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BMJ Open

PARK2 and proinflammatory/anti-inflammatory cytokine gene interactions contribute to the susceptibility to leprosy: a case–control study of North Indian population

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To cite: Chopra R, Kalaiarasan P, Ali S, *et al*. PARK2 and proinflammatory/anti-inflammatory cytokine gene interactions contribute to the susceptibility to leprosy: a case–control study of North Indian population. *BMJ Open* 2014;**4**:e004239. doi:10.1136/bmjopen-2013-004239

▸ Prepublication history and additional material for this paper is available online. To view these files please visit the journal online (http://dx.doi.org/10.1136/bmjopen-2013-004239).

Received 15 October 2013
Revised 22 December 2013
Accepted 10 January 2014

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ABSTRACT

Objectives: Cytokines and related molecules in immune-response pathways seem important in deciding the outcome of the host–pathogen interactions towards different polar forms in leprosy. We studied the role of significant and functionally important single-nucleotide polymorphisms (SNPs) in these genes, published independently from our research group, through combined interaction with an additional analysis of the in silico network outcome, to understand how these impact the susceptibility towards the disease, leprosy.

Design: The study was designed to assess an overall combined contribution of significantly associated individual SNPs to reflect on epistatic interactions and their outcome in the form of the disease, leprosy. Furthermore, in silico approach was adopted to carry out protein–protein interaction study between PARK2 and proinflammatory/anti-inflammatory cytokines.

Setting: Population-based case–control study involved the data of North India. Protein–protein interaction networks were constructed using cytoscape.

Participants: Study included the data available from 2305 Northern Indians samples (829 patients with leprosy; 1476 healthy controls), generated by our research group.

Primary and secondary outcome measures: For genotype interaction analysis, all possible genotype combinations between selected SNPs were used as an independent variable, using binary logistic regression with the forward likelihood ratio method, keeping the gender as a covariate.

Results: Interaction analysis between PARK2 and significant SNPs of anti-inflammatory/proinflammatory cytokine genes, including BAT1 to BTNL2-DR spanning the HLA (6p21.3) region in a case–control comparison, showed that the combined analysis of: (1) PARK2, tumour necrosis factor (TNF), BTNL2-DR, interleukin (IL)-10, IL-6 and TGFBR2 increased the risk towards leprosy (OR=2.54); (2) PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB provided increased protection (OR=0.26) in comparison with their individual contribution.

Conclusions: Epistatic SNP–SNP interactions involving PARK2 and cytokine genes provide an additive risk towards leprosy susceptibility. Furthermore, in silico protein–protein interaction of PARK2 and important proinflammatory/anti-inflammatory molecules indicate that PARK2 is central to immune regulation, regulating the production of different cytokines on infection.

Strengths and limitations of this study

- Many of the genetic studies lack replication in different population groups, explaining the heterogeneity in associated genes and genomic regions. This may be due to the complex nature of a disease. The complexity, however, could partly be delineated at genetic level by assessing the quantum of the contribution of different loci to the disease in a combined manner instead of their individual role.
- Our study highlights the importance of a combined effect of the important cytokine and other immune-regulatory genes, whose combined effect in diverse genotype combinations provides either increased risk or protection towards the complex genetic disease, leprosy.
- Genetic interaction and an additional in silico pathway analysis provided an overall perspective on how PARK2 gene product, parkin, acts as a centrally placed molecule, playing an important role in regulating different pathways of the immune response and susceptibility to leprosy. This conclusion needs further support with future experiments in vitro or in vivo of T cell responses in different genetic backgrounds of the identified networks in this study.

INTRODUCTION

Leprosy caused by *Mycobacterium leprae* is a chronic infectious disease, characterised by clinically defined polar forms in which pathology and immunology are inextricably related, providing a critical model to explore the immunoregulatory mechanisms in humans. At one pole, tuberculoid form is associated with a strong cell-mediated immunity (CMI) and T helper 1 (Th1) cytokine profile, and, at the other end of the spectrum, the lepromatous form is associated with a strong humoral response and Th2 cytokine profile. Cytokines and other related molecules of the immunological pathways thus seem to be a part of significant group of candidates that are apparently critical for the host–pathogen interactions, where the outcome of the disease is majorly dependent on the host factors controlling the immune response, especially when *M leprae* possesses the lowest level of genetic diversity.[1] This is supported by various studies of familial clustering,[2] twin studies,[3] complex segregation analysis,[4 5] test of analysis with the HLA genes[6] including recent genome-wide association studies,[7 8] and studies of several genes that modulate CMI, with a role in either susceptibility to leprosy per se or to leprosy types.[9] Various candidate gene studies and genome-wide approaches have implicated polymorphisms in cytokine genes, whose protein products are part of important immune modulatory molecules, playing a major role in influencing host–pathogen interactions and determine the outcome of many infectious and autoimmune diseases.[10–16] However, only a few observations have been replicated unequivocally in different population groups, suggesting the polygenic nature of the disease with a high degree of heterogeneity among different populations.

We, recently, have studied various candidate genes of proinflammatory/anti-inflammatory cytokines in two independent population groups, North and East India-Orissa, and found a strong association with interleukin (IL)-10, IL-10RB, TGFBR2, IL-6[14] and IL-12B.[17] Fine-mapping of a specific 6p (HLA) chromosomal region revealed a significant association of important candidates, BAT1, LTA, tumour necrosis factor (TNF) and BTNL2.[16] A subsequent study of the 6q chromosomal region, involving the overlapping regulatory domain of PARK2-PACRG genes, revealed an involvement of significant single-nucleotide polymorphisms (SNPs) and presence of a differential LD structure in Indian populations as compared with Vietnamese.[18] The latter observation and the functional role of PARK2, as a ubiquitin ligase, has recently been shown in providing resistance to intracellular pathogens[19] through ubiquitin-mediated autophagy. Furthermore, the involvement of parkin in regulating production of cytokines upon infection,[20] indeed, provides a strong hint for any functional variations in the gene having a profound effect in modulating the expression of the immune-regulatory genes. The importance of all the studied genes[14–18] in the network of immune-response necessitated the analysis of an interaction between these genes as a whole to understand their contribution together towards the susceptibility of the complex disease, leprosy, where the outcome of the infection in all probabilities depends on the nature of gene interactions between the genes with the potential of contributing to the immune pathology.

Therefore, the aim of this study was to assess an overall interaction between the significant and functionally important SNPs studied in a case–control comparison of the samples from New Delhi, in Northern India, where most of these SNPs were replicated in an unrelated East Indian-Orissa population. These included an overall interaction of the PARK2 gene significant SNPs[18] with the significant SNPs of anti-inflammatory cytokine genes (IL-10, IL-10RB, TGFBR2, IL-6),[14] proinflammatory cytokine genes (TNFα, LT-α, IL-12B) and the genes spanning the HLA region of the chromosome 6p21.3, that is, BAT1 to BTNL2-DR[16 17] to evaluate their combined contribution towards the outcome of the complex infectious disease, leprosy.

METHODS

The study involved the revisit of our published work on individual candidate genes and regions, studied in North Indian population groups in case–control comparison, for a combined genotype interaction and for in silico protein–protein interaction (PPI) and network analysis. The data compiled were of 2305 samples from Northern India (including 829 patients with leprosy and 1476 unrelated healthy control participants from North India)[14 16–18] with a complete coverage of genes belonging to proinflammatory, anti-inflammatory cytokines, selected HLA regions in 6p21.3 and common regulatory region of PARK2/PACRG genes located at 6q26 region.

The patients' group was classified according to the WHO guidelines. An individual was regarded as having leprosy if he or she showed skin lesion consistent with leprosy and with definite sensory loss, with or without thickened nerves and positive skin smears test. Furthermore, patients were classified as paucibacillary (PB) or multibacillary (MB) according to the Ridley and Jopling criteria,[21] including 421 patients with PB and 408 patients with MB, with a mean age of 32.30 ±3.2 years (range 6–80 years). All these patients were under treatment with multidrug therapy specific for MB and PB leprosy, as recommended by the WHO.

For genotype interaction analysis, all possible genotype combinations between selected SNPs (pairwise or multiple genes) were ascertained from a MassArray platform for the given genotypes of SNPs. However, only the combinations of significantly associated SNP genotypes were presented in the Ms for convenience. These interactions were tested using binary logistic regression with the forward likelihood ratio-based selection method, considering all variables independently and keeping gender as a covariate. In this selection method, entry testing based on the

Table 1 Genotype interaction analysis of PARK2 SNPs with proinflammatory /anti-inflammatory cytokines gene SNPs providing risk/protection towards leprosy susceptibility

PARK2 (SNPs are within 63.8 kb upstream gene region)		Gene	SNPs providing risk			Samples (n)		Significance	OR	95% CI for EXP(B)	
						Patients	Controls			Lower	Upper
rs9365492; minor, risk allele-C TC+CC	rs9355403; minor, risk allele-A GA+AA	IL-10	rs1800871 (−819); minor, risk allele-T CT+TT	rs1554286 (intron 3 boundary); minor, risk allele-T TT	rs1800872 (−592); minor, risk allele-A CA+AA	82	84	3.22E-05	1.997	1.441	2.767
		TGFBR2	rs2228048 (3’ UTR downstream); minor, risk allele-T CT+CC	rs744751 (3′ UTR downstream); minor, risk allele-G GG		287	400	1.04E-02	1.293	1.062	1.575
		IL-6	rs1800797 (−718); minor, risk allele-G GG			320	432	2.90E-03	1.333	1.103	1.611
		TNF	rs1800629 (−308); minor, risk allele-G GG	rs1800610 (Intron-1); minor, risk allele-G GG		311	420	2.06E-09	2.103	1.649	2.682
		BTNL2-DRA interval	rs3135365; minor, risk allele-C CA+CC	rs7773756; minor, risk allele-T CT+TT		272	269	1.22E-21	5.4	3.821	7.631
			SNPs providing protection								
TT	GG	LTA	rs13192469 (13 kb upstream); minor, risk allele-C TT	rs36221459 (−1409); minor, risk allele-DEL GTTT		240	571	3.56E-07	0.616	0.512	0.743
		IL-10RB2	rs3171425 (3’ UTR); major, risk allele-G GA+AA	rs7281762 (3′ UTR downstream); minor, risk allele-A GA+GG		164	391	1.10E-05	0.61	0.489	0.76
		BAT1	rs2523504 (−603); minor, risk allele-T CC			192	475	4.15E-05	0.645	0.523	0.795
		NFKBIL	rs2230365 (exon-3); minor, risk allele-T CC			195	486	1.01E-07	0.589	0.484	0.715
		TNF-LTB	rs769178 (gene downstream); major, risk allele-G GT+TT			66	211	8.93E-05	0.546	0.404	0.739
		IL12B	rs2853694; major, risk allele-A CA+CC			233	519	5.03E-04	0.705	0.579	0.858

IL, interleukin; SNP, single-nucleotide polymorphism; TNF, tumour necrosis factor.

significance of the score statistics and removal testing based on the probability of a likelihood ratio statistics were applied. Furthermore, in multiple gene interaction analysis, all interactions with either risk or protection were combined against other interactions to observe the overall effect of all risk versus protective interactions. These analyses were performed using statistical software package SPSS V.17.0 (SPSS, Chicago, Illinois, USA) for Windows. p Value was considered significant at and below 0.05.

In silico approach to assess the network of the genes in a PPI of PARK2, using Agile Protein Interaction Database (APID), a comprehensive resource for protein interaction data, automatically accessed by cytoscape[22] through the dedicated plugin APID2NET,[23] was carried out to understand the involvement of the studied interactome. APID integrates in a single web-based tool all known experimentally validated PPI from BIND,[24] BioGRID,[25] DIP,[26] HPRD,[27] IntAct[28] and MINT[29] databases.

RESULTS

The interaction analysis carried out between PARK2 gene regulatory region SNPs (rs9365492 and rs9355403)[18] and SNPs of the anti-inflammatory cytokines[14] provided a significant risk towards the leprosy susceptibility, combining individually with SNPs of IL-10 (OR=1.99), IL-6 (OR=1.33) and TGFBR2 (OR=1.29) cytokine genes. However, with IL10RB (receptor β), the result showed a significant protection towards the disease (OR=0.61). Similar analysis between PARK2 SNPs with proinflammatory cytokine genes TNFα and BTNL2-DRA interval (showing strong LD with the BTNL2 promoter SNPs)[16] provided a significant risk towards leprosy susceptibility with OR=2.10 and 5.40, respectively. However, the SNPs of BAT-1, NFKABIL1, LTA, TNF-LTB and IL12B[16 17] provided a significant protection towards leprosy with OR=0.65, 0.58, 0.61, 0.54 and 0.71, respectively (table 1 and see online supplementary figure S1).

In the second step of combined interaction analysis with all the genes, providing either protection or risk towards leprosy, showed that the combined genotypic interaction analysis of the SNP loci PARK2, TNF, BTNL2-DR, IL10, IL-6 and TGFBR2 further increased the risk of leprosy (OR=2.54), and a similar combined analysis for loci PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB increased the protection towards leprosy (OR=0.26) in comparison with their individual contribution (table 2A,B). Dividing the patients into PB and MB subtypes of leprosy revealed PB subtype to carry a higher risk (OR=3.02) and protection (OR=0.11) towards leprosy in comparison with MB subtype, for respective combinations.

We further performed in silico analysis to identify the PPI of PARK2. We used APID2NET and cytoscape tools for PARK2 interaction Data retrieval, providing a total of 43 PARK2 interacting proteins. However, the result did not provide any direct interaction of the PARK2 with the cytokines studied by us in North Indian population.[14 16–18] Furthermore, we considered 43 PARK2 interacting

Table 2 Combined interaction analysis of all the SNPs providing either protection or risk towards leprosy susceptibility

(A) Analysis of SNPs providing protection

	Samples (n) Pat	Samples (n) Cont	PARK2 rs9365492	PARK2 rs9355403	BAT1 promoter rs2523504	NFKBIL rs2230365	LTA 13 kb upstream rs13192469	LTA promoter rs36221459	TNF-LTB rs769178	IL10RB rs3171425	IL10RB rs7281762	IL12B rs2853694	Significance	OR	95% CI for EXP(B) Lower	95% CI for EXP(B) Upper
Alleles			T/C	G/A	C/T	C/T	T/C	GTTT/DEL	G/T	G/A	G/A	A/C				
Risk allele			C	A	T	T	C	DEL	G	G	A	A				
TOTAL	10	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	1.15E-04	0.263	0.133	0.518
PB/HC	2	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	2.36E-03	0.111	0.027	0.458
MB/HC	NA															

(B) Analysis of SNPs providing risk

	Samples (n) Pat	Samples (n) Cont	PARK2 rs9365492	PARK2 rs9355403	IL-10 rs1800871	IL-10 rs1554286	IL-10 rs1800872	IL-6 rs1800797	TGFBR2 rs2228048	TGFBR2 rs744751	TNF (-308) promoter rs1800629	TNF intron 1 rs1800610	BTNL2-DRA interval rs3135365	BTNL2-DRA interval rs7773756	Significance	OR	95% CI for EXP(B) Lower	95% CI for EXP(B) Upper
Alleles			T/C	G/A	C/T	C/T	C/A	G/A	C/T	G/A	G/A	G/A	A/C	C/T				
Risk allele			C	A	T	T	A	G	T	G	G	G	C	T				
Total	57	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GA+AA	GG	GG	CA+CC	CT+TT	5.77E-06	2.543	1.699	3.806
PB/HC	32	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.73E-06	3.028	1.894	4.843
MB/HC	25	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.16E-03	2.133	1.29	3.528

IL, interleukin; MB, multibacillary; PB, paucibacillary; SNP, single-nucleotide polymorphism; TNF, tumour necrosis factor.

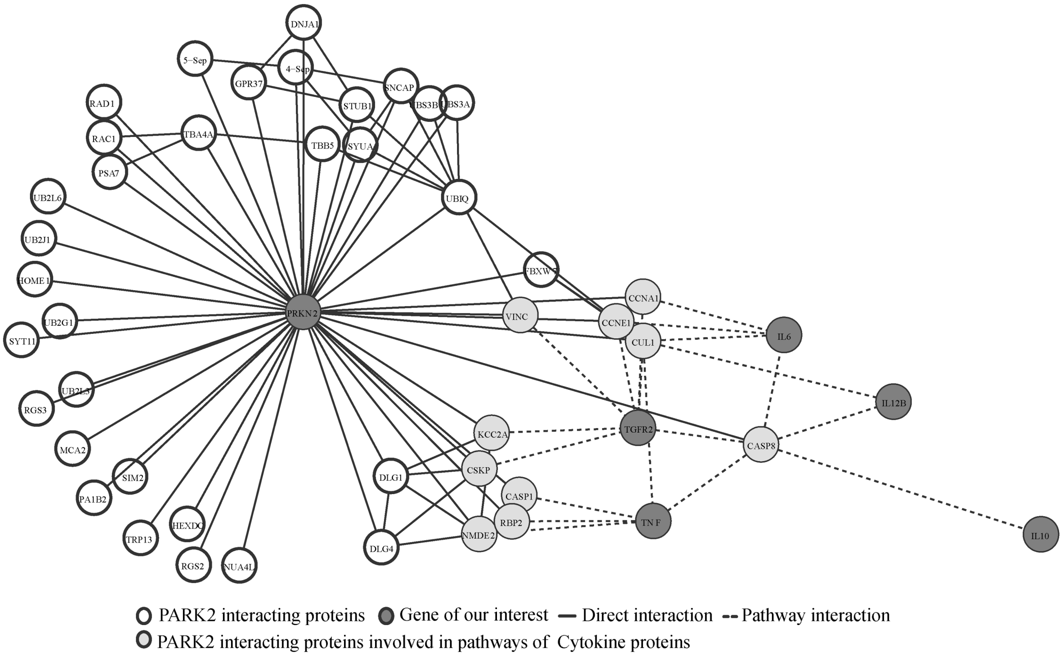

Figure 1 PARK2 interaction analysis: unfilled circles showing the PARK2 interacting proteins. Light grey circles showing protein links between the PARK2 interacting protein and five cytokines protein (dark grey circles) study by us in North India population.

proteins for pathways analysis by using KEGG, BioCarta, Nci-Nature and Reactome tools, which confirmed these 43 proteins to be involved in 253 different pathways (without removing overlapping pathways). Similarly, in the second step of pathway analysis, we considered 11 cytokine proteins studied by us in North Indian population,[14 16–18] and the results revealed the involvement of five cytokine proteins; IL12B, IL6, TNF, TGFR2 and IL10 in 94 pathways, not involving BTNL2, BAT1, NFKBIL, LTA, IL10RB2 and BTNL2-DR in any pathways. Comparing both pathways, 253 PARK2 interacting proteins pathways and 94 cytokine proteins pathways revealed 27 commonly involved pathways, via CASP8, CUL1, CCNE1 and CCNA proteins, involving only 5 (IL12B, IL6, TNF, TGFR2 and IL10) of 11 cytokine proteins studied in North Indian population (figure 1), connecting majorly through Toll-like receptor (TLR) signalling pathways (figure 1, see online supplementary table S1).

DISCUSSION

Leprosy, an ideal model of a chronic human complex infectious disease, provides an opportunity to dissect the components of the host-dependent polygenic susceptibility to this disease. Many loci have been shown to be individually associated and providing the risk towards the disease; justifying to find out interesting gene–gene interactions at different risk loci which may prove to provide a strong association towards the disease susceptibility. In order to understand the role of multiple genes together, an interaction analysis was carried out between the genotype status of functionally different variants of different genetic loci involved in immune response, with an expected combined effect on the outcome of the disease in different polar forms of the disease.

Considering the above facts, we first carried out pairwise interaction analysis of PARK2 gene with proinflammatory/anti-inflammatory cytokine genes (table 1), followed by multiple gene interaction analysis (table 2). Analysis of PARK2 with TNF, BTNL2-DR, IL10, IL-6 and TGFBR2 showed an increased risk towards leprosy (OR=2.54 (1.69 to 3.80), p=5.77e-06), while the combined analysis of PARK2 with BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB showed protection towards the disease (OR=0.26 (0.13 to 0.51), p=1.15e-04). PARK2, encoding E3 ubiquitin ligase protein-parkin, has been shown to be involved in the cellular ubiquitination metabolism,[30] providing resistance to intracellular pathogen via ubiquitin-mediated autophagy,[19] essentially shown to be involved in the host responses to *M leprae*[31] and for pathogenesis of the disease.[8 32] Recently, parkin protein has shown to be involved to respond to infection in a regulated way by producing important cytokines,[20] suppressing molecules that limit proinflammatory-IL-2,[33] TNFα cytokine production and enhancing the production of anti-inflammatory cytokines, IL-4, IL-10 and IL-13.[34–39] All these observations indicate the in vivo importance of PARK2 gene product, parkin, to be centrally involved in regulating the production of critical cytokines during immune response against the invading mycobacterium and justifying our study, where combination of risk genotype at different loci of important immune response gene with PARK2 provides increased and significant risk towards this complex disease. These interesting results of gene–gene interaction analysis suggest the in vivo effect of the invading mycobacterium in future, where immune response to specific antigens is assessed in cells with different background of important variations in the PARK2 promoter region followed by the effect on the expression levels of proinflammatory/anti-inflammatory cytokines.

An in silico approach was used to understand the role of immune-regulatory PPI between PARK2 and other cytokine genes, and an indirect interaction was observed between PARK2 and IL12B, IL6, TNF, TGFR2 and IL10 genes. All these interactions were found to be connected with TLR signalling pathway (see online supplementary table S1). As already known, the polymorphisms in different TLRs, important molecules of innate immune response, are associated with leprosy and its subtypes,[7 40–44] influencing recognition of *M leprae*. A simultaneous involvement of PARK2, a ubiquitin ligase protein involved in innate immunity by modulating the production of important cytokines, including IL6,[20] hints at the involvement of all these important molecules to be interconnected through a TLR receptor signalling pathway to fight against the invading mycobacterium.

The above interaction and pathway analysis allows us to propose that the complex genetic background is the predominant factor for the outcome of the disease, where the combined effect of the variant risk alleles of the PARK2 gene, responsible for affecting transcription binding site and lowering the expression of the reporter gene by in vitro experiment,[18] along with the risk alleles of the anti-inflammatory cytokine genes—IL-10, IL-6, TGFBR2, responsible for lowering the CMI response towards the invading bacteria and proinflammatory cytokines—TNFα, is responsible in providing highly significant risk towards leprosy. The study opens a way for future in vivo work of immune-response readouts in complex variant genomic backgrounds to understand the wide gap in understanding the balance in the network of all the immune regulatory molecules operational in providing either susceptibility or resistance towards disease.

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Acknowledgements The authors would like to thank all who participated in the study. They also thank Dr Shobit Caroli and Dr Sudhanshu, Dr Shalu the attending physicians at LNJP Hospital and Professor Jason P Sinnwell for his invaluable support in providing Haplo.Stats package.

Contributors RNKB and RC contributed in planning, designing and execution of work and wrote the article; RC, SA, PK, SA and AKS contributed in biostatistics and in silico analysis; VKG and SNB contributed in patient evaluation, clinical categorisation and discussion. All authors critically reviewed the manuscript. RNKB led the research effort.

Funding Financial and Infrastructural support for the production of manuscript was provided by Shri Mata Vaishno Devi University, University Grants Commission; and high-throughput project from DBT to the National Center of Applied Human Genetics.

Competing interests None.

Patient consent Obtained.

Ethics approval Approved by Jawaharlal Nehru University ethics committee, New Delhi, India.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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