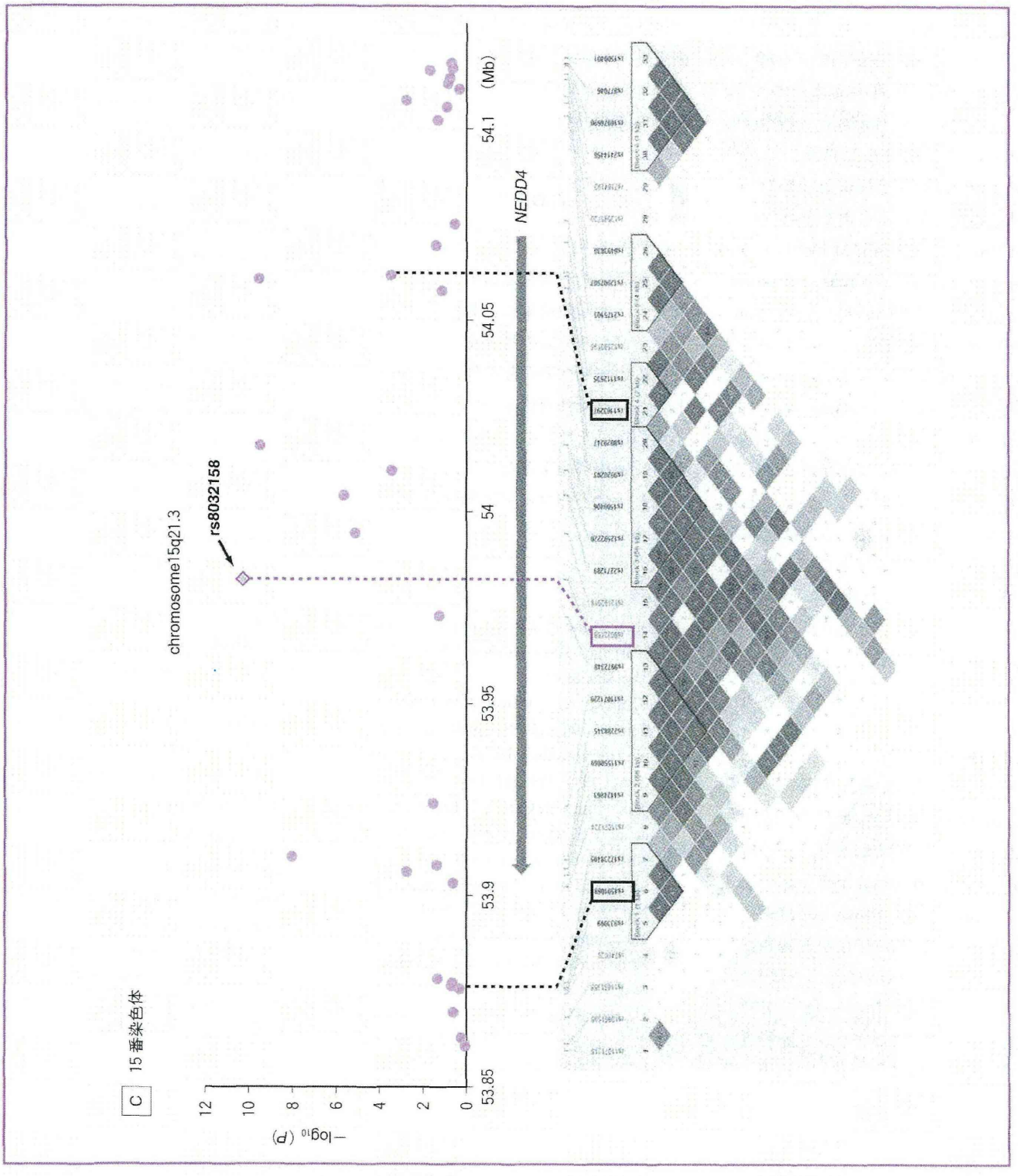

図2 ケロイドに関連する3領域

rs1511412のあいだには中等度の連鎖不平衡が認められるが（D' = 0.89, r^2 = 0.56），両SNP間の効果をロジスティック回帰分析（logistic regression analysis）にて検討した結果，この2つのSNPsはそれぞれ独立した2つの領域に存在しているものと考えられた。rs940187を含む領域にはblepharophimosis, epicanthus inversus and ptosis, candidate 1（*BPESC1*）というnon-coding RNAの存在が[5]，rs1511412を含む領域にはforkhead box L2（*FOXL2*）遺伝子とproline rich 23B（*PRR23B*）遺伝子という2つの遺伝子が報告されている。*FOXL2*は性腺ホルモン産生に深く関与していることが知られており[6][7]，ケロイドは思春期に発症あるいは増悪すること，閉経後に消退するといった報告[8]があることから，*FOXL2*が女性ホルモンの産生を上昇させ，ケロイドの形成に関与している可能性が考えられる。

3　15番染色体

15番染色体はrs8032158を含む185 kbの連鎖不平衡領域（53.87-54.06 Mb）との相関があり，同領域内にはneural precursor cell expressed, developmentally down-regulated 4（*NEDD4*）遺伝子の存在が報告されている（図2C）。*NEDD4*遺伝子はE3ユビキチンリガーゼの一種であり，PTENやp27のユビキチン化を行うことで，その安定性を制御していることが報告されており[9]-[11]，細胞増殖促進や接触阻害の異常を引き起こし，また，細胞外マトリックスであるフィブロネクチンやⅠ型コラーゲンの産生促進に働く可能性が示唆されている。

おわりに

GWASを用いることで新規のケロイド疾患感受性候補遺伝子の同定に成功した。今回の結果は，ケロイドのリスク予測システムの構築やケロイド発生機序の解明への寄与が期待できる。しかし，感受性遺伝子の機能とケロイドへの関与を解明し，新たな治療標的として臨床応用するためには，これらの遺伝子のさらなる機能解析が必要と考えられる。

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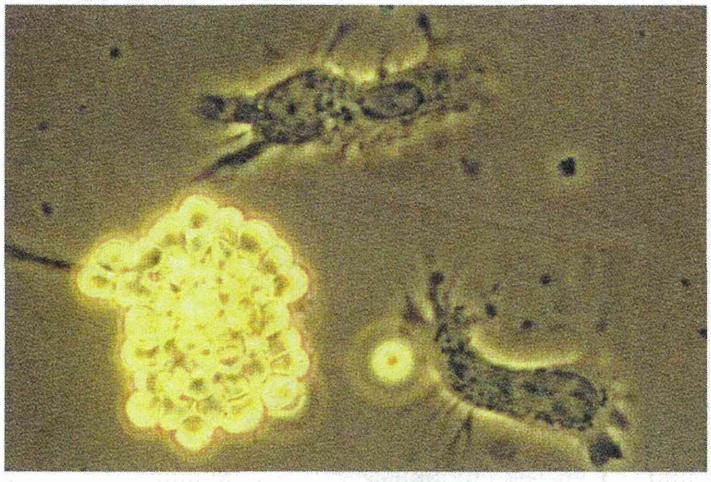

写真1　プラスチック付着性のDC(右2つの細胞)と抗体被覆赤血球をFcレセプターを介して結合したマクロファージ

➡ p47

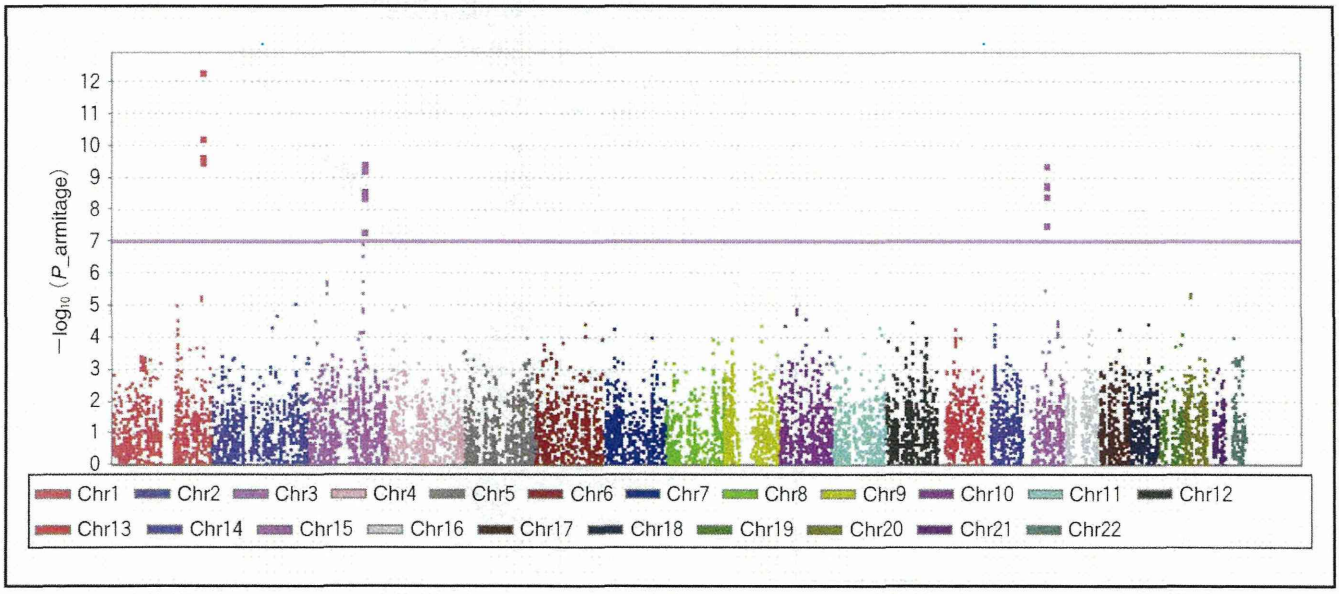

写真2　ケロイドのゲノムワイド関連解析の結果

➡ p67

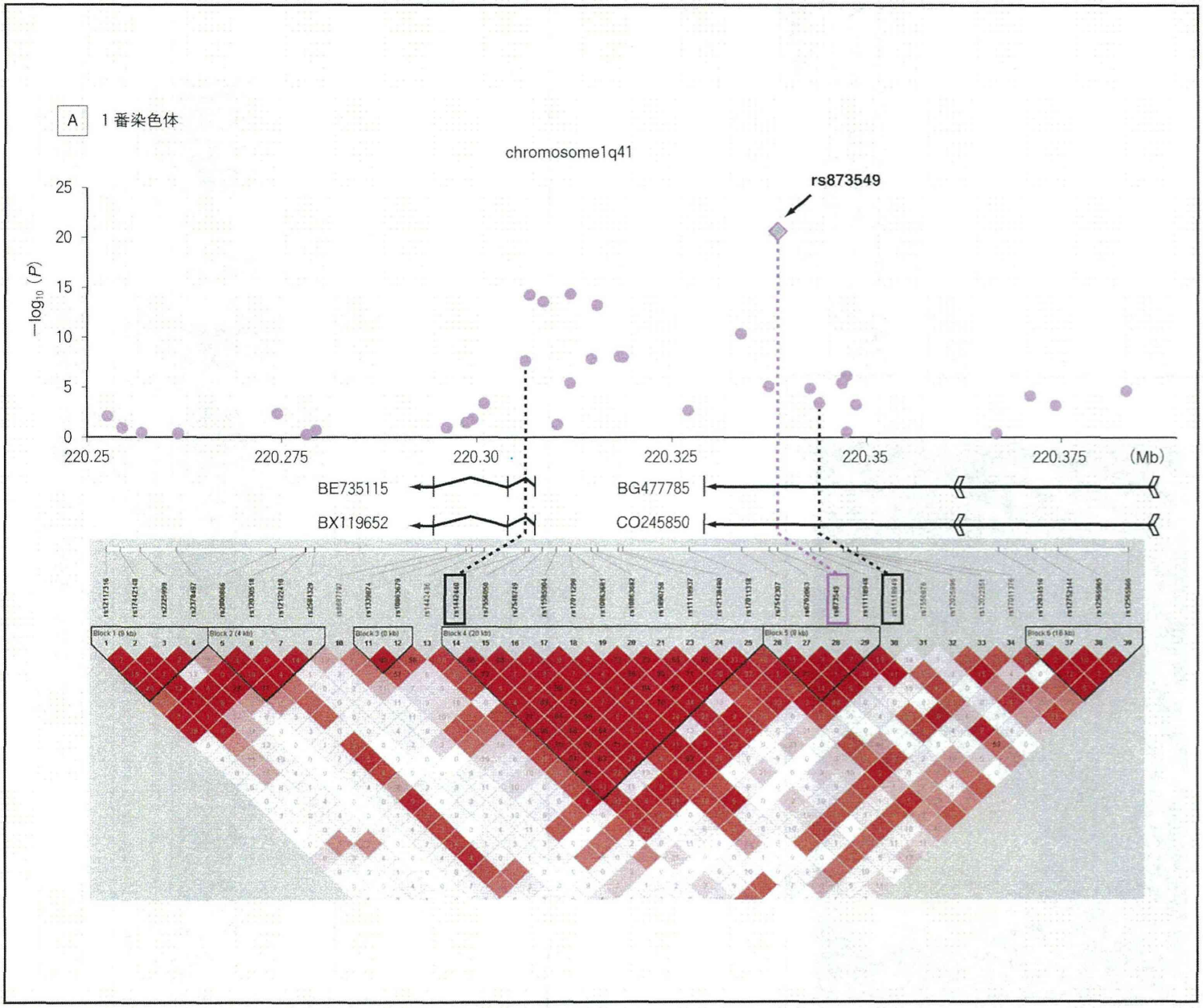

写真3 ケロイドに関連する3領域

➡ p68–70

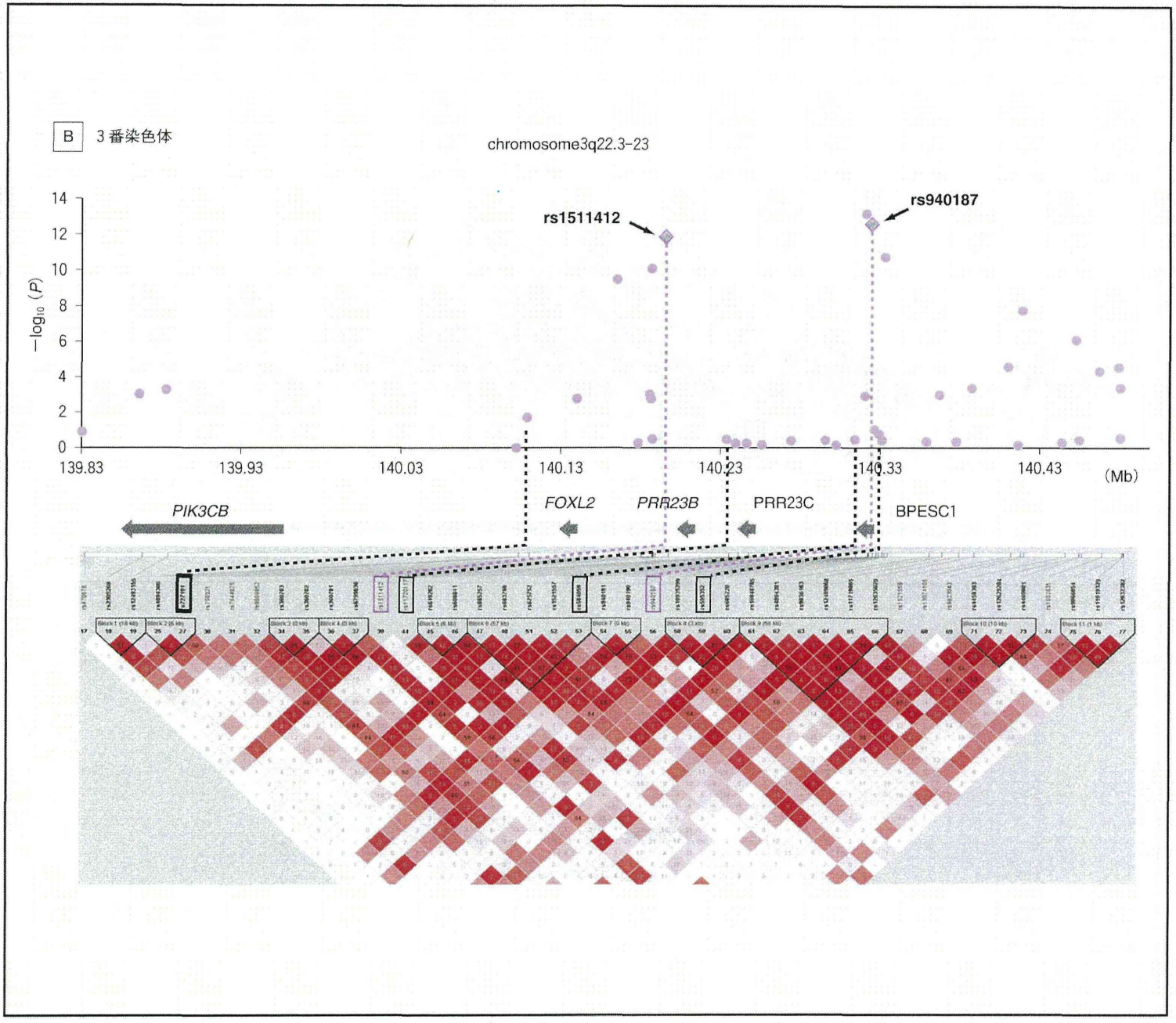

写真3　ケロイドに関連する3領域

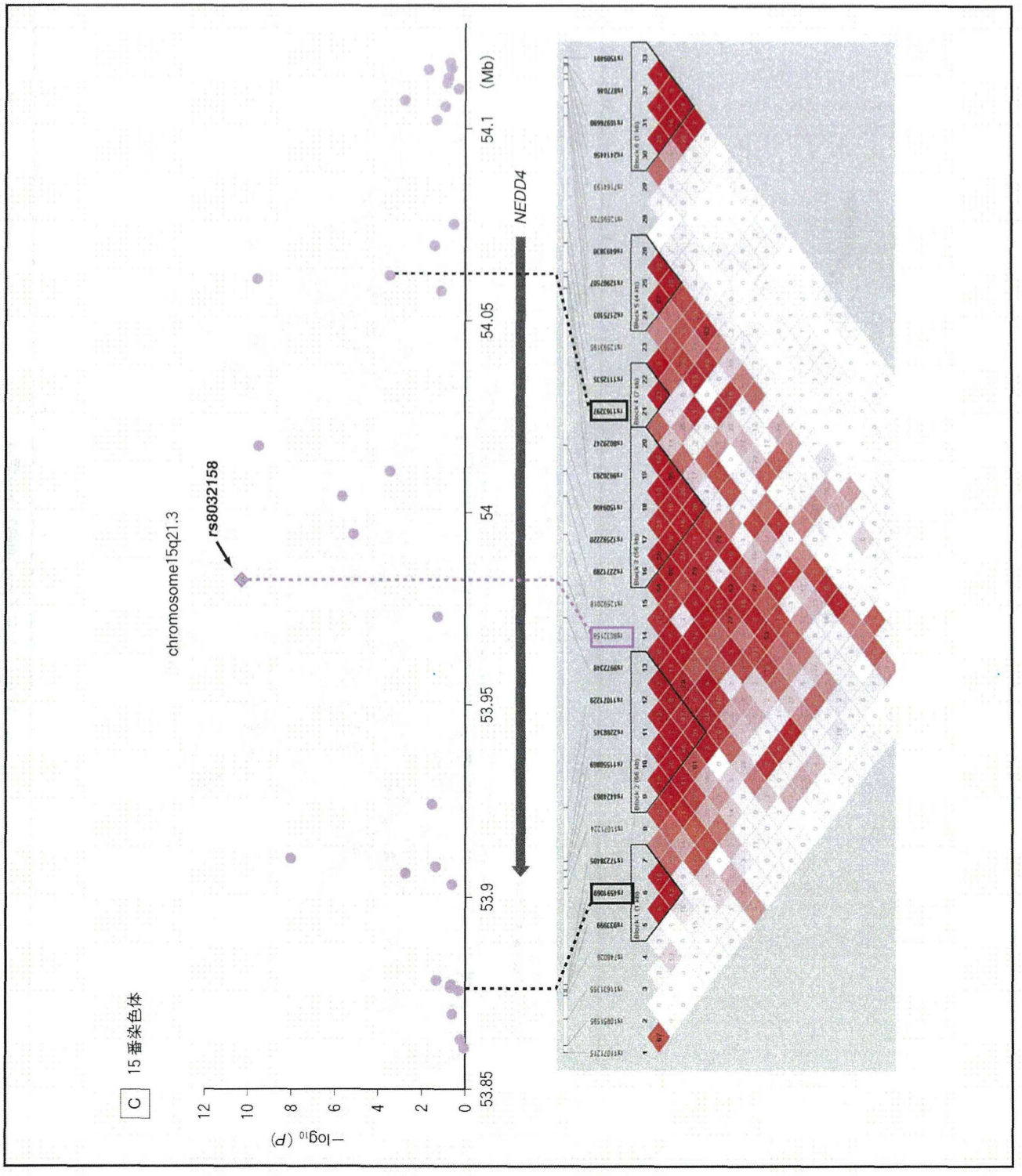

写真3　ケロイドに関連する3領域

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Clinical Cancer Research

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Clinical Cancer Research

Integration of Cell Line and Clinical Trial Genome-Wide Analyses Supports a Polygenic Architecture of Paclitaxel-Induced Sensory Peripheral Neuropathy

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Abstract

Purpose: We sought to show the relevance of a lymphoblastoid cell line (LCL) model in the discovery of clinically relevant genetic variants affecting chemotherapeutic response by comparing LCL genome-wide association study (GWAS) results to clinical GWAS results.

Experimental Design: A GWAS of paclitaxel-induced cytotoxicity was conducted in 247 LCLs from the HapMap Project and compared with a GWAS of sensory peripheral neuropathy in patients with breast cancer ($n = 855$) treated with paclitaxel in the Cancer and Leukemia Group B (CALGB) 40101 trial. Significant enrichment was assessed by permutation resampling analysis.

Results: We observed an enrichment of LCL cytotoxicity-associated single-nucleotide polymorphisms (SNP) in the sensory peripheral neuropathy-associated SNPs from the clinical trial with concordant allelic directions of effect (empirical $P = 0.007$). Of the 24 SNPs that overlap between the clinical trial ($P < 0.05$) and the preclinical cytotoxicity study ($P < 0.001$), 19 of them are expression quantitative trait loci (eQTL), which is a significant enrichment of this functional class (empirical $P = 0.0447$). One of these eQTLs is located in *RFX2*, which encodes a member of the DNA-binding regulatory factor X family. Decreased expression of this gene by siRNA resulted in increased sensitivity of Neuroscreen-1(NS-1; rat pheochromocytoma) cells to paclitaxel as measured by reduced neurite outgrowth and increased cytotoxicity, functionally validating the involvement of *RFX2* in nerve cell response to paclitaxel.

Conclusions: The enrichment results and functional example imply that cellular models of chemotherapeutic toxicity may capture components of the underlying polygenic architecture of related traits in patients. *Clin Cancer Res; 19(2); 491–9. ©2012 AACR.*

Introduction

Paclitaxel is a tubulin-targeting agent, widely used in the treatment of malignant disease, including ovarian, breast, lung, and head and neck cancers. Its long-term use is often limited by sensory peripheral neuropathy, although the mechanism of this toxicity is poorly understood. In one recent large study of more than 1,500 patients with breast cancer, severe (grade 3) sensory peripheral neuropathy occurred in 4% of patients treated with 4 cycles and 10% of patients treated with 6 cycles of single-agent paclitaxel (1). Currently, genetic prediction of which patients with cancer may experience severe side effects induced by paclitaxel treatment is not possible (2), but several preliminary genetic associations have been made (3–8). If patients likely to experience such toxicities could be identified before beginning a paclitaxel regimen, patient care might be improved by implementing a reduced dose or an alternative treatment.

Because accruing large patient cohorts receiving the same drug regimen for discovery genome-wide and replication studies in oncology is challenging, several groups of investigators have used lymphoblastoid cell lines (LCL) as a discovery tool and for follow-up functional studies (9–13). LCLs are easy to experimentally manipulate and the genetic background and expression environment is known. The LCL model also permits functional validation studies of

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Lymphoblastoid cell lines (LCL) have been used in chemotherapeutic pharmacogenomic marker discovery due to their ease of experimental manipulation, extensive genotype catalogs, and lack of the *in vivo* confounders present in clinical samples. One important question is how well these cell-based models generate clinically relevant single-nucleotide polymorphisms (SNP) associated with patient toxicity. We compared genome-wide association study (GWAS) results of paclitaxel-induced cytotoxicity in LCLs and paclitaxel-induced peripheral neuropathy in patients with breast cancer. We observed significant overlap between the clinical and LCL studies, thus confirming a role for the LCL model in the analysis of at least a subset of genes involved in patient paclitaxel response. One overlap gene, *RFX2*, was functionally validated in a nerve cell model of paclitaxel response. Peripheral neuropathy is an often dose-limiting toxicity induced by paclitaxel treatment. If physicians could predict which patients are more likely to experience this severe toxicity, lower doses or alternative treatments could be prescribed.

candidate markers and genes discovered in both preclinical and clinical studies (14, 15). However, a critical question is how well this cell-based model generates clinically relevant markers and genes associated with patient response to drug. Recently, a few chemotherapeutic response single-nucleotide polymorphisms (SNP) discovered in LCLs have been replicated in patient populations by associating with phenotypes such as tumor response and overall survival in patients receiving the same drug (16–19); however, these studies tested the individual variants most associated with the LCL phenotypes. We sought to understand to what extent the overall genetic architecture of patient response to chemotherapy can be captured by LCLs by investigating beyond just the top few signals. In contrast to previous studies that tested single SNPs, we use an enrichment method (20) to determine in a systematic manner whether top genome-wide association study (GWAS) SNPs for paclitaxel-induced sensory peripheral neuropathy in patients with breast cancer (3) are more likely to also be paclitaxel-induced cytotoxicity SNPs identified in LCLs than expected by chance.

In this study, we found that SNPs associated with patient paclitaxel-induced neuropathy are enriched for SNPs associated with paclitaxel-induced cytotoxicity in HapMap LCLs. This significant enrichment confirms that LCLs are a useful model in the study of a subset of shared genes involved in patient toxicity. The overlap SNPs are predominantly expression quantitative trait loci (eQTL) as defined previously (21), therefore supporting an enriched functional role for these significant SNPs. We show a functional role for one eQTL host gene (*RFX2*) in paclitaxel toxicity, using a cellular model of peripheral neuropathy. These results are consistent with the hypothesis that the cell-based models capture components of the underlying genetic architecture for paclitaxel-induced sensory peripheral neuropathy.

Materials and Methods

Cytotoxicity assays

HapMap LCLs from a population with Northern and Western European ancestry from Utah (HAPMAPPT01, CEU, $n = 77$), a Yoruba population in Ibadan, Nigeria (HAPMAPPT03, YRI, $n = 87$), and an African-American population from the Southwest of the United States (HAPMAPPT07, ASW, $n = 83$) were treated with 12.5 nmol/L paclitaxel and cytotoxicity was determined using an AlamarBlue (Invitrogen) cellular growth inhibition assay as described (22). The cytotoxicity phenotype used in the LCL GWAS was mean percentage survival at 12.5 nmol/L paclitaxel determined from 6 replicates from 2 independent experiments. Percentage survival values for each cell line were $\log_2$-transformed before statistical analysis to form an approximately normal distribution in each population.

LCL genome-wide meta-analysis

A GWAS of paclitaxel-induced cytotoxicity was conducted on each of the 3 populations separately. Greater than 2 million SNPs from HapMap r27 [minor allele frequency (MAF) > 0.05 within the panel, no Mendelian errors and in Hardy–Weinberg equilibrium ($P > 0.001$)] were tested for association with paclitaxel cytotoxicity in each population, using the quantitative trait disequilibrium test total association model (23). To control for population structure in the admixed ASW population, local ancestry at each genotyped SNP locus was estimated using HAPMIX (24) and to increase genome coverage of the ASW, ungenotyped markers were imputed using BEAGLE (25) as previously described (26). Genomic control lambda (λ_{GC}) values (27) were calculated for the GWAS of each population. Studies with λ_{GC} values greater than 1 were corrected for residual inflation of the test statistic by dividing the observed test statistic at each SNP by the λ_{GC} (27), and then the corresponding P values were carried through the meta-analysis.

Using the software METAL, we combined SNP P values across the 3 population studies, taking into account a study-specific weight (sample size) and direction of effect (positive or negative β; ref. 28). This approach converted the direction of effect and P value observed in each study into a signed Z-score, such that very negative Z-scores indicate a small P value and an allele associated with higher drug sensitivity, whereas large positive Z-scores indicate a small P value and an allele associated with higher drug resistance. Z-scores for each SNP were combined across studies in a weighted sum, with weights proportional to the square-root of the sample size for each study (28).

Patient samples and GWAS

Cancer and Leukemia Group B (CALGB) 40101 is a phase III trial comparing the efficacy of standard therapy cyclophosphamide and doxorubicin with single-agent paclitaxel

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as adjuvant therapy for breast cancer in women with 0 to 3 positive axillary lymph nodes. All study participants were enrolled in CALGB 40101 and gave their additional consent to participate in the pharmacogenetic companion study (CALGB 60202), which has been published (3). All patient research met state, federal, and Institutional Review Board guidelines. Germline DNA was isolated from 1,040 patients on the paclitaxel arm of CALGB 40101 and genotyped using the Illumina 610-Quad platform as described previously (3). Following quality control analysis, genotypes were available for 520,679 SNPs. Principal component (PC) analysis identified 855 genetic Europeans that were used in a GWAS of sensory peripheral neuropathy (3). A dose-to-event analysis was conducted, with an event defined as grade 2 or greater sensory peripheral neuropathy. The Cox score test, powered for additive genetic effects, was used to test these marginal associations. Only SNPs with MAFs more than 0.05 in the patient population and in Hardy–Weinberg equilibrium in the CEU ($P > 0.001$) were used in the LCL GWAS comparisons.

Enrichment analysis

We conducted a permutation resampling analysis (29) to test for an enrichment of cytotoxicity-associated SNPs (LCLs) among the paclitaxel-induced sensory peripheral neuropathy-associated SNPs (patients). To this end, the patient outcomes (cumulative dose and event indicator vectors) were randomly shuffled while keeping the genotype data fixed to preserve linkage disequilibrium. On the basis of this permutation replicate, the standardized Cox score statistics were recalculated for all the SNPs. This process was conducted 1,000 times. For each of the 1,000 permutation replicates, the number of SNPs that had $P < 0.05$ in the patient data, $P < 0.001$ in the LCL data, and the same direction of effect (the same allele associated with increased neuropathy and increased cytotoxicity) was calculated. The overlap distribution from the permutations was compared with the observed SNP overlap to generate an empirical *P* value, calculated as the proportion of permutations in which the number of LCL/patient overlap SNPs exceeds the observed number. To test the robustness of our findings, we calculated an empirical *P* value across a range of inclusion thresholds from $P < 0.001$ to $P < 0.1$. We also tested for enrichment of patient SNPs among the LCL SNPs by generating 1,000 randomized SNP sets the same size and MAF distribution as the observed LCL data at a range of *P* value thresholds to calculate empirical *P* values. In addition to the paclitaxel LCL cytotoxicity data, we compared the patient sensory peripheral neuropathy data with LCL cytotoxicity GWAS data from capecitabine (30) and carboplatin (13) as negative controls.

To test for eQTL enrichment in the LCL, patient, and LCL/patient overlap SNPs, we generated 10,000 randomized SNP sets each of the same size as the observed set of LCL cytotoxicity ($P < 0.001$), patient neuropathy ($P < 0.05$), or LCL/patient overlap SNPs. The randomized SNP sets were matched on MAF distribution of the observed list and sampled (without replacement) from the set of SNPs on the Illumina 610-Quad platform, similar to the method of Gamazon and colleagues (31). We grouped the platform SNPs into discrete MAF bins of a width of 5%, from which the SNPs used in the simulations were selected. For each of the 10,000 sets, we determined the number of eQTLs ($P < 10^{-4}$) and calculated an empirical *P* value for enrichment. The eQTLs were defined previously and are available in the SCAN database (21, 31).

Filtering procedure for functional analysis

First, we determined which of the LCL/patient overlap SNPs from the enrichment analysis were located in or near (within 2 kb) gene transcripts (dbSNP build 129, human genome assembly build 36). Eleven of 24 overlap SNPs were in or near genes and genotyping intensity plots for these SNPs in the patient data are available in Supplementary Fig. S1. Second, we determined which SNPs within genes were also eQTLs (31) and prioritized by which had the most target genes ($P < 10^{-4}$). We also tested whether the expression of the eQTL target genes associated with paclitaxel-induced cytotoxicity ($P < 0.05$) using previously published exon array data (32). A general linear model was constructed between gene expression and paclitaxel-induced cytotoxicity with growth rate (33) and population as covariates. A Toeplitz covariance structure with 2 diagonal bands was used to allow for familial dependencies in the data as previously described (9).

siRNA

Neuroscreen-1 (NS-1) rat pheochromacytoma cells (Cellomics Inc.) were maintained in NS-1 media (RPMI supplemented with 10% horse serum, 5% fetal calf serum and 1% L-glutamine). Cells were seeded at a density of 1×10^5 cells/mL on collagen I–coated plates and induced to differentiate by adding 20 ng/mL nerve growth factor (NGF, BD Biosciences) to the media 24 hours before transfection. Cells for cytotoxicity assays were plated in 96-well collagen I–coated plates, whereas cells for expression quantification and neurite-outgrowth assays were plated in 6-well collagen I–coated plates. Pooled *Rfx2* siRNA (25 nmol/L; Qiagen; S101639659, S101639666, S101639673, and S101639680) or nontargeting control siRNA (Qiagen; 1027292) was transiently transfected into the NS-1 cells using DharmaFECT Reagent #1 (Dharmacon). Quantitative reverse transcription PCR (qRT-PCR) was conducted for *Rfx2* (Rn00501380_m1) and control gene *Gapdh* (4352338E) using TaqMan Gene Expression Assays (Applied Biosystems) 24 hours posttransfection in the neurite-outgrowth assays and 24, 48, 72, and 96 hours posttransfection in the cytotoxicity assays to assess *Rfx2* knockdown in NS-1 cells. Expression of the potential *Rfx2* target genes *Cyp51* (Rn01526553_m1), *Bach1* (Rn01477344_m1), and *Cbara1* (Rn01644475_m1) was also measured by qRT-PCR at 24 hours post-*siRfx2* transfection. Each qRT-PCR was run in duplicate and individual samples were run in triplicate on each plate. Percentage knockdown was calculated by dividing the relative *Rfx2* expression levels in the *siRfx2* sample by those in the nontargeting control sample.