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Multi-Gene Interactions and the Prediction of Depression in the Wisconsin Longitudinal Study

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ABSTRACT

Objectives: Single genetic loci offer little predictive power for the identification of depression. This study examined whether an analysis of gene-gene interactions of 84 single nucleotide polymorphisms in genes associated with depression and age-related diseases would identify significant interactions with increased predictive power for depression.

Design: A retrospective cohort study.

Setting: A survey of participants in the Wisconsin Longitudinal Study.

Participants: A total of 4,792 persons (2,459 females and 2,333 males) who provided saliva for genotyping; the group comes from a randomly selected sample of Wisconsin high school graduates from the class of 1957 as well as a randomly selected sibling, almost all of whom are non-Hispanic white.

Primary outcome measure: Depression as determine by the Composite International Diagnostic Interview short-form (CIDI-SF).

Results: Using a classification tree approach (recursive partitioning (RP)) we identified a number of candidate gene-gene interactions associated with depression. The primary SNP splits revealed by RP (*ANKK1* rs1800497 in men and *DRD2* rs224592 in women) were found to be significant as single factors by logistic regression (LR) after controlling for multiple testing (P=0.001 for both). Without considering interaction effects, only 1 of the 5 subsequent RP splits reached nominal significance in logistic regression (*FTO* rs1421085 in women; P-value=0.008). However, after controlling for gene-gene interactions by running logistic regression on RP-specific subsets, every split became significant and grew larger in magnitude (OR [before]→[after]: Men: *GNRH1* novel SNP: [1.43 → 1.57]; Women: *APOC3* rs2854116: [1.28 → 1.56], *ACVR2B* rs3749386: [1.11 → 2.16], *FTO* rs1421085: [1.32 → 1.63], *IL6* rs1800795: [1.12 → 1.85]).

Conclusions: Our results suggest that examining gene-gene interactions improves the identification of genetic associations predictive of depression. Four of the SNPs identified in these interactions were located in two pathways well-known to impact depression: neurotransmitter (*ANKK1* and *DRD2*) and

neuroendocrine (*GNRH1* and *ACVR2B*) signaling. This study demonstrates the utility of RP analysis as an efficient and powerful exploratory analysis technique for uncovering genetic and molecular pathway interactions associated with disease etiology.

INTRODUCTION

Depression is a widespread mental disorder associated with a host of undesirable health, social, and economic outcomes. One in six Americans is diagnosed with depression in his or her lifetime (1). While many environmental factors—such as socioeconomic status, childhood abuse, and major life events—have important ties with depression, so too does gender and many genetic and epigenetic factors, making the disorder heterogeneous in nature (2). Another major risk factor for depression is age, with depression reaching its highest levels in adults over 80 years of age (3).

It has been demonstrated from twin studies that genetic factors typically account for 40–70% of the risk for developing major depressive disorder (MDD), and adoption studies have confirmed the role of genetic risk factors in the development of MDD (see (4) and references therein). Genetic studies, including recent genome-wide association studies (GWAS), have identified genetic alterations in over 50 genes known to be associated with depression (5). However, individually, the genetic alterations found within these genes (primarily single nucleotide polymorphisms (SNPs)) have little predictive value. There is a similar lack of predictive value from GWAS of other major age-related diseases (6).

Given this lack of predictive power among individual genetic alterations for depression together with the complex nature of aging-related diseases, it would seem prudent to examine epistatic effects on this age-related condition. In this respect, we have previously demonstrated that G x G interactions greatly modulate risk for complex age-related diseases (7, 8). Recent studies of depression also have identified epistatic effects. In particular, associations have been identified between *BDNF* Val66Met (brain-derived neurotrophic factor; rs6265) and *5-HTTLPR* (serotonin transporter linked promoter region (9); *GSK3B* rs6782799 (glycogen synthase kinase 3β), *BDNF* rs7124442 and *BDNF* Val66Met (10); *BDNF* Val66Met

and SNPs in *NTRK2* (neurotrophic tyrosine kinase receptor 2; (11)), and *5-HTTLPR* short allele and a chromosome 4 gene (12).

In this study, we have assessed the epistatic effects of known genetic alterations that link to depression and age-related diseases in the Wisconsin Longitudinal Study (WLS). Using recursive partitioning (RP) and logistic regression (LR) we identified associations between dopaminergic genes and depression in men and women, as well as G x G interactions involving neuroendocrine signaling pathways, with increased significance compared with single genetic associations.

METHODS

Study Participants and Surveys

Data were collected from the WLS, a random sample originally comprised of 10 317 men and women who graduated from Wisconsin high schools in 1957. Later in 1977, the WLS began interviewing one randomly selected sibling of each graduate, when possible. The cohort consists almost entirely of non-Hispanic white persons whose average level of educational attainment was 1.5 years of post-high school education at the time of interview in 2004. Ages of participants in the WLS ranged from 35 to 90 years old at this time, with 83% of participants being between 60 and 70 years old. Further characteristics of the WLS cohort may be found in detail elsewhere (13). Health and psychological well-being phenotypic data was taken from mail and phone surveys given in 2004-2005. Our main measure of depression is based on a variation of the Composite International Diagnostic Interview short-form (CIDI-SF). All participants answered a single stem question: “Have you ever had a time in life lasting two weeks or more when nearly every day you felt sad, blue, depressed, or when you lost interest in most things like work, hobbies, or things you usually liked to do for fun?” Only those who answered YES and whose depression was not always caused by alcohol, drugs, medications, or physical illness were asked further depression symptom questions. Symptom questions asked whether the two week period was accompanied with any weight loss, trouble sleeping, feeling tired, feeling bad upon waking, losing interest, trouble concentrating, or thoughts about death. Those answering YES to 3 or more of these symptom questions were classified as having depression (14).

Genotyping

7 101 participants (4 569 graduates & 2 532 siblings) provided saliva samples in Oragene DNA sample collection kits from which DNA was extracted and genotyped for 84 SNPs that were selected based on their association with depression and age-related conditions and diseases. Genotyping was performed by KBioscience (Hoddesdon, UK) with use of a homogeneous Fluorescent Resonance Energy Transfer

technology coupled to competitive allele specific PCR. All SNP genotypes described in our results were in Hardy-Weinberg equilibrium and their frequencies matched those reported in the literature for European samples.

Statistical Analysis

Of those participants that provided DNA and that also completed the survey depression questions (4 792), the following analyses were performed:

Recursive Partitioning (RP). RP is a data mining tool for revealing trends that relate a dependent variable (depression incidence) to various predictor variables (SNPs). Zhang and Bonney have shown how RP can be used in genetic association studies to identify disease genes (15). RP helps control for heterogeneity in the population and confounding factors by allowing for the segregation of the sample population according to any condition. Thus, RP is a useful way to handle complex datasets that might confound regression analysis due to the complexity of the relationship between the independent and dependent variables and due to missing information.

RP classification trees (using R package rpart) were used to identify potential interactions among the 84 SNPs in relation to depression. The trees split the data along branches according to criteria determined by the rpart package algorithm, which is originally based off the work of Breiman's classification and regression trees (CART) algorithm (16). Basically, the CART algorithm first considers all depressed and non-depressed subjects pooled together in a heterogeneous root node. Based on considering every possible "yes-no" binary partition that can be made by each independent variable, the single split which maximizes homogeneity between the two resulting sub-nodes as compared to the root node is made. Each sub-node can then be treated independently as a new root node for all subsequent splits, and the pattern continues until every subject constitutes a terminal node, resulting in a very large and complex tree. A 10-part cross validation procedure seeking to minimize misclassification and complexity

determines optimal pruning. See Therneau and Atkinson (17) for specific details of the rpart package. Tree nodes were re-created in Microsoft Visio to display depression incidence (in %) and total number of participants rather than the default number of controls/cases as presented by rpart.

Logistic Regression (LR). Variables found in association with depression based on RP analysis were considered in single factor LR models, separate by gender, using the specific dichotomous splitting of genotypes as designated by RP trees. Regression models for all seven SNP splits were first run on the full dataset to represent single main factor effects. Then each split was run on the respective subset of data as represented by the preceding RP split criteria. Thus, we attempt to mirror RP splits within a more formal LR framework in order to measure the significance of interactions presented by the trees. Multiple testing of 84 SNPs in RP for both male and females followed by 14 LR models resulted in a modified FDR significance level of 0.008.

RESULTS

Of the 4,792 participants with complete survey information on CIDI-SF depression (2 459 females and 2 333 males), we identified 711 participants (481 females and 230 males) with depression. Given that the independent variable gender (when included as a factor in the full dataset) was the primary split on RP trees; that women are over two times as likely to be diagnosed with depression than men; and since the female etiology of depression has been reported to be associated with unique social, psychological, and biological factors (18), all subsequent analyses were performed by gender.

Recursive Partition Analysis

To examine multi-gene interactions for association with depression we screened our dataset using RP. The two-factor RP tree (*ANKK1*/*GNRH1*) was the optimized pruning for men (Fig. 1), while the five-factor tree (*DRD2*/*APOC3*/*ACVR2B*/*FTO*/*IL6*) was the optimized pruning for women (Fig. 2). The best overall split for men was *ANKK1* rs1800497, where the incidence of depression increased 2.3-fold in those with no C-alleles compared to those with one or two C-alleles. Considering interaction between *ANKK1* and *GNRH1* widened the disparity in incidence, where those with at least one C-allele in both *ANKK1* rs1800497 and the novel SNP in *GNRH1* had a 3-fold lower incidence than those without a C-allele in *ANKK1* rs1800497.

For women, the best overall split was *DRD2* rs2242592, where those with one or two C-alleles had 1.3-fold higher incidence of depression compared to those without any C-alleles. G x G interactions associated with the highest incidence of depression included: *DRD2* rs2242592 T/T + *APOC3* rs45537037 T/T + *ACVR2B* rs3749386 C/C or T/T, accounting for a 1.4-fold increase in depression compared to baseline incidence.

Single Main-Factor Effects

Specific SNP interactions identified by RP were next analyzed by LR (see Table 1, Full Data). The

primary SNP splits in males and females were significant at the modified FDR level. Men with no C-alleles for *ANKK1* rs1800497 had 2.6 times higher odds [P=0.001 (1.5, 4.6)] of depression compared with men with at least 1 C-allele. Women with at least 1 C-allele for *DRD2* rs2242592 had 1.3 times higher odds [P=0.006 (1.1-1.6)] of depression compared with women with no C-alleles. One other split reached nominal significance; women homozygous (C/C or T/T) for *FTO* rs1421085 had 1.32 times higher odds [P=0.008 (1.1-1.6)] for depression than women with a heterozygous genotype. SNP splits of *GNRH1*, *APOC3*, *ACVR2B*, and *IL6* did not significantly associate with depression.

Gene-Gene Interactions Enhance Predictability for Depression

Specific SNP interactions identified by RP were next analyzed by LR as RP-specific subsets (see Table 1, RP-Subsetted Data). All 5 of the secondary and tertiary RP splits were found to be significant at the modified FDR level when considered as subsets. Among only men with at least one C-allele in *ANKK1* rs1800497, those with no C-allele in the novel SNP of *GNRH1* had 1.57 times higher odds [P=0.002 (1.2-2.1)] for depression than men with 1 or 2 C-alleles. For the subset of women in the first right-hand split of Fig. 2, those homozygous for *FTO* rs1421085 had 1.63 times higher odds [P=0.0006 (1.2-2.2)] for depression than women with a heterozygous genotype. For the remaining subset of women in the second right-hand split of Fig. 2, those homozygous for *IL6* rs1800795 had 1.85 times higher odds [P=0.007 (1.2-2.9)] for depression than women with a heterozygous genotype. For the subset of women in the first left-hand split of Fig. 2, those with no C-alleles for *APOC3* rs45537037 had 1.56 times higher odds [P=0.004 (1.2-2.1)] for depression than women with 1 or 2 C-alleles. For the subset of women in the second left-hand split of Fig. 2, those homozygous for *ACVR2B* rs3749386 had 2.16 times higher odds [P=0.001 (1.4-3.4)] for depression than women with a heterozygous genotype.

DISCUSSION

Utilizing RP as a screening tool to find potential multi-gene interactions, followed by verification of multi-gene interactions with LR, our data demonstrate that multi-gene interactions predict depression with a greater certainty than single main factor associations. RP provided us with primary dichotomous genotype splits in men and women (*ANKK1* rs1800497 and *DRD2* rs2242592, respectively) that were both significant in LR models at the modified FDR level (Table 1). Considering the 5 subsequent RP splits in LR over the entire dataset, only 1 reached a nominal level of significance (barely), which was *FTO* rs1421085 in women. However, after running LR on specific subsets of data according to the pattern of RP branches, every split was found to be significant and every odds ratios grew larger (Table 1; P-values [before]→[after]: Male Left: 1.43 → 1.57, Female Left 1: 1.28 → 1.56, Female Left 2: 1.11 → 2.16, Female Right 1: 1.32 → 1.63, Female Right 2: 1.12 → 1.85). Thus, RP provides two unique and important criteria: dichotomous genotype splitting instructions and gene-gene interaction patterns. These criteria go beyond the traditional single factor SNP approach to genetic association studies and allow identification of important multi-gene pathways that more suitably characterize the etiology of complex diseases.

The Utility of Recursive Partitioning, Multi-factor Dimensionality Reduction and Logistic Regression for Identification of Gene-Gene Interactions

With recent advances in genotyping allowing for high-dimensional SNP identification, it is now possible to examine genetic datasets not only for single main factor effects, but also G x G interactions. The requirement for G x G analyses as a better predictor of age-related diseases is obvious from the standpoint that humans are complex biological systems composed of numerous molecular interactions, and from recent studies indicating disease risk is modulated by G x G interactions (7). Notwithstanding this, the development of analytical tools for the identification of G x G interactions has not kept pace with the technological advances in identifying genetic alterations among individuals. In this respect, we have previously used MDR, LR and LD to identify G x G interactions among a small set of SNPs (7). However,

large datasets require a screening tool to identify potential multi-gene interactions. In this study, we have used RP to screen for multi-gene interactions, a data-mining technique that is currently under-utilized in genetic studies. RP serves as an efficient and powerful exploratory analysis technique, especially when looking for interactions in data sets with a large number of independent variables. This screening allows for the identification of G x G interactions (with greater explanatory power), that might otherwise not have been identified, and that can then be confirmed using more traditional statistical techniques. As illustrated in this paper, this data-mining methodology has the advantage of identification of genetic interactions *between* pathways involved in the etiology of depression, in keeping with the etiological heterogeneity of this disorder (see later).

Our study provides proof of principle for the use of RP in higher-dimensional analyses such as GWAS, where a comprehensive list of SNPs may fully explore genetic predisposition to depression and other age-related disease. The WLS is an ideal candidate for future GWAS studies given its large sample size, rich covariate composition and longitudinal nature.

In this genetic study we aimed to identify underlying genetic predispositions to depression and thus have not yet tested environmental/phenotypic data. Future analyses using RP to examine the impact of phenotypic and environmental factors on the development of depression would be anticipated to identify gene-phenotype/environment and multi-phenotype/environment interactions. Indeed, the predictive gains of G x G analyses were stronger for men than women, despite the fact that depression occurs disproportionately in women (~2:1 female-to-male; (19-23)). This suggests that environmental factors may be needed in addition to genetic factors in understanding the etiological pathways for women. Indeed, biological factors such as hormonal changes related to reproductive status (24, 25) may impact environmental factors such as psychosocial experiences (trauma, stress, interpersonal relationships, etc) and general health issues in the development of depression.

Genetic and Biological Correlates of Depression

Numerous studies have identified SNPs that associate with depression. Many of the SNPs associated with depression from other studies were not significantly associated in our study. This is perhaps not surprising, since a single factor is unlikely to provide consistent association especially in a complex condition such as depression, where multiple pathways intersect in regulating the risk of the disease. For example, if a SNP within the serotonin pathway also requires a SNP in the glutamatergic pathway in order for the patient to present with depression, the presence of either SNP in the absence of the other will not be predictive of depression. Moreover, as indicated by Shi and Weinberg, since the human genome contains genetic redundancy, disruption of a single gene may be selectively neutral, but the malfunction of several genes in a pathway might result in expression of a particular phenotype (26).

Both the primary splits in men and women were SNPs linked with *DRD2* (dopamine receptor D2), a gene that has previously been linked with depression and social phobia (27-29). The primary male genotype split rs1800497, technically found in gene *ANKK1*, is historically known as the *DRD2* Taq1A allele because of its known association with decreased dopamine receptor D2 density (in those with T alleles) (30-33). The Taq1A allele has also been previously associated with depressive symptoms in children, where those with the A1 allele (T) were more likely to have depressive symptoms (34). We saw a similar association between A1 and depression in WLS men, where those with two A1 alleles had 2.6 times higher odds for depression compared to those with one or no A1 alleles. The primary split in women (DRD2 rs2242592) has previously been found to be associated with schizophrenia, where the C-allele was associated with higher susceptibility for schizophrenia (35). Interestingly, this same study also found the Taq1A allele to also associate with schizophrenia.

The secondary and tertiary right-hand splits in the female RP tree—*FTO* (fat mass and obesity associated) rs1421085 and *IL6* (interleukin 6) rs1800795—have also been found to relate with mental illness and depression in previous studies (36)(37). There is evidence that activin receptor signaling also is involved in affective disorders, especially when considering interaction with GABAergic pathways (38). Although we did not see an interaction between SNPs in GABA/activin receptor genes and depression,

ACVR2B was associated with depression in women. No previous associations between depression and *APOC3*, *ACVR2B*, or *GNRH1* have been reported.

That these genetic variants are associated with *neuroendocrine* pathways (*GnRH1, ACVR2B*) that are known to regulate *neurotransmitter* release and cognitive behavior (39-40) supports these associations as relevant to the etiology of depression and underlines the benefits of using RP to identify meaningful G x G interactions associated with disease.

Limitations

Given the numerous genetic, phenotypic and environmental influences that are linked to depression, and the small number of SNPs analyzed, it is not surprising that predictability from our models was low (although our predictability was superior to previous studies examining only single main factors). Also, the predictive value of our statistical models was further limited due to user bias in selection of SNPs (from nearly two-million SNPs in the human genome) used in this study. As a result of this, interactions we have found could potentially be moderated by another gene that we have not considered in this study. Nonetheless, we identified significant G x G interactions between known, and newly identified, loci associated with depression. Importantly, 4 of the 7 SNPs identified in these interactions were primarily located in two pathways well-known to impact depression: neurotransmitter and neuroendocrine signaling.

The results from the RP analyses conducted in this study were confirmed by LR, demonstrating the utility of RP as a screening tool for identifying meaningful G x G interactions. Future development of algorithms for RP analysis should not only maximize the distance between branches of the next best split (i.e. rpart), but consider subsequent future split combinations that could potentially result in trees with "better" overall predictability.

Summary

Our data indicate that G x G interaction analyses allows for enhanced predictability of conditions and

diseases of aging. RP is an efficient and powerful exploratory analysis technique for elucidating G x G interactions in large datasets and combined with LR provides an important statistical analysis for the identification of well supported G x G interactions. We predict that such analytical methods will play an increasingly important role in the identification of epistatic effects in future large GWAS. Finally, our studies illustrate how RP analyses can be used to find interacting pathways involved in the etiology of a disease or condition such as depression.

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Contributorship Statement

CSA, RMH and TSH conceptualized the study. RMH, TSH, CLR, NSR, CL and CSA collected saliva samples and performed genotyping analyses. NSR, JAY, CL, VC and JB performed statistical analyses on the WLS dataset. CSA and RMH directed the statistical analyses. NSR and CSA drafted the manuscript. All authors critically reviewed the manuscript and approved the final version.

Data Sharing

Genetic and environmental data for the WLS is available online at http://www.ssc.wisc.edu/wlsresearch/

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Competing Interests

None

FIGURE LEGENDS

Figure 1. Recursive Partitioning Tree of CIDI-SF Depression in Males of the WLS. Upper and lower numbers in nodes represent the proportion of participants with depression and the number of participants in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. M1 is subset of data referenced in Table 1. Sensitivity: 0.526, Specificity: 0.598, Accuracy: 0.591.

Figure 2. Recursive Partitioning Tree of CIDI-SF Depression in Females of the WLS. Upper and lower numbers in nodes represent the proportion of participants with depression and the number of participants in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. F1-F4 are subsets referenced in Table 1. Sensitivity: 0.615, Specificity: 0.549, Accuracy: 0.562.

Table 1.
Single-factor logistic regression models based directly off male and female RP tree split criteria (see Figures 1 & 2). Each SNP split was first run on the full dataset to represent single main factor effects ("Full Data") for both males and females. Then the same SNP splits were run on specific subsets of data per RP tree splits (M1, F1-F4; "RP-Subsetted Data").

					Full Data		RP-Subsetted Data		
Gender	RP Split	Gene	SNP	Genotypes	OR (95% CI)	P-value	Subset	OR (95% CI)	P-value
Male	Primary	*ANKK1*	rs1800497	T/T vs. C/C + C/T	2.60 (1.47-4.61)	0.001 *	--	----	----
	Left	*GNRH1*	novel SNP	T/T vs. C/C + T/C	1.43 (1.08-1.88)	0.011	M1	1.57 (1.18-2.09)	0.002 *
Female	Primary	*DRD2*	rs2242592	C/C + T/C vs. T/T	1.33 (1.09-1.62)	0.001 *	--	----	----
	Left 1	*APOC3*	rs2854116	T/T vs. C/C + T/C	1.28 (1.05-1.57)	0.017	F1	1.56 (1.16-2.10)	0.004 *
	Left 2	*ACVR2B*	rs3749386	C/C + T/T vs. T/C	1.11 (0.91-1.36)	0.302	F2	2.16 (1.36-3.42)	0.001 *
	Right 1	*FTO*	rs1421085	C/C + T/T vs. T/C	1.32 (1.07-1.61)	0.008 *	F3	1.63 (1.23-2.17)	0.0006 *
	Right 2	*IL6*	rs1800795	C/C + G/G vs. C/G	1.12 (0.91-1.36)	0.283	F4	1.85 (1.18-2.88)	0.007 *

RP, recursive partitioning; OR, odds ratio; CI, confidence interval
M1: LR analysis was run for only those with genotype *DRD2* rs1800497 C/C or C/T
F1: LR analysis was run for only those with genotype *DRD2* rs2242592 T/T
F2: LR analysis was run for only those with genotypes *DRD2* rs2242592 T/T and *APOC3* rs2854116 T/T
F3: LR analysis was run for only those with genotype *DRD2* rs2242592 C/C or T/C
F4: LR analysis was run for only those with genotypes *DRD2* rs2242592 C/C or T/C and *FTO* rs1421085 T/C

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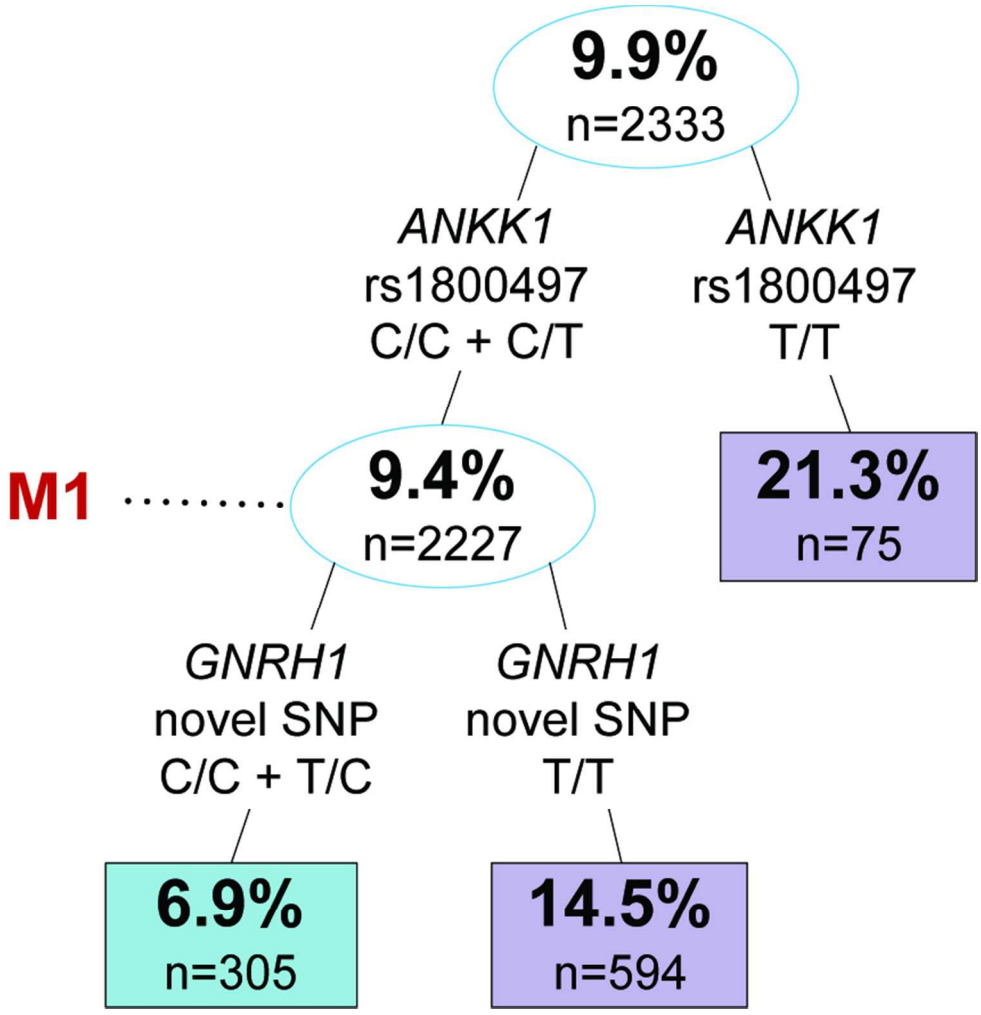

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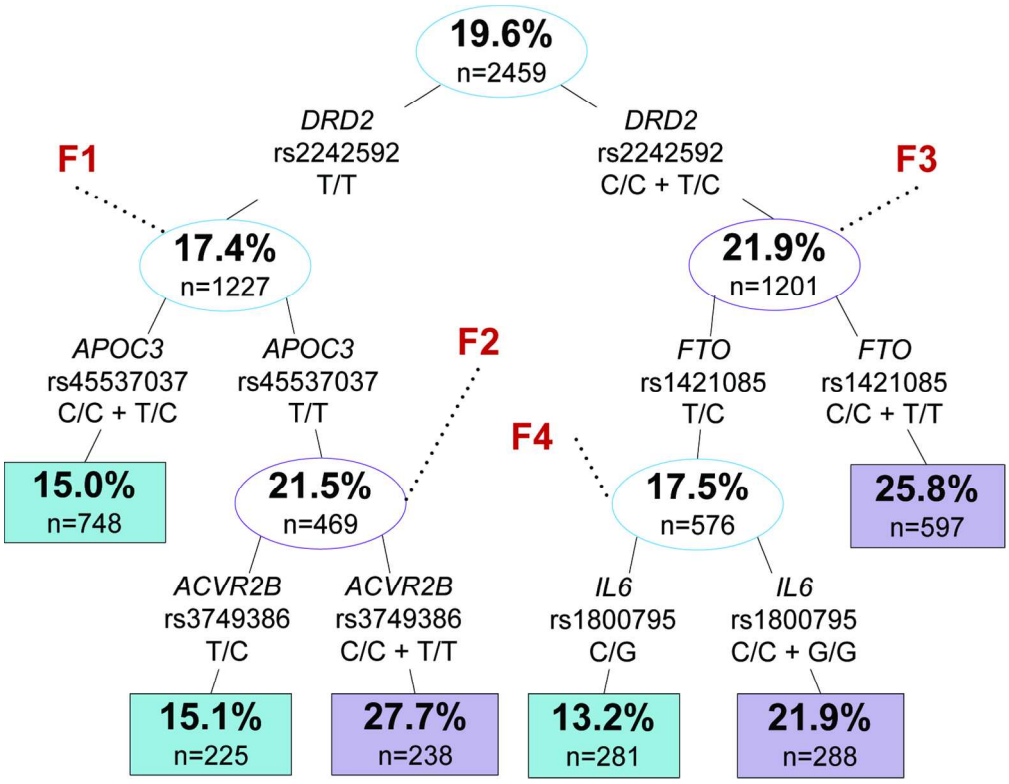

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Supplementary Table 1. Depression-Associated SNP Identified in the WLS

Gene	SNP	Name	Alleles	Chr#/Location	Residue	Associated disease/behavior
ACVR2B	rs3749386	activin receptor IIB	T/C	3/intron 1	--	left-right axis malformations*, (1)
APOC3	rs2854116	apolipoprotein C-III	T/C	11/promoter (-455)	--	nonalcoholic fatty liver disease, insulin resistance[2]
DRD2/ANKK1	rs1800497	dopamine receptor D2/ankyrin repeat and kinase domain containing 1	C/T	11/exon (ANKK1)	Glu713Lys	obesity, drug addiction[3]
DRD2	rs2242592	dopamine receptor D2	T/C	11/3'	--	Schizophrenia[4]
FTO	rs1421085	fat mass and obesity associated	T/C	16/intron 1	--	Obesity[5-7]; mental disorders (in men)[8]
GNRH1	novel SNP	gonadotropin-releasing hormone promoter	T/C	8/promoter	--	Alzheimer's disease[9]
IL6	rs1800795	interleukin 6 (interferon, beta 2)	C/G	7/promoter (-174)	--	Arthritis[10], breast cancer[11]; type II diabetes[12]; depression[13]

*Gene association only

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STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

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Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how loss to follow-up was addressed
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
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		(c) Summarise follow-up time (eg, average and total amount)
Outcome data	15*	Report numbers of outcome events or summary measures over time
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
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		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
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Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

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Multi-Gene Interactions and the Prediction of Depression in the Wisconsin Longitudinal Study

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Multi-Gene Interactions and the Prediction of Depression in the Wisconsin Longitudinal Study

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Running title: Gene interactions and depression

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ABSTRACT

Objectives: Single genetic loci offer little predictive power for the identification of depression. This study examined whether an analysis of gene-gene interactions of 78 single nucleotide polymorphisms in genes associated with depression and age-related diseases would identify significant interactions with increased predictive power for depression.

Design: A retrospective cohort study.

Setting: A survey of participants in the Wisconsin Longitudinal Study.

Participants: A total of 4,811 persons (2,464 females and 2,347 males) who provided saliva for genotyping; the group comes from a randomly selected sample of Wisconsin high school graduates from the class of 1957 as well as a randomly selected sibling, almost all of whom are non-Hispanic white.

Primary outcome measure: Depression as determine by the Composite International Diagnostic Interview short-form (CIDI-SF).

Results: Using a classification tree approach (recursive partitioning (RP)) we identified a number of candidate gene-gene interactions associated with depression. The primary SNP splits revealed by RP (*ANKK1* rs1800497 (also known as *DRD2* Taq1A) in men and *DRD2* rs224592 in women) were found to be significant as single factors by logistic regression (LR) after controlling for multiple testing (P=0.001 for both). Without considering interaction effects, only 1 of the 5 subsequent RP splits reached nominal significance in logistic regression (*FTO* rs1421085 in women; P-value=0.008). However, after controlling for gene-gene interactions by running logistic regression on RP-specific subsets, every split became significant and grew larger in magnitude (OR [before]→[after]: Men: *GNRH1* novel SNP: [1.43 → 1.57]; Women: *APOC3* rs2854116: [1.28 → 1.55], *ACVR2B* rs3749386: [1.11 → 2.17], *FTO* rs1421085: [1.32 → 1.65], *IL6* rs1800795: [1.12 → 1.85]).

Conclusions: Our results suggest that examining gene-gene interactions improves the identification of genetic associations predictive of depression. Four of the SNPs identified in these interactions were located in two pathways well-known to impact depression: neurotransmitter (*ANKK1* and *DRD2*) and

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neuroendocrine (*GNRH1* and *ACVR2B*) signaling. This study demonstrates the utility of RP analysis as an efficient and powerful exploratory analysis technique for uncovering genetic and molecular pathway interactions associated with disease etiology.

INTRODUCTION

Depression is a widespread mental disorder associated with a host of undesirable health, social, and economic outcomes. One in six Americans is diagnosed with depression in his or her lifetime (1). While many environmental factors—such as socioeconomic status, childhood abuse, and major life events—have important ties with depression, so too does gender and many genetic and epigenetic factors, making the disorder heterogeneous in nature (2). Another major risk factor for depression is age, with depression reaching its highest levels in adults over 80 years of age (3).

It has been demonstrated from twin studies that genetic factors typically account for 40–70% of the risk for developing major depressive disorder (MDD), and adoption studies have confirmed the role of genetic risk factors in the development of MDD (see (4) and references therein). Genetic studies, including recent genome-wide association studies (GWAS), have identified genetic alterations in over 50 genes known to be associated with depression (5). However, individually, the genetic alterations found within these genes (primarily single nucleotide polymorphisms (SNPs)) have little predictive value. There is a similar lack of predictive value from GWAS of other major age-related diseases (6).

Given this lack of predictive power among individual genetic alterations for depression together with the complex nature of aging-related diseases, it would seem prudent to examine epistatic effects on this age-related condition. In this respect, we have previously demonstrated that G x G interactions greatly modulate risk for complex age-related diseases (7, 8). Recent studies of depression also have identified epistatic effects. In particular, associations have been identified between *BDNF* Val66Met (brain-derived neurotrophic factor; rs6265) and *5-HTTLPR* (serotonin transporter linked promoter region (9); *GSK3B* rs6782799 (glycogen synthase kinase 3β), *BDNF* rs7124442 and *BDNF* Val66Met (10); *BDNF* Val66Met and SNPs in *NTRK2* (neurotrophic tyrosine kinase receptor 2; (11)), and *5-HTTLPR* short allele and a chromosome 4 gene (12).

The goals of this study were therefore to 1) explore G x G interactions that might better predict the genetic factors involved in the etiology of depression, and 2) to determine the utility of machine learning

algorithms (recursive partitioning) to identify genetic interactions. Using genotypic data from the ~~In this study, we have assessed the epistatic effects of known genetic alterations that link to depression and age-related diseases in the~~ Wisconsin Longitudinal Study (WLS)~~. Using recursive partitioning (RP) and logistic regression (LR)~~ we identified associations between dopaminergic genes and depression in men and women, as well as G x G interactions involving neuroendocrine signaling pathways, with increased significance compared with single genetic associations.

METHODS

Study Participants and Surveys

Data were collected from the WLS, a random sample originally comprised of 10,317 men and women who graduated from Wisconsin high schools in 1957. Later in 1977, the WLS began interviewing one randomly selected sibling of each graduate, when possible. The cohort ~~consists~~ reflects the ancestral makeup of the late-1950s Wisconsin population in that participants are almost entirely ~~of~~ non-Hispanic white ~~persons~~males and females. ~~whose average level of educational attainment was 1.5 years of post-high school education at the time of interview in 2004. Ages of participants in the WLS ranged from 35 to 90 years old at this time, with 83% of participants being between 60 and 70 years old~~. In general, the sample is broadly representative of older white Americans with at least a high school education (13). Further characteristics of the WLS cohort may be found in detail elsewhere (14). Health and psychological well-being phenotypic data was taken from mail and phone surveys given in 2004-2005. Our main measure of depression is based on a variation of the Composite International Diagnostic Interview short-form (CIDI-SF). All participants answered a single stem question: "Have you ever had a time in life lasting two weeks or more when nearly every day you felt sad, blue, depressed, or when you lost interest in most things like work, hobbies, or things you usually liked to do for fun?" Only those who answered YES and whose depression was not always caused by alcohol, drugs, medications, or physical illness were asked further depression symptom questions. Symptom questions asked whether the two week period was accompanied with a) any weight loss, b) trouble sleeping, c) feeling tired, d) feeling bad upon waking, e) losing interest, f) trouble concentrating, or g) thoughts about death. Those answering YES to 3 or more of these symptom questions were classified as having depression (15). Those answering YES to 2 or fewer symptom questions and all those answering NO to the initial stem question were classified as controls.

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Genotyping

7,101 participants (4,569 graduates & 2,532 siblings) provided saliva samples in Oragene DNA sample

collection kits from which DNA was extracted and genotyped for 78 SNPs that were selected based on their association with depression and age-related conditions and diseases (see Supplementary Information 1). Genotyping was performed by KBioscience (Hoddesdon, UK) with use of a homogeneous Fluorescent Resonance Energy Transfer technology coupled to competitive allele specific PCR. All SNP genotypes described in our results were in Hardy-Weinberg equilibrium and their frequencies matched those reported in the literature for European samples.

Statistical Analysis

Analyses were limited to the 4,811 pooled graduates and siblings for whom we have depression and genotype information (Note: individuals with more than 10% missing genotype data were not included). The average age among this sample was just under 65 years in 2004. 80% were married, and the average amount of post-high school educational attainment was 2 years. Median household income in 1993 was $56,700.

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Recursive Partitioning (RP). RP is a data mining tool for revealing trends that relate a dependent variable (~~depression incidence~~depressed vs. non-depressed) to various predictor variables (SNPs). Zhang and Bonney have shown how RP can be used in genetic association studies to identify disease genes (16). RP helps control for heterogeneity in the population and confounding factors by allowing for the segregation of the sample population according to any condition. Thus, RP is a useful way to handle complex datasets that might confound regression analysis due to the complexity of the relationship between the independent and dependent variables and due to missing information.

RP classification trees (using R package rpart) were used to identify potential interactions among the 78 SNPs in relation to depression. The trees split the data along branches according to criteria determined by the rpart package algorithm, which is originally based off the work of Breiman's classification and regression trees (CART) algorithm (17). Basically, the CART algorithm first considers all depressed and

non-depressed subjects pooled together in a heterogeneous root node. Based on considering every possible "yes-no" binary partition that can be made by each independent variable, the single split which maximizes homogeneity between the two resulting sub-nodes as compared to the root node is made. Each sub-node can then be treated independently as a new root node for all subsequent splits, and the pattern continues until every subject constitutes a terminal node, resulting in a very large and complex tree. A 10-part cross validation procedure seeking to minimize misclassification and complexity determines optimal pruning. See Therneau and Atkinson (18) for specific details of the rpart package. Priors were set to 0.5, 0.5. The usesurrogate parameter was set to 0 so that subjects missing the primary split variable do not progress further down the tree, and maxsurrogate was set to 0 to cut computation time in half. The threshold complexity parameter (cp) was set to 0.01. Tree nodes were re-created in Microsoft Visio to display percentage depressed ~~depression incidence (in %) and total number of participants rather than~~and the default number of controls/cases as presented by rpart.

Logistic Regression (LR). Variables found in association with depression based on RP analysis were considered in single factor LR models, separate by gender, using the specific dichotomous splitting of genotypes as designated by RP trees. Regression models for all seven SNP splits were first run on the full dataset to represent single main factor effects. Then each split was run on the respective subset of data as represented by the preceding RP split criteria. Thus, we attempt to mirror RP splits within a more formal LR framework in order to measure the significance of interactions presented by the trees. Multiple testing of 78 SNPs in RP for both male and females followed by 14 LR models resulted in a modified FDR significance level of 0.009.

RESULTS

Of the 4,811 participants ~~with complete survey information on CIDI-SF depression~~ (2,464 females and 2,347 males) under examination in this study, we identified 713 participants (481 females and 232 males) with depression (14.8 %). Given that the independent variable gender (when included as a factor in the full dataset) was the primary split on RP trees; that women are over two times as likely to be diagnosed with depression than men; and since the female etiology of depression has been reported to be associated with unique social, psychological, and biological factors (19), all subsequent analyses were performed by gender.

Recursive Partition Analysis

To examine multi-gene interactions for association with depression we screened our dataset using RP. The two-factor RP tree (*ANKK1*/*GNRH1*) was the optimized pruning for men (Fig. 1), while the five-factor tree (*DRD2*/*APOC3*/*ACVR2B*/*FTO*/*IL6*) was the optimized pruning for women (Fig. 2). For more detailed information on the 7 SNPs found by RP, see Supplementary Information 2. The best overall split for men was *ANKK1* rs1800497 (historically known as the *DRD2* Taq1A allele), where the incidence of depression increased 2.2-fold in those with no C-alleles compared to those with one or two C-alleles. Considering interaction between *ANKK1* and *GNRH1* widened the disparity in incidence, where those with at least one C-allele in both *ANKK1* rs1800497 and the novel SNP in *GNRH1* had a 2.7-fold lower incidence than those without a C-allele in *ANKK1* rs1800497.

For women, the best overall split was *DRD2* rs2242592, where those with one or two C-alleles had 1.3-fold higher incidence of depression compared to those without any C-alleles. G x G interactions associated with the highest incidence of depression included: *DRD2* rs2242592 T/T + *APOC3* rs45537037 T/T + *ACVR2B* rs3749386 C/C or T/T, accounting for a 1.4-fold increase in depression compared to baseline incidence.

Single Main-Factor Effects

Specific SNP interactions identified by RP were next analyzed by LR (see Table 1, Full Data). The primary SNP splits in males and females were significant at the modified FDR level. Men with no C-alleles for *ANKK1* rs1800497 had 2.55 times higher odds [P=0.001 (1.44, 4.51)] of depression compared with men with at least 1 C-allele. Women with at least 1 C-allele for *DRD2* rs2242592 had 1.32 times higher odds [P=0.006 (1.08-1.62)] of depression compared with women with no C-alleles. One other split reached nominal significance; women homozygous (C/C or T/T) for *FTO* rs1421085 had 1.32 times higher odds [P=0.008 (1.08-1.62)] for depression than women with a heterozygous genotype. SNP splits of *GNRH1*, *APOC3*, *ACVR2B*, and *IL6* did not significantly associate with depression.

Gene-Gene Interactions Enhance Predictability for Depression

Specific SNP interactions identified by RP were next analyzed by LR as RP-specific subsets (see Table 1, RP-Subsetted Data). All 5 of the secondary and tertiary RP splits were found to be significant at the modified FDR level when considered as subsets. Among only men with at least one C-allele in *ANKK1* rs1800497, those with no C-allele in the novel SNP of *GNRH1* had 1.57 times higher odds [P=0.002 (1.18-2.08)] for depression than men with 1 or 2 C-alleles. For the subset of women in the first right-hand split of Fig. 2, those homozygous for *FTO* rs1421085 had 1.65 times higher odds [P=0.0005 (1.24-2.18)] for depression than women with a heterozygous genotype. For the remaining subset of women in the second right-hand split of Fig. 2, those homozygous for *IL6* rs1800795 had 1.85 times higher odds [P=0.006 (1.19-2.89)] for depression than women with a heterozygous genotype. For the subset of women in the first left-hand split of Fig. 2, those with no C-alleles for *APOC3* rs45537037 had 1.55 times higher odds [P=0.004 (1.15-2.09)] for depression than women with 1 or 2 C-alleles. For the subset of women in the second left-hand split of Fig. 2, those homozygous for *ACVR2B* rs3749386 had 2.17 times higher odds [P=0.001 (1.37-3.44)] for depression than women with a heterozygous genotype.

DISCUSSION

Utilizing RP as a screening tool to find potential multi-gene interactions, followed by verification of multi-gene interactions with LR, our data demonstrate that multi-gene interactions predict depression with a greater certainty than single main factor associations. RP provided us with primary dichotomous genotype splits in men and women (*ANKK1* rs1800497 and *DRD2* rs2242592, respectively) that were both significant in LR models at the modified FDR level (Table 1). Considering the 5 subsequent RP splits in LR over the entire dataset, only 1 reached a nominal level of significance (barely), which was *FTO* rs1421085 in women. However, after running LR on specific subsets of data according to the pattern of RP branches, every split was found to be significant and every odds ratios grew larger (Table 1; OR [before]→[after]: Male Left: 1.43 → 1.57, Female Left 1: 1.28 → 1.55, Female Left 2: 1.11 → 2.17, Female Right 1: 1.32 → 1.65, Female Right 2: 1.12 → 1.85). Thus, RP provides two unique and important criteria: dichotomous genotype splitting instructions and gene-gene interaction patterns. These criteria go beyond the traditional single factor SNP approach to genetic association studies and allow identification of important multi-gene pathways that more suitably characterize the etiology of complex diseases.

The Utility of Recursive Partitioning, ~~Multi-factor Dimensionality Reduction~~ and Logistic Regression for Identification of Gene-Gene Interactions

With recent advances in genotyping allowing for high-dimensional SNP identification, it is now possible to examine genetic datasets not only for single main factor effects, but also G x G interactions. The requirement for G x G analyses as a better predictor of age-related diseases is obvious from the standpoint that humans are complex biological systems composed of numerous molecular interactions, and from recent studies indicating disease risk is modulated by G x G interactions (7). Notwithstanding this, the development of analytical tools for the identification of G x G interactions has not kept pace with

the technological advances in identifying genetic alterations among individuals. In this respect, we have previously used MDR, LR and LD to identify G x G interactions among a small set of SNPs (7). However, large datasets require a screening tool to identify potential multi-gene interactions. In this study, we have used RP to screen for multi-gene interactions, a data-mining technique that is currently under-utilized in genetic studies. RP serves as an efficient and powerful exploratory analysis technique, especially when looking for interactions in data sets with a large number of independent variables. This screening allows for the identification of G x G interactions (with greater explanatory power), that might otherwise not have been identified, and that can then be confirmed using more traditional statistical techniques. As illustrated in this paper, this data-mining methodology has the advantage of identification of genetic interactions *between* pathways involved in the etiology of depression, in keeping with the etiological heterogeneity of this disorder (see later).

Our study provides proof of principle for the use of RP in higher-dimensional analyses such as GWAS, where a comprehensive list of SNPs may fully explore genetic predisposition to depression and other age-related disease. The WLS is an ideal candidate for future GWAS studies given its large sample size, rich covariate composition and longitudinal nature.

In this genetic study we aimed to identify underlying genetic predispositions to depression and thus have not yet tested environmental/phenotypic data. Future analyses using RP to examine the impact of phenotypic and environmental factors on the development of depression would be anticipated to identify gene-phenotype/environment and multi-phenotype/environment interactions. Indeed, the predictive gains of G x G analyses were stronger for men than women, despite the fact that depression occurs disproportionately in women (~2:1 female-to-male; (20-24)). This suggests that environmental factors may be needed in addition to genetic factors in understanding the etiological pathways for women. Indeed, biological factors such as hormonal changes related to reproductive status (25, 26) may impact environmental factors such as psychosocial experiences (trauma, stress, interpersonal relationships, etc) and general health issues in the development of depression.

Genetic and Biological Correlates of Depression

Numerous studies have identified SNPs that associate with depression. Many of the SNPs associated with depression from other studies were not significantly associated in our study. This is perhaps not surprising, since a single factor is unlikely to provide consistent association especially in a complex condition such as depression, where multiple pathways intersect in regulating the risk of the disease. For example, if a SNP within the serotonin pathway also requires a SNP in the glutamatergic pathway in order for the patient to present with depression, the presence of either SNP in the absence of the other will not be predictive of depression. Moreover, as indicated by Shi and Weinberg, since the human genome contains genetic redundancy, disruption of a single gene may be selectively neutral, but the malfunction of several genes in a pathway might result in expression of a particular phenotype (27).

Both the primary splits in men and women were SNPs linked with *DRD2* (dopamine receptor D2), a gene that has previously been linked with depression and social phobia (28-30). The primary male genotype split rs1800497, technically found in gene *ANKK1*, is historically known as the *DRD2* Taq1A allele because of its known association with decreased dopamine receptor D2 density (in those with T alleles) (31-34). The Taq1A allele has also been previously associated with depressive symptoms in children, where those with the A1 allele (T) were more likely to have depressive symptoms (35). We saw a similar association between A1 and depression in WLS men, where those with two A1 alleles had 2.6 times higher odds for depression compared to those with one or no A1 alleles. The primary split in women (DRD2 rs2242592) has previously been found to be associated with schizophrenia, where the C-allele was associated with higher susceptibility for schizophrenia (36). Interestingly, this same study also found the Taq1A allele to also associate with schizophrenia.

The secondary and tertiary right-hand splits in the female RP tree—*FTO* (fat mass and obesity associated) rs1421085 and *IL6* (interleukin 6) rs1800795—have also been found to relate with mental illness and depression in previous studies (37, 38). There is evidence that activin receptor signaling also

is involved in affective disorders, especially when considering interaction with GABAergic pathways (39). Although we did not see an interaction between SNPs in GABA/activin receptor genes and depression, *ACVR2B* was associated with depression in women. No previous associations between depression and *APOC3*, *ACVR2B*, or *GNRH1* have been reported.

That these genetic variants are associated with *neuroendocrine* pathways (*GnRH1, ACVR2B*) that are known to regulate *neurotransmitter* release and cognitive behavior (39-40) supports these associations as relevant to the etiology of depression and underlines the benefits of using RP to identify meaningful G x G interactions associated with disease.

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Limitations

Given the numerous genetic, phenotypic and environmental influences that are linked to depression, and the small number of SNPs analyzed, it is not surprising that predictability from our models was low (although our predictability was superior to previous studies examining only single main factors). Also, the predictive value of our statistical models was further limited due to user bias in selection of SNPs (from nearly two-million SNPs in the human genome) used in this study. As a result of this, interactions we have found could potentially be moderated by another gene that we have not considered in this study. Nonetheless, we identified significant G x G interactions between known, and newly identified, loci associated with depression. Importantly, 4 of the 7 SNPs identified in these interactions were primarily located in two pathways well-known to impact depression: neurotransmitter and neuroendocrine signaling.

The results from the RP analyses conducted in this study were confirmed by LR, demonstrating the utility of RP as a screening tool for identifying meaningful G x G interactions. Future development of algorithms for RP analysis should not only maximize the distance between branches of the next best split (i.e. rpart), but consider subsequent future split combinations that could potentially result in trees with "better" overall predictability.

Summary

Our data indicate that G x G interaction analyses allows for enhanced predictability of conditions and diseases of aging. RP is an efficient and powerful exploratory analysis technique for elucidating G x G interactions in large datasets and combined with LR provides an important statistical analysis for the identification of well supported G x G interactions. We predict that such analytical methods will play an increasingly important role in the identification of epistatic effects in future large GWAS. Finally, our studies illustrate how RP analyses can be used to find interacting pathways involved in the etiology of a disease or condition such as depression.

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FIGURE LEGENDS

Figure 1. Recursive Partitioning Tree of CIDI-SF Depression in Males of the WLS. Upper and lower numbers in nodes represent the ~~proportion~~ percentage of participants with depression and the number of ~~participants~~ controls/cases in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. M1 is subset of data referenced in Table 1. Sensitivity: 0.526, Specificity: 0.598, Accuracy: 0.591. Due to missing genotype information, we lose approximately 1.5% of participants per split. *rs1800497 is historically known as the *DRD2* Taq1A allele

Figure 2. Recursive Partitioning Tree of CIDI-SF Depression in Females of the WLS. Upper and lower numbers in nodes represent the ~~proportion~~ percentage of participants with depression and the number of ~~participants~~ controls/cases in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. F1-F4 are subsets referenced in Table 1. Sensitivity: 0.607, Specificity: 0.563, Accuracy: 0.572. Due to missing genotype information, we lose approximately 1.4% of participants per split.

Table 1.
Single-factor logistic regression models based directly off male and female RP tree split criteria (see Figures 1 & 2). Each SNP split was first run on the full dataset to represent single main factor effects ("Full Data") for both males and females. Then the same SNP splits were run on specific subsets of data per RP tree splits (M1, F1-F4; "RP-Subsetted Data").

					Full Data		RP-Subsetted Data		
Gender	RP Split	Gene	SNP	Genotypes	OR (95% CI)	P-value	Subset	OR (95% CI)	P-value
Male	Primary	*ANKK1**	rs1800497	T/T vs. C/C + C/T	2.55 (1.44-4.51)	0.001 *	--	----	----
	Left	*GNRH1*	novel SNP	T/T vs. C/C + T/C	1.43 (1.09-1.88)	0.011	M1	1.57 (1.18-2.08)	0.002 *
Female	Primary	*DRD2*	rs2242592	C/C + T/C vs. T/T	1.32 (1.08-1.62)	0.006 *	--	----	----
	Left 1	*APOC3*	rs2854116	T/T vs. C/C + T/C	1.28 (1.04-1.57)	0.018	F1	1.55 (1.15-2.09)	0.004 *
	Left 2	*ACVR2B*	rs3749386	C/C + T/T vs. T/C	1.11 (0.91-1.36)	0.302	F2	2.17 (1.37-3.44)	0.001 *
	Right 1	*FTO*	rs1421085	C/C + T/T vs. T/C	1.32 (1.08-1.62)	0.007 *	F3	1.65 (1.24-2.18)	0.0005 *
	Right 2	*IL6*	rs1800795	C/C + G/G vs. C/G	1.12 (0.92-1.37)	0.269	F4	1.85 (1.19-2.89)	0.006 *

RP, recursive partitioning; OR, odds ratio; CI, confidence interval
M1: LR analysis was run for only those with genotype *DRD2* rs1800497 C/C or C/T
F1: LR analysis was run for only those with genotype *DRD2* rs2242592 T/T
F2: LR analysis was run for only those with genotypes *DRD2* rs2242592 T/T and *APOC3* rs2854116 T/T
F3: LR analysis was run for only those with genotype *DRD2* rs2242592 C/C or T/C
F4: LR analysis was run for only those with genotypes *DRD2* rs2242592 C/C or T/C and *FTO* rs1421085 T/C
*rs1800497 is historically known as the *DRD2* Taq1A allele

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Comment [NR1]: Note that we have added 1 reference (#13).

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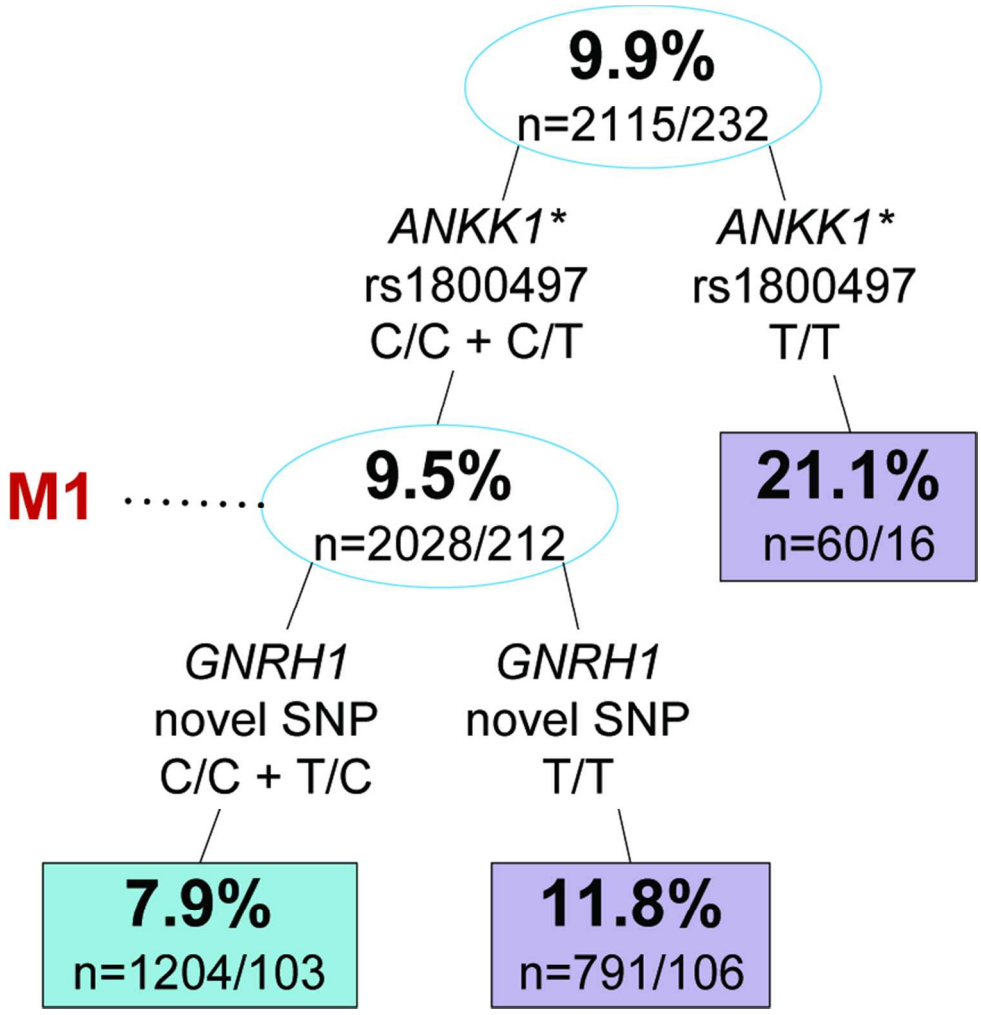

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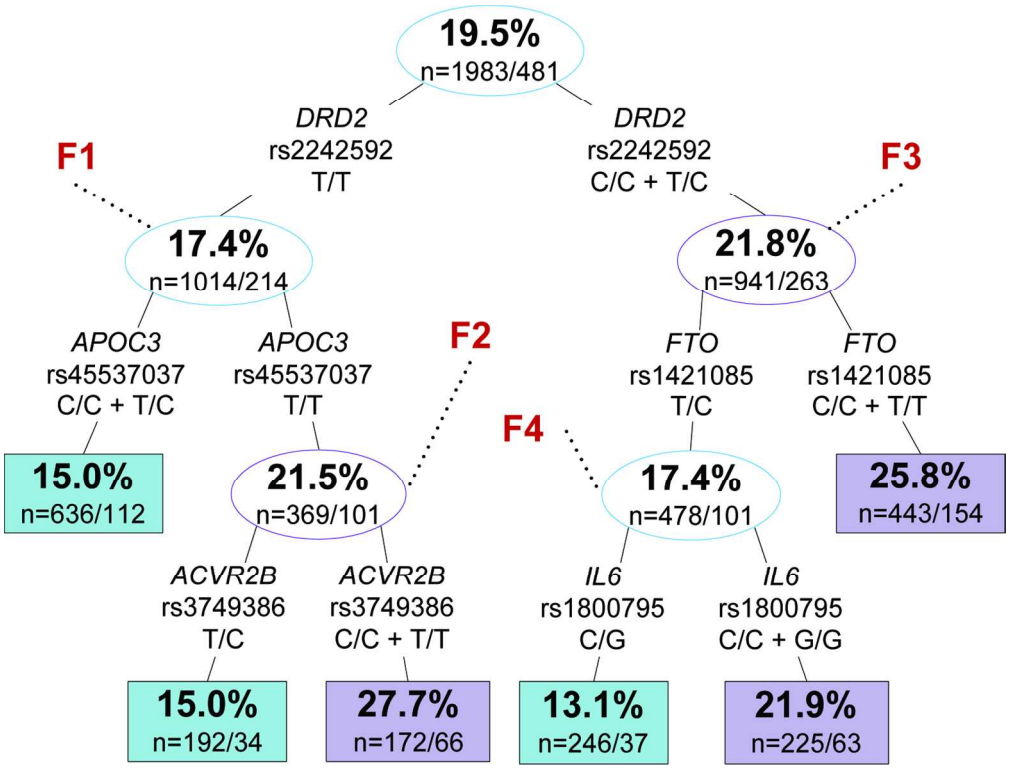

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Supplementary Table 1. Single Nucleotide Polymorphisms Assessed in the WLS

Gene	Encodes	SNP	Associated disease/behavior
A2M	alpha-2-macroglobulin	rs669	Alzheimer's disease (1)
ACVR2A	activin receptor IIA	rs1424954	pre-eclampsia (2)
ACVR2B	activin receptor IIB	rs3749386	--
ADIPOQ	adiponectin, C1Q and collagen domain containing	rs1501299	diabetes II (3, 4), obesity (5, 6), breast cancer (7)
ADIPOQ	adiponectin, C1Q and collagen domain containing	rs2241766	diabetes II (3, 4), obesity (8), breast cancer (7)
ACVRL1	activin receptor-like kinase 1	rs2071219	brain arteriovenous malformations (9)
APOC-3	apolipoprotein C-III	rs2854116	nonalcoholic fatty liver disease (10)
ApoE	apolipoprotein E	rs429358	Alzheimer's disease (11, 12)
ApoE	apolipoprotein E	rs7412	Alzheimer's disease (11, 12)
AR	androgen receptor	rs6152	male pattern baldness (13)
BCKDHB	branched chain keto acid dehydrogenase E1, beta polypeptide	rs4502885	premature ovarian failure (14)
BDNF	brain-derived neurotrophic factor	rs6265	depression (15-17), alcohol dependence-related depression (18), bipolar disorder (19), schizophrenia (20), cognition (21), BMI (22)
BDNF	brain-derived neurotrophic factor	rs908867	antidepressant response (23)
BRCA1	breast cancer 1, early onset	rs1799966	breast cancer (24)
BRCA2	breast cancer 2, early onset homolog	rs144848	breast cancer (24)
CH25H	cholesterol 25-hydroxylase	rs3802657	--
CHRM2	cholinergic receptor, muscarinic 2	rs2061174	alcohol dependence, depression (25)
CHRM2	cholinergic receptor, muscarinic 2	rs8191992	cognition (26)
COMT	catechol-O-methyltransferase	rs4680	ADHD (27), substance abuse (28-31), depression (32), antidepressant response (33), bipolar disorder (34), cognition (35)
CTSD	cathepsin D	rs17571	Alzheimer's disease (36)
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	rs8039957	breast cancer (37)
CYP11B2	cytochrome P450, family 11, subfamily B, polypeptide 2	rs1799998	stroke (38), cardiovascular disease (39)
DAT1	human dopamine transporter	rs11564774	ADHD (40)
DAT1	human dopamine transporter	rs2963238	alcohol-withdrawal seizures (41)
DISC1	disrupted in schizophrenia 1	rs821616	schizophrenia (42), cognitive aging (43)
DRD2	dopamine receptor D2	rs17529477	--
DRD2/ANKK1	dopamine receptor D2/ ankyrin repeat and kinase domain containing 1	rs1800497	obesity, drug addiction (44)
DRD2	dopamine receptor D2	rs2242592	schizophrenia (45)
DRD2	dopamine receptor D2	rs4245147	--
DRD2	dopamine receptor D2	rs6277	schizophrenia (46), PTSD (47)
DRD4	dopamine receptor D4	rs1800955	ADHD (48), heroine addiction (49)
DTNBP1	dystrobrevin-binding protein 1	rs1018381	schizophrenia (50), cognitive ability (51)
DTNBP1	dystrobrevin-binding protein 1	rs760761	schizophrenia (52)
ESR1	estrogen receptor 1	rs7761133	--
ESR1	estrogen receptor 1	rs3853248	--
FADS2	fatty acid desaturase 2	rs1535	breastfeeding & IQ (53)

FADS2	fatty acid desaturase 2	rs174575	breastfeeding & IQ (53)
FMR1	fragile X mental retardation 1	rs1805420	--
FSH	follicle stimulating hormone	rs6169	--
FSHR	follicle stimulating hormone receptor	rs6166	sterility (54), osteoporosis (55)
FST	follistatin	rs12152850	--
FST	follistatin	rs3797297	--
FTO	fat mass and obesity associated	rs1421085	obesity (56-58), mental disorders (59)
GABBR2	γ-aminobutyric acid B receptor 2	rs1435252	nicotine addiction (60)
GABBR2	γ-aminobutyric acid B receptor 2	rs2779562	nicotine addiction (60)
GNRH1	gonadotropin-releasing hormone	novel SNP	Alzheimer's disease (61)
HERC	hect domain and RLD 2	rs12913832	eye color (62, 63)
HFE	hemochromatosis	rs1799945	hemochromatosis(64)
HSD17B1	estradiol 17β-dehydrogenase 1	rs12602084	steroid metabolism (65)
HSD17B1	estradiol 17β-dehydrogenase 1	rs592389	vasomotor symptoms (66), cognition (67)
5-HTR1A	5-hydroxytryptamine (serotonin) receptor 1A	rs878567	mood disorders (68)
5-HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	rs6312	--
5-HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	rs6314	antidepressant response (69), bipolar disorder (70)
5-HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	rs7997012	antidepressant response (71)
5-HTR2C	5-hydroxytryptamine (serotonin) receptor 2C	rs6318	bipolar disorder (72), depression (73)
5-HTT	5-hydroxytryptamine transporter	rs25533	antidepressant response (74)
5-HTT	5-hydroxytryptamine transporter	rs8076005	depressive symptoms (75)
IGF1	insulin-like growth factor 1	rs12313279	--
IL1A	interleukin 1, alpha	rs17561	chronic rhinosinusitis (76), BMI (77)
IL6	interleukin 6	rs1800795	arthritis (78), breast cancer (79), diabetes (80), depression (81)
INHA	inhibin alpha	rs2059693	testicular cancer (82)
INHA	inhibin alpha	rs35118453	--
INHBA	inhibin beta A	rs2237436	--
INHBB	inhibin beta B	rs11902591	--
KIBRA	kidney and brain protein (WWC1)	rs17070145	Alzheimer's disease (83), episodic memory (84)
LEPR	leptin receptor	rs1137100	diabetes II (85), atherosclerosis (86)
LHR	luteinizing hormone receptor	rs4073366	Alzheimer's disease (87)
MAOA	monoamine oxidase A	rs3788862	pain (88)
OXTR	oxytocin receptor	rs2254298	autism (89, 90), social loneliness (91), depressive symptoms & anxiety (92)
PCK1	phosphoenolpyruvate carboxykinase 1	rs707555	diabetes II (93)
PGR	progesterone receptor	rs1042838	ovarian cancer (94), migraine (95), menstruation (96), pregnancy loss (97)
SNAP25	synaptosomal-associated protein 25	rs363050	intelligence (98, 99)
SSADH	succinic semialdehyde dehydrogenase	rs2760118	--
StAR	steroidogenic acute regulatory protein	rs3990403	--
TFAM	transcription factor A, mitochondrial	rs1937	Alzheimer's disease (100)
TFAM	transcription factor A, mitochondrial	rs2306604	Parkinson's disease (101)
TPH1	first tryptophan hydroxylase isoform	rs1799913	heroine addiction (102)

TPH2	first tryptophan hydroxylase isoform	rs11178997	bipolar disorder (103), PTSD symptoms (104)

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Supplementary Table 2. Depression-Associated SNP Identified in the WLS

Gene	Encodes	SNP	Alleles	Chr#/Location	Residue	Associated disease/behavior
ACVR2B	activin receptor IIB	rs3749386	T/C	3/intron 1	--	left-right axis malformations*(1)
APOC3	apolipoprotein C-III	rs2854116	T/C	11/promoter (-455)	--	nonalcoholic fatty liver disease(2)
DRD2/ANKK1	dopamine receptor D2/ankyrin repeat and kinase domain containing 1	rs1800497	C/T	11/exon (ANKK1)	Glu713Lys	obesity, drug addiction (3)
DRD2	dopamine receptor D2	rs2242592	T/C	11/3'	--	schizophrenia (4)
FTO	fat mass and obesity associated	rs1421085	T/C	16/intron 1	--	obesity (5-7), mental disorders (8)
GNRH1	gonadotropin-releasing hormone	novel SNP	T/C	8/promoter	--	Alzheimer's disease (9)
IL6	interleukin 6	rs1800795	C/G	7/promoter (-174)	--	arthritis (10), breast cancer (11), diabetes (12), depression (13)

*Gene association only

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STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how loss to follow-up was addressed
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) Summarise follow-up time (eg, average and total amount)
Outcome data	15*	Report numbers of outcome events or summary measures over time
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Multi-Gene Interactions and the Prediction of Depression in the Wisconsin Longitudinal Study

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ABSTRACT

Objectives: Single genetic loci offer little predictive power for the identification of depression. This study examined whether an analysis of gene-gene interactions of 78 single nucleotide polymorphisms in genes associated with depression and age-related diseases would identify significant interactions with increased predictive power for depression.

Design: A retrospective cohort study.

Setting: A survey of participants in the Wisconsin Longitudinal Study.

Participants: A total of 4,811 persons (2,464 females and 2,347 males) who provided saliva for genotyping; the group comes from a randomly selected sample of Wisconsin high school graduates from the class of 1957 as well as a randomly selected sibling, almost all of whom are non-Hispanic white.

Primary outcome measure: Depression as determine by the Composite International Diagnostic Interview short-form (CIDI-SF).

Results: Using a classification tree approach (recursive partitioning (RP)) we identified a number of candidate gene-gene interactions associated with depression. The primary SNP splits revealed by RP (*ANKK1* rs1800497 (also known as *DRD2* Taq1A) in men and *DRD2* rs224592 in women) were found to be significant as single factors by logistic regression (LR) after controlling for multiple testing (P=0.001 for both). Without considering interaction effects, only 1 of the 5 subsequent RP splits reached nominal significance in logistic regression (*FTO* rs1421085 in women; P-value=0.008). However, after controlling for gene-gene interactions by running logistic regression on RP-specific subsets, every split became significant and grew larger in magnitude (OR [before]→[after]: Men: *GNRH1* novel SNP: [1.43 → 1.57]; Women: *APOC3* rs2854116: [1.28 → 1.55], *ACVR2B* rs3749386: [1.11 → 2.17], *FTO* rs1421085: [1.32 → 1.65], *IL6* rs1800795: [1.12 → 1.85]).

Conclusions: Our results suggest that examining gene-gene interactions improves the identification of genetic associations predictive of depression. Four of the SNPs identified in these interactions were located in two pathways well-known to impact depression: neurotransmitter (*ANKK1* and *DRD2*) and

neuroendocrine (*GNRH1* and *ACVR2B*) signaling. This study demonstrates the utility of RP analysis as an efficient and powerful exploratory analysis technique for uncovering genetic and molecular pathway interactions associated with disease etiology.

INTRODUCTION

Depression is a widespread mental disorder associated with a host of undesirable health, social, and economic outcomes. One in six Americans is diagnosed with depression in his or her lifetime (1). While many environmental factors—such as socioeconomic status, childhood abuse, and major life events—have important ties with depression, so too does gender and many genetic and epigenetic factors, making the disorder heterogeneous in nature (2). Another major risk factor for depression is age, with depression reaching its highest levels in adults over 80 years of age (3).

It has been demonstrated from twin studies that genetic factors typically account for 40–70% of the risk for developing major depressive disorder (MDD), and adoption studies have confirmed the role of genetic risk factors in the development of MDD (see (4) and references therein). Genetic studies, including recent genome-wide association studies (GWAS), have identified genetic alterations in over 50 genes known to be associated with depression (5). However, individually, the genetic alterations found within these genes (primarily single nucleotide polymorphisms (SNPs)) have little predictive value. There is a similar lack of predictive value from GWAS of other major age-related diseases (6).

Given this lack of predictive power among individual genetic alterations for depression together with the complex nature of aging-related diseases, it would seem prudent to examine epistatic effects on this age-related condition. In this respect, we have previously demonstrated that G x G interactions greatly modulate risk for complex age-related diseases (7, 8). Recent studies of depression also have identified epistatic effects. In particular, associations have been identified between *BDNF* Val66Met (brain-derived neurotrophic factor; rs6265) and *5-HTTLPR* (serotonin transporter linked promoter region (9); *GSK3B* rs6782799 (glycogen synthase kinase 3β), *BDNF* rs7124442 and *BDNF* Val66Met (10); *BDNF* Val66Met and SNPs in *NTRK2* (neurotrophic tyrosine kinase receptor 2; (11)), and *5-HTTLPR* short allele and a chromosome 4 gene (12). The machine learning tool recursive partitioning has recently been used by Wong (13) to assess complex gene-gene interactions in depression. Wong notes that recursive partitioning is useful in that it quickly explores high dimensional data for non-linear effects that are non-

biased and easily interpretable.

The goals of this study were therefore to 1) explore G x G interactions that might better predict the genetic factors involved in the etiology of depression, and 2) to further demonstrate the utility of machine learning algorithms (recursive partitioning) to identify genetic interactions. Using genotypic data from the Wisconsin Longitudinal Study (WLS) we identified associations between dopaminergic genes and depression in men and women, as well as G x G interactions involving neuroendocrine signaling pathways, with increased significance compared with single genetic associations.

METHODS

Study Participants and Surveys

Data were collected from the WLS, a random sample originally comprised of 10,317 men and women who graduated from Wisconsin high schools in 1957. Later in 1977, the WLS began interviewing one randomly selected sibling of each graduate, when possible. The cohort reflects the ancestral makeup of the late-1950s Wisconsin population in that participants are almost entirely non-Hispanic white males and females. . In general, the sample is broadly representative of older white Americans with at least a high school education (14). Further characteristics of the WLS cohort may be found in detail elsewhere (15). Health and psychological well-being phenotypic data was taken from mail and phone surveys given in 2004-2005. Inclusion criteria for depression included any member of the WLS cohort who was depressed according to the Composite International Diagnostic Interview short-form (CIDI-SF). Individuals who answered YES to the question “Have you ever had a time in life lasting two weeks or more when nearly every day you felt sad, blue, depressed, or when you lost interest in most things like work, hobbies, or things you usually liked to do for fun?” and whose depression was not caused by alcohol, drugs, medications, or physical illness were asked further depression symptom questions. Symptom questions asked whether the two week period was accompanied with a) any weight loss, b) trouble sleeping, c) feeling tired, d) feeling bad upon waking, e) losing interest, f) trouble concentrating, or g) thoughts about death. Those answering YES to 3 or more of these symptom questions were classified as having depression (16). Those answering YES to 2 or fewer symptom questions and all those answering NO to the initial stem question were classified as controls.

Genotyping

7,101 participants (4,569 graduates & 2,532 siblings) provided saliva samples in Oragene DNA sample collection kits from which DNA was extracted and genotyped for 78 SNPs that were selected based on their association with depression and age-related conditions and diseases (see Supplementary

Information 1). Genotyping was performed by KBioscience (Hoddesdon, UK) with use of a homogeneous Fluorescent Resonance Energy Transfer technology coupled to competitive allele specific PCR. All SNP genotypes described in our results were in Hardy-Weinberg equilibrium and their frequencies matched those reported in the literature for European samples.

Statistical Analysis

Analyses were limited to the 4,811 pooled graduates and siblings for whom we have depression and genotype information (Note: individuals with more than 10% missing genotype data were not included). The average age among this sample was just under 65 years in 2004. 80% were married, and the average amount of post-high school educational attainment was 2 years. Median household income in 1993 was $56,700.

Recursive Partitioning (RP). RP is a data mining tool for revealing trends that relate a dependent variable (depressed vs. non-depressed) to various predictor variables (SNPs). Zhang and Bonney have shown how RP can be used in genetic association studies to identify disease genes (17). RP helps control for heterogeneity in the population and confounding factors by allowing for the segregation of the sample population according to any condition. Thus, RP is a useful way to handle complex datasets that might confound regression analysis due to the complexity of the relationship between the independent and dependent variables and due to missing information.

RP classification trees (using R package rpart) were used to identify potential interactions among the 78 SNPs in relation to depression. The trees split the data along branches according to criteria determined by the rpart package algorithm, which is originally based off the work of Breiman's classification and regression trees (CART) algorithm (18). Basically, the CART algorithm first considers all depressed and non-depressed subjects pooled together in a heterogeneous root node. Based on considering every possible "yes-no" binary partition that can be made by each independent variable, the single split which

maximizes homogeneity between the two resulting sub-nodes as compared to the root node is made. Each sub-node can then be treated independently as a new root node for all subsequent splits, and the pattern continues until every subject constitutes a terminal node, resulting in a very large and complex tree. A 10-part cross validation procedure seeking to minimize misclassification and complexity determines optimal pruning. See Therneau and Atkinson (19) for specific details of the rpart package. Priors were set to 0.5, 0.5. The usesurrogate parameter was set to 0 so that subjects missing the primary split variable do not progress further down the tree, and maxsurrogate was set to 0 to cut computation time in half. The threshold complexity parameter (cp) was set to 0.01. Tree nodes were re-created in Microsoft Visio to display percentage depressed and the default number of controls/cases as presented by rpart.

Logistic Regression (LR). Variables found in association with depression based on RP analysis were considered in single factor LR models, separate by gender, using the specific dichotomous splitting of genotypes as designated by RP trees. Regression models for all seven SNP splits were first run on the full dataset to represent single main factor effects. Then each split was run on the respective subset of data as represented by the preceding RP split criteria. Thus, we attempt to mirror RP splits within a more formal LR framework in order to measure the significance of interactions presented by the trees. Multiple testing of 78 SNPs in RP for both male and females followed by 14 LR models resulted in a modified FDR significance level of 0.009.

RESULTS

Of the 4,811 participants (2,464 females and 2,347 males) under examination in this study, we identified 713 participants (481 females and 232 males) with depression (14.8 %). Given that the independent variable gender (when included as a factor in the full dataset) was the primary split on RP trees; that women are over two times as likely to be diagnosed with depression than men; and since the female etiology of depression has been reported to be associated with unique social, psychological, and biological factors (20), all subsequent analyses were performed by gender.

Recursive Partitioning Analysis

To examine multi-gene interactions for association with depression we screened our dataset using RP. The two-factor RP tree (*ANKK1*/*GNRH1*) was the optimized pruning for men (Fig. 1), while the five-factor tree (*DRD2*/*APOC3*/*ACVR2B*/*FTO*/*IL6*) was the optimized pruning for women (Fig. 2). For more detailed information on the 7 SNPs found by RP, see Supplementary Information 2.

The best overall split for men was *ANKK1* rs1800497 (historically known as the *DRD2* Taq1A allele), where the incidence of depression increased 2.2-fold in those with no C-alleles compared to those with one or two C-alleles. Considering interaction between *ANKK1* and *GNRH1* widened the disparity in incidence, where those with at least one C-allele in both *ANKK1* rs1800497 and the novel SNP in *GNRH1* had a 2.7-fold lower incidence than those without a C-allele in *ANKK1* rs1800497.

For women, the best overall split was *DRD2* rs2242592, where those with one or two C-alleles had 1.3-fold higher incidence of depression compared to those without any C-alleles. G x G interactions associated with the highest incidence of depression included: *DRD2* rs2242592 T/T + *APOC3* rs45537037 T/T + *ACVR2B* rs3749386 C/C or T/T, accounting for a 1.4-fold increase in depression compared to baseline incidence.

Single Main-Factor Effects

Specific SNP interactions identified by RP were next analyzed by LR (see Table 1, Full Data). The primary SNP splits in males and females were significant at the modified FDR level. Men with no C-alleles for *ANKK1* rs1800497 had 2.55 times higher odds [P=0.001 (1.44, 4.51)] of depression compared with men with at least 1 C-allele. Women with at least 1 C-allele for *DRD2* rs2242592 had 1.32 times higher odds [P=0.006 (1.08-1.62)] of depression compared with women with no C-alleles. One other split reached nominal significance; women homozygous (C/C or T/T) for *FTO* rs1421085 had 1.32 times higher odds [P=0.008 (1.08-1.62)] for depression than women with a heterozygous genotype. SNP splits of *GNRH1*, *APOC3*, *ACVR2B*, and *IL6* did not significantly associate with depression.

Gene-Gene Interactions Enhance Predictability for Depression

Specific SNP interactions identified by RP were next analyzed by LR as RP-specific subsets (see Table 1, RP-Subsetted Data). All 5 of the secondary and tertiary RP splits were found to be significant at the modified FDR level when considered as subsets. Among only men with at least one C-allele in *ANKK1* rs1800497, those with no C-allele in the novel SNP of *GNRH1* had 1.57 times higher odds [P=0.002 (1.18-2.08)] for depression than men with 1 or 2 C-alleles. For the subset of women in the first right-hand split of Fig. 2, those homozygous for *FTO* rs1421085 had 1.65 times higher odds [P=0.0005 (1.24-2.18)] for depression than women with a heterozygous genotype. For the remaining subset of women in the second right-hand split of Fig. 2, those homozygous for *IL6* rs1800795 had 1.85 times higher odds [P=0.006 (1.19-2.89)] for depression than women with a heterozygous genotype. For the subset of women in the first left-hand split of Fig. 2, those with no C-alleles for *APOC3* rs45537037 had 1.55 times higher odds [P=0.004 (1.15-2.09)] for depression than women with 1 or 2 C-alleles. For the subset of women in the second left-hand split of Fig. 2, those homozygous for *ACVR2B* rs3749386 had 2.17 times higher odds [P=0.001 (1.37-3.44)] for depression than women with a heterozygous genotype.

DISCUSSION

Utilizing RP as a screening tool to find potential multi-gene interactions, followed by verification of multi-gene interactions with LR, our data demonstrate that multi-gene interactions predict depression with a greater certainty than single main factor associations. RP provided us with primary dichotomous genotype splits in men and women (*ANKK1* rs1800497 and *DRD2* rs2242592, respectively) that were both significant in LR models at the modified FDR level (Table 1). Considering the 5 subsequent RP splits in LR over the entire dataset, only 1 reached a nominal level of significance (barely), which was *FTO* rs1421085 in women. However, after running LR on specific subsets of data according to the pattern of RP branches, every split was found to be significant and every odds ratios grew larger (Table 1; OR [before]→[after]: Male Left: 1.43 → 1.57, Female Left 1: 1.28 → 1.55, Female Left 2: 1.11 → 2.17, Female Right 1: 1.32 → 1.65, Female Right 2: 1.12 → 1.85). Thus, RP provides two unique and important criteria: dichotomous genotype splitting instructions and gene-gene interaction patterns. These criteria go beyond the traditional single factor SNP approach to genetic association studies and allow identification of important multi-gene pathways that more suitably characterize the etiology of complex diseases.

The Utility of Recursive Partitioning and Logistic Regression for Identification of Gene-Gene Interactions

With recent advances in genotyping allowing for high-dimensional SNP identification, it is now possible to examine genetic datasets not only for single main factor effects, but also G x G interactions. The requirement for G x G analyses as a better predictor of age-related diseases is obvious from the standpoint that humans are complex biological systems composed of numerous molecular interactions, and from recent studies indicating disease risk is modulated by G x G interactions (7). Notwithstanding this, the development of analytical tools for the identification of G x G interactions has not kept pace with the technological advances in identifying genetic alterations among individuals. In this respect, we have previously used MDR, LR and LD to identify G x G interactions among a small set of SNPs (7). However, large datasets require a screening tool to identify potential multi-gene interactions. In this study, we have

used RP to screen for multi-gene interactions, a data-mining technique that is currently under-utilized in genetic studies. RP serves as an efficient and powerful exploratory analysis technique, especially when looking for interactions in data sets with a large number of independent variables. This screening allows for the identification of G x G interactions (with greater explanatory power), that might otherwise not have been identified, and that can then be confirmed using more traditional statistical techniques. As illustrated in this paper, this data-mining methodology has the advantage of identification of genetic interactions *between* pathways involved in the etiology of depression, in keeping with the etiological heterogeneity of this disorder (see later).

Our study provides proof of principle for the use of RP in higher-dimensional analyses such as GWAS, where a comprehensive list of SNPs may fully explore genetic predisposition to depression and other age-related disease. The WLS is an ideal candidate for future GWAS studies given its large sample size, rich covariate composition and longitudinal nature.

In this genetic study we aimed to identify underlying genetic predispositions to depression and thus have not yet tested environmental/phenotypic data. Future analyses using RP to examine the impact of phenotypic and environmental factors on the development of depression would be anticipated to identify gene-phenotype/environment and multi-phenotype/environment interactions. Indeed, the predictive gains of G x G analyses were stronger for men than women, despite the fact that depression occurs disproportionately in women (~2:1 female-to-male; (21-25)). This suggests that environmental factors may be needed in addition to genetic factors in understanding the etiological pathways for women. Indeed, biological factors such as hormonal changes related to reproductive status (26, 27) may impact environmental factors such as psychosocial experiences (trauma, stress, interpersonal relationships, etc) and general health issues in the development of depression.

Genetic and Biological Correlates of Depression

Numerous studies have identified SNPs that associate with depression. Many of the SNPs associated

with depression from other studies were not significantly associated in our study. This is perhaps not surprising, since a single factor is unlikely to provide consistent association especially in a complex condition such as depression, where multiple pathways intersect in regulating the risk of the disease. For example, if a SNP within the serotonin pathway also requires a SNP in the glutamatergic pathway in order for the patient to present with depression, the presence of either SNP in the absence of the other will not be predictive of depression. Moreover, as indicated by Shi and Weinberg, since the human genome contains genetic redundancy, disruption of a single gene may be selectively neutral, but the malfunction of several genes in a pathway might result in expression of a particular phenotype (28).

Both the primary splits in men and women were SNPs linked with *DRD2* (dopamine receptor D2), a gene that has previously been linked with depression and social phobia (29-31). The primary male genotype split rs1800497, technically found in gene *ANKK1*, is historically known as the *DRD2* Taq1A allele because of its known association with decreased dopamine receptor D2 density (in those with T alleles) (32-35). The Taq1A allele has also been previously associated with depressive symptoms in children, where those with the A1 allele (T) were more likely to have depressive symptoms (36). We saw a similar association between A1 and depression in WLS men, where those with two A1 alleles had 2.6 times higher odds for depression compared to those with one or no A1 alleles. The primary split in women (DRD2 rs2242592) has previously been found to be associated with schizophrenia, where the C-allele was associated with higher susceptibility for schizophrenia (37). Interestingly, this same study also found the Taq1A allele to also associate with schizophrenia.

The secondary and tertiary right-hand splits in the female RP tree—*FTO* (fat mass and obesity associated) rs1421085 and *IL6* (interleukin 6) rs1800795—have also been found to relate with mental illness and depression in previous studies (38, 39). There is evidence that activin receptor signaling also is involved in affective disorders, especially when considering interaction with GABAergic pathways (40). Although we did not see an interaction between SNPs in GABA/activin receptor genes and depression, *ACVR2B* was associated with depression in women. No previous associations between depression and

APOC3, *ACVR2B*, or *GNRH1* have been reported.

That these genetic variants are associated with *neuroendocrine* pathways (*GnRH1, ACVR2B*) that are known to regulate *neurotransmitter* release and cognitive behavior (39-40) supports these associations as relevant to the etiology of depression and underlines the benefits of using RP to identify meaningful G x G interactions associated with disease.

Limitations

Given the numerous genetic, phenotypic and environmental influences that are linked to depression, and the small number of SNPs analyzed, it is not surprising that predictability from our models was low (although our predictability was superior to previous studies examining only single main factors). Also, the predictive value of our statistical models was further limited due to user bias in selection of SNPs (from nearly two-million SNPs in the human genome) used in this study. As a result of this, interactions we have found could potentially be moderated by another gene that we have not considered in this study. Nonetheless, we identified significant G x G interactions between known, and newly identified, loci associated with depression. Importantly, 4 of the 7 SNPs identified in these interactions were primarily located in two pathways well-known to impact depression: neurotransmitter and neuroendocrine signaling.

The results from the RP analyses conducted in this study were confirmed by LR, demonstrating the utility of RP as a screening tool for identifying meaningful G x G interactions. Future development of algorithms for RP analysis should not only maximize the distance between branches of the next best split (i.e. rpart), but consider subsequent future split combinations that could potentially result in trees with "better" overall predictability.

Summary

Our data indicate that G x G interaction analyses allows for enhanced predictability of conditions and diseases of aging. RP is an efficient and powerful exploratory analysis technique for elucidating G x G

interactions in large datasets and combined with LR provides an important statistical analysis for the identification of well supported G x G interactions. We predict that such analytical methods will play an increasingly important role in the identification of epistatic effects in future large GWAS. Finally, our studies illustrate how RP analyses can be used to find interacting pathways involved in the etiology of a disease or condition such as depression.

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FIGURE LEGENDS

Figure 1. Recursive Partitioning Tree of CIDI-SF Depression in Males of the WLS. Upper and lower numbers in nodes represent the percentage of participants with depression and the number of controls/cases in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. M1 is subset of data referenced in Table 1. Sensitivity: 0.526, Specificity: 0.598, Accuracy: 0.591. Due to missing genotype information, we lose approximately 1.5% of participants per split. *rs1800497 is historically known as the *DRD2* Taq1A allele

Figure 2. Recursive Partitioning Tree of CIDI-SF Depression in Females of the WLS. Upper and lower numbers in nodes represent the percentage of participants with depression and the number of controls/cases in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. F1-F4 are subsets referenced in Table 1. Sensitivity: 0.607, Specificity: 0.563, Accuracy: 0.572. Due to missing genotype information, we lose approximately 1.4% of participants per split.

Table 1.
Single-factor logistic regression models based directly off male and female RP tree split criteria (see Figures 1 & 2). Each SNP split was first run on the full dataset to represent single main factor effects ("Full Data") for both males and females. Then the same SNP splits were run on specific subsets of data per RP tree splits (M1, F1-F4; "RP-Subsetted Data").

					Full Data		RP-Subsetted Data		
Gender	RP Split	Gene	SNP	Genotypes	OR (95% CI)	P-value	Subset	OR (95% CI)	P-value
Male	Primary	*ANKK1**	rs1800497	T/T vs. C/C + C/T	2.55 (1.44-4.51)	0.001 *	--	----	----
	Left	*GNRH1*	novel SNP	T/T vs. C/C + T/C	1.43 (1.09-1.88)	0.011	M1	1.57 (1.18-2.08)	0.002 *
Female	Primary	*DRD2*	rs2242592	C/C + T/C vs. T/T	1.32 (1.08-1.62)	0.006 *	--	----	----
	Left 1	*APOC3*	rs2854116	T/T vs. C/C + T/C	1.28 (1.04-1.57)	0.018	F1	1.55 (1.15-2.09)	0.004 *
	Left 2	*ACVR2B*	rs3749386	C/C + T/T vs. T/C	1.11 (0.91-1.36)	0.302	F2	2.17 (1.37-3.44)	0.001 *
	Right 1	*FTO*	rs1421085	C/C + T/T vs. T/C	1.32 (1.08-1.62)	0.007 *	F3	1.65 (1.24-2.18)	0.0005 *
	Right 2	*IL6*	rs1800795	C/C + G/G vs. C/G	1.12 (0.92-1.37)	0.269	F4	1.85 (1.19-2.89)	0.006 *

RP, recursive partitioning; OR, odds ratio; CI, confidence interval
M1: LR analysis was run for only those with genotype *DRD2* rs1800497 C/C or C/T
F1: LR analysis was run for only those with genotype *DRD2* rs2242592 T/T
F2: LR analysis was run for only those with genotypes *DRD2* rs2242592 T/T and *APOC3* rs2854116 T/T
F3: LR analysis was run for only those with genotype *DRD2* rs2242592 C/C or T/C
F4: LR analysis was run for only those with genotypes *DRD2* rs2242592 C/C or T/C and *FTO* rs1421085 T/C
*rs1800497 is historically known as the *DRD2* Taq1A allele

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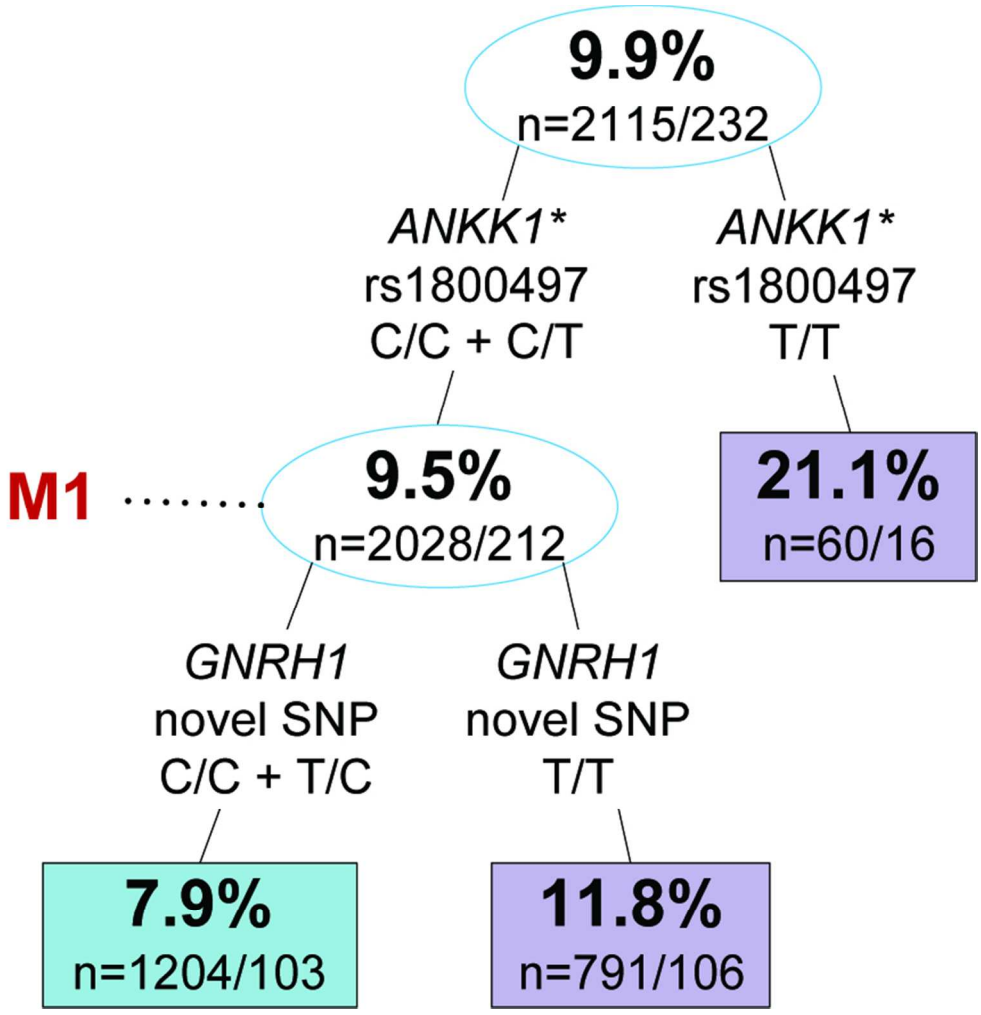

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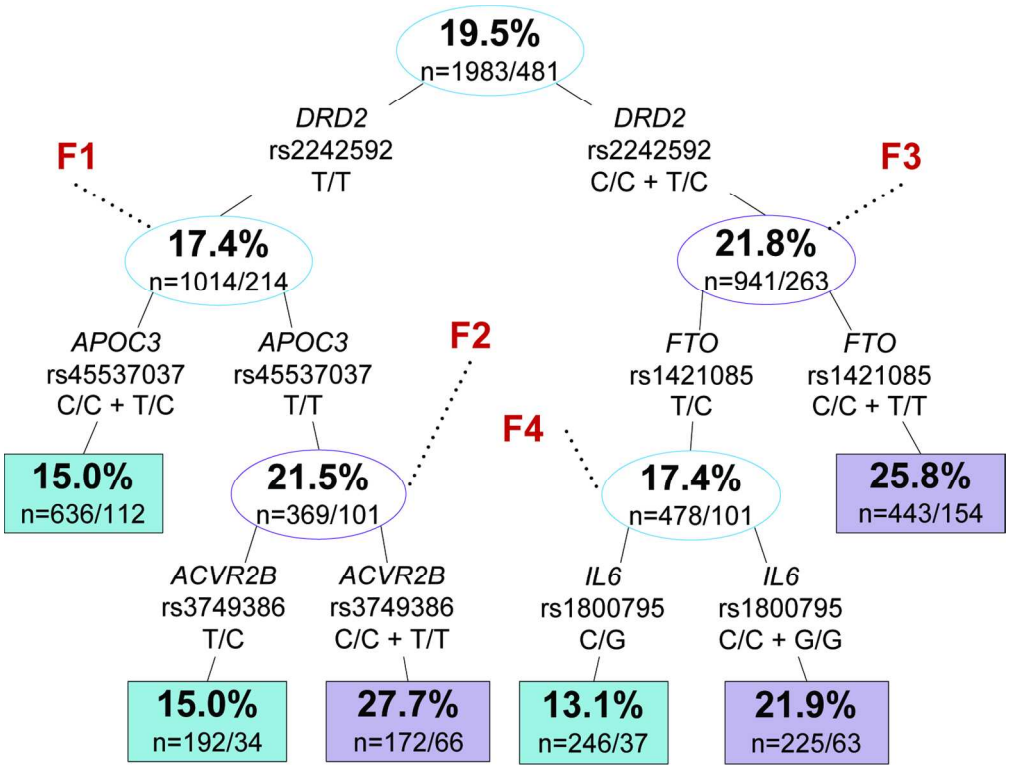

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Supplementary Table 1. Single Nucleotide Polymorphisms Assessed in the WLS

Gene	Encodes	SNP	Associated disease/behavior
A2M	alpha-2-macroglobulin	rs669	Alzheimer's disease (1)
ACVR2A	activin receptor IIA	rs1424954	pre-eclampsia (2)
ACVR2B	activin receptor IIB	rs3749386	--
ADIPOQ	adiponectin, C1Q and collagen domain containing	rs1501299	diabetes II (3, 4), obesity (5, 6), breast cancer (7)
ADIPOQ	adiponectin, C1Q and collagen domain containing	rs2241766	diabetes II (3, 4), obesity (8), breast cancer (7)
ACVRL1	activin receptor-like kinase 1	rs2071219	brain arteriovenous malformations (9)
APOC-3	apolipoprotein C-III	rs2854116	nonalcoholic fatty liver disease (10)
ApoE	apolipoprotein E	rs429358	Alzheimer's disease (11, 12)
ApoE	apolipoprotein E	rs7412	Alzheimer's disease (11, 12)
AR	androgen receptor	rs6152	male pattern baldness (13)
BCKDHB	branched chain keto acid dehydrogenase E1, beta polypeptide	rs4502885	premature ovarian failure (14)
BDNF	brain-derived neurotrophic factor	rs6265	depression (15-17), alcohol dependence-related depression (18), bipolar disorder (19), schizophrenia (20), cognition (21), BMI (22)
BDNF	brain-derived neurotrophic factor	rs908867	antidepressant response (23)
BRCA1	breast cancer 1, early onset	rs1799966	breast cancer (24)
BRCA2	breast cancer 2, early onset homolog	rs144848	breast cancer (24)
CH25H	cholesterol 25-hydroxylase	rs3802657	--
CHRM2	cholinergic receptor, muscarinic 2	rs2061174	alcohol dependence, depression (25)
CHRM2	cholinergic receptor, muscarinic 2	rs8191992	cognition (26)
COMT	catechol-O-methyltransferase	rs4680	ADHD (27), substance abuse (28-31), depression (32), antidepressant response (33), bipolar disorder (34), cognition (35)
CTSD	cathepsin D	rs17571	Alzheimer's disease (36)
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	rs8039957	breast cancer (37)
CYP11B2	cytochrome P450, family 11, subfamily B, polypeptide 2	rs1799998	stroke (38), cardiovascular disease (39)
DAT1	human dopamine transporter	rs11564774	ADHD (40)
DAT1	human dopamine transporter	rs2963238	alcohol-withdrawal seizures (41)
DISC1	disrupted in schizophrenia 1	rs821616	schizophrenia (42), cognitive aging (43)
DRD2	dopamine receptor D2	rs17529477	--
DRD2/ANKK1	dopamine receptor D2/ ankyrin repeat and kinase domain containing 1	rs1800497	obesity, drug addiction (44)
DRD2	dopamine receptor D2	rs2242592	schizophrenia (45)
DRD2	dopamine receptor D2	rs4245147	--
DRD2	dopamine receptor D2	rs6277	schizophrenia (46), PTSD (47)
DRD4	dopamine receptor D4	rs1800955	ADHD (48), heroine addiction (49)
DTNBP1	dystrobrevin-binding protein 1	rs1018381	schizophrenia (50), cognitive ability (51)
DTNBP1	dystrobrevin-binding protein 1	rs760761	schizophrenia (52)
ESR1	estrogen receptor 1	rs7761133	--
ESR1	estrogen receptor 1	rs3853248	--
FADS2	fatty acid desaturase 2	rs1535	breastfeeding & IQ (53)

FADS2	fatty acid desaturase 2	rs174575	breastfeeding & IQ (53)
FMR1	fragile X mental retardation 1	rs1805420	--
FSH	follicle stimulating hormone	rs6169	--
FSHR	follicle stimulating hormone receptor	rs6166	sterility (54), osteoporosis (55)
FST	follistatin	rs12152850	--
FST	follistatin	rs3797297	--
FTO	fat mass and obesity associated	rs1421085	obesity (56-58), mental disorders (59)
GABBR2	γ-aminobutyric acid B receptor 2	rs1435252	nicotine addiction (60)
GABBR2	γ-aminobutyric acid B receptor 2	rs2779562	nicotine addiction (60)
GNRH1	gonadotropin-releasing hormone	novel SNP	Alzheimer's disease (61)
HERC	hect domain and RLD 2	rs12913832	eye color (62, 63)
HFE	hemochromatosis	rs1799945	hemochromatosis(64)
HSD17B1	estradiol 17β-dehydrogenase 1	rs12602084	steroid metabolism (65)
HSD17B1	estradiol 17β-dehydrogenase 1	rs592389	vasomotor symptoms (66), cognition (67)
5-HTR1A	5-hydroxytryptamine (serotonin) receptor 1A	rs878567	mood disorders (68)
5-HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	rs6312	--
5-HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	rs6314	antidepressant response (69), bipolar disorder (70)
5-HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	rs7997012	antidepressant response (71)
5-HTR2C	5-hydroxytryptamine (serotonin) receptor 2C	rs6318	bipolar disorder (72), depression (73)
5-HTT	5-hydroxytryptamine transporter	rs25533	antidepressant response (74)
5-HTT	5-hydroxytryptamine transporter	rs8076005	depressive symptoms (75)
IGF1	insulin-like growth factor 1	rs12313279	--
IL1A	interleukin 1, alpha	rs17561	chronic rhinosinusitis (76), BMI (77)
IL6	interleukin 6	rs1800795	arthritis (78), breast cancer (79), diabetes (80), depression (81)
INHA	inhibin alpha	rs2059693	testicular cancer (82)
INHA	inhibin alpha	rs35118453	--
INHBA	inhibin beta A	rs2237436	--
INHBB	inhibin beta B	rs11902591	--
KIBRA	kidney and brain protein (WWC1)	rs17070145	Alzheimer's disease (83), episodic memory (84)
LEPR	leptin receptor	rs1137100	diabetes II (85), atherosclerosis (86)
LHR	luteinizing hormone receptor	rs4073366	Alzheimer's disease (87)
MAOA	monoamine oxidase A	rs3788862	pain (88)
OXTR	oxytocin receptor	rs2254298	autism (89, 90), social loneliness (91), depressive symptoms & anxiety (92)
PCK1	phosphoenolpyruvate carboxykinase 1	rs707555	diabetes II (93)
PGR	progesterone receptor	rs1042838	ovarian cancer (94), migraine (95), menstruation (96), pregnancy loss (97)
SNAP25	synaptosomal-associated protein 25	rs363050	intelligence (98, 99)
SSADH	succinic semialdehyde dehydrogenase	rs2760118	--
StAR	steroidogenic acute regulatory protein	rs3990403	--
TFAM	transcription factor A, mitochondrial	rs1937	Alzheimer's disease (100)
TFAM	transcription factor A, mitochondrial	rs2306604	Parkinson's disease (101)
TPH1	first tryptophan hydroxylase isoform	rs1799913	heroine addiction (102)

TPH2	first tryptophan hydroxylase isoform	rs11178997	bipolar disorder (103), PTSD symptoms (104)

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Supplementary Table 2. Depression-Associated SNP Identified in the WLS

Gene	Encodes	SNP	Alleles	Chr#/Location	Residue	Associated disease/behavior
ACVR2B	activin receptor IIB	rs3749386	T/C	3/intron 1	--	left-right axis malformations*(1)
APOC3	apolipoprotein C-III	rs2854116	T/C	11/promoter (-455)	--	nonalcoholic fatty liver disease(2)
DRD2/ANKK1	dopamine receptor D2/ankyrin repeat and kinase domain containing 1	rs1800497	C/T	11/exon (ANKK1)	Glu713Lys	obesity, drug addiction (3)
DRD2	dopamine receptor D2	rs2242592	T/C	11/3'	--	schizophrenia (4)
FTO	fat mass and obesity associated	rs1421085	T/C	16/intron 1	--	obesity (5-7), mental disorders (8)
GNRH1	gonadotropin-releasing hormone	novel SNP	T/C	8/promoter	--	Alzheimer's disease (9)
IL6	interleukin 6	rs1800795	C/G	7/promoter (-174)	--	arthritis (10), breast cancer (11), diabetes (12), depression (13)

*Gene association only

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Multi-Gene Interactions and the Prediction of Depression in the Wisconsin Longitudinal Study

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Running title: Gene interactions and depression

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ABSTRACT

Objectives: Single genetic loci offer little predictive power for the identification of depression. This study examined whether an analysis of gene-gene interactions of 78 single nucleotide polymorphisms in genes associated with depression and age-related diseases would identify significant interactions with increased predictive power for depression.

Design: A retrospective cohort study.

Setting: A survey of participants in the Wisconsin Longitudinal Study.

Participants: A total of 4,811 persons (2,464 females and 2,347 males) who provided saliva for genotyping; the group comes from a randomly selected sample of Wisconsin high school graduates from the class of 1957 as well as a randomly selected sibling, almost all of whom are non-Hispanic white.

Primary outcome measure: Depression as determine by the Composite International Diagnostic Interview short-form (CIDI-SF).

Results: Using a classification tree approach (recursive partitioning (RP)) we identified a number of candidate gene-gene interactions associated with depression. The primary SNP splits revealed by RP (*ANKK1* rs1800497 (also known as *DRD2* Taq1A) in men and *DRD2* rs224592 in women) were found to be significant as single factors by logistic regression (LR) after controlling for multiple testing (P=0.001 for both). Without considering interaction effects, only 1 of the 5 subsequent RP splits reached nominal significance in logistic regression (*FTO* rs1421085 in women; P-value=0.008). However, after controlling for gene-gene interactions by running logistic regression on RP-specific subsets, every split became significant and grew larger in magnitude (OR [before]→[after]: Men: *GNRH1* novel SNP: [1.43 → 1.57]; Women: *APOC3* rs2854116: [1.28 → 1.55], *ACVR2B* rs3749386: [1.11 → 2.17], *FTO* rs1421085: [1.32 → 1.65], *IL6* rs1800795: [1.12 → 1.85]).

Conclusions: Our results suggest that examining gene-gene interactions improves the identification of genetic associations predictive of depression. Four of the SNPs identified in these interactions were located in two pathways well-known to impact depression: neurotransmitter (*ANKK1* and *DRD2*) and

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neuroendocrine (*GNRH1* and *ACVR2B*) signaling. This study demonstrates the utility of RP analysis as an efficient and powerful exploratory analysis technique for uncovering genetic and molecular pathway interactions associated with disease etiology.

INTRODUCTION

Depression is a widespread mental disorder associated with a host of undesirable health, social, and economic outcomes. One in six Americans is diagnosed with depression in his or her lifetime (1). While many environmental factors—such as socioeconomic status, childhood abuse, and major life events—have important ties with depression, so too does gender and many genetic and epigenetic factors, making the disorder heterogeneous in nature (2). Another major risk factor for depression is age, with depression reaching its highest levels in adults over 80 years of age (3).

It has been demonstrated from twin studies that genetic factors typically account for 40–70% of the risk for developing major depressive disorder (MDD), and adoption studies have confirmed the role of genetic risk factors in the development of MDD (see (4) and references therein). Genetic studies, including recent genome-wide association studies (GWAS), have identified genetic alterations in over 50 genes known to be associated with depression (5). However, individually, the genetic alterations found within these genes (primarily single nucleotide polymorphisms (SNPs)) have little predictive value. There is a similar lack of predictive value from GWAS of other major age-related diseases (6).

Given this lack of predictive power among individual genetic alterations for depression together with the complex nature of aging-related diseases, it would seem prudent to examine epistatic effects on this age-related condition. In this respect, we have previously demonstrated that G x G interactions greatly modulate risk for complex age-related diseases (7, 8). Recent studies of depression also have identified epistatic effects. In particular, associations have been identified between *BDNF* Val66Met (brain-derived neurotrophic factor; rs6265) and *5-HTTLPR* (serotonin transporter linked promoter region (9); *GSK3B* rs6782799 (glycogen synthase kinase 3β), *BDNF* rs7124442 and *BDNF* Val66Met (10); *BDNF* Val66Met and SNPs in *NTRK2* (neurotrophic tyrosine kinase receptor 2; (11)), and *5-HTTLPR* short allele and a chromosome 4 gene (12). The machine learning tool recursive partitioning has recently been used by Wong (13) in order to assess complex gene-gene interactions in depression. Wong notes that recursive partitioning is useful in that it quickly explores high dimensional data for non-linear effects that are non-

biased and easily interpretable.

The goals of this study were therefore to 1) explore G x G interactions that might better predict the genetic factors involved in the etiology of depression, and 2) to further demonstrate the utility of machine learning algorithms (recursive partitioning) to identify genetic interactions. Using genotypic data from the Wisconsin Longitudinal Study (WLS) we identified associations between dopaminergic genes and depression in men and women, as well as G x G interactions involving neuroendocrine signaling pathways, with increased significance compared with single genetic associations.

METHODS

Study Participants and Surveys

Data were collected from the WLS, a random sample originally comprised of 10,317 men and women who graduated from Wisconsin high schools in 1957. Later in 1977, the WLS began interviewing one randomly selected sibling of each graduate, when possible. The cohort reflects the ancestral makeup of the late-1950s Wisconsin population in that participants are almost entirely non-Hispanic white males and females. . In general, the sample is broadly representative of older white Americans with at least a high school education (14). Further characteristics of the WLS cohort may be found in detail elsewhere (15). Health and psychological well-being phenotypic data was taken from mail and phone surveys given in 2004-2005. Inclusion criteria for depression included any member of the WLS cohort who was depressed according to the Composite International Diagnostic Interview short-form (CIDI-SF). Individuals who answered YES to the question "Have you ever had a time in life lasting two weeks or more when nearly every day you felt sad, blue, depressed, or when you lost interest in most things like work, hobbies, or things you usually liked to do for fun?" and whose depression was not caused by alcohol, drugs, medications, or physical illness were asked further depression symptom questions. Symptom questions asked whether the two week period was accompanied with a) any weight loss, b) trouble sleeping, c) feeling tired, d) feeling bad upon waking, e) losing interest, f) trouble concentrating, or g) thoughts about death. Those answering YES to 3 or more of these symptom questions were classified as having depression (16). Those answering YES to 2 or fewer symptom questions and all those answering NO to the initial stem question were classified as controls.

~~Data were collected from the WLS, a random sample originally comprised of 10,317 men and women who graduated from Wisconsin high schools in 1957. Later in 1977, the WLS began interviewing one randomly selected sibling of each graduate, when possible. The cohort consists reflects the ancestral makeup of the late-1950s Wisconsin population in that participants are almost entirely of non-Hispanic white personsmales and females. whose average level of educational attainment was 1.5 years of post-~~

high school education at the time of interview in 2004. Ages of participants in the WLS ranged from 35 to 90 years old at this time, with 83% of participants being between 60 and 70 years old. In general, the sample is broadly representative of older white Americans with at least a high school education (14). Further characteristics of the WLS cohort may be found in detail elsewhere (15). Health and psychological well-being phenotypic data was taken from mail and phone surveys given in 2004-2005. Our main measure of depression is based on a variation of the Composite International Diagnostic Interview short-form (CIDI-SF). All participants answered a single stem question: "Have you ever had a time in life lasting two weeks or more when nearly every day you felt sad, blue, depressed, or when you lost interest in most things like work, hobbies, or things you usually liked to do for fun?" Only those who answered YES and whose depression was not always caused by alcohol, drugs, medications, or physical illness were asked further depression symptom questions. Symptom questions asked whether the two week period was accompanied with a) any weight loss, b) trouble sleeping, c) feeling tired, d) feeling bad upon waking, e) losing interest, f) trouble concentrating, or g) thoughts about death. Those answering YES to 3 or more of these symptom questions were classified as having depression (16). Those answering YES to 2 or fewer symptom questions and all those answering NO to the initial stem question were classified as controls.

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Genotyping

7,101 participants (4,569 graduates & 2,532 siblings) provided saliva samples in Oragene DNA sample collection kits from which DNA was extracted and genotyped for 78 SNPs that were selected based on their association with depression and age-related conditions and diseases (see Supplementary Information 1). Genotyping was performed by KBioscience (Hoddesdon, UK) with use of a homogeneous Fluorescent Resonance Energy Transfer technology coupled to competitive allele specific PCR. All SNP genotypes described in our results were in Hardy-Weinberg equilibrium and their frequencies matched those reported in the literature for European samples.

Statistical Analysis

Analyses were limited to the 4,811 pooled graduates and siblings for whom we have depression and genotype information (Note: individuals with more than 10% missing genotype data were not included). The average age among this sample was just under 65 years in 2004. 80% were married, and the average amount of post-high school educational attainment was 2 years. Median household income in 1993 was $56,700.

Recursive Partitioning (RP). RP is a data mining tool for revealing trends that relate a dependent variable (depressed vs. non-depressed) to various predictor variables (SNPs). Zhang and Bonney have shown how RP can be used in genetic association studies to identify disease genes (17). RP helps control for heterogeneity in the population and confounding factors by allowing for the segregation of the sample population according to any condition. Thus, RP is a useful way to handle complex datasets that might confound regression analysis due to the complexity of the relationship between the independent and dependent variables and due to missing information.

RP classification trees (using R package rpart) were used to identify potential interactions among the 78 SNPs in relation to depression. The trees split the data along branches according to criteria determined by the rpart package algorithm, which is originally based off the work of Breiman's classification and regression trees (CART) algorithm (18). Basically, the CART algorithm first considers all depressed and non-depressed subjects pooled together in a heterogeneous root node. Based on considering every possible "yes-no" binary partition that can be made by each independent variable, the single split which maximizes homogeneity between the two resulting sub-nodes as compared to the root node is made. Each sub-node can then be treated independently as a new root node for all subsequent splits, and the pattern continues until every subject constitutes a terminal node, resulting in a very large and complex tree. A 10-part cross validation procedure seeking to minimize misclassification and complexity determines optimal pruning. See Therneau and Atkinson (19) for specific details of the rpart package.

Priors were set to 0.5, 0.5. The usesurrogate parameter was set to 0 so that subjects missing the primary split variable do not progress further down the tree, and maxsurrogate was set to 0 to cut computation time in half. The threshold complexity parameter (cp) was set to 0.01. Tree nodes were re-created in Microsoft Visio to display percentage depressed ~~depression incidence (in %) and total number of participants rather than~~and the default number of controls/cases as presented by rpart.

Logistic Regression (LR). Variables found in association with depression based on RP analysis were considered in single factor LR models, separate by gender, using the specific dichotomous splitting of genotypes as designated by RP trees. Regression models for all seven SNP splits were first run on the full dataset to represent single main factor effects. Then each split was run on the respective subset of data as represented by the preceding RP split criteria. Thus, we attempt to mirror RP splits within a more formal LR framework in order to measure the significance of interactions presented by the trees. Multiple testing of 78 SNPs in RP for both male and females followed by 14 LR models resulted in a modified FDR significance level of 0.009.

RESULTS

Of the 4,811 participants (2,464 females and 2,347 males) under examination in this study, we identified 713 participants (481 females and 232 males) with depression (14.8 %). Given that the independent variable gender (when included as a factor in the full dataset) was the primary split on RP trees; that women are over two times as likely to be diagnosed with depression than men; and since the female etiology of depression has been reported to be associated with unique social, psychological, and biological factors (20), all subsequent analyses were performed by gender.

Recursive Partitioning Analysis

To examine multi-gene interactions for association with depression we screened our dataset using RP. The two-factor RP tree (*ANKK1*/*GNRH1*) was the optimized pruning for men (Fig. 1), while the five-factor tree (*DRD2*/*APOC3*/*ACVR2B*/*FTO*/*IL6*) was the optimized pruning for women (Fig. 2). For more detailed information on the 7 SNPs found by RP, see Supplementary Information 2. Note that subjects are lost in every step down each tree due to missing genotype information. We lose approximately 1.5% of data per split in men and 1.4% of data per split in women.

The best overall split for men was *ANKK1* rs1800497 (historically known as the *DRD2* Taq1A allele), where the incidence of depression increased 2.2-fold in those with no C-alleles compared to those with one or two C-alleles. Considering interaction between *ANKK1* and *GNRH1* widened the disparity in incidence, where those with at least one C-allele in both *ANKK1* rs1800497 and the novel SNP in *GNRH1* had a 2.7-fold lower incidence than those without a C-allele in *ANKK1* rs1800497.

For women, the best overall split was *DRD2* rs2242592, where those with one or two C-alleles had 1.3-fold higher incidence of depression compared to those without any C-alleles. G x G interactions associated with the highest incidence of depression included: *DRD2* rs2242592 T/T + *APOC3* rs45537037 T/T + *ACVR2B* rs3749386 C/C or T/T, accounting for a 1.4-fold increase in depression compared to baseline incidence.

Single Main-Factor Effects

Specific SNP interactions identified by RP were next analyzed by LR (see Table 1, Full Data). The primary SNP splits in males and females were significant at the modified FDR level. Men with no C-alleles for *ANKK1* rs1800497 had 2.55 times higher odds [P=0.001 (1.44, 4.51)] of depression compared with men with at least 1 C-allele. Women with at least 1 C-allele for *DRD2* rs2242592 had 1.32 times higher odds [P=0.006 (1.08-1.62)] of depression compared with women with no C-alleles. One other split reached nominal significance; women homozygous (C/C or T/T) for *FTO* rs1421085 had 1.32 times higher odds [P=0.008 (1.08-1.62)] for depression than women with a heterozygous genotype. SNP splits of *GNRH1*, *APOC3*, *ACVR2B*, and *IL6* did not significantly associate with depression.

Gene-Gene Interactions Enhance Predictability for Depression

Specific SNP interactions identified by RP were next analyzed by LR as RP-specific subsets (see Table 1, RP-Subsetted Data). All 5 of the secondary and tertiary RP splits were found to be significant at the modified FDR level when considered as subsets. Among only men with at least one C-allele in *ANKK1* rs1800497, those with no C-allele in the novel SNP of *GNRH1* had 1.57 times higher odds [P=0.002 (1.18-2.08)] for depression than men with 1 or 2 C-alleles. For the subset of women in the first right-hand split of Fig. 2, those homozygous for *FTO* rs1421085 had 1.65 times higher odds [P=0.0005 (1.24-2.18)] for depression than women with a heterozygous genotype. For the remaining subset of women in the second right-hand split of Fig. 2, those homozygous for *IL6* rs1800795 had 1.85 times higher odds [P=0.006 (1.19-2.89)] for depression than women with a heterozygous genotype. For the subset of women in the first left-hand split of Fig. 2, those with no C-alleles for *APOC3* rs45537037 had 1.55 times higher odds [P=0.004 (1.15-2.09)] for depression than women with 1 or 2 C-alleles. For the subset of women in the second left-hand split of Fig. 2, those homozygous for *ACVR2B* rs3749386 had 2.17 times higher odds [P=0.001 (1.37-3.44)] for depression than women with a heterozygous genotype.

DISCUSSION

Utilizing RP as a screening tool to find potential multi-gene interactions, followed by verification of multi-gene interactions with LR, our data demonstrate that multi-gene interactions predict depression with a greater certainty than single main factor associations. RP provided us with primary dichotomous genotype splits in men and women (*ANKK1* rs1800497 and *DRD2* rs2242592, respectively) that were both significant in LR models at the modified FDR level (Table 1). Considering the 5 subsequent RP splits in LR over the entire dataset, only 1 reached a nominal level of significance (barely), which was *FTO* rs1421085 in women. However, after running LR on specific subsets of data according to the pattern of RP branches, every split was found to be significant and every odds ratios grew larger (Table 1; OR [before]→[after]: Male Left: 1.43 → 1.57, Female Left 1: 1.28 → 1.55, Female Left 2: 1.11 → 2.17, Female Right 1: 1.32 → 1.65, Female Right 2: 1.12 → 1.85). Thus, RP provides two unique and important criteria: dichotomous genotype splitting instructions and gene-gene interaction patterns. These criteria go beyond the traditional single factor SNP approach to genetic association studies and allow identification of important multi-gene pathways that more suitably characterize the etiology of complex diseases.

The Utility of Recursive Partitioningand Logistic Regression for Identification of Gene-Gene Interactions

With recent advances in genotyping allowing for high-dimensional SNP identification, it is now possible to examine genetic datasets not only for single main factor effects, but also G x G interactions. The requirement for G x G analyses as a better predictor of age-related diseases is obvious from the standpoint that humans are complex biological systems composed of numerous molecular interactions, and from recent studies indicating disease risk is modulated by G x G interactions (7). Notwithstanding this, the development of analytical tools for the identification of G x G interactions has not kept pace with the technological advances in identifying genetic alterations among individuals. In this respect, we have

previously used MDR, LR and LD to identify G x G interactions among a small set of SNPs (7). However, large datasets require a screening tool to identify potential multi-gene interactions. In this study, we have used RP to screen for multi-gene interactions, a data-mining technique that is currently under-utilized in genetic studies. RP serves as an efficient and powerful exploratory analysis technique, especially when looking for interactions in data sets with a large number of independent variables. This screening allows for the identification of G x G interactions (with greater explanatory power), that might otherwise not have been identified, and that can then be confirmed using more traditional statistical techniques. As illustrated in this paper, this data-mining methodology has the advantage of identification of genetic interactions *between* pathways involved in the etiology of depression, in keeping with the etiological heterogeneity of this disorder (see later).

Our study provides proof of principle for the use of RP in higher-dimensional analyses such as GWAS, where a comprehensive list of SNPs may fully explore genetic predisposition to depression and other age-related disease. The WLS is an ideal candidate for future GWAS studies given its large sample size, rich covariate composition and longitudinal nature.

In this genetic study we aimed to identify underlying genetic predispositions to depression and thus have not yet tested environmental/phenotypic data. Future analyses using RP to examine the impact of phenotypic and environmental factors on the development of depression would be anticipated to identify gene-phenotype/environment and multi-phenotype/environment interactions. Indeed, the predictive gains of G x G analyses were stronger for men than women, despite the fact that depression occurs disproportionately in women (~2:1 female-to-male; (21-25)). This suggests that environmental factors may be needed in addition to genetic factors in understanding the etiological pathways for women. Indeed, biological factors such as hormonal changes related to reproductive status (26, 27) may impact environmental factors such as psychosocial experiences (trauma, stress, interpersonal relationships, etc) and general health issues in the development of depression.

Genetic and Biological Correlates of Depression

Numerous studies have identified SNPs that associate with depression. Many of the SNPs associated with depression from other studies were not significantly associated in our study. This is perhaps not surprising, since a single factor is unlikely to provide consistent association especially in a complex condition such as depression, where multiple pathways intersect in regulating the risk of the disease. For example, if a SNP within the serotonin pathway also requires a SNP in the glutamatergic pathway in order for the patient to present with depression, the presence of either SNP in the absence of the other will not be predictive of depression. Moreover, as indicated by Shi and Weinberg, since the human genome contains genetic redundancy, disruption of a single gene may be selectively neutral, but the malfunction of several genes in a pathway might result in expression of a particular phenotype (28).

Both the primary splits in men and women were SNPs linked with *DRD2* (dopamine receptor D2), a gene that has previously been linked with depression and social phobia (29-31). The primary male genotype split rs1800497, technically found in gene *ANKK1*, is historically known as the *DRD2* Taq1A allele because of its known association with decreased dopamine receptor D2 density (in those with T alleles) (32-35). The Taq1A allele has also been previously associated with depressive symptoms in children, where those with the A1 allele (T) were more likely to have depressive symptoms (36). We saw a similar association between A1 and depression in WLS men, where those with two A1 alleles had 2.6 times higher odds for depression compared to those with one or no A1 alleles. The primary split in women (DRD2 rs2242592) has previously been found to be associated with schizophrenia, where the C-allele was associated with higher susceptibility for schizophrenia (37). Interestingly, this same study also found the Taq1A allele to also associate with schizophrenia.

The secondary and tertiary right-hand splits in the female RP tree—*FTO* (fat mass and obesity associated) rs1421085 and *IL6* (interleukin 6) rs1800795—have also been found to relate with mental illness and depression in previous studies (38, 39). There is evidence that activin receptor signaling also is involved in affective disorders, especially when considering interaction with GABAergic pathways (40).

Although we did not see an interaction between SNPs in GABA/activin receptor genes and depression, *ACVR2B* was associated with depression in women. No previous associations between depression and *APOC3*, *ACVR2B*, or *GNRH1* have been reported.

That these genetic variants are associated with *neuroendocrine* pathways (*GnRH1, ACVR2B*) that are known to regulate *neurotransmitter* release and cognitive behavior (39-40) supports these associations as relevant to the etiology of depression and underlines the benefits of using RP to identify meaningful G x G interactions associated with disease.

Limitations

Given the numerous genetic, phenotypic and environmental influences that are linked to depression, and the small number of SNPs analyzed, it is not surprising that predictability from our models was low (although our predictability was superior to previous studies examining only single main factors). Also, the predictive value of our statistical models was further limited due to user bias in selection of SNPs (from nearly two-million SNPs in the human genome) used in this study. As a result of this, interactions we have found could potentially be moderated by another gene that we have not considered in this study. Nonetheless, we identified significant G x G interactions between known, and newly identified, loci associated with depression. Importantly, 4 of the 7 SNPs identified in these interactions were primarily located in two pathways well-known to impact depression: neurotransmitter and neuroendocrine signaling.

The results from the RP analyses conducted in this study were confirmed by LR, demonstrating the utility of RP as a screening tool for identifying meaningful G x G interactions. Future development of algorithms for RP analysis should not only maximize the distance between branches of the next best split (i.e. rpart), but consider subsequent future split combinations that could potentially result in trees with "better" overall predictability.

Summary

Our data indicate that G x G interaction analyses allows for enhanced predictability of conditions and diseases of aging. RP is an efficient and powerful exploratory analysis technique for elucidating G x G interactions in large datasets and combined with LR provides an important statistical analysis for the identification of well supported G x G interactions. We predict that such analytical methods will play an increasingly important role in the identification of epistatic effects in future large GWAS. Finally, our studies illustrate how RP analyses can be used to find interacting pathways involved in the etiology of a disease or condition such as depression.

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FIGURE LEGENDS

Figure 1. Recursive Partitioning Tree of CIDI-SF Depression in Males of the WLS. Upper and lower numbers in nodes represent the percentage of participants with depression and the number of controls/cases in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. M1 is subset of data referenced in Table 1. Sensitivity: 0.526, Specificity: 0.598, Accuracy: 0.591. Due to missing genotype information, we lose approximately 1.5% of participants per split. *rs1800497 is historically known as the *DRD2* Taq1A allele

Figure 2. Recursive Partitioning Tree of CIDI-SF Depression in Females of the WLS. Upper and lower numbers in nodes represent the percentage of participants with depression and the number of controls/cases in that node, respectively. Blue and purple boxes/circles indicate lower and higher rates of depression relative to the primary node, respectively. Split information indicates gene, SNP, and genotype criteria, respectively. F1-F4 are subsets referenced in Table 1. Sensitivity: 0.607, Specificity: 0.563, Accuracy: 0.572. Due to missing genotype information, we lose approximately 1.4% of participants per split.

Table 1.
Single-factor logistic regression models based directly off male and female RP tree split criteria (see Figures 1 & 2). Each SNP split was first run on the full dataset to represent single main factor effects ("Full Data") for both males and females. Then the same SNP splits were run on specific subsets of data per RP tree splits (M1, F1-F4; "RP-Subsetted Data").

					Full Data		RP-Subsetted Data		
Gender	RP Split	Gene	SNP	Genotypes	OR (95% CI)	P-value	Subset	OR (95% CI)	P-value
Male	Primary	*ANKK1**	rs1800497	T/T vs. C/C + C/T	2.55 (1.44-4.51)	0.001 *	--	----	----
	Left	*GNRH1*	novel SNP	T/T vs. C/C + T/C	1.43 (1.09-1.88)	0.011	M1	1.57 (1.18-2.08)	0.002 *
Female	Primary	*DRD2*	rs2242592	C/C + T/C vs. T/T	1.32 (1.08-1.62)	0.006 *	--	----	----
	Left 1	*APOC3*	rs2854116	T/T vs. C/C + T/C	1.28 (1.04-1.57)	0.018	F1	1.55 (1.15-2.09)	0.004 *
	Left 2	*ACVR2B*	rs3749386	C/C + T/T vs. T/C	1.11 (0.91-1.36)	0.302	F2	2.17 (1.37-3.44)	0.001 *
	Right 1	*FTO*	rs1421085	C/C + T/T vs. T/C	1.32 (1.08-1.62)	0.007 *	F3	1.65 (1.24-2.18)	0.0005 *
	Right 2	*IL6*	rs1800795	C/C + G/G vs. C/G	1.12 (0.92-1.37)	0.269	F4	1.85 (1.19-2.89)	0.006 *

RP, recursive partitioning; OR, odds ratio; CI, confidence interval
M1: LR analysis was run for only those with genotype *DRD2* rs1800497 C/C or C/T
F1: LR analysis was run for only those with genotype *DRD2* rs2242592 T/T
F2: LR analysis was run for only those with genotypes *DRD2* rs2242592 T/T and *APOC3* rs2854116 T/T
F3: LR analysis was run for only those with genotype *DRD2* rs2242592 C/C or T/C
F4: LR analysis was run for only those with genotypes *DRD2* rs2242592 C/C or T/C and *FTO* rs1421085 T/C
*rs1800497 is historically known as the *DRD2* Taq1A allele

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