

Mucin Family Genes are Downregulated in Colorectal Cancer Patients

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Abstract

Mucins are very well known to be associated with different types of cancer. Their role in colorectal cancer has been extensively studied without direct correlation with their change in expression levels. In the present study we employed the human exon array from Affymetrix to provide evidence that mucin family genes are downregulated in colorectal cancer tumor samples. We analyzed 92 samples taken from normal and tumor tissues. All mucin family genes except MUC1 were downregulated with the fold change value ranging from -3.53 to 1.78 as calculated using AltAnalyze software. Maximum drop in RNA transcripts were observed for MUC2 with a fold change of -3.53. Further, we carried out Integromics analysis to analyze mucin genes using hierarchical clustering. MUC1 and MUC4 were found to be potential biomarkers for human colorectal cancer. Top upstream regulators were identified for mucin genes. Network analyses were carried out to further our understanding about potential mechanisms by which mucins can be involved in causing colorectal cancer.

Keywords: Microarray; Colorectal cancer; Mucins

Introduction

The differential expression of mucins has been related to altered risk of metastasis and death. Recently, mucins have been used as targets for molecular therapy and as a source of immune therapy [1]. The mucin family includes proteins that contain tandem repeat structures with a high proportion of prolines, threonines and serines. The human mucin (MUC) family consists of members-designated MUC1 to MUC21-that have been sub-classified into secreted and transmembrane forms. The secreted mucins (for example, MUC2, MUC5AC, MUC5B and MUC6) form a physical barrier, which as a mucous gel provides protection for epithelial cells that line the respiratory and gastrointestinal tracts and form the ductal surfaces of organs such as the liver, breast, pancreas and kidney. The transmembrane mucins (for example, MUC1, MUC4, MUC13 and MUC16) have a single membrane-spanning region and contribute to the protective mucous gel through their ectodomains of O-glycosylated tandem repeats that form rod-like structures [2]. Mucins would play a crucial role in forming the defense structure of the gut and could thus be well associated with its diseases. Inflammatory bowel disease [3], crohn's disease [4,5] and colorectal cancer [1,6] are known to have the involvement of mucins via different mechanisms. As much as it is established that mucins play a crucial role in causing colorectal cancer, the mechanism by which they do so is very complex. There are expression level studies that link mucins with colorectal cancer, but there are conflicting reports regarding their differential expression with respect to normal tissue.

There is evidence of upregulation of some mucins in breast, pancreatic and ovarian cancers especially the transmembrane ones. But there are reports about downregulation of mucins in colorectal cancer [7].

The posttranslational modification of mucins through glycosylation could be crucial in determining their role in cancer. Hence, most of the studies were intended to study the functional aspects of mucins and their association with cancer. This has also led to the identification of target mucins for cancer therapy.

The need for fully understanding the expression pattern of the mucin family genes is strongly felt especially in colorectal cancer where the mucin lined gut is the site of action. The present amount of evidence is not sufficient to establish the expression pattern of mucin family of

genes in colorectal cancer at human level. In the present study, we attempt to fill this gap of knowledge by undertaking a comprehensive study focused at analyzing the mucins at transcript level. We studied the expression pattern of tumor tissues and compared it with the normal tissues derived from the same patients and found downregulation of all mucin family genes. This is the first report that comprehensively provides microarray based evidence for downregulation of mucins in colorectal cancer.

Materials and Methods

Sample collection

We collected 92 matched tumor-normal samples from 48 patients diagnosed with colorectal cancer. Each Hematoxylin and Eosin (H&E) stained case was reviewed by a board-certified pathologist to confirm the specimen histological consistency with colon adenocarcinoma and that normal specimen contained no tumor cells. The sections were required to contain >60% tumor cell nuclei for inclusion in the study. The study was granted the institutional review Board (IRB) approval after due review. The necessary procedural and ethical consent forms were signed by the patients prior to sample collection.

Sample processing and DNA/RNA extraction

Each specimen weighed between 10-30 mg. The tissue sample was stored in RNA later (Ambion) at 4°C for 24 hrs; followed by freezing and further storage at -20°C. DNA and RNA extraction was carried out from the same sample by NucleoSpin Trio Kit (Macherey-Nagel, Germany). Quality and quantity checks were done using Nanodrop (Thermo Fischer Scientific).

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Data generation using human exon arrays

For gene expression studies, Human Gene Chip Exon 1.0 ST arrays were obtained from Affymetrix. The recommended WT expression kit was obtained from Ambion. Recommended protocol for amplification, labeling and hybridization was followed. Arrays were scanned using 3000 7G scanner from Affymetrix. Data was exported using Expression Console in the form of .CEL files.

Gene expression analysis

'AltAnalyze' program (v2.8.0.1 -64bit) was used for exon level gene expression analysis. The exon expression intensities were summarized based on constitutive probe set expression to generate gene expression values. Finally, these values were utilized to calculate the differential expression of genes. Fold change values were calculated by creating a ratio using the signal intensities in tumor vs. normal samples.

Integromics analysis

Omics Office for biomarker discovery from TIBCO spotfire was used to carry out statistical analyses. Linear Models for Microarray Analysis (LIMMA) and Hierarchical clustering were carried out using Integromics.

Ingenuity pathway analysis

Data from AltAnalyze for expression levels of all mucin family genes was subjected to Ingenuity Pathway Analysis (IPA). Biomarker and Core analyses were carried out with a selection for 'Human' and 'colorectal cancer'.

Results

Mucin family genes are downregulated in colorectal cancer.

Definition	Symbol	Fold-Change Tumor vs. Normal	Adj p-value Tumor vs. Normal	ANOVA
mucin-like 1	MUCL1	1.177928928	0.324880848	0.33269532
mucin 15, cell surface associated	MUC15	-1.008115774	0.928082382	0.927222838
mucin 16, cell surface associated	MUC16	-1.021494911	0.810097704	0.808270374
mucin 6, oligomeric mucus/gel-forming	MUC6	-1.059016589	0.272918223	0.258507815
mucin 19, oligomeric	MUC19	-1.088384059	0.287600842	0.286939379
mucin 7, secreted	MUC7	-1.088559983	0.455603832	0.459317975
mucin 8	MUC8	-1.090471283	0.070469366	0.062986147
mucin 21, cell surface associated	MUC21	-1.09606114	0.168972271	0.090273566
mucin 22	MUC22	-1.097767715	0.079416992	0.090273566
mucin 1, cell surface associated	MUC1	-1.139269043	0.168972271	0.172474892
mucin 3A, cell surface associated	MUC3A	-1.161091911	0.259114609	0.266051645
mucin 20, cell surface associated	MUC20	-1.240464797	0.207167498	0.215117389
mucin 13, cell surface associated	MUC13	-1.248437908	0.631733467	0.638485312
mucin 5B, oligomeric mucus/gel-forming	MUC5B	-1.743234796	0.005887836	0.007046705
mucin 12, cell surface associated	MUC12	-1.890092833	0.000218802	0.000293135
mucin 4, cell surface associated	MUC4	-2.209820943	0.000460973	0.000609726
mucin 2, oligomeric mucus/gel-forming	MUC2	-3.531477506	0.000350047	0.000476223

Table 1: All mucins except MUCL1 are downregulated in colorectal cancer patient samples.

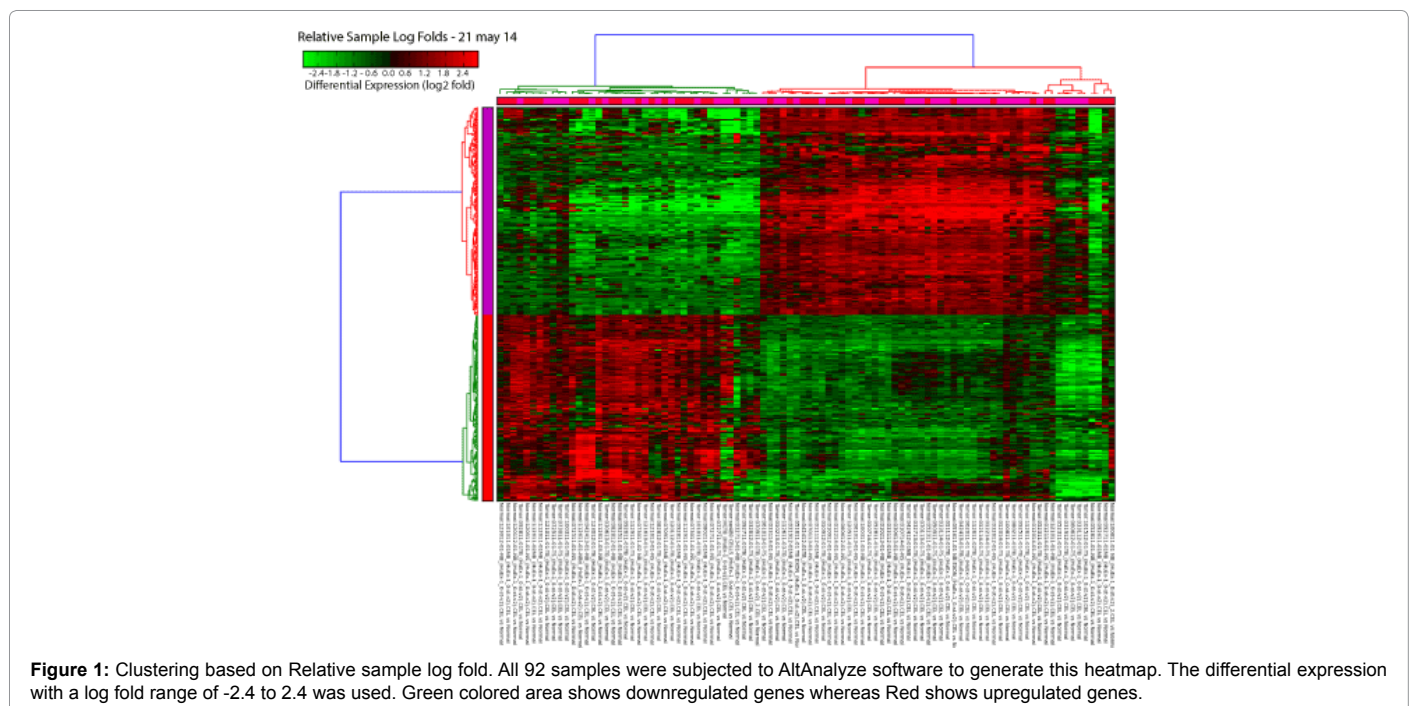


Figure 1: Clustering based on Relative sample log fold. All 92 samples were subjected to AltAnalyze software to generate this heatmap. The differential expression with a log fold range of -2.4 to 2.4 was used. Green colored area shows downregulated genes whereas Red shows upregulated genes.

Analysis using AltAnalyze

Analysis of 45,475 probe IDs available on exon array, made possible the composite analysis of mucin genes. 17 mucin genes were found in the database out of which 16 were downregulated. Only MUC1 was upregulated with a fold change value of 1.17 (Table 1). Differential expression heat map analysis shows two distinct groups from the 92 samples studied (Figure 1).

Analysis using Integromics

Only Nine mucin genes were mapped by Integromics software. Hierarchical clustering analysis for these nine mucin genes using complete Euclidean linkage shows MUC20, MUC4, MUC1 and MUC13 with maximum scores (Figure 2).

LIMMA analysis was carried out to find out the most significant differentially expressed genes. Of all the nine mucin genes, MUC1 and MUC4 were found to be statistically most significant in terms of differential expression (Figure 3).

Ingenuity pathway analysis

In order to comprehensively analyze the functional significance of the downregulated mucin genes, we carried out ingenuity pathway analysis. Biomarker analysis for colorectal cancer in humans yielded MUC2 and MUC4 to be the putative genes (Table 2). They were

found to be biomarkers for diagnosis, disease progression, efficacy and prognosis.

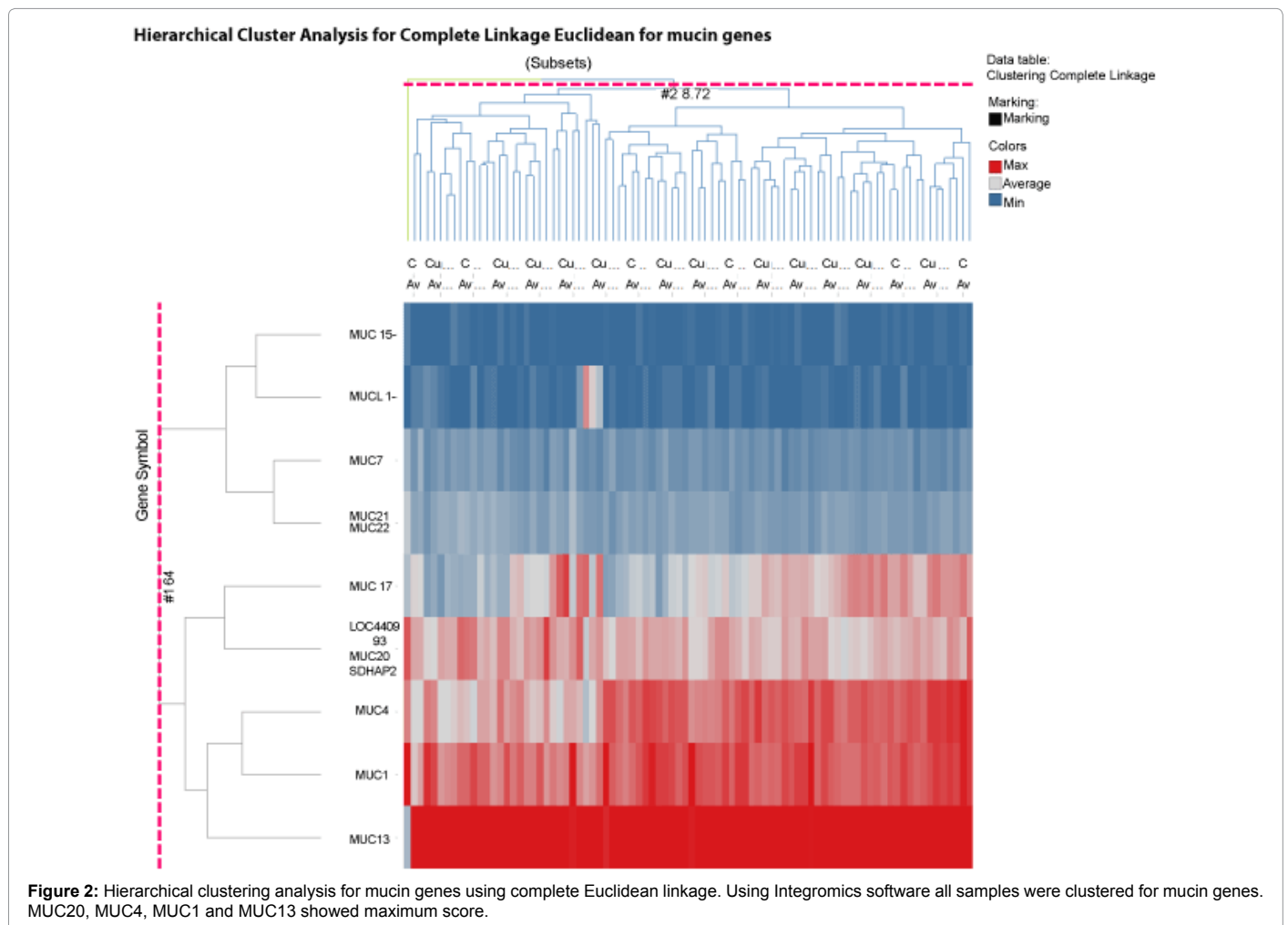
An upstream target analysis for mucin genes gave us a list of 35 genes, which could be affecting mucins through different pathways (Table 3). Top upstream regulators were CTCF, IL22, GAB2, IL1B and TSC2 genes.

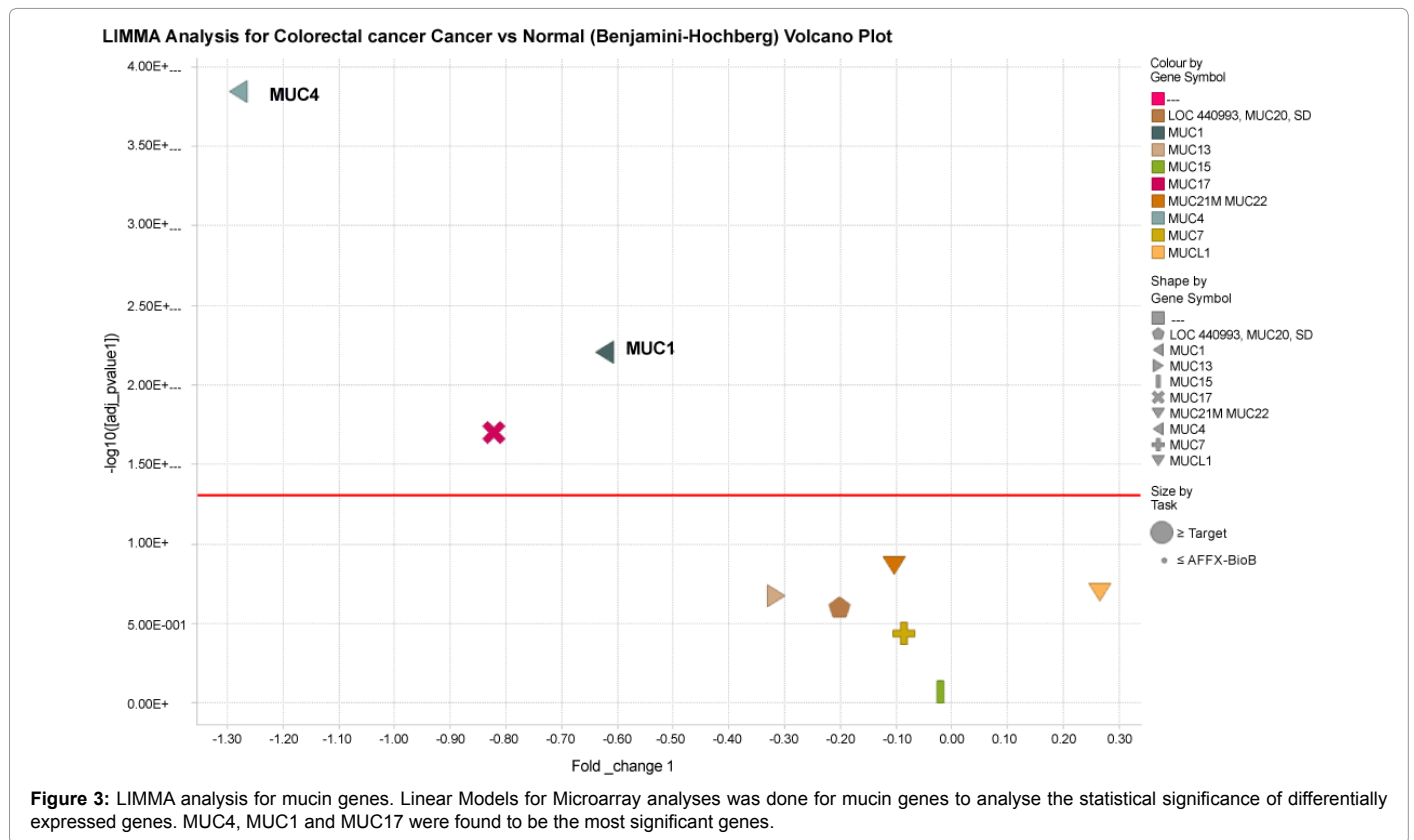
Seven mucin genes viz., MUC1, MUC2, MUC3A, MUC4, MUC5B, MUC6 and MUC8 were part of a network with a score of 17 (The score is a numerical value used to rank networks according to their degree of relevance to the network eligible molecules, which are mucin genes mentioned above). Genes from this network could be involved in cellular development, cellular growth and proliferation, cell death and survival (Figure 4).

A network generated out of the mucin genes and overlaid with different cancer related functions showed involvement of MUC1, MUC2 and MUC4. However, MUC 3A, MUC 5B, MUC12, MUC19, MUC21 and MUC1 were not associated with any known colorectal cancer related functions (Figure 5).

Discussion

Mucins are known to be involved in different types of cancer. There have been many reports about their role in causation or spread of cancer. However, there is no comprehensive attempt to measure the





Location	Plasma Membrane transcription regulator	Extracellular Space growth factor
Family	HuHMFG1	
Drug(s)		
Fold Change	-1.139	-2.210
p-value	4.40E-02	1.49E-05
Human	x	x
Blood	x	
BL Fluid	x	
Plasma/Serum	x	
Sputum	x	
Tears		x
Urine	x	
Memory B cells	x	
Naive B cells	x	
Epidermis	x	
Kidney	x	
Large Intestine		x
Lung	x	
Lymph node	x	
Mammary Gland	x	
Ovary	x	
Pancreas	x	
Retina		x
Skin	x	
Small Intestine	x	
Stomach	x	
Thyroid Gland		x
Uterus		x
COLO205	x	
HCC-2998	x	
HT29	x	
KM-12	x	
SW-620	x	

Table 2: Putative biomarker molecules among the family of mucin genes.

RNA expression level analysis for the entire mucin family genes in the same setting. In this report we have attempted to study the expression level of all mucin family genes in colorectal cancer patient samples. We employed the exon microarray to interrogate the expression levels in 92 samples. AltAnalyze and Integromics softwares produced consistent results providing a confident analysis of mucin family genes.

MUC1 has been implicated in colorectal and other cancers mainly by studies involving post transcriptional events. Some studies suggest the gene expression changes of its variants in cancer as well. MUC1 is being found to be an important molecule in the immunobiology of adenocarcinomas [8]. The role of MUC1 in cancer progression is still controversial and somewhat confusing. While Mukherjee and colleagues developed MUC1 specific immune therapy in a CRC model, Lillehoj and co-investigators showed recently that MUC1 inhibits cell proliferation by a β -catenin-dependent mechanism [9]. MUC1 has been suggested as a potential target for developing anticancer agents including vaccines, antibodies and small molecules [10,11]. Several lines of evidence point towards a biological role of MUC1 in colorectal cancer. A positive correlation was described between mucin secretion, proliferation, invasiveness, metastasis and bad prognosis [12-14]. In another report, mucinous CRC is associated with a higher expression of MUC2 and MUC5AC, but a lower expression of MUC1 [1]. Mucin 1 (Muc1) is a tumor-associated glycoprotein and has an important role in cancer progression and metastasis. Recent evidence suggests inhibition of Muc1 could significantly suppress the growth of HCC2998 in nude mice models [15]. Our results show MUC1 to be downregulated in tumor samples, which is consistent with some studies but not all.

Studies at protein level show overexpression of MUC2, MUC5B, MUCAC and MUC6 in colorectal cancer [16]. Immunohistochemical

Upstream Regulator	Molecule Type	p-value of overlap	Target molecules in dataset
CTCF	transcription regulator	1.43E-08	MUC2,MUC5B,MUC6
IL22	cytokine	4.90E-08	MUC1,MUC4,MUC5B
GAB2	other	1.77E-06	MUC2,MUC5B
IL1B	cytokine	2.06E-04	MUC1,MUC3A,MUC4
TSC2	other	7.93E-04	MUC2
FOXA1	transcription regulator	7.93E-04	MUC4
AGR2	other	1.59E-03	MUC1
DDIT4	other	1.59E-03	MUC2
CDX1	transcription regulator	1.59E-03	MUC4
SP1	transcription regulator	2.58E-03	MUC2,MUC5B
NFATC3	transcription regulator	3.17E-03	MUC2
RPS6KB1	kinase	3.17E-03	MUC1
Secretase gamma	complex	3.96E-03	MUC2
DNMT3A	enzyme	3.96E-03	MUC4
HNF1B	transcription regulator	3.96E-03	MUC4
SIM2	transcription regulator	4.75E-03	MUC2
ATF1	transcription regulator	6.33E-03	MUC5B
miR-145-5p (and other miRNAs w/seed UCCAGUU)	mature microRNA	7.12E-03	MUC1
ERBB2	kinase	7.12E-03	MUC1
IL13	cytokine	8.66E-03	MUC2,MUC5B
MTOR	kinase	9.48E-03	MUC1
HDAC3	transcription regulator	9.48E-03	MUC4
PRKCD	kinase	1.26E-02	MUC5B
HNF4A	transcription regulator	1.26E-02	MUC4
Akt	group	1.50E-02	MUC1
CAMP	other	1.81E-02	MUC2
CDX2	transcription regulator	2.82E-02	MUC4
GATA4	transcription regulator	2.90E-02	MUC4
HDAC1	transcription regulator	2.90E-02	MUC4
SP3	transcription regulator	3.28E-02	MUC2
HIF1A	transcription regulator	3.67E-02	MUC1
TLR2	transmembrane receptor	3.89E-02	MUC2
ERK	group	4.28E-02	MUC8
DNMT3B	enzyme	4.35E-02	MUC4
Histone h3	group	4.73E-02	MUC4

Table 3: Upstream regulators for mucin genes.

studies have been attempted to use the expression pattern for diagnostic value. However, it came with a heterogeneous pattern for MUC1, MUC2 and MUC25AC [17]. Studies with knockout mice suggest the tumor suppressing nature for MUC2 [18] and is supported by other studies [19]. This also resonates in our results which show downregulation of MUC2 in tumor samples. Using in situ hybridization and immunohistochemistry, it was shown that MUC2 and MUC3 were downregulated in colorectal cancer [20]. MUC 11 and 12 were also found downregulated in colorectal cancer [7]. Immunohistochemical patterns have also shown that MUC4 was downregulated in hyperplastic polyps and completely lost in serrated adenomas [21].

MUC12 gene expression was also earlier reported to be downregulated in colorectal cancers and its probable association with metastasis [22]. MUC12 was also found to be an independent marker

for prognosis in stage II and III colorectal cancer patients. We also report the downregulation of MUC12 in a significant manner.

The current level of evidence linking mucins to colorectal cancer underlies the importance of studying these molecules in detail. However, the disparity in evidences regarding the expression and post translational modification of these mucins in colorectal cancer may be limiting factor. These disparities could be because of difference in sampling and the method of investigation. This generates a big scope to study these mucins in a composite manner. Our study attempts to address this using a single snapshot taken from tumor and normal samples using exon level microarray. Ingenuity pathway analysis put these mucins in a different perspective with spotlight on MUC1 and MUC4 for their use as biomarkers. There are still some unexplored mucins with highly significant differential expression (e.g. MUC5B) that deserve further studies to prove or rule out their importance in colorectal cancer.

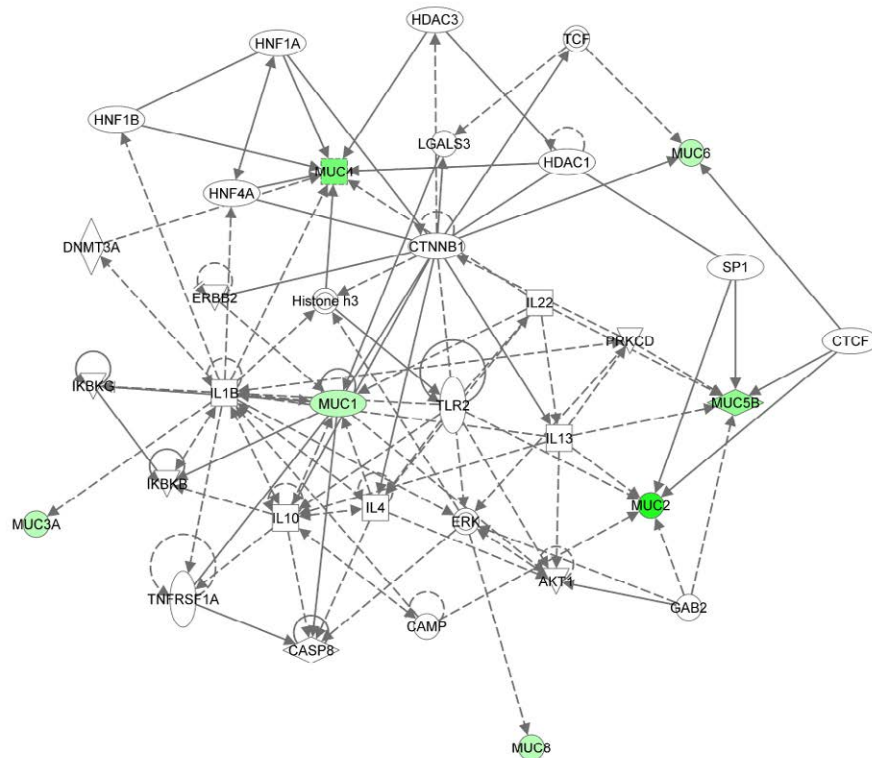


Figure 4: Network analysis of mucin genes. Using Ingenuity Pathway analysis all mucin genes were subjected to core analysis. 7/17 genes were included in the network with other molecules.

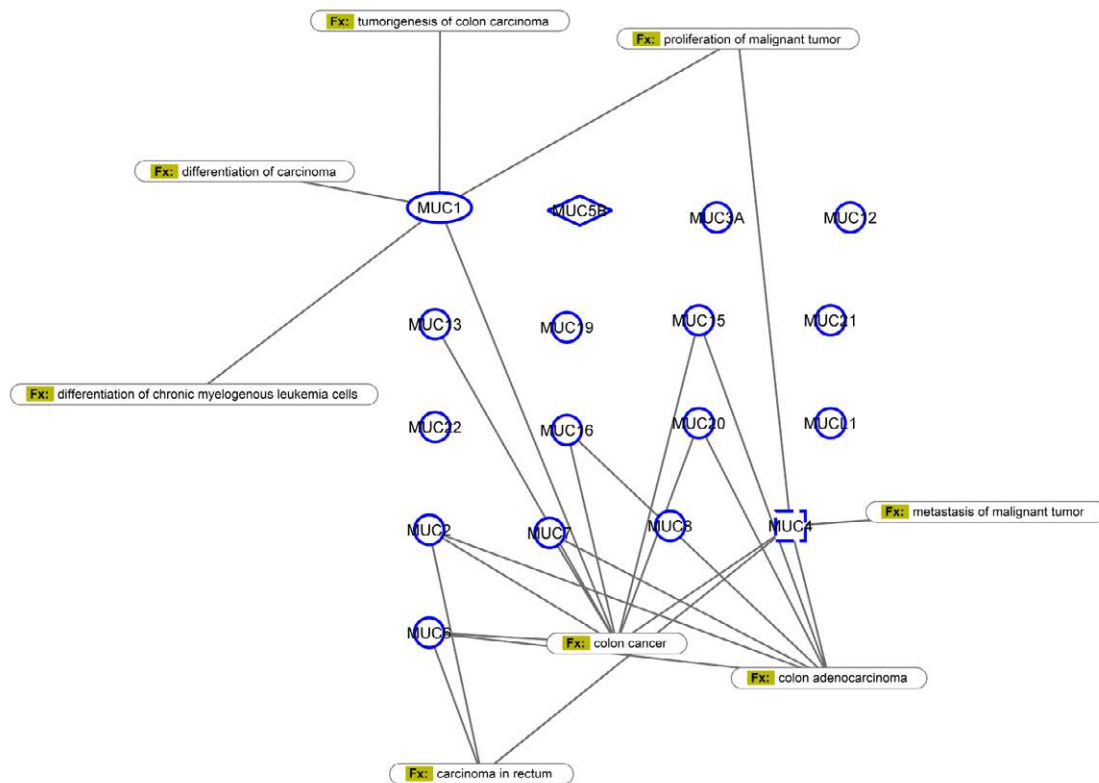


Figure 5: Mucin genes associated with colorectal cancer related functions. Mucin genes were overlaid with known functions associated with different aspects of colorectal cancer. Some of these mucin genes show little or no known role in colorectal cancer. Genes with high significance and unknown role would be the molecules with priority to be studied further.

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