Identification of genetic modifiers and its study in modulation of disease severity in cystic fibrosis patients

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Abstract: Cystic Fibrosis (CF) is a common, phenotypically variable disease caused by mutations in the CFTR gene, which encodes a cAMP-dependent chloride channel found in many cell types. Symptoms are primarily gastrointestinal and pulmonary in nature; with the pulmonary disease accounting for the majority of the mortality in CF. There are reports dedicated toward the characterization of the genetic contribution to the disease variance, with many modifiers gene for Cystic Fibrosis. In this study we have undertaken a novel approach to the identification of cystic fibrosis modifier genes by establishing the Gene Modifier Study (GMS), which utilizes a candidate gene approach to compare the genetic profile of the most severe CF patients to that of the mildest CF patients. We present probable causes for CF due to modifiers prominently variants within HLA-DQA1 and HLA-DQA2 which are associated with CF patient with long-term disease severity and survival.

1. INTRODUCTION

Cystic Fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population, affecting nearly 1 in 2500 newborns. Cystic Fibrosis is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a cAMP regulated Cl⁻ channel[1, 2, 3] expressed in the apical (luminal) membrane of epithelia. The cystic fibrosis gene Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) encodes for a protein of 1480 amino acids CFTR is also known as ABCC7 and belongs to a large family of ATPbinding cassette (ABC) transporters [1, 4]. The proteins consists of two transmembrane domains (TM's), each spanning the membrane six times, two nucleotide binding domains (NBD's) and a highly charged cytoplasmic domain (theR domain) containing consensus sequences for phosphorylation.

The protein CFTR functions as a channel across the membrane of cells that produce mucus, sweat, saliva, tears, and digestive enzymes [5]. Under normal conditions the CFTR protein functions as a chloride channel pumping Cl⁻ ions out of cell. CFTR also functions as regulator of other channels. The transport of chloride ions helps control the movement of water in tissues, which is necessary for the production of thin, freely flowing mucus. The CFTR protein also regulates the transport of sodium ion across cell membranes. These channels are necessary for the normal function of organs such as the lungs and pancreas[6].

Mutations which result in a loss of channel activity in CFTR can be classified into four major categories, class I-IV. \blacktriangle F508 is a class II mutation found in more than 90% of all CF alleles. A single amino acid deletion of a phenylalanine at position 508 (\blacktriangle F508) causes a defect in CFTR maturation. This results in an incorrect targeting to the apical membrane; the mutant protein is retained and degraded in the endoplasmic reticulum(ER) by the quality control mechanism[7].

Variability in pulmonary disease progression in CF cannot be explained by the CFTR defect alone. The CFTR defect in association with gene defects in other genes involved in modulating the severity of the inflammatory response and response to infection may contribute to more severe and rapid phenotypic progression of pulmonary disease in CF.

Although the gene defective in CF patients has been identified, CF disease pathogenesis is still a not well understood process. In order to explain the CF pathogenesis, a detailed understanding of the CFTR protein is essential.

For this, we have studied the regulation of CFTR, its physiological functions, and the effects of the \blacktriangle F508 CFTR mutation. Based on the functions of CFTR and the specific

properties of CFTR mutants, strategies towards a therapy for CF can be developed. With an intent to better treatment to CF patients, present study focusses on the understanding the upregulating and downregulating genes during the CF disease progression. The study addresses whether there are genes other than CFTR gene responsible for variability in frequency of pulmonary infections requiring intravenous therapy seen in CF patients and susceptibility to recurrent pulmonary infection.

2. MATERIALS AND METHODOLOGY

2.1. Gene datasets

The Gene Expression Omnibus (GEO) is a public repository that archives and freely distributes microarray, nextgeneration sequencing, and other forms of high- throughput functional genomic data submitted by the scientific community. Two gene datasets were obtained from GEO viz. GDS2142 and GDS2143. This database stores curated gene expression Datasets, as well as original series and platform records in the Gene Expression Omnibus (GEO) repository. Dataset records contain additional resources including cluster tools and differential expression queries.

2.2. DNA microarray

DNA microarray known as gene chips, bio- chips or siliconchips, are solid support usually made up of glass or silicon, holding DNA that represent thousands of genes that act as probes for mRNA.

2.3. Analyzing the datasets

R is a free software environment for statistical computing and graphics. It comprises and runs on a wide variety of UNIX platforms, Windows and MacOS. R provides a wide variety of statistical and graphical techniques, including linear and nonlinear modelling, classical statistical tests, time-series analysis, classification, clustering, and others. It is an environment within which statistical techniques were implemented. Bioconductor is a free, open source and open development software project for the analysis and comprehension of genomic data generated by wet lab experiments in molecular biology. Bioconductor is based primarily on the statistical R programming.

MATLAB is high level language and interactive environment for numerical computation visualization, and programming. Using MATLAB, we can analyze data, develop algorithms, and create models and applications. Normalization was done for resolving the systematic errors and bias introduced by the microarray experimental platform. Normalization is useful for a number of situations including within-slide comparison, multiple-slide comparison, and paired-slide comparison for dye exchange experiments. Normalization removes unwanted systematic variability in hybridization and to adjust the spatial effects from microarray data by adjusting the intensities of hybridization to balance them appropriately to make decisive analysis. It identifies the differences introduced in labelling or detection efficiencies when different fluorescent dyes are used. It also adjusts to bias introduced because of scanner settings. The microarray data generated by the feature extraction software is typically in the form of one or more text files.

Multi Experiment Viewer (MeV) is an application that allows the user to view processed microarray slide representations and identifies genes and expression patterns of interest. This Crude Analysis was used to perform the back validation of genes which were obtained by using SAM test and T-test. In this, the selection of upregulated and down regulated genes was done using MS Excel.

3. RESULTS

3.1. Box plot analysis

Box plot analysis is method for visualizing the distribution of the data values throughout the dataset providing information about the spread and skewness in the dataset. Fig. 3.1 and Fig. 3.2 represent the box plot analysis of the gene datasets GDS2142 and GDS2143 respectively as before and after normalization.



Fig. 3.1: Boxplot of Dataset GDS2142 (GSE2395) before normalization



normalization



Fig. 3.3: Boxplot of Dataset GDS2143 (GSE2395) before normalization



Fig. 3.4: Boxplot of Dataset GDS2143 (GSE2395) after normalization

3.2. Histogram analysis

A histogram is a representation of a statistical graph of a frequency distribution in which vertical rectangles of different heights are proportionate to corresponding frequencies. Fig. 3.3 and Fig. 3.4 represent the histogram curve of the datasets each before and after normalization. The microarray data shows histogram as frequency on y-axis and log transform of pixel intensities along the x-axis.



Fig. 3.5: Histogram of GDS2142 (GSE2395) before normalization



Fig. 3.6: Histogram of GDS2142 (GSE2395) after normalization



Fig. 3.7: Histogram of GDS2143 (GSE2395) before normalization



Fig. 3.8: Histogram of GDS2143 (GSE2395) after normalization

3.3. Visualizing the chip data image

The normalized data is viewed in MeV. The respective chip used and organism are selected for all the datasets for automatic annotation loading. When the data is loaded we can visualize all the samples at the top of the image display. Each row of the image is a single gene represented by a Probe ID towards the right. Each column represents a sample. When either of the boxes is clicked, the information about the intensity value of that gene in the respective sample along with its gene name, symbol, and Probe IDare displayed. Fig. 3.5 shows the level of gene expression across all the samples of the data set experiment in normal and diseased state.



Fig. 3.9: Data image of GDS2143 (GSE2395)

3.4. Sam Results

When SAM test is performed on the normalized data, we obtained a set of positively significant genes and negatively significant genes. The genes were listed along with their respective fold change. Fig. 3.6 represents the positive and negative significant genes inferred by SAM test.



Fig. 3.10: SAM graph showing upregulated genes in red of HG-U133-A chip: GSE39843

3.5. T-test analysis

On performing T-test, we obtained a set of differentially expressed genes characterized by specific p-value for each gene as is observed in Fig. 3.8 and Fig. 3.9. If the p-values are low, the gene is highly significant and vice versa. Volcano plot was also used to visualize the genes differentiated as positively or negatively regulated by considering the fold change as well.



Fig. 3.11: Cluster information of HG-U133-A chip: GSE39843

ustering	Solistics		Classifi	cation	•	Neta Reductio	n	- Nota	Analysis	•	Susication		•	Miscellaneous	-
Sample Table View	A Stored Col	or Expression	-	GENE_TITL	E TX_END	CHR_LOC.	STRAND	TX_START	GO_TERMS	GENE_SY.				Z_ID UNGENE_	GENBANK.
Gene Table View			201909_at		2734995	chrif 27096.	+	2709622	G0:000641.		NH_00100	201909_at	6192		NM_00100E
Cluster Manager	1			eukaryotic t.		drff:22737.	*	22737610	G0:000641.		NII_00458			Hs.451178	BC005248
Sample Clusters				X (inactive)	73072588	chr(c73040.		73040494	NA)0ST	NR_001564		7503	Hs.529901	AA167449
		_		DEAD (Asp.	15032390	chr/.15016.	+	15016018	GO:000588.		NH_00112.		8653	Hs.99120	NM_004660
Gene Clusters	H	-		X (nactive)		chr(c73040.	-	73040494	NA	XIST	NR_001564		7503	Hs.529901	AV646597
Analysis Results		-	201859_at		70864565	chr10/7084.	*	70847827	GO:000591.		NM_00272		5552	Hs.1908	NM_002727
Data Source Selection		~	209683_at		16847134	chr2:16730. chr1:15295		16730731	GD:0005622 GD:000854		NM_03079		81553	Hs.467769 Hs.46320	AA243659 Al923984
Data Filter - Variance Filte		-		small proli-	152958289	drr1.15295. drr10.5154.	*	152906056 51549552	GD:000854.		NH_00598 NH 00244			HS.46320 HS.255462	A923984 NM 002443
				solute carri	230933619	chr10/5/154. chr2/23089.	•	230899697	GD:000815.		NW 15252			Hs.200402 Hs.504317	R15072
 Expression Image 				microsemi		chr10.5154		230899097	GO 000815		NU 00244.			Hs 255462	U22178
 Gana/Row Count 273. 		\rightarrow		eukanotic t.		dir 10.5 154.		22737610	G0.000641		NH 00458.			Hs.461178	NM 004681
 Parameters 		-		NLR family	55512503	dir19:5547.	-	55477770	GD:000591		NM_01785.			Hs.369279	AF298547
SAM (1)		\rightarrow	236694 at		21752308	drff 21729		21729234	NA NA	CYof15A	NH 00100			Hs 522863	A/1468885
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- Delta table			201858_s		70864565	dr107084	+	73847827	GD 000691		NH_00272_			Hs 1908	103223
				gap junctio		dr132079		20796101	GO:000691		NH 00111			Hp.511757	4694073
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Centroid Graphs	4			glutathione.	24384284	chr22:2437		24376140	GO 000573	GSTT1	NH_00085			Hs 268573	NM 000853
Expression Graphs				hyakronan.		chr16:6914		69140159	GO-000597	HAS3	NH 00532			Hs.592069	AF232772
- Table Views			206700 s	lysine (K)-s.	21906825	chrif:21857.		21857302	G0:005511	KDM5D	NH_00114.	205700 s	8284	Hs.80358	NM 004653
Cluster Information			203924_at	NA	-1	chrNA-1-1	NA	-1	NA	NA	NA	203924_at	NA	NA	NM_000846
				alpha-2-ply.		chr7.99564.		99564351	GO:000715.		NIL_00118	209309_M	563	Hs.546239	D90427
– 🕄 Results (#,%)				P1MA-like 1	46974820	chr19:4696.		45959748	NA	PNMAL1	NH_00110		55228	Hs.8395	NM_018215
- 🕒 General Information			230760_at		2850546	chrf:28031.	+	2903111	G0:004544.		NIL_00114		7544		EF592062
T Tests (2)			40665_M	flavin conta.		dhr1:17106.	+		GO:005511.		NIL_00100		2328	Hs.445350	M83772
Expression Images		~	235892_at		-1	chrNA-1-1	NA	-1	NA	NA	NA		NA	NA	AI620881
Centroid Graphs			221748_s_		218808796	chr2:21866.			GO:000704.		NII_02254		7145		AL046979
			205001_8_	DEAD (Asp.	15032390	chrr 15016.	+	15016018	GO 000588		NII_00112			Hs.99120	AF000985
			232618_at		21752308	chrf.21729.	*	21729234	NA	CYorf15A	NH_00100		245126	Hs 522863 Hs 528305	AF332224
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- El Significant Genes	HI	- >-		protease, s.		chr118651.	+	85511490 1253286	GD 000650		NH_00717			Hs.25338	NM_007173
Non-significant G		+	1565271_a. (213920_at	telomerase.	1290162	drif5:12532 drif2:1114	-	1253286	G0:000072		NU_19825			Hs.492203 Hs.124953	AB085628 AB006631
- Cluster Information		\mathbf{H}	213620_at 213680_at		52845910	dr121114. dr125284		52840435	GD 004589		NM_01526 NM_00555		23316	HS.124953 Hs.708950	AB006631 A831452
		\rightarrow		arachidona.		chr12:5284. chr10:4585	1	45859528	G0:000739		NM_00059.			Hs. 708950 Hs. 89499	M831452 NM_00069E
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3.12: Significant genes from T-TEST of HG-U133-A chip: GSE39843

Oustering -	Statistics		Cassifi	cation	•	Dura Reductio		-	ratis		A	,	•	liscellaneous	
	Stored Cold	r Fungesion		GENE TITLE		CHR_LOC.	STRAND	TX_START	GO_TERMS	CENE SY	REFSEQ A	PROBE_ID		UNIGENE_	GENBANK
	N COLLEGE COLL	- Concostore	218990 s	small proli	152976331	dr1:15297		152974222	G0:000854		NM 00109	218990 s	6707	Hs.139322	NW 00541
Gene Table View			205207 at	interleukin	22771619	drr7.22766		22766765	GO:000178		NM 00060	205207 at	3569		NM 00050
Cluster Manager		-		NA	-1	ch/NA-1-1		-1	NA	NA	NA	202409_M	NA	NA	X07868
Sample Clusters		<u> </u>	211122 s	chemokine.	76957233	chr4:76954		76954841	GO:000716	CXCL11	NM 00540.	211122 8	6373	Hs 632592	AF002985
Gene Clusters		- N	210163 at	chemokine	76957233	chr4:76954		76954841	GO:000716				6373	Hs 632592	AF030514
Analysis Results		~	208025_8_	high mobili	66309299	dr126621.	•	66218239	GO:000632	HMGA2	NM_01348	208025_5_	8091	Hs 505924	NM_00348
			209395_at	chilinase 3	203155822	chr1:20314.	-	203148058	GO:000597	CHI3L1	NM_00127	209395_at	1116	Hs 382202	M80927
Data Source Selection		\sim	209396_8_	chitinase 3.	203155922	chr1:20314.		203148058	GO 000597.	CHI3L1	NU_00127_	209396_5_	1116	Hs 382202	M80927
Data Filter - Variance Filte		M	214974_X_8	t chemokine	74864416	chr4:74861.		74861359	GO:000716.		NM_00299.	214974 x a	8 6374	Hs.89714	AK026546
- Expression Image		M	205403_at	interleukin	102644880	chr2:10250.	+	102608305			NM_00463	205403_at	7850	Hs.25333	NV_00463
GeneRow Count 2733		M.	218468_s_		33026866	dr:15:3301.	+	33010204	GO:000165.		NM_01337	218468_s_	26585	Hs.40098	AF154054
► ☐Parameters		M.			76944650	chr4:76942.		76942272	GO:000693.		NM_00156	204533_at	3627		NM_00156
		K.	206157_at	pentraxin-r	157161416		+	157154579	GO:000187		NM_00285		5806	Hs.591286	NN_00285
- 🔳 SAM (1)		M.	207850_at	chemokine	74904490	chr4:74902.		74902313	GO:000693	CXCL3	NM_00209	207850_at	2921	Hs.89590	NM_00209
- 🛃 SAM Graph		FA.		hyaluronan	122653630	chr8:12262.		122625270		HAS2	NM_00532	205432_M	3037	Hs.159226	NW_00532
- Deita table		n		fatly acid bi	82395473	chr8:82390.		82390731	GO:000181.		NM_00144	203980_M	2167	Hs.391561	NM_00144
Expression Images	1	M.	202238_\$	nicotinami	114183238	dr11.1141.		114166534		NNMT	NM_00616	202238_6_	. 4837		NN_00616
Centroid Graphs		M-	218469_at	gremin 1,	33026866	dhr15:3301.		33010204	GO:000165.	GREM1	NM_01337_	218469_at	26585	Hs.40098	NM_01337
	I	~		thrombosp.	39889667	dhr15/3987	+	39873279	GO:000018		NM_00324	201109_s_	7057	Hs.164226	AI/726673
 Expression Graphs 	I	The		thrombosp.	39889667	dhr15:3987.	+	39873279	GO:000018.	THBS1		201110_s_	7057	Hs.164226	NW_00324
► Table Views			228057_at	DNA-dama.	101111613	chr4:10110.		101107028	GO:000996.		NM_14524	228057_at	115265	Hs.480378	AA528140
Cluster information	<u> </u>	~~	215101_8_	chemokine.	74854416	chr4:74861.	-	74861359	GO:000716.	CKCL5		215101_s_	. 6374	Hs.89714	BG165705
Results (#,%)		-			114183238	dr11.1141. dr/2.15221.	•	114166534 152214104	GO:000573 GO:000715		NM_00516	202237_at	4837		NM_00616
General Information		\sim		tumor neor dvcerol-3	152236559	dh/2 96687	•	152214104	GO1000715. GO1000815		NM_00711 NM_20732	206026_s_	150763	Hs.437322 Hs.348529	NM_00711 AN082827
		8			42234436	dr/2.9068/	.	42212529			NM_20732.	201884 at	1048	Hs.709196	NN 00436
T Tests (2)		-	201064_at	dusterin	*223**30	dir18.4221. dir8.27454.	ſ	27454450	GO 000183		NM 00117_	201684_at	1191	Hs.436657	M25915
🗠 📱 Expression Images		->-			76076799	dv1:75672	£	75672075	GO:001602		NM 00113	235763 at	204962	Hs.654821	AA001450
Centroid Graphs		MA -	207173 x a		65155919	dy16.6498	f	64980684	GO 000150		NM_00179	207173 1 2		Hs.116471	D21254
Expression Graphs		Ex-		S-nudeotid.	86205496	chr6:86159	<u>. </u>	86159301	GO:000616		NM 01252	203939 at	4917	Hs.153952	NW 00252
Table Views		2		radical S-a	7038363	dr/270177.		7017795	GO:003027		NM 08065		91543	Hs.17518	AW189843
		1		radical S-a	7038363	dr/2.70177		7017795	GO 003027			213797 at	91543	Hs.17518	AI337069
Significant Genes			229290 at	death asso.	159672494			159651828		DAPL1	NM_00101_	229290 at	92196	Hs.59761	AI692575
III Non-significant Ge		X		tumor necr.	152236559	dh/2.15221.		152214104	GO:000715		NM 00711_	206025 s	7130	Hs 437322	AA/188198
 El Cluster Information 		-	205863 at	S100 calci	153348075	dv1:15334		153346183		\$100A12	NM 00562		6283	Hs.19413	NM 00562
Results (#.%)			230372 at	hyaluronan		chr8:12262.		122625270		HAS2	NM 00532	230372 at	3037	Hs 159226	A(374739

Fig. 3.13: Non-significant genes from T-TEST of HG-U133-A chip: GSE39843

3.6. Back validation analysis

This analysis was used for selection of upregulated and Downregulated genes using Ms.Excel. We segregated the samples of Schizophrenia and Cystic Fibrosis from datasets GSE12649 and GSE12654 and performed their back validation separately. The results were then compared to other datasets in order to identify upregulated and down regulated genes.

Table 1: Downregulated genes associated with cystic fibrosis

Gene	Probe ID	Fold
symbol		change
HLA-DQA1	212671_s_at	0.4
HLA-DQB1	212998_x_at	0.4
HLA-DQA2	212671_s_at	0.4
IRF1	238725_at	0.5
STAT1	AFFXHUMISGF3A/M	0.5
	97935_3_at	
FCGBP	203240_at	0.4

4. DISCUSSIONS

In cystic fibrosis, chronic bacterial infection (Pseudomonas aeruginosa) and an excessive inflammatory response is the primary cause of chronic respiratory infection which is responsible for most of the morbidity and mortality of these patients. Nitric oxide (NO) having antibacterial property acts in defense of lung epithelial cells by inhibiting the bacterial ribonucleotide reductase. This results into double stranded break in bacterial DNA thereby inhibiting proliferation of bacteria. Synthesis of NO is product of Interferon Regulatory Factor 1 (IRF1) mediated activation of NOS2 gene. Expression of the two genes is activated by Signal transducer for and activator of transcription 1 (STAT1). Study shows that STAT1 level were found to be increased in CF cells but the active phosphorylated form of STAT1 was bound to the protein inhibitor (PIAS1). In CF, the level of STAT1 inhibitor PIAS1(protein inhibitor of activated STAT1) is increased in CF patients which leads to interruption in normal STAT1 signaling. This results in reduction of IRF1 and NOS2 expression level in CF epithelial cell.

Major cause of death in patients with cystic fibrosis (CF) is colonization with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The wide phenotypic variation in CF patients suggests that genes other than the cystic fibrosis transmembrane conductance regulator (CFTR) gene modify the disease. Another set of downregulated genes HLA-DQA1, HLA-DQA2 and HLA-DQB1 belonging to the 8.1ancestral Major Histocompatibility Complex (MHC) haplotype modify the disease. The MHC Class II antigens are found on antigen presenting cells (APC). The APC flag the class II antigens to T-cells each with unique T-cell receptor (TCR) variants. A few TCR variants that recognize these DQ/antigen complexes are on CD4+ T-cells (helper T-cells) which can promote the amplification of B-cells. These active B-cells then recognize a different portion on the same antigen.

Downregulated Fc fragment of IgG binding protein (FCGBP) also contributes in modifying the CF disease. More than 90% CF deaths occur due to IgG antibodies inefficient in reacting with the *Pseudomonas* infection. Current studies suggest the severity in Cystic Fibrosis is apparently due to defective opsonophagocytosis. This may be caused either by cleavage of IgG and complement complex mediated by immune system or change from opsonic to monophonic antibody isotope.

Pseudomonas aeruginosa produce a protease Elastase, possessing lactase activity is a major virulence factor in Pseudomonas aeruginosa that is believed to cause extensive tissue damage during infection in the human host. Elastase is capable of degrading elastin, collagens, immunoglobulins, complement components, laminin and fibrin. In accordance with substrates it utilizes, elastase of P. aeruginosa is also able to inhibit several processes involved in defense mechanisms of hosts. Elastase stimulates the production of proinflammatory mediators such as IL-8, which further induces neutrophil influx. Elastase also impairs mucociliary clearance by direct effects on ciliary function and by stimulating increased mucus production. Elastase produced by Pseudomonas aeruginosa cleaves human IgG at hinge region into immune fragments, Fab and Fc that significantly inhibit opsonophagocytosis and uptake of immune complexes by phagocytes. Since macrophages use antibodies to ingest P. aeruginosa, opsonophagocytosis is reduced in the presence of excess Elastase. It has been shown that the Pseudomonas uptake and killing were improved in absence of cleavage fragments.

The experimental evidence for the hypothesis is reported [8, 9, 10]. This proves the significance of the study.

5. ACKNOWLEDGEMENT

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