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CXCR4 pathway associated with family history of melanoma

Wen-Qing Li¹, Jiali Han^{1,2,3}, Hans R. Widlund¹, Mick Correll⁴, Yaoyu E. Wang⁴, John Quackenbush⁴, Martin C. Mihm⁵, Alvaro Laga Canales⁶, Shaowei Wu¹, Todd Golub⁷, Yujin Hoshida⁷, David J. Hunter^{2,3}, George Murphy⁶, Thomas S. Kupper¹, and Abrar A. Qureshi^{1,2}

¹Department of Dermatology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA.

²Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA.

³Department of Epidemiology, Harvard School of Public Health, Boston, MA.

⁴Center for Cancer Computational Biology, Dana-Farber Cancer Institute, Boston, MA.

⁵Department of pathology, Massachusetts General Hospital, Boston, MA.

⁶Department of Pathology, Brigham and Women's Hospital, Boston, MA.

⁷Broad Institute, Harvard Medical School, Boston, MA.

Abstract

Purpose—Genetic predisposition plays a major role in the etiology of melanoma, but known genetic markers only account for a limited fraction of family history-associated melanoma cases. Expression microarrays have offered the opportunity to identify further genomic profiles correlated with family history of melanoma. We aimed to distinguish mRNA expression signatures between melanoma cases with and without a family history of melanoma.

Methods—Based on the Nurses' Health Study, family history was defined as having one or more first-degree family members diagnosed with melanoma. Melanoma diagnosis was confirmed by reviewing pathology reports and tumor blocks were collected by mail from across the United States. Genomic interrogation was accomplished through evaluating expression profiling of formalin-fixed paraffin-embedded tissues from 78 primary cutaneous invasive melanoma cases, on either a 6K or whole-genome (24K) Illumina gene chip. Gene Set Enrichment Analysis was performed for each batch to determine the differentially enriched pathways and key contributing genes.

Results—The CXC chemokine receptor 4 (CXCR4) pathway was consistently up-regulated within cases of familial melanoma in both platforms. Leading edge analysis showed four genes from the CXCR4 pathway, including *MAPK1*, *PLCG1*, *CRK*, and *PTK2*, were among the core

Corresponding author: Abrar A. Qureshi, MD, MPH, Harvard Medical School, Department of Dermatology, Brigham and Women's Hospital, 45 Francis St, 221L, Boston, MA 02115. aqureshi@bics.bwh.harvard.edu Telephone: 617-264-5913, Fax: 617-264-3021.

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members that contributed to the enrichment of this pathway. There was no association between the enrichment of *CXCR4* pathway and *NRAS*, *BRAF* mutation, or Breslow thickness of the primary melanoma cases.

Conclusions—We found that the *CXCR4* pathway might constitute a novel susceptibility pathway associated with family history of melanoma in first-degree relatives.

Keywords

melanoma; genetic pathway analysis; genome-wide expression profiling

Introduction

Malignant melanoma accounts is one major public health concern in Western countries(1). Both genetic and environmental risk factors play roles in the development of melanoma. The molecular understanding of melanoma tumorigenicity has been substantially strengthened by the identification of oncogenes such as *BRAF* and *NRAS*, with somatic mutations in these genes directly associated with melanoma development (2–5). Family history of melanoma, multiple benign or atypical nevi, sun sensitivity and pigmentation have also been linked to melanoma predisposition (5–7). Approximately 10% of melanoma cases have at least one first-degree relative with a diagnosis of melanoma, which has offered an opportunity to look for genetic predisposition of melanoma (6).

Evidence for genomic biomarkers related to melanoma cases with family history is yet sparse. The cyclin dependent kinase (CDK) inhibitor 2A (*CDKN2A*) is the first identified melanoma-predisposing gene (8–9), and a few rare kindreds have mutations in cyclindependent kinase 4 (*CDK4*) (8). Genetic variants in pigmentation genes have been identified to confer susceptibility to sporadic and familial melanoma (10–12). These genes only contribute to a small fraction of family-history associated melanomas. In addition, little is known on the expression profiling of family history-associated melanoma, suggesting a large gap in our molecular understanding of predisposition to familial melanoma.

Expression profiles from quantitative RNA expression arrays have been correlated with disease phenotypes. Few studies have comprehensively evaluated the expression signatures in familial melanoma. Based on primary melanoma cases obtained from the Nurses' Health Study (NHS), we have assembled expression data sets from formalin-fixed paraffin-embedded (FFPE) tissues using the 6K and Whole-Genome (24K) cDNA-mediated Annealing, Selection, extension and Ligation assays (DASL, Illumina, San Diego, CA) and aimed to distinguish signatures between melanoma cases with and without a family history of melanoma in this study.

Materials and Methods

Study participants

The NHS was established in 1976 when 121,700 married, female registered nurses aged 30–55 years in the US returned a questionnaire enquiring about their medical history and lifestyle practices. Biennially, information on lifestyle factors and medical history was

collected by mailed questionnaires. For self-reported melanoma cases, medical records and pathology reports were requested and reviewed by physicians blinded to study design.

A question regarding melanoma among father, mother, or siblings (i.e., a first-degree relative) was included in the 1982 questionnaire, and updated in 1992, 1996, and 2000. Information on age, mole count on the left arm, natural hair color, and childhood tan and sunburn reaction, and UV index at birth, age 15, and age 30 was collected through the questionnaires.

For individuals who had a diagnosis of melanoma confirmed by medical record review, we obtained tissue blocks from pathology laboratories based on information from the original pathology reports, with the consent of study participants. For this study, we initially included 48 FFPE blocks from 36 melanoma cases with or without family history in the first batch of analysis on expression profiling. In this batch, we have two duplicate blocks each for seven cases, three duplicates for one case, and four duplicates for one case. For the second batch, 49 blocks from another 44 melanoma cases with or without family history of melanoma were included. We had two blocks from five cases in this second batch. The duplicate blocks were created from the pathology laboratory where the tumor was grossed and processed to accommodate large tissue specimens that would not fit on a single block. Duplicate blocks were deliberately included in our study to ascertain expression concordance across duplicate specimens from the same melanoma tissue. The study protocol was approved by the Human Research Committee of Brigham and Women's Hospital.

FFPE Tissue preparation

We cut FFPE sections to a thickness of 4 μm and mounted on to a positively charged glass slide. Sections were evaluated by a board-certified dermatopathologist and if there was adequate tumor tissue (>50% of the tissue) available, we then proceeded to cut the remaining 10 sections, each 10 μm . Of the 10 sections, six sections were used to extract RNA and four sections were used to prepare DNA. Breslow thickness (in continuous variable, mm) and Clark's level (I, II, III, IV, and V) of melanoma was determined by experienced pathologists blinded to the characteristics and expression profiling of the study subjects.

Analysis of gene expression

Gene-expression profiling analysis was performed by using the DASL assay (Illumina, San Diego, CA), which has been considered a valid approach to profile randomly fragmented mRNA extracted from FFPE tissue (FFPE-RNA) (13–15). One study demonstrated a similar sensitivity in gene detection by DASL between matched fresh frozen and FFPE samples (13–15). With as little as 250 pg of intact total RNA (~25 cell equivalent), the assay still maintains good reproducibility ($R^2=0.92$) (13–15). Fragmented FFPE-RNA was converted into cDNA using random primers. For each target site on the cDNA, a pair of query oligos separated by a single nucleotide was annealed to the cDNA, and, the gap between the query oligos was extended and ligated to generate a PCR template. A pair of universal PCR primers was used for amplification, and linearly amplified PCR products were hybridized to a bead microarray. The array was then scanned by a BeadArray Reader (Illumina). We initially used the 6K DASL in the first batch for which 6100 transcriptionally informative

genes were selected for analysis (NCBI's Gene Expression Omnibus, <http://www.ncbi.nlm.nih.gov/geo/>, platform ID GPL5474, for the full list of genes) (14). We then applied the 24K DASL in the second batch to expand the expression differences which combines the unique PCR and labeling steps of the original DASL assay with gene-based hybridization and the whole-genome probe set of Illumina's Direct Hybridization Assay. The 24K panel has 24,526 wellannotated transcripts associated with 18,401 unique gene symbols (15).

Analysis of somatic *BRAF*/*NRAS* mutations

We utilized pyrosequencing to determine somatic mutations in *BRAF* and *NRAS*. FFPE tissues were hand-dissected under a microscope after pathology review to enrich for tumor and genomic DNA extracted using Qiagen DNeasy FFPE kit. For somatic mutations of *BRAF* V600E, a 228-bp region of human *BRAF* exon 15 spanning the mutation site was amplified by PCR using the following primers: FV600: ATGCTTGCTCTGATAGGAA; RV600-BIO: biotin-GCATCTCAGGGCCAAA, followed by pyrosequencing using sequencing primer (sV600: GGTGATTTTGGTCTAGCTAC) essentially as previously described (16). For evaluation of *NRAS* Q61R somatic mutation, a 136-bp region was amplified using the following primers: FQ61: GATTCTTACAGAAAACAAGTGGTTATAGAT; and RQ61- BIO: biotin-GCAAATACACAGAGGAAGCCTTCG, followed by pyrosequencing using sequencing primer (Q61: GACATACTGGATACAGCTGG) essentially as described (17). The samples were processed and analyzed on the Qiagen PyroMark24 and allele/mutation call cutoffs were set at 15% signal intensities.

Statistical analysis

The raw expression values for each sample were examined for distribution, and samples with large number of outliers (as indicated by the circles deviated from the box-plot) were removed. Outliers are defined as 1.5-fold inter-quartile range away from the middle 50% of the data. Five blocks from five subjects were removed from the 6K platform and two blocks from one subject were removed from the 24K platform (Figure 1a). Eventually, 43 blocks from 35 samples in the 6K platform and 47 blocks from another 43 samples in the 24K platform remained. We compared the distribution of major characteristics between the two platforms using Student's t-test or chi-square test. Of them, there were two duplicate blocks each for six cases, and three duplicates for one case in the 6K platform. In the 24K platform, there were two duplicate blocks from the same melanoma tissues for four cases. The DASL results across duplicate blocks were used as quality control and to evaluate consistency of the platform (Figure 1b). Concordance between calls of duplicate blocks was ascertained by the distribution of expression, as annotated by the diagonal cluster of the dots, as well as a correlation coefficient (R^2). Duplicate blocks were merged by average for down-stream analysis. Quantile normalization was performed within each platform respectively to minimize experimental bias (18). Within each batch, every expression value for any one probe was mapped to the corresponding quantile of the standard distribution (18). Linear models for microarray data were constructed by using the Bioconductor or LIMMA package to determine and rank differential gene expression statistics (19), and the analysis was performed in R (version 2.15.0, <http://www.r-project.org>).

For each batch, Gene Set Enrichment Analysis (GSEA) was then performed separately on the pre-ranked differential gene expression statistics determined by the linear models (20–21). To evaluate the significant enriched canonical pathways for familial melanoma cases in comparison with sporadic cases, we performed the GSEA using the stand-alone version of GSEA software from the Broad Institute (<http://www.broadinstitute.org/gsea>), across the 638 curated canonical pathways (gene sets) available from the Molecular Signatures Database (MSigDB) database C2-CP collection (release 3.0). For the top pathways in familial melanoma that met the false discovery rate (FDR) q -value ≤ 0.25 across the two batches, we sought to extract the core members of genes that contribute to the enrichment score of the pathways by using leading edge analysis for each batch. The leading-edge gene subsets were defined as genes that appear in the ranked list at, or before, the point where the running sum reaches its maximum deviation from 0 (20). We evaluated the association between the top pathway in analysis of familial melanoma and hair color (read/blonde, or brown/black), moles count (≤ 3 , or > 3), tanning reaction (average/deep, or none/light tanning), or tendency to burn/painful burn (yes or no), by using GSEA. We also sought to evaluate the enrichment of the top pathway in analysis of familial melanoma by *BRAF*/*NRAS* mutations and Breslow thickness (≤ 1 or > 1 mm). Given the moderate sample size, we used two categories of Breslow thickness rather than treat it as a continuous variable or four categories.

Results

We initially included 97 annotated melanoma blocks as interrogated in two batches; a total of 48 blocks were analyzed on a 6K DASL platform, and a subsequent series of 49 samples analyzed on a 24K platform. Overall raw gene expression value distributions for all arrays, including duplicates, were examined for data quality, and ineligible arrays were removed to yield 43 blocks of 35 samples and 47 blocks of 43 samples in each batch (Figure 1a). High concordance across array results for duplicate samples suggested data consistency of the DASL platform, with R^2 for correlation more than 0.60 for all duplicated sample; an example of the consistency across duplicates are shown in Figure 1b. Characteristics of study participants remaining in the analysis are presented in Table 1. The proportion of melanoma cases with family history as determined with at least one first-degree relative diagnosed with melanoma was similar between the 6K (11.4%) and 24K platforms (11.6%).

For the duplicate blocks, we took the average expression value for each probe. Using GSEA, we initially identified five pathways (gene sets) enriched in familial melanoma in comparison with sporadic cases, from the 6K and 24K array platforms respectively (FDR $q \leq 0.25$) (Table 2 and Figure 2). Overlapping the two batches, the CXCR4 pathway was the only one pathway consistently enriched in melanoma sections from cases with a family history of melanoma (FDR q 0.179 in batch 0, and 0.087 in batch 1). As defined by the MSigDB database, there are 24 genes constituting the CXCR4 pathway; wherein the 6K DASL covered 20 genes of these, while the 24K DASL array platform contained all (Supplementary Table S1).

Through the leading-edge analysis, we observed that 8 genes in the 6K batch and 13 genes in 24K batch were driving the enrichment of the CXCR4 pathway (Table 3, Figure 3a and

b). Across the two platforms, we found four individual genes common to both 6K and 24K DASL, including *PTK2*, *CRK*, *PLCG1*, and *MAPK1*. Among the other assumed important genes in the 24K DASL array, *GNGT1*, *PIK3C2G*, and *GNAQ* were not covered in the 6K DASL array. Enrichment plot for CXCR4 signature showed the profile of the enrichment score and positions of gene set members on the rank. The leading-edge gene sets are composed of genes in CXCR4 pathway up-regulated in melanoma cases with family history.

We evaluated the association between CXCR4 pathway and hair color, moles count, tanning ability, or burning ability, but did not observe significant enrichment of CXCR4 pathway by these characteristics. We also performed subgroup analysis to determine if *BRAF*-V600E or *NRAS*-Q61R somatic mutations, or Breslow thickness was confounding our results. CXCR4 pathway was not found to be enriched in any of the categories for both batches; hence there were no obvious confounding effects of these conditions that resulted in CXCR4 signature enrichment. Further analysis for the whole genome expression profiling did not find consistently up-regulated or down-regulated pathways across the two arrays by any characteristics.

Discussion

In our study, initially we used a 6K DASL assay to evaluate the gene-expression differences in melanoma with and without family history. Once the 24K platform became available, we further utilized this platform to expand potential expression differences. By performing GSEA, we were able to identify a signature associated with a chemokine receptor, CXCR4, and its downstream signaling consistently up-regulated in melanoma cases with a family history within both the 6K and 24K DASL datasets. Further analysis found four genes in this pathway common to the two platforms contributing to the enrichment. To our knowledge, this is the first study reporting an association between expression profiling of CXCR4 pathway and family history of melanoma.

CXCR4 is a G protein-coupled receptor that responds exclusively to the ligand CXCL12 and transduces intracellular signaling modulating cell migration, proliferation, survival, increase in intracellular calcium, and gene transcription (22–24), and has been reported as the most widely expressed chemokine receptor among various cancers (23–28). In melanoma, CXCR4 expression has been reported to be up-regulated and associated with proliferation, angiogenesis, metastasis and poor prognosis (27, 29–31). However, no previous studies have reported the significance of CXCR4 pathway in melanoma in a pathway analysis. Although we cannot tell the variation that could be explained by the identification of this pathway, our study adds on the adverse effect of CXCR4, which consistently showed the up-regulation of CXCR4 pathway across two batches of gene-expression profiling analysis.

Our leading edge analysis emphasizes the contribution of *PTK2*, *CRK*, *PLCG1* and *MAPK1* to the enrichment of CXCR4 pathway in familial melanoma. Protein tyrosine kinase (PTK2) is a non-receptor tyrosine kinase linked to tumorigenesis, proliferation, migration, and survival of skin cancer and other tumors (32–34). CRK shares with PLCG1 the Src homology 2 domain, by which they couple growth factor stimulation to intracellular signal transduction pathways (35). *PLCG1* encodes a tyrosine kinase and controls the cellular

transduction of receptor-mediated tyrosine kinase activators (39). MAPK1 was also reported to be a downstream constituent of CXCL12 signaling in controlling chemotaxis and survival of dendritic cells (36). The over-expression of MAPK1 has been indicated in melanoma.

Increased number of moles, excessive sun exposure and sun sensitivity have been shown to increase risk for melanoma in Caucasians (5–7). Mole counts also were associated with family history of melanoma in NHS (data not shown). Because we did not observe any association between expression profile of CXCR4 pathway and mole counts, it is less likely that this pathway could have an effect on familial melanoma by promoting the progression nevi. Cases with familial melanoma were more likely to sunburn and less likely to tan in our cohort (data not shown). The CXCR4 pathway may therefore be a surrogate marker for the host response to sun exposure. However, we did not find expression enrichment of CXCR4 pathway by tanning or burning ability. Our analysis did not lend support to the role of CXCR4 pathway in melanoma mediated by known phenotypic factors.

NRAS and *BRAF* somatic mutations are common in cutaneous melanomas⁵, although rarely detected mutually in the same tumor (37). In our analysis, expression profiling of CXCR4 pathway was not associated with *BRAF* or *NRAS* mutation, demonstrating the effect independent of *NRAS* and *BRAF*.

We detected the genome wide expression profiling of FFPE tissues by using a DASL-based approach. In the first batch, we applied the 6K DASL assay on selected genes across the genome. We then used the whole-genome DASL in another set of samples which greatly increases the assay target to capture the bulk of transcriptional differences. It has been recognized that genome-wide association studies on genetic variants or expression for complex and heterogeneous diseases are limited in their ability to detect new single marker, perhaps due to the extreme levels of statistical significance necessary to control for multiple comparisons (20, 38). The pathway-based approach has been suggested to utilize the genome-wide data more efficiently and holds the potential to yield novel findings (39). GSEA, a knowledge-based approach for interpreting genome-wide expression profiles, is able to conclude information on a biological process from the DASL data (20). Our results in the 24K DASL replicated that from the 6K DASL in the pathway-level and gene-level for the CXCR4 gene sets; therefore offering new insight into the role of CXCL12/CXCR4 axis in melanoma cases with family history. Although we did not indicate *CXCR4* and *CXCL12* gene as the core contributors of expression enrichment, we observed an overlap of four individual genes in two platforms.

We acknowledge limitations of our study. First, we lack a large replication set to validate our results. For the other four significantly enriched pathways in the 24K DASL, we did not observe a similar enrichment in the 6K DASL. The differences may be due to the limited coverage of core genes in the pathways in the 6K DASL. Further data on a 24K DASL using independent samples is warranted to validate these four pathways. For the other four significantly enriched pathways in the 6K DASL, we did not observe a similar enrichment in the 24K DASL, which may be partly due to the considerably differential gene coverage of 24K array and the algorithms of GSEA. During the beadchip upgrade for development of the 24K DASL array, the raw intensities and expression profiles between the 6K and 24 K were

not directly comparable; however, the relative differences between samples should correlate and validate between the two platforms. In addition, at the probe level, the shared probes are identical between the 6K and the 24K platforms; therefore, the sensitivities of the two platforms should be similar in capturing the expression signals. At least for the CXCR4 pathway, using 24K DASL is a valid approach to replicate the results from 6K DASL. Second, because our study was conducted among female Caucasians, our results require further confirmation in other populations. Third, GSEA did not control for other covariates in this gene-set (profile) based analysis. Although some correlations may exist between family history of melanoma and other known melanoma risk factors, for example, childhood sun reactivity and mole counts, we did not find consistent enrichment of CXCR4 pathway by other factors. We also did not find any consistent enrichment of other gene-sets by these factors. Therefore, this would not be a concern influencing our results.

In conclusion, our genome-wide expression profiling analysis demonstrates CXCR4 pathway as a potential signature associated with familial risk of melanoma. Further data are warranted to elucidate the mechanisms underlying the observed association between the core attributing genes in the CXCR4 pathway and melanoma with a family history.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a

b

Figure 1. Quality control for the raw expression values from cDNA-mediated Annealing, Selection, extension and Ligation assays

a. Boxplot of the raw gene expression values from 6K (batch 0) and 24K (batch 1) arrays.

Samples with high number of outliers, highlighted with *, were removed from further analysis. b. Example of concordance plot for duplicate blocks from the same melanoma tissues. The x and y axes are gene expression values for duplicate blocks for a case from the 6K array shown as an example. Diagonal cluster indicates strong data correlation across duplicates.

Figure 2. Enriched Signaling Pathways in 6K (batch 0) and 24K (batch 1) cDNA-mediated Annealing, Selection, extension and Ligation assays
The enrichment scores for significantly enriched pathways identified in either 6K or 24K array are shown by the color ranged from red to green. Green and red color indicates high to low ES scores respectively. The significant pathways are noted by (*).

a.

b.

Figure 3.

a. Enrichment profile for the CXCR4 pathway in the 6K cDNA-mediated Annealing, Selection, extension and Ligation assay. The plot of the running sum for the gene sets, including the location of the maximum enrichment score (blue line) and the leading-edge subset (red circle). The contrast was set up as experiment-control (i.e. patients with family history compared with sporadic patients). The group of cases with family history is over-represented by the CXCR4 gene set, so that “na_pos” means up-regulated genes in familial

patients (hence down-regulated genes in sporadic patients). In contrast, na_neg indicates those down-regulated genes in familial melanoma cases.

b. Enrichment profile for the CXCR4 pathway in the 24K cDNA-mediated Annealing, Selection, extension and Ligation assay. The plot of the running sum for the gene sets, including the location of the maximum enrichment score (blue line) and the leading-edge subset (red circle). The contrast was set up as experiment-control (i.e. patients with family history compared with sporadic patients). The group of cases with family history is over-represented by the CXCR4 gene set, so that “na_pos” means up-regulated genes in familial patients (hence down-regulated genes in sporadic patients). In contrast, na_neg indicates those down-regulated genes in familial melanoma cases.

Table 1

Characteristics of the melanoma cases in the 6K and 24K cDNA-mediated Annealing, Selection, extension and Ligation (DASL) arrays

	6K DASL (n=35, batch 0)	24K DASL (n=43, batch 1)	<i>P</i>[*]
Age at diagnosis, years, n (median)	33 (60)	42 (61)	0.29
Red or blonde hair (%)	5/28 (17.9)	6/35 (17.1)	0.94
3 moles (%)	6/25 (24.0)	8/32 (25.0)	0.93
Childhood tendency to Average/deep tan (%)	16/28 (57.1)	19/35 (54.3)	0.82
Childhood tendency to burn/severe burn (%)	14/28 (50.0)	15/35 (42.9)	0.76
Family history of melanoma (%)	4/35 (11.4)	5/43 (11.6)	0.88
Breslow thickness 1 mm (%)	17/31 (45.9)	18/38 (47.4)	0.71
Clark's level IV-V (%)	11/28 (39.3)	21/37 (56.8)	0.25

* *P*-values were calculated by Student's t-test or chi-square test.

Pathway enrichments results (with false discovery rate 0.25) from the 6K and 24K cDNA-mediated Annealing, Selection, extension and Ligation (DASL) arrays by using gene set enrichment analysis

Table 2

Name of pathways	Total No. of genes ^a	No. of Genes covered	Enrichment score (ES)	Normalized ES	False discovery rate q-value
6K DASL (batch 0)					
HSA03022 Basal Transcription Factors pathway	34	17	0.68	2.09	0.037
ARF pathway	16	13	0.69	1.95	0.077
mRNA Processing Reactome pathway	121	63	0.46	1.95	0.113
CXCR4 pathway	24	20	0.57	1.85	0.179
HSA00240 Pyrimidine Metabolism pathway	63	45	0.45	1.80	0.241
24K DASL (batch 1)					
CXCR4 pathway	24	24	0.64	1.95	0.087
ATIR pathway	34	32	0.56	1.81	0.249
FBW7 pathway	9	9	0.77	1.79	0.143
GH pathway	27	27	0.55	1.70	0.226
CCR3 pathway	23	22	0.59	1.79	0.250

^aNumber of total genes for this pathway in the Molecular Signatures Database.

Table 3

Leading edge analysis of genes in the CXCR4 pathway, from the 6K and 24K cDNA-mediated Annealing, Selection, extension and Ligation (DASL) arrays

	Rank in gene list	Rank metric score	Running enrichment score
6K DASL (batch 0)			
PTK2	162	1.74	0.12
GNAI1	259	1.57	0.24
CRK	421	1.34	0.32
PLCG1	681	1.08	0.37
RELA	896	0.94	0.42
MAPK3	899	0.93	0.50
PIK3CA	1208	0.76	0.51
MAPK1	1213	0.75	0.57
24K DASL (batch 1)			
PTK2B	124	3.23	0.11
GNGT1	331	2.69	0.19
PIK3C2G	485	2.46	0.27
PRKCA	826	2.12	0.32
CXCR4	1391	1.79	0.36
PIK3R1	1521	1.73	0.41
GNAQ	1627	1.68	0.46
PTK2	2004	1.54	0.50
PRKCB1	2387	1.40	0.52
PLCG1	2653	1.32	0.56
MAPK1	2721	1.31	0.60
MAP2K1	2941	1.25	0.63
CRK	3521	1.11	0.64