emphasised that such Performance Standards are intended for providing an accelerated validation only for versions of the LLNA that involve only minor modifications to the standard assay as described in OECD TG 429 (1).

Both ECVAM and ICCVAM (46) have been working on developing Performance Standards for the LLNA. Their two draft documents were presented and discussed at the ECVAM workshop.

Against this background, the workshop participants debated and suggested the criteria and a list of reference chemicals that should be considered in developing Performance Standards for the LLNA. Consideration was given, not only to the performance of the test as it was assessed in the peer review of the LLNA, but also to the experience gained from the use of the test during the years that have followed that evaluation.

The proposed Performance Standards, as outlined in this workshop, were subsequently considered by ECVAM and ICCVAM as a contribution to the ongoing process of harmonisation of the two documents.

Proposed Performance Standards

Essential test method components

For these Performance Standards to apply, the modified LLNA must comply fully with the OECD TG 429 (1), with the exception of the way in which the lymph node cell proliferation is measured. Ideally, there should be no alteration to the prediction model, but it is accepted that this may be necessary, dependent on how the proliferation is measured. Significant changes to the prediction model may, of course, trigger the need for a more substantial assessment than is provided for by this proposed set of Performance Standards.

Recommended reference chemicals

Reference chemicals should be used to assess whether the performance of modified LLNA methods is comparable with that of the standard LLNA. A set of 20 chemicals, which comprise 13 sensitisers, 5 non-sensitisers, 1 false positive and 1 false negative in the standard LLNA, are suggested for the purpose (Table 1). For the selection of the relevant substances, the following criteria have been considered:

a) The chemicals should be readily available from one or more commercial suppliers.

b) Ideally, for each of the selected chemicals, LLNA and guinea-pig data (from either the GPMT or the Buehler test) should be available, as well as evidence for the elicitation of contact sensitisation in humans.

c) For the majority of the chemicals, there should be a clear consensus about the presence or absence of skin sensitising potential, where the results of all the existing predictive test results match the human data. The exceptions to this are sodium lauryl sulphate (SLS) and nickel sulphate, which have been selected as being false positive and false negative, respectively, in the standard LLNA.

d) There should be no doubt that the skin sensitising activity is attributable to the defined chemical structure, rather than to a contaminant.

e) Defined EC3 values should be available for the selected chemicals.

f) The selected chemicals should not be unstable or require unusual storage or transport conditions.

Within the pool of chemicals selected, attempts have been made to represent the variety of chemical classes and properties associated with skin sensitisation, although it must be recognised that, with a limited number of chemicals, not all the variables can be fully represented.

Furthermore, the list of chemicals has been selected to reflect the range of skin sensitisation potencies which are known to exist, but with emphasis on those chemicals considered to show weak or moderate potency, since these will provide the best assurance of the retention of test sensitivity, commensurate with the absolute minimum of additional animal testing.

In additional, in any evaluations of this type, it is important to define, in advance, whether a chemical is sufficiently sensitising to be formally classified according to the current regulations as a skin sensitiser. Only if this is the case, should it be identified as a positive in a predictive test, with the consequence that the existence of positive human data (e.g. in clinical case reports) is not, of itself, sufficient to mean that a chemical should be regarded as a substance which should test positive in a predictive assay.

The number of chemicals proposed should be sufficient to permit an assessment of the performance of modified versions of the LLNA, while minimising the number of animals required for such an evaluation.

Performance requisites: predictive capacity and reliability

Predictive capacity is defined as the capacity of a method to predict the accepted reference values

Table 1: Reference chemicals recommended for the evaluation of modified LLNA methods for the identification of skin sensitisation hazard

Chemical name	CAS No.	Physical form	Vehicle	EC3 LLNA	Value	n	Reference
Benzoquinone	106-51-4	Solid	AOO	+	0.01[a]	1	Gerberick *et al.* (54)
2,4-Dinitrochlorobenzene	97-00-7	Liquid	AOO	+	0.04[b]	13	Basketter *et al.* (20)
4-Phenylenediamine	106-50-3	Solid	AOO	+	0.11[b]	10	Basketter *et al.* (20)
Isoeugenol	97-54-1	Liquid	AOO	+	1.5[b]	31	Basketter *et al.* (20)
2-Mercaptobenzothiazole	149-30-4	Solid	DMF	+	1.7[a]	1	Gerberick *et al.* (54)
Diethyl maleate	141-05-9	Liquid	AOO	+	3.9[b]	2	Basketter *et al.* (55) Ryan *et al.* (56)
α-Hexyl cinnamic aldehyde	101-86-0	Liquid	AOO	+	9.9[b]	15	Basketter *et al.* (20)
Eugenol	97-53-0	Liquid	AOO	+	10.1[b]	4	Basketter *et al.* (20)
Citral	5392-40-5	Liquid	AOO	+	13[a]	1	Gerberick *et al.* (54)
Phenyl benzoate	93-99-2	Solid	AOO	+	20[a]	1	Gerberick *et al.* (54)
Cinnamic alcohol	104-54-1	Solid	AOO	+	21[a]	1	Gerberick *et al.* (54)
Imidazolidinyl urea	39236-46-9	Solid	DMF	+	24[a]	1	Gerberick *et al.* (54)
Ethyl acrylate	140-88-5	Liquid	AOO	+	32.4[b]	2	Dearman *et al.* (57) Warbrick *et al.* (58)
Methyl salicylate	119-36-8	Liquid	AOO	–			Gerberick *et al.* (54)
Isopropanol	67-63-0	Liquid	AOO	–			Gerberick *et al.* (54)
Salicylic acid	69-72-7	Solid	AOO	–			Gerberick *et al.* (54)
Lactic acid	50-21-5	Solid	DMSO	–			Gerberick *et al.* (54)
Hexane	110-54-3	Liquid	AOO	–			Gerberick *et al.* (54)
Sodium lauryl sulphate	151-21-3	Solid	DMF	False positive			Gerberick *et al.* (54)
Nickel sulphate	10101-98-1	Solid	DMSO	False negative			Basketter & Scholes (59)

[a]single EC3 values; [b]mean EC3 values; AOO = acetone/olive oil (4:1 v/v); DMF = dimethylformamide; DMSO = dimethylsulphoxide. + = sensitiser in the standard LLNA; – = non-sensitiser in the standard LLNA.

(47). This is also referred to as the accuracy of a test method. The predictive capacity of a proposed test method should, when evaluated by using the recommended reference chemicals, at least meet or exceed that of the validated reference method. For this purpose, the threshold concentration at which a test chemical is positive in a new test method must fall within the range of 0.5–2.0× the published threshold (EC3) values from the standard LLNA in the relevant vehicle.

Reliability refers to the extent to which a test method can be performed reproducibly within and between laboratories and over time, when conducted by using the same protocol (26).

For the assessment of the inter-laboratory reproducibility, it is recommended that, when tested in at least three different laboratories, the threshold concentrations at which both α-hexyl cinnamic aldehyde (HCA) and 2,4-dinitrochlorobenzene (DNCB) are positive, fall within a range of 0.5× and 2.0× the published threshold (EC3) values in the standard LLNA.

Intra-laboratory reproducibility should be demonstrated by using HCA on four separate occasions over at least a 12-week period. Again, the threshold concentration should fall between 0.5× and 2.0× that of the expected threshold (EC3) value in the standard LLNA.

Alternative Endpoints to the Standard LLNA: Examples

This section describes three examples of alternative endpoints that are currently being developed for the LLNA.

1. A non-radioactive (non-RI) method, in which ATP content measurement is used as the endpoint

Principle

ATP is the principal energy source for all living organisms, and the amount of ATP determined is

known to correlate with the number of living cells (48, 49). Therefore, ATP content is considered to be a possible alternative endpoint to ^{3}H-thymidine incorporation as an index of cell number. An alternative LLNA involving the use of this endpoint as a non-RI method, termed the LLNA-DA (LLNA modified by Daicel, based on ATP content), has been developed (by Daicel Chemical Industries Ltd, Niigata, Japan; 50, 51). Measuring ATP contents has several advantages. The procedure for determining ATP content is easy and rapid, with a wide dynamic range for its stimulation index (SI).

Description of the method

In the protocol for the LLNA-DA, groups of female CBA/JNCrlj mice are treated by the topical application of 25µl of the test chemical (or the vehicle control) to the dorsum of both ears on days 1, 2, 3, and 7. Pretreatment with 1% SLS is carried out 1 hour before each application. On day 8, the auricular lymph nodes are excised. After recording the lymph node weight, single cell suspensions in phosphate-buffered saline are immediately prepared for each individual animal. The ATP content is determined by a luciferin–luciferase assay after appropriate sample dilution. For the determination of the ATP content, several measurement kits are commercially available (e.g. the Via Light™ HS Kit [Lonza Rockland, Inc., Rockland, ME, USA] and CheckLite™ 250 Plus [Kikkoman Corporation, Chiba, Japan]). A SI relative to the concurrent vehicle control is derived, and a SI of 3 is set as the cut-off value.

Summary of results

Thirty-one well-known chemicals were tested by the lead laboratory, and clear dose–response results were observed for weak sensitisers, as well as for strong or extreme sensitisers. The performance of this method showed good agreement with that of the standard LLNA or other test systems (Table 2). The accuracy of LLNA-DA compared to the standard LLNA was 93% (28/30), LLNA-DA *versus* guinea-pig tests was 80% (20/25), and LLNA-DA *versus* human tests was 79% (15/19). Similarly to the LLNA, the LLNA-DA uses a SI of 3 to discriminate between positive and negative responses. The EC3 values of the LLNA-DA, calculated from the positive tested chemicals, were compared with those of the original LLNA (Figure 1). This demonstrated that the EC3 values obtained with LLNA-DA were very similar to those obtained with the standard LLNA.

To evaluate inter-laboratory reproducibility, two large scale inter-laboratory validation studies were conducted in Japan. The results from 17 laboratories, with 14 chemicals under blind conditions in the two studies, showed that this method is sensitive, with little intra-laboratory variation. In the first study, acceptably small inter-laboratory variations in SI values were obtained for 10 chemicals, with the exception of two metallic salts. In the second study, with five chemicals, including three metallic salts, acceptably small variations for all the chemicals were obtained.

Conclusions

Compared with the standard LLNA, the LLNA-DA not only has a modified endpoint measurement, but also requires an adjustment to the dosing schedule and a pretreatment with SLS — changes which are necessary to enable the SI cut-off value of the LLNA-DA to retain that of standard the LLNA. As a consequence, this method cannot be considered to be a minor modification of the standard LLNA. However, as described above, the evidence concerning its sensitivity and reliability suggests that this approach looks promising.

2. A non-RI modification of the LLNA, based on BrdU incorporation

Principle

This modification of the LLNA has been developed as an alternative to the standard LLNA. It is based on BrdU (5-bromo-2´-deoxyuridine) incorporation in place of ^{3}H-thymidine incorporation, to measure lymph node cell proliferation (41).

Description of the method

The method based on BrdU incorporation is practically identical to the standard LLNA methodology, apart from the use of BrdU and colorimetric detection, for which a single intraperitoneal injection (5mg/mouse per injection) of BrdU was made on day 4. This administration schedule was found to be the most effective labelling protocol for yielding maximum SI values, based on preliminary study data with several different protocols. Approximately 24 hours after the BrdU injection, the auricular lymph nodes were removed, weighed, and stored at –20°C until analysed by using an enzyme-linked immunosorbent assay (ELISA) to measure the level of BrdU incorporation. In the current form of the test, cellular proliferative responses are measured by using a commercial BrdU detection

Table 2: Comparison of the concordances for 31 chemicals

Chemicals	LLNA-DA	LLNA*	GPMT/BA*	HMT/HPTA*
2,4-Dinitrochlorobenzene	+	+	+	
p-Phenylenediamine	+	+	+	+
Toluene diisocyanate	+	+		
Glutaraldehyde	+	+		
$K_2Cr_2O_7$	+	+	+	+
Phthalic anhydride	+	+	+	
Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
Cobalt chloride	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	–	+
Benzocaine	+	+/–	+	+/–
Abietic acid	+	+	+	+
α-Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazole	–	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	–	–
$NiSO_4$	–	–	+	+
Benzalkonium chloride	+	–	–	+
Propyl paraben	–	–	–	+/–
Diethylphthalate	–	–		
1-Bromobutane	–	–		
Methylsalicylate	–	–	–	–
Chlorobenzene	–	–	–	
Lactic Acid	–	–	–	
Hexane	–	–		–
Isopropanol	–	–	–	

*GPMT = guinea-pig maximisation test; BA = Buehler assay; HMT = human maximisation test; HPTA = human patch test allergen. *Results of the LLNA, GPMT/BA and HMT/HPTA are taken from references 18, 20, 54 and 60. + = sensitiser; – = non-sensitiser; +/– = ambiguous results.*

kit (e.g. one provided by Roche Diagnostics, Indianapolis, IN, USA; Cat. No. 11647229001). To perform the BrdU ELISA, the lymph nodes were crushed, passed through a #70 nylon mesh, and individual LNCs were suspended in 15ml of physiological saline. The cell suspension was added to the wells of a flat-bottomed microplate, in triplicate. After fixation and denaturation of the LNCs, anti-BrdU antibody was added to each well, and after rinsing, a substrate solution containing tetramethylbenzidine (TMB) was added and allowed to produce the chromogen. Absorbance at 370nm, with a reference wavelength of 492nm, was defined as the BrdU labelling index (41).

Summary of results

This method can display clear dose-related responses, and the potency class prediction is obtained with similar concentrations of test chemicals to those required in the standard LLNA (42, 43).

However, the potential lower sensitivity of non-RI alternative methods was also recognised as an issue. To evaluate the assay performance of this non-RI LLNA, 23 well-known chemicals, categorised as human contact allergens from class 1 to class 5, were tested, and the results were analysed to identify the best prediction model (Table 3).

Consequently, the conditions that set the cut-off point as SI > 1.5, with statistical significance between the treated and concurrent vehicle control or the cut-off point as SI > 1.5 with > 3 SD of the mean SI of the concurrent vehicle control, were adopted as the best endpoints for this non-RI LLNA method. By using these decision criteria, the highest concordance and lowest number of false negatives in the non-RI LLNA were obtained, as compared with the standard LLNA (52).

In addition, a novel approach to predicting the sensitisation potency of chemicals by comparison with known human contact allergens has been proposed as a useful application of this method (Table 4; 43). In this approach, nine well-known chemicals, categorised as human contact allergens from class 1 to class 5, were tested by the non-RI LLNA, with the reference allergens, 2,4-dinitrochlorobenzene (DNCB) as the class 1 human contact allergen, isoeugenol as the class 2 human contact allergen, and HCA as the class 3 human contact allergen. All the chemicals were assigned to the correct or adjacent classes.

Conclusions

The results suggested that this new strategy for a non-RI LLNA could provide both hazard identification and sensitisation potency data, which are prerequisites for judging the sensitisation risk for humans represented by new chemical products. These results indicate that a non-RI LLNA based on the BrdU incorporation, may be one of the promising alternatives to the standard LLNA.

3. The measurement of cell numbers and ear thickness as alternative non-radioactive endpoints in the LLNA

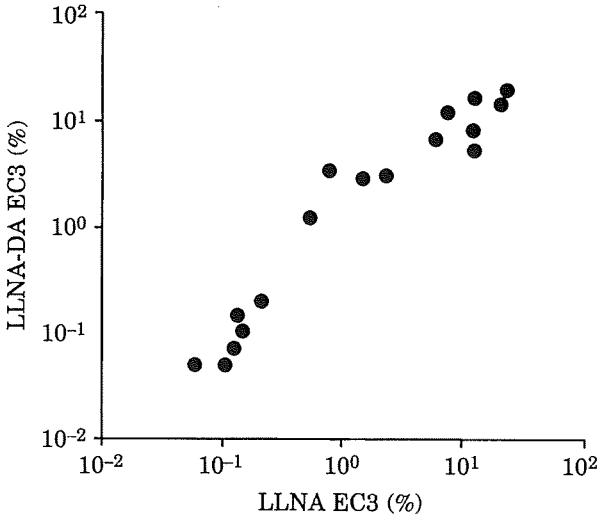
Principle

In the LLNA, lymphocyte proliferation in the auricular lymph node is measured by analysing ^{3}H-thymidine incorporation in the lymph node as a whole, after intravenous injection in the test mice. Based on the notion that the process of proliferation is induced within the lymph node, and

Table 3: Positive (+) and negative (–) classifications by different SI cut-off values in the non-RI LLNA based on BrdU incorporation

	Cut-off SI value																		
Chemical name	**3.0**	**2.9**	**2.8**	**2.7**	**2.6**	**2.5**	**2.4**	**2.3**	**2.2**	**2.1**	**2.0**	**1.9**	**1.8**	**1.7**	**1.6**	**1.5**	**1.4**	**1.3**	**RI**
2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,4-Benzoquinone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diphencyclopropenone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glutaraldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,4-Phenylenediamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2-Mercaptobenzothiazole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Cinnamic aldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isoeugenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,3-Aminophenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-(4-Isopropylphenyl) isobutyraldehyde	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
Citral	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eugenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydroxycitronellal	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
α-Hexyl cinnamic aldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isopropyl myristate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4-Chloroaniline	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aniline	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–
2-Hydroxypropylmethacrylate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isopropanol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phthalic acid diethyl ester	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Propylene glycol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dimethyl isophthalate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Figure 1: Correlation of EC3 values in the standard LLNA and the LLNA-DA



that the proliferation of lymphocytes results in increased numbers of lymphocyte after application of the sensitiser on the ear, assessment of lymphocyte numbers rather than lymphocyte proliferation as the read-out, has been proposed (33, 34). Many studies show that the ear-draining lymph nodes increase in size after the application of sensitisers on the ear. In addition, the number of lymphocytes is also increased after such treatment. There is good correlation between lymph node cell number counts and ^{3}H-thymidine incorporation (Figure 2).

Description of the method

This alternative non-RI test is based on the standard LLNA described in OECD TG 429 (1). Instead of assessing proliferative reactions directly, the method involves the removal of the lymph nodes, the preparation of single cell suspensions according to routine procedures, and the subsequent counting of cell numbers by using automated cell-counting devices. However, the proposed test also involves a number of other deviations from the standard LLNA: the use of BALB/c or NMRI mice, the excision of lymph nodes at day 4 instead of day 6, and the use of DAE433 as a vehicle. Also, a cut-off at a stimulation index of 1.4, subject to the provision of statistical significance, is used.

Some chemicals that have irritant capacity, also appear to be capable of inducing cell proliferation, and thus increase numbers of cells in the lymph nodes draining the ears on to which the test chemical is applied. Therefore, an additional endpoint on irritancy, i.e. the measurement of ear thickness, was added to this non-RI approach. Ear thickness can be measured in the same mice in which the modified LLNA is carried out. At the same time as when the lymph nodes are removed, punch biopsies of the ears are made and weighed (53). An increment in weight amounting to 1.2-times the control value, and which is statistically significant, is considered a positive result. The classification of chemicals is then based on both types of information (Table 5).

Summary of results

In a collaborative study, carried out by nine laboratories in Europe with 12 chemicals (including four irritants and one fully negative control) and BALB/c mice, the proposal to use lymph node cell numbers as the read-out for assessing the sensitising capacities of chemicals was put to the test (33, 34). The majority of the laboratories correctly identified the chemicals that were characterised as sensitisers, on the basis of statistically-significant increases in cell numbers. The results for HCA are shown in Table 6. The results from a sin-

Table 4: Classification criteria for the sensitisation potency of chemicals tested by the non-RI LLNA based on BrdU incorporation

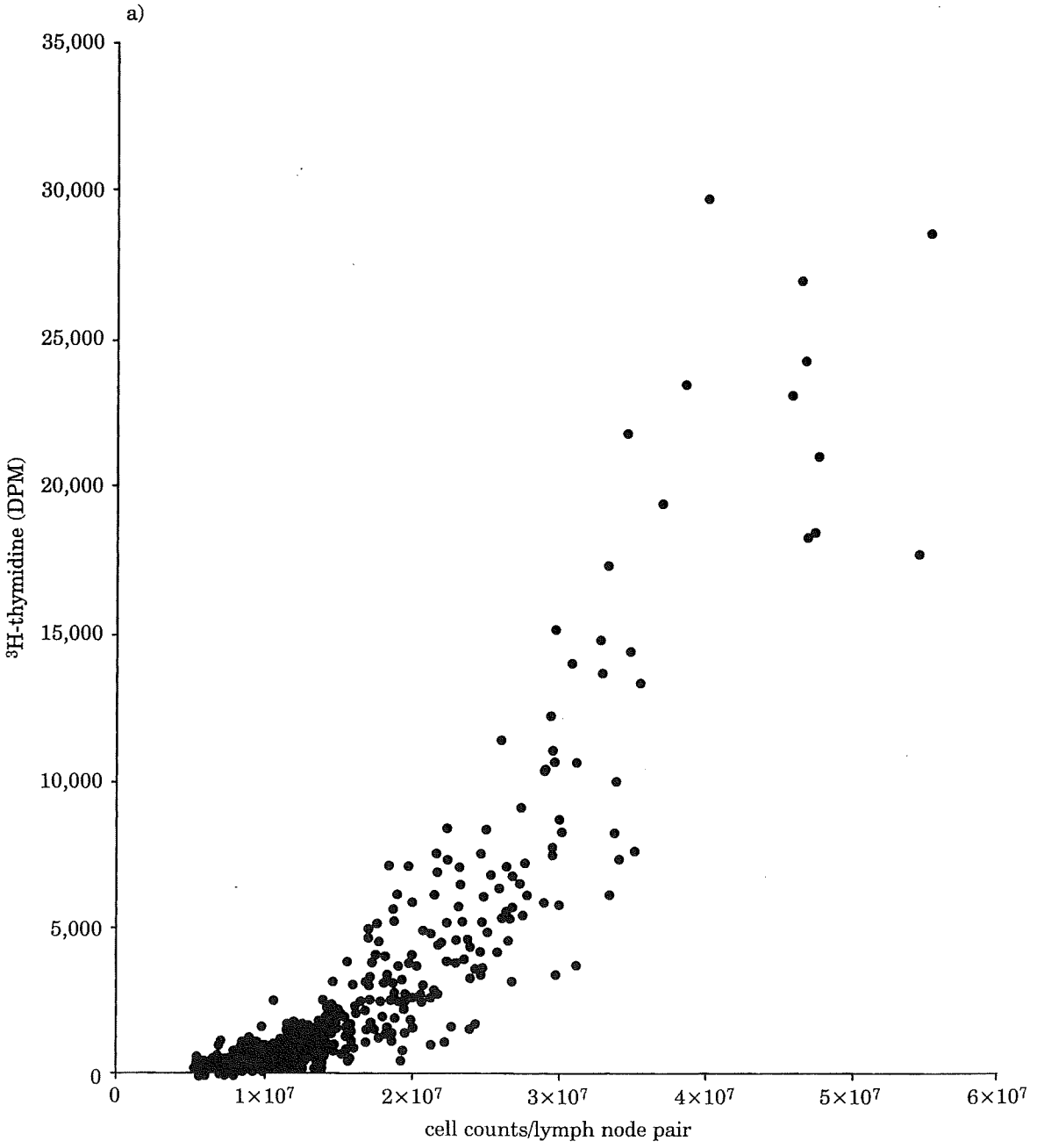
Human class	Requirements	Sensitisation class
1	SI for 2% test chemical ≥ SI for 2% DNCB	Strong sensitiser
2	SI for 2% test chemical < SI for 2% DNCB SI for 10% test chemical ≥ SI for 10% isoeugenol	Moderate sensitiser Moderate sensitiser
3	SI for 10% test chemical < SI for 10% isoeugenol SI for 50% test chemical ≥ SI for 50% HCA	Weak sensitiser Weak sensitiser
4–5	SI for 50% test chemical < SI for 50% HCA	Extremely weak or non-sensitiser

gle laboratory indicated that the assessment of potency by using this method was reproducible.

Conclusions

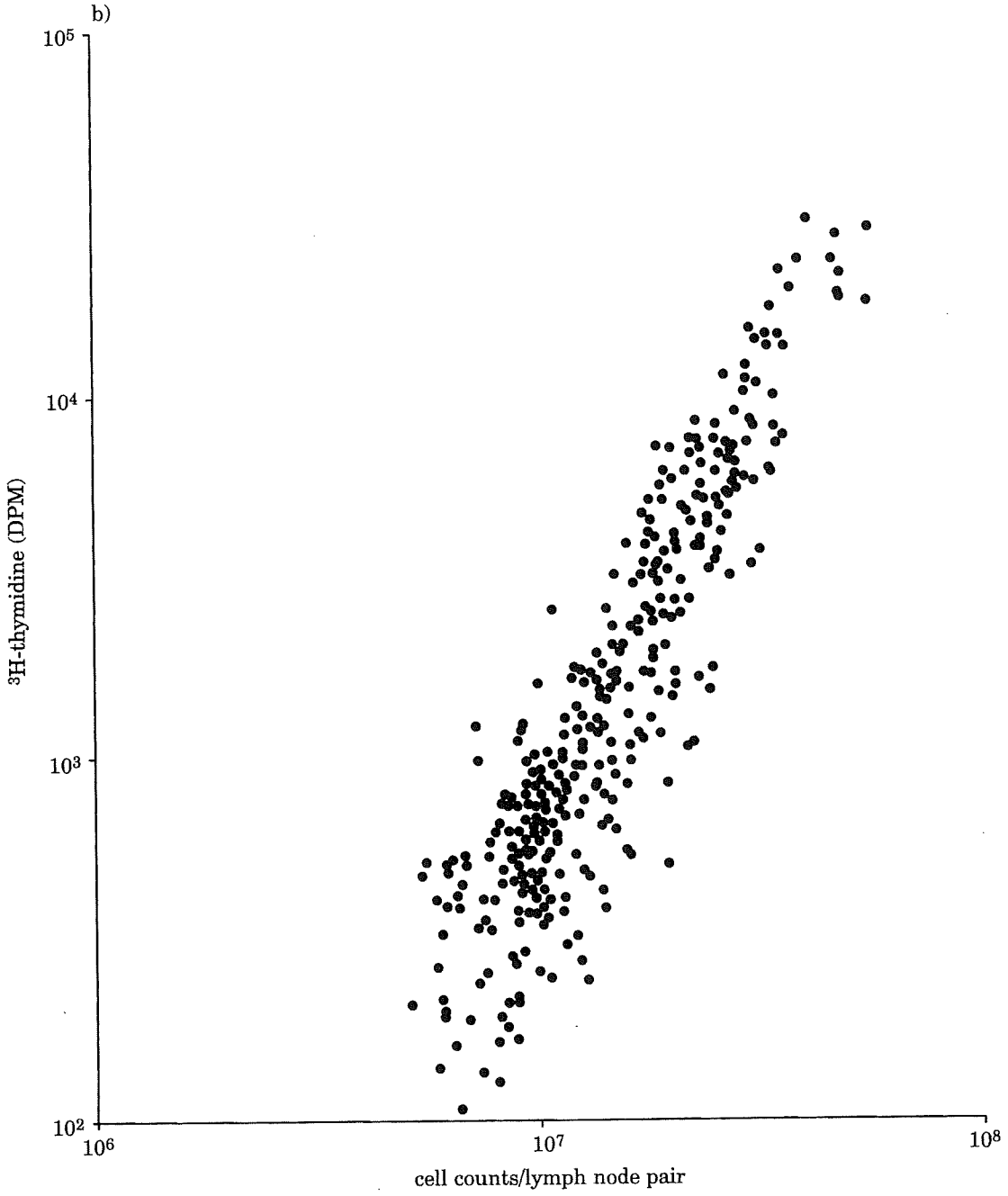
This proposed non-RI LLNA uses cell number as a correlate of cell proliferation, but, as other modifications to the standard LLNA were also made, the method constitutes a major change. There is value in the proposed test, in that it would, if validated, eliminate the use of a radioactive label. Moreover, the measurement of ear thickness may provide a means of providing further information that can help to discriminate true sensitisers from chemicals that induce increase lymphocyte numbers or proliferation in lymph nodes for other reasons. With regard to the counting of cell numbers,

Figure 2: Lymph node cell proliferation: cell counts compared with ^{3}H-thymidine uptake values



Data kindly provided by BGIA, Bonn, Germany. (b) shows an expansion of the data points in the bottom-left area of graph (a).

Figure 2: continued



Data kindly provided by BGIA, Bonn, Germany. (b) shows an expansion of the data points in the bottom-left area of graph (a).

further experience needs to be gained with this approach, in a setting where there are no other deviations from the standard LLNA, and formal comparisons of the same endpoints in the same animals are performed. Further experience in the interpretation of ear thickness measurements is also required, so that criteria can be set for judging when a positive ear swelling response can reverse the conclusion of sensitisation, based on proliferation of cells (numbers) in the LLNA. It should be noted that the additional endpoint of measuring ear thickness could also be used in combination with other (non-radioactive) modifications of the LLNA.

Table 5: A classification scheme for evaluating tests based on cell counting and the measurement of ear thickness

Endpoints	Results							
Lymph node weight	+	+	+	+	–	–	–	–
Lymph node cell counts	+	+	–	–	+	+	–	–
Assessment I	A/I	A/I	I	I	A	A	–	–
Acute skin reaction	–	+	+	–	–	+	+	–
Assessment II	–	I	I	–	–	I	I	–
Overall assessment	A	A/I	I	I?	A	A/I	I	–

+ = statistically-significant increase; A = allergen; I = irritant; Assessment I = evaluation on the basis of lymph nodes only; Assessment II = evaluation on the basis of ear thickness only.

Summary and Conclusions

The LLNA is becoming the skin sensitisation assay of first choice for many regulatory authorities. Consequently, there is an increasing drive to replace its radiolabel-based endpoint. A number of alternatives to the standard LLNA, which do not employ radiolabel, have already been developed. However, several of these efforts involve major changes to the standard LLNA protocol, so a substantial validation process will be required, to ensure that they retain the degree of sensitivity and specificity afforded by the standard method (1). Where the changes to the LLNA are only minor, retaining the same endpoint measure (proliferation in draining auricular lymph nodes), ECVAM and some other authorities (e.g. ICCVAM) have decided to consider the establishment of Performance Standards to allow for a streamlined validation process.

The ECVAM draft Performance Standards have been reviewed in this workshop, and recommendations have been made, which cover both strategic issues, such as the harmonisation of standards between validation authorities, and suggestions for the selection of chemicals and how the modified LLNA protocols and associated prediction models should perform in relation to them. In addition, three examples of modified LLNAs which avoid the use of radiolabelling have been considered (but not reviewed in detail). These have been presented in a common format, to display the principles on which they are founded, the current status of their evaluation, and brief conclusions on their performances. Whilst some of them may be relatively close to acceptability for use, the workshop participants agreed that none of them have yet been adequately validated as substitutes for the standard LLNA, either because they do not fit the criteria of "minor change" or because their data package is at present insufficient.

Recommendations

1. Wherever possible, Performance Standards should be harmonised between validation authorities.

2. Minor modifications to the assay can be assessed by using Performance Standards such as those recommended in this report.

Table 6: Comparisons of observed stimulation indices between different laboratories, for HCA in the non-RI LLNA based on cell counting

	Lab. 1	Lab. 2	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 6	Lab. 7
Vehicle	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3% HCA	1.05	1.20	0.92	1.03	1.21	1.06	1.05	1.30	0.98
10% HCA	1.11	**1.90**	**1.69**	1.15	**1.53**	1.29	1.24	**1.64**	**1.64**
30% HCA	**1.59**	**2.01**	**1.57**	**2.02**	**2.37**	**1.90**	**2.03**	**2.10**	**3.10**

Statistically-significant stimulation indices are marked in bold; laboratories 1 and 2 performed repeat experiments.

3. Minor modifications to the OECD TG 429 should be assessed by using a carefully selected set of chemicals and against a clear set of criteria, both of which have been detailed in this report.

4. For modifications to the OECD TG 429 which are not regarded as minor, it may also be the case that the information needed would not be as comprehensive as for a completely new test. Such data requirements should be judged on a case-by-case basis.

5. The progress that has been made in developing realistic alternative read-outs for the standard LLNA should be welcomed, and there should be further investment in research in this area.

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Clinical/Scientific Notes

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DISCORDANT CLINICOPATHOLOGIC PHENOTYPES IN A JAPANESE KINDRED OF FATAL FAMILIAL INSOMNIA

The GAC→AAC mutation at codon 178 (D178N) of prion protein (PrP) gene (*PRNP*) results in 2 distinct clinicopathologic phenotypes dependent on codon 129 polymorphism of the mutant allele: fatal familial insomnia (FFI) with methionine encoded in codon 129 and familial Creutzfeldt-Jakob disease (CJD) with valine.[1,2] However, some D178N patients who had homozygosity for methionine at codon 129 (D178N-129MM) were reported recently to have the CJD phenotype.[3-5] The cause for these clinicopathologic diversities is unclear. We report a Japanese son–mother pair who presented with FFI and CJD phenotypes.

Patient 1 (proband). A 54-year-old man, born to nonconsanguineous parents, developed dysphagia and loss of appetite. Later, he showed peculiar movement in sleep, followed by insomnia and hypersomnolence. Diplopia, intention tremor, ataxic gait, sleep apnea, fluctuant low-grade fever, tachycardia, hyperhidrosis, constipation, and impotence also followed. He was admitted to hospital 7 months after the onset of symptoms.

Neurologic examination showed mild memory disturbance, cerebellar ataxia, myoclonus, brisk deep tendon reflexes, sleep apnea, and dysautonomia. He did not show akinetic mutism. He moved continuously during sleep. EEG showed no periodic synchronous discharge (PSD), and polysomnography showed loss of deep sleep and marked reduction of REM sleep. Brain MRI showed only mild atrophy. In the cerebral cortex and thalamus, hypoperfusion and hypometabolism were detected by SPECT with ^{99m}Tc-ECD and PET with ^{18}F-2-fluorodeoxy-D-glucose. He died 13 months after the onset of symptoms. *PRNP* analysis, with informed consent, on leukocyte DNA showed D178N-129MM. Histologic examination showed spongiform changes limited to the cingulate gyrus and subiculum, and severe neuronal loss and fibrillary gliosis in the centromedian and dorsomedial nucleus of the thalamus and in the inferior olivary nucleus (figure, A, D, and G). Immunohistochemical analysis showed no pathologic PrP (PrP^{Sc}) deposition in the cerebral cortex, thalamus, or inferior olivary nucleus. Western blot analysis (WB) showed very small amount of type 2 PrP^{Sc} and the same glycosylation pattern of PrP^{Sc} as FFI (figure, I).[6,7]

Patient 2 (mother). A 60-year-old woman showed rapidly progressive dementia. She became mute 5 months later. Neurologic examination showed rigidity and brisk deep tendon reflexes, but no ataxia. EEG showed no PSD. She developed akinetic mutism and died in 1987, 14 months after the onset of symptoms. Histopathologic examination showed spongiform changes throughout the cerebral cortex, and mild neuronal loss and fibrillary gliosis in the dorsomedial nucleus of the thalamus and moderate neuronal loss in the inferior olivary nucleus (figure, B, E, and H). Immunohistochemical analysis of PrP^{Sc} visualized punctate and coarse granular deposits throughout the cerebral cortex (figure, C), and coarse granular deposits in thalamus (figure, F). Although the clinical course and histopathologic findings of patient 2 were compatible with CJD phenotype, the type and glycosylation pattern, including glycoform ratio, of PrP^{Sc} analyzed by WB were the same as patient 1, who presented as typical FFI (figure, I). However, a larger amount of PrP^{Sc} was detected than that of patient 1. *PRNP* analysis on preserved frozen brain tissue, performed with informed consent, revealed exactly the same as patient 1, D178N-129MM.

Discussion. A few reports describe the CJD phenotype in a genetically confirmed D178N-129MM kindred,[3-5] although one kindred showed both FFI and CJD phenotypes within the same family.[5] That kindred and ours indicate that certain factors other than codon 129 polymorphism in the normal allele determine the clinicopathologic phenotype of D178N-129MM.

Our WB suggests the causes of this clinicopathologic diversity. The detection of the larger amount of PrP^{Sc} in patient 2 than in patient 1 may indicate that the amount of PrP^{Sc} is related to the clinicopathologic D178N-129MM phenotype. However, the mechanism by which there is an increased amount of

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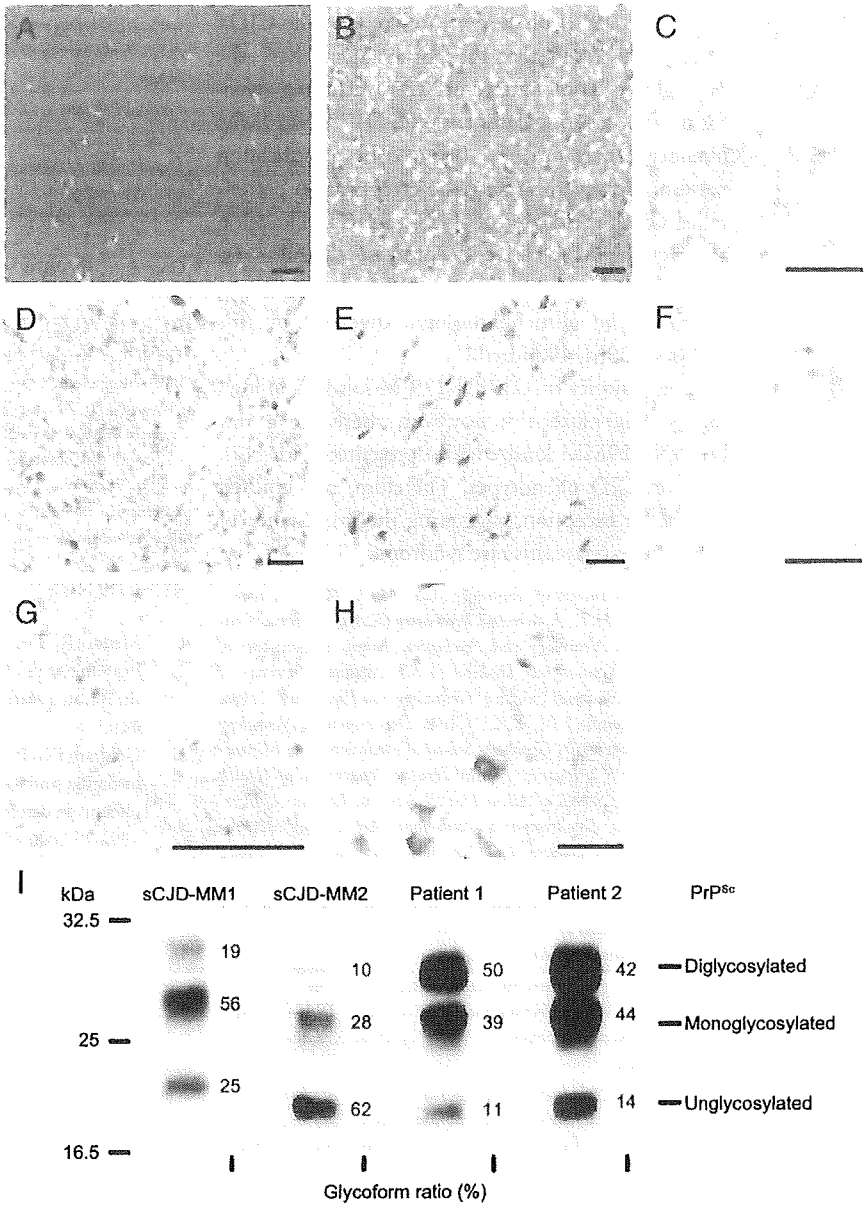
Figure Histopathologic analysis and Western blot analysis



(A–H) Histopathologic findings of patient 1 (A, D, and G) and patient 2 (B, C, E, F, and H); hematoxylin and eosin staining of the frontal cortex (A and B), immunohistochemistry using anti-prion antibody (3F4; Signet Laboratories, Dedham, MA) of the frontal cortex (C) and thalamus (F), Klüver-Barrera staining of the thalamus (D and E) and inferior olivary nucleus (G and H). No spongiform changes were detected in the frontal cortex of patient 1 (or at best partial changes; not shown). In comparison, severe and fine spongiform changes and neuronal loss were detected in patient 2. Patient 1 showed severe neuronal loss and gliosis in the thalamus (D) and inferior olivary nucleus (G). In contrast, patient 2 showed neuronal loss and gliosis, which were mild in the thalamus (E) and moderate in the inferior olivary nucleus (H). Immunohistochemistry in patient 2 showed punctate and coarse granular deposits of pathologic prion protein (PrP^{Sc}) (C) and of the thalamus (F). There also was perivacuolar staining of PrP^{Sc} in places of the cerebral cortex. Coarse granular deposits of PrP^{Sc} in the thalamus were more prominent than that of the cerebral cortex in patient 2. However, immunohistochemistry did not show any PrP^{Sc} deposit throughout the brain in patient 1 (not shown). PrP^{Sc} plaques were not found in either patient. Bar = 50 μm (A, B, D, E, G, and H), 20 μm (C and F). (I) Western blot analysis of patient 1, patient 2, and 2 sporadic Creutzfeldt-Jakob disease (sCJD) patients as disease control was performed with 3F4. Samples of the left two lanes, sCJD-MM1 and sCJD-MM2, were extracted from the sCJD patients who were homozygous for methionine at codon 129 and the types of PrP^{Sc} were type 1 and type 2. The applied amount of frontal cortex tissue was 1.667 mg wet weight in patient 1 and 0.025 mg wet weight in patient 2. Note that both patients 1 and 2 had type 2 PrP^{Sc} as sCJD-MM2. The smallest fragment of PrP^{Sc}, which represents unglycosylated fragment, is weaker than the other 2 fragments in both patients. The PrP^{Sc} glycoform ratio, quantified with Quantity One software using an imaging device, Vasa Doc 5000 (BioRad Laboratories), clarifies that the glycosylation pattern of patient 1 and patient 2 is different from that of sCJD-MM1 and sCJD-MM2. Note the strong band of PrP^{Sc} in patient 2 although a smaller amount of brain tissue was applied than that of patient 1.

PrP^{Sc} in this kindred is unknown. In addition, that both patients have the same type 2 PrP^{Sc} indicates that the type of PrP^{Sc} may not be related to the D178N-129MM phenotype, unlike sporadic CJD.[6] Finally, the result that the both patients have the same glycosylation pattern of PrP^{Sc}, including glycoform ratio, also indicates that the D178N-129MM phenotype may not be influenced by glycosylation pattern. However, because the glycosylation pattern reflects both the degree of glycosylation and location at which the PrP is cleaved by protease, careful investigation of the glycosylation of PrP is necessary to interpret the clinicopathologic diversity of this D178N-129MM kindred.

The majority of D178N-129 M kindred follows the genetic pattern[1,2]; however, there are a few D178N-129MM kindred who presented with both FFI and CJD phenotypes. Therefore, our kindred may be an exception, suggesting the importance of WB to investigate this rare syndrome.

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Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease

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To identify susceptibility variants for Parkinson's disease (PD), we performed a genome-wide association study (GWAS) and two replication studies in a total of 2,011 cases and 18,381 controls from Japan. We identified a new susceptibility locus on 1q32 ($P = 1.52 \times 10^{-12}$) and designated this as *PARK16*, and we also identified *BST1* on 4p15 as a second new risk locus ($P = 3.94 \times 10^{-9}$). We also detected strong associations at *SNCA* on 4q22 ($P = 7.35 \times 10^{-17}$) and *LRRK2* on 12q12 ($P = 2.72 \times 10^{-8}$), both of which are implicated in autosomal dominant forms of parkinsonism. By comparing results of a GWAS performed on individuals of European ancestry, we identified *PARK16*, *SNCA* and *LRRK2* as shared risk loci for PD and *BST1* and *MAPT* as loci showing population differences. Our results identify two new PD susceptibility loci, show involvement of autosomal dominant parkinsonism loci in typical PD and suggest that population differences contribute to genetic heterogeneity in PD.

Parkinson's disease (MIM168600) is one of the most common neurodegenerative diseases worldwide, affecting 1–2% of individuals aged ≥65 years[1]. Clinical features of PD result primarily from loss of dopaminergic neurons in the substantia nigra. Various medical treatments improve PD symptoms but do little to deter disease progression. Identifying genetic risk factors for PD will be helpful in elucidating the pathogenesis of the disease. Linkage studies have been successful in mapping genes for mendelian forms of parkinsonism: *SNCA* (encoding α-synuclein)[2] and *LRRK2* (refs. 3,4) in autosomal dominant forms, and *PARK2* (encoding parkin), *PINK1*, *PARK7* (encoding DJ-1) and *ATP13A2* in autosomal recessive forms[5,6]. However, mendelian forms of parkinsonism are rare compared to the far more common typical PD, a complex disorder caused by multiple genetic and environmental factors[7]. Association studies have evaluated variants in many candidate genes for PD[7], but only a few, such as common variants of *SNCA*[8–10] and rare mutations of *GBA*[11], have been identified as PD-susceptibility genes with genome-wide significance. Recently, GWASs in PD have provided association evidence at several loci, but not at the genome-wide significant level[12–14].

We conducted a GWAS and two subsequent replication studies for PD to identify further common variants that contribute to disease. In the GWAS stage, we genotyped 561,288 SNPs on autosomal and sex chromosomes using the HumanHap550 array (Illumina). The GWAS stage included 1,078 PD cases and 2,628 controls in the Japanese population (**Supplementary Note**). After SNP and sample quality control analyses, we used genotype data from 435,470 SNPs in 988 cases and 2,521 controls in the GWAS analysis (see Online Methods).

We tested for association between each SNP and PD using the Cochran-Armitage trend test with 1 d.f. The quantile-quantile plot showed a close match to test statistics expected under the null distribution (genomic inflation factor $\lambda = 1.055$ for PD) (**Fig. 1a,b**). This indicates minimal overall inflation of genome-wide statistical results due to population stratification and also reveals a number of SNPs whose *P* values exceed those expected under the null hypothesis. Seventeen SNPs showed $P < 5 \times 10^{-7}$, the threshold for genome-wide significance suggested by the Wellcome Trust Case Control Consortium[15] (**Fig. 1c**). All these SNPs were located on 4q22, a region harboring *SNCA* that was previously identified by us and others as a definite susceptibility gene for PD[8–10].

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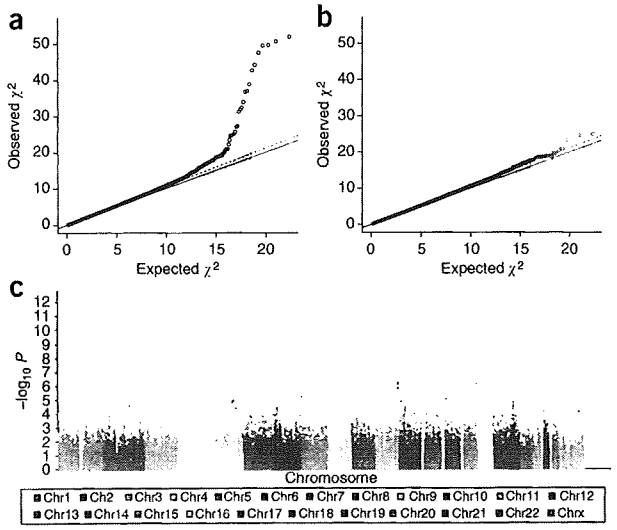

Figure 1 Genome-wide association results from the discovery phase. (**a**) Quantile-quantile plot for test statistics (Cochran-Armitage trend test) for 435,470 SNPs passing quality control. The solid line represents concordance of observed and expected values. Slope of the dashed line represents the genomic inflation factor ($\lambda = 1.055$). The shaded region is the 95% concentration band formed by calculating, for each order statistic, the 2.5th and 97.5th percentiles of the respective distribution under the null hypothesis. (**b**) Quantile-quantile plot for test statistics (Cochran-Armitage trend test) after the removal of the four loci with strong associations in this study (1q32, 4p15, 4q22 and 12q12). (**c**) Manhattan plot presenting the *P* values across the genome. The $-\log_{10} P$ (Cochran-Armitage trend test) from 435,470 SNPs in 988 Parkinson's disease cases and 2,521 controls is plotted according to its physical position on successive chromosomes.

For fast-track replication, we selected the 337 most associated SNPs ($P \leq 0.000533$) from analysis of GWAS data and genotyped them in a sample set of replication 1, which consisted of 612 cases and 14,139 controls from Japan (**Supplementary Note**). Thirty-two SNPs showed association of $P < 0.05$ in replication 1 (**Supplementary Fig. 1**). Combined analyses of the GWAS and replication 1 showed that 12 SNPs in 3 loci (1q32, 4p15 and 4q22) surpassed $P < 5 \times 10^{-7}$. Furthermore, we found association signals ($P = 3.06 \times 10^{-6}$, OR = 1.36) on 12q12, harboring *LRRK2*, which is a causative gene for autosomal dominant parkinsonism (**Table 1**).

In replication 2, we tested 24 SNPs at these four loci for association with PD. An independent sample set (321 cases and 1,614 controls) recruited from Japan was used in replication 2 (**Supplementary Note**). Association evidence was again found at these four loci: 1q32, $P = 2.80 \times 10^{-4}$, OR = 1.37; 4p15, $P = 7.70 \times 10^{-3}$, OR = 1.26; 4q22, $P = 0.02$, OR = 1.22; and 12q12, $P = 6.43 \times 10^{-4}$, OR = 1.57 (**Table 1**). The disease associations on 1q32 and 12q12 exceeded the conservative Bonferroni-corrected threshold for significance ($P = 0.0021$; calculated as 0.05/24). All the SNPs showed allele frequency differences in the same direction in the GWAS, replication 1 and replication 2. Furthermore, combined analysis of the GWAS and two replication stages provided strong evidence of association in the four regions with a significance level of $P = 2.72 \times 10^{-8}$ or less (**Table 1**).

We identified two new susceptibility loci with genome-wide significance on 1q32 and 4p15, which have not been reported to be associated with PD in previous studies[12–14]. On 1q32, seven SNPs (rs16856139, rs823128, rs823122, rs947211, rs823156, rs708730 and rs11240572) reached $P < 5 \times 10^{-7}$ in the overall analysis (**Fig. 2a**). rs947211 showed the strongest association to PD ($P = 1.52 \times 10^{-12}$, OR = 1.30) and is located 8.5 kb upstream of *RAB7L1* and 5.6 kb downstream of *SLC41A1*. Linkage disequilibrium (LD) analysis revealed that SNPs with significant associations to PD lie within several LD blocks containing the following five genes: *SLC45A3*, *NUCKS1*, *RAB7L1*, *SLC41A1* and *PM20D1* (also called *FLJ32569*) (**Table 1** and **Fig. 2a**). Three genes (*NUCKS1*, *RAB7L1* and *SLC41A1*) were contained in the same LD block as rs947211. rs947211 was weakly correlated with the other six SNPs ($r^2 = 0.07$–0.25), and we observed residual association signals when rs947211 and each of the other six SNPs were paired in conditional analyses of our overall data. This result suggests that this locus has multiple independent association signals (**Supplementary Table 1**). These data provide the first evidence, to our knowledge, of an association between 1q32 and PD susceptibility, and we designated this region as *PARK16*.

On 4p15, four SNPs (rs11931532, rs12645693, rs4698412 and rs4538475) reached $P < 5 \times 10^{-7}$ in the combined analysis (**Fig. 2b**). These four SNPs showed strong disease association with almost the same significance levels (ranging from $P = 3.94 \times 10^{-9}$ to $P = 1.78 \times 10^{-8}$, all OR = 1.24); among them, rs4538475 was the most strongly associated. The four SNPs were located from intron 8 to 4.1 kb downstream of *BST1* (bone marrow stromal cell antigen). LD analysis revealed that the four SNPs were correlated with $r^2 > 0.78$ and lie within a 15 kb LD block containing a single gene, *BST1*.

The remaining two intervals (4q22 and 12q12) harbored genes previously found to be causal for autosomal dominant forms of parkinsonism, specifically, *SNCA* and *LRRK2*, respectively. On 4q22, seven SNPs (rs3733449, rs11931074, rs3857059, rs2736990, rs3796661, rs6532194 and rs12233759) throughout the *SNCA* region showed genome-wide significant association in the combined analysis (**Fig. 2c**). The most significantly associated SNPs, rs11931074 ($P = 7.35 \times 10^{-17}$, OR = 1.37) and rs3857059 ($P = 5.68 \times 10^{-16}$, OR = 1.36), are approximately 35.7 kb apart, located 7.2 kb downstream from and in intron 4 of *SNCA*, respectively. The entire *SNCA* gene was divided into two LD blocks at intron 4. Both SNPs were positioned on the 3′ side of the LD block and showed a high degree of LD ($r^2 = 0.98$). Three SNPs (rs2736990, rs3796661 and rs6532194) were moderately correlated with rs11931074 ($r^2 = 0.81$, 0.76 and 0.63, respectively). The remaining two SNPs (rs3733449 and rs12233759) were weakly correlated with rs11931074 ($r^2 = 0.05$ and 0.24, respectively), and residual association signals were marginally observed when rs11931074 and each of these two SNPs were paired in conditional analyses of our overall data (**Supplementary Table 1**). These data confirm *SNCA* as a susceptibility gene for PD.

On 12q12, five SNPs (rs1994090, rs7304279, rs4768212, rs2708453 and rs2046932) surpassed $P < 5 \times 10^{-7}$ in the overall analysis (**Fig. 2d**). The five SNPs showed strong disease association with almost the same significance (ranging from $P = 2.72 \times 10^{-8}$ to $P = 1.09 \times 10^{-7}$, OR = 1.37–1.39); among them, rs1994090 was the most strongly associated to PD. These five SNPs were located from intron 2 of *SLC2A13* to 38.4 kb upstream of *LRRK2*. These SNPs were highly correlated with $r^2 > 0.83$ and were positioned within several LD blocks defined by the method of Gabriel *et al.*[16]. This is the first evidence that common variants proximal to *LRRK2* are associated with PD at genome-wide significance level.

Variants with the highest significance at the four loci detected in this study were common SNPs with risk allele frequencies of 0.50 (rs947211 on 1q32), 0.38 (rs4538475 on 4p15), 0.58 (rs11931074 on 4q22) and 0.08 (rs1994090 on 12q12) (**Table 1**). Population attributable risks for rs947211, rs4538475, rs11931074 and rs1994090 were estimated to be 13%, 8%, 18% and 3%, respectively.

Table 1 Summary of association results for representative SNPs that characterize the association of Parkinson's disease with 1q32 (*PARK16*), 4p15 (*BST1*), 4q22 (*SNCA*) and 12q12 (*LRRK2*)

				GWAS			Replication 1			GWAS + Replication 1		Replication 2			GWAS + Replication 1+2	
			Allele	MAF			MAF					MAF				
Locus	SNP	Pos (Mb)	Minor/ major	Case Ctrl	P_{trend}	OR (95% CI)	Case Ctrl	P_{trend}	OR (95% CI)	P_{cmh}	OR (95% CI)	Case Ctrl	P_{trend}	OR (95% CI)	P_{cmh}	OR (95% CI)
New PD loci																
1q32	rs16856139	203.91	T/C	0.10	2.55×10^{-6}	1.50	0.11	0.067	1.19	2.15×10^{-6}	1.35	0.10	0.015	1.42	1.02×10^{-7}	1.36
(*PARK16*)				0.14		(1.26–1.77)	0.13		(0.99–1.44)		(1.19–1.54)	0.13		(1.07–1.88)		(1.22–1.53)
	rs823128	203.98	G/A	0.10	2.09×10^{-5}	1.43	0.11	0.0056	1.31	4.67×10^{-7}	1.38	0.09	0.0028	1.53	4.88×10^{-9}	1.41
				0.14		(1.21–1.69)	0.13		(1.08–1.59)		(1.22–1.57)	0.14		(1.16–2.03)		(1.26–1.58)
	rs823122	203.99	C/T	0.10	7.98×10^{-5}	1.39	0.11	0.013	1.27	3.87×10^{-6}	1.34	0.09	0.0034	1.52	5.22×10^{-8}	1.37
				0.14		(1.18–1.64)	0.13		(1.05–1.54)		(1.18–1.52)	0.14		(1.15–2.01)		(1.22–1.54)
	rs947211	204.02	A/G	0.43	1.15×10^{-4}	1.23	0.42	1.35×10^{-6}	1.35	1.12×10^{-9}	1.28	0.42	2.80×10^{-4}	1.37	1.52×10^{-12}	1.30
				0.48		(1.11–1.37)	0.50		(1.19–1.52)		(1.18–1.38)	0.50		(1.16–1.63)		(1.21–1.39)
	rs823156	204.03	G/A	0.13	1.20×10^{-5}	1.40	0.14	0.012	1.25	6.45×10^{-7}	1.33	0.12	0.0013	1.52	3.60×10^{-9}	1.37
				0.17		(1.20–1.62)	0.17		(1.05–1.48)		(1.19–1.49)	0.17		(1.17–1.95)		(1.23–1.52)
	rs708730	204.04	G/A	0.14	2.60×10^{-5}	1.37	0.15	0.022	1.22	2.89×10^{-6}	1.30	0.12	0.0019	1.48	2.43×10^{-8}	1.33
				0.18		(1.18–1.59)	0.17		(1.03–1.44)		(1.17–1.46)	0.18		(1.15–1.89)		(1.21–1.48)
	rs11240572	204.07	A/C	0.13	1.66×10^{-4}	1.34	0.13	0.016	1.24	9.78×10^{-6}	1.30	0.12	0.0024	1.49	1.08×10^{-7}	1.33
				0.16		(1.15–1.56)	0.16		(1.04–1.48)		(1.16–1.46)	0.17		(1.15–1.92)		(1.20–1.48)
4p15	rs11931532	15.33	T/C	0.45	2.75×10^{-4}	1.22	0.47	1.86×10^{-4}	1.26	2.02×10^{-7}	1.23	0.47	0.0077	1.26	5.13×10^{-9}	1.24
(*BST1*)				0.40		(1.09–1.35)	0.42		(1.11–1.42)		(1.14–1.34)	0.41		(1.06–1.49)		(1.15–1.33)
	rs12645693	15.34	G/A	0.45	3.06×10^{-4}	1.21	0.47	3.00×10^{-4}	1.25	3.42×10^{-7}	1.23	0.47	0.0077	1.26	8.65×10^{-9}	1.24
				0.40		(1.09–1.35)	0.42		(1.11–1.41)		(1.14–1.33)	0.41		(1.06–1.49)		(1.15–1.33)
	rs4698412	15.35	A/G	0.38	5.28×10^{-5}	1.25	0.40	4.91×10^{-4}	1.24	1.03×10^{-7}	1.25	0.38	0.055	1.19	1.78×10^{-8}	1.24
				0.33		(1.12–1.40)	0.35		(1.10–1.40)		(1.15–1.35)	0.34		(1.00–1.42)		(1.15–1.33)
	rs4538475	15.35	A/G	0.41	4.05×10^{-5}	1.25	0.43	3.48×10^{-4}	1.25	5.98×10^{-8}	1.25	0.42	0.022	1.22	3.94×10^{-9}	1.24
				0.36		(1.12–1.40)	0.38		(1.10–1.41)		(1.15–1.35)	0.37		(1.03–1.46)		(1.16–1.34)
Loci located in or near autosomal dominant parkinsonism genes																
4q22	rs11931074	90.86	G/T	0.32	6.17×10^{-13}	1.50	0.36	2.12×10^{-5}	1.31	2.19×10^{-16}	1.41	0.38	0.034	1.21	7.35×10^{-17}	1.37
(*SNCA*)				0.42		(1.34–1.68)	0.42		(1.16–1.48)		(1.30–1.53)	0.43		(1.01–1.44)		(1.27–1.48)
	rs3857059	90.89	A/G	0.32	1.17×10^{-12}	1.49	0.36	6.92×10^{-5}	1.29	1.54×10^{-15}	1.40	0.38	0.041	1.20	5.68×10^{-16}	1.36
				0.41		(1.34–1.67)	0.42		(1.14–1.45)		(1.29–1.52)	0.43		(1.01–1.43)		(1.26–1.46)
	rs894278	90.95	G/T	0.43	7.68×10^{-5}	1.24	0.39	0.46	1.05	4.77×10^{-4}	1.15	0.42	0.020	1.22	3.28×10^{-5}	1.17
				0.38		(1.11–1.37)	0.38		(0.93–1.18)		(1.07–1.25)	0.37		(1.03–1.45)		(1.09–1.25)
	rs6532194	91.00	C/T	0.31	6.93×10^{-11}	1.44	0.36	0.0014	1.22	1.77×10^{-12}	1.35	0.37	0.040	1.21	4.15×10^{-13}	1.32
				0.40		(1.29–1.61)	0.41		(1.08–1.39)		(1.24–1.46)	0.41		(1.01–1.44)		(1.22–1.42)
12q12	rs1994090	38.71	G/T	0.11	4.45×10^{-5}	1.43	0.10	0.018	1.26	3.06×10^{-6}	1.36	0.12	0.0019	1.51	2.72×10^{-8}	1.39
(*LRRK2*)				0.08		(1.20–1.70)	0.08		(1.04–1.54)		(1.20–1.55)	0.08		(1.16–1.97)		(1.24–1.56)
	rs7304279	38.75	T/C	0.11	5.17×10^{-5}	1.42	0.10	0.026	1.25	5.10×10^{-6}	1.35	0.12	0.0022	1.50	5.06×10^{-8}	1.38
				0.08		(1.20–1.69)	0.08		(1.03–1.52)		(1.19–1.54)	0.09		(1.15–1.95)		(1.23–1.55)
	rs4768212	38.76	C/T	0.11	3.98×10^{-5}	1.43	0.10	0.057	1.21	1.10×10^{-5}	1.34	0.12	0.0020	1.51	1.09×10^{-7}	1.37
				0.08		(1.20–1.70)	0.08		(0.99–1.48)		(1.18–1.52)	0.08		(1.16–1.97)		(1.22–1.54)
	rs2708453	38.76	T/G	0.11	7.46×10^{-5}	1.41	0.10	0.063	1.21	2.04×10^{-5}	1.33	0.13	6.43×10^{-4}	1.57	9.67×10^{-8}	1.38
				0.08		(1.19–1.68)	0.08		(0.99–1.48)		(1.17–1.52)	0.08		(1.21–2.04)		(1.22–1.55)
	rs2046932	38.87	T/C	0.11	3.24×10^{-5}	1.44	0.10	0.039	1.23	5.47×10^{-6}	1.35	0.13	0.0017	1.52	4.34×10^{-8}	1.39
				0.08		(1.21–1.71)	0.08		(1.01–1.51)		(1.19–1.54)	0.09		(1.17–1.97)		(1.23–1.56)

Nucleotide positions refer to NCBI build 36. *P* values obtained in the case-control analysis using the Cochran-Armitage trend test (1 d.f.) are listed (P_{trend}). Combined *P* values (P_{cmh}) and combined ORs of the Cochran-Mantel-Haenzel test statistics are shown. MAF, minor allele frequency.

Next, we exchanged data with colleagues performing a GWAS of PD in individuals of European ancestry[17]. Their study found a strong association at the *MAPT* (microtubule-associated protein tau) region on 17q21. We genotyped our samples for six SNPs at the *MAPT* locus to evaluate these associations in the Asian population; however, the association with *MAPT* was not replicated in our study (**Supplementary Table 2** and **Supplementary Fig. 2**). Conversely, despite strong association signals in our scan of the samples from the Asian population, the association with *BST1* on 4p15 was not detected among individuals of European ancestry[17]. In contrast, the associations we found with *PARK16* and *LRRK2* were replicated among individuals of European ancestry[17]. These data provide evidence that *PARK16* and *LRRK2*, in addition to *SNCA*, are PD risk loci common to Asian- and European-descent populations and indicate that there is population genetic heterogeneity in the *MAPT* region and 4p15 (*BST1*) for PD susceptibility.

The *PARK16* region contains functionally interesting candidate genes for PD etiology. SLC41A1 is a magnesium (Mg^{2+}) transporter[18]. It is of interest that Mg^{2+} deficiency is thought to be an environmental risk factor for the amyotrophic lateral sclerosis

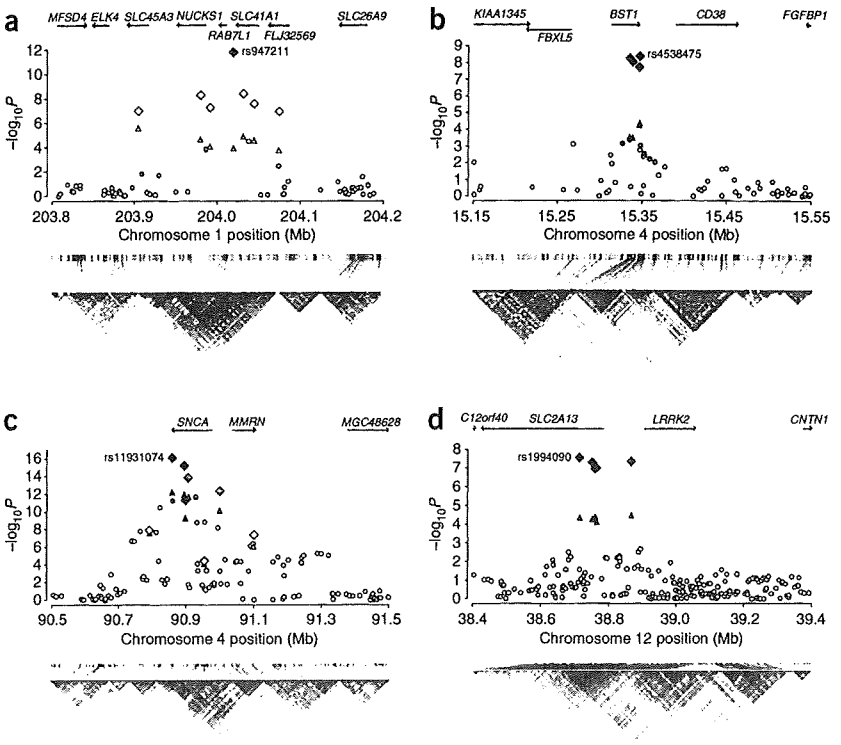

Figure 2 Regional association plots and linkage disequilibrium structure for the four PD risk loci. (**a**) 1q32 (*PARK16*). (**b**) 4p15 (*BST1*). (**c**) 4q22 (*SNCA*). (**d**) 12q12 (*LRRK2*). The $-\log_{10}$ *P* (Cochran-Armitage trend test) for association in the GWAS stage of SNPs across each region are shown as small triangles for SNPs that were selected for replication and as small circles for SNPs not selected. The $-\log_{10}$ combined *P* values (Cochran-Mantel-Haenszel test) for association in overall samples of SNPs selected for replication are shown as large diamonds. In each panel, the SNP with the most significant association in the combined analysis is listed. Proxies are indicated with colors determined from their pairwise r^2 from the JPT and CHB HapMap data (red, $r^2 > 0.8$; orange, $r^2 = 0.5–0.8$; yellow, $r^2 = 0.2–0.5$; white, $r^2 < 0.2$ or no information available). Positions are NCBI build 36 coordinates.

(ALS)-parkinsonism/dementia complex (MIM105500)[19]. Furthermore, RAB7L1 is a small GTP-binding protein that plays an important role in regulation of exo- and endocytotic pathways[20], and NUCKS1 is a nuclear protein containing several consensus phosphorylation sites for casein kinase II and cyclin-dependent kinases of unknown function[21]. We evaluated the relationships between the PD-associated SNPs and the transcript levels of genes in an available genome-wide gene expression database[22]. We found that rs947211 and ten tightly linked HapMap SNPs ($r^2 > 0.9$) were strongly associated with transcript levels of *NUCKS1* (rs947211, $P = 6.0 \times 10^{-15}$; rs823114, $P = 2.7 \times 10^{-34}$). These PD-susceptibility variants are the principal genetic determinants of variation in expression levels of *NUCKS1* (**Supplementary Fig. 3**). These data highlight *NUCKS1* as a promising candidate for association with PD that is worthy of additional follow-up.

The product of *BST1* on 4p15 catalyses formation of cyclic ADP-ribose (cADPR)[23]. cADPR mobilizes calcium (Ca^{2+}) from ryanodine-sensitive intracellullar Ca^{2+} stores in the endoplasmic reticulum[24]. Disruption of Ca^{2+} homeostasis has recently been proposed as a possible cause of selective vulnerability of dopaminergic neurons in PD[25–27]. Associated SNPs in the *BST1* region may modify ADP-ribosylcyclase activity, thus leading to Ca^{2+} dyshomeostasis in dopaminergic neurons.

Two of the four susceptibility loci detected in our scan contained genes linked to autosomal dominant forms of parkinsonism. Gene overdosage is a potential mechanism for the influence of *SNCA* on PD because triplication and duplication of the *SNCA* locus has been seen in families with autosomal dominant parkinsonism[28]. SNPs with prominently low *P* values compared to other SNPs in the region were around the 3′ region of *SNCA*; these SNPs may function as enhancer or silencer elements, improve RNA stability or influence alternative splicing. The associated interval on 12q12 contains *SLC2A13* and the region upstream of *LRRK2*. Given prior evidence, *LRRK2* stands out as the most likely susceptibility gene for PD, although it remains possible that *SLC2A13*, which encodes a H^+-myoinositol cotransporter, may be the PD-related gene in this region[29]. Previous reports have investigated the association of SNPs in *LRRK2* with PD, but the results are a subject of dispute[30,31]. In the present study, it is noteworthy that the PD-associated intervals lie upstream of *LRRK2*. Increased kinase activity of mutant LRRK2 mediates neuronal toxicity[32,33]. PD-associated SNPs may play a role in transcriptional upregulation of *LRRK2*, leading to loss of dopaminergic neurons.

SNCA is a main component of Lewy bodies, a pathological hallmark of typical PD. The clinical features of individuals with *SNCA* duplication or *LRRK2* mutation similar to typical PD. 1.6% of sporadic PD cases among individuals of European ancestry have heterozygous *LRRK2* G2019S mutations[34]. These data support the close involvement of these genes with sporadic PD. Our data clearly show that the genes involved in autosomal dominant parkinsonism play a large part in the complex etiology of typical PD. Genes that cause autosomal dominant parkinsonism through their causative mutations also confer risk of typical PD through their common variants. Although further research is needed, this relationship between rare single-gene disorders and common multifactorial disorders may also be applicable for other disorders beyond PD.

Finally, *MAPT* mutations cause hereditary frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), a type of autosomal dominant parkinsonism[35], and the *MAPT* H1 haplotype has been reported to be associated with several tauopathies[36–38]. Although the *MAPT* region is divided into two major haplotypes, H1 and H2, in Europeans, the H2 haplotype is absent in East Asians. Therefore, we believe that the differences observed between our study and the findings in populations of European descent reflect population differences in the genetic heterogeneity of PD etiology, although differences in allele frequencies and LD structure and a possible difference in the effect size between the European and East Asian populations may influence the detection power of the two scans.

Further increases in sample sizes for SNP-GWAS efforts and searches for copy number variation and rare variants will reveal additional genetic risk factors and further enhance our understanding of PD.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

T. Toda conceived the study. W.S., I.M. and T. Toda designed the study. W.S., Y.N., C.I., M.K. and T.Y. performed genotyping. W.S. and T. Toda wrote the manuscript. W.S., T.K. and T. Tsunoda performed data analysis. W.S., I.M., Y.H., M.W., A.T., H.T., K.N., K.H., F.O., H.K., S.S., M.Y., N.H., M.M. and T. Toda managed Parkinson clinical information and DNA samples. M.K. and Y.N. managed DNA samples belonging to BioBank Japan. T. Toda obtained funding for the study.

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