Introduction to pathway analysis based on high-throughput genomic data

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NHRI

Based on

- Mootha et al. (2003) PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature Genetics*, 34, 3, 267-273
- Subramanian et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. PNAS, 102, 15545-15550
- Subramanian et al. (2007) GSEA-P: A desktop application for Gene Set Enrichment Analysis. Bioinformatics, doi: 10.1093/bioinformatics/btm369.

Mootha et al. (2003)

- In a follow-up study, 17 NGT(normal glucose tolerance), 8 IGT(impaired glucose tolerance, 18 DM2. Age-matched.
- Diagnosed by hyperinsulinemic clamp. At time of diagnosis, thus before treatment, skeletal muscle biopsy samples were taken.
- Microarray expression experiments.
- No single gene had a significant difference in expression between the diagnostic categories.
- Given a gene set (pathway), they ask if it is associated with the disease status.

Basic ideas

- Consider only NGT and DM2 to simplify the matter.
- Order the genes according to their differences in expression.
- A given gene set whose members contain more low ranking or high ranking genes than expected by chance.
- Collected databases of gene sets, including pathways.
- Developed statistical methods.

The findings

- 149 gene sets were prepared without knowledge of the expression data.
- The gene set getting highest score is OXPHOS (oxidative phosphorylation)
- The next four highest scoring gene sets overlap with the top one. This overlap explains the enrichments greatly.
- OXPHOS has 106 genes. 94 of them showed a decrease of about 20% in DM2 subjects.
- A subset of OXPHOS genes was strongly upregulated in response to PGC-1a.

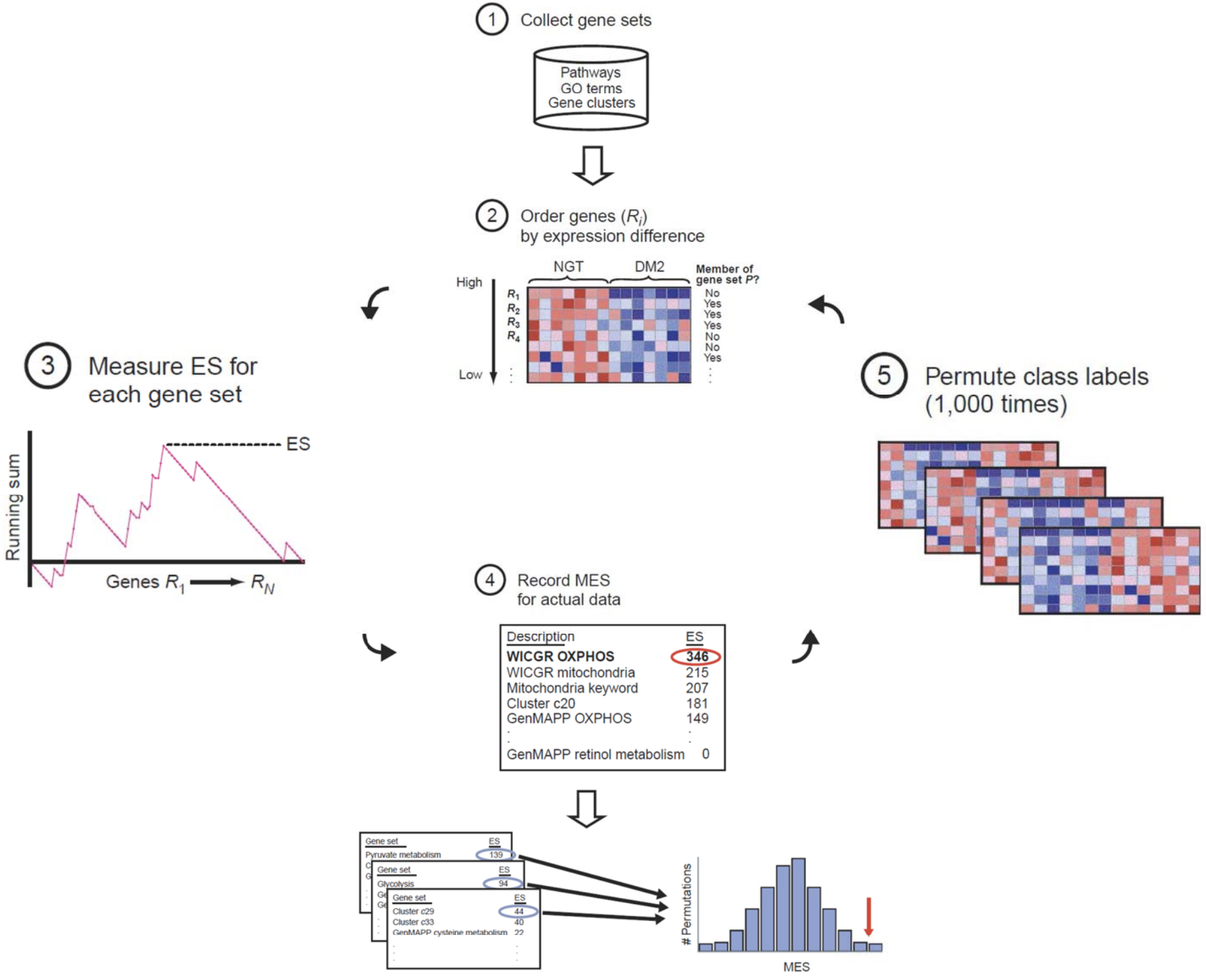
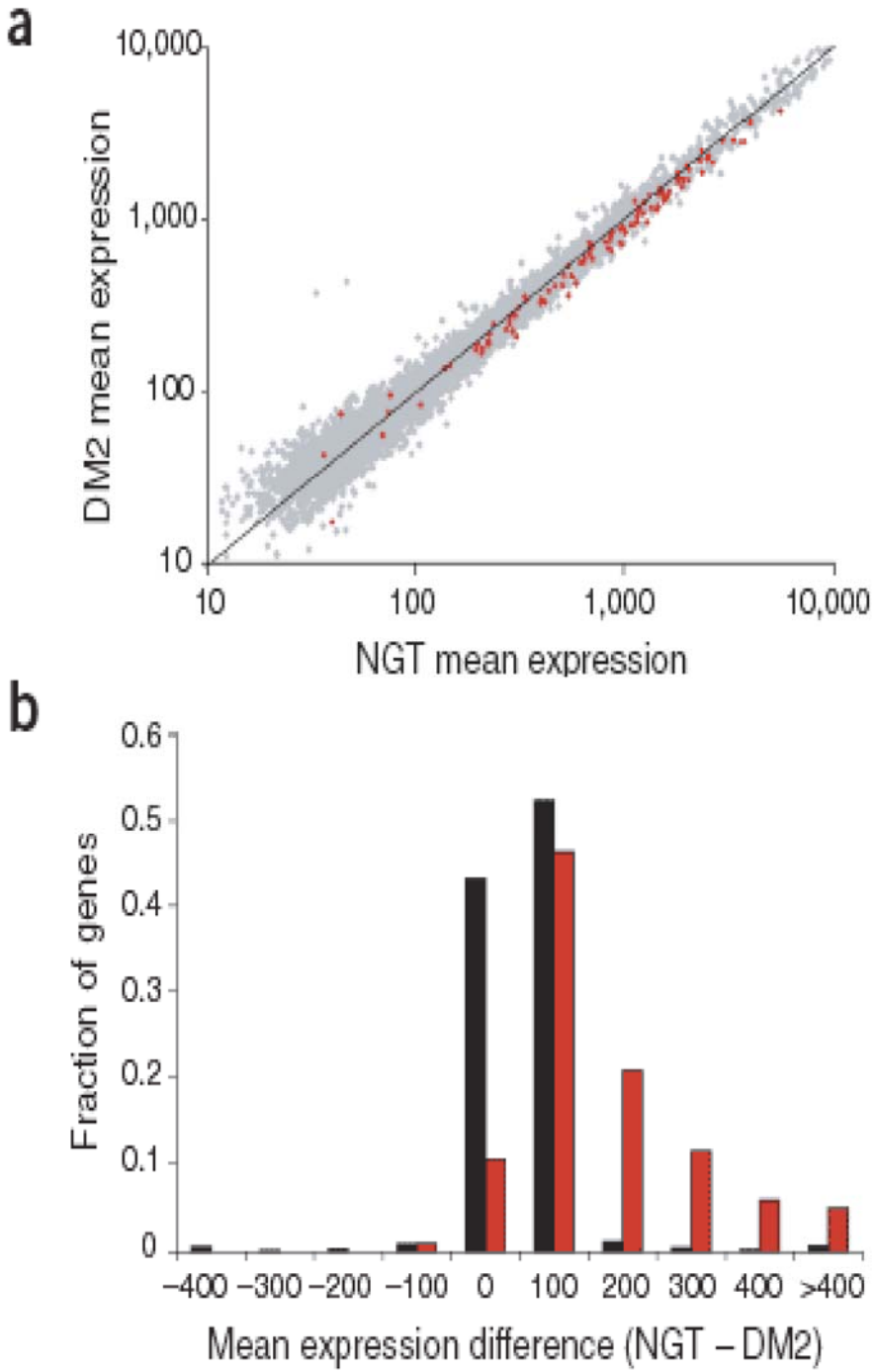
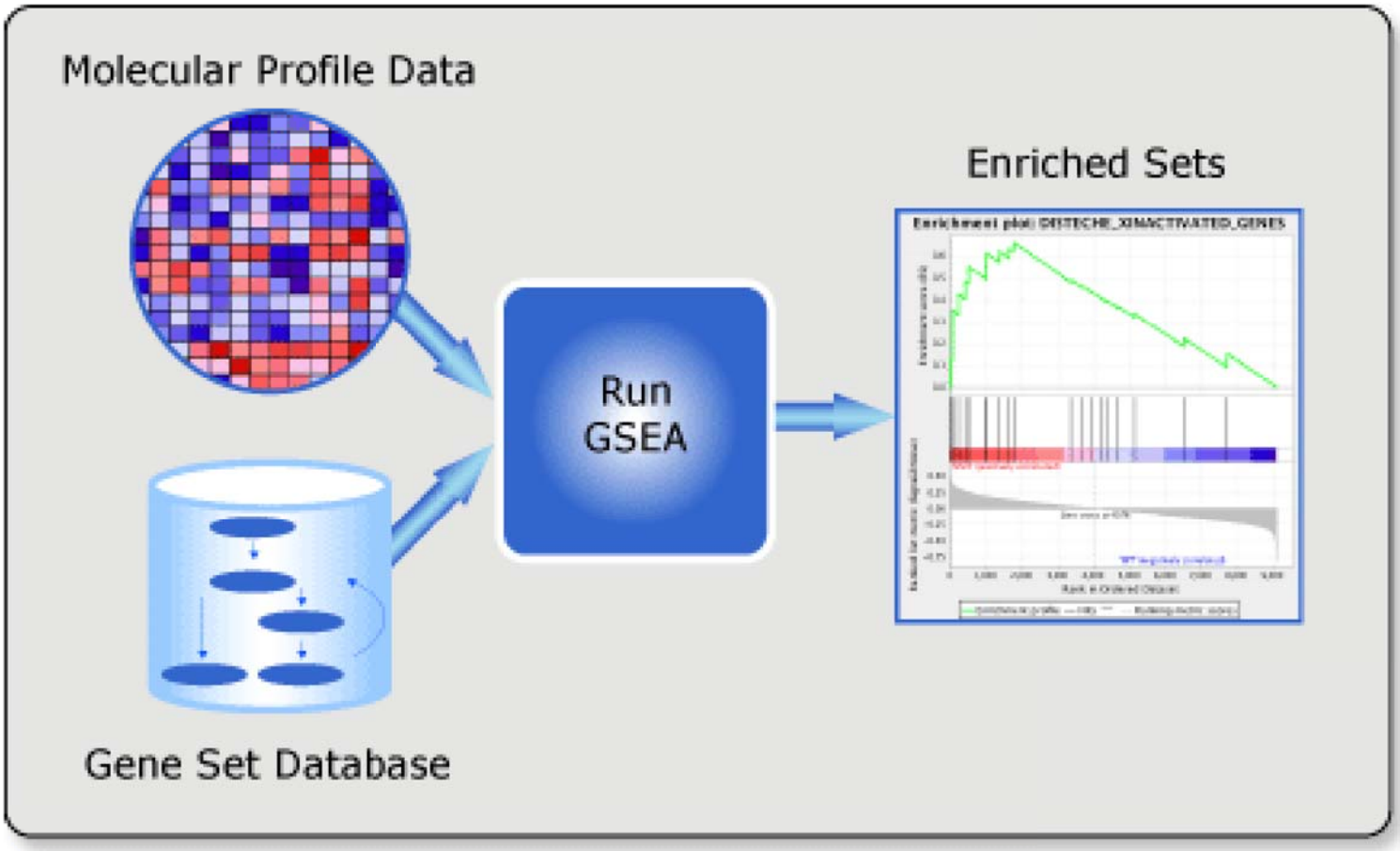

1 Collect gene sets
Pathways
GO terms
Gene clusters
2 Order genes (R_i) by expression difference
NGT
DM2
Member of gene set P?
High
Low
R_1 No
R_2 Yes
R_3 Yes
R_4 Yes
No
No
Yes
3 Measure ES for each gene set
ES
Running sum
Genes R_1 → R_N
4 Record MES for actual data
Description ES
WICGR OXPHOS 346
WICGR mitochondria 215
Mitochondria keyword 207
Cluster c20 181
GenMAPP OXPHOS 149
GenMAPP retinol metabolism 0
Gene set ES
Pyruvate metabolism 139
Gene set ES
Glycolysis 94
Gene set ES
Cluster c29 44
Cluster c33 40
Permutations
MES
5 Permute class labels (1,000 times)
6 Evaluate significance of actual MES against 1,000 permuted MES

Table 1 Clinical and biochemical characteristics of male subjects with NGT, IGT and DM2

	Class			*P* value		
	NGT	IGT	DM2	NGT versus IGT	IGT versus DM2	NGT versus DM2
n	17	8	18			
Age, y	66.1 (1.0)	66.4 (1.6)	65.5 (1.8)			
BMI, kg/m^2	23.6 (3.4)	27.1 (4.8)	27.3 (4.0)			5.70×10^{-3}
WHR	0.91 (0.09)	0.97 (0.04)	0.99 (0.03)	3.00×10^{-2}		3.83×10^{-3}
Triglycerides, mmol/l	1.03 (0.40)	1.83 (1.60)	2.04 (1.13)			2.63×10^{-3}
Cholesterol, mmol/l	5.39 (0.09)	4.60 (1.48)	5.77 (0.97)			
OGTT						
Glucose 0 min, mmol/l	4.67 (0.50)	5.05 (0.46)	7.83 (2.3)		9.22×10^{-5}	2.01×10^{-5}
Insulin 0 min, μU/ml	5.41 (3.3)	13.38 (8.9)	12.0 (6.0)	4.05×10^{-2}		4.10×10^{-4}
Glucose 120 min, mmol/l	6.58 (0.94)	9.15 (0.8)	14.9 (4.0)	2.51×10^{-6}	8.91×10^{-6}	4.90×10^{-8}
Insulin 120 min, μU/ml	33.5 (19.3)	125.1 (66.1)	43.5 (25.6)	5.47×10^{-3}	9.73×10^{-3}	
M value, mg/kg/min	8.74 (3.15)	6.32 (3.08)	4.22 (1.72)			2.30×10^{-5}
VO2max, ml O_2/kg/min	32.1 (5.46)	26.5 (4.6)	24.3 (5.6)	1.72×10^{-2}		3.09×10^{-4}
Glycogen, mmol/kg	371.1 (77.0)	326.5 (88.0)	350.6 (97.8)			
Type I fibers						
Number, %	37.2 (13.5)	33.5 (3.6)	36.4 (9.3)			
Area, %	39.1 (14.4)	32.7 (0.91)	40.1 (10.7)		2.35×10^{-2}	
Capillaries/fiber	3.91 (0.72)	4.05 (1.04)	4.14 (0.75)			
Type IIb fibers						
Number, %	73.8 (42.1)	60.2 (51.4)	72.2 (36.7)			
Area, %	31.3 (18.0)	24.7 (18.3)	36.2 (15.4)			
Capillaries/fiber	2.97 (0.71)	3.05 (0.87)	3.02 (0.65)			

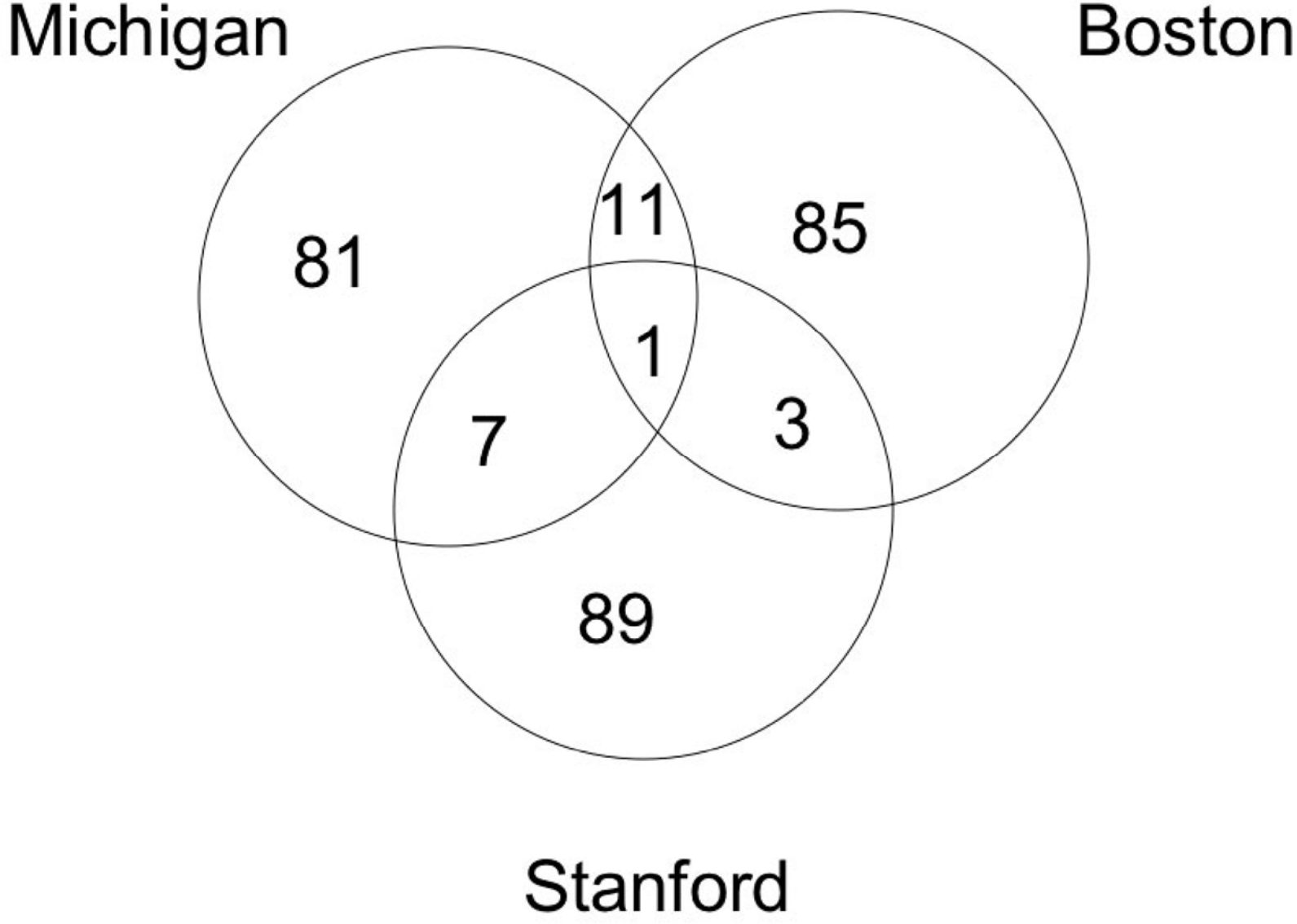
Values are mean (s.d.). OGTT, oral glucose tolerance test. M value is the total body glucose uptake. VO2max is the total body aerobic capacity. Only values of $P < 0.05$ are shown for pairwise comparisons, using a two-sided *t*-test.


a
10,000
1,000
100
10
DM2 mean expression
10
100
1,000
10,000
NGT mean expression
b
0.6
0.5
0.4
0.3
0.2
0.1
0
Fraction of genes
−400 −300 −200 −100 0 100 200 300 400 >400
Mean expression difference (NGT – DM2)


Molecular Profile Data
Enriched Sets
Run
GSEA
Gene Set Database

Lung ADC studies

- Microarray gene expression data from tumor samples of LADC patients and their clinical outcome data, classified as “good” or “poor” outcome.
- Boston: 62 samples. Michigan: 86 samples. Stanford: 24 samples.
- BS denotes the set of top 100 genes correlated with poor outcome in Boston study. Similarly the notation MS and SS.
- The overlap is distressingly small.


Michigan
Boston
81
11
85
1
7
3
89
Stanford

GSEA vs single-gene methods

- Looking for differentially expressed gene sets.
- Gene sets, defined by pathways or biological processes, are more reproducible and interpretable than single-gene in a large scale experiment.
- When members in a gene set exhibit strong correlation among them, GSEA is more signal-revealing.
- Leading-edge analysis helps to elucidate the results.

Enrichment Score

Given microarray data from cancer cell samples and normal cell samples. Two-sample test to look for differentially genes. Let $t_i = t(g_i)$ denote the statistic for g_i. Order these genes by $t_i = t(g_i)$. Assume L=$(g_1,...,g_N)$ is ordered by t_i.

Enrichment score (Kolmogrov-Siminov test)

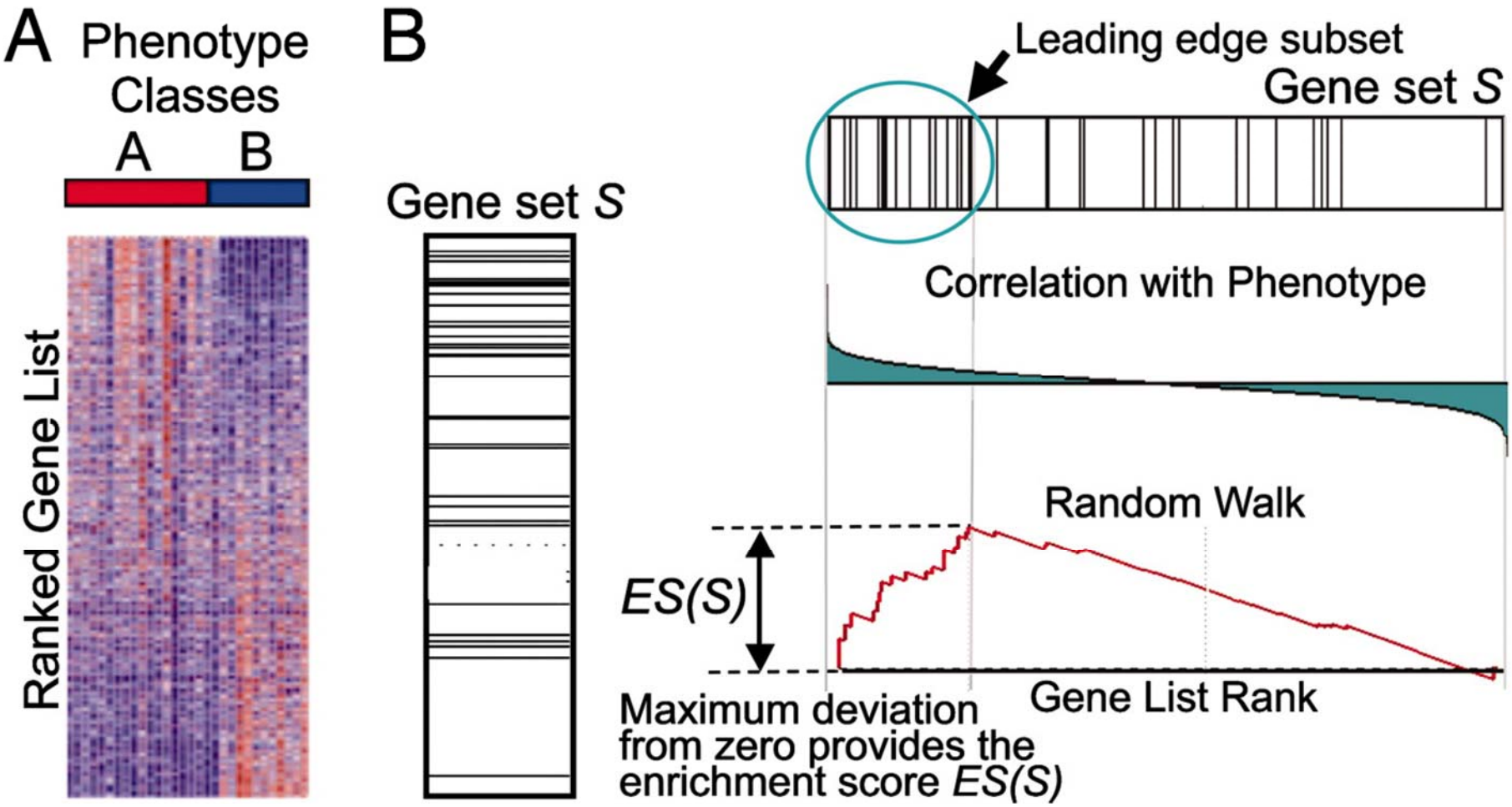
Let S be a given gene set. To know if S is associated with cancer status. Define

$$\mathrm{P}_{hit}(S,i) = \sum_{\substack{j \le i \\ g_i \in S}} \frac{|t_j|^p}{N_R}, \quad \text{where} \quad N_R = \sum_{g_i \in S} |t_i|^p$$

$$\mathrm{P}_{miss}(S,i) = \sum_{\substack{j \le i \\ g_i \notin S}} \frac{1}{N - N_R}.$$

$$ES(S) = \max\{\mathrm{P}_{hit}(S,i) - \mathrm{P}_{miss}(S,i) \mid i = 1,\ldots,N\}$$

A GSEA overview illustrating the method.



Subramanian A et al. PNAS 2005;102:15545-15550

Significance of Enrichment Score

- Randomize the labels among the samples, compute the enrichment score based on this randomized sample.
- Do it 1000 times.
- Use the positive or negative portion of the null enrichment scores to get the p-value, depending on the sign of ES(S).

Multiple hypothesis testing

To adjust for the size of gene sets.

Given S and 1000 permutation, compute $E(S, \pi_k)$, for k=1,..., 1000.

If $E(S, \pi)$ is positive (negative), divide it by the mean of the positive (negative) $E(S, \pi_k)$. Denote it by $NES(S, \pi)$

Divide by the same number to get $NES(S)$.

False Discovery Rate

Given S_*, denote $NES(S_*)$ by NES^*.

Suppose it is positive.

$$\frac{\dfrac{\#\{(S,\pi) \mid NES(S,\pi) > NES^*\}}{\#\{(S,\pi) \mid NES(S,\pi) > 0\}}}{\dfrac{\#\{S \mid NES(S) > NES^*\}}{\#\{S \mid NES(S) > 0\}}}$$

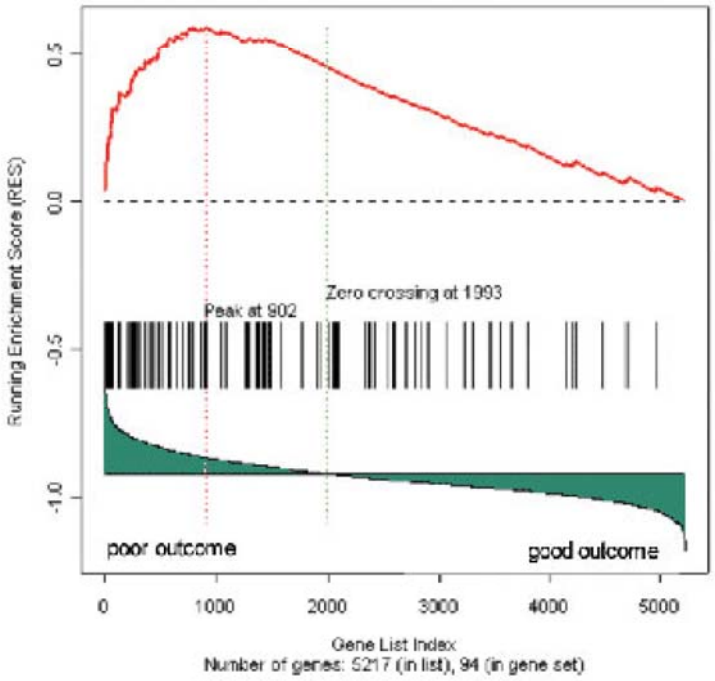
Negative case treated similarly.

Lung ADC Studies

- Given the gene set BS and consider the entire ranked gene list from Michigan array data. BS has NES=1.90, p-value <0.001.
- Conversely, the gene set MS has NES =2.13, p-value<0.001.
- For GSEA using C2, at FDR 0.25, there are good overlaps.
- Greater biological insight can be obtained at lower threshold.

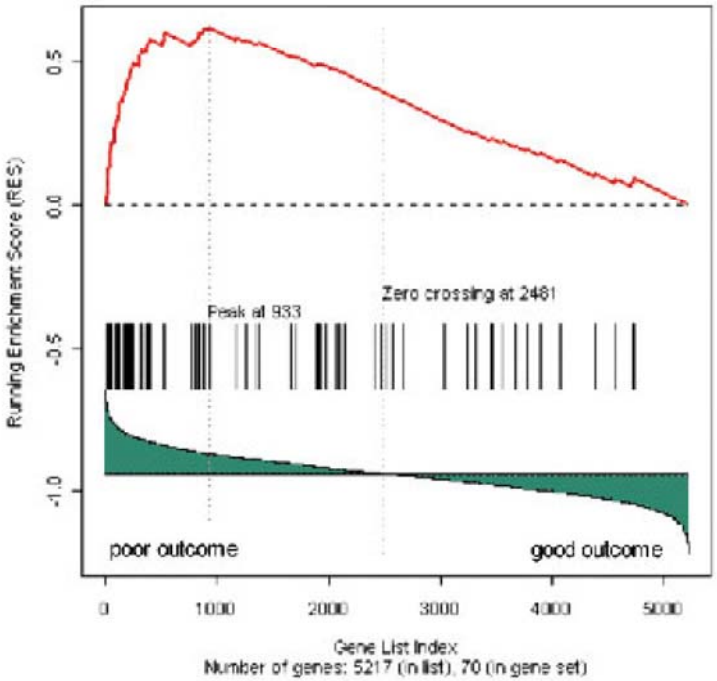
Boston Dataset

Gene Set: $S_{Michigan}$



Michigan Dataset

Gene Set: S_{Boston}



Molecular Signatures Database

- C1: Positional gene sets for each human chromosome and each cytogenetic band (326 gene sets)
- C2: Curated gene sets collected from various sources such as online pathway databases, publications in PubMed, and knowledge of domain experts. 3272 gene sets in total; CGP, 2392; CP, 880. BIOCARTA, 217; KEGG,186; REACTOME, 430 .
- C3: Motif gene sets, 836. MicroRNA targets, 221; TFT, 615.
- C4: Computational gene sets, 881.
- C5: GO gene set, 1454. Biological process, 825; Celleur components,233; Molecular function, 396 gene sets.

MSigDB
Molecular Signatures Database

Molecular Signatures Database v4.0

Overview

The Molecular Signatures Database (MSigDB) is a collection of annotated gene sets for use with GSEA software. From this web site, you can

- **Search** for gene sets by keyword.
- **Browse** gene sets by name or collection.
- **Examine** a gene set and its annotations. See, for example, the ANGIOGENESIS gene set page.
- **Download** gene sets.
- **Investigate** gene sets:
  - **Compute overlaps** between your gene set and gene sets in MSigDB.
  - **Categorize** members of a gene set by gene families.
  - **View the expression profile** of a gene set in any of the three provided public expression compendia.

Registration

Please register to download the GSEA software and view the MSigDB gene sets. After registering, you can log in at any time

Collections

The MSigDB gene sets are divided into 7 major collections:

c1 **positional gene sets** for each human chromosome and cytogenetic band.

c2 **curated gene sets** from online pathway databases, publications in PubMed, and knowledge of domain experts.

c3 **motif gene sets** based on conserved cis-regulatory motifs from a comparative analysis of the human, mouse, rat, and dog genomes.

c4 **computational gene sets** defined by mining large collections of cancer-oriented microarray data.

c5 **GO gene sets** consist of genes annotated by the same GO terms.

c6 **oncogenic signatures** defined directly from microarray gene expression data from cancer gene perturbations.

c7 **immunologic signatures** defined directly from microarray gene expression data from immunologic studies.

Citing the MSigDB

Data set: Lung cancer outcome, Boston study	FDR
Enriched in poor outcome	
Hypoxia and p53 in the cardiovascular system	0.050
Aminoacyl tRNA biosynthesis	0.144
Insulin upregulated genes	0.118
tRNA synthetases	0.157
Leucine deprivation down-regulated genes	0.144
Telomerase up-regulated genes	0.128
Glutamine deprivation down-regulated genes	0.146
Cell cycle checkpoint	0.216

Data set: Lung cancer outcome, Michigan study	FDR
Enriched in poor outcome	
Glycolysis gluconeogenesis	0.006
vegf pathway	0.028
Insulin up-regulated genes	0.147
Insulin signalling	0.170
Telomerase up-regulated genes	0.188
Glutamate metabolism	0.200
Ceramide pathway	0.204
p53 signalling	0.179
tRNA synthetases	0.225
Breast cancer estrogen signalling	0.250
Aminoacyl tRNA biosynthesis	0.229

- This analysis shows **much greater consistency** across the three lung data sets by using GSEA than by **single-gene analysis**. Moreover, it helps to generate compelling hypotheses for further exploration. In particular, 40 of the 60 top scoring gene sets across these three studies give a consistent picture of underlying biological processes in poor outcome cases.

- Striking evidence in all three studies of the effects of rapid cell proliferation, including sets related to **Ras activation** and the **cell cycle** as well as responses to hypoxia including **angiogenesis**, **glycolysis**, and **carbohydrate metabolism**. More than one-third of the gene sets (23 of 60) are related to such processes. These responses have been observed in *malignant tumor microenvironments where enhanced proliferation of tumor cells leads to low oxygen and glucose levels*.The **leading-edge subsets** of the associated significant gene sets include hypoxia-response genes such as HIF1A, VEGF, CRK, PXN, EIF2B1, EIF2B2, EIF2S2, FADD, NFKB1, RELA, GADD45A, and also Ras/MAPK activation genes (HRAS, RAF1, and MAP2K1).

- Strong evidence for the simultaneous presence of increased amino acid biosynthesis, ***mTor* signaling**, and up-regulation of a set of genes down-regulated by both amino acid deprivation and rapamycin treatment. Supporting this finding are 17 gene sets associated with amino acid and nucleotide metabolism, immune modulation, and *mTor* signaling. Based on these results, one might speculate **that rapamycin treatment might have an effect on this specific component of the poor outcome signal**. There is evidence of the efficacy of rapamycin in inhibiting growth and metastatic progression of non-small cell lung cancer in mice and human cell lines.

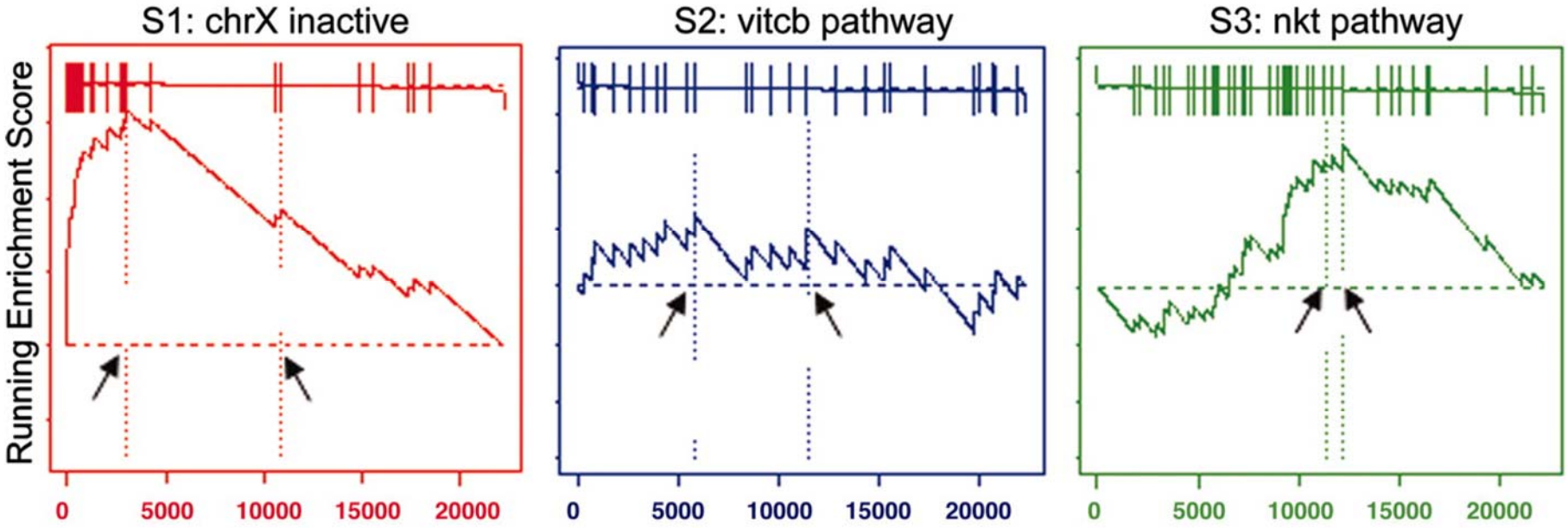
Choosing the value of p

Male vs Female Lymphoblastoid Cells

- 15 males and 17 females to identify gene sets correlated with the distnction “M>F” and “F>M”.

Gene set	**Original method nominal P value**	**New method nominal P value**
S1: chrX inactive	0.007	<0.001
S2: vitcb pathway	0.51	0.38
S3: nkt pathway	0.023	0.54

Original (4) enrichment score behavior.



Subramanian A et al. PNAS 2005;102:15545-15550

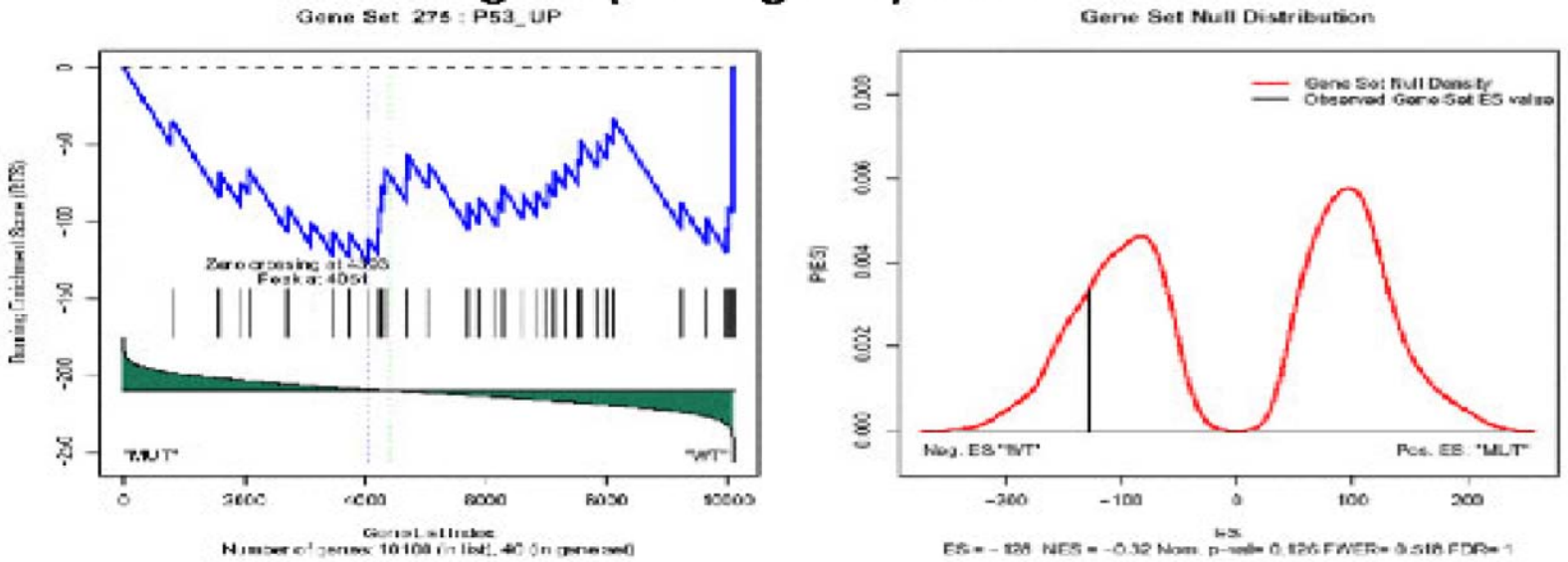
Data set: Lymphoblast cell lines	FDR
Enriched in males	
chrY	<0.001
chrYp11	<0.001
chrYq11	<0.001
Testis expressed genes	0.012
Enriched in females	
X inactivation genes	<0.001
Female reproductive tissue expressed genes	0.045

p53 status in cancer cell lines

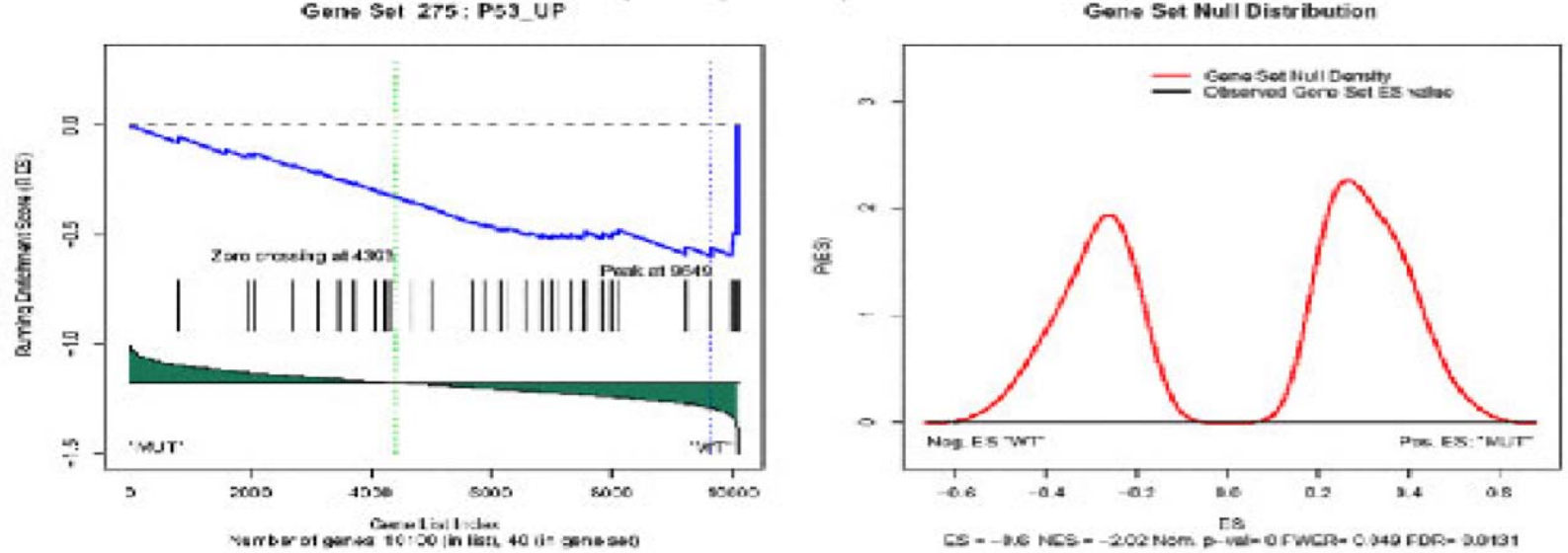
- 17 are normal and 33 carry mutations in p53, among NCI-60 cell lines.

p53 Upregulated Genes in p53 Wild Type Phenotype

Original (unweighted) GSEA Method



Current (weighted) GSEA Method

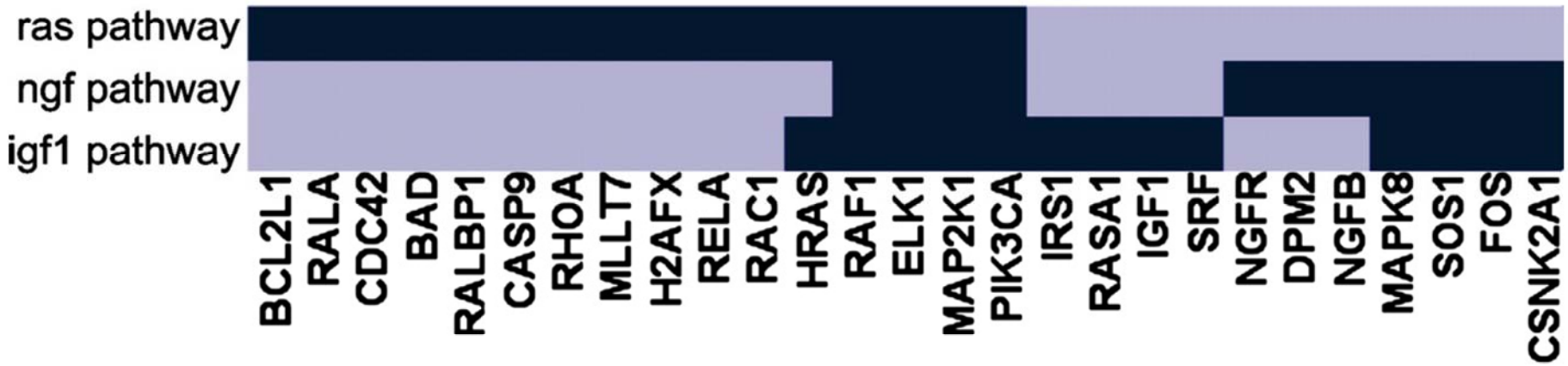


Data set: p53 status in NCI-60 cell lines	**FDR**
Enriched in p53 mutant	
Ras signaling pathway	0.171
Enriched in p53 wild type	
Hypoxia and p53 in the cardiovascular system	<0.001
Stress induction of HSP regulation	<0.001
p53 signaling pathway	<0.001
p53 up-regulated genes	0.013
Radiation sensitivity genes	0.078

Enriched in P-53 mutant

- Core genes.
- Only Ras signaling pathway is significant at FDR .25.
- The next significant ones are Ngf and Igf1 signaling pathways.
- They share core genes, component of MAPK.

Leading edge overlap for p53 study.



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Many pathways:
Leading edge analysis

Remarks

- Kolmogorov-Smirnov type statistic. The value of p. p=1 is default. Larger p penalize genes having small correlation with the phenotype.
- Many pathways, leading edge analysis.
- The permutation method based on label shuffling is computational demanding but more biologically relevant and may produce larger FDR than other simple method. Tamayo et al. (2012)
- Over-representative methods (IPA). Aggregation methods. Third generation methods. IPA.

More systematic approaches

- Genomic data from experiments and exogenous functional information from projects like GO and KEGG.
- Measurements are at the gene level; inference is at the functional category level.
- Leading analysis implies that these pathways are related.
- Quantitative responses.
- Both expression data and SNP data.

- Efron and Tibshirani (2007) ON TESTING THE SIGNIFICANCE OF SETS OF GENES, Annals of Applied Statistics

- Newton, He, Kendziorski (2012) A model-based analysis to infer the functional content of a gene list. Stat Appl Genet Mol Biol.

Thank you