Anti-osteopontin autoantibodies in various types of cancer

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Abstract. The aberrant processing of biomolecules by cancer cells may give rise to autoimmune phenomena. The metastasis gene osteopontin is alternatively spliced only in transformed cells, and the variants promote tumor progression. They may also serve as tumor-associated antigens, and their neutralization by autoantibodies could favorably affect prognosis. A competitive solid-phase ELISA format was applied to determine reactivity toward recombinant osteopontin in human serum or plasma samples. Approximately 35% of thyroid cancer patients and 15% of other cancer patients were found to harbor autoantibodies directed to osteopontin variants, averaging 21% of patients across all cancers studied. The reactivity of the autoantibodies was consistent with the differential appearance of splice variants in individual malignancies. In thyroid cancer, autoantibodies were found to be more frequently associated with a milder form of the disease. The junctions of osteopontin splice variants produced by cancers represent tumor-associated neo-epitopes. Whereas the uniqueness of their sequences renders the acquisition of tolerance during immune maturation improbable, their immunogenicity is predicted to be low. The rare occurrence of antibodies to either osteopontin-b or osteopontin-c suggests that the breaking of tolerance to full-length osteopontin is more prevalent in tumor autoimmunity than a reaction to a poorly immunogenic neo-epitope.

Introduction

Biomarkers are important for guiding the diagnosis and treatment of cancer. One such marker is the metastasis gene osteopontin (OPN), the expression of which supports progression and dissemination (1,2), and reduces the prospects for survival (3,4). However, OPN is also secreted from immune system cells and physiologically acts as a cytokine. The gene product is subject to alternative splicing in transformed cells, which generates three distinct forms (5). While in studies prior to 2006 total OPN was measured, the splice variants (the expression of which varies by cancer type) may be more sensitive markers than pan-OPN. Further, any additional information that can put into context the reliability of OPN splice variants in diagnosis, prognosis, and prediction may be of value. In this regard, autoantibodies that neutralize OPN function could be a modulating influence.

Being highly expressed in malignant tumors, the OPN forms have the potential to act as tumor-associated antigens. This possibility is particularly given for the splice variants OPN-b and OPN-c, which are absent from healthy tissue. Therefore, the host immune system is not tolerized to these potential autoantigens. In various immune diseases, anti-OPN autoantibodies have been described to arise (6,7-9), even though they do not occur in healthy individuals (10). Cancer may also generate anti-OPN autoimmunity (11,12). It is conceivable that such autoantibodies (to OPN or its splice-variants) will neutralize OPN function. Taken together, these elements raise the possibility that antibodies to OPN forms, produced by some cancer patients, are capable of inactivating their targets and thus having a propensity for improving the prognosis. It is not known which splice forms may be responsible for stimulating the generation of autoantibodies.

Here we tested the prevalence of antibodies to the three variant forms of OPN in the blood of cancer patients. We hypothesized that the occurrence of OPN splice variants in these patients may stimulate an autoimmune response, and that patients who have autoantibodies to OPN splice variants experience a milder course of the disease. We found autoantibodies in a variable fraction of patients, averaging 21% across all cancers studied. While the reactivity of the autoantibodies is consistent with the differential appearance of splice variants in individual malignancies, autoantibodies are much more common to full-length osteopontin than to either osteopontin-b or osteopontin-c.

Materials and methods

Patients. This study contained serum samples from patients enrolled at the Tumor Bank of the University of Cincinnati Cancer Institute (UCCITB). Plasma samples from healthy donors were obtained from CHTN Midwestern Division (Columbus, OH, USA). Serum from thyroid tumors and controls was provided by Johns Hopkins Medical Institute (JHMI). Of 175 patients, we tested serum. Of 29 patients we tested plasma, among whom 14 were non-cancer controls.

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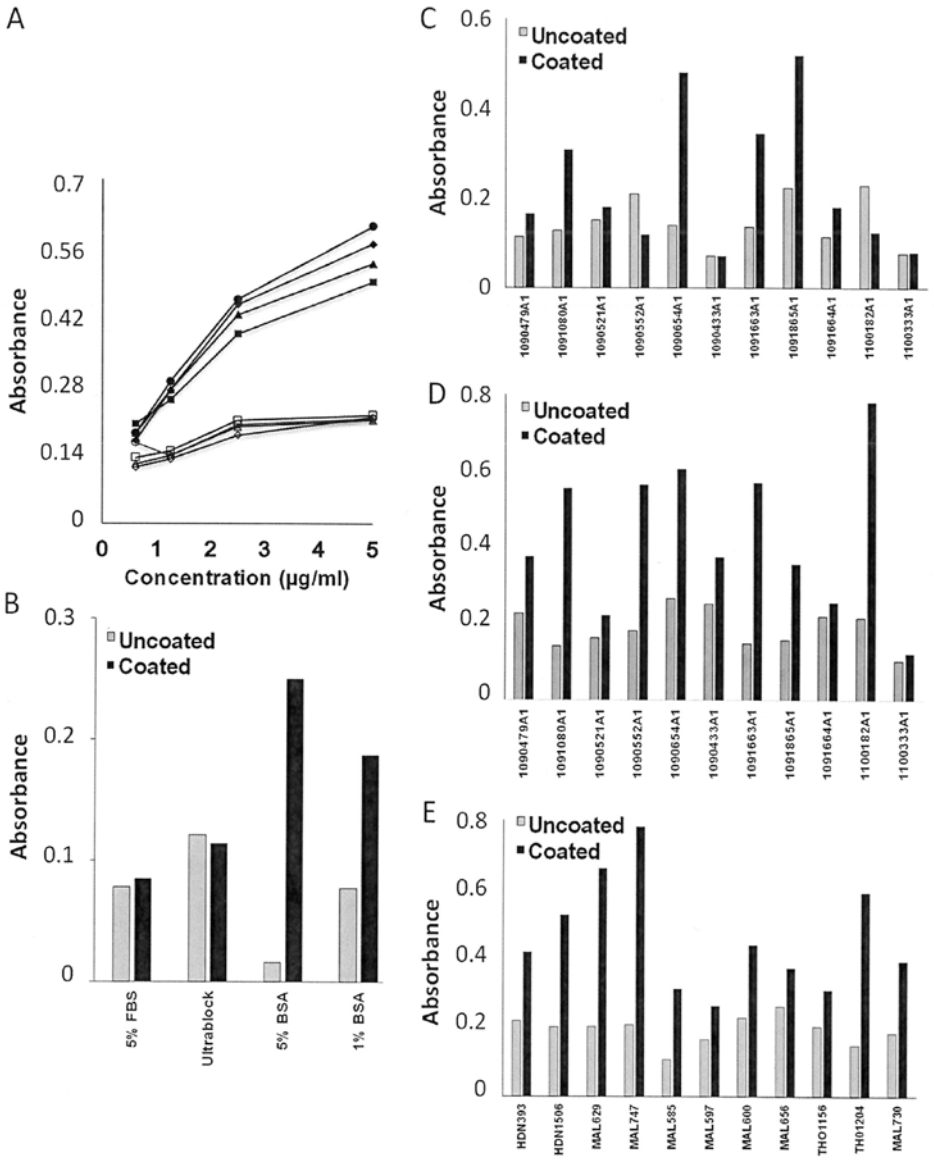

Figure 1. Effects of various blocking modalities in ELISA. (A) Purified Ig is inert to the modality of blocking. The plate was coated with GST-OPN-c 40 ng/100 μl/well. The blocking buffers compared included 5% FBS (circles), Ultrablock (diamonds), 5% BSA (triangles) and 1% BSA (squares). Probing commenced with anti-hOPNc IgY (1:3,200, 1:6,400, 1:12,800 and 1:25,600), followed by HRP-conjugated goat anti-human IgG (1:2,000), and specificity of the signal was corroborated by comparing competition with soluble GST-OPN-c 2 μg/100 μl/well (open symbols) vs. no competition (filled symbols). (B) Various blocking buffers impact signal and background absorbance of plasma samples. The plate was coated with GST-OPN 40 ng/100 μl/well. On the next day, the plate was blocked with 5% FBS, Ultrablock, 5% BSA or 1% BSA in distinct wells. A representative plasma sample was added (1:400) to the antigen-coated as well as the antigen-uncoated wells for detection with HRP-anti-human Ig. (C and D) Testing 1% BSA (C) or 5% BSA (D) with the same set of plasma samples. The plate was coated with or without GST-OPN-a, then blocked with the indicated concentrations of BSA. Plasma samples were added (1:400) to the antigen-coated wells and antigen-uncoated wells. (E) BSA at 5% was also found to be suitable for blocking of serum samples. Serum samples were added (1:400) to the antigen-coated wells (OPN-a) and antigen-uncoated wells. Each analysis was performed at least twice; one representative experiment is shown.

Competitive ELISA. The antibodies used in this study were O-17 (cat. no. 18625; IBL America, Minneapolis, MN, USA), anti-hOPNc IgY (cat. no. AhOPNc; Gallus Immunotech, Cary, NC, USA; Exalpha Biologicals, Shirley, MA, USA) and LF161 (Dr Larry Fisher, NIH). The polyclonal rabbit antibody O-17 recognizes an epitope upstream of the splice junctions and thus is common to all three forms of OPN (anti-pan-OPN). The IgY antibody recognizes the OPN-c splice junction. The polyclonal rabbit antibody LF161 has reactivity selectively with exon 4 (present in OPN-a and -b).

We assessed the presence of anti-OPN autoantibodies by competitive solid-phase enzyme-linked immunosorbent assay (ELISA). For the detection of autoantibodies to OPN splice variants, the plates were coated with GST-OPN (-a or -b or -c). Specific autoantibodies in the serum can bind to the plated antigens, but the binding is reduced if soluble antigen is added in excess together with the serum to compete with the plated antigen for the autoantibodies. The soluble Ag-Ab complex formed with the soluble antigen is eliminated in the washes, and the absorbance measured is reflective of the autoantibody bound to the coated antigen. We avoided setting up a sandwich ELISA, because of previously reported heterogeneity in the results with this format (13).

GST fusion proteins of the human OPN splice variants -a, -b, and -c were generated from pGEX-6P constructs and purified over GSH-agarose columns. High-binding plates

Table I. Patient characteristics.

Cancer type	N	Sex (male/female)	Average age (range)	Race	Follow-up
Thyroid	5	2/3	43.6 (19-65)	3 W 2 B	0 a; 1 b; 1 c
Thyroid	50	(unknown)	(unknown)	(unknown)	None
Non-melanoma skin	5	4/1	73.8 (70-78)	5 W	0 a; 1 b; 4 c
Lung	52	27/25	62.1 (29-84)	45 W 6 B 1 O	0 a; 15 b; 20 c
Pancreas	25	13/12	61.6 (28-83)	12 W 4 B	0 a; 5 b; 12 c
Breast	49	0/49	58.9 (30-86)	40 W 7 B 1 A 1 O	7 a; 15 b; 10 c
Breast cancer remission	2	0/2	57.5 (49-66)	2 W	None
Benign breast tumor	2	0/2	48.5 (39-58)	2 W	None
Healthy controls	14	4/7 (3 unknown)	43.1 (18-65)	13 W 1 B	None
Total	204				

The patient populations are described according to select clinical variables. In the race column, W indicates white or Caucasian, B indicates Black or African-American, A indicates Asian, O indicates other. In the follow-up column, a is less than 6 months, b is 6-12 months, c is more than 12 months. Information was not available for the 50 thyroid cancers provided from JHMI.

were coated with 40 ng/100 μl/well of recombinant osteopontin in phosphate buffer saline (PBS) overnight at 4˚C. On the following day, the plates were blocked with 200 μl/well 5% bovine serum albumin (BSA) in PBS for 2 h at room temperature with gentle shaking. Following three washes with PBST (PBS containing 0.5% Tween-20), serum samples (200-, 400-, or 800-fold final dilution) were added in PBST/1% BSA. For competition wells, the same GST-OPN form as had been plated was added at 2 μg/100 μl/well. Otherwise, PBST/1% BSA was added to a volume of 100 μl/well. As reference standards, anti-osteopontin antibodies (O-17, LF161, or anti-hOPNc IGY) were used (with and without competing GST-OPN) in separate wells. The plates were incubated for 2 h at room temperature with gentle shaking, before washing five times. This was followed by addition of 100 μl/well of HRP-conjugated goat anti-human IgG (1:2,000 diluted; cat. no. 5220-0279; KPL/SeraCare, Milford, MA, USA) for 1 h. The reference wells received their appropriate secondary antibodies. After 3 washes, 100 μl/well of the detection reagent TMB (3,3',5,5'-tetramethylbenzidine; Surmodics BioFx, Eden Prairie, MN, USA) was provided and the reaction was terminated with stopping reagent (Surmodics TMB Stop Solution) at the appropriate time. The optical density (OD) of each well was read at a wavelength of 450 nm.

A main challenge in using human serum samples in ELISA is a high and variable background of non-specific binding. To minimize this effect, we tested various blocking buffers, including 5% FBS (fetal bovine serum), ELISA Ultrablock (AbD Serotec), 5% BSA (bovine serum albumin), and 1% BSA. Whereas ultra-block (which contains predominantly fish protein) increased the non-specific absorbance, the others had some efficacy in suppressing it. BSA (5%) was determined to be the most suitable choice (Fig. 1).

The format of a competitive ELISA may benefit from a preincubation of the antibody with the competing antigen. However, we found that preincubation of the serum with the competing GST-OPN did not make a significant difference in relation to adding the reagents directly to the coated wells. The absorbance values between preincubated and directly added wells differed by only ±0.92% (data not shown). Two protocols of competition were evaluated.

i) We applied competition with the GST-OPN form that was identical to the plated form (Fig. 2A-E). This modality of competition assesses the specificity of the absorbance signal, it does not identify the epitope or splice variant specificity of auto-antibodies present.
ii) The alternative competition with anti-osteopontin antibodies O-17 (a polyclonal rabbit antibody recognizes all forms of OPN), LF161 (a polyclonal rabbit antibody that recognizes exon-4 in OPN-a and OPN-b) or IgY (anti-hOPN antibody recognizes OPN-c) became problematic. The secondary anti-hIg antibody cross-reacted with the rabbit polyclonal antibodies O-17 and LF161. This left the competition with IgY as a viable option for testing reactivity against osteopontin-c (Fig. 2F-H).

Statistical analysis. To account for sample-to-sample variation in background absorbance, the ratio of uncompeted to competed absorbance (reflective of the specific signal) was calculated and used in the biostatistical analysis. For each cancer specimen and each of the OPN splice variants, the ratios from repeat experiments were compared for significant differences to the mean values of ratios from all healthy controls using a one-tailed t-test for samples with unequal variance (accounting for heteroscedasticity).

Results

Patient characteristics. Specimens from 204 patients and controls were obtained from three sources: The University of Cincinnati Tumor Bank, Collaborative Human Tissue Network (CHTN), and Johns Hopkins Medical Institute. They covered thyroid cancers, skin cancers, lung cancers, pancreatic cancers, and breast cancers, as well as remission from breast cancers, benign breast tumors, and no-tumor controls (Table I).

Presence of autoantibodies. Of the 186 cancer patients, 2 patients in remission from breast cancer, and 2 patients

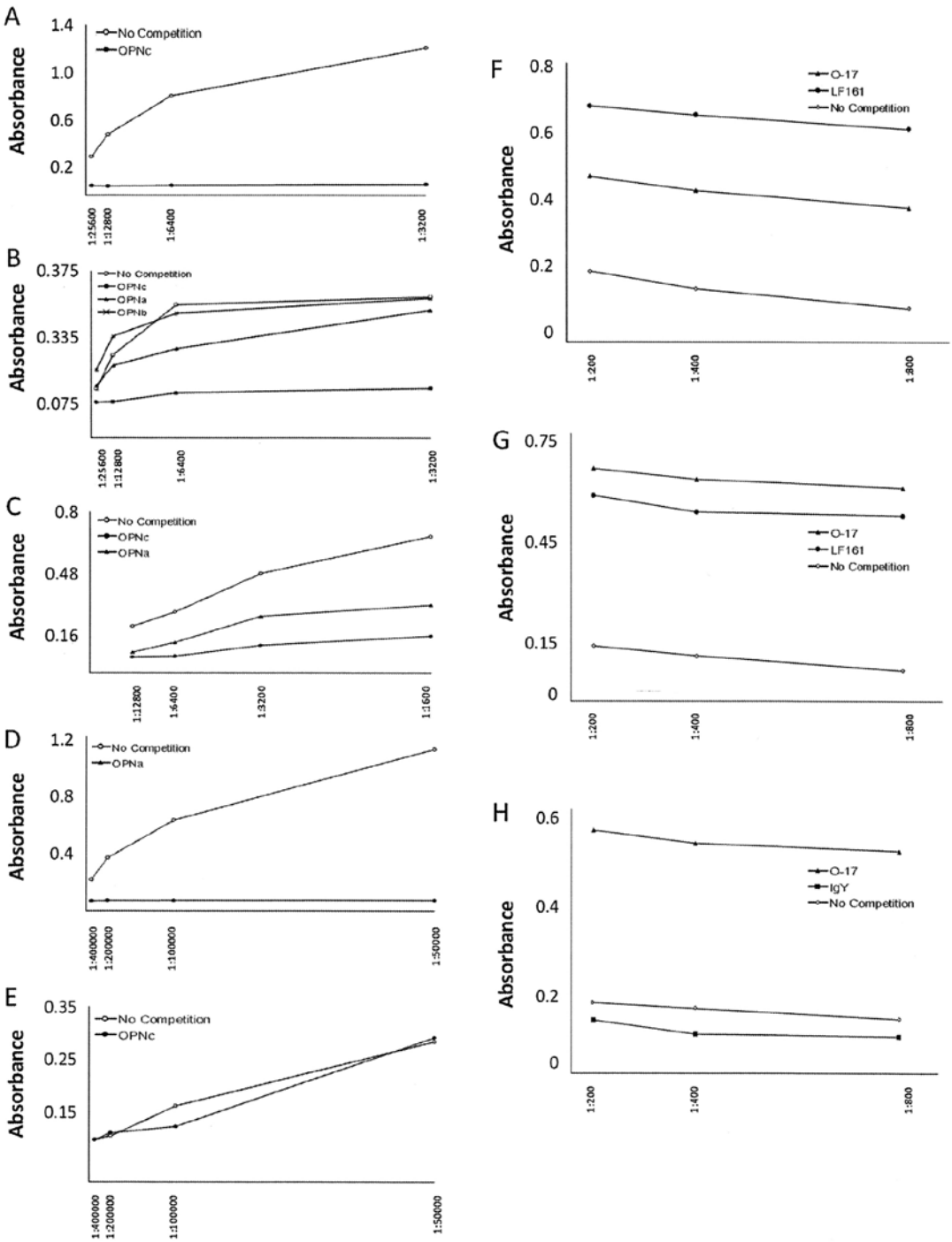

Figure 2. ELISA validation. In all cases, the plate was coated with the suitable form of GST-OPN overnight. The next day, the plate was blocked with 5% BSA. Serial dilutions of the primary antibody were added in the absence of competition or in the presence of soluble GST-OPN. Detection was accomplished by binding of secondary HRP-conjugated anti-Ig antibody and color development with TMZ reagent. The graphs show one representative experiment of many independent replicates. (A-E) Competition with GST-OPN utilizing positive control antibodies. LF161 and IgY recognize distinct forms of bound GST-OPN, while O-17 binds to a common epitope. The absorbance signal generated in solid-phase ELISA can be competed by the addition of a suitable form of soluble GST-OPN at 2 μg/100 μl/well. (A) Testing the competition with GST-OPN-c as an indicator for the binding specificity by anti-osteopontin-c IgY, added in a dilution series (1:3,200, 1:6,400, 1:12,800, 1:25,600). (B) Testing the competition with OPN-a and OPN-b (the plate was coated with GST-OPN-c.) vs. anti-osteopontin-c IgY. No competitor or soluble GST-OPN-a, GST-OPN-b or GST-OPN-c were added in separate wells. (C) Testing the competition by GST-OPN-a or GST-OPN-c against the binding of the anti-pan-OPN antibody O-17 (diluted at 1:1,600, 1:3,200, 1:6,400, 1:12,800). The plate was coated with GST-OPN-b. (D) Testing the competition with GST-OPN-a as an indicator for the binding specificity by anti-osteopontin-a/b LF161, added in a dilution series (1:50,000, 1:100,000, 1:200,000, 1:400,000). The plate was coated with GST-OPN-b. The curves reflect either no competition of competition with soluble GST-OPN-a. (E) Testing the competition with GST-OPN-c as an indicator for the binding specificity by anti-osteopontin-a/b LF161. The plate was coated with GST-OPN-a. (F-H) ELISA with antibody competition. Secondary anti-human antibody crossreacts with rabbit Ig (O-17, LF161), but not with chicken IgY (anti-hOPNc). (F) The plate was coated with GST-OPN-a. On the following day, the wells were blocked with 5% BSA for 2 h. A human serum sample was added for measurement at serial dilution (1:100, 1:200, 1:400 and 1:800). For the competition wells LF161, O-17 or no competition were added in separate wells. Detection was achieved with HRP-conjugated goat anti-human IgG (1:2,000), followed by the color reagents. (G) The plate was coated with GST-OPN-b. A human serum sample was added for measurement at serial dilution. For the competition wells LF161, O-17 or no competition were added in separate wells. (H) The plate was coated with GST-OPN-c. A human serum sample was added for measurement at serial dilution. For the competition wells LF161, IgY or no competition were added in separate wells.

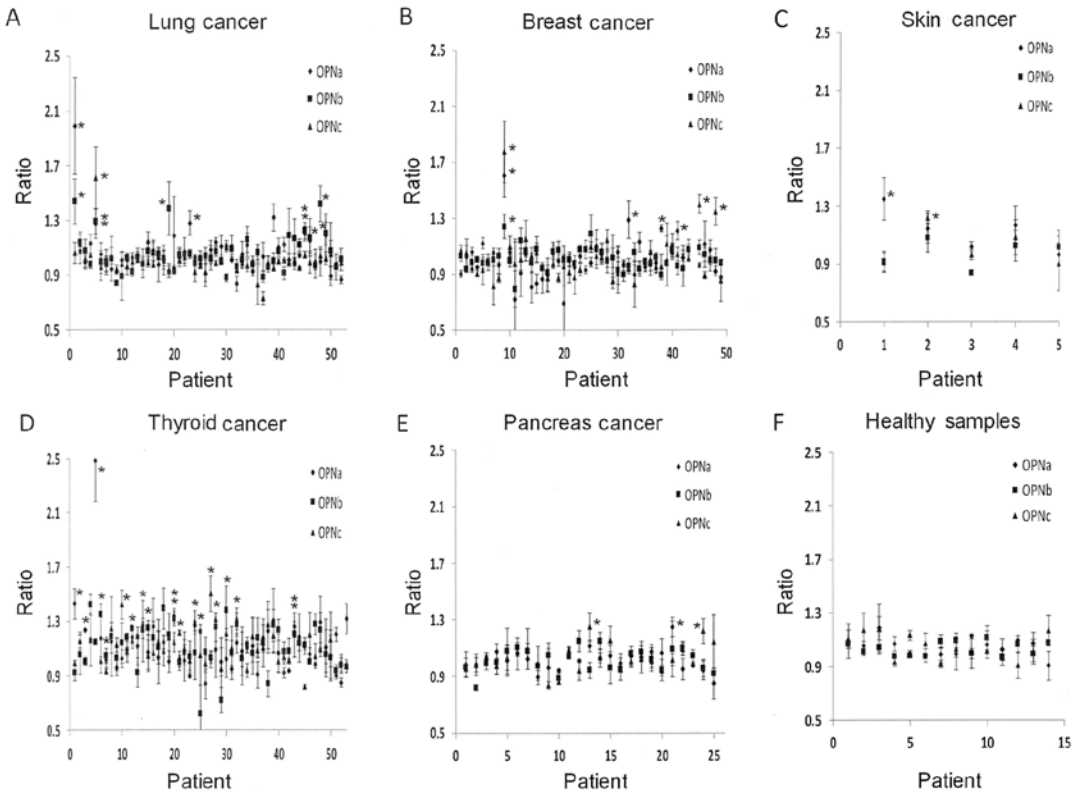

Figure 3. Competitive ELISA for osteopontin splice variant autoantibodies. Absorbance ratios are displayed for lung cancer (n=52) (A), breast cancer (n=49) (B), thyroid cancer (n=55) (C), pancreatic cancer (n=25) (D), non-melanoma skin cancer (n=5) (E), and healthy controls (n=14) (F). The data show mean values ± SEM of relative ratios (uncompleted signal/competed signal). For healthy controls, these ratios ranged from 0.906 to 1.167. Note that for all graphs, the y-axes have identical scale to facilitate the quantitative comparison of autoantibody reactivity in the cancer specimens. *P≤0.05.

with benign breast tumors (190 in total), 39 (21%) had auto-antibodies to OPN. The largest fraction of autoantibodies was found in thyroid cancers at 19 of 55 patients (35%). Among all other tumor patients, 20 of 135 patients (15%) had autoantibodies to OPN. Only 2 had pan-OPN reactivity, 4 were specific for OPN-a/b, 1 was specific for OPN-a/c, 13 were selectively specific for OPN-a, 7 for OPN-b, and 12 for OPN-c (Fig. 3, summarized in Table II). Consistent with the preferential expression of certain splice variants in individual tumors (14,15), only autoantibodies were observed that matched the occurrence of the cognate OPN form (no anti-OPNb in breast cancer, from which OPN-b is absent; no anti-OPNc in lung cancer, which does not produce this form).

While competition with the same form of GST-OPN that was plated indicated specificity of the auto-antibody signal, it gave no indication of the affected epitope. Our anti-hOPNc chicken antibody had been raised to the splice junction of OPN-c. We therefore used it as a competing agent. Expectedly, the antibody had only a minor effect on serum reactivity when GST-OPNa or GST-OPNb was plated in the ELISA. Where GST-OPNc was plated, and competition with GST-OPNc had previously indicated the presence of OPN-c-specific autoantibodies, the competition with anti-hOPNc confirmed that the autoantibodies were directed at the splice junction. The effect was similar for a serum sample with reactivity to OPN-a and OPN-c, however, against serum with anti-pan-OPN autoantibodies, the competition with anti-hOPNc was not effective, likely because the autoantigenic epitope was located away from the spice junction (Fig. 4).

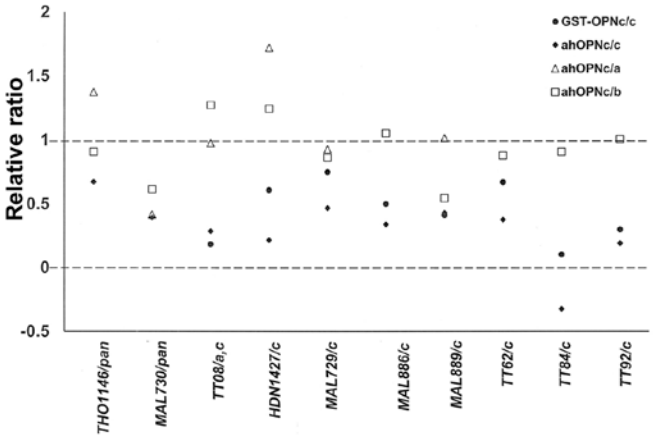

Figure 4. Competitive ELISA for autoantibodies reactive to OPN-c. Shown are relative absorbances under competition, after the uncompeted absorbance was set to 1 and the background absorbance to 0. The ratios are reflective of inhibition by the indicated competitors. Each specimen on the x-axis is indicated with its name and associated antibody reactivity (as shown in Fig. 3). The data series are GST-OPNc=competition with GST-OPNc, ahOPNc=competition with anti-OPNc, /a=plated GST-OPNa, /b=plated GST-OPNb, /c=plated GST-OPNc. One representative experiment of two is shown.

Autoantibodies and disease severity. Papillary (PTC) and follicular thyroid carcinoma (FTC) are distinct forms of differentiated thyroid carcinoma. Within PTC, the follicular variant (PTC-FV) is common. While it displays variations in clinical presentation, the long-term outcome is similar to

Table II. Splice variant-specific osteopontin autoantibodies in cancer.

Cancer type	N	Anti-panOPN	Anti-OPNa/b	Anti-OPNa/c	Anti-OPNb/c	Anti-OPNa	Anti-OPNb	Anti-OPNc	Total	Percentage
Thyroid	55	-	2	1	-	6	3	7	19	35
Non-melanoma skin	5	-	-	-	-	1	-	1	2	40
Lung	52	1	2	-	-	2	4	-	9	17
Pancreas	25	-	-	-	-	1	-	2	3	12
Breast	49	1	-	-	-	3	-	2	6	12
Breast cancer remission	2	-	-	-	-	-	-	-	0	0
Benign breast tumor	2	-	-	-	-	-	-	-	0	0
Total	190	2	4	1	0	13	7	12		
Percentage		1	2	1	0	7	4	6		
Non-thyroid total	135	2	2	0	0	7	4	5		
Non-thyroid percentage		1	1	0	0	5	3	4		

Autoantibodies may recognize all osteopontin forms (anti-pan-OPN) or may be specific for select splice variants.

Figure 5. Anti-OPN antibodies and disease subtype of thyroid cancer. The scatter plots show the relative absorbance ratios for autoantibodies to OPN-a (top panel), OPN-b (middle panel), or OPN-c (bottom panel) indicated as relative absorbance ratios. The inserts show a tighter scale, which has the highly reactive outlier removed from the OPN-a and OPN-c panels. The horizontal bars are mean values, which are lowest in the FTC variant for OPN-a and OPN-c reactivity (even after the outlier removal). PTC, papillary thyroid carcinoma; PTC-TV, tall cell variant of PTC; FTC, follicular thyroid carcinoma.

classical PTC (16). Here, the two manifestations are grouped together. The tall cell variant (PTC-TV) is more aggressive and has intermediate prognosis between other forms of PTC and FTC (17). FTC is associated with poorer survival than PTC (18). For the thyroid cancers, the autoantibody reactivities to OPN-a and OPN-c were higher in mild (PTC) vs. aggressive (FTC) disease (Fig. 5). We found no anti-OPNa or anti-OPNc antibodies in the FTC specimens. This is consistent with the possibility that neutralization of OPN-a and/or OPN-c may counteract progression and ameliorate the course of the disease. By contrast, there was no correlation of autoantibody reactivity with the levels of TG (thyroglobulin), the presence of anti-TG antibodies, TSH (thyroid-stimulating hormone), or RAI (radioactive iodine) uptake (data not shown). This indicates that anti-OPN auto-antibodies provide distinct information on the disease status from the conventional molecular indicators.

Table III. Prevalence of sequences that match the splice junctions in osteopontin.

A, pBlast search was conducted for the splice junctions of OPN-b

Sequence	Description	Score	Query cover (%)	E value	Ident. (%)	Accession no.	Uncon-firmed
AAV38944.1	Secreted phosphoprotein 1 (synthetic construct)	47.3	100	5.00E-05	94	AAV38944.1	n/a
AAP36151.1	*Homo sapiens* secreted phosphoprotein 1 (synthetic construct)	47.3	100	5.00E-05	94	AAP36151.1	n/a
XP_023053890.1	Osteopontin isoform X3 (*Piliocolobus tephrosceles)*	47.3	100	5.00E-05	94	XP_023053890.1	*
NP_001126162.1	Osteopontin precursor (*Pongo abelii*)	47.3	100	5.00E-05	94	NP_001126162.1	*
XP_021567722.1	Osteopontin isoform X2 (*Carlito syrichta*)	47.3	100	5.00E-05	94	XP_021567722.1	*
BAA05950.1	OPN-b (*Homo sapiens*)	47.3	100	5.00E-05	94	BAA05950.1	
NP_000573.1	Osteopontin isoform OPN-b precursor (*Homo sapiens*)	47.3	100	5.00E-05	94	NP_000573.1	
ABM86681.1	Secreted phosphoprotein 1 (synthetic construct)	47.3	100	5.00E-05	94	ABM86681.1	n/a
XP_004039151.2	PREDICTED: osteopontin isoform X2 (*Gorilla gorilla gorilla*)	47.3	100	5.00E-05	94	XP_004039151.2	
XP_003950415.1	PREDICTED: osteopontin isoform X2 (*Pan troglodytes*)	47.3	100	5.00E-05	94	XP_003950415.1	
XP_011807212.1	PREDICTED: osteopontin isoform X3 (*Colobus angolensis palliat*us)	47.3	100	5.00E-05	94	XP_011807212.1	
XP_011846123.1	PREDICTED: osteopontin isoform X3 (*Mandrillus leucophaeus*)	47.3	100	5.00E-05	94	XP_011846123.1	
XP_008961899.1	PREDICTED: osteopontin isoform X2 (*Pan paniscus*)	47.3	100	5.00E-05	94	XP_008961899.1	
XP_008141789.1	PREDICTED: osteopontin isoform X2 (*Eptesicus fuscus*)	47.3	100	5.00E-05	94	XP_008141789.1	
XP_007997396.1	PREDICTED: osteopontin isoform X2 (*Chlorocebus sabaeus*)	47.3	100	5.00E-05	94	XP_007997396.1	
XP_004661666.1	PREDICTED: osteopontin isoform X2 (*Jaculus jaculus*)	43.9	100	8.00E-04	88	XP_004661666.1	
XP_008828427.1	PREDICTED: osteopontin isoform X2 (*Nannospalax galili*)	43.9	100	8.00E-04	88	XP_008828427.1	
XP_006867814.1	PREDICTED: osteopontin isoform X2 (*Chrysochloris asiatica*)	43.1	100	0%	88	XP_006867814.1	
XP_003790145.1	PREDICTED: osteopontin (*Otolemur garnettii*)	43.1	100	0%	88	XP_003790145.1	

Search sequence: SQKQNLLAPQETLPSKS

Table III. Continued.

B, pBlast search was conducted for the splice junctions of OPN-c

Sequence	Description	Score	Query cover (%)	E value	Ident. (%)
XP_023053891.1	Osteopontin isoform X4 (*Piliocolobus tephrosceles*)	50.3	100	4.00E-06	100
XP_021567723.1	Osteopontin isoform X3 (*Carlito syrichta*)	50.3	100	4.00E-06	100
NP_001035149.1	Osteopontin isoform OPN-c precursor (*Homo sapiens*)	50.3	100	4.00E-06	100
BAA05951.1	OPN-c (*Homo sapiens*)	50.3	100	4.00E-06	100
XP_005341953.1	Osteopontin isoform X2 (*Ictidomys tridecemlineatus*)	44.3	93	5.00E-04	93
XP_006931039.1	Osteopontin isoform X2 (*Felis catus*)	44.3	100	5.00E-04	94
XP_021066705.1	Osteopontin (*Mus pahari*)	42.2	93	0.003	93
XP_003434071.1	Osteopontin isoform X2 (*Canis lupus familiaris*)	41.8	100	0.004	88
ABA40758.1	Osteopontin (*Canis lupus familiaris*)	41.8	100	0.004	88
EPY87927.1	Osteopontin isoform OPN-c precursor (*Camelus ferus*)	40.1	100	0.015	81
AEA49027.1	Osteopontin-C (*Homo sapiens*)	40.1	75	0.016	100
XP_003950416.1	PREDICTED: osteopontin isoform X3 (*Pan troglodytes*)	50.3	100	4.00E-06	100
XP_014994236.1	PREDICTED: osteopontin isoform X1 (*Macaca mulatta*)	50.3	100	4.00E-06	100
XP_011807213.1	PREDICTED: osteopontin isoform X4 (*Colobus angolensis palliatus*)	50.3	100	4.00E-06	100
XP_011846124.1	PREDICTED: osteopontin isoform X4 (*Mandrillus leucophaeus*)	50.3	100	4.00E-06	100
XP_008961900.1	PREDICTED: osteopontin isoform X3 (*Pan paniscus*)	50.3	100	4.00E-06	100
XP_017652830.1	PREDICTED: osteopontin isoform X3 (*Nannospalax galili*)	50.3	100	4.00E-06	100
XP_012415080.1	PREDICTED: osteopontin isoform X3 (*Trichechus manatus latirostris*)	50.3	100	4.00E-06	100
XP_014334291.1	PREDICTED: osteopontin isoform X3 (*Bos mutus*)	47.7	100	3.00E-05	94
XP_004266185.1	PREDICTED: osteopontin isoform X3 (*Orcinus orca*)	47.3	100	4.00E-05	94
XP_007450745.1	PREDICTED: osteopontin isoform X3 (*Lipotes vexillifer*)	47.3	100	4.00E-05	94
XP_016016596.1	PREDICTED: osteopontin isoform X2 (*Rousettus aegyptiacus*)	44.3	100	5.00E-04	88
XP_008141790.1	PREDICTED: osteopontin isoform X3 (*Eptesicus fuscus*)	44.3	100	5.00E-04	88
XP_015354628.1	PREDICTED: osteopontin isoform X2 (*Marmota marmota marmota*)	44.3	93	5.00E-04	93
XP_019320322.1	PREDICTED: osteopontin isoform X2 (*Panthera pardus*)	44.3	100	5.00E-04	94
XP_014929118.1	PREDICTED: osteopontin isoform X2 (*Acinonyx jubatus*)	44.3	100	5.00E-04	94
XP_007074758.1	PREDICTED: osteopontin isoform X2 (*Panthera tigris altaica*)	44.3	100	5.00E-04	94
XP_006867815.1	PREDICTED: osteopontin isoform X3 (*Chrysochloris asiatica*)	41.4	93	0.005	87
Search sequence: SGSSEEKQNAVSSEET					

Using the default search parameters, an alignment score >40 was accepted (for the OPN-b sequence, unspliced matches were deleted manually). Authentic and predicted sequences are separated. Of note, however, several of the sequences not marked as predicted in NCBI are nevertheless 'derived from a genomic sequence [and] predicted by automated computational analysis' or 'conceptually translated' (marked by *). n/a indicates that validation of expression is not applicable for synthetic sequences. The existence of spliced osteopontin RNA and protein has thus far only been corroborated in *Homo sapiens*.

Autoantibodies and prognosis. We hypothesized that the presence of autoantibodies would generate a more favorable disease outcome. For the non-thyroid cancers, no correlation was discernible with disease stage or grade (data not shown). This could be due to a lack of power in 20 patients with autoantibodies and 135 patients without autoantibodies. The available follow-up information was very limited (see Table I), with only 47 patients having been tracked for more than 12 months. Further research will test the prognostic power of OPN variants in the presence or absence of auto-antibodies in cancer.

Discussion

In the present study, we identified autoantibodies to spliced forms of OPN. Approximately 21% of cancer patients were found to harbor such antibodies. The proportion was particularly high in thyroid cancers (35%) as compared to other tumor types (15%). The reactivity of the autoantibodies was consistent with the appearance of splice variants in individual cancers. Breast cancer expresses OPN-a and -c, but not OPN-b (15,19,20). Liver cancer has elevated levels of OPN-a and -b, but not -c (14).

Without therapeutic intervention, the immunogenicity of most cancers is low, and some even engage active mechanisms of immune escape. Other cancers, by contrast, may express tumor-associated antigens that can act in an immunostimulatory fashion. This mechanism typically requires the presentation, via MHC class I or class II, of epitopes to which the host is not tolerized. It then leads to the production of tumor-reactive effector T-cells or antibodies. Tumors considered to have strong antigenicity include melanoma, lung cancer, renal carcinoma, and colon cancer. By contrast, the paucity of breast antigenicity has been a longstanding disappointment in the immunotherapy community. For OPN variants, the ability to act as tumor-associated antigens is uncertain. The unspliced form is present in the healthy body, where it acts as an inducer of the cellular immune defense (21) but does not stimulate autoimmunity (7,10,11). By contrast, the splice variants, which seem to have arisen very recently in evolution (Table III), are not synthesized without transformation and their junctions represent tumor-associated neo-epitopes. Whereas the uniqueness of their splice junction sequences renders the acquisition of tolerance during immune maturation improbable, their inherent immunogenicity is predicted to be low. The rare occurrence of autoantibodies, specific to either OPN-b or OPN-c, suggests that the breaking of tolerance to full-length OPN is more prevalent in tumor antigenicity than a reaction to a poorly immunogenic neo-epitope.

The association of disease with deviations of the immune system is common. At the fringes of the phenotypical spectrum, this can manifest as immune suppression or as autoimmunity. In cancer, the affliction of an organ such as the thymus, which is involved in the development of the immune system, predisposes to autoimmune reactions. Once the immune system has turned against its host, epitope spreading may occur and lead to the generation of autoantibodies against multiple antigens, regardless of their roles in disease generation. Cytokines may be the targets of autoantibodies in tumorous as well as in autoimmune diseases.

Autoantibodies to multiple cytokines are ubiquitous in healthy individuals, although their presence has been reported

Table IV. Literature data on the frequency of anti-osteopontin autoantibodies.

Healthy % (n/total)	Osteoarthritis % (n/total)	Rheumatoid arthritis % (n/total)	Autoimmune uveitis % (n/total)	Insulin-dependent diabetes % (n/total)	Hashimoto thyroiditis % (n/total)	Chronic hepatitis % (n/total)	Liver cirrhosis % (n/total)	Hepatocellular carcinoma % (n/total)	Benign prostate hyperplasia % (n/total)	Prostate cancer % (n/total)	(Refs.)
0% (0/15)											(10)
0% (0/67)	8.1% (11/136)										(7)
	9.5% (11/105)	15% (13/88)									(8)
0% (0/19)			25.7% (19/74)								(6)
3.9% (3/76)				7.3% (5/68)	100% (1/1)						(9)
0% (0/75)						3.1% (1/32)	15.6% (5/32)	12.8% (19/148)			(11)
10% (3/30)									33% (6/18)	62% (18/29)	(12)

Publications on anti-OPN autoantibodies have invariably focused on the unspliced, full-length form. In healthy individuals, autoantibodies to OPN are rare or absent.

to be variable. Focusing on the detection of cytokine/autoantibody complexes in 15 healthy individuals, reactivity was detected to IL-2, IL-8, TNF-α, VEGF and G-CSF, but not to OPN, IL-3 or M-CSF (10). In patients with early-stage osteoarthritis of the knee, the prevalence of autoantibodies to cartilage OPN, intermediate layer protein, YKL-39, and cyclic citrullinated peptide suggests that autoimmune processes are involved in the disease that is widely considered to be caused by wear and tear. Anti-OPN antibodies were detected in 0% (0/67) of healthy controls and in 8.1% (11/136) of osteoarthritis patients, specifically with grades II and III but not with grade IV (7). OPN is one of the autoantigens in osteoarthritis and rheumatoid arthritis, with major epitopes being conformational. Autoantibodies to native OPN were present in 9.5% (11/105) of osteoarthritis serum samples and 15% (13/88) of rheumatoid arthritis serum samples. The existence of anti-OPN antibodies may be linked to disease severity in rheumatoid arthritis as it correlates with high serum levels of rheumatoid factor and C-reactive protein and with accelerated erythrocyte sedimentation rate (8). Insulin-dependent diabetes mellitus has an autoimmune origin, for which autoantigens include insulin, glutamic acid decarboxylase, and the tyrosine phosphatases IA-2ic, and IA-2b. About 7% of Insulin-dependent diabetes mellitus sera may contain anti-OPN antibodies. In the condition, OPN is an autoantigen, which is located in the somatostatin cells of the islets (9). The blinding disease autoimmune uveitis is caused by autoreactive T-cells attacking the inner eye, and is accompanied by autoantibodies. In recurrent uveitis, autoantibodies were identified to the three autoantigens OPN, extracellular matrix protein 1, and metalloproteinase inhibitor 2. OPN reactivity was present in 25.7% (19/74) of recurrent uveitis vitreous samples, whereas all controls were negative (0/19) (6) (Table IV).

Autoimmunity may be associated with cancer. The frequency of anti-OPN autoantibodies in hepatocellular carcinoma was found to be 12.8% (19/148), compared to liver cirrhosis at 15.6% (5/32), and chronic hepatitis at 3.1% (1/32). There was 0% reactivity (0/75) in serum from healthy donors. The association of autoantibodies with prognosis was not assessed (11). By immunoblotting, arguably a less quantitative and less reliable technique, the frequency of anti-OPN autoantibodies was described as 66% in prostate cancer, compared to 33% in benign prostate hyperplasia and 10% in healthy serum donors (12).

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

Authors' contributions

GFW designed the research. LKA mainly collected the data. GFW and LKA performed the statistical analysis and analyzed the data. Both authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of all parts of the study are appropriately addressed.

Ethics approval and consent to participate

The study was covered by University of Cincinnati/IRB Protocol 04-01-29-01: Osteopontin splice variants in human breast cancer, Exemption 4.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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