The Oxidative Modification of COL6A1 in Membrane Proteins of Ovarian Cancer Patients

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ABSTRACT

Ovarian cancer is the most lethal gynecological malignancy, and specific biomarkers are important needed to improve diagnosis, prognosis, and to forecast and monitor treatment efficiency. There are a lot of pathological factors, including reactive oxygen species (ROS), involved in the process of cancer initiation and progression. The oxidative modification of proteins by ROS is implicated in the etiology or progression of disorders and diseases. In this study, a labeling experiment with the thiol-modifying reagent biotinylated iodoacetamide (BIAM) revealed that a variety of proteins were differentially oxidized between normal and tumor tissues of ovarian cancer patients. To identify cysteine oxidation-sensitive proteins in ovarian cancer patients, we performed comparative analysis by nano-UPLC-MS^E shotgun proteomics. We found oxidation-sensitive 22 proteins from 41 peptides containing cysteine oxidation. Using Ingenuity program, these proteins identified were established with canonical network related to cytoskeletal network, cellular organization and maintenance, and metabolism. Among oxidation-sensitive proteins, the modification pattern of Collagen alpha-1(VI) chain (COL6A1) was firstly confirmed between normal and tumor tissues of patients by 2-DE western blotting. This result suggested that COL6A1 might have cysteine oxidative modification in tumor tissue of ovarian cancer patients.

(Key words : Ovarian cancer, Shotgun proteomics, Cysteine oxidation, Collagen alpha-1(VI), Ingenuity pathway)

INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer death in women, the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy (Jemal *et al*., 2006). Ovarian cancer at its early stages (Ⅰ/Ⅱ) is difficult to diagnose until it spreads and advances to later stages (Ⅲ/Ⅳ), because most of the common symptoms are nonspecific. The 5-year survival rate exceeds 90% and most patients are cured by surgery alone when presented at an early stage (Bhoola and Hoskins, 2006). However, the underlying pathophysiology is not clearly understood. Malignant cells are resistant to apoptosis through a mechanism that may involve alterations in their redox balance (Jiang *et al*., 2011). Cancer states have been associated with oxidative stress produced through either an increased free radical generation and/or a decreased antioxidant level in the target cells and tissue (Klaunig *et al*., 1998). Reactive oxygen species (ROS) are considered as a significant class of carcinogens participating in cancer initiation, promotion and progression (Sangeetha *et al*., 2010). Also, it is now well known that ROS play a key role in human cancer development (Kumar *et al*., 2009). In addition to the ROS produced as a byproduct of oxidative phosphorylation in mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, a flavoenzyme family member, generates a significant amount of endogenous ROS through the reduction of O_2 to superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and other ROSs (Ushio-Fukai and Alexander, 2004; Bedard and Krause, 2007). Aerobic organisms include human are constantly exposed to one or more systems, which generates ROS that can damage proteins, nucleic acids and lipids. Thus, ROS are involved protein oxidative damage that can result in the modifications in structure, enzyme activity, and signaling pathways (Stadtman and Levine, 2000).

The oxidative modification of proteins by ROS is implicated in the etiology or progression of disorders and diseases. For the most part, oxidatively modified proteins are not repaired and must be removed by proteolytic degradation, and a decrease in the efficiency of pro-

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teolysis will cause an increase in the cellular content of oxidatively modified proteins (Grune *et al*., 1996). Proteins oxidation may result in modification of their enzymatic and binding properties and lead to diverse functional changes (Levine *et al*., 1994). Accumulation of modified proteins disrupts cellular function either by loss of catalytic and structural integrity or by interruption of regulatory pathways (Stadtman and Levine, 2000). Compared with other protein oxidation, cysteine oxidation was particularly regulated in specific sites of amino acids because of the third structure and physiological condition. Furthermore, thiol oxidation in cysteines showed various form such as disulfide bond (S-S), sulfenic acid (-SOH), sulfinic acid (-SOOH), and sulfonic acid (-SOOOH). And also cysteine oxidation is influence by different form of posttranslational modification, such as phosphorylation and acetylation (Seo *et al*., 2009; Dansen *et al*., 2009; Woo *et al*., 2010). Like this, the redox state of cysteines could motivate protein-protein interactions, and these oxidatively modified cysteines could attend mediators of cellular signaling in that way. However, most of these are unclear, although some mammalian proteins have been identified (Kwon *et al*., 2004; Phalen *et al*., 2006; Lei *et al*., 2008).

Recently, an approach of functional proteomics, redox proteomics, has been employed by coupling mass spectrometry (MS) with cysteine oxidation-sensitive chemicals under oxidative stress conditions (Choi *et al*., 2006; Charles *et al*., 2007; Fu *et al*., 2009; Yang *et al*., 2012). Here, we identified differentially oxidized proteins between normal and tumor tissues of ovarian cancer patients using nano-UPLC-MS^E shotgun proteomics. These results suggest that oxidation-dependent modified proteins will help to understand the environment of ovarian cancer with redox-imbalanced mechanisms.

MATERIALS AND METHODS

Tissue Specimens

Tissue specimens were obtained with the approval of the committee on the Ethical Research involving Human Subjects from the Samsung medical center and Chung-Ang University Hospital. The age of the patients ranged from 38 to 70 years. For comparative proteomics, 3 tumor specimens belong to tumor stage Ⅲ~Ⅳ. For western blotting, the specimens consist of 3 patients of tumor stage I and 3 patients of tumor stage Ⅲ. All normal ovarian tissues were the opposite side of tumor tissues. The specimen samples were obtained from resected specimens within 30 min after surgical resection, snap frozen in liquid nitrogen, and stored at −80℃. A histological diagnosis of all ovarian patients was confirmed after microscopic examination of hematoxylin/eosin-stained sections by a board certified pathologist.

Two-Dimensional Gel Electrophoresis (2-DE) and Western Blotting

To extract the membrane fraction containing cytoskeletal proteins, the frozen tissues were homogenizd in labeling buffer (10 mM MES, pH 6.5, 1 mM EDTA, 0.1 mM AEBSF, 2.0 μg/ml leupeptin, and aprotinin) containing 40 μM N-(biotinoyl)-N′-(iodoacetyl) ethylenediamine (biotinylated odoacetamide, BIAM) (Molecular Probes) and incubated for 4 h at 4℃. The buffer was bubbled with nitrogen gas for 1 h to remove oxygen. The labeling reaction was quenched by the addition of 4 mM dithiothreitol (DTT). And then, the homogenized samples were centrifuged at 13,000 rpm for 20 min at 4℃, and the supernatant (cytosolic fraction) was discarded. The pellet (membrane fraction) was dissolved in Urea buffer (7 M urea, 2 M thiourea and 0.49% CHAPS, pH 7.5), and protein concentration was determined using the Bradford assay (BIO-RAD).

One hundred microgram of membrane fraction mixed with DeStreak™ rehydration solution (GE Healthcare Bio-Sciences AB) was applied to rehydrate gel strips with an immobilized nonlinear pH gradient from 3 to 10 (Immobiline DryStrip pH 3~10 NL, 13 cm; GE Healthcare Bio-Sciences AB). Isoelectric focusing was carried out in four steps as follows: 50 V, 12 h; 500 V, 1 h; 1,000 V, 1 h; and 8,000 V for a total of 60,000 V-h. After reduction and alkylation, second dimensional electrophoresis was conducted on a 5~12% SDS-polyacrylamide gradient gel using an SE-260 vertical unit (Amersham Biosciences). After electrophoresis, proteins were transferred to PVDF membrane and detected with horseradish peroxidase (HRP)-conjugated streptavidin (Pierce, Rockford, IL) using ECL system (iNtRON, South Korea).

For detection of COL6A1 modification, 60 μg of membrane proteins was used to 2-DE electrophosis, and loaded on 8% SDS-polyacrylamide gel. And then the transferred membrans were incubated with anti-COL6A1 antibody (Santa Cruz Biotechnology), and exposured with anti-rabbit antibody (Cell Signaling Technology) using ECL system.

Nano-UPLC MS^E Shotgun Proteomics

Iodoacetamide Labeling

The frozen tissues were homogenizd in labeling buffer (1×PBS, pH 7.4, 1 mM EDTA, 0.1 mM AEBSF, 2.0 μg/ml leupeptin, and aprotinin) containing 220 μM iodoacetamide (Sigma-Aldrich) and incubated for 4 h at 4℃. The buffer was bubbled with nitrogen gas for 1 h to remove oxygen. And then, the homogenized samples were centrifuged at 13,000 rpm for 20 min at 4℃, and the supernatant (cytosolic fraction) was discarded. The

pellet (membrane fraction) was dissolved in Urea buffer (7M urea, 2M thiourea and 0.49 % CHAPS, pH 7.5), and protein concentration was determined using the Bradford assay (BIO-RAD).

In-gel Tryptic Digestion

Six hundred microgram of membrane proteins that had been labeled with IAM at free thiol residues were subjected to 8% SDS-PAGE. The separated proteins were then visualized by staining with Coomassie Blue (R-250). For efficient trypsin treatment of proteins, the separated gels were excised by divided into 10 gel slices. Proteins were reduced with 10 mM DTT and alkylated with 55 mM N-ethylmaleimide (NEM; Sigma) in 100 mM ammonium bicarbonate. Following tryptic digestion (2 μg/sliced gel; Promega, Madison, WI) for 16 hr at 37℃, the peptides were recovered and extracted from the sliced gels using 5% formic acid and 50% acetonitrile. The combined samples were desalted using a solid phase Oasis HLB C18 microelution plate (Waters, Inc., Milford, MA) and then stored at −80℃ before being subjected to analysis.

Nano-UPLC-MS^E Tandem Mass Spectrometry

The separations were performed on a 75 μm×250 mm nano-ACQUITY UPLC 1.7 μm BEH300 C18 RP column and a 180 μm×20 mm Symmetry C18 RP 5 μm enrichment column using a nano-ACQUITY Ultra Performance LC Chromatography™ System (Waters Corporation)

Tryptic digested peptides (5 μl) were loaded onto the enrichment column with a mobile phase that contained 3% acetonitrile in water with 0.1% formic acid. Using a flow rate of 300 nl/min, a step gradient was applied: 3~40% mobile phase B (97% acetonitrile in water with 0.1% formic acid) over 95 min, 40~70% mobile phase B over 20 min, and 80% mobile phase B within 10 min. Sodium formate (1 μmol/min) was used to calibrate the TOF analyzer in the 50~2,000 m/z range and [Glu^1]-fibrinopeptide (m/z 785.8426) at 600 nl/min was used for lock mass correction. During data acquisition, the collision energy of low energy MS mode and high-energy mode (MS^E) were set to 4 eV and 15 ~40 eV energy ramping, respectively. One cycle of MS and MS^E mode of acquisition was performed every 3.2 s. In each cycle, MS spectra were acquired for 1.5 s with a 0.1 s inter-scan delay (m/z 300~1,990), and the MS^E fragmentation (m/z 50~2,000) data were collected in triplicate (Yang *et al*., 2010).

Protein and Peptide Identification

The continuum LC-MS^E data were processed and searched using the IDENTITYE algorithm in PLGS (ProteinLynx Global Server) version 2.3.3 (Waters Corporation). Acquired data from alternating low and elevated energy mode in the LC-MS^E were automatically smoothed, background subtracted, centered, deisotoped, and charge state reduced, and then the alignment of precursor and fragmentation data was combined with retention time tolerance, ±0.05 min. Processed ions were mapped against the IPI mouse database (version 3.44) using the following parameters: peptide tolerance, 100 ppm; fragment tolerance, 0.2 Da; missed cleavage, 1; and variable modifications including carbamidomethylation (IAM binding), and NEM from artificial reducing agent-treated states. Peptide identification was performed using the tryptic digestion rule with one missed cleavage. All proteins identified had a >95% probability of significance. The false positive rate for protein identification was set to 5% in the databank search query options based on the automatically generated reverse database. Protein identification required the assignment of at least two peptides with a total of seven or more fragments.

Bioinformatics Analysis

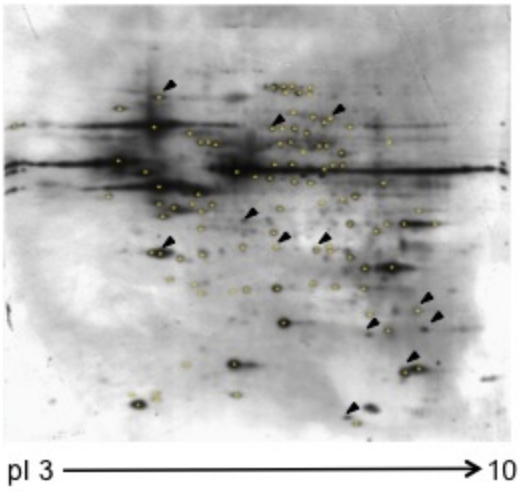
Ingenuity Pathway Analysis (IPA version 9.0; Ingenuity Systems Inc., www.ingenuity.com) was used to perform a knowledge-based network and canonical pathway analysis. Results of network analysis were produced from membrane proteins containing different cysteine oxidation-pattern between normal and tumor tissues of ovarian cancer patients.

RESULTS

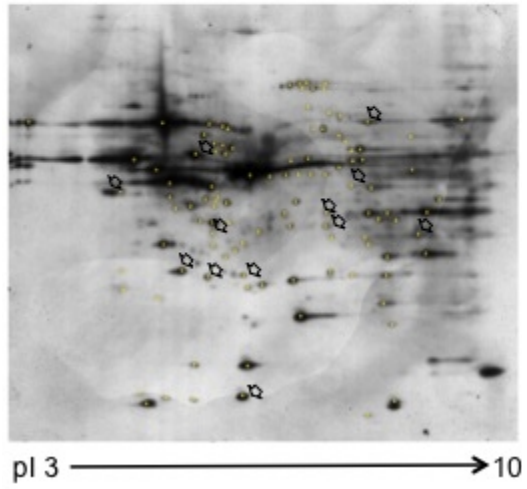
Differential Oxidative Modification of Ovarian Cancer Patients

To check cysteine oxidation in ovarian cancer patients, membrane proteins were prepared with BIAM as an alkylating agent specific for reactive thiols (-SH). 2-DE using non-linear IPG ranging from pH 3~10 was performed to separate the membrane proteins extracted from normal and tumor tissue in ovarian cancer patients. The representative 2DE results in normal versus tumor tissues were shown in Fig. 1. The pattern was showed different cysteine-oxidation between normal and tumor of ovarian cancer patients.

For identification of oxidative modified cysteine, membrane proteins were subjected to MS/MS analysis with IAM binding. Because other cysteinyl thiol-binding chemical such as BIAM interfere ionization peptides during LC-MS analysis, we pretreated IAM to detect the endogenous free cysteinyl thols. Within three independent experiments, we identified 2948 and 1939 peptides in normal and tumor tissues of patients, respectively (with confidence level ≥95%). According to combination of identified peptides, we found 204 and 152 of in



(A)



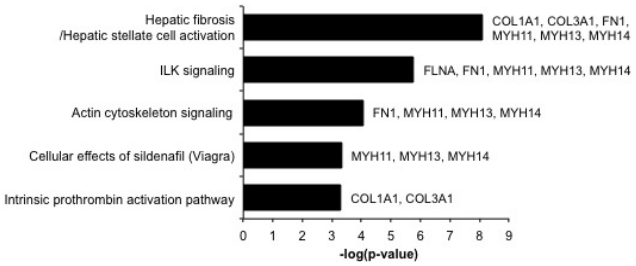
(B)

Fig. 1. Detection of proteins containing oxidative-sensitive cysteine in membrane fraction of ovarian cancer patients. Oxidative cysteine modification in membrane fraction of normal (A) and tumor (B) tissues of ovarian cancer using BIAM-streptavidin western blotting. Membrane proteins were separated by 2D gel electrophoresis using 13 cm Immobiline Drystrip with a pH 3~10 nonlinear gradient, as described in detail in materials and methods. Arrowhead, spot detected only in normal tissues; arrow, spot detected only in tumor tissues. Samples were run in triplicate. This is the representative figure.

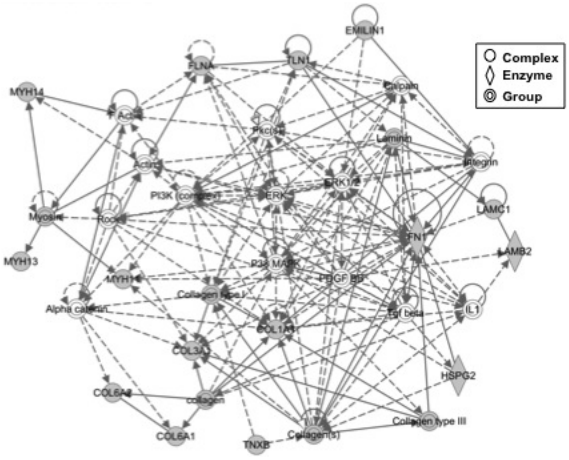
normal and tumor tissues of patients (data not shown). Total peptides and proteins in tumor tissue were reduced than in normal tissue of ovarian cancer patient. Of these proteins, we compared and selected peptides containing different pattern of cysteine oxidation. As a result of the comparison, we found 41 pepetides containing oxidatively modified-cysteines within 22 proteins. Table 1 summarizes the oxidation sensitive-proteins identified in our study, which exhibited a significant difference of cysteine oxidation between normal and tumor tissues of ovarian cancer patients.

Reciprocal Networks of Proteins Containing Cysteine Oxidation

The identified oxidation-sensitive proteins were classified as cytoskeletal proteins, stress-responsive proteins, and so forth. To provide insight into the mechanisms of oxidation-sensitive proteins in ovarian cancer patients, pathway analysis was carried out on the datasets of identified proteins using Ingenuity Pathway Analysis software. Fig. 2B shows a multidirectional interaction network among the target proteins determined to have the most significantly related functions. This canonical network was established with cytoskeletal network, cellular organization and maintenance, and metabolism (Table 2). The components of this network were analyzed for their association with connective tissue disorder, dermatological disease and conditions, genetic and developmental disorders, and metabolic di-



(A)



(B)

Fig. 2. Functional annotation of cysteine oxidation-sensitive proteins using IPA. To categorize identified proteins, *p*-values were used to show the ratio between the number of identified proteins classified and the total number of proteins referenced in the linked function by the software. (A) Canonical network among cysteine oxidation-sensitive proteins. ILK, integrin-linked kinase. (B) The representative annotation of reciprocal network. The most significant connection in the global network of identified cysteine oxidation-sensitive proteins (Table 2) are indicated. The identified proteins in this study are shown as gray-filled figures. Solid lines indicate direct protein-protein interactions; solid arrow lines, membership or protein-DNA interactions; and dotted arrow lines, direct expressional activation.

Table 1. A protein list containing different oxidation-sensitive cysteines between normal and tumor tissues of ovarian cancer patients

No.	IPI No.	Protein name	Gene	Score (PLGS)	MS/MS spectra	Binding status	IAM binding peptide[a)]
1	IPI00007858	Myosin-13	MYH13	194	9	N	**C**NGVLEGIRI**C**RKGFPSRILYA
2	IPI00010951	Epiplakin	EPPK1	50	4	N	D**C**L**C**QMLD
3	IPI00013079	Emilin-1	EMILIN1	328	19	T	VAYKTVTDMEWR**CC**QGYGGDEG**C**GA**C**GGVQEELGRLRDGVER
4	IPI00021033	Collagen alpha-1(III) chain	COL3A1	120	3	N	DDII**C**DD
5	IPI00024284	Basement membrane-specific 18 eparin sulfate proteoglycan core protein	HSPG2	343	10	N	DAMKATATS**C**RP**C**P**C**PYID
						T	ATATS**C**RP**C**P**C**PYI
						T	**C**DERGSMGTSGEA**C**R
6	IPI00025276	Isoform XB of Tenascin-X	TNXB	174	9	N	S**C**PGD**C**NQR
						N	**C**RGHGL**C**EDGV**C**V**C**D
						N	**C**STRT**C**PRD**C**RGR
						T	A**C**PRD**C**R
7	IPI00076042	Short heat shock protein 60	HSP60s2	44	7	N	IAECKKQILQAKR
8	IPI00216230	Lamina-associated polypeptide 2, isoform alpha	TMPO	47	7	T	DLAL**C**RAYEAAASALQIATHTAFVAKAMQA
9	IPI00291136	Collagen alpha-1(VI) chain	COL6A1	885	46	N	E**C**EILDIIMKM**C**S**CC**E**C**K
						T	AVAFQD**C**PVD
10	IPI00296922 /IPI00743203	Laminin subunit beta-2	LAMB2	61	14	T	GS**C**YPATGD
						T	AGYTGLR**C**EA**C**APGHFG
						T	FTGH**C**SCRPGVSGVR**C**DQ**C**AR
						N	MAVYLGSGNVSGGV**C**D
11	IPI00298281	Laminin subunit gamma-1 precursor	LAMC1	165	9	N	FAVGGR**C**K**C**NGHASE**C**MKNEF
						N	D**C**QP**C**P**C**PGGSS**C**AVVPKTKE
						T	ENFFRLGNNEA**C**SS**C**H**C**SPVGSLSTQ**C**D
						T	NIDPNAVGN**C**NRLTGE**C**LK**C**IYNTAGFY**C**D
						T	E**C**NVETGR**C**V**C**KDNVEGFN**C**ER**C**KPGFFNLESSNPR
						T	**C**KA**C**N**C**NPYGTMKQQSS**C**NPVTGQ**C**E**C**LPHVTGQD**C**GA**C**
						T	TPPEEY**C**VQTGVTGVTKS**C**HL**C**
12	IPI00298994	Talin-1	TLN1	75	14	T	AIADMLRA**C**KEAAYHPEVAPDSINQLITM**C**TQQAPGQKE**C**D
13	IPI00302592	Filamin A	FLNA	160	7	T	**C**SKAGNNMLLVGVHGPRTP**C**EEILVKHVGSRLYSVSYLLK
14	IPI00304840	Isoform 2C2 of Collagen alpha-2(VI) chain	COL6A2	623	30	N	**C**EKR**C**GALDVVFVI
						T	TINRIIKVMKHEAYGE**C**YK
15	IPI00305166	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	SDHA	162	4	N	**CCC**VADR
16	IPI00337335	Isoform 1 of Myosin-14	MYH14	219	13	N	A**C**RVAEQAAND
17	IPI00339225	Isoform 5 of Fibronectin precursor	FN1	1,664	91	N	Q**C**QDSETGTFYQIG
						N	DADQKFGF**C**PMAAHEEI**C**TTNEGVMYR
						T	NRGNLLQ**C**I**C**TGNGRGEWK**C**ER
18	IPI00387144	Tubulin alpha-1B chain	TUBA1B	358	10	N	DI**C**RRNL
19	IPI00472724	Elongation factor 1-alpha 1	EEF1A1	97	9	T	**C**ILPPTRPTDKPLR
20	IPI00739539	ANKRD26-like family C member 1B	A26C1B	50	10	T	AVQ**C**QEDE**C**ALMLLEHGT
21	IPI00740545	POTE ankyrin domain family member 1	POTEI	16	7	N	TALTKAVQ**C**QEDE**C**ALMLLEHGTDPNIP
22	IPI00873982	Myosin heavy chain 11 smooth muscle isoform	MYH11	174	8	T	QA**C**ILMIKALEL
						T	AFLVLEQLR**C**NGVLEGIRI**C**R

N, normal tissues; T, tumor tissues. a) **C** represents an IAM-labeled cysteine

Table 2. The associated top networks generated by the Ingenuity pathway analysis

Network	Molecules in network	Score	Focus molecules	Functions
1	Actin, Alpha catenin, Calpain, **COL1A1, COL3A1, COL6A1, COL6A2**, collagen, CollagentypeI, CollagentypeIII, Collagen(s), **EMILIN1**, ERK, ERK1/2, FActin, **FLNA, FN1, HSPG2**, IL1, Integrin, **LAMB2, LAMC1**, Laminin, **MYH11, MYH13, MYH14**, Myosin, P38MAPK, PDGFBB, PI3K(complex), Pkc(s), Rock, Tgfbeta, **TLN1, TNXB**	42	15	Cellular Movement, Cellular Assembly and Organization, Cellular Function and Maintenance
2	AGT, BMP3, CD3, CD6, CDC42SE1, COX7A1, DDAH2, DDX18, **EEF1A1, EPPK1**, FUT8, GFM2, Insulin, Jnk, KLK1, MAS1, MYC, NGFRAP1, NKTR, PCYOX1, Pka, PREP, PSG9, RAB13, RPL15, RRS1, **SDHA(includesEG:157074)**, SMAD2, TBC1D1, **TMPO**, TRDMT1, TUBA8, **TUBA1B**, TWSG1, vitaminK1	11	5	Carbohydrate Metabolism, Cardiovascular System Development and Function, Tissue Morphology

* Bold indicates the identified cysteine oxidation-sensitive proteins in membrane and cytosol

sease. Thus, these results suggest that the highly interconnected network likely represents significantly interconnected biological functions involved in the pathogenesis of numerous diseases.

Different Modification Pattern of COL6A1

Peptides of several proteins including HSPG2 (IPI00024284), TNXB (IPI00025276), COL6A1 (IPI00291136), LAMB2 (IPI00296922/IPI00743203), LAMC1 (IPI00298281), COL6A2 (IPI00304840) and FN1 (IPI00339225), possessed more than one cysteine that was sensitive to oxidative stress both in normal and tumor tissues (Table 1). But, Some proteins showed IAM-binding cysteines, which have the reduced forms in tumor tissue of ovarian cancer patient: EMILIN1 (IPI00013079), TMPO (IPI00216230), TLN1 (IPI00298994), FLNA (IPI00302592), EEF1A1 (IPI00472724), A26C1B (IPI00739539), and MYH11 (IPI00873982). Thus, a variety of proteins might be sensitive to cysteine oxidation, and be affected by alteration of the functional activity and structure.

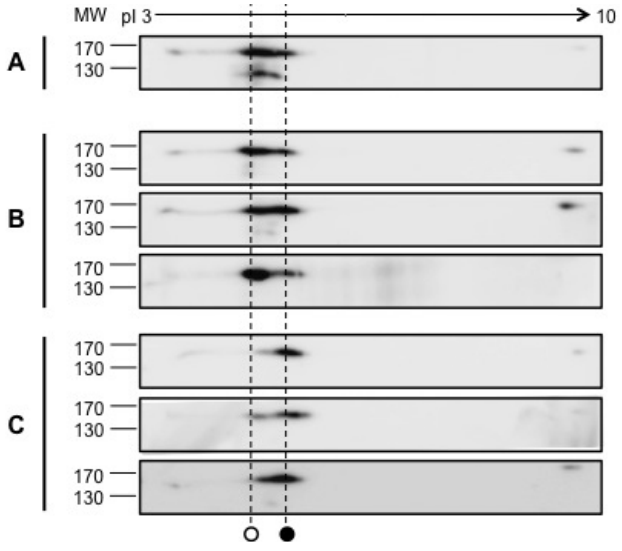


Fig. 3. Analysis of the modification of COL6A1 in normal and tumor tissues of ovarian cancer patients using 2-DE and western blotting. (A) Normal tissues, (B) tumor stage I, and (C) tumor stage Ⅲ of independent ovarian cancer specimens. Membrane proteins were resolved by 2D electrophoresis, transferred on PVDF, and immunoblotted with antibody against anti COL6A1. White circle, acidic location; block circle, reducing location.

Among cysteine oxidation-sensitive proteins, we confirmed the modification pattern of COL6A1 on independent ovarian cancer specimens with 2-DE and western blotting (Fig. 3). The spot of COL6A1 tends to be acidic in specimen of normal tissue (Fig. 3A) and tumor stage I (Fig. 3B). However, most of COL6A1 that appeared at the reducing migrated location under tumor stage Ⅲ was supposed to be modified (Fig. 3B). This result showed that cysteine oxidation might be important factor to regulate the functionality of COL6A1.

DISCUSSION

Redox proteomics is an emerging branch of proteomics aimed at investigating oxidative-stress induced modifications of proteins (D'Alessandro *et al*., 2011). Several experimental approaches have been developed for the systematic and exhaustive characterization of the so-called thiol proteome (Poole *et al*., 2005; Leitner and Lindner, 2006; Leichert *et al*., 2008). Amongst the many kinds of amino acid residues susceptible to oxidative stress, cysteine is by far one of the most sensitive. Oxidation of its -SH groups can have functional significance by regulating protein function and can be the target of oxidative insult. Therefore, redox proteomics aims at detecting and analyzing redox-based changes within the proteome both in redox signaling scenarios and in oxidative stress. In recent years, though only preliminary results are currently available, a role for redox proteomics has progressively emerged in turning over alterations of redox balance which target protein species in

several diseases (Dai *et al*., 2005; Dalle-Donne *et al*., 2005; Bouayed *et al*., 2009; Toda *et al*., 2010). Above all, redox proteomics might represent a valid tool to explore protein targeting oxidative modifications prior to or upon drug administration, which could accelerate the whole process of drug designing and testing up. In upcoming years, redox proteomics will likely play a pivotal role in the quest for new therapeutic targets and their validation, in the process of determining oxidative stress-triggered cellular alteration upon drug treatments and thus in the very heart of the design and testing of new drugs and their metabolites against those pathologies relying on altered redox homeostasis.

In the present study, the extracted proteins were subjected to in-gel trypsin digestion, after which the digested peptides were separated and analyzed using a nano-UPLC coupled Q-TOF tandem mass spectrometer (Yang *et al*., 2010). Elevated chromatography performance by nano-UPLC coupled to fast MS/MS significantly increases sensitivity and in-depth analysis of peptides and proteins. And also, the mass accuracy achieved with this system reduces redundancy of multiple peptide assignments, because of a sequential low and high collision energy data acquisition cycle and the development of specific software (PLGS) (Xu *et al*., 2008).

Collagen type Ⅵ is ubiquitously distributed in the connective tissues and is particularly abundant around cells, associated with interstitial collagen fibres types Ⅰ~Ⅲ, with a possible role as substrate for the attachment of cells and in anchoring collagen fibers, nerves and blood vessels to the surrounding connective tissue (Kuo *et al*., 1997). It has a characteristic beaded filamentous structure of tetrameric units that consists of three different α-chains, α1(Ⅵ), α2(Ⅵ), and α3(Ⅵ) (in human). Collagen VI has high affinity with numerous extracellular matrix components (i.e., biglycan, decorin, hyaluronan, fibronectin, perlecan, and heparin) as well as with the cell membrane (Tillet *et al*., 1994). Thus, it has been hypothesized that collagen VI plays important roles in mediating cell-matrix interactions as well as intermolecular interactions in various tissues as well as cell cultures (Alexopoulos *et al*., 2009). In human, two inherited muscle disorders have been linked to collagen VI genes: Bethlem myopathy, an early onset myopathy with benign course, and Ullrich congenital muscular dystrophy, a severe and rapidly progressive muscle disease causing respiratory failure and death between the first and third decade of life. In the mouse, inactivation of the Col6a1 gene induces a myopathic syndrome with histological and functional features much milder than those found in human (Braghetta *et al*., 2008). Like this, the defects about COL6A1 mostly were analyzed in myogenic and muscular disorders because of its localization and structure. Recently, two studies reported the expressional increase of COL6A1 in ovarian cancer cell lines under hormone responsibility (Parker *et al*., 2009; Zhao *et al*., 2011). COL6A1 has been implicated in cell migration and differentiation, and thus changes in COL6A1 gene expression have implications for cancer cell growth. COL6A1 gene expression has been related to cell growth and cancer development and progression (Wan *et al*., 2004). However, the mechanism related to COL6A1 in cancer metastasis is still unclear, and there have been few studies that have reported the relationship between COL6A1 and cancers.

Though we systematically performed the identification of cysteine oxidation patterns, information regarding the oxidation of cysteine in ovarian cancer is still lacking. With redox proteomics based on our data, the study of cellular oxidation network may contribute to understanding how redox homeostasis is regulated in ovarian cancer patients. In the future, the role of COL6A1 with oxidation sensitive modification will need to be determined for clarification of its relationship to cancer progression (metastasis).

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