Diabetic Complications Consortium

Application Title: "Genomic Characterization of Diabetic Gastroparesis"

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1. Project Accomplishments:

Our grant titled "Genomic Characterization of Diabetic Gastroparesis" was funded by an NIH-sub award with a projected start date of July 16, 2012. However, due to delays, funding was not available until the end of September 2012 and our sample request to the GpCRC repository was therefore not made until early February 2013. We received the first 44 serum samples for our study in late April of 2013. Of these 44 initial samples, 3 samples were removed from the study because they failed to meet the sample criteria stated in our proposal and another 4 samples were removed because they failed to pass quality control measures necessary for RNA-seq. The remaining 37 diabetic, gastroparetic microRNA samples have been sequenced and analyzed. Additionally, as of the end of April 2014, we have received 13 diabetic-only serum controls matching our criteria, which we have since processed and sequenced appropriately.

Upon receipt of the diabetic, gastroparetic serum samples from the GpCRC consortium, it was observed that many of these samples showed significant levels of visible irregularities (i.e. hemolysis, particulates, fat deposition, etc.). As even small traces of red blood cells and other contaminants in serum may dramatically affect the microRNA profile, it was decided that we should modify our protocol to take this additional complication of sample contamination into consideration. RNA-seq offers more quantitative information regarding microRNA expression than standard microarray hybridization techniques offers. Additionally, carryover of some contaminants (i.e. salts, lipids, etc.) can prevent hybridization of microRNA probes to arrays by altering the probe chemistry. Thus, it was decided that we should isolate the microRNAs from these samples and interrogate microRNA expression profiles with RNA-seq rather than microRNA microarrays, as RNA-seq would be more conducive to normalizing the samples for blood-based microRNA and other contamination.

In addition to altering the method to interrogate microRNA profiles in these serum samples, we also made changes to the microRNA isolation protocol itself. When we initially proposed this study, a means to isolate microRNA which was optimized for biofluids (including serum and plasma) was not available. By the time we received these serum samples, Exiqon had optimized just such an isolation system which we then used with great success (miRCURY RNA Isolation Kit – Biofluids (cat# 300112). This system incorporates the use of multiple synthetic microRNA-like spike-ins in 100-fold incremental concentrations to monitor microRNA isolation efficacy and examines quality control of isolated samples.

Following microRNA isolation of each sample, quality control analysis was done using realtime pcr to interrogate each sample. For this, we used the Exiqon miRCURY microRNA QC PCR Panel (cat# 203844) which analyzes the uniformity of the RNA

isolation process and the quality of isolated microRNA. This QC panel contains primer sets for the synthetic spike-ins to identify inconsistencies of microRNA isolation across all samples and may reveal presence of nucleases in samples which would then be excluded. Additionally, the QC panel detects the presence of cel-39-3p and UniSp3 to determine if cDNA synthesis and realtime pcr analysis were successful or if contaminants within the sample prevented amplification. Finally, the QC panel evaluates the samples for microRNAs that are expressed in most human tissues (miR-103 and miR-191), are indicative of hemolysis (miR-451 and miR-23a), or found in other human biofluids such as cerebrospinal fluid and urine samples (miR-124 and miR-30c). Based on analysis of these results, 4 diabetic, gastroparesis samples were removed from further analysis; while all 13 diabetic-only serum passed quality control measures.

After quality analysis was completed on serum microRNAs, we generated two separate microRNA libraries consisting of the remaining 37 diabetic gastroparesis samples in the study, and an additional library for the 13 diabetic-only control serum samples. Libraries were generated using Illumina TruSeq Small RNA Sample Kit (cat# RS-200-0012 and RS-200-0024), samples were then indexed, multiplexed into one of three separate libraries and run on Illumina MiSeq next generation sequencer on MiSeq standard 50 cycle flow cells (cat# MS-102-2001). Samples were run at a concentration of 10pM library in the presence of 10% PhiX control spike-in library to add basecall diversity and enhance sequencing call-rate efficiency. Sequencing reads averaged between samples to over 200,000 reads per sample.

2. Specific Background and Aims:

Gastroparesis is defined as delayed gastric emptying in the absence of physical blockage, and is a contributing factor to high inpatient hospital costs and increased patient mortality. Gastroparetic patients experience nausea, vomiting, bloating, and heartburn. Moreover, 43% of gastroparetic patients report pain that impairs their quality of life [1]. Diabetic, idiopathic, and post-surgical patients are the most common individuals to suffer from this condition that affects 9.6 males and 37.8 females per 100,000 people, respectively [2]. Gastroparesis is reported in 27-65% of patients with longstanding Type I Diabetes and 30% of patients with type 2 diabetes, and extragastric factors such as loss of glycemic control, vagal neuropathic damage, and the mitochondrial DNA mutation 3243 predispose diabetic patients to gastroparesis [5-7]. Recent studies have identified clinical and pathological factors associated with gastroparesis, including obesity, dietary intake and nutritional deficiencies, and pathology of gastric tissue from these patients reveals reduced/dysmorphic interstitial cells of Cajal, abnormal infiltration of macrophages, and increased connective tissue [3-6]. However, no genetic characterization of diabetic gastroparesis has been done before now.

We hope that with this new knowledge of the molecular changes associated with gastroparesis, we will be capable of identifying targetable pathways that can further therapeutic treatment of diabetic gastroparesis

Aims

The goal of this study was to identify changes in microRNA expression from serum and gastric tissues of human diabetic gastroparetic patients in order to identify the molecular pathways that lead to gastric dysfunction

3. Results

I) Results of microRNA Characterization of Diabetic Gastroparetic Serum Samples

MicroRNAs modify RNA degradation and silence translation of target messenger RNAs and this process is intrinsic to both normal and disease processes. It is estimated that upwards of 60% of all genes encoding human proteins contain microRNA recognition sites in their 3' untranslated region capable of silencing messenger RNA expression. Often microRNAs will act locally, either by carrying out their biological function within the cell of its origin or by acting on neighboring cells. In addition to local regulation, most cells can package microRNAs into nano-sized plasma membrane-bound vesicles called microvesicles (also known as exosomes) and shed them into the circulation. Once distributed in the circulation, microvesicles containing miRNAs bind to remote cells allowing cell-to-cell communication at a distance via transfer of microRNAs. Secreted microvesicles containing microRNAs can be found in every human tissue type and bodily fluids such as blood, serum, plasma, saliva, ascites fluid, urine, and spinal fluid [7], suggesting that remote cell-to-cell communication is a common biological process. A number of researchers have taken advantage of this remote communication process with great success, utilizing differentially expressed serum-derived microRNAs as non-invasive diagnostic markers for multiple cancers, heart disease, and high blood pressure. Our study marks the first characterization of circulating microRNA profiles in the serum of diabetic gastroparetic patients. We hope that this research will not only help to identify a circulating microRNA signature unique to diabetic gastroparesis patients, but also may reveal novel targets for therapeutic intervention and candidate biomarkers for gauging efficacy of patient treatment.

Aberrant expression of microRNAs has previously been reported in diabetics, suggesting their potential for as biomarkers for disease diagnosis [8, 9]. There are a number of known diabetes-associated miRNAs, including miR-29b, miR-15a, miR-28-3p, miR-223, and miR-126 which are currently being evaluated for their potential as biomarkers of aberrant insulin signaling, fibrinolysis or as druggable targets in diabetic patients [10, 11]. By comparing circulating microRNA profiles in the serum of diabetic, gastroparetic patients to that of diabetic-only patient serum controls, we found a number of interesting candidates, which with additional validation studies may also be useful as biomarkers for gastroparesis susceptibility and/or severity as well as potential therapeutic targets.

A. Up-regulated miRNAs identified in Diabetic Gastroparetic Serum Samples

Our sequencing efforts resulted in the identification of 14 microRNA (Table 1), many of which are already known to play a role in diabetes, lipid metabolism, pancreatic cell proliferation/differentiation, wound healing and insulin signaling. One such microRNA,

miR-126 is believed to be expressed predominantly in endothelial cells and is known to target expression of various transcripts which regulate angiogenesis [12]. MiR-126 has also been identified as a possible early prediction biomarker for type 2 diabetes mellitus [10]. In our deep sequencing experiments on diabetic gastroparetic serum and diabetic-only serum, we saw a 1.8-fold increase in levels of miR-126*, which is the complementary strand to miR-126. Although much less is known regarding the function of miR-126* than its complement, recent studies have demonstrated a possible role of miR-126* in silencing proteins, including prostein, in non-endothelial cells [13]. Our sequencing also identified an approximately 2.6-fold increase in expression of miR-26a in the diabetic gastroparetic samples as compared to the diabetic serum samples. MiR-26a is induced in response to hypoxia and is often seen up-regulated in both smooth muscle cells and throughout neurogenesis [14]. MiR26a has recently been implicated in the regulation of ten eleven translocation (TET) enzymes which mediate the DNA demethylation necessary for promoting pancreatic cell differentiation in vitro and in vivo [15]. Additionally, miR-26a has been shown to demonstrate properties of both a tumor suppressor and an oncogene depending on the cellular context by regulating apoptosis and protein regulators of invasion and proliferation [16, 17].

The expression of miR-191 was also shown to be dramatically differentially regulated in diabetic, gastroparetic samples compared to diabetic-only samples (3.2-fold increase over control). Previous studies have shown that miR-191 is down-regulated in the regulatory T-cells of patients with Type I Diabetes [18]. MiR-191 actively regulates cyclins and apoptosis, and high levels of miR-191 expression have been correlated with lower survival probability in patients with acute myeloid leukemia [19]. We feel that because miR-191 shows significant increases in expression in diabetic, gastroparetic patients over the diabetic-only controls, miR-191 may be a useful prognostic indicator of gastroparesis and patient health and hope to begin validation studies soon. Our studies also identified miR-192 as a potentially useful biomarker. Activation of miR-192 induces cell cycle arrest, having dozens of direct target transcripts that regulate G1 and G2 checkpoints [20]. MiR-192 is also currently being used as a biomarker for drug-induced liver damage [21]. Studies on cultured mesangial cells and glomeruli from diabetic mice indicated that miR-192 could be induced by TGF-beta1 to target degradation of E-box repressors Zeb 1 and Zeb 2, which in turn, allow for the increased production of collagen, TGF-beta and fibronection, which are mediators of renal fibrosis [22]. Consequently, reduction of renal miR-192 decreased renal fibrosis and improved proteinuria, supporting the use of anti-miR-192 for the treatment of diabetic nephropathy [22].

Table 1. Up-regulated microRNAs and validated Target Genes

microRNA (miR)	Fold Change	Validated miR Targets
hsa-miR-151-5p	+3.8	ARHGDIA, NTRK3, MPL, E2F6, CASC4
hsa-miR-375	+3.6	JAK2, MTPN, INSM1, SP1, MAP3K8, FZD8, BCL2L11, IF16
hsa-miR-27b	+3.6	NOTCH1, MMP13, CYP1B1, PPARG, CYPA4, PAX3, FASN

hsa-miR-143	**+3.2**	**KRAS, MYO6, DNMT3A, MAPK7, COX2, HRAS, SERPINE1, AKT1, MDM2, BCL, JAG1**
hsa-miR-191	**+3.2**	**MDM4, CDK6, IRS4, ATF6, SOX4, IL1A**
hsa-miR-361-5p	**+2.9**	**VEGF, CD46, DUSP3, RPUSD4, ARF4**
hsa-miR-148a	**+2.8**	**DNMT1, HLA-G, TGIF2, DNMT3B, IRS1, ACVR1, BCL2, HSP90B1, PTPN4**
hsa-miR-26a	**+2.6**	**CDK8, CDC6, LIF, PTEN, SERBP1, SMAD1, MAP3K2, RB1, Myc, IFNB1, GSK3B**
hsa-miR-142-5p	**+2.1**	**NFE2L2**
hsa-miR-126*	**+1.8**	**SLC45A3**
hsa-miR-151-3p	**+1.8**	**MCL1, CASC4 ZNF763**
hsa-miR-21*	**+1.8**	**RASGRP1, CDC25A, BCL2, WNT1, JAG1**
hsa-miR-192	**+1.7**	**EGR1, TGF, MAP3K1, PiK3R4, RABGAP**
hsa-miR-146a	**+1.6**	**CXCR4, KIT, FADD, TRAF6, ROCK1, BRCA2, BRAC1, CCNA2, IL8, NFKB1, CDKN1A, EGFR**

MiR-148a was also found to be more than 2.8-fold up-regulated in diabetic, gastroparetic samples than in diabetic, only controls. Recent knockdown experiments in β-cell culture revealed that miR-148 was partially responsible for insulin production through regulation of transcriptional repressors of insulin synthesis [23]. MiR-148 is also known to promote cell proliferation and cell cycle progression as well as target p27, a key inhibitor of the cell cycle, for degradation, leading to progression of gastric cancer proliferation [24]. In addition to finding microRNAs with known roles in modulating insulin sensitivity, our study also identified miR-143, a modulator of adipogenic differentiation, as differentially expressed in gastroparetic patients (3.2-fold). High levels of miR-143 correlate to higher levels of terminal adipogenic differentiation as seen in adipose tissue-derived stroma cells, in which overexpression of miR-143 promoted adipose tissue formation by directly repressing MAP2K5, a key member of the MAPKK family in the MAPK signaling pathway [25]. This up-regulation of miR-143 has been demonstrated in pre-diabetic patients, diabetic patients, obese patients and patients with pancreatic cancer [26]. MiR-27b was also found to be up-regulated in our study (3.6-fold), and it is also known to impair human adipocyte differentiation [27]. And, expression of hepatic miR-27b has been found to be inversely proportional to levels of dyslipidemia and atherosclerosis in mouse models [28]. Additionally, expression of miR-27B represses migration of mouse mesenchymal stem cells from bone marrow and prolongs wound repair [29].

Recently, miR-21 was identified as a regulator of immune cells. MiR-21, which was found up-regulated (1.8-fold), plays a crucial role in a plethora of biological functions and diseases including development, cancer and inflammation, and is especially correlated with the pathogenesis of autoimmune diseases and is known to inhibit insulin secretion in vitro [30, 31]. Studies have also recently identified a unique regulatory pathway of β-cell death involving miR-21, in which miR-21 targets the tumor suppressor gene PDCD4 and its upstream transcriptional activator nf-kappaB [32]. Thus, targeting the miR-21−PDCD4 pathway has already been identified as a potentially useful strategy for treating autoimmune Type I diabetes [32].

MiR-375 is one of the earliest identified and most functionally characterized microRNAs in literature. miR-375, which was up-regulated 3.6-fold in our study, is believed to be specifically expressed in the pituitary gland and the pancreatic islets, where it regulates insulin [33]. Knockout mice lacking miR-375 have been shown to have a lower pancreatic β-cell mass due to impaired proliferation, resulting in hyperglycemia [34]. Overexpression of miR-375 is also known to reduce insulin secretion through inhibition of exocytosis of insulin granules via translational repression of the cytoplasmic protein myotrophin, a pivotal factor implicated in the regulation of a variety of genes responsible for cellular apoptosis and proliferation [34]. Recently researchers demonstrated that a combinatory dysregulation of miR-142-5p and miR-375 was a useful prognostic marker for recurrence risk in gastric cancer following surgical resection [35]. Signaling pathway analyses showed that most of the targeted genes that were regulated by miR-375 and miR-142-5p were both involved in the same pathways, such as cell cycle, focal adhesion, MAPK, transforming growth factor-b, Wnt and vascular endothelial growth factor (VEGF). MiR-142-5p has also been found up-regulated in the peripheral blood mononuclear cells from renal transplant patients and that this aberrantly high miR-142-5p expression could be used prognostically to determine the likelihood of organ transplant rejection [36]. Additionally, miR-142-3p was shown to demonstrate deregulated expression in plasma of morbidly obese men and is currently under investigation for use as a biomarker for risk estimation and classification of morbidly obese patients [37].

Our studies identified an association of diabetic, gastroparesis with some lesser known microRNAs including miR-151-5p, miR-361-5p and miR 151-3p. These microRNAs are among several known to be consistently up-regulated in the progression of esophageal cancer [38]. Additionally, miR-361-5p is believed to target the 3'untranslated region of VEGF under hypoxic conditions [39] and acts as a tumor suppressor in prostate cancer where it targets STAT6 [40]. Little is also known in regards to miR-146a, however, it has been founds that miR-146a increases in monocytes and macrophages upon induction of lipopolysaccharide and negatively regulates innate immune response by targeting TRAF6 [41].

Gene set enrichment analysis for messenger RNA (www.broad.mit.edu/gsea/) and target scanning for microRNAs (www.targetscan.org) was performed to examine how expression profiles of validated microRNA targets fit into known and archived biological signaling pathways in order to identify putative signaling pathways that are altered between the disease states. Here we identified the known validated targets of these 14 up-regulated microRNAs using the TargetScan database, and by running STRING analysis on this list of

targeted genes, we were able to identify the most affected signaling networks which these microRNAs regulate (Figure 1 A-C). Unsurprisingly, the most impacted signaling networks involved AKT signaling (Figure 1A), CREB signaling (Figure 1B) and NF-kappa B signaling (Figure 1C), all of which are heavily implicated in both diabetes as well as gastroparesis.

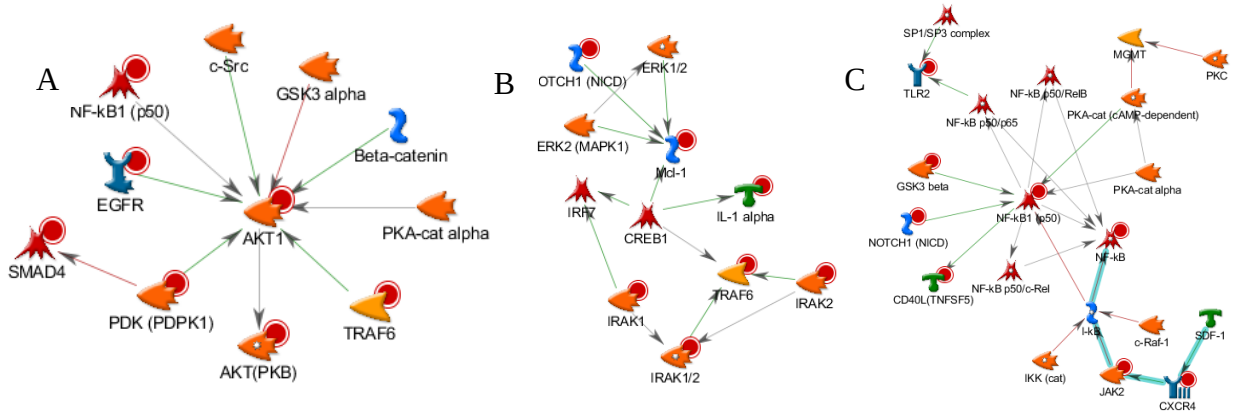

Figure 1 STRING diagram of microRNA target genes of differentially regulated microRNAs. Genes marked with red dot indicate validated target of microRNA, while those lacking are related nodes based on published datasets. (A) STRING indicating heavy involvement of microRNA targets on AKT expression, (B) Mcl-1 and TRAF expression, and (C) NF-kappa B expression.

B. Down-regulated miRNAs identified in Diabetic Gastroparetic Serum Samples

Our deep sequencing studies also indicated that some circulating microRNAs were down-regulated in diabetic, gastroparetic serum as compared to diabetic-only control serum. Of these 11 down-regulated microRNAs, miR-19b showed the most drastic change of 6 fold reduction in expression. Low level of expression of mouse miR-19b has been demonstrated previously in apoE-/- mouse aortic tissues than in healthy controls [42]. MiR-19b is believed to indirectly regulate SOCS3 expression in atherosclerotic vascular disease and loss of miR-19b has been correlated to the pathogenesis of disease [42]. In addition to the reduced expression of miR-19b, we also saw a reduced level of expression miR-let-7a, miR-let-7i and miR-let-7g. In addition to the miR-let-7 family of microRNAs being key regulators throughout development, let-7 microRNAs are repressors of IL-13 secretion [43]. Skeletal muscle of type II diabetic patients demonstrates higher expression of let-7 microRNAs which in turn reduces IL-13 levels and inhibits glucose uptake and incorporation to glycogen [43]. Very little information is currently available on the roles of miR-425, miR-30b, miR-103 and miR-532-5p, although recent reports suggest that miR-425, miR-103 and miR-30b may act as a possible oncogenes [44-46]. Finally, our studies suggested that miR-320a was differentially down-regulated in diabetic, gastroparetic samples as compared to diabetes-only control (1.7-fold decreased expression). Recent studies have shown that several exosomally derived miRs, including miR-320, play an active role in protecting the heart against ischemia and reperfusion injury through exosomal transfer into endothelial cells [47]. MiR-320 has been shown to drive the synthesis of important cardioprotective proteins including heat shock protein (HSP)-70, endothelial nitric oxide synthase, inducible nitric oxide synthase, HSP-20, Sirt1, and hypoxia-inducible factor 1a [47].

Table 2. Down-regulated microRNAs and validated Target Genes

microRNA (miR)	Fold Change	Validated miR Targets
hsa-miR-19b	-6.0	PTEN, ATXN1, MYLIP, SOCS1, BCL2L1, TGFBR2, BMPR2, FGFR2, CCND1, KDR, CASP8
hsa-miR-532-5p	-1.9	RUNX3, TRAPPC2P1, CPNE1, ZFHX3
hsa-miR-320a	-1.7	TFRC, HSPB6, MAPK1, IGF2BP1, ATPAF2, IGF2BP3, NPR1
hsa-let-7a	-1.6	CDK6, CDC25A, MYC, BCL2, ITGB3, NF2, NRAS, KRAS, VDR, DICER1, IGF2, HRAS, NFKB1
hsa-let-7i	-1.6	TLR4, SOCS1, IL13, FANCD2, BMP2K, CDC25A, EGLN3, PIK3C2A
hsa-miR-103	-1.5	CCNE1, CDK2, CREB1, DICER1, PTEN, TIMP3, ID2, GPD1
hsa-miR-30b	-1.4	CCNE2, SMAD1, BCL6, SOCS1, SNAI1, MYOIE, CLDN12
hsa-miR-425	-1.3	MAP2K6, DICER1, PIP4K2A, DHCR7
hsa-let-7g	-1.3	CDK6, CDC25A, KRAS, MYC, IGF2BP1, CDKN2A, BCK2L1, FN1, IL13, AKAP11
hsa-miR-26b	-1.3	CDK6, CCNE1, PARP4, FKBP2, TIMP1, FOXE1, SERBP1
hsa-let-7f	-1.2	KLK10, KLK6, IL13, MPL, MYC, CDKN1A, VIM, TG

Additionally, we identified the known validated targets of these 11 down-regulated microRNAs using the TargetScan database. STRING analysis on these targeted genes, identified the most affected signaling networks which these microRNAs regulate (Figure 2 A-B). The signaling pathways most heavily modulated by expression of these microRNAs were mitogen activation and cell cycle (G1/S transition) modulation (Figure 2A) and immune response regulating cell surface receptor signaling pathway (Figure 2B).

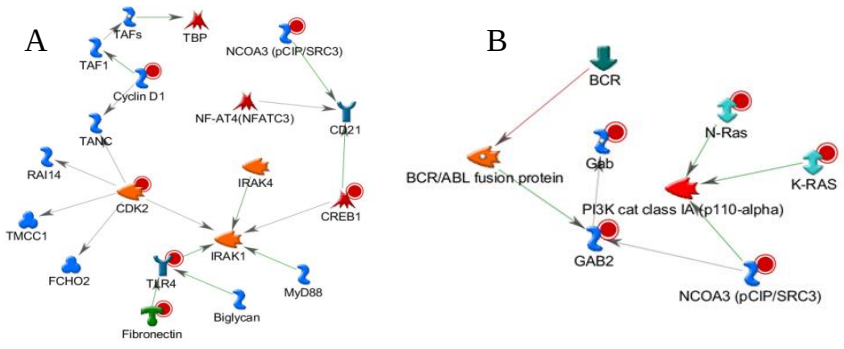

Figure 2 STRING diagram of microRNA target genes of differentially regulated microRNAs. Genes marked with red dot indicate validated target of microRNA, while those lacking are related nodes based on published datasets. (A) STRING indicating heavy involvement of microRNA targets on cell cycle progression and (B) immune system response.

C. Future Validation studies on Differentially Expressed circulating microRNAs in Serum

Deep sequencing of serum samples was completed this month, following the receipt of control serum samples in late April 2014. Currently, validation studies are planned to verify the altered expression levels of these 25 microRNAs in diabetic gastroparetic serum samples. As indicated in our original proposal, validation will entail real-time PCR analysis to be performed using micro-RNA specific probes. We expect these results to be available within the next 6-8 weeks.

II. Genomic Characterization of Diabetic Gastroparesis in FFPE Gastric Biopsies Samples

In March 2014, we received 32 paraffin-embedded gastric tissues (both diabetic gastroparetic and control tissues) to complete the second half of our grant proposal. These tissues were graciously provided by Dr. Gianrico Farrugia who has kindly allowed us access to these rare tissue samples. As indicated previously, total RNA isolation and microRNA isolation, RNA microarray analysis, microRNA library construction, and analysis of all 32 samples will take a total length of time of 5-6 months. As we have received these samples less than two months ago, results from this portion of the study are not yet available. Results, however, should be ready within the next 6-8 weeks.

III. References:

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3. Publications:

We anticipate submitting manuscript with results of our study in August 2014.