# Estrogen-regulated proteins cathepsin D and pS2 in breast carcinoma

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## ABSTRACT

*In addition to classical prognostic/predictive factors, significant biological markers have been identified to provide potentially relevant information regarding natural or clinical course of breast cancer. Steroid receptor status of the primary breast cancer have been proven to be a predictor of response to endocrine therapy since up to 80 % of patients with steroid receptor-positive tumors respond to endocrine treatment. In order to improve the predictive value of steroid receptor status, attention has been paid to estrogen-regulated proteins, including pS2 and cathepsin D among others that may be indicators of a functional signal transduction pathway through which tumor cells respond to estrogen stimulation. It has been shown that pS2 protein may be constitutive product as well as estrogen-regulated product in breast carcinoma. pS2 appears to be positively correlated with ER, associated with a good prognosis and a predictor of response to endocrine treatment of primary and metastatic breast cancer. The expression cathepsin D may be both constitutive and overexpressed as a result of estrogen-induced transcription. It was believed that the main role of cathepsin D was to degrade protein, but many other biological functions of cathepsin D were recognized. Cathepsin D level in primary breast cancer has been demonstrated as an independent marker of poor prognosis associated with increased risk for metastasis and shorter survival times. Our recent results show direct correlation of cathepsin D positivity with pS2 expression. Additionally, we found that cathepsin D is statistically significantly associated with pS2 both in node-negative and node-positive patients bearing tumors smaller than 2 cm.*

**KEY WORDS:** *Breast Neoplasms; Carcinoma; Cathepsin D; Receptors, Estrogen; Neoplasm Proteins; Tumor Markers, Biological*

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## INTRODUCTION

Breast cancer represents the most frequent female malignant tumor with more than a million new cases diagnosed in 2002, in the entire world (1). The increase of incidence that is evident may be partially explained with improved diagnosis, at earlier stage of disease. However, the other factors such as genetics and environment must be taken into account to play an important role. In addition to classical prognostic factors, such as age, tumor size, axillary node status, histological tumor grade and type, significant biological factors, termed prognostic and/or predictive markers, have been identified that have shown to provide potentially relevant information regarding natural or clinical course of disease. Since the hormonal treatment is much better tolerated by the breast cancer patients than chemotherapy, an important goal in the treatment of these patients is to determine the expression of relevant prognostic markers identifying those tumors that will most likely respond in a favorable manner to anti-hormonal treatment. One such prognostic/predictive marker is cellular estrogen receptor, the molecule responsible for specifically binding, concentrating and retaining of estrogen in target cells. It is well documented that 50 % to 80 % of breast cancer patients are estrogen receptor positive (2). At present, expression of estrogen receptor represents the most significant factor related to results of anti-hormonal treatment and, due to its presence, approximately 60 % of breast cancer patients respond favorable to tamoxifen or other forms of endocrine therapies (3). But, knowledge of estrogen receptor status is not sufficient to

accurately predict response to endocrine therapy in a significant number of patients. In order to improve the predictive value of estrogen receptor status, attention has been paid to estrogen-regulated proteins. It was supposed that estrogen-regulated proteins, including progesterone receptor, pS2 and cathepsin D among others, may be indicators of a functional signal transduction pathway through which tumor cells respond to estrogen stimulation. The measurement of the expression of progesterone receptor is usually on the premise that this steroid receptor is regulated by estrogen and will be expressed only in tissues in which the estrogen regulatory pathway, mediated by estrogen receptor, is intact. As demonstrated by clinical studies, patients with slower course of disease and more sensitive to tamoxifen as anti-estrogen treatment were characterized by coexpression of estrogen and progesterone receptors (4). Along with the presence of estrogen and progesterone receptors, pS2 protein is considered to reflect the intactness of regulatory mechanism by which the tumor cells are able to respond to estrogen stimulation.

## pS2 GENE/PROTEIN

pS2 gene was discovered during the search for estrogen-regulated genes in MCF-7 breast cancer cell lines derived from human breast cancer. The pS2 gene is located on chromosome 21q (5). It comprises three exons of 125, 153 and 212 base pairs interrupted by two introns of 3.1 Kb (intron A) and 0.77 Kb (intron B) (5). The gene encode an 84 amino-acid

precursor protein of 9.14 kDa that is cleaved, after processing, to a 6.4 kDa polypeptide of 60 amino-acids secreted by MCF-7 breast cancer cells and many human breast cancer (5). pS2 gene was assumed to comprise two sites for initiation of transcription of which one is mainly used (6). The 5' flanking region of the pS2 gene contains a complex promoter/enhancer region responsive to epidermal growth factor (EGF), the c-Ha-ras oncoprotein, the c-jun protein as well as to estrogens (7). Extensive studies revealed specific sequences in 5' flanking region of the pS2 gene involved in regulating gene expression. In vitro binding studies support idea that, in the absence of hormone, unoccupied estrogen receptor is bound to the consensus estrogen-response element (ERE) half site that is protected (8). Binding of hormone or anti-hormone to estrogen receptor is accompanied by the changes in estrogen receptor conformation that probably results in the presentation of different functional estrogen receptor surfaces thus providing the basis for the recruitment of specific sets of transcription factors to the promoter. Differential occupation of the ERE may be influenced by the presence of tissue-specific accessory factors, the inaccessibility of protein binding sites due to promoter organization or the transient nature of protein-DNA interactions and may be involved in silencing, activation and maintenance of the pS2 gene expression.

A number of methods exist to measure pS2 and cathepsin D levels in breast carcinoma samples including: enzyme-linked immunoassay (ELISA), immunohistochemistry (IHC), radioimmunoassay (RIA), northern blot and reverse transcription-polymerase chain reaction (RT-PCR). It has been shown that the pS2 protein may be constitutive product as well as estrogen-regulated product in breast carcinoma. The pS2 protein is not highly expressed protein in normal breast tissue and clinical studies revealed that approximately 50 % of all breast carcinomas express pS2 (9). The physiological role of this cysteine-rich protein in breast tissue remains unclear to date. High levels of pS2 expression have been found in the mucosal linings of the stomach and intestines suggesting a possible protease-protective role (10). Owing to its structural similarities with insulin-like growth factors (IGF-1 and IGF-2), pS2 protein was suggested to be a growth factor (11).

### Association of pS2 expression with prognostic/predictive markers

Although several studies have failed to demonstrate correlation between expressions of pS2 and ER or PR (12,13), most studies have found positive association between expression of pS2 and ER and/or PR expression (14,15). It has been argued that estrogen-dependent tumors will express both progesterone receptor and pS2 thus making dual measurements redundant. However, several studies have indicated that the presence of pS2 protein results in differences between subsets of breast cancer that exhibit different responses to endocrine treatments and, therefore, may help in identifying those human breast cancers that will respond favorable to anti-hormonal therapy even in the absence of either estrogen receptor or progesterone receptor (16,17). The higher concentration of pS2 protein has been observed in premenopausal breast cancer patients (18). One study have demonstrated that elevated levels of pS2, determined by IHC, are typical for postmenopausal patients with tumors of lower grades, in whom no progression of the neoplastic process has developed (19) while several studies found no association between pS2 and neither menopausal status nor age (15,20). Initially, no relationship between pS2 expression and TNM status as well as histological grade was observed in most of the studies (19, Refs within 20). Recent studies reported equivocal associations between: a) pS2 and tumor size and histological grade (14); b) pS2 and tumor grade but not size (refs. within 14,19); c) pS2 and tumor size only (16); d) pS2 and lymph node status alone (15) or combined with tumor size (20). Numerous studies have investigated relationship between pS2 protein and other biological markers. Expression of pS2 was found inversely correlated with expression levels of EGF receptor (3), p53 (15), proliferating associated index MIB1 (15). On the other hand, positive correlations were observed between pS2 and expression of heat shock protein 27 (hsp27) and bcl-2 (15). One study failed to demonstrate their association (11), but strong correlation between pS2 and cathepsin D was demonstrated in several studies investigating these markers (20,21).

Our recent results show direct correlation of cathepsin D positivity (with the cut-off value of 39 pmol/mg) with pS2 expression, as presented in Figure 1. Additionally, we found that cathepsin D is statistically significantly associated with pS2 both in node-negative and node-positive patients bearing tumors smaller than 2 cm (pT1), as shown in Figures 2 and 3. These results are partially in accordance with findings of Marsigliante et al. (22) who reported significant correlation between pS2 and cathepsin D in the whole group of pT1 breast carcinomas as well as in node-positive but not node-negative pT1 breast carcinomas. Correlation between two markers may indicate a control of pS2 on cathepsin D expression in small tumors, i.e. pS2 and cathepsin D cooperation may be an early event in tumor development.
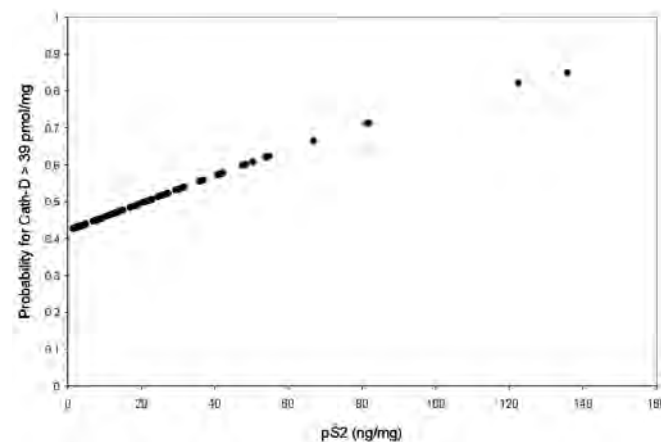


**Figure 1.** Direct correlation between probability for cathepsin D to be greater than 39 pmo/mg and pS2 levels in patients with breast carcinoma
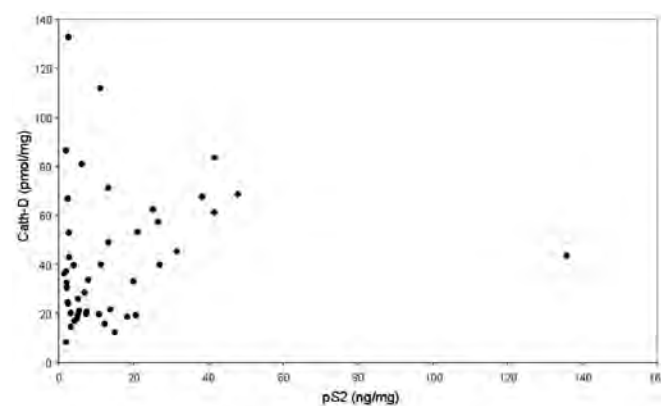


**Figure 2.** Correlation between cathepsin D and pS2 levels in node-negative patients bearing breast tumors smaller than 2 cm
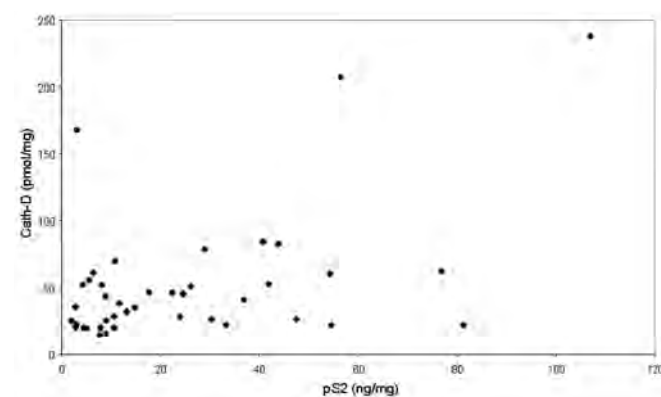


**Figure 3.** Correlation between cathepsin D and pS2 levels in node-positive patients bearing breast tumors smaller than 2 cm

Clinical evidence has indicated that pS2 appears to be associated with a good prognosis and predictive of response to endocrine therapy (23). There was a significant direct relationship between higher ER, PR and pS2 determined by IHC and increasing response to tamoxifen whereas in logistic regression model, only ER and pS2 retained significance for predicting tamoxifen response (24). However, most studies using IHC could not demonstrate correlation between pS2 status and disease-free interval (DFI) or overall survival (OS) (17,25). In opposition to IHC studies, the majority of cytosol assay-based studies have found a better outcome in terms of both DFI and OS in patients bearing pS2-positive tumors (19, Refs within 15).

## CATHEPSIN-D GENE/PROTEIN

Cathepsin D is an aspartic endoprotease that is ubiquitously distributed in all cells at low concentration (26). Cathepsin D gene promoter has a mixed structure with the general features of housekeeping genes (with multiple start sites, high G+C content and several potential Sp1-binding sites) and those of a hormone-regulated tissue-specific genes that include a TATA sequence (27). Transcription from the cathepsin D gene is started at five sites spanning 52 base pairs and mapping at -20, -44, -51, -60 and -72 base pairs from the first base of the initiation codon (27). Although cathepsin D gene is controlled by a mixed promoter, it was demonstrated that estrogens stimulate only TATA-dependent transcription in breast cancer cells (27) that is transcription only from site located downstream from TATA box at -20 base pairs (TATA-dependent), while transcription from other sites is TATA-independent (27). Several sites for binding of transcription factor have been recognized within the cathepsin D gene promoter that include potential site for estrogen receptor, activator protein 2 (AP2) and Simian virus-40 protein-1 (Sp1) (28). Experiments have demonstrated that the proximal 356 base pairs of the cathepsin D promoter are sufficient to produce a maximal induction of transcription (28).

A mechanism proposed to explain the processing and activation of cathepsin D combines partial autoactivation and enzyme-assisted activation yielding mature enzyme (Reviewed in 29). Synthesis of cathepsin D is initiated on the rough endoplasmic reticulum as a pre-pro-enzyme. Following the co-translational removal of the signal peptide, a 52 kDa pro-cathepsin D is glycosylated and transported to Golgi stacks. Pro-enzyme binds to mannose-6-phosphate (M6P) receptor and the complex is directed to lysosomes where its processing yields an active intermediate single-chain molecule of 48 kDa. In some cell types, targeting of cathepsin D to lysosomes is mediated independently of M6P receptors. Cleavage of intermediate enzyme in lysosomes produces a mature two-chain molecule comprising of a light (14 kDa) amino-terminal domain and a heavy (34 kDa) carboxyl-terminal domain. During the conversion from intermediate single-chain to mature two-chain enzyme, 7 amino-acid residues between light and heavy chains as well as several amino acids from carboxyl terminus of heavy chain are removed. In this way, cathepsin D protein contains tree distinct regions, typical for aspartic proteases: an N-terminal domain (residues 1-188), a C-terminal domain (residues 189-346) and an interdomain composed of the N-terminus (residues 1-7), the C-terminus (residues 330-346) and the interdomain-linking residues (160-200) (30). The latter region links pseudo-twofold-related N-terminal and C-terminal domains. Each of these terminal domains comprises one catalytic site, aspartic amino-acid residues 33 on a light and 231 on a heavy chain.

Since the cathepsin D gene is controlled by a mixed promoter, this gene has the advantage of being both constitutively expressed from TATA-independent start sites, possibly as a result of a constitutive production of autocrine or paracrine factors responsible for the induction of protease in estrogen receptor-negative breast cancer, and overexpressed, in some physiological or pathological conditions, as a consequence of transcription stimulation by estrogens and by some growth factors, such as EGF, IGF-1, in estrogen receptor-positive breast cancer (27). Its overexpression was observed both in breast cancer cell lines and in human breast tumors, at the mRNA and protein level (31, 32). The mechanism of overexpression of cathepsin D gene does not appear to be associated with gene ampli-

fication or remodeling of chromatin structure. Cathepsin D mRNA overexpression, induced by estrogen, is considered mainly as a result of increased initiation of transcription (27). Different methods that have been used in order to determine the overexpression of cathepsin D protein in breast cancer includes IHC, *in situ* hybridization, cytosol assay, northern and western blot analysis, and microdialysis. They revealed that in breast cancer tissue cells cathepsin D is overexpressed by a factor 2 up to 50 in comparison to its expression in other cell types or normal mammary gland cells (33).

The optimal pH for cathepsin D action, although acidic, varies according to the nature of the substrate. For the activity of cathepsin D *in vitro* an acidic pH is required with an optimum pH of 4.5 – 5.0 (34). Although, in contrast to other proteases, no endogenous cathepsin D tissue inhibitor is known in mammals, its activity is specifically inhibited by pepstatin, a natural inhibitor that binds to and blocks the active site of cathepsin D protein. Previously, it was considered that its normal physiological function was to degrade proteins in lysosomes at an acidic pH (35).

In metastasis, cathepsin D protein was supposed to facilitate the invasion of cancer cells by digestion of extracellular matrix and the basement membrane components (34). Several studies have suggested the role of cathepsin D in stimulating proliferation of cancer cells thus acting as a mitogen (36). Various mechanisms have been proposed to explain mitogenic effect of cathepsin D. It has been speculated that cathepsin D interacts with M6P receptor or its pro-fragment (amino-acid residues 27-44) interacts with an unknown cell surface receptor (37). Indeed, mutated cathepsin D, devoid of its catalytic activity, was demonstrated to be mitogenic both *in vitro* and *in vivo* which suggests that cathepsin D may act as an extracellular ligand protein by triggering directly or indirectly an as yet unidentified membrane receptor (38). On the other hand, catalytic activity of cathepsin D seems to be associated with activation of growth factors or prevention of growth inhibitors' secretion (39). Independently of its proteolytic activity, cathepsin D stimulates tumor angiogenesis as indicated by immunohistochemical studies (36). Contrary to other proteases, degradation of the extracellular matrix was not assumed to be the mechanism involved since catalytically-inactive cathepsin D mutant was as potent as wild-type cathepsin D for inducing angiogenesis. Release of bFGF that is bound to extracellular matrix in breast cancer cells following cathepsin D action may be a possible explanation (39). Besides, cathepsin D may stimulate the growth of endothelial cells via paracrine loop acting as a extracellular binding protein triggering an as yet unidentified cell surface receptor that could be present on endothelial cells as well as on epithelial cancer cells (29). Interaction between stromal and epithelial cancer cells appears to be important for both normal development and neoplasia. Stromal and tumor cells interchange growth factors and proteases in order to activate adjacent extracellular matrix and in turn induce selection and expansion of neoplastic cells (40). Overexpressed cathepsin D may be captured by stromal cells resulting, independently of proteolytic activity of cathepsin D, in a significant increase of proliferation, motility and invasive capacity of fibroblasts via paracrine loop by triggering an unknown membrane receptor that in turn may increase indirectly the levels of other proteases involved in the degradation of extracellular matrix (29). Hypersecreted cathepsin D might act either as a protease after its extracellular activation at an acidic pH or as a binding protein and its action could take place extracellularly or intracellularly. Degradation of the basement membrane after secretion and extracellular activation is unlikely to be a major mechanism for cathepsin D action since its activation requires an acidic pH which is found intracellularly. Results strongly favor the hypothesis of cathepsin D acting as a secreted ligand protein possibly due to interaction with an unknown membrane receptor. Alternative mechanism for mitogenic effect of cathepsin D on cancer cells involves binding to M6P receptor. The role of cathepsin D in apoptosis is controversial and depends upon environmental conditions. It has been assumed that in such physiological conditions as cancer, cathepsin D is capable to inhibit apoptosis (41) while it was recognized as a key positive promoter (mediator) of apoptosis induced by apoptotic agents (42).

### Association of cathepsin D with prognostic/predictive markers

Clinical studies have revealed that overexpression of cathepsin D in primary breast cancer is an independent prognostic parameter correlated with the incidence of clinical metastases and shorter survival times thus confirming it as a marker of aggressiveness (43). There have been controversies regarding clinical importance of this protease, mostly based on mixing results of well standardized methodology with various studies using non-validated or non-quantified methods. Tumor cathepsin D expression showed a marginal relationship with ER status while stromal cathepsin D was inversely correlated with ER status and positively correlated with histological grade (15). The importance of measurement of cathepsin D, and generally proteins, in all compartments where they are biologically active is therefore reasonable to suggest. As revealed by a large study of Foekens et al. (43), cathepsin D expression was not associated with tumor grade but was correlated with ER, PR, menopausal, node status as well as age and tumor size. Although weak, these correlations were statistically significant because of the size of the study. Some of these associations were confirmed by other investigators reporting that cathepsin D levels were considerably higher in large tumors (pT2-4) than in smaller ones (pT1) as well as in node-positive than in node-negative breast tumors (44). In addition, no association between axillary lymph node metastasis and cathepsin D positivity was reported (45). Several studies have indicated that increased levels of cathepsin D in node-negative breast cancer patients were able to predict a shorter DFI and OS, independently of the classical prognostic parameters such as steroid receptors' status, tumor size and histological grade (43, 46) whereas Ferno et al. (47) reported prognostic importance of cathepsin D only for node-positive breast cancer patients. On the other hand, it was reported that cathepsin D positivity showed no significant correlation with DFI and OS when determined by IHC (45).

Expression of pS2 protein has been found to be a good indicator of the response of breast cancer patients to endocrine treatment. In combination with estrogen and progesterone receptors' levels, the presence of pS2 protein was shown to define a group of breast cancer patients with a very good overall prognosis.

Cathepsin D level in primary breast cancer has been demonstrated as an independent marker of poor prognosis associated with increased risk for metastasis and shorter survival times.

It should be emphasized that apparently contradictory results regarding pS2 and cathepsin D prognostic/predictive value, obtained by various methods with different endpoints measured (mRNA or protein level) and based on different cut-off values, complicate direct comparisons between studies and may, partially, explain the reluctance to fully use pS2 and cathepsin D expression levels as prognostic/predictive markers in clinical settings.

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