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ARTICLE in JOURNAL OF PROTEOME RESEARCH · JUNE 2010

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## Mass Spectrometry Analysis of the Post-Translational Modifications of $\alpha$ -Enolase from Pancreatic Ductal Adenocarcinoma Cells

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Received December 3, 2009

Enolase is a key glycolytic enzyme that catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. Recently, enolase was revealed as an important protein in pathophysiological processes since it was found on the surface of hematopoietic cells and overexpressed in several tumor cells. Our previous studies demonstrated that  $\alpha$ -enolase is up-regulated in pancreatic ductal adenocarcinoma (PDAC). In this present work, we further characterized the  $\alpha$ -enolase from PDAC and normal pancreatic duct cells by mass spectrometry using LTQ-Orbitrap and identified multiple post-translational modifications of  $\alpha$ -enolase, such as phosphorylation, acetylation, and methylation. The result showed that more acetylated lysines, methylated aspartic acids, and glutamic acids were found in PDAC cells than that of normal pancreatic duct cells.

**Keywords:** mass spectrometry • LTQ-Orbitrap • enolase • post-translational modification • phosphorylation • acetylation • methylation • pancreatic ductal adenocarcinoma

### 1. Introduction

Enolase, discovered in 1934 by Lohman and Mayerhof, is one of the most abundantly expressed cytosolic proteins in many organisms, and it is a key glycolytic enzyme that catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate, which in turn is converted by pyruvate kinase to pyruvate.<sup>1</sup> Enolase is a metalloenzyme that requires metal ion magnesium ( $Mg^{2+}$ ) activation.<sup>2</sup> In vertebrates, the enzyme occurs as three isoforms:  $\alpha$ -enolase is found in almost all human tissues, whereas  $\beta$ -enolase is predominantly found in muscle tissues and  $\gamma$ -enolase is only found in neuron and neuroendocrine tissues.<sup>3</sup> All enolases are made up of either homo- or heterodimers.<sup>4–6</sup> The crystal structure of enolase from yeast and human has been defined and catalytic mechanism of the enzyme has been proposed.<sup>7–9</sup>

Recently, accumulating evidence revealed that, in addition to its innate glycolytic function, enolase plays an important role in several biological and pathophysiological processes: (1) by using an alternative start codon, the  $\alpha$ -enolase mRNA can be translated into a 37 kDa protein which lacks the first 96 amino acid residues. This protein, named Myc Promoter-Binding Protein 1 (MBP-1), is localized in the nucleus and can bind to

the c-myc P2 promoter and negatively regulate transcription of the proto-oncogene;<sup>10</sup> (2)  $\alpha$ -enolase has been detected on the surface of hematopoietic cells such as monocytes, T cells and B cells, neuronal cells, and endothelial cells as a strong plasminogen-binding receptor, modulating the pericellular and intravascular fibrinolytic system. The expression of  $\alpha$ -enolase on the surface of a variety of eukaryotic cells has been found to be dependent on the pathophysiological conditions of these cells;<sup>11–15</sup> however, how  $\alpha$ -enolase is displayed on the cell surface remains unknown; (3) increased expression of enolase has been reported to correlate with progression of tumors, such as neuroendocrine tumors, neuroblastoma and lung cancers, and enolase has been considered to be a potential diagnostic marker for many tumors.<sup>16–22</sup>

Our previous studies demonstrated that  $\alpha$ -enolase is up-regulated at both the mRNA and protein levels in pancreatic ductal adenocarcinoma (PDAC).<sup>23</sup> PDAC is the fourth leading cause of cancer-related deaths in Western countries. The mean life expectancy is 15–18 months for patients with local and regional disease, and only 3–6 months for those with metastatic disease.<sup>24</sup> In this present work, we further characterized the  $\alpha$ -enolase from PDAC and normal pancreatic duct cells by reversed-phase liquid chromatography nanospray tandem mass spectrometry (LC–MS/MS) analysis using an LTQ-Orbitrap instrument, and identified multiple post-translational modifications of  $\alpha$ -enolase, such as phosphorylation, acetylation, and methylation. The LTQ-Orbitrap provides high accuracy mass measurement that is essential for the validation of modified peptide identifications and the reduction of false positive identifications.

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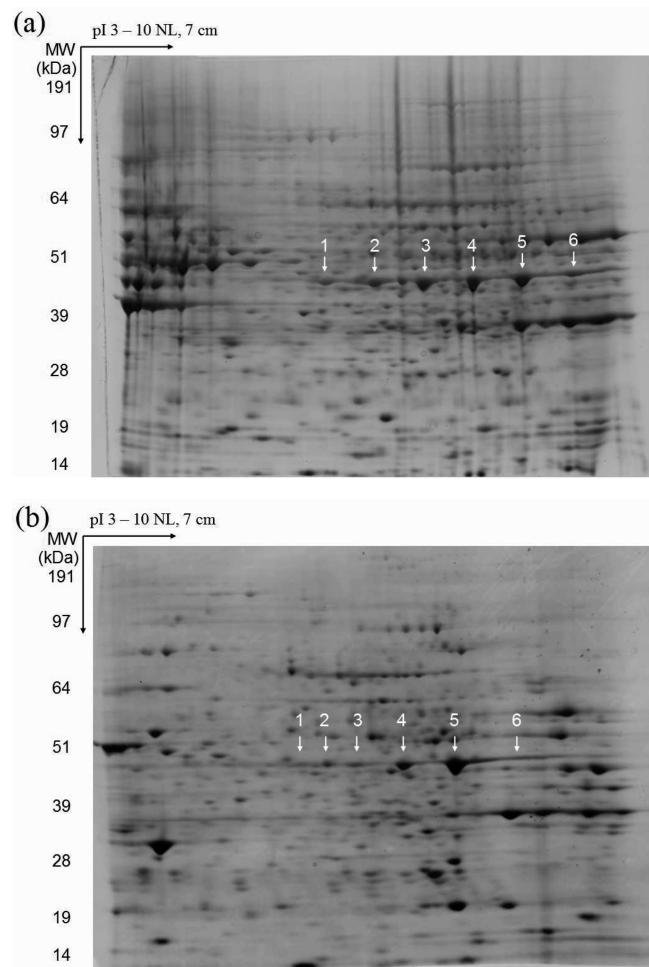
## 2. Materials and Methods

**2.1. Sample Preparation.** CF-PAC-1 cells (metastatic cell line derived from PDAC patients, ECACC ref. No: 91112501) were cultured at 37 °C in Dulbecco modified Eagle's medium (DMEM) (Invitrogen) supplemented with 20 mM glutamine, 10% fetal calf serum (FCS), and 40 µg/mL Gentamycin with humidified 5% CO<sub>2</sub>. The cells were harvested and washed with Hank's balanced salt solution (Sigma-Aldrich). The cell pellet was freeze-dried overnight and stored at -80 °C until use. The cells were resuspended in rehydration buffer consisting of 5 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer (nonlinear pH 3–10, GE Healthcare Bio-Sciences), 80 mM dithiothreitol (DTT), 10 µL/mL protease inhibitory cocktails (Sigma-Aldrich), 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 µL/mL Nuclease Mix (GE Healthcare Bio-Sciences), lysed by sonication for 30 s, and centrifuged at 16 000× g for 10 min. The supernatant was transferred to another centrifugation tube, and the protein concentration was measured by Bradford Assay (BioRad).

Normal human pancreatic duct cells were obtained from brain death donor under IRB approval (San Raffaele Scientific Institute, Italy).

**2.2. Two-Dimensional Electrophoresis.** Two-dimensional electrophoresis (2-DE) was performed as previously described.<sup>25</sup> Briefly, protein lysate (400 µg) was diluted in rehydration buffer and applied to Immobiline Dry strips (pH 3–10 NL, 13 cm, GE Healthcare Bio-Sciences) by in-gel rehydration. For the first dimension, an IPGphor IEF unit system (GE Healthcare Bio-Sciences) was used. Isoelectric focusing (IEF) was performed with a voltage gradient up to 8000 V for a total of 32 000 Vh. Prior to SDS-PAGE, the IPG strips were equilibrated for 15 min with a solution of 5 mM Tris/HCl buffer, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 2% DTT, and then for a further 5 min in the same buffer containing 2.5% iodoacetamide and Bromophenol Blue instead of DTT. For the second dimension, strips were run on small NuPAGE Novex 4–12% Bis-Tris Zoom precast gels (Invitrogen) using Novex X-Cell II Mini-cell system (Invitrogen) at a constant 200 V and stained with colloidal Coomassie Brilliant Blue G-250 (Sigma-Aldrich) stain. 2-DE gel images were acquired with ProXPRESS 2D Proteomic Imaging System (Perkin-Elmer Life and Analytical Sciences), with a 16-bit slow scan CCD camera cooled to -35 °C and recorded in TIFF format.

**2.3. Mass Spectrometry Analysis.** Protein spots were excised from the 2-DE gel and in-gel digested with trypsin.<sup>26</sup> The extracted tryptic peptides were analyzed by LC-MS/MS using an LTQ-Orbitrap mass spectrometer (ThermoFisher).<sup>27</sup> The reversed-phase LC column was slurry-packed in-house with 5 µm, 200 Å pore size C<sub>18</sub> resin (Michrom BioResources, CA) in a 100 µm i.d. × 10 cm long piece of fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a laser-pulled tip. After sample injection, the column was washed for 5 min with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid, 80% acetonitrile) to 45% mobile phase B in 120 min at 200 nL/minute, then to 100% B in an additional 5 min. The LTQ-Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60 000 resolving power) was followed by eight MS/MS scans where the eight most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. The "FT master scan preview mode", "Charge state screening", "Monoisotopic precursor selection",



**Figure 1.** Coomassie-stained 2-DE gel of separated proteins. (a) Proteins from PDAC cells; (b) proteins from normal pancreatic duct cells. Six spots (~47 kDa) were excised from the gel and proteins were in-gel digested with trypsin for LC-MS/MS analysis.

and "Charge state rejection" were enabled so that only the 2+ and 3+ ions were selected and fragmented by CID. Tandem mass spectra collected by Xcalibur (version 2.0.2) were searched against the NCBI human protein database using SEQUEST (Bioworks software from ThermoFisher, version 3.3.1) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable methionine oxidation. Mass tolerance for precursor ions was 10 ppm and mass tolerance for fragment ions was 0.2 Da. The SEQUEST search results were filtered using the following criteria: "Xcorr versus charge 2.0, 3.0 for 2+, 3+ ions; ΔCn > 0.1; mass accuracy 3 ppm; probability of randomized identification of peptide <0.05". For identification of post-translational modifications, variable modifications such as phosphorylation of Ser/Thr/Tyr, acetylation of Lys, methylation of Asp and Glu were allowed. Confident peptide identifications were determined using stringent filter criteria for database match scoring followed by manual evaluation of the results.

## 3. Results

Proteins from PDAC cells were separated by two-dimensional electrophoresis and stained by Coomassie Brilliant Blue (Figure 1). We picked 6 spots that have molecular weight ~47 kDa, corresponding to monomer α-enolase. Proteins in the spots

**Table 1.** Summary of Identification of α-Enolase from 2-DE Gel by LTQ-Orbitrap<sup>a</sup>

	total matched spectra	total nonenolase spectra (% total)	total α-enolase spectra (% total)	amino acid coverage of α-enolase (%)
PDAC				
Spot 1	2115	977 (46.2%)	1138 (53.8%)	75.7%
Spot 2	2789	1328 (47.6%)	1461 (52.4%)	78.4%
Spot 3	2346	951 (40.5%)	1395 (59.5%)	84.6%
Spot 4	3082	1389 (45.1%)	1693 (54.9%)	79.8%
Spot 5	3024	1316 (43.5%)	1708 (56.5%)	75.7%
Spot 6	2241	1144 (51.1%)	1097 (48.9%)	78.4%
Control <sup>b</sup>				
Spot 1	1269	875 (69.0%)	394 (31.0%)	72.1%
Spot 2	1399	949 (67.8%)	450 (32.2%)	75.7%
Spot 3	1884	1186 (63.0%)	698 (37.0%)	75.7%
Spot 4	1978	816 (41.3%)	1162 (58.7%)	84.6%
Spot 5	2041	744 (36.4%)	1297 (63.6%)	84.6%
Spot 6	1538	826 (53.7%)	712 (46.3%)	79.8%

<sup>a</sup> All results were obtained using the following criteria to filter the SEQUEST search results: "Xcorr versus charge 2.0, 3.0 for 2+, 3+ ions; ΔCn > 0.1; mass accuracy 3 ppm; probability of randomized identification of peptide <0.05". The estimated false discovery rate by searching combined forward and reversed database is lower than 1%. <sup>b</sup> Control sample is the cells from normal pancreatic duct.

were in-gel digested and the extracted peptides were identified by LC-MS/MS. The SEQUEST search results were filtered by stringent criteria as described above and yielded 2115 matched MS2 spectra from spot 1. Among these, 1138 (53.8%) spectra were matched to α-enolase with 75.7% protein coverage by amino acid, and 977 (46.2%) spectra were matched to 152 proteins other than α-enolase, indicating that α-enolase was the most abundant protein in spot 1 based on semiquantitative MS2 spectra counts. The estimated false discovery rate (FDR) is lower than 1% by searching a combined forward-reversed database as described by Elias.<sup>28</sup> MS analysis of spot 2–6 yielded results similar to spot 1 (Table 1), confirming that α-enolase was the most abundant protein in spot 1–6 with similar molecular weight and isoelectric point ranging from 6 to 9. Since no small protein modifiers such as ubiquitin or ubiquitin-like proteins were found in these spots, the α-enolase proteins in these spots were likely modified by small molecules that could potentially alter the pI of α-enolase without significant change of molecular weight.

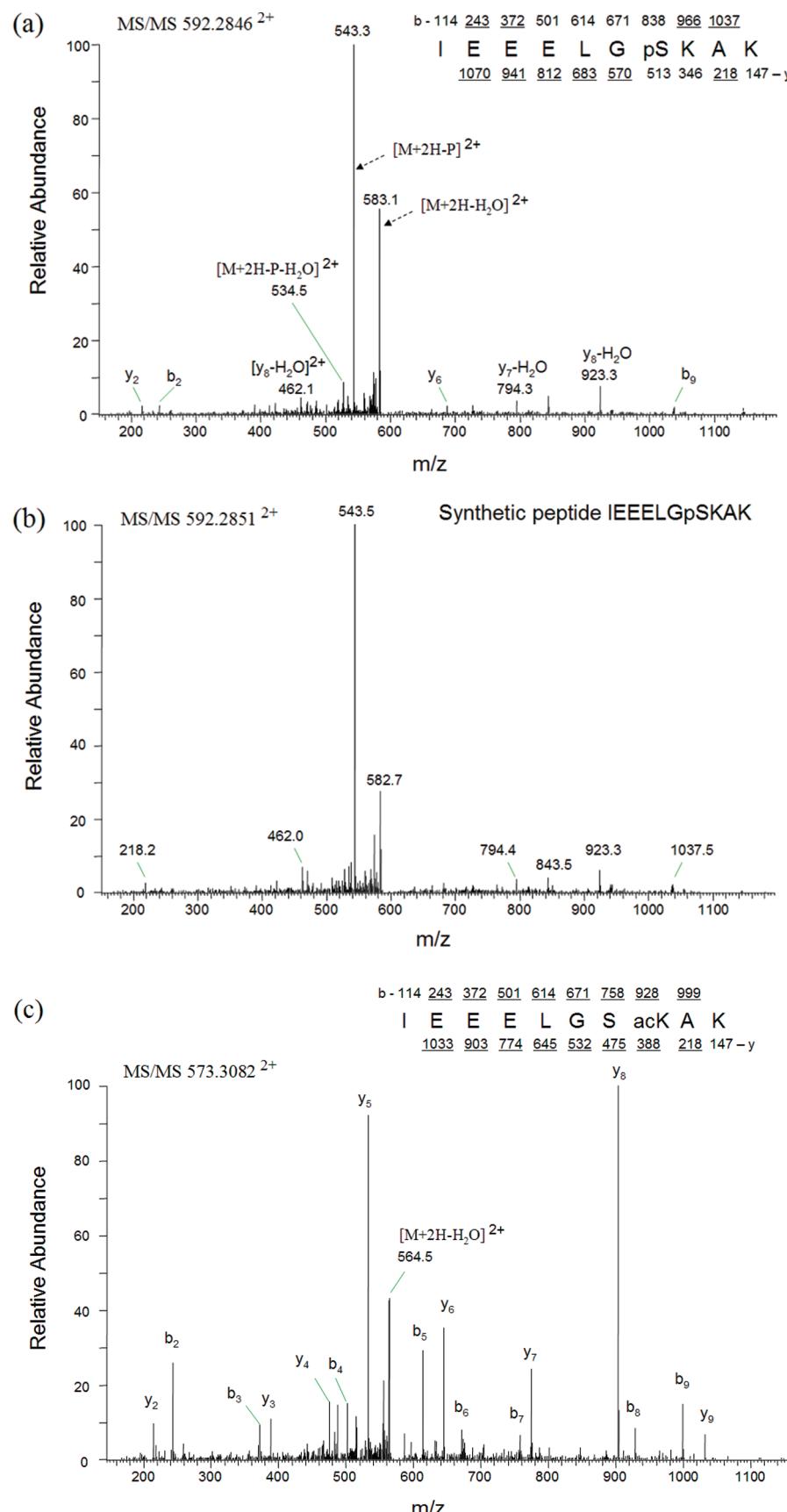
We then searched the MS raw data to identify potential phosphorylation of α-enolase by changing the SEQUEST search parameter with variable phosphorylation of Ser/Thr/Tyr. Indeed, one phosphopeptide IEEELGpSKAK was identified from spot 1 and 2, but not from 3–6. The phosphopeptide, containing phosphorylated Ser-419, is close to the C-terminus of α-enolase primary sequence. The CID spectrum of this phosphopeptide was shown in Figure 2. To confirm the validity of the spectral findings, a synthetic phosphopeptide corresponding to the tryptic fragment was synthesized, and its MS2 spectrum was acquired using the LTQ-Orbitrap. The MS2 spectrum of this phosphopeptide obtained from cell lysate was almost identical to the MS2 spectrum of synthesized peptide (Figures 2), indicating accurate identification of phosphorylation at Ser-419.

Next, we examined whether the α-enolase was acetylated. We searched the MS raw data by changing the SEQUEST search parameter with variable acetylation of protein amino terminus and the side chain of lysine. As a result, acetylation of protein amino terminus was not found, but 26 unique peptides were identified with N-acetylation of lysine side chain, such as K<sup>Ac</sup>LNVTEQEKK from spots 1 and 2, LAK<sup>Ac</sup>YNQLLR from spots 1–5, and SCNCLLLK<sup>Ac</sup>VNQIGSVTESLQACK from all 6 spots (Table

2). Interestingly, peptide IEEELGSKAK, whose phosphorylated form was characterized as above, was also found acetylated at Lys-420 from spot 1 and spot 2. As shown in Figure 2 of the CID spectrum of acetylated peptide IEEELGSK<sup>Ac</sup>AK, most fragmented b-ions and y-ions from this peptide are matched, supporting correct identification. One acetylated peptide LAK<sup>Ac</sup>YNQLLR was further obtained as synthetic peptide, and its MS2 spectrum was acquired using the LTQ-Orbitrap. The MS2 spectrum of this peptide obtained from cell lysate was almost identical to the MS2 spectrum of synthesized peptide (Supporting Information Figures 1S), indicating accurate identification of this acetylated peptide.

We further investigated the potential methylation of α-enolase from PDAC cells. It is known that proteins can undergo post-translational methylation at one or more nucleophilic side chains, such as N-methylation (protein residues methylated on nitrogen include the ε-amine of lysine, the imidazole ring of histidine, the guanidino moiety of arginine, and the side chain amide nitrogens of glutamine and asparagines), O-methylation (proteins methylated on oxygen atoms of side chain carboxylates of glutamates and aspartates to create methyl esters), and S-methylation (proteins methylated on the thiolate side chain of cysteine).<sup>29</sup> After searching the MS raw data by changing the SEQUEST search parameter with variable methylation of amino acids, we identified 35 unique modified peptides containing O-methylation from these 6 spots, whereas N-methylation and S-methylation of α-enolase were not detected (Table 2). Again, one methylated peptide LMIEMDGTE<sup>NM</sup>NK was further obtained as synthetic peptide, and its MS2 spectrum was acquired using the LTQ-Orbitrap. The MS2 spectrum of this peptide obtained from PDAC cell lysate was almost identical to the MS2 spectrum of synthesized peptide (Supporting Information Figures 2S), indicating accurate identification of this methylated peptide.

As a control, we also assessed if α-enolase from normal pancreatic duct cells were post-translationally modified. Using similar approach, proteins from normal pancreatic duct cells were separated by 2-DE and 6 spots were picked for LC-MS/MS analysis (Figure 1). The MS result confirmed that α-enolase was the most abundant protein in spot 1–6 (Table 1). As shown in Table 2, the phosphopeptide IEEELGpSKAK was also identified from spot 2 and 3; 17 unique



**Figure 2.** Example CID spectra of post-translationally modified peptide IEEELGSKAK. (a) CID spectrum of the phosphopeptide IEEELGpSKAK (2+ ion  $m/z$  592.2846) obtained from PDAC cells; (b) CID spectrum of synthetic phosphopeptide IEEELGpSKAK; (c) CID spectrum of acetylated peptide IEEELGS#AK (2+ ion  $m/z$  573.3082) from PDAC cells. The spectra were labeled to show singly charged b and y ions, as well as ions corresponding to neutral losses of the phosphate group and water.

**Table 2.** Summary of Identification of Post-Translational Modifications of α-Enolase by LTQ-Orbitrap

modified peptides identified by MS	position <sup>a</sup>	spot from PDAC						spot from control					
		1	2	3	4	5	6	1	2	3	4	5	6
Phosphorylation													
IEEELGpSKAK	413–422	✓	✓							✓	✓		
Acetylation													
GNPTVEVDLFTSK#GLFR <sup>b</sup>	16–32		✓	✓									
GVSK#AVEHINK	61–71	✓	✓							✓			
AVEHINK#TIAPALVSK <sup>b</sup>	65–80	✓	✓	✓				✓	✓		✓	✓	
TIAPALVSK#K	72–81	✓	✓										✓
K#LNVTEQEKE	81–89	✓	✓							✓	✓	✓	✓
KLNVTEQEKE#IDK	81–92	✓	✓	✓	✓	✓	✓	✓	✓		✓		✓
IDK#LMIEMDGTENK	90–103	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓
LMIEMDGTENK#SK	93–105	✓	✓			✓	✓	✓					
SK#FGANAILGVSLAVCK	104–120					✓							
AGAVEK#GVPLYR	121–132	✓	✓		✓	✓	✓	✓	✓			✓	✓
IGAEVYHNLK#NVIK	184–197	✓	✓	✓	✓	✓	✓	✓				✓	
YGK#DATNVGDEGGFAPNILENK	200–221	✓	✓	✓	✓	✓	✓	✓	✓				
DATNVGDEGGFAPNILENK#EGLELLK	203–228	✓		✓	✓	✓	✓	✓	✓				
EGLELLK#TAIGK	222–233	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓
TAIGK#AGYTDK	229–239	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓
AGYTDK#VVIGMDVAASEFFR	234–253	✓	✓	✓	✓	✓	✓	✓	✓				
SGK#YDLDFK	254–262	✓		✓					✓				
YDLDFK#SPDDPSR	257–269		✓	✓	✓								
YISPDQLADLYK#SFIK	270–285	✓	✓	✓	✓	✓	✓	✓					✓
SFIK#DYPVVSIEDPFDQDDWGAWQK	282–306	✓	✓	✓	✓	✓	✓	✓	✓				
IAK#AVNEK	328–335	✓											
AVNEK#SCNCLLLK	331–343						✓					✓	
SCNCLLLK#VNQIGSVTESLQACK	336–358	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
VNQIGSVTESLQACK#LAQANGWGVMVSHR	344–372	✓	✓	✓	✓	✓	✓	✓	✓				
LAK#YNQLLR	404–412	✓	✓	✓	✓	✓	✓	✓			✓	✓	✓
IEEELGSK#AK	413–422	✓	✓							✓	✓	✓	✓
Methylation													
GNPTVE*VDLFTSK <sup>c</sup>	16–28			✓		✓					✓		✓
GNPTVEVD*LFTSK	16–28			✓	✓	✓		✓		✓	✓	✓	✓
AAVPSGASTGIYE*ALELR	33–50	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓
AAVPSGASTGIYEALR	33–50	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓
KLNVTE*QEKE	81–89		✓							✓	✓		✓
KLNVTEQE*K	81–89	✓	✓							✓	✓		✓
KLNVTEQEKE#IDK	81–92			✓	✓	✓	✓	✓					
LMIE*MDGTEENK	93–103	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
LMIEMDGTENK#NK	93–103	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
LAMQE*FMILPVGAANFR	163–179		✓	✓	✓	✓	✓	✓					
IGAE*VYHNLK	184–193	✓	✓					✓	✓	✓	✓	✓	✓
DATNVGDE*GGFAPNILENK	203–221	✓			✓	✓	✓	✓	✓				
DATNVGDEGGFAPNILENK	203–221	✓	✓	✓	✓	✓	✓	✓	✓				
D*ATNVGD*E*GGFAPNILENK	203–221	✓	✓	✓	✓	✓	✓	✓	✓				
DATNVGDEGGFAPNILENK*NKE*GLE*LLK	203–228			✓		✓							
DATNVGDEGGFAPNILENKKE*GLELLK	203–228				✓								
VVIGMDVAASE*FFR	240–253	✓			✓	✓	✓				✓	✓	
SGKYDLDKFSPDD*PSR	254–269			✓									
YISPD*QLADLYK	270–281		✓			✓				✓	✓		✓
DYPVVSIE*DPPFDQDDWGAWQK	286–306	✓	✓	✓	✓	✓	✓	✓					
DYPVVSIED*PFDQDDWGAWQK	286–306	✓	✓	✓	✓	✓	✓	✓	✓				
DYPVVSIEDPFD*QDDWGAWQK	286–306						✓						
DYPVVSIEDPFDQD*WGAWQK	286–306						✓	✓	✓				
DYPVVSIEDPFDQD*D*WGAWQK	286–306						✓	✓	✓				
DYPVVSIEDPFD*QD*D*WGAWQK	286–306							✓					
DYPVVSIEDPFD*QD*D*WGAWQK	286–306	✓						✓					
D*YPVVSIE*D*PFDQDDWGAWQK	286–306	✓											
VNQIGSVTE*SLQACK	344–358	✓	✓	✓	✓	✓	✓	✓	✓				
SGE*TEDTFIADLVVGLCTGQIK	373–394	✓	✓	✓	✓	✓	✓	✓					
SGETE*TDFIADLVVGLCTGQIK	373–394	✓						✓					
SGETED*TDFIADLVVGLCTGQIK	373–394	✓	✓	✓	✓	✓	✓	✓	✓				
SGETEDTFIAD*LVVGLCTGQIK	373–394	✓	✓	✓	✓	✓	✓	✓	✓				
IE*EELGSKAK	413–422	✓	✓										
IEE*ELGSKAK	413–422		✓	✓	✓	✓				✓	✓	✓	✓
IEEE*LGSKAK	413–422		✓	✓	✓								

<sup>a</sup> Position of the initial and final peptide amino acids in the protein sequence. <sup>b</sup> N-acetylated lysine is labeled with #. <sup>c</sup> O-methylated glutamate and aspartate are labeled with \*.



**Figure 3.** Schematic of post-translational modification sites of  $\alpha$ -enolase. The primary sequence of  $\alpha$ -enolase is presented with phosphorylated serine highlighted in red, acetylated lysine highlighted in green, and methylated aspartic acid and glutamic acid highlighted in pink. (a)  $\alpha$ -enolase from PDAC cells. Phosphorylation at Ser-419, acetylation at 26 lysine residues Lys-28, 64, 71, 80, 81, 89, 92, 103, 105, 126, 193, 202, 221, 228, 233, 239, 256, 262, 281, 285, 330, 335, 343, 358, 406, 420, methylation at 13 aspartic acid residues Asp-23, 91, 203, 209, 266, 274, 286, 294, 297, 299, 300, 378, 383, and methylation at 21 glutamic acid residues Glu-21, 45, 48, 86, 88, 96, 101, 167, 187, 210, 219, 222, 225, 250, 293, 352, 375, 377, 414, 415, 416 were identified; (b)  $\alpha$ -enolase from normal pancreatic duct cells. Phosphorylation at Ser-419, acetylation at 17 lysine residues Lys-64, 71, 80, 81, 89, 92, 126, 193, 202, 228, 233, 281, 335, 343, 358, 406, 420, methylation at 8 aspartic acid residues Asp-23, 91, 203, 209, 274, 299, 300, 378, and methylation at 16 glutamic acid residues Glu-21, 45, 48, 86, 88, 96, 101, 187, 210, 219, 250, 293, 375, 377, 415, 416 were identified.

peptides were identified with N-acetylation of lysine side chain, and 22 unique peptides were identified with O-methylation of aspartates and glutamates. Notably, several acetylated peptides, such as LMIEDGTENK<sup>#</sup>SK, AGYTDK<sup>#</sup>VVIGMDVAASEFFR, and several methylated peptides, such as LAMQE<sup>#</sup>FMLPVGAANFR, VNQIGSVTE\*SLQACK, were found in PDAC cells but not in normal pancreatic duct cells.

Lastly, searching for other potential post-translational modifications on  $\alpha$ -enolase from PDAC and normal pancreatic duct cells, such as hydroxylation, S-nitrosylation, were performed but yielded no identification. All of the post-translational modifications on  $\alpha$ -enolase identified by LC-MS/MS from this study are presented schematically in Figure 3. For instance, phosphorylation at Ser-419 was found in both PDAC and normal pancreatic duct cells; 26 lysine residues in PDAC cells and 17 lysine residues in normal pancreatic duct cells were acetylated. In addition, 13 aspartic acid and 21 glutamic acid residues were methylated in PDAC cells, whereas 8 aspartic acid and 16 glutamic acid residues were methylated in normal pancreatic duct cells. Hence, the number of acetylated and methylated residues in normal pancreatic duct cells is smaller than that of PDAC cells. It is noteworthy that the unmodified counterparts of these post-translational modified peptides were also identified from each spot, indicating that the  $\alpha$ -enolase was a heterogeneous mixture in each spot. It is likely that dozens of different forms of  $\alpha$ -enolase, each containing variable phosphorylation, acetylation, and methylation, may exist in the 6 spots and the smear regions around these spots. Taken together, we revealed multiple post-translational modifications of  $\alpha$ -enolase from PDAC and normal pancreatic duct cells, such as phosphorylation, acetylation, and methylation, thus providing important biological information toward further understanding of the  $\alpha$ -enolase modifications and their functional significance in disease.

#### 4. Discussions

To date, there have been several reports of post-translational modification of  $\alpha$ -enolase.<sup>30–39</sup> These 26 previously known modifications are listed in Protein Knowledgebase (UniProtKB), including 1 N-acetylserine, 15 N6-acetyllysines, 5 phosphoserines, 3 phosphotyrosines and 2 phosphothreonines (<http://www.uniprot.org/uniprot/P06733>). Here, using high resolution mass spectrometry, we efficiently profiled the post-translational modifications of  $\alpha$ -enolase from PDAC and normal pancreatic duct cells, and identified 1 phosphoserine, 26 acetyllysines, 13 methylaspartates, and 21 methylglutamates. Specifically, the identified phosphoserine Ser-419 is different from the phosphoserine Ser-37, 63, 254, 263, 272 listed in Protein Knowledgebase. On the other hand, we did not identify any phosphotyrosine and phosphothreonine in this study. Interestingly, among the 26 identified acetyllysines, 12 (Lys-64, 71, 89, 126, 193, 228, 233, 256, 281, 285, 406, 420) are listed in Protein Knowledgebase. The existence of phosphorylation at Ser-419 and O-methylation at multiple aspartates and glutamates, to our knowledge, has never been reported before. More importantly, the data indicate that the  $\alpha$ -enolase in PDAC cells was subjected to more acetylation and methylation than that of normal pancreatic duct cells. These novel findings, as usual, require further study to determine the significance of this initial work.

Post-translational protein modifications, such as phosphorylation, acetylation, and methylation, are common and important mechanisms of acute and reversible regulation of protein function in mammalian cells, and largely control cellular signaling events that orchestrate biological functions. At physiological pH, the side chains of Ser/Thr/Tyr are not charged, the side chain of lysine is cationic, and the side chains of Asp/Glu are negatively charged. Thus, phosphorylation of Ser/Thr/Tyr will introduce negative charge to these amino acid residues, N-acetylation of lysine side chain will quench the positive charge, and methylation on carboxylate side chain will

cover up a negative charge and add hydrophobicity. Dynamic phosphorylation, acetylation, and methylation of protein function together in signal transduction pathways to induce rapid changes in response to hormones, growth factors, and neurotransmitters and are recognized as key modes of regulating cell cycle, cell growth, cell differentiation, and metabolism in eukaryotic cells.<sup>29,39–41</sup>

The results described in the current study suggest that the “old” enzyme enolase is actually a complicated enzyme with a potential myriad of isoforms. It remains to be determined how the phosphorylation, acetylation, and methylation of  $\alpha$ -enolase can affect its catalytic activity, localization in the cell, protein stability, and the ability to dimerize or form a complex with other molecules. Furthermore, investigation of these modification patterns in different human cancer cells will provide insights into its important role in pathophysiological processes.

**Acknowledgment.** This work was supported in part by grants from the Associazione Italiana Ricerca sul Cancro (AIRC nr. 5548), Fondazione San Paolo (Special Project Oncology), Ministero della Salute: Progetto integrato Oncologia, Regione Piemonte: Ricerca Industriale e Sviluppo Precompetitivo (BIOPRO and ONCOPROT), Ricerca Industriale “Converging Technologies” (BIOTHER), Progetti strategici su tematiche di interesse regionale o sovra regionale (IMMONC), Ricerca Sanitaria Finalizzata, Ricerca Sanitaria Applicata, and the College of Science at George Mason University.

**Supporting Information Available:** Supplementary Figures 1S and 2S. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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PR901109W