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Autoantigens in Vitiligo Identified by the Serological Selection of a Phage-Displayed Melanocyte cDNA Expression Library

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Vitiligo is an acquired idiopathic hypomelanotic disorder characterized by circumscribed depigmented macules resulting from the loss of cutaneous melanocytes. Although the exact cause of vitiligo remains obscure, autoimmunity may play a role in the development of the disease. The present study was undertaken to investigate the applicability of phage display technology to identify B-cell autoantigens in vitiligo. A melanocyte cDNA phage display library was subjected to rounds of enrichment with vitiligo patient IgG. Subsequently, enriched IgG-binding peptides representing putative autoantigens were identified by sequencing their encoding cDNAs. Radioimmunoassays were used to confirm the immunoreactivity of vitiligo patient ($n=61$) and control ($n=28$) sera to several of the putative autoantigens. Non-segmental vitiligo patient sera ($n=53$) were positive for antibody (Ab) reactivity to gamma-enolase (8%); alpha-enolase (9%); heat-shock protein 90 (13%); osteopontin (4%); ubiquitin-conjugating enzyme (15%); translation-initiation factor 2 (6%); and GTP-binding protein, Rab38 (15%). Ab reactivity to at least one of the previously unknown autoantigens was detected in 51% of patients with non-segmental vitiligo. In contrast, Ab reactivity in a group of patients with segmental vitiligo ($n=8$) was not demonstrated. Overall, the study indicated that the targets of autoantibodies in vitiligo patients can be revealed by employing the methodology of phage display.

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INTRODUCTION

Vitiligo is an acquired idiopathic hypomelanotic disorder characterized by circumscribed depigmented macules resulting from the loss of cutaneous melanocytes. Two major clinical subtypes are distinguished, namely, segmental vitiligo, which follows dermatomal lines, and non-segmental (generalized) vitiligo, which includes symmetrical and acrofacial manifestations of the disease (Taieb, 2000). Although the exact cause of vitiligo remains to be established, autoimmunity has been suggested to play a role in the development of the disease, as it is frequently associated with autoimmune disorders (Alkhateeb *et al.*, 2003). Furthermore, autoantibodies (Naughton *et al.*, 1983) and autoreactive T lymphocytes (Ogg *et al.*, 1998; Lang *et al.*, 2001; Palermo *et al.*, 2001; Van den Boorn *et al.*, 2009) that target pigment cells have been detected in patients with vitiligo. Several genes that have a role in regulating the immune response have been associated with susceptibility to vitiligo, including *AIS1, AIS2, AIS3*, *NALP1*, and *PTPN22,* and certain antigen (Ag) specificities of the major histocompatibility complex (Spritz, 2007). In addition to the autoimmune hypothesis, the theory that metabolic dysregulation can lead to the production of toxic metabolites that damage melanocytes with resultant vitiligo is well documented (Schallreuter *et al.*, 2005; Dell'Anna and Picardo, 2006). Notably, *in vitro* studies have provided a link and a temporal sequence connecting cellular oxidative stress and the immune response in vitiligo: stressed melanocytes were found to mediate dendritic cell activation with the consequent dendritic cell effector functions having a role in the destruction of pigment cells (Kroll *et al.*, 2005). Conceivably, autoimmune responses may arise as a secondary phenomenon following melanocyte destruction with aberrant immune reactivities then amplifying the damage to pigment cells.

The role of autoantibodies in the destruction of melanocytes in vitiligo is uncertain, although vitiligo-associated autoantibodies are able to destroy melanocytes *in vitro* by complement-mediated damage and antibody (Ab)-dependent cellular cytotoxicty, and *in vivo* after passive immunization of

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Abbreviations: Ab, antibody; Ag, antigen; HSP, heat-shock protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SC, scleroderma; UCE, ubiquitin-conjugating enzyme

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nude mice grafted with human skin (Norris *et al.*, 1988; Gilhar *et al.*, 1995). Studies have revealed that autoantibodies in vitiligo patients are commonly directed against pigment cell Ags that are located on the surface of the cell, although these remain unidentified (Cui *et al.*, 1992). In addition, specific vitiligo-associated autoantigens have been identified, including tyrosinase (Song *et al.*, 1994), tyrosinase-related protein-1 (Kemp *et al.*, 1998a), tyrosinase-related protein-2 (Okamoto *et al.*, 1998), Pmel17 (Kemp *et al.*, 1998b), transcription factor SOX10 (Hedstrand *et al.*, 2001), and the melanin-concentrating hormone receptor 1 (Kemp *et al.*, 2002). Although Abs to pigment cells may not be the direct etiological agents of vitiligo, further studies of anti-melanocyte Ab reactivity are particularly valuable as they may reveal autoantigens that are important for pathological T-cell responses in vitiligo patients (Ogg *et al.*, 1998; Lang *et al.*, 2001; Palermo *et al.*, 2001; Van den Boorn *et al.*, 2009).

The aim of the present study was to identify vitiligo-associated autoantigens using the phage display technique based on the pJuFo cDNA expression system (Crameri and Suter, 1993). This methodology permits both the expression of cDNA libraries and the covalent attachment of the expressed products as fusion proteins on the surface of filamentous phage particles, thus allowing the selective enrichment of phage particles that display IgG-binding peptides in rounds of biopanning with patient IgG samples.

RESULTS

Enrichment of phage particles displaying IgG-binding peptides by vitiligo patient IgG

A melanocyte cDNA phage display library was subjected to five rounds of biopanning (Figure 1) against a pool of biotinylated IgG. Each of the 10 IgG samples included in the pool was prepared from an individual vitiligo patient selected from a cohort of patients who had no other autoimmune disease (Table 1). After each round of enrichment, the number of phage particles eluted was estimated by infecting *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) and plating on a selective medium. A 20-fold increase in the ratio of phage particles eluted to the phage particles applied for enrichment was noted after the fifth round of biopanning indicating enrichment of the phage display library (Table 2). There was no significant change in the range of insert sizes from the multiple rounds of enrichment (Table 2), suggesting that there was no apparent negative selection on the basis of size constraints of the cDNAs.

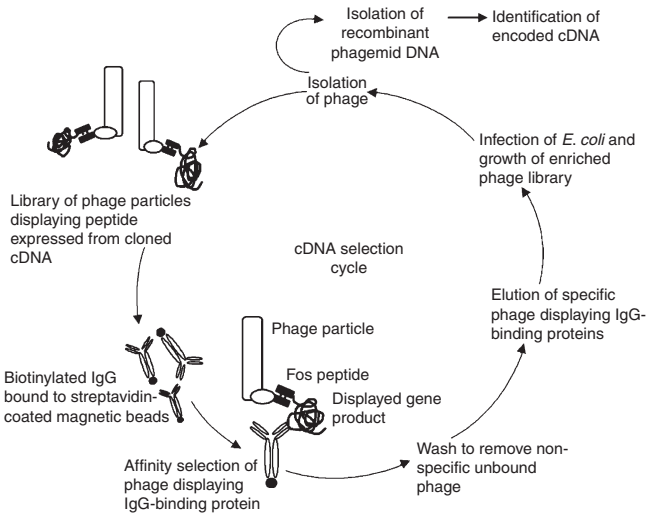

Figure 1. Enrichment of the melanocyte cDNA phage display library with vitiligo patient IgG. The melanocyte cDNA phage display library was incubated with biotinylated vitiligo patient IgG. The phage–antigen–IgG complexes were captured on Dynabeads M-280 Streptavidin. Non-specifically bound and unbound phage particles were removed by washing. IgG-bound phage particles were eluted and used to infect *E. coli* XL1-Blue and amplified for use in further rounds of selection.

Identification of IgG-binding proteins enriched by vitiligo patient IgG

After the fifth round of enrichment, 60 randomly chosen individual clones were grown, phagemid DNA isolated from each culture, and this analyzed by polymerase chain reaction (PCR) amplification with 1192 and 1500 primers. The PCR amplification products were purified and sequenced to identify cDNA inserts by BLAST searches of international databases. Of the 60 clones sequenced, 28 cDNAs encoded proteins of known function (Table 3) and 10 encoded proteins with undetermined functions (data not shown). All proteins were in-frame with pJuFo-Sfi-encoded Fos peptide that would allow for expression and display on phage particles. Of the remainder, 16 cDNAs encoded proteins of known or undetermined function that were not in-frame for expression and display on phage particles, 4 clones had no significant

Table 1. Characteristics of patients from whom IgG was used for selective enrichment of the melanocyte cDNA phage display library

Patient[1]	Sex	Age (years)	Disease duration (years)	Vitiligo type
V1	F	52	6	Symmetrical
V2	F	40	9	Symmetrical and periorificial
V3	F	54	33	Symmetrical and periorificial
V4	M	77	7	Symmetrical and periorificial
V5	F	43	12	Symmetrical
V6	M	23	1	Periorificial
V7	F	58	4	Segmental
V8	F	37	27	Symmetrical with segmental patch
V13	M	68	10	Symmetrical and periorificial
V17	F	70	<1	Symmetrical

F, female; M, male; MCHR1, melanin-concentrating hormone receptor 1; TRP, tyrosinase-related protein.
[1]No patient had clinical manifestations of any other autoimmune disease. All patients were negative for autoantibodies to tyrosinase (Kemp *et al.*, 1997a), TRP-1 (Kemp *et al.*, 1998a), TRP-2 (Kemp *et al.*, 1997b), Pmel17 (Kemp *et al.*, 1998b), MCHR1 (Kemp *et al.*, 2002), and SOX10 (Hedstrand *et al.*, 2001), except patient V7 who was weakly positive for tyrosinase autoantibodies (Kemp *et al.*, 1997a), and patients V6 and V13 who were positive for MCHR1 autoantibodies (Kemp *et al.*, 2002).

Table 2. Analysis of rounds of biopanning of the melanocyte cDNA phage display library with vitiligo patient IgG

Round	Phage applied (cfu)	Phage eluted (cfu)	Ratio phage eluted:phage applied (E:A)	Fold increase in ratio of E:A[1]	Recombinant phage (%)	Size range of cDNA inserts (bp)
1	1.3×10^{11}	7.2×10^{3}	5.5×10^{-8}	1.0	19/24 (79%)	1000–2300
2	3.3×10^{10}	1.2×10^{3}	3.6×10^{-8}	0.7	19/24 (79%)	1000–2500
3	2.3×10^{10}	1.0×10^{3}	4.4×10^{-8}	0.8	20/24 (83%)	800–2500
4	1.8×10^{10}	8.4×10^{3}	4.8×10^{-7}	8.8	21/24 (88%)	800–2500
5	1.8×10^{10}	20.9×10^{3}	1.1×10^{-6}	20.7	20/24 (83%)	800–2500

cfu, colony-forming unit
[1]The ratios of E:A phage are normalized against the ratio of E:A phage in the first round of biopanning.

Table 3. Enriched cDNA-encoded proteins with known function isolated from biopanning of the melanoctye cDNA phage display library with vitiligo patient IgG

Protein identity	Accession number	No. of clones[1]	First amino acid[2]	Previously reported as an autoantigen
Ubiquitin-conjugating enzyme	BC000468	2/60	1	No
Translation-elongation factor 6	NM002212	2/60	1	No
Osteopontin	XM011125	2/60	1	Type I diabetes mellitus (Fierabracci *et al.*, 1999); Rheumatoid arthritis (Sakata *et al.*, 2001)
Translation-elongation factor 1 alpha	NM005348	2/60	1	Systemic lupus erythematosus (Frampton *et al.*, 2000); Atopic dermatitis (Ohkouchi *et al.*, 1999)
Heat-shock protein 90	NM005348	1/60 2/60	206 71	Systemic lupus erythematosus (Conroy *et al.*, 1994); Melanoma with melanoma-associated hypopigmentation (Kiniwa *et al.*, 2001)
Alpha-enolase	BC004458	1/60	20	Systemic lupus erythematosus (Pratesi *et al.*, 2000); Premature ovarian failure (Sundblad *et al.*, 2006)
Gamma-enolase	BC002745	1/60 1/60	1 23	Systemic lupus erythematosus (Pratesi *et al.*, 2000)
SAR1 gene homolog A (GTPase)	BC003658	1/60	1	No
Melanin-concentrating hormone receptor 1	NM005297	1/60	1	Vitiligo (Kemp *et al.*, 2002)
Ribosomal protein L9	NM000661	2/60	1	No
Ribosomal protein L24	NM145729	1/60	1	No
Translation-initiation factor 2	BC002513	1/60	1	Melanoma with melanoma-associated hypopigmentation (Kiniwa *et al.*, 2001)
GTP-binding protein Rab38	NM022377	2/60	1	Melanoma (Zippelius *et al.*, 2007)
Ribosomal protein S18B	NM014046	2/60	1	Colorectal cancer (Somers *et al.*, 2002)
Tyrosine hydroxylase	NM199292	1/60	214	Autoimmune polyglandular syndrome type 1 (Hedstrand *et al.*, 2000)
Triosphosphate isomerase 1	NM000365	1/60	1	No
Integrin beta 4	NM000213	1/60	1	No
Ribosomal RNA processing protein	BC020641	1/60	1	No

[1]Number of clones representing the identified cDNA sequence.
[2]The first amino acid of the identified cDNA coded for in the clone. The ATG start codon of the identified cDNA is designated as encoding amino acid residue number 1.

matches, and 2 clones did not contain a cDNA insert (data not shown). More than one copy of several putative Ags was present in the final selected library (Table 3), a further indication that the biopanning process with vitiligo patient IgG had enriched the phage display library with Ab-binding peptides.

Seven potential autoantigens enriched from the biopanning procedure were chosen for further analysis in

radioimmunoassays to determine Ab reactivity in a panel of vitiligo patient sera. These included alpha-enolase, gamma-enolase, osteopontin, and heat-shock protein (HSP) 90, all of which had been identified as the targets of autoantibodies in autoimmune disease (Table 3), and translation-initiation factor 2 and GTP-binding protein, Rab38, previously reported as autoantigens in melanoma (Table 3). Putative autoantigens having specific expression and function in pigment cells were GTP-binding protein, Rab38, and ubiquitin-conjugating enzyme (UCE) (Table 3), and these were therefore analyzed further. The cDNA encoding each putative autoantigen was cloned into pcDNA3 using Ag-specific primers (Supplementary Table S1), as described in Materials and Methods.

Results of radioimmunoassays with vitiligo patient and control sera

Plasmids containing putative autoantigen-encoding cDNAs (Supplementary Table S1) were translated *in vitro*, as detailed in Materials and Methods, to provide radiolabelled proteins. Sera from 61 vitiligo patients and 28 healthy controls were then tested for immunoreactivity to each putative autoantigen in radioimmunoassays. For each serum, an Ab index was assigned, this being the mean Ab index of at least two experiments (SD of $<10\%$) (Figure 2). The upper limit of normal for each assay was calculated using the mean Ab index $+3$SD of the population of 28 healthy individuals (Figure 2). Any serum with an Ab index above the upper limit of normal was designated as positive for Ab reactivity.

The results of the radioimmunoassay are summarized in Table 4. Sera from 28 controls were negative for Abs to all the Ags tested, except for two individuals who had immunoreactivity to HSP90 and alpha-enolase in one case, and to HSP90 and osteopontin in the second case (Table 4). No patient with segmental vitiligo was positive for Abs to any of the autoantigens analyzed (Table 4). In the patients with non-segmental vitiligo, Ab reactivity to the individual Ags ranged from 6 to 15% of the total patients tested (Table 4). In addition, Ab responses to the individual Ags in patients with and without an autoimmune disease, ranged from 0 to 38 and 3 to 18%, respectively (Table 4).

A significant difference in the prevalence of Abs in the control and non-segmental vitiligo patient groups was noted

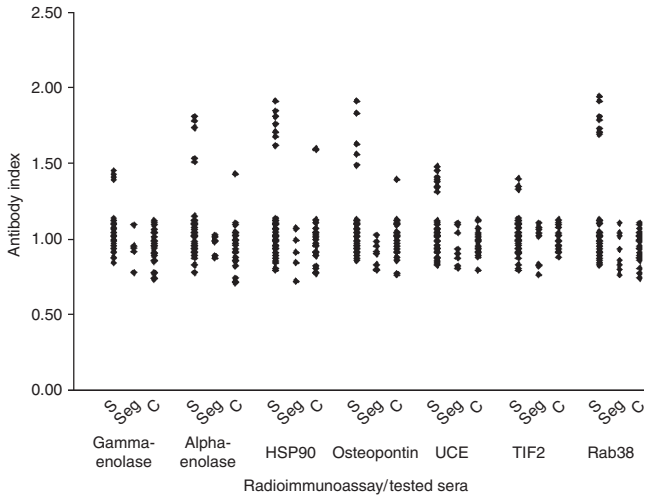

Figure 2. Antibody (Ab) indices of vitiligo patient and control sera in radioimmunoassays. Non-segmental vitiligo patient ($n=53$), segmental vitiligo patient ($n=8$) and control ($n=28$) sera were analyzed in radioimmunoassays for Ab reactivity to gamma-enolase, alpha-enolase, HSP90, osteopontin, ubiquitin-conjugating enzyme (UCE), translation-initiation factor 2 (TIF2), and GTP-binding protein, Rab38, as detailed in Materials and Methods. The Ab index shown for each serum sample is the mean of at least two experiments. The upper limits of normal for each radioimmunoassay were Ab indices of gamma-enolase, 1.30; alpha-enolase, 1.40; HSP90, 1.59; osteopontin, 1.38; UCE, 1.22; TIF2, 1.23; GTP-binding protein, Rab38, 1.64. S, symmetrical vitiligo patients; Seg, segmental vitiligo patients; C, healthy controls.

Table 4. Immunoreactivity of vitiligo patient and control sera to putative autoantigens

Antigen	Controls[1] (n=28)	Non-segmental vitiligo[1] (n=53)	P-value[2]	Segmental vitiligo[1] (n=8)	P-value[2]	Non-segmental vitiligo without autoimmune disease[1] (n=40)	P-value[2]	Non-segmental vitiligo with autoimmune disease[1] (n=13)	P-value[2]	P-value[3]
Gamma-enolase	0 (0)	4 (8)	0.29	0 (0)	—	3 (8)	0.27	1 (8)	0.32	1.00
Alpha-enolase	1 (4)	5 (9)	0.66	0 (0)	1.00	1 (3)	1.00	4 (31)	0.03	0.01
HSP90	2 (7)	7 (13)	0.49	0 (0)	1.00	2 (5)	1.00	5 (38)	0.02	0.01
Osteopontin	1 (4)	5 (9)	0.66	0 (0)	1.00	1 (3)	1.00	4 (31)	0.03	0.01
Ubiquitin-conjugating enzyme	0 (0)	8 (15)	0.04	0 (0)	—	7 (18)	0.04	1 (8)	0.32	0.66
Translation-initiation factor 2	0 (0)	3 (6)	0.55	0 (0)	—	3 (8)	0.26	0 (0)	—	—
GTP-binding protein Rab38	0 (0)	8 (15)	0.04	0 (0)	—	7 (18)	0.04	1 (8)	0.32	0.66

[1]The number of antibody-positive sera with percentage in parentheses.
[2]P-value calculated using Fisher's exact test for comparing the prevalence of antibodies in vitiligo patient and in control groups. $P<0.05$ was considered significant.
[3]P-value calculated using Fisher's exact test for comparing the prevalence of antibodies in non-segmental vitiligo (with or without autoimmune disease) patient groups. $P<0.05$ was considered significant.

Table 5. Analysis of antibody reactivity to the identified autoantigens in vitiligo patients and controls

Antibody reactivity	Controls (*n*=28)	Non-segmental vitiligo (*n*=53)	*P*-value[1]	Segmental vitiligo (*n*=8)	*P*-value[1]	Non-segmental vitiligo without autoimmune disease (*n*=40)	*P*-value[1]	Non-segmental vitiligo with autoimmune disease (*n*=13)	*P*-value[1]	*P*-value[2]
Negative	26 (93%)	26 (49%)	<0.0001	8 (100%)	1.00	23 (58%)	0.002	3 (23%)	<0.0001	0.06
Positive	2 (7%)	27 (51%)		0 (0%)		17 (43%)		10 (77%)		

[1]*P*-value calculated using Fisher's exact test for comparing the prevalence of antibody reactivity in vitiligo patient and control groups. $P<0.05$ was considered significant.
[2]*P*-value calculated using Fisher's exact test for comparing the prevalence of antibodies in non-segmental vitiligo (with or without autoimmune disease) patient groups. $P<0.05$ was considered significant.

only for anti-UCE and anti-GTP-binding protein, Rab38, Abs (Table 4). In the cohort of patients with non-segmental vitiligo, but no autoimmune disease, a significant difference from the controls was apparent again only in the case of the prevalence of Abs against anti-UCE and anti-GTP-binding protein, Rab38, Abs (Table 4). A significant difference in the prevalence of Abs in patients with non-segmental vitiligo and with autoimmune disease when compared with both the controls and the non-segmental vitiligo (without autoimmune disease) patient group was noted for alpha-enolase, HSP90, and osteopontin (Table 4).

Analysis of Ab reactivity to the identified autoantigens in vitiligo patients

An overall analysis of Ab reactivity to the identified autoantigens in the vitiligo patient and control groups is shown in Table 5. Ab reactivity to at least one of the reported autoantigens was detected in 27 of 53 (51%) patients with non-segmental vitiligo, but in only 2 of 28 (7%) controls and in 0 of 8 (0%) patients with segmental vitiligo. In the non-segmental patient groups with and without autoimmune disease, humoral immune responses to the analyzed autoantigens were demonstrated in 10 of 13 (77%) and in 17 of 40 (43%) individuals tested, respectively.

A significant difference in the prevalence of immunoreactivity in the controls and patients was evident only in the non-segmental vitiligo patient group (Table 5). The prevalence of Ab reactivity was also increased significantly in the non-segmental vitiligo patient groups (with or without autoimmune disease) when compared with controls (Table 5). No significant difference was apparent when comparing the non-segmental vitiligo patient groups with or without autoimmune disease (Table 5).

The details of the 27 non-segmental vitiligo patients who were positive for Ab reactivity to the identified autoantigens are summarized in Table 6. A total of 14 and 13 patients showed humoral immune responses to one and to two Ags, respectively (Table 6). In addition, in the 17 patients without an autoimmune disease, 10 and 7 had immunoreactivity to 1 and to 2 Ags, respectively, whereas in the 10 patients with an autoimmune disorder, 4 and 6 had Ab targeted against 1 and 2 Ags, respectively (Table 6). The majority of Abs against alpha-enolase, HSP90, and osteopontin were identified in non-segmental vitiligo patients with autoimmune disease with 13/17 of the detected reactivities (Table 6). In contrast, the majority of Abs to UCE, translation-initiation factor 2, GTP-binding protein, Rab38, were revealed as targets of the humoral immune response in non-segmental vitiligo patients without an autoimmune disorder with 17/19 of the detected reactivities (Table 6).

For comparison, the details of the 26 non-segmental vitiligo patients who were negative for Ab reactivity to the autoantigens identified in this study are given in Table 7. These details are compared with those of patients positive for Ab responses in Table 8. There was no apparent correlation between the detection of autoantibodies to the reported autoantigens and either disease duration, the age of disease onset, or the clinical sub-type of non-segmental vitiligo (Table 8). In addition, a correlation was not evident between the extent and/or activity of vitiligo and the number of autoantibodies detected. The prevalence of autoimmune disorders and of Abs to the melanin-concentrating hormone receptor 1 was increased in the group of patients with immunoreactivity to the identified autoantibody targets (Table 8).

DISCUSSION

The combination of affinity selection and biological amplification employed in phage display technology makes it a particularly useful technique for the isolation of previously unknown autoantigens. Large cDNA libraries can be screened in a single experiment, and even if their expression in the original library is rare, IgG-binding peptides can be identified through repeated rounds of enrichment. Furthermore, displayed proteins may assume their native structure (Skerra and Pluckthun, 1988), and the libraries are screened in liquid-phase in contrast to conventional immunoscreening on nitrocellulose membranes. Both these attributes are important for detecting Ab binding to conformational rather than linear epitopes. In this study, the technique of phage display based on the pJuFo cDNA expression system (Crameri and Suter, 1993) was employed to identify melanocyte autoantigens in vitiligo.

Several putative autoantigens were enriched from the melanocyte cDNA phage display library by their ability to bind to IgG from vitiligo patients. Four of the autoantibody

Table 6. Details of non-segmental vitiligo patients with antibody reactivity to the identified autoantigens

	Antibody reactivity							Patient details				
Patient	Gamma-enolase	Alpha-enolase	HSP90	Osteopontin	Ubiquitin-conjugating enzyme	Translation-initiation factor 2	GTP-binding protein Rab38	Vitiligo type	Autoimmune disease[1]	Age at vitiligo onset (years)	Disease duration (years)	MCHR1 antibodies[2]
V1 (F)	+	+	–	–	–	–	–	S	–	46	6	–
V2 (F)	–	–	–	–	+	–	–	S/P-o	–	31	9	–
V3 (F)	–	–	–	–	+	–	+	S/P-o	–	21	33	–
V4 (M)	–	–	–	–	–	+	+	S/P-o	–	70	7	–
V6 (M)	–	–	+	+	–	–	–	P-o	–	22	1	+
V12 (M)	–	–	–	–	+	–	–	S	–	17	10	+
V13 (M)	–	–	–	–	+	–	–	S/P-o	–	58	10	+
V18 (M)	–	–	–	–	–	+	+	S	–	72	2	+
V25 (F)	–	–	–	–	–	–	+	S	–	6	13	–
V29 (M)	–	–	–	–	+	–	–	S	–	73	2	+
V31 (M)	–	–	–	–	+	–	+	S	–	37	9	–
V33 (F)	–	–	–	–	+	–	–	S	–	51	3	–
V40 (M)	–	–	–	–	–	–	+	S	–	41	<1	–
V46 (F)	–	–	–	–	–	+	–	S/Seg	–	<1	37	+
V49 (M)	–	–	–	–	–	–	+	S	–	51	5	–
V50 (F)	+	–	–	–	–	–	–	S	–	20	3	+
V52 (M)	+	–	+	–	–	–	–	S	–	14	16	+
V14 (M)	–	+	–	+	–	–	–	S	ATD	58	1	–
V20 (M)	–	–	–	–	+	–	–	S	AA	20	50	–
V21 (M)	–	–	–	–	–	–	+	S	AA	41	2	–
V34 (F)	–	–	+	+	–	–	–	S	ATD	19	40	–
V42 (F)	–	+	+	–	–	–	–	S	SLE	51	4	–
V43 (F)	–	–	+	–	–	–	–	S	ATD	50	8	–
V47 (F)	+	+	–	–	–	–	–	S	SC	50	8	–
V48 (F)	–	–	–	+	–	–	–	S	ATD	19	21	–
V54 (F)	–	–	+	+	–	–	–	S	ATD	40	10	+
V55 (F)	–	+	+	–	–	–	–	S	ATD	48	10	+

+, positive for antibody reactivity; –, negative for antibody reactivity; AA, alopecia areata; ATD, autoimmune thyroid disease; F, female; M, male; P-o, periorificial; S, symmetrical; SC, scleroderma; SLE, systemic lupus erythematosus; S/Seg, symmetrical with segmental patch.
[1] –, No autoimmune disease
[2]Data from Kemp *et al.*, 2002 and Gottumukkala *et al.*, 2006.

targets analyzed, namely, alpha-enolase, gamma-enolase, osteopontin, and HSP90, have been defined as autoantigens in several autoimmune diseases (Conroy *et al.*, 1994; Fierabracci *et al.*, 1999; Pratesi *et al.*, 2000; Sakata *et al.*, 2001; Sundblad *et al.*, 2006); hence they are likely to be markers of autoimmunity, but not specific to vitiligo. Indeed, the majority of vitiligo patients with Ab reactivity to alpha-enolase, osteopontin, and HSP90 also had an autoimmune disorder. Interestingly, autoantibody responses to HSP70 and HSP90 have been detected in a single melanoma patient with concurrent melanoma-associated hypopigmentation (Kiniwa *et al.*, 2001), and recent studies have demonstrated the release of HSP70 from stressed melanocytes (Denman *et al.*, 2008). The anti-HSP Abs found in some vitiligo patients may therefore result from an immune response to extracellular HSPs.

Translation-initiation factor 2 has been reported as an autoantigen in a single melanoma patient with concurrent melanoma-associated hypopigmentation (Kiniwa *et al.*, 2001). Abs to this protein were detected at a low frequency in only 3 of 53 (6%) non-segmental vitiligo patients. The reason for the autoantigenicity of this protein remains undetermined, but other translation–initiation and translation–elongation factors have been reported as autoantigens in other conditions (Ohkouchi *et al.*, 1999; Frampton *et al.*, 2000; Kiniwa *et al.*, 2001).

Table 7. Details of non-segmental vitiligo patients without antibody reactivity to the identified autoantigens

	Patient details				
Patient	Vitiligo type	Autoimmune disease	Age at vitiligo onset (years)	Disease duration (years)	MCHR1 antibodies[1]
V5 (F)	S	–	31	12	–
V8 (F)	S/Seg	–	10	27	–
V9 (F)	S	–	40	36	+
V10 (M)	S	–	56	4	–
V15 (F)	S	–	39	9	–
V16 (M)	S	–	24	45	–
V17 (F)	S	–	70	<1	–
V19 (F)	S	–	53	5	–
V22 (M)	S	–	13	26	–
V23 (M)	U	–	15	28	–
V24 (F)	S/P-o	–	21	20	–
V26 (F)	S	–	12	23	–
V27 (F)	S	–	19	4	–
V28 (F)	S	–	39	5	–
V30 (F)	S	–	52	1	–
V32 (M)	S/Seg	–	37	49	–
V35 (F)	S	–	23	<1	–
V36 (F)	P-o	–	20	9	–
V38 (M)	S	–	31	<1	–
V39 (M)	S	–	13	19	–
V41 (F)	S	–	39	9	–
V45 (F)	S	–	9	5	–
V51 (M)	S	–	67	<1	–
V11 (M)	S	ATD	48	1	–
V44 (F)	S	ATD	30	5	+
V53 (F)	S	ATD	45	6	–

–, No autoimmune disease; ATD, autoimmune thyroid disease; F, female; M, male; P-o, periorificial; S, symmetrical; S/Seg, symmetrical with segmental patch; U, universal.
[1]Data from Kemp *et al.*, 2002 and Gottumukkala *et al.*, 2006.

Table 8. Comparison of non-segmental vitiligo patients with and without immunoreactivity to the identified autoantigens

Patient detail	Patients with immunoreactivity (*n*=27)	Patients with no immunoreactivity (*n*=26)
Sex (M/F)	13/14	9/17
Mean age (range)	45 years (14–76 years)	50 years (19–77 years)
Mean age at onset of vitiligo (range)	38 years (<1–73 years)	33 years (9–70 years)
Mean disease duration (range)	12 years (<1–50 years)	14 years (<1–49 years)
Vitiligo subtype:		
Symmetrical	21 (78%)	21 (81%)
Symmetrical/ periorificial	4 (15%)	1 (4%)
Symmetrical/ segmental	1 (4%)	2 (8%)
Periorificial	1 (4%)	1 (4%)
Universal	0 (0%)	1 (4%)
Autoimmune disease	10 (37%)	3 (12%)
MCHR1 autoantibodies	10 (37%)	2 (8%)

F, female; M, male; MCHR1, melanin-concentrating hormone receptor 1

Autoantigens with an association to melanocyte function were also isolated from the biopanning procedure. The melanocyte-expressed GTP-binding protein, Rab38, proposed as a regulator of trafficking of melanogenic enzymes from the trans-Golgi network to melanosomes (Wasmeier *et al.*, 2006), is expressed at an increased level in vitiligo melanocytes (Stromberg *et al.*, 2007). Earlier, Rab38 was reported as an autoantigen in melanoma, but not in patients with vitiligo (Zippelius *et al.*, 2007). This contrast to our study may reflect differences in the patients included, such as the total number of individuals, and in the technique used for Ab identification. The UCE, which mediates degradation of the transcriptional regulator of genes involved in melanogenesis, microphthalmia-associated transcription factor (Xu *et al.*, 2000), has not been previously characterized as an autoantigen. However, another transcription-related protein that has been identified as a vitiligo-associated autoantigen, is the melanocyte-specific transcription factor SOX10 (Hedstrand *et al.*, 2000).

Further autoantigens of note that were not analyzed in radioimmunoassays against the panel of vitiligo patient sera include integrin beta 4 and tyrosine hydroxylase. Integrin beta 4 is an adhesion molecule vital for the attachment of epithelial cells to the basal layer of the epidermis (Van der Neut *et al.*, 1996). How this specific protein relates to melanocytes has not been reported, but the expression of several adhesion molecules has been shown to be down-regulated in vitiligo melanocytes (Stromberg *et al.*, 2007), and the detachment and transepidermal loss of melanocytes have been demonstrated in vitiligo (Gauthier *et al.*, 2003). Tyrosine hydroxylase has already been revealed as an autoantigen in patients with autoimmune polyglandular syndrome type 1 (Hedstrand *et al.*, 2000), but has not been studied as an immunological target in vitiligo.

Our earlier studies have detected Abs to several autoantigens in vitiligo patients. Usually, these have been present at a low frequency, for example, tyrosinase, 11% (Kemp *et al.*, 1997a); tyrosinase-related protein-1, 5% (Kemp *et al.*, 1998a); tyrosinase-related protein-2, 5%, (Kemp *et al.*, 1997b); Pmel17, 5% (Kemp *et al.*, 1998b); transcription factor SOX10, 3% (Hedstrand *et al.*, 2000); and

melanin-concentrating hormone receptor 1, 16% (Kemp *et al.*, 2002). Likewise, the prevalence of Abs to each specific target identified here was generally low in the population of patients with non-segmental vitiligo reaching 15% only in the case of two Ags, UCE and GTP-binding protein, Rab38. The low prevalence of different Abs may indicate that vitiligo represents a spectrum of disease pathologies, which lead to a similar clinical manifestation rather than a disorder with a single pathogenicity.

Autoimmunity has been suggested to play a role in the development of non-segmental vitiligo: it is frequently associated with autoimmune disorders (Taieb, 2000; Alkhateeb *et al.*, 2003), responds less well to autologous melanocyte grafting (Gauthier and Surleve-Bazeille, 1992; Taieb, 2000), and is responsive to treatment with immunosuppressive agents (Lepe *et al.*, 2003). In this study, Ab reactivity to at least one of the identified autoantigens was detected in 51% (27/53) of patients with non-segmental vitiligo. Interestingly, no significant difference in the overall prevalence of immunoreactivity to the cohort of autoantigens was found between patients with non-segmental vitiligo and autoimmune disease and those without an associated autoimmune disorder. However, the majority of Abs against alpha-enolase, HSP90, and osteopontin were identified in non-segmental vitiligo patients with autoimmune disease, whereas the majority of Abs to UCE, translation-initiation factor 2, GTP-binding protein, Rab38, were revealed as targets of the humoral immune response in non-segmental vitiligo patients without an autoimmune disorder. In contrast, we could not detect Ab reactivity in patients with segmental vitiligo to any of the autoantigens analyzed, although only eight samples were available for testing. Notably, it has been suggested that segmental vitiligo has a different etiology from the non-segmental type, not involving autoimmune pathomechanisms (Taieb, 2000).

Our results indicate that putative vitiligo autoantigens can be initially identified by phage display technology, including those proteins with previously demonstrated IgG-binding properties in association with autoimmune disease and candidate autoantigens with proposed specific functions in melanocytes. It is possible that pooling patient IgG samples for enrichment experiments, as done here, may dilute a positive autoantibody for which the other pooled IgG are negative. This may work against the identification of uncommon but important autoantigens and hence, the biopanning of single patient IgG samples may be an alternative in further studies.

Future work will be required to determine whether the Abs to the specific autoantigens reported here have any pathological significance in vitiligo. Although vitiligo-associated autoantibodies have been shown to damage melanocytes by complement-mediated mechanisms and Ab-dependent cellular cytotoxicty (Norris *et al.*, 1988; Gilhar *et al.*, 1995), the role of the humoral immune response in vitiligo pathogenesis remains uncertain. In contrast, several studies have underlined the role of T lymphocytes in the initiation and progression of vitiligo (Ogg *et al.*, 1998; Lang *et al.*, 2001; Palermo *et al.*, 2001; Van den Boorn *et al.*, 2009). Melanocyte Ag-specific T cells are present in perilesional vitiligo skin and have cytotoxic activity against pigment cells (Ogg *et al.*, 1998; Lang *et al.*, 2001; Palermo *et al.*, 2001; Van den Boorn *et al.*, 2009). Furthermore, perilesional T cells also have the capacity to destroy melanocytes within skin tissue itself (Van den Boorn *et al.*, 2009). The Ags recognized by T cells with anti-melanocyte activity include tyrosinase, MART-1 (MelanA), and gp100 (Pmel17) (Ogg *et al.*, 1998; Lang *et al.*, 2001; Palermo *et al.*, 2001; Van den Boorn *et al.*, 2009). Previously, autoantibodies to tyrosinase (Song *et al.*, 1994) and Pmel17 (Kemp *et al.*, 1998b) have been detected in vitiligo patients, and autoantibodies may therefore be markers of important T-cell responses in vitiligo. With regard to this, studies to identify T-cell reactivity to the autoantigens characterized here are needed. Overall, the continued characterization of anti-melanocyte Ab reactivity in vitiligo patients is particularly valuable, as this may reveal autoantigens that are also the targets for T cells, which have adverse effects on pigment cells.

MATERIALS AND METHODS

Patient and control data

Sera from 61 vitiligo patients were used in this study. In 53 patients (21 male, 32 female; mean age: 48 years with range 14–77 years; mean disease duration: 13 years with range <1–50 years; mean age at onset: 35 years with range <1–73 years), vitiligo was classified as non-segmental (symmetrical, 42; periorificial, 2; symmetrical/periorificial, 5; universal, 1; symmetrical with segmental patch, 3). Of these patients, 40 had no other autoimmune disorders and no family history of autoimmune disease, and 13 had one other autoimmune disorder: autoimmune thyroid disease 9; alopecia areata, 2; systemic lupus erythematosus, 1; scleroderma, 1. In eight patients, vitiligo was classified as segmental (three male, five female; mean age: 33 years with range 16–58 years; mean disease duration: 2 years with range <1–4 years; mean age at onset: 33 years with range 14–54 years). Of these patients, six had no other autoimmune disorders and no family history of autoimmune disease, and two had alopecia areata. Sera from 28 healthy individuals (10 male, 18 female; mean age: 34 years with range 21–59 years) were used as controls. All sera were kept at −20 °C. The study was approved by the South Sheffield Research Ethics Committee, Sheffield, UK and all patients gave an informed consent. The study was conducted in accordance with the Declaration of Helsinki Principles.

IgG biotinylation

Immunoglobulin-G was isolated from the sera of patients using protein G Sepharose 4 Fast Flow affinity column chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). Eluted IgG fractions were dialysed extensively against phosphate-buffered saline (PBS; pH 7.4; Sigma, Poole, UK) and concentrated using an Amicon Concentrator (Amicon, Beverley, MA). The concentrated IgG was filter-sterilized with a Millex Filter Unit (Millipore, Bedford, MA) and the final concentration measured by photometry at 280 nm. Samples were stored at a concentration of 10 mg ml^{-1} at −20 °C. Biotinylation of the IgG was performed using EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce, Rockford, IL), according to the manufacturer's protocol. All biotinylated IgG samples were stored at a concentration of 2 mg ml^{-1} at 4 °C.

Melanocyte cDNA phage display library

Construction of the melanocyte cDNA phage display library in vector pJuFo-Sfi was described previously (Kemp *et al.*, 2002). The library was stored in PBS at −80 °C.

Biopanning experiments

In biopanning experiments, 15-µl aliquots of biotinylated IgG were incubated with 200 µg of Dynabeads M-280 Streptavidin (Dynal Biotech, Oslo, Norway), prepared according to the manufacturer in 235 µl of sterile water, and incubated at 4 °C for 30 minutes on a rotating platform to permit Ab–bead binding. To block any non-specific phage particles from binding to the beads later in the procedure, 300 µl of 2% (w/v) dried milk powder in PBS containing 10% (w/v) glycerol were added to the bead–IgG suspension and incubation continued for 1 hour at 4 °C. The bead–IgG complexes were separated from the blocking buffer using a Dynal Magnetic Particle Concentrator (Dynal Biotech), washed twice and finally resuspended in 150 µl of PBS/0.05% (w/v) Tween 20 before the addition of a 100-µl sample of phage display library. The suspension was then incubated overnight at 4 °C to allow interaction of the Ab–bead complexes with peptides displayed on the surface of the phage particles. The bead–IgG complexes were washed extensively with PBS/0.05% (w/v) Tween 20 to remove any unbound phage particles. Bound phage particles were eluted from the bead–IgG complexes with 150 µl of 100 mM HCl (adjusted to pH 2.2 with solid glycine) and the beads magnetically separated from the supernatant, which was neutralized using 9 µl of 2 M Tris. The phage particle suspension was subsequently used to infect 2 ml of exponentially growing *E. coli* XL1-Blue (Stratagene) for 15 minutes at room temperature. Aliquots of the infected cells were then plated onto selective medium to allow the recovery of individual bacterial clones for analysis and to determine the numbers of phage particles eluted.

To generate a phage display library for a further round of selection, the infected *E. coli* XL1-Blue culture was superinfected with 1×10^{12} plaque-forming units of VCMS13 helper phage (Stratagene) at room temperature for 30 minutes. The culture was subsequently transferred to 100 ml of LB (Luria–Bertani) medium (Sambrook *et al.*, 1989) supplemented with 50 µg ml^{-1} ampicillin, 10 µg ml^{-1} tetracycline, and 10 µg ml^{-1} kanamycin. After overnight incubation at 37 °C, the culture was centrifuged and phage particles precipitated from the supernatant with 0.2 volumes of 20% (w/v) polyethylene glycol 4000/2.5 M NaCl. The phage particles were resuspended in 2–3 ml of PBS and stored at −20°C. This first round library enriched in phage particles displaying IgG-binding peptides was used in a second round of selective enrichment, as detailed above. In all, five rounds of biopanning were undertaken.

Throughout, phage particle titers were determined by infecting log-phase *E. coli* XL1-Blue with an aliquot of phage display library and then plating out samples onto LB agar containing 50 µg ml^{-1} ampicillin and 10 µg ml^{-1} tetracycline.

For analysis, individual bacterial clones were cultured and phagemid DNA prepared using a Wizard Minipreps DNA Purification System (Promega, Southampton, UK). To confirm the presence of a cDNA insert, phagemid DNA (50 ng samples) was subjected to 36 cycles of PCR amplification in a DNA Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT) using previously detailed reaction conditions (Kemp *et al.*, 2001) with primers 1192 (5′-CCGCTGGATTGTTATTACTCGCTG-3′) and 1500 (5′-TGCAAGGC GATTAAGTTGGGTAAC-3′) (Life Technologies, Paisley, UK), which flank the *Sfi*I cloning site in pJuFo-Sfi (Kemp *et al.*, 2002). The PCR amplification products were analyzed by agarose gel electrophoresis (Sambrook *et al.*, 1989) and purified according to a Wizard PCR Preps DNA Purification System (Promega). Sequencing of PCR amplification products with primer 1192 was carried out using a BigDye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 3730 capillary sequencer (Applied Biosystems). The cDNA sequences were compared with international databases using the BLAST network service of the National Centre for Biotechnology Information (Bethesda, MD).

Cloning of putative autoantigens

Total melanocyte RNA (1 µg) was used to prepare cDNA in a 30-µl reaction containing 10 mM dithiothreitol, 1 mM dNTPs (Promega), 250 ng random primers (Promega), 600 U M-MLV reverse transcriptase (Promega) and 1 × M-MLV reverse transcriptase buffer (Promega). The cDNA was subjected to PCR amplification using conditions as described previously (Kemp *et al.*, 2001) with Ag-specific primers (Supplementary Table S1). Restriction sites for *Eco*RI and *Xba*I were incorporated into the forward and reverse primers, respectively, to allow subcloning of the PCR amplification product into pcDNA3 (Invitrogen, Abingdon, UK) and subsequent expression of the cDNA from the T7 promoter in the vector. Recombinant plasmids (Supplementary Table S1) were purified with a Qiagen Plasmid Maxi Kit (Qiagen, Crawley, UK) and verified by sequencing as above with T7 and SP6 primers (Promega).

Radioimmunoassays assays

In vitro translation of putative autoantigens cloned in pcDNA3 (Supplementary Table S1) was performed according to a TnT T7 Coupled Reticulocyte Lysate System (Promega) with translation-grade [^{35}S]-methionine (1000 Ci mmol^{-1}; 10 mCi ml^{-1}; Amersham Pharmacia Biotech, Little Chalfont, UK). SDS-PAGE of *in vitro* translated Ags was carried out in 10% (w/v) polyacrylamide gels using standard protocols (Sambrook *et al.*, 1989). Gels were processed and autoradiographed at −70 °C, as detailed elsewhere (Kemp *et al.*, 1998a).

Assays were executed as previously detailed (Kemp *et al.*, 1998a) with serum at a final dilution of 1:100 and with each sample in duplicates. An Ab index for each serum tested in the radioimmunoassays was calculated as counts per minute immunoprecipitated by tested serum/mean counts per minute immunoprecipitated by 28 healthy control sera. Each serum was tested in at least two experiments and the mean Ab index was calculated. The upper limit of normal for the assay was calculated using the mean Ab index + 3 SD of the population of 28 healthy individuals. Any serum with an Ab index above the upper limit of normal was designated as positive for Ab reactivity.

Statistical analysis

The frequency of Abs was compared between patients and controls using Fisher's exact test for 2 × 2 contingency tables. *P*-values <0.05 (two-tailed) were regarded as significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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