α-enolase: a promising therapeutic and diagnostic tumor target

α-ENOLASE: A PROMISING THERAPEUTICAL AND DIAGNOSTIC TUMOR TARGET

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ABSTRACT

α-enolase is a metabolic enzyme involved in the synthesis of pyruvate. It also acts as a plasminogen receptor and thus mediates activation of plasmin and extracellular matrix degradation. In tumor cells α-enolase is up-regulated and supports anaerobic proliferation (Warburg effect), it is expressed at the cell surface, where it promotes cancer invasion, and is subjected to a specific array of post-translational modifications, namely acetylation, methylation and phosphorylation. Both α-enolase overexpression and its post-translational modifications could be of diagnostic and prognostic value in cancer. This review will discuss recent information on the biochemical, proteomics and immunological characterization of α-enolase, particularly its ability to trigger a specific humoral and cellular immune response. In our opinion, this information can pave the way for effective new therapeutic and diagnostic strategies to counteract the growth of the most aggressive human disease.

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INTRODUCTION

Enolase is a metalloenzyme that catalyzes the dehydration of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) in the second half of the glycolytic pathway. In the reverse reaction (anabolic pathway) that occurs during gluconeogenesis, the enzyme catalyzes the hydration of PEP to PGA [1, 2]. Enolase is found from archaebacteria to mammals, and its sequence is highly conserved [3]. In mammals, three genes, *ENO1*, *ENO2* and *ENO3* encode for three isoforms of the enzyme: α-enolase (ENOA), γ-enolase (ENOG) and β-enolase (ENOB) respectively, with high sequence identity [4-6]. The expression of these isoforms is tissue-specific: α-enolase is present in almost all adult tissues, β-enolase is expressed in muscle tissues and γ-enolase is found in neurons and neuroendocrine tissues [1, 7-9]. The monomer of ENOA consists of a smaller N-terminal domain (residues 1-133) and a larger C-terminal domain (residues 141-431). In eukarya, enzymatically active enolase consists of a dimeric form in which two subunits face each other in an antiparallel manner [1, 10]; some eubacterial enolases, by contrast, are octameric [11]. Enolase can form homo- or heterodimers such as αα, αβ, ββ, αγ and γγ [1].

Besides its enzymatic activity, in many prokaryotic and eukaryotic cells, ENOA is expressed on the cell surface where it acts as a plasminogen receptor promoting cell migration and cancer metastasis [12-23]. Moreover, *ENO1* can be translated into a 37-kDa protein, c-myc promoter-binding protein (MBP-1), by using an alternative start codon [24]. MBP-1 lacks the first 96 residues of ENOA and localizes in the nucleus where it binds to the *c-myc* P2 promoter and acts as a transcription repressor, leading to tumor suppression [25-27]. ENOA associates with MBP-1 in the transcriptional regulation of the oncogene *c-myc* [28].

ENOA IS A SURFACE PLASMINOGEN-BINDING RECEPTOR IN TUMORS

In breast, lung and pancreatic neoplasia, ENOA is localized on the surface of cancer cells [29-31], while in melanoma and non-small cell lung carcinoma (NSCLC) cells it can be also secreted by exosomes [32, 33]. How ENOA is displayed on the cell surface remains unknown. Many glycolytic enzymes and cytosolic proteins that lack N-terminal signal peptide reach the surface of eukaryotic cells [34]. In mammal cells, some export routes of unconventional protein secretion have been postulated: membrane blebbing, membrane flip-flop, endosomal recycling or a plasma membrane transporter [35]. One possibility is that phosphoinositides recruit ENOA and translocate it to the cell surface [36]. It is not known if surface ENOA is also present as a monomer. As the monomeric form is catalytically inefficient it could be available to interact with other proteins that mediate its transport to the cell surface [37]. However, in breast cancer cells, surface ENOA maintains its catalytic activity, suggesting that cell surface localization does not affect this function [31].

Cell-surface ENOA is one of the many plasminogen-binding molecules that include actin [38], gp330 [39], cytokeratin 8 [40], histidine-proline rich glycoprotein (HPRG) [41], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [42], annexin II [43], histone H2B [44] and gangliosides [14]. ENOA and most of these proteins have carboxyl terminal lysines predominantly responsible for plasminogen activation [45]. Interaction of the plasminogen lysine-binding sites with ENOA is dependent upon recognition of ENOA C-terminal lysines K420, K422 and K434 [14]. In view of the surface potential of the human ENOA crystal structure, an additional plasminogen binding site that includes K256 has been proposed [10].

Binding with ENOA lysyl residues leads to activation of plasminogen to plasmin by the proteolytic action of either tissue-type (tPA) or urokinase-type (uPA) plasminogen activators [19, 46]. Plasmin is a serine-protease with a broad spectrum

substrate, including fibrin, extracellular matrix (ECM) components (laminin, fibronectin) and proteins involved in ECM degradation (matrix metalloproteinases, such as MMP3) [47-50]. Binding of plasminogen to the cell surface has profibrinolytic consequences: enhancement of plasminogen activation, protection of plasmin from its inhibitor α_2-antiplasmin, and enhancement of the proteolytic activity of cell-bound plasmin [13, 51]. Proteolysis mediated by cell-associated plasmin contributes to both physiological processes, such as tissue remodelling and embryogenesis, and to pathophysiological processes, such as cell invasion, metastasis and inflammatory response [19, 45]. A noteworthy positive correlation exists between elevated levels of plasminogen activation and malignancy [46, 52]. Higher expression levels of uPA and/or plasminogen activator inhibitor-1 (PAI-1) in tumor tissues correlate with aggressiveness and poor prognosis. ENOA takes part, together with uPAR (urokinase plasminogen activator receptor), integrins and some cytoskeletal proteins, in a multiprotein complex, called metastasome, responsible for adhesion, migration and proliferation in ovarian cancer cells [53]. In human follicular thyroid carcinoma cells, retinoic acid causes a decrease in ENOA levels that coincides with their reduced motility [54], and cell-surface ENOA is enhanced in breast cancer cells rendered superinvasive following paclitaxel treatment [55].

In pancreatic cancer patients, deregulated expression of many proteins involved in the plasminogen pro-fibrinolytic cascade (annexin A2, PAI-2, uPA, uPAR, MMP-1 and MMP-10) correlates with survival [56-59]. In the same tumor, tPA activates a mitogenic signal mediated by ERK-1/2 through the EGFR and annexin A2 [60, 61]. These proteins probably form a complex that also includes ENOA, as it has been pulled down with annexin A2, cytokeratin 8 and tPA in raft membrane fractions of pancreatic cancer cells [62].

ENOA IS A TUMOR-ASSOCIATED ANTIGEN

Tumor-associated antigens (TAAs) are self proteins that can trigger multiple specific immune responses in the autologous host [63]. Activation of the immune system against TAAs occurs at an early stage of tumorigenesis, as illustrated by the detection of high titers of autoantibodies in patients with early-stage cancer [64], and correlates with the progression of malignant transformation [65]. It is not entirely clear how TAAs are able to trigger humoral responses, especially as many of those discovered so far are intracellular proteins, but are thought to be altered in a way that renders them proteins immunogenic [66, 67]. Several hypotheses have been proposed: these self-proteins could be overexpressed, mutated, misfolded, aberrantly degraded or localized so that autoreactive immune responses in cancer patients are induced [65, 68, 69]. Moreover TAAs that have undergone post-translational modifications (PTMs) (e.g. glycosylation, phosphorylation, acetylation, oxidation and proteolytic cleavage) may be perceived as foreign by the immune system [66-68]. The immune response against such immunogenic epitopes of TAAs induces the production of autoantibodies as serological biomarkers for cancers [70].

Both its overexpression in tumors and its ability to induce a humoral and/or cellular immune response in cancer patients classify ENOA as a true TAA.

ENOA expression is increased in tumors

The overexpression of ENOA is associated with tumor development through a process known as aerobic glycolysis or the Warburg effect [71]. Warburg observed that cancer cells consume more glucose than normal cells and generate ATP by converting pyruvate to lactic acid, even in the presence of a normal oxygen supply [72]. The mechanism of the Warburg effect was uncertain until the recent identification of up-regulation of glycolytic enzymes by hypoxia-inducible factor

(HIF). When a solid tumor exceeds 1 mm^3, its cells face hypoxic stress due to slow angiogenesis [73, 74]. Because the *ENO1* promoter contains a Hypoxia Responsive Element (HRE) [75, 76], ENOA is up-regulated at the mRNA and/or protein level in several tumors, including brain [77], breast [78-83], cervix [77, 84, 85], colon [77, 86, 87], eye [77], gastric [77, 88, 89], head and neck [90, 91], kidney [77], leukaemia [92], liver [77, 93, 94], lung [77, 95-99], muscle [77], ovary [77, 100], pancreas [29, 77, 101, 102], prostate [77, 103], skin [104] and testis [77] (Table 1). Results from a bioinformatic study support a correlation between ENOA expression and tumorigenicity [52, 77]. Moreover ENOA's enzymatic activity may also be increased in breast tumor tissue, especially in metastatic sites [82, 83]. Increased ENOA expression can influence chemotherapy treatments, as shown in estrogen receptor-positive breast tumors where it induces tamoxifen resistance [78], and in colorectal carcinoma cells where it is overexpressed after 5-fluorouracil administration [87].

ENOA post-translational modifications in tumors

PTMs are common mechanisms that control signal transduction, protein-protein interaction and translocation [105, 106].

Reversed-phase liquid chromatography, nanospray tandem mass spectrometry (LC-MS/MS) has been used to characterize ENOA PTMs in several cancer and normal cell lines (Table 2) (http://www.uniprot.org/uniprot/P06733) [107-115].

Acetylation, methylation and phosphorylation were the main PTMs (Table 2). Acetylation was found in cervix and colon cancer, leukaemia, normal pancreatic ducts and tumoral pancreatic cells. Fourteen acetylated lysine residues are common to leukemia, pancreatic cancer and normal pancreas, and one of them is the only acetylated residue in cervix tumor. Three acetylations are common to both leukemia and pancreatic cancer, whereas three are specific for normal and tumoral pancreatic

cells. However, six specific acetylated lysines were found in pancreatic cancer cells, and three in leukemia. The only acetylated serine identified is specific for colon cancer (Table 2).

Methylation has been assessed in normal and tumoral pancreas only. Twenty-four aspartate and glutamate residues were found in both cell types. However, five aspartates and five glutamates are specifically methylated only in pancreatic cancer (Table 2).

Phosphorylation is the PTM that displays the most specific pattern in each cell line. Two serine and one threonine residues were specifically found in cervix cancer, one threonine and one serine in embryonic kidney, three serines and two threonines in leukemia; while two tyrosine residues were found in both leukemia and lung cancer and one serine in both tumoral and normal pancreas.

ENOA in tumor cells is subjected to more acetylation, methylation and phoshorylation than in normal tissues, indicating that many PTMs are associated with cancer development and some are specific for each kind of tissue or cancer. This can reflect the specific activation of pro-mitogenic signalling pathways in tumor cells. In many cases PTMs regulate the stability and functions of proteins; for example, in metabolic enzymes, acetylation acts as a on/off switch mechanism [116], while methylation on carboxylate side chains enhances hydrophobicity by increasing the affinity of proteins for phospholipids [115]. We speculate that PTMs are important mechanisms in the regulation of ENOA functions, localization and immunogenicity.

ENOA induces a specific immune response in tumors

Several TAAs induce the production of IgG autoantibody in cancer patients via an integrated immune response triggered by $CD4^+$ T cells, $CD8^+$ T cells and B cells. TAAs released by secretion, shedding or tumor cell lysis are captured by Antigen

Presenting Cells (APCs), processed and presented by either MHC class I or MHC class II molecules for priming and activation of $CD8^+$ and $CD4^+$ T cells respectively. Uptake of antigen by B cells also occurs and is driven by membrane Ig, leading to MHC class II antigen presentation to $CD4^+$ T cells. Activated $CD4^+$ T cells, through the secretion of appropriate cytokines, trigger B cells to produce IgG against the same TAA [117], and $CD8^+$ T cells to differentiate into TAA-specific cytotoxic T lymphocytes (CTL). *In vivo* maintenance and survival of TAA specific CTL is also dependent on cytokines released by $CD4^+$ T cells [118]. This coordinated immune response suggests that IgGs against TAA are not only a diagnostic tool but also allow the selection of TAAs for cancer immunotheraphy.

In many cancer patients, including pancreatic [119], leukemia [120, 121], melanoma [104, 122], head and neck [123-125], breast [126] and lung [30, 96, 99, 127-130], ENOA has been shown to induce autoantibody production (Table 1). In pancreatic cancer patients, autoantibodies to ENOA are directed against two up-regulated isoforms phosphorylated in Ser-419 [119] (Table 2). Protein phosphorylation increases the affinity of peptides for MHC molecules that can be recognized by T cells [131].

In pancreatic cancer, ENOA elicits a $CD4^+$ and $CD8^+$ T cell response both *in vitro* and *in vivo* [29]. In pancreatic ductal adenocarcinoma patients production of anti-ENOA IgG is correlated with the ability of T cells to be activated in response to the protein [29], thus confirming the induction of a T and B cell integrated antitumor activation against ENOA. In oral squamous cell carcinoma, an MHC class II-restricted peptide of human ENOA recognized by $CD4^+$ T cell and able to confer cytotoxic susceptibility has been identified [132].

Clinical correlations

The diagnostic and prognostic value of ENOA expression and production of autoantibodies to it has been illustrated in several tumors (Table 1). In breast cancer, enhanced ENOA expression is correlated with greater tumor size, poor nodal status and shorter disease-free interval [78]. In head and neck and non-small cell lung cancer, patients with high ENOA expression had significantly poorer clinical outcomes than low expressers, including shorter overall- and progression-free survival [99, 123]. In hepatocellular cancer, expression of ENOA increased with tumor de-differentiation and correlated positively with venous invasion [93, 94]. In pancreatic cancer, detection of autoantibodies against Ser-419 phosphorylated ENOA usefully complemented the diagnostic performance of serum CA19.9 levels up to 95%. The presence of this humoral response was also correlated with a longer progression-free survival upon gemcitabine treatment and overall survival, supporting the clinical significance of phosphorylated ENOA autoantibodies [119]. The concept that autoantibody levels can also function as markers for the diagnosis and prognosis of cancers has been extensively pursued [69, 133].

CONCLUSIONS

Taken as a whole, these findings illustrate the multifunctional properties of ENOA in tumorigenesis, and its key implications in cancer proliferation, invasion and immune response. In cancer cells, ENOA is over-expressed and localizes on their surface. where it acts as a key protein in tumor metastasis, promoting cellular metabolism in anaerobic conditions and driving tumor invasion through plasminogen activation and extracellular matrix degradation, and also displays a characteristic pattern of PTMs, namely acetylation, methylation and phosphorylation, that regulate protein functions and immunogenicity. In several kinds of tumors, patients develop an integrated

response of $CD4^+$, $CD8^+$ T cells and B cells against ENOA, along with anti-ENOA autoantibodies in their sera. Clinical correlations propose ENOA as a novel target for cancer immunotherapy. In pancreatic cancer, for example, the pancreas- specific Ser-419 phosphorylated ENOA is up-regulated and induces the production of autoantibodies with diagnostic and prognostic value (Figure 1).

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Figure Legend

Figure 1. Production of autoantibodies to phosphorylated-ENOA in pancreatic cancer. ENOA is overexpressed in tumor cells compared to normal tissues and it is present on the surface of different cell types where it acts as a plasminogen receptor. ENOA is phosphorylated on Ser-419 only in pancreatic tissues, the overexpression of this post-translationally modified ENOA in tumor condition induces the production of autoantibodies with clinical relevance in pancreatic cancer patients.

Table 1. Expression of ENOA, immune response to it and clinical correlations in cancer. m: mRNA; p: protein; e: enzymatic activity; Ab: antibody production; T: T cell response; DFI: Disease-Free Interval; M: Malignancy; OS: Overall Survival; PFS: Progression-Free Survival.

Cancer	ENOA enhanced expression	Immune response to ENOA	Clinical correlations
Brain	m [77]		
Breast	m, p, e [78-83]	Ab [126]	DFI, M [78]
Cervix	m, p [77, 84, 85]		
Colon	m, p [77, 86, 87]		
Eye	m [77]		
Gastric	m, p [77, 88, 89]		
Head and neck	p [90, 91]	Ab [123-125], T [132]	OS, PFS [123]
Kidney	m [77]		
Leukemia	p [92]	Ab [120, 121]	
Liver	m, p [77, 93, 94]		M [93, 94]
Lung	m, p [77, 95-99]	Ab [30, 96, 99, 127-130]	OS, PFS [99]
Muscle	m [77]		
Ovary	m, p [77, 100]		
Pancreas	m, p [29, 77, 101, 102]	Ab [119], T [29]	OS, PFS [119]
Prostate	m, p [77, 103]		
Skin	m [104]	Ab [104, 122]	
Testis	m [77]		