratio from all analyses was calculated. Only protein spots that exhibited statistically significant alterations were defined as altered by TNIK and subjected to protein identification. For protein identification, a sample of 500 mg protein, of which 100 mg was labeled with CyDyes, was subjected to two-dimensional separation. Proteins in the gels were transferred to polyvinylidene difluoride (PVDF) membrane (ProBlott PVDF, Applied Biosystems) followed by Colloidal Gold Total Protein staining (Bio-Rad). Protein spots defined as altered by TNIK were excised (1 mm x 1 mm square) from the membrane. Immobilized proteins were reduced, S-carboxymethylated, and digested *in situ* with *Achromobacter* protease I (a Lys-C), which cleaves after Lys[13]. Molecular mass analyses of Lys-C fragments were performed by MALDI-TOF mass spectrometry, using Applied Biosystems Voyager-DE/STR[14]. Identification of proteins was carried out by the peptide mass fingerprinting technique and screening the NCBI non-redundant protein database using the MASCOT software with a mass tolerance setting of ± 0.03 Da.

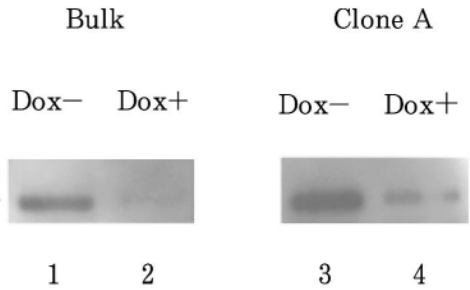

Fig. 1 Strong but leaky expression of HA-TNIK in cloned cells

Strong but leaky expression of HA-TNIK in cloned cells. Bulk and cloned cells were cultured either in the absence (−) or presence (+) of Dox (1 μg/ml) for 72 hours. After cell harvesting, 10 μg of total cell proteins were subjected to Western immunoblotting using mouse monoclonal anti-HA antibody. Arrows indicate positions of HA-TNIK.

RESULTS

As one way to analyze the role of signaling pathways mediated by TNIK in a context of squamous cell carcinoma, we sought to identify proteins whose expression levels were altered by over-expression of HA-TNIK in a squamous cell carcinoma cell line, Pam212, using the 2-D DIGE/MALDI-TOF/MS proteomic approach. To this end, we utilized the Rev-Tet-Off system to engineer the cells so that they express HA-TNIK when cultured in the absence of a tetracycline derivative Dox. Two cell lines (A and B) that express high level of HA-TNIK were established by cloning from bulk culture. However, during the cloning procedure, we noticed that cells expressing large amont of HA-TNIK when cultured in the absence of Dox also express some amount of HA-TNIK even when cultured in the presence of Dox (so called "leaky expression"). In Fig. 1., HA-TNIK expression of bulk and clone A cells are compared. Bulk cells expressed modest amount of HA-TNIK in the absence of Dox (lane 1) but negligible amount of HA-TNIK in the presence of Dox (lane 2). On the other hand, clone A cells expressed large amount of HA-TNIK in the absence of Dox (lane 3). However, they also expressed some amount of HA-TNIK in the presence of Dox (lane 2). To avoid potential effect of this leaky expression on protein expression profile, we used parental Pam212 cells as "control cells" and cloned cells expressing induced HA-TNIK as "TNIK cells" for comparison. Potential effect of cloning was avoided by

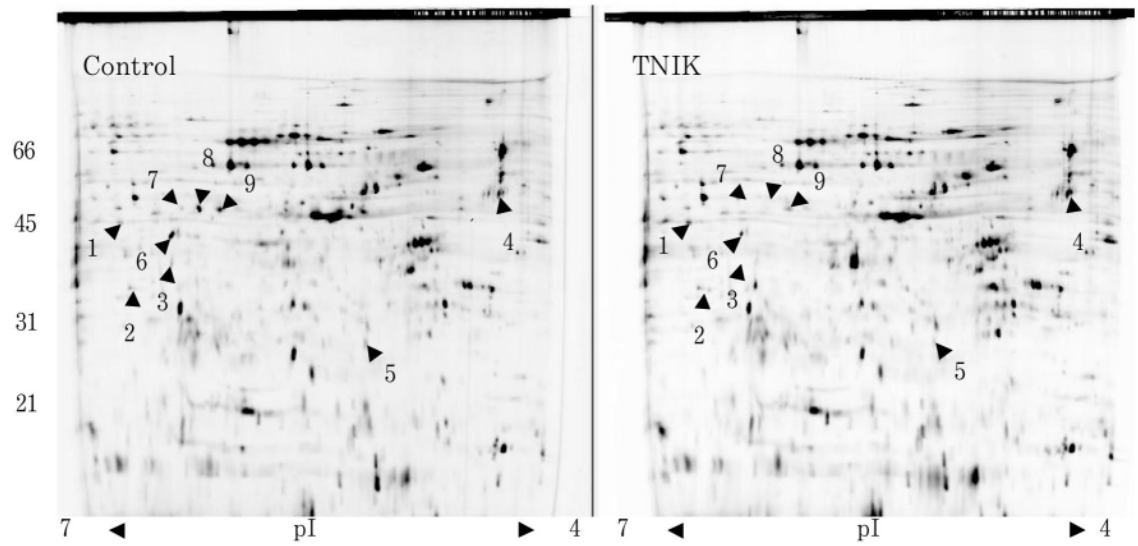

Fig. 2 Representative 2-D DIGE images

Representative 2-D DIGE images. Protein samples from control and TNIK cells (50 mg protein each) were labeled with Cy3 and Cy5 fluorescent dyes, respectively, or vice versa. Samples were mixed and separated in the same gel on the basis of pI (x-axis) and molecular mass (y-axis). Four analyses using independent pairs of control and TNIK cells gave essentially the same results. Shown are representative fluorescent images from one of the four analyses (grayscale images for each individual channel for Cy3 and Cy5). Both Cy3 (control) and Cy5 (TNIK) images are shown. Arrows with numbers indicate protein spots whose intensities were consistently altered by TNIK in all four analyses (spots #1, #2 and #4, up-regulated; spots #3, #5, #6, #7, #8 and #9, down-regulated).

excluding findings not common to both clones.

For screening of proteins that are differentially expressed between the control and TNIK cells, we have carried out the Dox-controlled over-expression experiments of HA-TNIK four times: twice for each of the cloned cell lines, A and B. Experiments were carried out on different occasions, generating TNIK-A and TNIK-B cells twice each (TNIK-A#1, TNIK-A#2, TNIK-B#1 and TNIK-B#2). Control cells were also prepared each at the same time (control #1 to #4). We extracted proteins from control and TNIK cells and examined global protein expression profiles using 2-D DIGE, a powerful two-dimensional differential gel electrophoresis system (Fig. 2). A pair of protein samples were labeled with different fluorescent CyDyes and mixed. Mixed labeled proteins were separated in the same gel, according to their isoelectric point (pI) in the first dimension and to their molecular mass in the second dimension. Separated proteins were represented by fluorescent spots on the gel, which were visualized by obtaining fluorescence images of the gel for each CyDye. Using this system, we analyzed protein expression profiles in four pairs of control and TNIK cell samples. We found nine protein spots whose intensities were consistently altered by over-expression of HA-TNIK in all analyses (designated spot #1 to spot #9 in Fig. 2).

As shown in Table 1, these protein spots exhibited various extents of HA-TNIK-induced quantitative alterations as revealed by "average ratio" calculation as follows: [1] The intensity of each spot in each analysis was expressed as %V, where volume (integrated area and fluorescence strength) of each spot is normalized to the sum of volume of all the spots in the gel. [2] In each analysis, %V of each spot in HA-TNIK-over-expressing samples was normalized to that of control samples to give a ratio of HA-TNIK-induced change. [3] The ratios in all four analyses were averaged to give the "average ratio": note that "average %V" values given in Table 1 are averages of all four analyses, and "aver age ratio" calculated as above ([1]-[3]) is not the ratio of "average %V" for TNIK cells to "average %V" for control cells (see notes on Table 1 for more detailed procedures and their reasons). Among the nine protein spots, the "average ratio" varied from 0.23 to 4.09, and all alterations were statistically significant ($p<0.01$ for spots #2, #4 and #8, $p<0.05$ for spots #1, #3, #5, #6, #7, #9 by paired t-test;

Table 1 Altered proteins

Spot	% vol		Average ratio	p-value
	Control	TNIK		
1	0.0876	0.1213	2.4644	0.0124
2	0.0168	0.0355	4.0949	0.0063
3	0.0945	0.0265	0.2261	0.0197
4	0.3174	0.5803	1.8540	0.0036
5	0.1726	0.0685	0.4255	0.0261
6	0.3392	0.2322	0.6489	0.0132
7	0.0783	0.0459	0.5615	0.0366
8	0.3377	0.1392	0.3585	0.0074
9	0.2594	0.1943	0.7468	0.0244

Four paired comparisons between control and either TNIK-A or TNIK-B cells were carried out [(1)-(4)]. However, actually 6 experiments were performed as follows:
(1a) control#1-Cy5 vs TNIK-A#1-Cy3
(1b) control#1-Cy3 vs TNIK-A#1-Cy5
(2) control#2-Cy5 vs TNIK-A#2-Cy3
(3a) control#3-Cy5 vs TNIK-B#1-Cy3
(3b) control#3-Cy3 vs TNIK-B#1-Cy5
(4) control#4-Cy5 vs TNIK-B#2-Cy3

In the fisrt pairing of control vs TNIK-A cells [(1)] or control vs TNIK-B cells [(3)], experiments were performed in duplicate by reversing CyDyes [(1a) and (1b); (3a) and (3b)]. Thus, %V of each spots in experiments (1a) and (1b) were averaged to give the %V of comparison (1), which were used for subsequent calculations. Similarly, %V of each spots in experiments (3a) and (3b) were averaged to give the %V for the comparison (3). On the other hand, in the second pairing [(2) and (4)], only single experiment each was performed. In these cases, %V of each spot from each experiments were used directly for subsequent calculations.

The reason why two experiments were performed for comparison (1) and (3) each by switching CyDyes is because we had found previously that a few proteins were labeled by Cy5 more efficiently than Cy3, or vice versa. Thus, any protein spot showing inconsistent behavior in different CyDye combinations, e.g., up-regulated in (1a) but down-regulated in (1b), were neglected in all comparisons as a potentially misleading spots. In addition, any spot that show different behavior in any comparison, e.g., up-regulated in all comparison except for in (4), was also neglected.

Table 1). Thus, these nine spots were defined as being altered by over-expression of HA-TNIK. As expressed by the "average ratio" values, three out of the nine spots were up-regulated by TNIK (spots #1, #2 and #4), while the other six spots were down-regulated (spots #3, #5, #6, #7, #8 and #9).

We succeeded to identify proteins contained in five out of these nine spots. Protein identification was carried out by analyzing peptides obtained by protease digestion of each protein within the spot. We have chosen our method for collecting peptides of highest concentration based on our own experiences[12,13], which we also found in the literature: [1] In 2-D DIGE gels, protein spots captured by fluorescence CyDye scanning are not always the point of highest protein concentration[7]. Most protein spots contain labeled as well as unlabeled protein: only a small fraction of proteins are fluorescence-labeled with our minimal labeling procedure. [2] Fluorescence-labeling causes retarded migration of proteins in the second dimension, an effect more marked for small proteins[7]. To subject the area of highest protein concentration to the protein identification procedures based on these

Table 2 Identified proteins

Spot No.	Protein Name (accession No.)	Molecular Mass (Da)	Total amino acid recidues	Matched Peptides (residue No.)	% Coverage
1	isovaleryl co-A dehydrogenase (NP_062800)	46296	424	57-65 77-83 154-178 195-211 212-230 244-260 327-346 400-424	32
5	Rho GDP dissociation inhibior alpha (NP_598577)	23393	204	34-43 100-105 106-113 114-127 128-135 142-167 168-178 187-199	47
6	stomatin-like protein 2 (NP_075720)	38361	353	115-140 141-145 179-187 188-200 201-221 255-288 292-321 322-351	47
7	ribosomal protein, mitochondrial, S22 (NP_079761)	41167	359	66-80 89-103 258-277 333-359	21
9	ornithine aminotransferase (NP_058674)	48324	439	33-49 50-66 103-107 108-129 136-151 363-374 375-386 422-434 435-439	27

information, we electro-blotted protein spots to PVDF membranes, visualized them by colloidal gold staining, and excised them. Additionally, transfer of protein spots from the gel to a membrane allowed efficient reduction and S-alkylation of disulfide bonds and digestion with Lys-C protease (on-membrane digestion). We obtained peptide mass spectra by MALDI-TOF mass spectrometry, and identified proteins using bioinformatics database searching (Mascot Search, peptide mass fingerprinting). For identified proteins, database accession number, theoretical molecular mass, total amino acid number, matched peptides (amino acid residue numbers), and percentage of coverage of protein sequence (%coverage, matched amino acid/total amino acid x 100) are shown in Table 2.

By combining the data in Table 1 and Table 2, the identified proteins included (spot number, and nature of alteration induced by TNIK, in parentheses): isovaleryl coenzyme-A dehydrogenase[15] (spot 1, up-regulated 2.46-fold); Rho GDP dissociation inhibitor alpha[16] (spot 5, down-regulated 2.35-fold); stomatin-like protein 2[17] (spot 6, down-regulated 1.57-fold); ribosomal protein, mitochondrial, S22[18] (spot 7, down-regulated 1.78-fold); ornithine aminotransferase[19] (spot 9, down-regulated 1.34-fold).

DISCUSSION

Proteomic consequence of TNIK activation in the context of squamous cell carcinoma was examined in the present study by using Pam212 cells. By comparing global protein expression profile between

the control Pam212 and HA-TNIK-over-expressing cell lines, we found that expression of nine proteins were consistently altered by TNIK over-expression (activation). Out of them, five proteins were identified: [1] isovaleryl coenzyme-A dehydrogenase; [2] Rho GDP dissociation inhibitor alpha; [3] stomatin-like protein 2; [4] ribosomal protein, mitochondrial, S22; [5] ornithine aminotransferase. Strikingly, down-regulation of Rho GDP dissociation inhibitor alpha ([2]), down-regulation of stomatin-like protein 2 ([3]), and down-regulation of ornithine aminotransferase ([5]) observed in HA-TNIK-over-expressing cell lines all made us to speculate that activation of TNIK would make Pam212 cell tumor more benign and sensitive to chemotherapy.

Rho family small G proteins, Rho, Rac and Cdc42, were initially linked to regulation of actin cytoskeleton[20]. However, now it is widely accepted that their roles extend beyond these initial observations and cover diverse aspects of cellular processes including gene transcription and cell cycle progression[21]. Small G proteins including Rho family act as molecular switches cycling between GDP-bound inactive and GTP-bound active forms. Rho GDP dissociation inhibitor alpha was identified as a negative regulator of Rho family members typified by its ability to prevent dissociation of GDP from them, thereby keeping them in GDP-bound inactive forms[16]. Despite such negative roles initially attributed to Rho GDP dissociation inhibitor alpha, accumulated evidence has come to suggest that it may also act as a positive regulator for Rho family members by regulating their spatial distribution[22,23]. For an example relevant to tumor biology, the transforming activity of constitutively active Cdc42 (F28L) mutant in NIH3T3 fibroblasts was suppressed by down-regulation of Rho GDP dissociation inhibitor through RNA interference[23]. The transforming activity was also suppressed by additional R66A mutation in Cdc42 that abolishes interaction with Rho GDP dissociation inhibitor, but the R66A mutation also abolished plasma membrane distribution of the Cdc42(F28L, R66A) mutant. In further support of tumorigenic role of Rho GDP dissociation inhibitor, it is highly expressed in multiple types of human cancers including ovarian cancer[24] and breast cancer[25]. More importantly, recent studies have shown that Rho GDP dissociation inhibitor is highly expressed in chemoresistant cancer cell lines, such as melanoma[26] and breast cancer cells[27]. Down-regulation of Rho GDP dissociation inhibitor by RNA interference (RNAi) sensitized the chemoresistant cells to drugs[27]. Thus, Rho GDP dissociation inhibitor likely plays some role in the development of chemoresistance.

Human stomatin is an erythrocyte membrane protein of unknown function. Stomatin-like protein 2 is unique in its structure among mammalian stomatin-like proteins and could play some role in the control of ion channel conductance or membrane lipid domain organization[17]. Zhang *et al.* first reported that stomatin-like protein 2 is highly expressed in human esophageal squamous cell carcinoma, lung cancer, laryngeal cancer, and endometrial adenocarcinoma[28]. Further, they showed that esophageal squamous cell carcinoma cell lines transduced with a stomatin-like protein 2 antisense construct produced slower-growing tumor in nude mice. Elevated expression of stomatin-like protein 2 was also found in human osteosarcoma[29], in laryngeal squamous cell carcinoma and breast cancer as prognostic factors with higher expression being found in patients with metastasis[30,31], and in paclitexel-resistant human breast cancer cell line[32]. Finally, Cui *et al.* reported that in human endometrial adenocarcinoma, sense and antisense constructs of stomatin-like protein 2 accelerated and delayed cell growth, respectively, *in vitro*[33]. Thus, stomatin-like protein 2 is also involved in tumorigenesis and malignant progression.

Ornithine aminotransferase has recently been found to be necessary for mitotic cell division of human cancer cells but not of normal cells[34]. Down-regulation of ornithine aminotransferase by RNAi in human cervical carcinoma HeLa cells and osteosarcoma U20S cells led to accumulation of cell arrested at the G_2/M phase of the cell cycle and an increase in mitotic spindle defects, subsequently causing massive cell death. This "second function" of ornithine aminotransferase is unrelated to its role in amino acid metabolism and is blocked by a naturally occurring anti-cancer compound diazonamide A and its synthetic analog AB-5[34,35]. *In vitro*, AB-5 inhibited growth of human colon, breast, ovarian, pancreatic, lung and prostate cancer cell lines and melanoma cell lines with nannomolar growth inhibitory 50 (GI50) values similar to two widely used antimitotics, vinblastine and paclitaxel[35]. *In vivo*, AB-5 inhibited growth of implanted tumors of HCT116 colon cancer cell line, PC3 prostate cancer

cell line and MDA-MB-435 breast cancer cell line with efficacies comparable to those of vinblastine and paclitaxel without any overt side effects such as weight loss and neutropenia in nude mice[35].

In conclusion, our proteomic analysis of TNIK-overexpressing Pam212 cell lines revealed down-regulation of three proteins that are recently under extensive study as candidate proteins for cancer prognositc marker and target proteins for chemotheraputic drug development. Although we need to be cautious in examining results of such studies, it would be interesting to examine whether implanted tumor of TNIK- overexpressing Pam212 cell lines and parental Pam212 cells behave differently in mice *in vivo*, since there exists a precedent in that TetOff system (feeding with Dox+ or Dox-water) was useful for induction of gene expression in implanted tumor[36].

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