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Nidogen-2: A new serum biomarker for ovarian cancer

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Abstract

Objectives: New ovarian cancer biomarkers suitable for early disease diagnosis, prognosis or monitoring could improve patient management and outcomes.

Design and Methods: Nidogen-2 was measured by immunoassay in serum of 100 healthy women, 100 women with benign gynecological conditions and 100 women with ovarian carcinoma.

Results: Serum nidogen-2 concentration between normal and benign disease patients was not different (median, 13.2 and 12.1 mg/L, respectively). However, nidogen-2 concentration in serum of ovarian cancer patients was elevated (median, 18.6 mg/L; $p<0.0001$). Both nidogen-2 and CA125 were elevated more in serous histotypes of ovarian cancer and late state disease. Nidogen-2 and CA125 concentrations were strongly correlated. ROC curve analysis for nidogen-2 had an area under the curve (AUC) ranging from 0.73 to 0.83 but CA125 was superior (AUC ranging from 0.87 to 0.99). There was no complementarity between the two markers.

Conclusions: Nidogen-2 is a new biomarker for ovarian cancer which correlates closely with CA125.
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Keywords: Ovarian cancer; Biomarker; Proteomics; Mass spectrometry; Nidogen-2; Basement membrane

Introduction

Ovarian cancer is the most lethal gynecological malignancy accounting for approximately 3% of all new cancer cases in 2008 [1]. Unfortunately, the majority of cases are presented at late stages where the 5-year survival rate is 25–40%. When presented at an early stage, the 5-year survival rate exceeds 90% and most patients are cured by surgery alone [2]. Currently, the best serum marker for ovarian cancer is carbohydrate antigen 125 (CA125), but its utility as a screening marker is limited due to its high false positive rates. CA125 could be elevated in other malignancies such as uterine, fallopian, colon and gastric cancer [3,4] as well as in non-malignant conditions such as pregnancy and endometriosis [5]. Thus, the need to identify new biomarkers with increased sensitivity and specificity for early diagnosis, prognosis or monitoring of ovarian cancer is crucial for optimal patient management.

We have previously performed an extensive proteomic analysis of ovarian cancer ascites, identifying over 450 proteins [6]. After applying a set of filtering criteria to reduce the number of potential biomarker candidates, we identified 52 proteins for which further clinical validation is warranted. Our proteomic approach for discovering novel ovarian cancer biomarkers [7] appears highly efficient since it was able to identify 25 known serum ovarian cancer biomarkers, according to literature searches. Of our 52 candidates, 18 of them had reagents available to develop an ELISA to measure the levels of these proteins in biological fluids. Through analysis of serum of healthy individuals, patients with ovarian cancer and patients with benign gynecological conditions, we were able to identify one promising candidate molecule: nidogen-2, a basement membrane protein.

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Basement membranes are thin extracellular sheets of protein matrix layers separating epithelial, endothelial, muscle and other cells from underlying connective tissue, thus serving as a major filtration barrier, maintaining proper tissue organization and compartmentalization [8]. In addition, the basement membrane controls a large number of cellular processes including adhesion, migration, differentiation, gene expression and apoptosis [9,10]. The major components of the basement membrane include collagen IV, laminins, heparan sulfate proteoglycan (perlecan) and nidogens and it is these proteins that allow for cell adhesion and the formation of networks to confer the mechanical stability of the basement membrane [11,12].

Among the components of the basement membrane, the nidogen family of two known basement membrane proteins has a major role in the supramolecular organization of the extracellular matrices. In humans, two nidogen proteins, nidogen-1 (150 kDa) and nidogen-2 (200 kDa), have been identified. The two proteins share a 46% primary sequence identity and a similar three-dimensional structure, consisting of three globular domains (G1, G2, G3) connected by a flexible link and a rod [13,14]. The nidogens bind and form a ternary complex with laminin-1 and collagen type IV, connecting the two networks and stabilizing and maintaining the structure of the basement membrane [13,15–17]. Both nidogens are co-expressed in various tissues and it has been proposed that they fulfill similar, if not identical functions and may also play a compensatory role [18,19].

Physiologically, nidogens have been shown to interact with cell receptor molecules and also control cell polarization, migration and invasion [20–23]. Through interactions with the leukocyte response integrin, nidogen favors neutrophil chemotaxis during inflammation. The interactions between cells and basement membranes regulate various cellular processes, including differentiation, proliferation and apoptosis.

In this study, we investigated the levels of nidogen-2 in serum of ovarian cancer patients and patients with benign gynecological conditions or normal controls. Elevation of nidogen-2 was identified in ovarian carcinoma serum samples, mostly associated with the serous histotype. Nidogen-2 expression correlates with levels of CA125. These data support the view that nidogen-2 is a new serological biomarker of ovarian carcinoma. Its clinical utility needs to be addressed in larger studies.

Methods

Patients and specimen

All patients in this study were of Japanese origin and were identified as part of a screening study in the region of Shizuoka, Hamamatsu, Japan, including 212 hospitals. All samples were

Table 1
Distribution of serum Nidogen-2, CA125 and age in normal, benign disease and ovarian cancer patients.

	Marker	Disease	*N*[a]	Min	Q1[b]	Median	Q3	Max	*p* Value[c]
	Age	Normal	94	25	39	52	66	88	<0.0001
		Benign	94	20	32	38	47	80	
		Cancer	94	33	50	58	66	82	
Normal vs. benign	CA125	Normal	100	6.0	10.2	12.3	17.2	44.7	0.32
		Benign	100	5.4	10.2	13.9	16.7	175.5	
	Nidogen-2	Normal	100	5.4	9.5	13.1	15.9	33.0	0.17
		Benign	100	4.4	9.5	12.1	14.4	27.3	
Benign vs. cancer	CA125	Benign	100	5.4	10.2	13.9	16.7	175.5	
		Cancer	100	7.2	100	351	1006	6490	<0.0001
		Early	57	7.2	60	171	675	6490	<0.0001
		Late	43	39	216	819	1590	3207	<0.0001
	Nidogen-2	Benign	100	4.4	9.5	12.1	14.4	27.3	
		Cancer	100	6.8	12.3	18.5	30.4	106.2	<0.0001
		Early	57	6.8	11.6	17.0	22.8	106.2	<0.0001
		Late	43	9.6	13.1	24.6	37.1	75.2	<0.0001
Normal vs. cancer	CA125	Normal	100	6.0	10.2	12.3	17.2	44.7	
		Cancer	100	7.2	100	351	1006	6490	<0.0001
		Early	57	7.2	60	171	675	6490	<0.0001
		Late	43	39	216	819	1590	3207	<0.0001
	Nidogen-2	Normal	100	5.47	9.5	13.1	15.9	33.0	
		Cancer	100	6.87	12.3	18.5	30.4	106	<0.0001
		Early	57	6.87	11.6	17.0	22.8	106	<0.0001
		Late	43	9.65	13.1	24.6	37.1	75	<0.0001
Early vs. late cancer[d]	CA125	Early	57	7.2	60	171	675	6490	<0.0001
		Late	43	39	216	819	1590	3207	
	Nidogen-2	Early	57	6.87	11.6	17	22.8	106.2	0.008
		Late	43	9.65	13.1	24.6	37.1	75.2	

[a] Number of samples.
[b] Quartile. All values are in KU/L for CA125 and μg/L for nidogen-2. Age is in years.
[c] Kruskal–Wallis test.
[d] Early = Stages 1–2; Late = Stages 3-4.

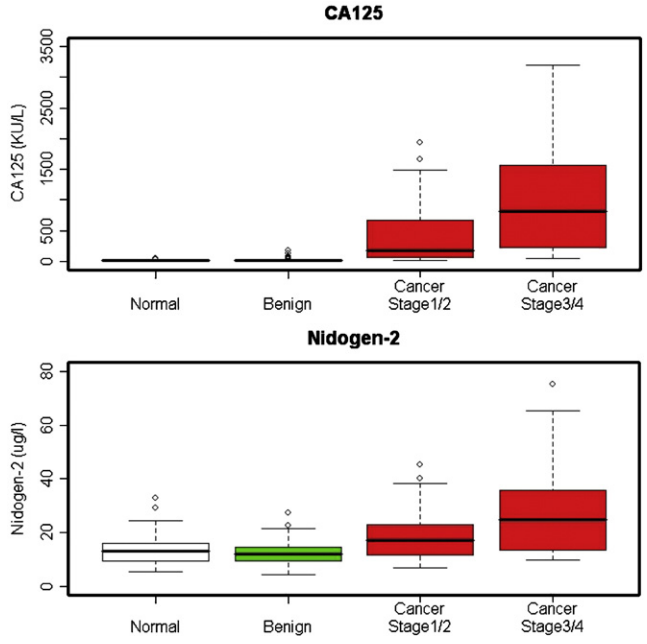

Fig. 1. Distribution of serum CA125 and nidogen-2 in the three groups of patients (normal females, benign gynecological cases, ovarian carcinoma) at early (1/2) and late (3/4) stage. The horizontal line indicates the median value for each group. The differences between normal and benign groups are not statistically significant. The differences between normal or benign groups and cancer (early or late) are highly significant ($p<0.001$) for both markers (see also Table 1).

collected and stored in an identical fashion (−80 °C) until analysis. Samples were collected with informed consent and Institutional Review Board approval. From the large number of samples available (>70 000), we selected 100 serum samples from ovarian cancer patients (ages 33 to 82 years; median, 57.5 years), 100 serum samples from normal, apparently healthy women (ages 25 to 88 years; median, 51.5 years), and 100 serum samples from women with benign gynecological malignancies (ages 20 to 80 years; median, 38 years). Of the 100 ovarian carcinoma patients, 38 were stage 1, 19 were stage 2, 31 were stage 3, and 12 were stage 4 and 1 case was unknown. Regarding histological types, 59 samples were from serous, 19 from mucinous, 11 from endometrioid and 10 from clear cell carcinomas of the ovary. Malignant tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Patients with benign gynecological conditions were diagnosed with uterine leiomyomas ($n=45$), adenomyosis ($n=18$) and ovarian cysts ($n=37$).

Measurement of CA125 and nidogen-2 in serum

CA125 was measured with a commercially available immunoassay method (Roche). The precision of this assay is <10%. The concentration of nidogen-2 in serum was measured using a non-competitive "sandwich-type" ELISA developed in-house with commercially available antibodies from R&D Systems (Minneapolis, MN). Goat polyclonal anti-human nidogen-2 antibody was immobilized in a 96-well white polystyrene plate by incubating 200 ng/100 μL/well in a coating buffer (50 mmol/L Tris, 0.05% sodium azide; pH 7.8) overnight. After washing three times with washing buffer (5 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween 20; pH 7.8), 50 μL of each serum sample (diluted 1:200 in 6% bovine serum albumin (BSA) solution) or 50 μL of nidogen-2 standards were pipetted into each well, in addition to 50 μL of assay buffer (50 mmol/L Tris, 6% BSA, 0.01% goat IgG, 0.005% mouse IgG, 0.1% bovine IgG, 0.5 mol/L KCl, 0.05% sodium azide; pH 7.8) and incubated for 90 min with shaking at room temperature. The plates were washed six times with the washing buffer, after which biotinylated detection antibody solution (100 μL; 25 ng goat polyclonal anti-human nidogen-2 antibody in assay buffer) was added to each well and incubated

Table 2
Estimated odds ratio (OR) with and without adjusting for age.

Comparison	Marker	No adjustment			Age adjusted		
		OR	95% CI	*p*-value	OR	95% CI [a]	*p*-value
Normal vs. benign	CA125	1.03	(1, 1.06)	0.063	1.03	(1, 1.06)	0.028
	NIDOGEN-2	0.95	(0.89, 1.01)	0.120	0.91	(0.84, 0.98)	0.010
Benign vs. cancer	CA125	1.04	(1.02, 1.05)	<0.001	1.03	(1.02, 1.05)	<0.001
	NIDOGEN-2	1.19	(1.12, 1.26)	<0.001	1.19	(1.1, 1.29)	<0.001
Benign vs. stage ½	CA125	1.03	(1.02, 1.05)	<0.001	1.03	(1.01, 1.04)	<0.001
	NIDOGEN-2	1.18	(1.1, 1.27)	<0.001	1.17	(1.06, 1.28)	0.001
Benign vs. stage	CA125	1.05	(1.03, 1.07)	<0.001	1.04	(1.02, 1.06)	<0.001
	NIDOGEN-2	1.22	(1.13, 1.32)	<0.001	1.25	(1.12, 1.39)	<0.001
Normal vs. cancer	CA125	1.08	(1.04, 1.12)	<0.001	1.08	(1.04, 1.13)	<0.001
	NIDOGEN-2	1.14	(1.08, 1.2)	<0.001	1.13	(1.07, 1.2)	<0.001
Normal vs. stage 1/2	CA125	1.07	(1.04, 1.11)	<0.001	1.07	(1.03, 1.11)	<0.001
	NIDOGEN-2	1.13	(1.06, 1.2)	<0.001	1.11	(1.05, 1.19)	0.001
Normal vs. stage 3/4	CA125	1.28	(1.01, 1.62)	0.038	1.25	(0.99, 1.57)	0.055
	NIDOGEN-2	1.17	(1.1, 1.25)	<0.001	1.18	(1.1, 1.27)	<0.001
Early vs. late cancer [b]	CA125	1	(1, 1)	0.015	1	(1, 1)	0.016
	NIDOGEN-2	1.04	(1.01, 1.07)	0.018	1.04	(1.01, 1.07)	0.019

[a] CI, Confidence interval.
[b] Early = Stages 1–2; Late = Stages 3–4.

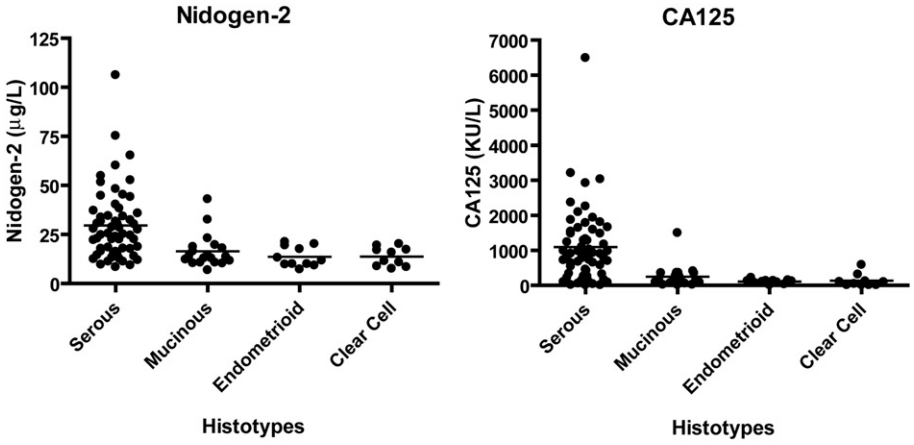

Fig. 2. Distribution of CA125 and nidogen-2 according to the histotype of ovarian cancer. The horizontal line indicates median values.

for 1 hour at room temperature with shaking. The plates were then washed six times with the washing buffer. Subsequently, alkaline phosphatase-conjugated streptavidin solution (5 ng/well; Jackson ImmunoResearch, Westgrove, PA) in 6% BSA buffer (in 50 mmol/L Tris, 0.05% sodium azide; pH 7.8) were added to each well and incubated for 15 min with shaking, at room temperature. The plates were washed six times with the washing buffer and substrate buffer (100 µL; 0.1 mol/L Tris buffer; pH 9.1) containing 1 mmol/L of the substrate diflunisal phosphate, 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$ was added to each well and incubated for 10 min with shaking at room temperature. After adding 50 µL of developing solution containing Tb^{3+}/EDTA complex, the fluorescence of each well was measured with the Perkin-Elmer Envision 2103 multilabel reader. The assay has a detection limit of 0.1 µg/L and a dynamic range of up to 100 µg/L. Precision was less than 10% within the measurement range. Serum samples were analyzed in duplicate.

Data analysis and statistics

Spearman's rank correlation coefficient was used to assess the correlation between CA125 and nidogen-2. Logistic regression was performed to calculate the odds ratio (OR) that defines the relation between biomarkers and cases, benign or control subjects. OR were calculated on log-transformed biomarkers and were represented with their 95% confidence interval (CI) and two-sided *p*-values. The diagnostic value of nidogen-2 was considered using receiver operating characteristic (ROC) curves. ROC curves were constructed by plotting sensitivity versus 1-specificity and the areas under the curve (AUC) were calculated. The bootstrap method was used to calculate the confidence intervals for AUC. All analyses were performed using Splus 8.0 software (Insightful Corp., Seattle WA).

Results

In Table 1 we present the distributions of age, CA125 and nidogen-2 in the three groups of patients. The comparisons between the groups are also shown. Neither nidogen-2 nor CA125 are different between normal and benign groups. However, CA125 and nidogen-2 are both elevated in the cancer group ($p<0.001$) in comparison to either normal or benign groups. The difference remained when the cancer group was separated into early or late cancer ($p<0.001$ in both cases). Within the cancer group of patients, both CA125 ($p<0.001$) and nidogen-2 ($p=0.008$) were higher in the late state group, in comparison to the early stage group.

The distributions of CA125 and nidogen-2 in the three groups of patients are further depicted in Fig. 1. There is a clear elevation of the two markers in both early and late stage disease, but especially in the latter group.

We then calculated the odds ratio (OR) of patients having either early or late stage ovarian cancer, in comparison to either the benign or normal groups, by using logistic regression. Elevation of either CA125 or nidogen-2 was

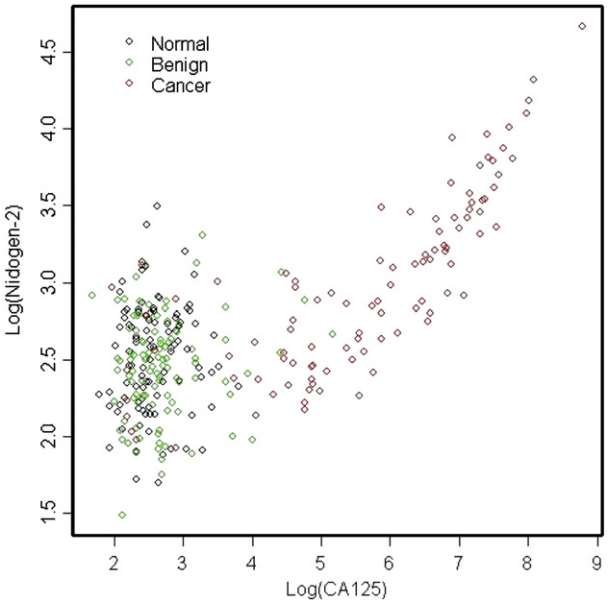

Fig. 3. Correlation between CA125 and nidogen-2. The Spearman correlation coefficient was highly significant ($r_s=0.46$; $p<0.001$).

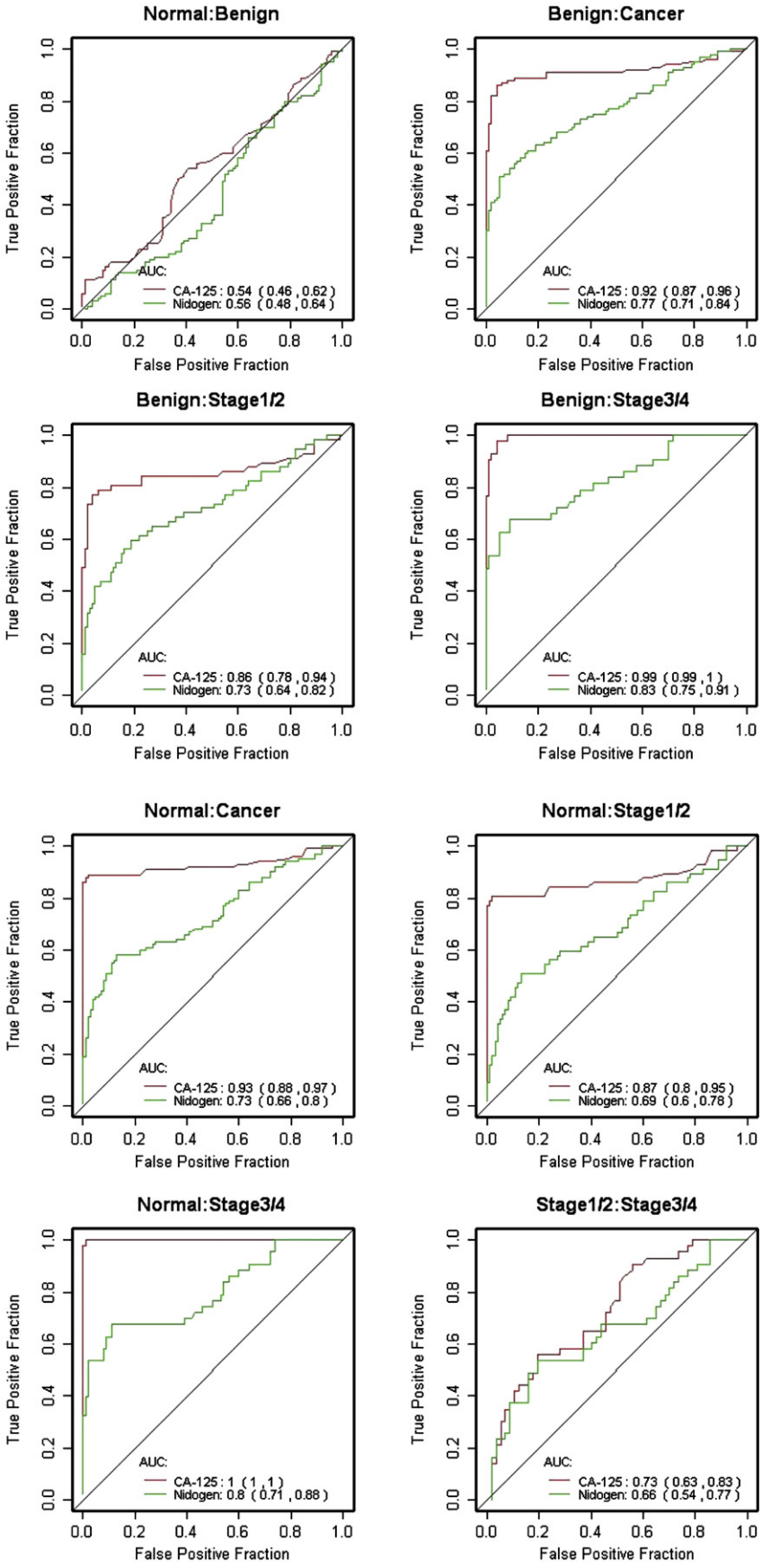

Fig. 4. ROC curves for nidogen-2 and CA125 with estimated AUC (95% CI). For discussion, see text.

associated with presence of cancer, even after adjustment for age (Table 2).

In Fig. 2 we present the distributions of CA125 and nidogen-2 according to the histotype of ovarian cancer. Both markers were elevated in the serous histotype.

We found a strong correlation between CA125 and nidogen-2 concentration (log-transformed data; $r_s = 0.46$, $p < 0.001$ for all samples) (Fig. 3). When we calculated OR by logistic regression, after adjusting for CA125, none of the comparisons (normal vs. benign; benign vs. cancer; normal vs. cancer; early vs. late cancer) was statistically significant.

In order to examine the diagnostic value of CA125 and nidogen-2 for either early or late stage ovarian cancer, we constructed ROC curves as shown in Fig. 4. None of the markers could discriminate normal from benign groups. However, both markers could discriminate benign or normal from cancer (both early and late cancer); the AUCs were maximal for comparisons of normal or benign groups with late stage cancer.

We further examined if we could combine the two markers by drawing ROC curves using the linear scores from the logistic model. The ROC curves of the combined markers were almost identical to those of CA125 alone under all comparison (data not shown). We thus concluded that nidogen-2 could not significantly supplement CA125 as an additional ovarian cancer biomarker.

Discussion

The discovery of new ovarian cancer biomarkers for early diagnosis, prognosis, monitoring and prediction of therapeutic response could significantly contribute to better clinical outcomes. Currently, the only well-accepted ovarian cancer biomarker is CA125, which was discovered over 20 years ago. A number of other potential ovarian cancer biomarkers have been identified, yet, most are without established clinical value [24–28]. CA125 is also unable to diagnose early ovarian cancer effectively [29]. In addition to its low sensitivity for early disease, CA125 suffers from low specificity as its levels are elevated in many benign gynecological diseases [29]. Thus, new biomarkers with increased sensitivity and specificity for ovarian cancer are needed to improve clinical outcomes.

The basement membrane plays a key role in maintaining tissue organization and compartmentalization [8]. Thus, removal or disruption of the integrity of the basement membrane creates an invasion-permissive environment, often promoting cancer cell proliferation and invasion, [30], a regulated process that occurs in trophoblast implantation, organogenesis, angiogenesis and cancer metastasis [31]. Basement membrane abnormalities may lead to an increase in tumor susceptibility [32] as the need for basement membrane degradation is bypassed and as interaction of tumor cells with stromal fibroblast growth factors, cytokines and/or matrix proteins triggers a pro-proliferative effect [33].

Previous studies have suggested that nidogen can protect laminin-1 against proteolysis [11], suggesting that nidogen absence may cause additional basement membrane protein degradation and remodeling. Thus, nidogen loss may affect basement membrane structural integrity by loosening cell interaction with basal membrane and by weakening the strength of the basement membrane itself, thereby facilitating the route to invasion for genetically altered cells, favoring metastasis and promoting angiogenesis. The loss of nidogen expression has been shown to have a potential pathogenic role in colon and stomach tumorigenesis [34].

In this study, nidogen-2 was elevated in serum of patients with ovarian carcinoma, compared to patients with benign gynecological diseases and normal controls. ROC curve analysis demonstrated that nidogen-2 has potential diagnostic value. Spearman correlation showed that nidogen-2 correlates highly with CA125 (Fig. 3). Similar to CA125, nidogen-2 is more frequently elevated in serous adenocarcinoma compared to other histotypes (Fig. 2). Serum nidogen-2 is also more frequently elevated in late-stage disease (Fig. 1). To our knowledge, this is the first to report on serum nidogen-2 elevation in ovarian cancer patients. The availability of reliable immunoassays, such as the one developed in this study for measuring serum nidogen-2, may facilitate further studies to establish the clinical usefulness of this marker in ovarian cancer.

Currently, it is accepted that no single cancer biomarker can provide all the necessary information for optimal cancer diagnosis and management. The current trend is to focus on the identification of multiple biomarkers that can be used in combination. Such approaches have already been shown to have clinical potential in ovarian cancer [26–28]. Unfortunately, the close correlation between CA125 and nidogen-2 precludes their combination in a panel which would perform better than CA125 alone.

Nidogen-2 has been shown to possess numerous glycosylation sites. Hexosamine analysis of nidogen-2 demonstrated 25 ± 2 glucosamine and 19 ± 2 galactosamine residues. This indicates that all five predicted N-glycosylation sites and a substantial number of O-glycosylation sites are occupied on nidogen-2 [14]. The abundant amount of glycosylation suggests that is may be possible for different glycosylated forms of nidogen-2 to be present in the serum, especially during certain pathological conditions, including ovarian cancer. This issue is worth examining in the future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2009.10.012.

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