Circulating Autoantibodies to Phosphorylated α-Enolase are a Hallmark of Pancreatic Cancer.

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Circulating autoantibodies to phosphorylated α-enolase are a hallmark of pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis and no diagnostic markers have, as yet, been defined. In PDAC patients, α-enolase (ENOA) is up-regulated and elicits the production of autoantibodies. Here, we analyzed the autoantibody response to post-translational modifications of ENOA in PDAC patients. ENOA isolated from PDAC tissues and cell lines was characterized by two-dimensional electrophoresis (2-DE) western blot (WB), revealing the expression of six different isoforms (named ENOA1,2,3,4,5,6) whereas only 4 isoforms (ENOA3,4,5,6) were detectable in normal tissues. As assessed by 2-DE WB, 62% of PDAC patients produced autoantibodies to the two more acidic isoforms (ENOA1,2) as opposed to only 4% of controls. Mass spectrometry showed that ENOA1,2 isoforms were phosphorylated on serine 419. ROC analysis demonstrated that autoantibodies to ENOA1,2 usefully complement the diagnostic performance of serum CA19.9 levels, achieving approximately 95% diagnostic accuracy in both advanced and resectable PDAC. Moreover, the presence of autoantibodies against ENOA1,2 correlated with a significantly better clinical outcome in advanced patients treated with standard chemotherapy. In conclusion, our results demonstrate that ENOA phosphorylation is associated with PDAC and induces specific autoantibody production in PDAC patients that may have diagnostic value.

Keywords: antibodies/ mass spectrometry/ pancreatic ductal adenocarcinoma/ serological proteome analysis/ two-dimensional electrophoresis/ tumor associated antigen

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in both sexes in United States and Europe.[1] Most patients die within 12 months and only 4% survive for five years after diagnosis. Surgical resection is the only potentially curative option. However, the absence of early symptoms or clinical-pathological markers results in diagnosis at a late, inoperable, stage in more than 80% of cases.[1, 2] Biomarkers for the early detection of PDAC or even its differentiation from chronic pancreatitis (CP) are lacking. The only serological marker described, CA19.9, may also be elevated in non-malignant conditions, such as acute and chronic pancreatitis, as well as hepatitis and biliary obstruction,[3] and has discriminating power only when high cut-off values are used.[4]

We have observed that PDAC patients have circulating $CD4^+$ T cells recognizing alpha-enolase (ENOA), a key glycolytic enzyme up-regulated in PDAC [5] and in other tumors.[6-10] The presence of ENOA-specific $CD4^+$ T cells suggests the existence of anti-ENOA-antibody-producing B cells. In addition, the over-expression of this enzyme may promote the overcoming of immune tolerance, and often results in autoantibody production.[11] Notably, ENOA has been suggested as a potential biomarker of pancreatic cancer.[12] By using a serological proteome analysis (SERPA) approach, we have identified, in serum of PDAC patients, autoantibodies directed against a number of metabolic enzymes that are often up-regulated in PDAC.[13] In this paper we have evaluated: i) the expression and phosphorylation status of ENOA in PDAC cells; ii) the autoantibody response to phosphorylated ENOA in a large cohort of PDAC patients and their ability, alone or in combination with CA19.9, to discriminate PDAC patients from controls; iii) the correlation of anti-phosphorylated ENOA autoantibody response and clinical-pathological variables of advanced PDAC patients treated with gemcitabine-based first-line chemotherapy.

Experimental Section

Sera Specimens. The study was conducted with ethical approval from the Ethical Committees of the Dept. of Internal Medicine, University of Turin and Regina Elena National Cancer Institute, Rome. Serum samples were isolated from venous blood with the informed consent of patients and healthy donors and stored at −80°C until use. De-identified numeric specimen codes were used to protect the identity of the individuals. Diagnosis of PDAC or any other cancer was always confirmed by histological or cytological analysis. Sera from 120 PDAC patients, with clinical features described in Table 1, were tested and the resulting reactivities were compared with those of control sera from the following sources: 40 healthy subjects (HS, M/F: 14/26; median age, 71 years; range, 57–87 years) without a prior history of cancer or autoimmune disease; 50 non-PDAC cancer patients (9 liver, 12 breast, 9 colon, 19 lung and 1 ovarian; M/F: 24/26; median age, 69 years, range, 44–86 years); 46 chronic pancreatitis patients (CP, M/F: 26/20; median age, 58 years; range, 22–74 years); 12 autoimmune diseases patients (AD, 3 Mixed Cryoglobulinemia, 2 Meniere's Syndrome, 4 Rheumatoid Arthritis, 2 Systemic Lupus Erythematosus, and 1 Autoimmune Pancreatitis; M/F: 3/9; median age, 49 years; range, 38–79 years).

Two-dimensional electrophoresis and western blot analysis. Normal pancreatic (NP) tissues from six patients surgically treated for diseases not related to the pancreas, and PDAC tissues from twelve patients surgically treated (stage II of PDAC) were obtained frozen from Regina Elena National Cancer Institute (Rome, Italy) and used for 2-dimensional electrophoresis (2-DE) western blot (WB). Frozen tissue was homogenized in 2-DE lysis buffer, subjected to 2-DE and electroblotted onto a nitrocellulose membrane (GE Healthcare Bio-Sciences, Uppsala, Sweden) as previously described.[13] Proteins from 10^7 CF-PAC-1, (ECACC ref. no. 91112501), MiaPaCa-2 (ECACC ref. no. 85062806) and BxPC-3 (ECACC ref. no. 93120816) PDAC cell lines and from 10^7 Saos-2 (ECACC ref. no. 89050205) human epithelial-like osteosarcoma cell line were

solubilized, subjected to 2-DE and electrotransferred onto a nitrocellulose membrane (GE Healthcare). ENOA isoforms were revealed with mouse anti-α-enolase monoclonal antibody (mAb) 19/12 [14] (for 1 h, 1:1000) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1 h, 1:2000, Santa Cruz Biotechnology, Santa Cruz, California, USA) as a secondary antibody. Sera from PDAC and controls were tested to determine IgG concentration by using a commercial kit (Human IgG ELISA Quantitation Set from Bethyl Laboratories, provided by Tema Ricerche, Bologna, Italy). Sera reactivity to ENOA was tested on PDAC cell lines by using each serum at working dilution of 0.1 mg/ml of IgG for 4 h, and incubated with HRP-conjugated rabbit anti-human IgG (90 min, 1:1000, Santa Cruz Biotechnology) as a secondary antibody. Each serum was screened on three replica blots from the CF-PAC-1 cell line, and reactivity was confirmed by screening once on blots from the MiaPaCa-2 and the BxPC-3 cell lines. Immunodetection was accomplished by ECL PLUS (Enhanced Chemiluminescence, GE Healthcare Bio-Sciences). The resulting chemifluorescent signals were scanned with "ProXPRESS 2D" (PerkinElmer) with an excitation/emission filter setting of 460/80, 530/30 respectively and for an exposure time of 12 s. Images were recorded in TIFF format. The volume of ENOA1,2 spots recognized by autoantibodies was calculated after background subtraction using "ProFinder 2D" (PerkinElmer) software and reported as arbitrary units (AU).

Nano-liquid chromatography tandem mass spectrometry analysis of ENOA phosphorylation. Six ENOA spots were excised from preparative Coomassie Brilliant Blue stained CF-PAC-1 2-DE gels and the in-gel digestion by trypsin was performed according to standard procedure.[15] The extracted peptides were analyzed by reversed-phase liquid chromatography nanospray tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap mass spectrometer (ThermoFisher).[16] The reversed-phase LC column was slurry-packed in-house with 5 μm, 200 Å pore size C_{18} 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/min, 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%. Tandem mass spectra collected by Xcalibur (version 2.0.2, ThermoElectron, San Jose, CA, USA) were searched against the NCBI human protein database (released on September 5, 2009, 34180 sequences) using Bioworks 3.3.1 (ThermoFisher, Extract_msn for peak picking software and SEQUEST for search engine) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable phosphorylation of Ser/Thr/Tyr and methionine oxidation. Mass tolerance for precursor ions was 10 ppm and mass tolerance for fragment ions was 0.5 Da. Confident phosphopeptide identifications were determined using stringent filter criteria "ranked top #1; Xcorr versus charge 1.8, 2.5 for 2+, 3+ ions; mass accuracy 3 ppm; probability of randomized identification of peptide < 0.05" for database match scoring followed by manual evaluation of the results. The estimated "false discovery rate (FDR)" is lower than 1% by searching a combined forward-reversed database.

"On-blot" phosphatase treatment. CF-PAC-1 2-DE gels were electroblotted onto nitrocellulose membranes and, after 2 h blocking with 5% nonfat dry milk in PBS, were either incubated or not for 15 h at 30°C on a rocker in PBS containing 600 U/ml of lambda protein phosphatase (λPPase, Sigma Chemical Co, St. Louis, Missouri, USA), 40 µl/ml of 20 mM $MnCl_2$ and 40 µl/ml of λPPase buffer. The reactivity of a pool of three ENOA1,2^+ sera (diluted in PBS to reach the final concentration of 0.1 mg/ml of IgG) was subsequently assessed.

Western blot with pre-adsorbed sera. A pool of three ENOA1,2^{+} sera was diluted in PBS to reach the final IgG concentration of 0.1 mg/ml and incubated for 15 h at 4°C on a rocker with 20 μg/ml of recombinant α-enolase (rENOA; supplementary data). The reactivity of these pre-adsorbed sera was evaluated by CF-PAC-1 2-DE WB as described.

Statistical analysis. All statistics were computed using the SPSS (17.0) and MedCalc softwares. Data ranges and 95% confidence intervals (95% CI) were reported for descriptive statistics. A 2-sided P value of <0.05 was considered to be statistically significant. Association between clinical-pathological variables and the presence of anti-ENOA1,2 autoantibodies was tested by the Pearson's χ^2 test, Mantel-Haen-Haenszel test or Fisher's exact test, as appropriate. Progression-free survival (PFS) was calculated by the Kaplan-Meier product-limit method from the first day of treatment to the date of progression or death for any cause. If a patient was not dead, survival was censored at the time of the last visit. The log-rank test was used to assess differences between subgroups. The Hazard Ratio (HR) and the confidence limits at 95% were estimated for each variable using the Cox univariate model and adopting the most suitable prognostic category as the reference group. A multivariate Cox proportional Hazard model was also developed using stepwise regression (forward selection) with predictive variables that were significant at univariate analysis. The cut-off P-values to enter in or to be removed from the multivariate model were set at 0.10 and 0.15, respectively. Multivariate logistic regression was performed to assess the diagnostic value of CA19.9 serum levels and the absence or presence of anti-ENOA1,2 autoantibodies, alone or combined, in a cohort of 98 PDAC patients and 63 controls (HS+CP) for whom both data were available. A logistic equation including the coefficients of the regression analysis was then constructed to calculate an estimation of individual patient's probability of having PDAC: probability of PDAC = $(Exp^{\Sigma(X \times B) + intercept(\alpha)})/1+(Exp^{\Sigma(X \times B) + intercept(\alpha)})$, where X x B is the coefficient B for each single confounding factor X. We then generated receiver operating characteristic (ROC)

curves and compared their area under the curves (AUC, c-statistics) to measure the diagnostic accuracy of the model.[17, 18]

Results

Two acidic ENOA isoforms are expressed in PDAC. In agreement with previous studies,[6-9] data obtained using mRNA microarrays, immunohistochemistry and WB indicate that ENOA is up-regulated in PDAC.[5] To characterize the expression of ENOA in PDAC, we used a 2-DE WB-based approach. In normal pancreatic tissues (NP, n=6), 2-DE WB using an anti-ENOA mAb identified four ENOA isoforms (named ENOA3,4,5,6). These isoforms had a similar 47 kDa molecular weight and pI ranging from 6.6 to 8 (Figure 1A). In surgical PDAC specimens (n=12), as well as in PDAC cell lines (CF-PAC-1, MiaPaca-2 and BxPC3), ENOA3,4,5,6 expression was increased approximately 2-fold, as compared to NP, and two more acidic isoforms (pI 6, named ENOA1,2), barely detectable in NP, became clearly apparent (fold increase > 3, Figure 1A). Conversely, ENOA1,2 isoforms were not detected in the Saos-2 osteosarcoma cell line (Figure 1B). In PDAC cell lines, MALDI-MS/MS analysis confirmed that all 2-DE WB-detected isoforms were indeed ENOA isoforms (Supplementary Table 1).

Sera from PDAC patients contain autoantibodies to ENOA1,2 isoforms. Using CF-PAC-1 cell lysates as the source of ENOA, the presence of circulating autoantibodies to PDAC-associated ENOA1,2 isoforms was analyzed by 2-DE WB in 120 sera from PDAC patients (clinical features described in Table 1) and reactivity was compared to that of sera from 40 HS, 46 CP, 12 AD, and 50 non-PDAC patients (Table 2 and Figure 2). Sixty-two percent of PDAC patients produced autoantibodies directed against ENOA1,2 (hereafter referred to as ENOA1,2^{+}); conversely, such reactivity was observed in only 4% of non-PDAC and 9% of CP patient sera (Table 2). A similar pattern of serum reactivity was observed using MiaPaCa-2 or BxPC-3 cell lines as the source of

ENOA (data not shown). Of note, sera from ENOA1,2^{+} patients were able to recognize ENOA1,2 not only in cell line lysates but also in lysates from PDAC biopsies (data not shown).

Sera from PDAC patients contain autoantibodies to phosphorylated ENOA1,2 isoforms. The 2-DE pattern of ENOA1,2 isoforms suggested that they are phosphorylated. Next, we investigated whether autoantibodies to ENOA1,2 are directed to phosphorylated epitopes. To this purpose nitrocellulose membranes blotted from CF-PAC-1 2-DE gels were treated with λPPase, that removes phosphate groups from proteins, and the ability of ENOA1,2^{+} sera to react with blotted ENOA was tested. This "on-blot" λPPase treatment abolished the reactivity of ENOA1,2^{+} sera (Figure 3), while the reactivity of anti-α-enolase mAb (which does not distinguish between phosphorylated and un-phosphorylated isoforms) was not affected (Figure 3, lower panels). This indicates that removal of phosphate groups selectively affects the reactivity of ENOA1,2^{+} patient sera, suggesting that they contain autoantibodies against phosphorylated ENOA1,2 isoforms. Conversely, pre-incubation of ENOA1,2^{+} sera with un-phosphorylated recombinant ENOA (rENOA) did not alter their WB reactivity with ENOA1,2 (Figure 3). These data indicate that in PDAC sera autoantibodies to ENOA1,2 isoforms are indeed directed to phosphorylated ENOA.

To further confirm the ENOA autoantibody reactivity observed in PDAC patients, phosphorylation of all six ENOA isoforms from CF-PAC-1 2-DE gels was analyzed by MS. Nano-LC-MS/MS analysis revealed the presence of phosphorylation on a serine residue at position 419 (^{413}IEEELGpSKAK^{422}) only in ENOA1,2 isoforms (accession number 4503571) (Figure 4), indicating that in PDAC ENOA is phosphorylated at serine 419. These data strongly suggest that autoantibodies that recognize ENOA1,2 are predominantly directed to serine 419-phosphorylated ENOA.

The presence of autoantibodies to ENOA1,2 improves the diagnostic performance of CA19.9 in PDAC. Autoantibodies against ENOA1,2 discriminated PDAC patients from controls (HS, non-PDAC, CP and AD) with 62% sensitivity (95% CI: 52.9-70.4%) and 97% specificity

(95% CI: 93.7-99.5%). In addition, anti-ENOA1,2 autoantibodies were significantly more frequent in patients with normal CA19.9 serum levels (< 37 IU/ml), as compared to patients with pathological elevation of CA19.9 (91% *vs* 59%, respectively, p=0.04, data not shown). This observation prompted us to test the diagnostic performance of combined CA19.9 serum levels (≥ 37 IU/mL) and anti-ENOA1,2 autoantibodies in a training set of 61 advanced PDAC patients at stage III and IV and 63 controls (HS+CP), using ROC analysis. As shown in Figure 5, combined CA19.9/ENOA1,2 discriminated PDAC patients from controls with 95.1% sensitivity and 94% specificity, with an overall diagnostic accuracy of 94±0.02% (p=0.0001). These results were further confirmed in a validation set of 37 resectable PDAC patients. This cohort includes stage II and III patients who underwent surgery with curative intent. In this setting, the combination of elevated CA19.9 serum levels and anti-ENOA1,2 autoantibodies resulted in an overall diagnostic accuracy of 95±0.03% (p=0.0001, Figure 5). Thus, in both settings, detection of anti-ENOA1,2 autoantibodies usefully complemented the diagnostic performance of serum CA19.9 levels.

The antibody response to ENOA1,2 correlates with clinical outcome in advanced PDAC patients. No significant associations were found between baseline PDAC patients' clinical-pathological characteristics (age, gender, previous surgery, histology, disease stage, Eastern Cooperative Oncology Group performance status-ECOG PS) and the presence or absence of autoantibodies to ENOA1,2. Interestingly, in a cohort of 77 advanced PDAC patients, at stage III and IV, who were evaluable for clinical outcome upon I-line gemcitabine-based chemotherapy, the presence of autoantibodies to ENOA1,2 and disease stage were the only independent predictors of disease control (complete or partial response and stable disease; p=0.017 and p=0.033, respectively) and longer progression-free survival (PFS) (p=0.002 for both variables, Table 3) at multivariate analysis. Moreover, when the analysis was restricted to patients who were able to mount a humoral immune response against any of the ENOA isoforms (n=51), ENOA1,2^{+} patients had a highly significant reduction in the risk of progression or death as compared to patients with autoantibodies

directed only against ENOA3,4,5,6 isoforms (p=0.0001 and p=0.004 for PFS and overall survival (OS), respectively), further supporting the clinical relevance of an autoantibody response to ENOA1,2 (Figure 6).

Discussion

The dismal prognosis and late presentation of PDAC in most patients emphasizes the need to improve the early detection of PDAC. To this purpose, starting from the observation that the expression of ENOA is up-regulated in PDAC (at mRNA and protein levels; http://www.genecards.org/index.shtml) [5-8, 19] compared with normal pancreatic tissues, we characterized ENOA expression in PDAC and the relative autoantibody response in PDAC patients' sera. As a result of these studies, we demonstrate that two out of six ENOA isoforms (ENOA1,2) detectable by 2-DE WB are preferentially expressed in PDAC and that such isoforms are phosphorylated on serine 419. Although it is known that ENOA is phosphorylated in Ser/Thr/Tyr residues,[20-24] this is the first evidence that in PDAC cells ENOA is phosphorylated in serine 419. The fact that the same phosphorylated peptide containing serine 419 was found in both ENO1,2 isoforms suggests that additional differential post-transcriptional modifications (*e.g.* acetylation, methylation) are responsible for their different isoelectric point. Most importantly, here we provide evidence that ENOA1,2 isoforms induce an *in vivo* humoral response in a substantial proportion of PDAC patients (62%). Such reactivity is presumably directed against the phosphorylated serine 419 residue and is specifically associated to PDAC, in that it is only sporadically observed in patients with inflammatory pancreatic diseases (CP), autoimmunity, and patients with non-PDAC malignancies. This would imply that the aberrant ENOA phosphorylation observed in PDAC may induce the overcoming of immune tolerance against this enzyme and trigger the antibody response.[25] Autoantibodies to ENOA3,4,5,6 isoforms have indeed been found in the serum of

patients with lung carcinoma,[26-28] acute leukemia,[29] and multiple sclerosis,[30] as well as in a small proportion of HS (this paper). Conversely, we found that autoantibody production against ENOA1,2 was almost exclusively observed in PDAC patient sera.

Of clinical relevance, ENOA1,2 autoantibodies are significantly more frequent in patients with normal CA19.9 levels, potentially complementing the performance of the most widespread serological test currently employed in the diagnostic workup of both cystic and solid pancreatic masses. CA19.9 immunoassay achieves high sensitivity and specificity only in the presence of a considerable tumor burden and when high cut-offs are used.[4] Moreover, it may be elevated in non-malignant conditions, such as acute and chronic pancreatitis, as well as hepatitis and biliary obstruction,[3] and in other gastrointestinal and non-gastrointestinal cancers. Here we demonstrate that the combination of CA19.9 and anti-ENOA autoantibodies yields a diagnostic accuracy of approximately 95% not only in advanced PDAC patients undergoing palliative treatment, but also in patients who may be amenable to surgery with curative intent. In resectable PDAC, where the rate of abnormal CA19.9 serum level falls considerably,[31] the simultaneous detection of autoantibodies to phosphorylated ENOA1,2 could thus provide a more finely-tuned diagnostic tool and warrants further confirmation in larger surgical series as well as a potential screening method for high risk populations.[32] By 2-DE approach we provide the proof-of-concept that phosphorylated ENOA at serine 419 is specifically recognized by autoantibodies in PDAC patients. Development of a handy assay based on the capture of autoantibodies to phosphorylated serine 419 residue will allow to validate the diagnostic potential in a large cohort of patients.

Finally, ENOA1,2^{+} patients exhibit a more favorable clinical course with a significantly lower proportion of disease progression and a consequently longer PFS upon gemcitabine treatment. Interestingly, this advantage seems to be restricted to patients who are able to mount a humoral immune response specifically directed against the phosphorylated isoforms ENOA1,2. This is clinically relevant in a disease, such as advanced PDAC, where only performance status and disease

stage have proved to reliably predict clinical outcome.[33] The mechanism involved in the production of autoantibodies to ENOA in PDAC patients is presently unclear. The combined effect of increased anaerobic metabolism and gemcitabine treatment enhances ENOA expression and induces resistance to apoptosis.[34] This, in turn, may stimulate the overcoming of immune tolerance to ENOA and trigger specific antibody production. Most interestingly, from a clinical perspective, ENOA is also expressed on the surface of many cell types including PDAC where it acts as a plasminogen receptor.[5, 14, 35] It is therefore a suitable target for anti-ENOA1,2 IgG antibodies, which might, in turn, efficiently trigger antibody or complement-dependent cytotoxicity. In addition, as cancer progression is often associated with the ability to escape immune responses,[36] the association of autoantibody production with a favorable clinical course may reflect a more efficient immune response in ENOA1,2$^+$ PDAC patients. This hypothesis is supported by the observation that the peripheral blood of ENOA1,2$^+$ PDAC patients contains ENOA-specific CD4$^+$ and CD8$^+$ T cells.[5] In this context, the presence of autoantibodies to ENOA1,2 in PDAC patients might directly reflect the activation of anti-ENOA CD4$^+$ T helper cells.

In conclusion, this study indicates that serine 419-phosphorylated ENOA1,2 isoforms and the autoantibody response against them are specifically associated with PDAC and appear to influence its clinical course. Further investigations are needed to validate PDAC sera reactivity against phosphorylated ENOA1,2 in a large-scale study, as well as to fully evaluate their clinical usefulness.

Conflicts of interests

FN, BT and PC are inventors of a PCT application n°: WO/2008/03779 entitled “Novel antigens and antibodies associated to pancreatic ductal adenocarcinoma”. Potential investigator conflict of interest has been disclosed to study participants.

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Figure Legends

Figure 1. Expression analysis of ENOA isoforms. (A) The protein extracts from PDAC (n=12) and normal tissues (NP, n=6) were separated by 2-DE, electroblotted onto nitrocellulose membranes and probed with anti-α-enolase mAb and with anti-actin polyclonal antibody as a loading control. 2-DE WB indicates the presence of six ENOA isoforms on cropped images from 2-DE gels. The arrows indicate the ENOA1,2 isoforms position. Quantisation of ENOA isoform intensities on 2-DE WB are measured as arbitrary units (AU) and normalised on the total actin

intensity. The fold between PDAC and NP are indicated at the bottom. (B) 2-DE WB of the ENOA isoform expression in CF-PAC-1, MiaPaCa-2, BxPC-3 and Saos-2 cell lines.

Figure 2. Sera reactivity against ENOA1,2. Colloidal Blue Coomassie stained image of 2-DE as compared with representative reactivity of sera from PDAC and HS by 2-DE WB on CF-PAC-1 cell line. Numbered arrows indicate the ENOA1,2 isoforms. To establish the serum positivity to ENOA1,2 isoforms the volume of corresponding spots were calculated in each 2-DE WB. When the spot volume with the same pI and MW of that recognized by the anti-ENOA mAb was equal to the background, the sample was considered ENOA1,2 negative. For the ENOA1,2 positive sera (n= 74) the range of volume of ENOA1 recognized by autoantibodies varied from 0.4 x 10^6 to 38 x 10^6 arbitrary units (AU) whereas that of ENOA2 varied from 1 x 10^6 to 30 x 10^6 AU.

Figure 3. Analysis of sera reactivity to phosphorylated ENOA1,2. ENOA isoforms from CF-PAC-1 cell line were separated by 2-DE and transferred onto nitrocellulose membrane. Representative reactivity of a pool of three ENOA1,2$^+$ PDAC sera assessed alone on untreated membrane, alone on membrane treated "on blot" with λPPase or mixed with rENOA on untreated membrane is shown in upper panels. The reactivity of anti-α-enolase mAb is present at the bottom of each panel. Graph shows the quantisation analysis of ENOA isoforms probed by ENOA1,2$^+$ sera. The relative intensities were normalised on the total ENOA intensity and expressed as arbitrary units (AU). The results are presented as means of three experiments ± SD.

Figure 4. CID spectrum of the doubly-charged phosphopeptide ^{413}IEEELGpSKAK^{422} from ENOA1,2 (accession number 4503571). The spectrum (upper panel) was labeled to show singly-

charged b and y ions, as well as ions corresponding to neutral losses of the phosphate group and water. The lower panel is the table of the fragment assignments of this phosphopeptides, in which the matched b ions are colored with red, the matched y ions are colored with blue, the matched a ions are colored with green, the matched z ions are colored with purple, the matched b ions with neutral loss of NH_3 or H_2O are colored with yellow, and the matched y ions with neutral loss of NH_3 or H_2O are colored with magenta.

Figure 5. ROC analysis of sensitivity and specificity of combined CA19.9 (≥ 37 IU/mL) and/or ENOA1,2 positivity. Curves reflect individual patient's probability of having PDAC calculated by a multivariate logistic regression model taking into account both CA19.9 serum levels and autoantibodies to ENOA1,2 in a set of advanced (n=61) and resectable (n=37) PDAC patients relative to HS+CP controls (n=63).

Figure 6. Survival analysis in advanced PDAC patients undergoing chemotherapy according to anti-ENOA reactivity. Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) relative to ENOA1,2$^+$ (thick line) and ENOA3,4,5,6-only positive (dotted line) PDAC patients undergoing first-line palliative chemotherapy. Log-rank p=0.0001 and 0.004 for PFS and OS, respectively.

Table 1. Clinical features of 120 PDAC patients

Characteristics			**N**	***%*** [a]
Gender				
	Male		67	56
	Female		53	44
Age (years)				
	Mean	67	-	-
	Range	32-86	-	-
Stage [b]				
	IIA		4	3
	IIB		21	17
	III		27	22
	IV		68	57
Grading				
	Not reported		64	53
	1		3	2
	2		32	27
	3		21	17
Primary Site				
	Not reported		21	17
	Head		60	50
	Isthmus		8	7
	Body		12	10
	Tail		9	7
	Body-Tail		10	8
Metastatic sites [c]				
	Abdominal Wall		1	-
	Bone		3	-

	Liver		57	-
	Lung		8	-
	Lymph Nodes		15	-
	Peritoneum		9	-
ECOG PS				
	Not reported		38	32
	0		34	28
	1		42	35
	≥2		6	5
Surgery with Radical Intent				
	Yes		38[d]	32
	No		82	68
Surgical Margin Status				
	R0		29	76[e]
	R1		9	24
Baseline CA19.9 (IU/ml)				
	Evaluable		98	82
	Mean	3069	-	-
	Median	511	-	-
	Range	2-26818	-	-
First-Line Chemotherapy[f]			79	-
	Evaluable		77	97[g]
	Gem		60	76
	Gem/Oxal		11	14
	Gem/5-FU		4	5
	Non-Gem		4	5

ECOG PS, eastern cooperative oncology group performance status; 5-FU, 5-fluorouracil; Gem, gemcitabine; Oxal, oxaliplatin.

[a]Percentages may not add up to 100 due to rounding.

[b]Classified according to the TMN classification of malignant tumor of the pancreas (UICC).[37]

[c]Twenty-two patients had multiple metastatic sites.

[d]Serum collection for anti-ENOA autoantibodies: analysis was performed at the time of surgery in 14 patients and at the time of relapse in 24 patients.

[e]Percentages are calculated on the total of thirty-eight patients undergoing surgery with radical intent.

[f]First-line chemotherapy refers to palliative chemotherapy administered for relapsed, locally advanced inoperable, or metastatic disease.

[g]Percentages are calculated on the total of 79 patients undergoing I-line chemotherapy.

Table 2. Frequencies of sera reactivity against ENOA1,2

Serum	Number of subjects	ENOA1,2 positive sera (%)	p Value*
PDAC	120	74 (62%)	-
HS	40	0 (0%)	P=0.0001
Non-PDAC	50	2 (4%)	P=0.0001
CP	46	4 (9%)	P=0.0001
AD	12	0 (0%)	P=0.0181

*Two-tailed p values are referred to PDAC and are determined by Mantel-Haenszel χ^2 test.

Table 3. Cox regression analysis of outcome-predicting factors

		Multivariate	
		HR (95% CI)	**p Value**
		DCR (logistic regression)	
Age (years)	<67 *vs* >67	2.55 (0.92-7.03)	0.071
Disease stage	II-III *vs* IV	3.86 (1.12-13.3)	0.033
Anti-ENOA1,2	Pos *vs* Neg	3.59 (1.26-10.2)	0.017
		PFS (Cox regression)	
Age (years)	>67 *vs* <67	1.61 (0.99-2.62)	0.051
Disease stage	IV *vs* II-III	2.77 (1.46-5.25)	0.002
Anti-ENOA1,2	Neg *vs* Pos	2.20 (1.34-3.62)	0.002
		OS (Cox regression)	
Disease stage	IV *vs* II-III	3.15 (1.61-6.14)	0.001
Surgery	No *vs* Yes	3.14 (1.58-6.24)	0.001
ECOG PS	1/2/3 *vs* 0	2.11 (1.22-3.63)	0.007
Anti-ENOA1,2	Neg *vs* Pos	1.56 (0.93-2.64)	0.095

CI, confidence interval; DCR, disease control rate; ECOG PS, eastern cooperative oncology group performance status; HR, hazards ratio.