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Aberrant tumor-associated antigen autoantibody profiles in healthy controls detected by multiplex bead-based immunoassay

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Abstract

There is an increasing amount of emphasis being placed on serological biomarkers as tools for early detection of various cancers. In addition to the tumor-related circulating antigens under current investigation, autoantibodies to tumor-associated antigens are emerging as alternative candidates due to their potential high sensitivity and specificity. Already a number of specific autoantibodies have been identified and several groups have reported on the ability of panels of autoantibodies to discriminate malignant from non-malignant conditions. In this investigation we evaluate tumor-associated antigen autoantibody profiles in a group of healthy individuals. We identify a subset of individuals that demonstrate high levels of autoantibody production across the spectrum of tumor-associated antigens tested. We conclude that this observation is a result of undefined non-malignant autoimmune stimulation. Our findings may be an indication of factors present in the general population that may confound multiplex autoantibody-based diagnostic tests by reducing assay specificity. Such factors will require further characterization and the development of adequate controls in order to improve the performance of diagnostic tests.

Keywords

Tumor-associated antigens; autoantibody profiles; autoimmunity; early detection

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Introduction

Immune surveillance of the early events of tumorigenesis may provide the key to early detection. Molecular alterations emanating from malignant cells stimulate a localized humoral immune response in nearby lymph nodes, which in turn leads to a systemic immune response. This response may be detected clinically in the form of circulating autoantibodies specific for tumor-associated antigens (TAA). An increasing amount of attention is being paid to the potential role of circulating antigens as cancer biomarkers. Such antigens are held to represent factors shed from the growing tumor as well as components of the host response. TAA autoantibodies offer several distinct advantages over antigenic biomarkers due to their inherent stability and specificity [1]. The nature of B-cell stimulation results in an amplified antibody response to a relatively small amount of antigen. This stimulation may stem from subtle changes such as antigen upregulation or altered glycosylation patterns [2]. Autoantibodies specific for TAAs have been detected in high titer in early stage cancer patients [3] and may indicate potential targets for immune therapy given their demonstrated activation of immune pathways [4–6].

Autoantibodies specific for oncogenic proteins such as p53 [7], her2/neu [3], MUC1 [8] and c-myc [9] have been previously detected in human cancers and considered as potential biomarkers. Serological expression cloning utilizing phage expression libraries, or SEREX, was first developed over 10 years ago [10] and has led to the identification of over 2000 autoantigens. While no single autoantibody has demonstrated the sensitivity and specificity required of a diagnostic test, advances based on the SEREX principle such as combinatorial phage display have enabled the development of multiplexed approaches. Investigations utilizing phage display have reported autoantibody panels that discriminate prostate [11], stage I NSCLC [12], and breast cancers [13] from controls with sensitivity/specificity of 88/82%, 90/90%, and 77/83% respectively. High throughput methods such as protein microarrays and glycan arrays, which screen for immunogenic alterations in glycosylation [14], have also been utilized to identify autoantibodies in ovarian [15] and breast cancers [2].

The greatest challenge encountered in the development of any diagnostic test based on serological biomarkers is the identification of individual markers highly specific for the malignant condition. The use of autoantibodies as biomarkers presents a unique set of challenges in that non-malignant conditions such as environmental factors, pathogen invasion, and autoimmune disease can trigger the production of a high-level of IgG and IgM autoantibodies which recognize various TAAs and thus reduce biomarker specificity [16–18]. We conducted an analysis of TAA autoantibodies in a large set of healthy control subjects. We identified a subgroup of individuals within our study set that demonstrated highly elevated levels of TAA autoantibodies for most of the antigens tested. We present these results as evidence of a potentially significant obstacle that must be overcome in order to advance the clinical relevance of autoantibody-based diagnostic methods.

Experimental

Materials and Methods

Study population and serum collection—Serum samples from 205 healthy controls were obtained for use in this study. 150 samples were collected as part of the Pittsburgh Lung Screening Study (PLuSS) [19] according to study protocols. An additional 55 samples were collected by the Early Detection Research Network (EDRN) according to a defined protocol [20]. Written and informed consent was obtained for each patient and all protocols were approved by the University of Pittsburgh IRB. The study group included active and non-active smokers and non-smokers. The characteristics of the study group are outlined in Table 1.

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Multiplexed bead-based TAA autoantibody assay development—Serum samples were tested for autoantibodies to 36 distinct tumor associated antigens chosen on the basis of published evidence (Table 2). The Luminex (Austin, TX) xMAP™ platform allows the simultaneous detection of up to 100 analytes based on the covalent attachment of specific capture molecules to internally-dyed spectrally distinct microbeads. Recombinant or native peptides corresponding to each target antigen were employed as capture probes and coupled to Luminex microbeads as previously described [21]. The individual microbead-antigen combinations were combined into multiplex panels in a stepwise fashion as each assay completed development and validation. Assay specificity was first evaluated by incubation of antigen-coupled microbeads with unrelated human IgG or IgM. Specificity was further evaluated by incubation of antigen-coupled microbeads with serum preincubated with antigen coated polystyrene beads (Sigma, St. Louis, MO) to remove specific autoantibodies, or Protein A/G Sepharose (EMD, Gibbstown, NJ) to absorb all IgG.

Data collection and analysis—Assays were performed and validated as described previously [22]. Briefly, antigen-coated microbeads were blocked with bovine serum albumin for 1 hour, washed, and then incubated with serum diluted 1:100 in blocking buffer for 30 min at 4°C. This dilution was deemed optimal for antibody recovery based on titration (data not shown). Following this incubation, microbeads were washed and bound antibodies detected by phycoerythrin-conjugated donkey anti-human IgG/IgM (Jackson Laboratories, West Grove, PA). Fluorescence was measured on a Luminex 100 analyzer. The data was analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) Standard statistical methods were used to establish relative fluorescence intensity distributions for each analyte and divide the data into percentiles. Values observed to be greater than the ninety-fifth percentile were considered exceptional.

Results

The analysis of our experimental results revealed a subset of individuals that demonstrated significantly elevated levels of autoantibodies to multiple TAAs tested (Figure1, Table 3). We established a statistical cutoff at the 95th percentile for each analyte tested and noted the distribution of results above that level. While outliers with respect to each analyte were observed intermittently throughout the study population, serum samples from nine individuals were found to contain autoantibody levels above the cutoff level for >40% of the antigens tested. Four of these samples were above cutoff levels for >75% of all tested antigens and many of the observed autoantibody intensities represented >10-fold increases over the cutoff levels. Each of these noteworthy subjects demonstrated autoantibody levels which fell below the cutoff value for at least one tested antigen, indicating that these results were not a product of sample evaporation. These observations in healthy control subjects suggest the influence of external stimuli of autoimmunity serving to confound our investigation.

Discussion

As was previously discussed, any effective serological biomarker-based screening test would require the use of combinations of biomarkers, as all individual biomarkers evaluated to date have lacked either sufficient sensitivity or specificity. The results we present here may represent a potential pitfall for multimarker approaches. We describe healthy normal individuals with elevated levels of numerous TAA autoantibodies which would serve to lower the specificity of any multimarker screening strategy based on those autoantibodies. Current clinical standards for cancer screening include stringent requirements for specificity. For example, given the low prevalence of ovarian cancer, it has been suggested that a screening strategy must achieve a minimum specificity of 99.6% and a sensitivity of >75% for early stage disease to avoid an unacceptable level of false-positive results [23,24]. In our investigation the 9 individuals

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demonstrating elevated levels of >40% of tested autoantibodies represent 4.39% of our study population. If our study population is representative of the general population, this suggests a maximum diagnostic specificity of <96% for a test based on these autoantibodies, a level which no published study of this type has yet achieved. Clearly this is an observation that must be incorporated into continuing efforts to develop TAA autoantibody-based screening.

The 205 healthy subjects considered in this investigation represent the control arm of a larger study group that includes 815 patients diagnosed with a variety of benign and malignant conditions. The diseased group was comprised of conditions of the liver, esophagus, pancreas, lung, ovary, breast, and prostate and also melanoma. We found that sera from 24 (~2.9%) of these patients contained TAA autoantibody profiles similar to those of the 9 control subjects discussed above (data not shown). Considered together, these findings support the notion that these aberrant autoantibody profiles arise independent of our current set of clinical variables.

The source of the background level of autoimmunity observed in our investigation is unclear. One aspect or our study population that distinguishes it from the general population is the prevalence of smokers. In our study almost 88% of subjects were current or former smokers, while the CDC estimates the equivalent prevalence in the entire US to be almost 42% [25]. Epidemiologists have established causal links between cigarette smoking and autoimmune disorders such as system lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), Graves' hyperthyroidism, and primary biliary cirrhosis (PBC) [26–29]. In attempts to characterize these links, researchers have shown that cigarette smoke has profound stimulatory effects on peripheral blood leukocytes, particularly neutrophils, macrophages and monocytes, and leads to increased cellular release of CRP, IL-6, fibrinogen, and matrix-metalloproteinases (reviewed in [30]). Although the role of smoking in autoimmunity is well established, its role in this investigation is not as straightforward, as four out of the nine exceptional subjects we identified are non-smokers. The nine-subject subset is equally nondescript with regards to gender and age, being comprised of five males and four females with ages ranging from 38–83. Thus, the observation we describe here is evidently multifactorial and warrants further investigation.

Conclusions

We report the findings outlined above not with the intent to discourage attempts to develop TAA autoantibody screening panels, but in the hope that our observation might lead to further refinement of those efforts. These refinements are certain to involve methods of controlling for background autoimmunity and the identification of tumor-associated antigens that interact with host immunity in a manner independent of any background humoral response. Given the inherent complexity and our limited understanding of the humoral response to tumorigenesis, it is not surprising that obstacles such as these will arise. However, the substantial promise and potential benefits of this type of diagnostic strategy remain clear.

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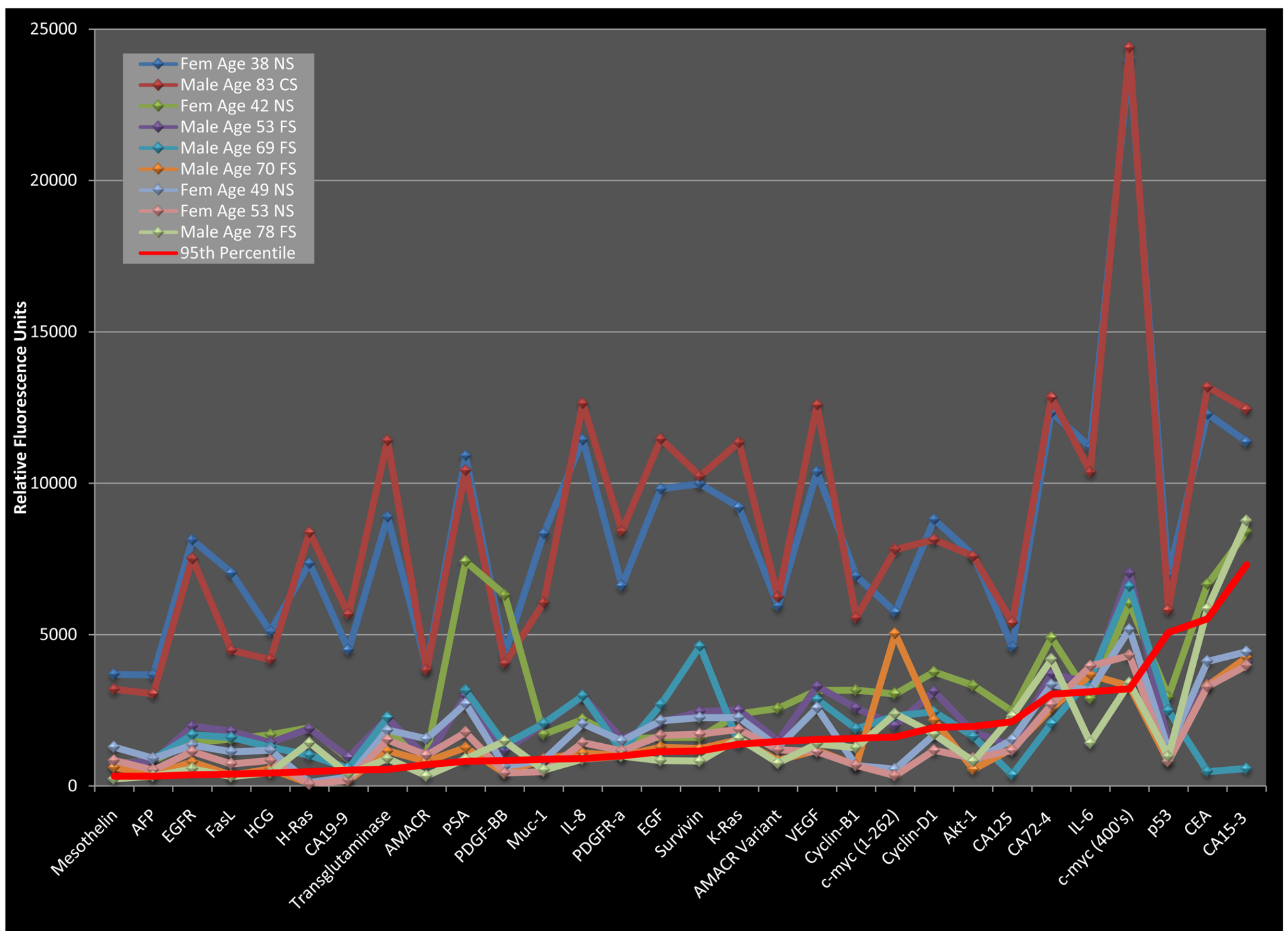

Figure 1. Autoantibodies to tumor associated antigens

Autoantibodies were measured by bead-based immunoassay in serum obtained from 205 healthy donors. Autoantibody levels from nine high-titer subjects are shown along with 95th percentile level for selected antigens. Solid lines connect measurements from individual subjects. Legend abbreviations: fem – female, NS – no smoking history, CS – current smoker, FS – former smoker.

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Table 1

Characteristics of Study Population

Description	Age	N	%
Male	34–83	101	49.3
Female	36–81	104	50.7
Smoking Status		N	%
Active		84	41.0
Former		96	46.8
Never/Unkown		25	12.2

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Table 2

Tumor Associated Antigens Antigen

Antigen	Supplier	Form	Antigen	Supplier	Form
AFP	Bio Processing	Native	HCG	Fitzgerald	Native
Akt1	Invitrogen	Rec	Her2/neu	R&D	Rec
AMACR*	Gift	Rec	H-Ras	Jena Bioscience	Rec
AMACR Variant*	Gift	Rec	IL-6	Peprotech	Rec
CA125	Bio Processing	Native	IL-8	Peprotech	Rec
CA 15-3	Bio Processing	Native	K-Ras	Jena Bioscience	Rec
CA 19-9	Bio Processing	Native	MART-1	Lab Vision	Rec
CA 72-4	Bio Processing	Native	Mesothelin	Genway	Rec
CEA	Bio Processing	Native	Muc-1	Gift	Rec
c-myc (1-262)	Santa Cruz	Rec	Osteopontin	R&D	Rec
c-myc (408-439)	EMD	Rec	P53	Santa Cruz	Native
CRP	Biodesign	Native	PDGF-BB	US Biological	Rec
Cydin-B1 (1-433)	Santa Cruz	Rec	PDGFR-α	Santa Cruz	Rec
Cydin-D1 (1-295)	Santa Cruz	Rec	PSA	Bio Processing	Native
EGF	Peprotech	Rec	Survivin	Alpha Diagnostics	Rec
EGFR	R&D	Rec	Transglutaminase	Sigma	Native**
FasL	Peprotech	Rec	Tyrosinase	Lab Vision	Rec
GplOO	Spring Bioscience	Rec	VEGF	ID Labs	Rec

* α-methyiacyi-CoA Racemase, see ref[31];

** guinea pig; Rec - recombinant

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Table 3

Autoantibody levels of high-titer individuals

		Subject #	1	2	3	4	5	6	7	8	9
		Description	**38 yof NS**	**83 yom CS**	**42 yof NS**	**53 yom FS**	**69 yom FS**	**70 yom FS**	**49 yof NS**	**53 yof NS**	**78 yom FS**
Antigen	**95th Pctl**										
AFP	322		**3665**	3039	835	829	882	369	905	523	319
Akt-1	1962		7624	7585	3320	1780	1630	542	805	910	814
AMACR	699		3920	3823	1024	927	NT	805	1558	1014	332
AMACR variant	1462		5950	6229	2561	1479	NT	838	1277	1190	756
CA125	2121		4587	5396	2465	1323	368	1235	1535	1171	2304
CA15-3	7302		11372	12445	8397	4099	575	4240	4429	3978	8756
CA19-9	524		4486	**5667**	892	948	583	177	289	194	382
CA72-4	3025		12336	12841	4890	3703	2070	2550	3346	2694	4174
CEA	5518		12287	13178	6655	3361	471	3310	4113	3278	5862
c-myc (1-262)	1614		5739	7811	3038	2001	2310	5028	552	335	2382
c-myc (408-439)	3208		23978	24386	6031	7037	6594	3297	5171	4308	3398
CRP	1202		NT	NT	NT	NT	1734	NT	NT	NT	NT
Cyclin-B1	1572		6938	5552	3162	2571	1884	678	673	679	1258
Cyclin-D1	1925		8794	8137	3762	3123	2445	2173	1697	1173	1714
EGF	1130		9813	**11461**	1596	2095	2681	1282	2148	1663	836
EGFR	362		**8131**	**7515**	1445	1973	1689	785	1349	1131	560
FasL	390		**7044**	**4480**	1536	1808	1601	351	1125	724	305
gp100	4208		NT	NT	2790	NT	NT	8604	3650	1734	2244
HCG	424		**5100**	4171	1692	1431	1281	560	1173	844	437
Her2/Neu	996		657	900	996	222	242	324	439	322	2754
H-Ras	467		**7360**	**8378**	1917	1892	1014	82	109	51	1426
IL-6	3108		11192	10372	2897	3395	3553	3667	3160	3955	1419
IL-8	897		**11435**	**12641**	2195	2957	2972	1089	2052	1427	882
K-Ras	1376		9209	11345	2381	2508	1400	1528	2254	1866	1572
MART-1	7387		NT	NT	11075	NT	NT	7102	5480	1632	8656
Mesothelin	317		**3683**	**3190**	728	723	NT	607	1283	841	230
Muc-1	876		8329	6051	1703	2027	2060	894	846	471	521

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		Subject #	1	2	3	4	5	6	7	8	9
		Description	38 yof NS	83 yom CS	42 yof NS	53 yom FS	69 yom FS	70 yom FS	49 yof NS	53 yof NS	78 yom FS
Antigen	**95th Pctl**										
Osteopontin	404		821	407	242	282	259	513	176	137	389
p53	5067		6973	5811	2943	1380	2485	806	1202	805	972
PDGF-BB	831		4358	4039	6312	1175	1369	415	644	424	1473
PDGFR-a	987		6613	8411	1543	1551	1128	989	1489	1154	980
PSA	809		**10909**	**10419**	7417	2975	3150	1268	2712	1766	893
Survivin	1150		9976	10230	1600	2454	4617	1225	2253	1712	817
Transglutaminase	541		**8890**	**11414**	1801	2238	2257	1158	1842	1520	898
Tyrosinase	1766		NT	NT	2406	NT	NT	2347	4550	3206	2238
VEGF	1533		10377	12583	3152	3287	2856	1177	2576	1132	1386
# Markers Tested			32	32	35	33	31	35	35	35	36
# Outlying Markers			31	31	31	26	23	20	18	16	16
% Outlying			**97**	**97**	**89**	**79**	**74**	**57**	**51**	**46**	**44**

All autoantibody values represent mean fluorescence intensity (MFI) determined by Luminex analysis. 95 percentile determined for each autoantibody by analysis of all subjects (N=205). Shaded values are above 95th percentile cutoff (outliers), shaded and bold values represent >10-fold increase over cutoff value. yom-year old male, yof-year old female. CS-current smoker, FS-former smoker, NS-no smoking history. NT-not tested.