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GESTATIONAL DIABETES MELLITUS ALTERS APOPTOTIC AND INFLAMMATORY GENE EXPRESSION OF TROPHOBLASTS FROM HUMAN TERM PLACENTA

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Abstract

AIM—Increased placental growth secondary to reduced apoptosis may contribute to the development of macrosomia in GDM pregnancies. We hypothesize that reduced apoptosis in GDM placentas is caused by dysregulation of apoptosis related genes from death receptors or mitochondrial pathway or both to enhance placental growth in GDM pregnancies.

METHODS—Newborn and placental weights from women with no pregnancy complications (controls; N=5), or with GDM (N=5) were recorded. Placental villi from both groups were either fixed for TUNEL assay, or snap frozen for gene expression analysis by apoptosis PCR microarrays and qPCR.

RESULTS—Maternal, placental and newborn weights were significantly higher in the GDM group vs. Controls. Apoptotic index of placentas from the GDM group was markedly lower than the Controls. At a significant threshold of 1.5, seven genes (BCL10, BIRC6, BIRC7, CASP5, CASP8P2, CFLAR, and FAS) were down regulated, and 13 genes (BCL2, BCL2L1, BCL2L11, CASP4, DAPK1, IκBκE, MCL1, NFκBIZ, NOD1, PEA15, TNF, TNFRSF25, and XIAP) were unregulated in the GDM placentas. qPCR confirmed the consistency of the PCR microarray. Using Western blotting we found significantly decreased placental pro-apoptotic FAS receptor and FAS ligand (FASL), and increased mitochondrial anti-apoptotic BCL2 post GDM insult. Notably, caspase-3, which plays a central role in the execution-phase of apoptosis, and its substrate poly (ADP-ribose) polymerase (PARP) were significantly down regulated in GDM placentas, as compared to non-diabetic Control placentas.

CONCLUSION— Women with gestational diabetes (GDM) are at increased risk for having macrosomic newborns, and larger placentas with reduced apoptosis. Decreased apoptosis

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subsequent to alterations in apoptotic and inflammatory genes may promote elevated weight in the GDM placentas.

Keywords

apoptosis; apoptotic gene microarray; gestational diabetes; placenta

INTRODUCTION

Gestational diabetes (GDM) is a state of glucose intolerance, and insulin resistance¹⁻³ beginning or first recognized during pregnancy. GDM is associated with significant fetal complications including an abnormal pattern of fetal growth,⁴ and adverse perinatal outcome such as macrosomia (large for gestational age).⁴⁻⁶ The infants with macrosomia are at an increased risk to developing glucose intolerance and obesity in adolescence and later life.⁷⁻⁹

Coincident with the risk of macrosomia, women with GDM have heavier placentas.¹⁰⁻¹¹ These placentas exhibit significantly increased central thickness and diameter⁵ that provide an enlarged surface areas of exchange on the maternal and fetal side.¹² The morphological changes were paralleled by a variety of gene alterations for key functions of biologic processes^{13,14} such as inflammatory response and immune cell activation.^{13,15} The nature of the alterations and their extent depend on a range of variables including the quality of glycemic control achieved during the critical periods in placental development, the modality of treatment, and the time period of severe departures from metabolic control.¹² Interestingly, no data on expression profiling of apoptotic genes in placentas of GDM pregnancies has so far been documented.

Apoptosis (physiological cell death) is vital for normal placental growth and development.^{16,17} Apoptosis occurs via two main caspase-dependent pathways; the death receptor pathways and the mitochondrial pathway.¹⁸ The best investigated death receptors include the tumor necrosis factor (TNF) and the Fas-ligand-mediated pathways, both involving receptors of the TNF receptor (TNFR) family¹⁹ coupled to extrinsic signals. Conversely, the mitochondrial pathway, a principal site for apoptotic regulation by the BCL2 family, is mediated by a complex interplay between mostly internal stimulus, and upstream pathways that converge on the mitochondria to disable this organelle.²⁰ The apoptosis associated regulatory mechanisms are pivotal to the maintenance of placental homeostasis; imbalance of these processes may compromise placenta function and subsequently, pregnancy success.²¹ The placental villous trophoblast bi-layer consists of the cytotrophoblast and the syncytiotrophoblast. Activation of death receptors and mitochondrial pathways increase the number of placental cytotrophoblasts available to fuse into syncytiotrophoblast to form the exchange zone between fetal and maternal circulations. Recently we have shown that apoptosis level is significantly lower in trophoblasts from GDM pregnancies, compared to non-diabetic pregnancies,¹⁰ potentially explaining the larger placental size in GDM pregnancies.

We hypothesize that decreased apoptosis in placentas from women with GDM stem from the dysregulation of an array of apoptotic associated genes from death receptors or mitochondrial pathway, or both. Hence, we sought to determine whether GDM modifies the

pattern of placental apoptotic transcriptome in human placenta obtained from GDM and non-diabetic pregnancies.

MATERIALS AND METHODS

Studies were approved by the Institutional Review Board (IRB) (IRB#00543-40-12) of the Los Angeles BioMedical Research Institute at Harbor-UCLA (LABioMed), and were in accordance with the Declaration of Helsinki and National Institutes of Health (NIH) guidelines. Informed consent for use of patient demographics and placental tissue was obtained at the time of delivery.

Participant demographics

A glucose screening test (also called a glucose challenge test or GCT) was performed on 10 patients at 24 weeks to check for GDM using a 50 g, 1 hour glucose load. GDM was defined as an abnormal glucose tolerance during the third trimester, according to the criteria defined by the American Diabetes Association (ADA).²² In the five patients that were tested positive (>140 mg/dL) (GDM, N=5), a 2-hour Oral Glucose Tolerance Test (OGTT) was carried out. All participants were term gestations, non-smokers, did not abuse alcohol and did not have concurrent medical conditions, except for GDM in the diabetic patient group. Among the five GDM participants, four were treated with diet and one patient was treated with metformin. The GDM women treated with diet receive nutritional counseling, by a registered dietitian consistent with the recommendations by the ADA. The aim of dietary therapy was to prevent single large meals and foods with a large percentage of simple carbohydrates. The diet is composed of 55–60% carbs, primarily complex; <30% fat, 20% protein with 3 meals, 3 snacks using a caloric prescription based on true body weight and on ADA recommendations. One GDM patient who did not achieving glucose targets on diet therapy (fasting < 90 mg/dL or 2-hour postprandial levels <120 mg/dL) was treated with metformin. Metformin was prescribed to begin at 500 mg twice daily and increased incrementally every 1–2 weeks by 500 mg with meals until a maximum total dose of 2500 mg/day. The participants were generally seen in clinic every other week until 34 weeks of gestation and weekly thereafter.

Placentas without membranes or umbilical cords and newborns to participants with no pregnancy complication (controls, N=5), or with GDM (N=5) were weighed within 10 minutes of delivery. To ensure the lack of placental inflammatory changes associated with vaginal delivery, and improve homogeneity and comparability only patients with planned Caesarean section were enrolled in the study.

Placental tissue collection

Basal and chorionic plates from the placentas that were close to being circular were removed from cotyledons within 2 cm of the insertion of the umbilical cord, and the placentas were washed in isotonic solution. Villous samples (4 mm diameter) were divided into a specimen that was fixed in 4% paraformaldehyde for 24 hour prior to embedding in paraffin for sectioning, while another was snap frozen in liquid nitrogen and stored at 80°C for gene expression analysis.

Terminal dUTP Nick-End Labeling (TUNEL) assay and apoptotic index

We used an ApopTag *in situ* apoptosis detection kit (Millipore Corporation, USA) as previously described by Belkacemi et al.²³. Apoptotic cells in non-diabetic (Controls, N=5) and GDM (N=5) placentas were detected by the brown precipitate overlying nuclei after incubation in 3,3'-diaminobenzidine (DAB) chromogen (Vector laboratories, USA) and counterstained with methyl green (Sigma, USA). Terminal deoxynucleotidyl transferase enzyme was replaced with phosphate buffer saline (PBS), in the negative control. All samples were run concurrently to ensure validity and reliability of the experiments.

All slides were scanned by AxioCam HRC light microscope (Carl Zeiss MicroImaging, USA). Five separate specimens from each placenta of GDM and non-diabetic Control women were assessed. Ten random fields from each section of the five placentas from each group were digitalized at 200× final magnification by an observer blinded to clinical history. The digitized images were stored in uncompressed tiff format (tagged image file format) with 24-bit RGB class and 640 x 480 pixel resolution. The level of apoptotic positive nuclei immunostaining within the GDM and the non-diabetic Control placentas were calculated using the Image Pro, version 4.5, analysis software system (MediaCybernetic Inc., USA). For each of the digitalized image, villi were drawn manually using a marker tool and selected areas of interest (AOI) were highlighted by an editable colored outline, then analyzed automatically with Image Pro software. The tissue sections were processed concurrently to ensure uniformity of immunostaining. For TUNEL immunolabeling, the nuclei were considered positive if their immunostains were equal or larger than 50% of the nuclear area. Cells were classified as immuno-positive or -negative based on pre-determined thresholds that evaluated color, intensity of staining, cell size, axis length, roundness, and compactness. The filtering used thresholds as follows: mean density (minimum=115; maximum=164–169, proportionally for labeling mean density per image), area (minimum=labeled nuclear mean area per image/2.3), axis (minimum=2 um), roundness (0.6–1.0), and perimeter ratio (0.5–1.0). Mean density and area thresholds were automatically defined based, on mean density and mean area of TUNEL labeled nuclei in the evaluated image, respectively. Our evaluation of the TUNEL labeled nuclei is per the protocol described by Konstantinidou et al.²⁴ Based on previous findings by high-resolution confocal microscopy, and immunofluorescence detection of the plasma membrane protein, E-cadherin that one-third of the cytotrophoblasts in term villi were interdigitated into the syncytiotrophoblasts,²⁵ and our examination of the trophoblast by light microscopy, we chose not to distinguish the cytotrophoblast from the syncytiotrophoblast and documented TUNEL positive nuclei in the trophoblast bi-layer as "trophoblast". Apoptotic stromal and endothelial cells were systematically excluded. The apoptotic index (AI) in each section was calculated as the percentage of trophoblasts nuclei stained TUNEL-positive divided by the total number of DAPI-stained nuclei found within the trophoblast.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of frozen human placenta using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Residual DNA was digested with Turbo DNase enzyme and inactivated with DNase removal reagent (Turbo DNase kit, Invitrogen, USA). Final RNA was resuspended in 100 µl DEPC-treated water, quantitated

using a Nanodrop spectrophotometer (Thermo Scientific, USA) and stored at -80°C until use. Complementary DNA (cDNA) was generated from 3 µg of total RNA using Superscript III reverse transcriptase kit (Invitrogen). The RNA was incubated in 20 µl of a reverse transcription reaction mixture (1×reverse transcription buffer, 6.25 mM MgCl₂, 10 mM DTT, 0.5 mM deoxyribonucleoside triphosphates, 50 ng random hexamers, 40 U RNaseOUT [RNase inhibitor], and 10 U Superscript III reverse transcriptase) at 50°C for 50 minutes followed by digestion of RNA:DNA hybrids with RNase H. Four reactions for each sample were performed and the cDNA was combined and diluted with nuclease free water to accommodate the apoptosis and real time qPCR assays.

PCR apoptosis microarrays

GDM placental cDNA samples were assayed for apoptotic gene expression using TaqMan Array Human Apoptosis 96-well Plates (Invitrogen). The PCR microarray consists of 92 assays to apoptosis-associated TaqMan primer/probes and four assays to endogenous control genes. This microarray allows expression analysis of the signaling pathways that initiate human apoptosis caspases dependent death receptor regulated pathways, and mitochondrial pathway along with four control housekeeping genes. In brief, a reaction mix consisting of 10 µl of 2 × TaqMan Universal Master Mix II (Applied Biosystems, USA), 1 µl of cDNA, and 9 µl of nuclease free water was run in each well of the 96 well plate. The PCR reaction was performed in a Step One Plus Real Time PCR System (Applied Biosystems) with cycling conditions of one cycle of 50°C for 2 minutes, one cycle 95°C, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Ct values and fold changes between non-diabetic Control and GDM arrays were calculated using the manufacturer's plate template file and software (StepOne software version 2.2.2, USA). For data analysis 18S was used as the endogenous control gene.

Ontology and pathway analysis of PCR microarrays

GDM genes that were up-regulated or down-regulated by 1.5-fold after log₂ ratio transformation of the fold change relative to the control were considered significantly expressed. The genes were allocated to specific ontology groups using G:Profiler (<http://biit.cs.ut.ee/gprofiler/>).²⁶ Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://www.david.niaid.nih.gov>) (version 6),²⁷ a set of functional annotation tools to understand biological meaning behind large list of genes, was used to assign genes to known Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic processes, and to view gene biochemical pathway maps.

Real-time reverse transcriptase polymerase chain reaction (qPCR)

The qPCR primer sequence sets were designed using the OligoPerfect Designer website (Invitrogen) (Table 1). PCR based on SYBR green binding was performed for target genes and the 18S ribosomal RNA (normalization). Ten microliter reactions consisted of 2×SYBR Green Master Mix (RT-SN2X-06+, Anaspec, San Jose, USA), 300 pmole primers, and 1 µl of cDNA per well were run on a Step One Plus Real Time PCR System (Applied Biosystems) with cycling conditions of one cycle of 50°C, 2 minutes, one cycle 95°C, 40 cycles of 95°C 15 seconds, 60°C 1 minute. All samples were run in duplicate. StepOne software version 2.2.2 (Applied Biosystems, USA) was used to select a threshold level of

fluorescence that was in the linear phase of the PCR product accumulation. Results were determined as the difference between the CT for a specific mRNA and the CT for a reference mRNA, normalized to 18S threshold expression, and was expressed as fold change with the formula 2^{-CT} . Real-time reverse-transcription PCR efficiencies were acquired by the amplification of dilution series of cDNA according to the equation $10(1/\text{slope})$ and consistent between target mRNA and 18S mRNA. Control PCR samples replaced cDNA with water and gave a threshold level (CT) value of 40, which indicated no detectable PCR product.

Protein isolation and Western blot analysis

Protein was extracted in radioimmunoprecipitation assay (RIPA, Pierce, USA) buffer that contained protease and phosphatase inhibitors (HALT cocktail, Pierce) by homogenization of placental tissue (N=10) from GDM and non-diabetic Control women using a Bullet Blender Homogenizer as per manufacturer's protocol (Next Advance, USA). Homogenates were centrifuged for 10 minutes at 12,000g and protein concentration was determined by bicinchoninic acid (BCA) solution (Pierce). Proteins were frozen at -80°C until use.

Protein expression was analyzed as previously performed^{28,29} and each blot was performed at least twice. Briefly, equal amounts of protein (50 µg) were mixed with Criterion sodium dodecyl sulfate sample buffer (Bio-Rad, USA), boiled for 5 minutes, and separated on a Criterion 4–12% Bis-Tris denaturing gel. The separated proteins were transferred electrophoretically to a nitrocellulose membrane (Bio-Rad) for 1.5 hours at 120 volts, 4°C. Nonspecific antibody binding was blocked by incubation for 1 hour at RT with 5% nonfat dry milk in Tris-buffered saline solution containing 0.1% Tween 20 (TBST; Bio-Rad) for 1 hour, and then in the same solution containing a primary antibody that reacts with FAS, FASL, BCL2, BAK1, activated caspase-3, or poly (ADP-ribose) polymerase (PARP) (see Table 2 for dilutions), at 4°C overnight. After washes in TBS-T, membranes were incubated with the corresponding horseradish peroxidase secondary antibody (Table 2) at room temperature for 1 hour followed by washes in TBS-T and a final TBS only wash, each 10 minutes. Chemi-Glow substrate (Alpha Innotech, USA) was used to detect the targeted protein. The band density on the X-ray film were scanned and the results compared by Alpha Imager software (Alpha Innotech, USA). To ensure equal loading, protein blots were stripped with Restore stripping buffer (Pierce), and reprobed with β-actin housekeeping gene (1:10,000; Cell Signaling Technologies, USA).

Statistical analysis

Differences between GDM and non-diabetic Control patient and infant demographics, PCR microarrays, qPCR and Western blotting were compared by two-tailed, Student's *t*-test using NCSS97 software (NCSS, USA) and data were presented as mean±SD. For the PCR apoptotic microarray results, analysis of variance (ANOVA) was first performed on five plates prior to ontology and pathway analyses. All comparisons with a P-value less than 0.05 were considered statistically significant.

RESULTS

Maternal and neonatal demographics

Maternal age and body weights were significantly higher in the GDM group, compared with those from the non-diabetic Control group (Table 3, $P=0.02$ and 0.004 ; respectively). Non-fasting mean serum glucose value was 80.5 ± 11.7 mg/dL in the non-diabetic Control group (Table 3). Fasting mean serum glucose value was elevated (94.3 ± 7.3 mg/dL) and consistent with the 2-hour OGTT mean serum glucose value (152.3 ± 34.3 mg/dL) in the GDM group (Table 3). However the fasting glucose values obtained from the GDM participants cannot be compared to the glucose values from non-diabetic patients since the non-diabetic Control patients were not fasting (Table 3).

Neonates of GDM and non-diabetic participants groups were composed of three females and two males each. Gestational age at delivery was comparable (Table 2, $P=0.81$), but the mean neonatal birth weight was significantly higher in the GDM group, compared with that from non-diabetic Controls (Fig. 1A, Table 3, $P<0.05$). We also observed a trend toward an increase in the neonatal body length in the GDM group, compared to the non-diabetic Controls but without reaching significance. Placental weight was markedly higher in the GDM group, compared to non-diabetic Control group (Figure 1B; Table 3, $P=0.04$). However, there was no significant difference between birth-weight to placenta ratio (measure of placental efficiency) of the GDM group, compared to the Control group ($P=0.95$).

Placental apoptotic index

Since placental cell death influences placental development,^{30,31} we used TUNEL assay to assess placental apoptosis, and predicted that GDM placentas would have lower levels of cell death in trophoblasts than the respective non-diabetic Controls placentas. As expected, compared to trophoblasts from non-diabetic Control placentas, trophoblasts from placentas of women with GDM had a significant 71% decrease in positive nuclei, compared to Controls (IA: 0.05 ± 0.01 vs. 0.17 ± 0.04 , Figure 2A-E; $P<0.04$) by quantification of TUNEL.

Analysis of GDM mRNA targets in placenta using TaqMan microarray

Placentas from GDM and non-diabetic control women were subjected to PCR apoptotic microarrays to determine the effect of diabetic insult on apoptotic gene expression. Among the 96 gene sequences surveyed, 94 genes were present in GDM and non-diabetic Control placental transcriptomes (Supplement Table 1). Among those, 31 transcripts from placentas of women with GDM were significantly different from placentas of non-diabetic Control women (Supplement Table 1; $P<0.05$). At a chosen significance threshold of 1.5, we detected 20 transcripts among which seven genes (BCL10, BIRC6, BIRC7, CASP5, CASP8P2, CFLAR, and FAS) were down regulated, and 13 genes (BCL2, BCL2L1, BCL2L11, CASP4, DAPK1, I κ B κ E, MCL1, NF κ BIZ, NOD1, PEA15, TNF, TNFRSF25 and XIAP) were unregulated (Table 4), implying that GDM insult induced rather than repressed apoptotic genes. The ratios relative to the respective Controls ranged from -2.4 decrease to 3.1 increase.

Specificity of the real-time PCR reaction (qPCR)

Ten genes from the death receptors and the BCL2 family of genes (BCL2, BCL2L2, BAK1, FAS, FASLG, TNFRSF1B1, NOD1, DAPK1, CASP8P, CASP3), were selected for qPCR to evaluate the consistency of PCR microarrays results. The results obtained from qPCR were comparable to that of the PCR microarrays except for caspase-3 expression, which was unchanged using microarrays, but significantly increased (3.0-fold) by qPCR (Table 5).

The melting-curve analysis proved that each reaction amplified a single predominant product with a distinct T_m . A single dissociation peak for the product of the qPCR reaction was obtained for every gene tested (data not shown). Our results indicate that the qPCR assay was gene specific and that the results were not confounded by nonspecific amplification of primer-dimers.

Gene ontology functional categories description

The 20 differentially expressed transcripts post GDM insult detected by microarray were assigned to specific groups using G:profiler. The groups which ranged between 3 to 18 members (Table 4) were associated with diverse cell functions that include (i) cell homeostasis, (ii) response to stimuli, in particular, response to cellular, external, biotic and abiotic stimuli, and (iii) regulation of cellular processes. Apoptosis process and regulation were also included in the G:profiler output (Table 6). A cluster of 17 genes were found in the apoptosis process, with lower number of genes allocated to cell-type specific apoptotic process, execution phase of apoptosis, and apoptosis regulation including positive, and negative programmed cell death, mitochondrial membrane potential, mitochondrial membrane permeability, mitochondrion organization, as well as autophagy (Table 6).

KEGG pathways

Using DAVID, we mapped the 20 differentially expressed transcripts to pathways in the KEGG database.³² A total of seven pathways demonstrating at least 3 genes each with >1.5-fold change in expression were detected (Table 7; $P < 0.05$). Among those, three pathways (apoptosis, NF κ B and NOD-like receptor signaling) were directly related to placental development and function. As anticipated, apoptosis had the highest number of modulated genes among which BAX, BAK1, BCL2, BCL2L2 were up regulated whereas BCL10, CASP8P2 were down regulated (Table 7, Figure 4). Pathways involved in immune response such as NOD1 receptor signaling and NF κ B were also represented with 3 and 5 genes respectively (Table 7). Interestingly, two of the differentially expressed genes were associated with diabetes type 1 (DT1) pathway, suggesting that GDM and (DT1) may share some common pathways.

Western blot validation of FAS receptor and mitochondrial apoptotic pathways

Microarray and RT-PCR data analysis demonstrated that both FAS receptor and mitochondrial apoptotic pathway genes from term placenta were negatively impacted by GDM insult. We assessed the expression of FAS, FASL, BCL2, BAK1, activated caspase-3, and PARP proteins that represent key players in the induction of apoptosis through FAS receptor or mitochondrial pathways or both using Western blot analysis.

As expected, we detected a single immunoreactive band of the predicted molecular weight on Western blots of diabetic and non-diabetic Control placental protein lysates for FAS, FASL, BCL2, BAK1 (Figures 3A, B, D, and E). In agreement with the microarray and RT-PCR results, densitometric analysis of FAS and FASL showed significantly decreased expression in placentas from GDM pregnancies, compared to non-diabetic Controls (Figure 3C; $P<0.0003$, and $P<0.05$). Conversely, BCL2 was markedly increased in placentas post GDM (Figure 3F; $P<0.05$); whereas BAK1 showed a trend upward but was not statistically significant (1.3-fold, Figure 3F; $P=0.5$).

Expression of cleaved caspase-3 in the total cell protein lysate from non-diabetic placentas revealed three polypeptides of 20, 15 and 11 kDa (Figure 3G), but the 15 kDa band was almost absent in lysates from GDM placentas (Figure 3G). This fragment is known to result from caspase-3 cleavage during apoptosis. Densitometric analysis revealed that combined levels of these active caspase-3 polypeptide fragments was significantly decreased in placentas of women with GDM, compared with placentas from non-diabetic Control women (Figure 3I; $P<0.04$).

Consistent with the down regulation of activated caspase-3 in placentas of women with GDM, densitometric analysis of the 85 kDa active fragment of PARP was significantly reduced in GDM placentas, compared to non-diabetic Control placentas (Figures 3H-I; $P<0.02$).

Collectively these data pointed to dysregulation of some key protein players of FAS receptor and mitochondrial pathways post GDM as represented in Figure 4.

DISCUSSION

Gestational diabetes (GDM) insult during pregnancy elicits changes in the expression profile of placental genes with a prominent modulation in mediators of death receptor and mitochondrial pathways, together with genes regulating inflammatory response. These gene alterations are reflective of a state of physiological cell death dysregulation and inflammation. Ontological analysis of differentially expressed genes by biological function categories showed that those genes may be involved in a range of functions vital to placental homeostasis. Using KEGG data analysis we detected a total of nine pathways in the GDM placentas that were affected by the GDM insult. Among those death receptor, and mitochondrial pathways and TNF α signaling, were the most relevant to placental growth in hyperglycemic environment, as compared to controls. Results obtained by microarrays analysis were comparable to that of qPCR, except for caspase-3 expression, which was unchanged by DNA microarray measurement but up regulated by qPCR. Analysis of proteins of FAS and mitochondrial pathways suggested a caspase- dependent placental cell survival mechanism potentially through concomitant inhibition of both pathways. We propose that placental transcriptome and protein dysregulation post GDM induced aberrant placental signaling and altered fetal nutrient supply¹⁰ ultimately leading to fetal overgrowth and heavier newborns. Newborns of women with GDM have an increased risk of macrosomia³³ hypoglycemia³⁴ as well as the potential of developing glucose intolerance and obesity in adolescence and later life.³⁵

We found that increased mass in placentas from women with GDM was associated with significantly reduced apoptosis in the trophoblast. The findings of our study are consistent with a previous report showing that increased placental growth in streptozotocin-induced diabetic rats was associated with a notable decrease in apoptotic index.³⁶ Controversially, data from the literature validates opposite results. For example Burleigh et al.³⁷ did not observed any increase in placental or found differences in cell death rates among trophoblast compartments of five diabetic and normoglycemic human placentas. Likewise Araujo et al.³⁸ did not detect any increase in placental or fetal weights from diabetic and normoglycemic patients, however when cultured *in vitro*, trophoblasts purified from placentas of diabetic patients showed marked increase in apoptosis after 1 day, compared to their normal matched Control placentas. A predominance of apoptosis in trophoblast cultures, or placental tissue from diabetic participants as in Sgarbosa et al.²¹ study may result from differences in the mode of delivery. Indeed in this study participants were subjected to labor that would more likely induce ischemia-reperfusion, stimulate oxidative stress, and increase activation apoptosis in the labored placentas,³⁹ compared with our non-labored placentas delivered by Cesarean section. Other technical considerations such as placental sampling, or differences in gestational age^{40,41} cannot be ruled out, or perhaps, the investigators were simply selecting for DNA damage response associated with forms of programmed cell death other than apoptosis such as necrosis.⁴²

In addition to glucose, insulin is a metabolic hormone highly implicated in the regulation of fetal and placental growth and development.⁴³ Such actions are mediated through insulin receptors (IRs), which are greatly expressed at the microvillous membrane of the syncytiotrophoblast at the beginning of pregnancy, and mainly found at the endothelium at term pregnancy.⁴⁴ In diet-controlled trophoblasts from GDM pregnancies, the number of IRs is lower than non-diabetic pregnancies,⁴⁵ but it is uncertain whether endothelial IRs are also altered.³ Insulin rescue various cell types from apoptotic cell death,^{46,47} and lesser IRs in placentas from diet-treated GDM patients may imply that maternal insulin plays a minor role in the impediment of trophoblast cell death. Conversely metformin, a therapeutic drug with anti-apoptotic properties,⁴⁸ can easily cross the placenta⁴⁹ and thus may mediate, at least partially, placental apoptosis inhibition in the GDM placenta. However a greater placental metformin treated sample size is required to address this possibility. Since neither insulin nor metformin seem to prevent apoptosis in the GDM placentas, we speculate that increased production or expression of growth factors such as insulin like growth factors 1 (IGF1) and or fibroblast growth factor 2 (FGF-2) may act as inhibitors of apoptosis to significantly decrease apoptosis leading to placental hypertrophy in the GDM pregnancies.^{50,37}

Parallel to decreased placental apoptosis in the GDM placentas we observed a variety of abnormal placental gene expression involved in apoptotic and inflammatory cell signaling using microarrays. Some limitations of our investigation include the fact that as a dynamic method microarrays technology allows a single measure of RNA expression that is dependent on gene sequence quality, the rate of initiation to degradation, and stability. Nonetheless, this method is valuable, as it allows the measurement of expression levels of

large numbers of genes simultaneously to obtain information on disease pathology and progression, and provides signatures of the state of activity of diseased cells.

To further substantiate the findings of the PCR microarrays, we performed qPCR. Concordance between expression measurements of our PCR microarray experiments and qPCR experiment results were generally confirmatory. Absence of full agreement between PCR microarrays and qPCR expression measurements as is the case of caspase-3 may have resulted from differences in experimental setup including differences in probe sequences and target location. Moreover, production of mRNA transcripts of multiple isoforms by splicing, or other post-transcriptional processing can cause discrepancies. Cleaved caspase-3 protein expression down regulation in the GDM placentas supports a post-translational modification of caspase-3, but this entails further investigation.

Changes in death receptor and mitochondrial pathway genes are associated with increased apoptosis in intrauterine growth restricted (IUGR) placentas;^{23,16,31} but little is known about the mechanism of GDM mediated placental decreased apoptosis. Because of the critical role of apoptosis in placental homeostasis, our study aimed at deciphering whether GDM changes the pattern of placental apoptotic transcriptome of death receptor and mitochondrial pathways to reduced placental apoptosis. Our findings revealed alterations in 20 transcripts at a significant threshold of 1.5 in either pathway in the GDM placentas. Thus, placental apoptotic transcriptome emerged as a primary target of the altered environment of diabetic pregnancy and may play a role in the mechanisms associated with reduced apoptosis in placentas of women with GDM. A parallel modulation of protein expression from some of these genes is consistent with this hypothesis.

The death receptors and mitochondrial pathways acting through members of the caspase cystein protease family are subdivided into three groups: effectors or activators of apoptosis and mediators in inflammation. As a central mechanism mediating apoptosis changes in caspase proteases⁵¹ would support an aberrant apoptotic signaling in the GDM placentas. Altered caspases expression has been hypothesized to contribute to differential activation and resistance to apoptosis.⁵² In our study, concomitant decrease in caspase-8-associated protein 2 (CASP8AP2) transcript facilitating death receptor-induced apoptosis through caspase-8 activation at the death-inducing signaling complex,⁵³ and significant up regulation of inhibitors of apoptosis (IAP) CFLAR and PEA15 transcripts binding caspase-8-like regulators of death ligand-induced apoptosis⁵⁴ may reflect a Fas-mediated apoptosis pathway dysfunction to promote placental trophoblast cell survival in the GDM placentas. Significant down regulation of FAS and FASL protein expression in the GDM placentas substantiates this premise. Simultaneous up regulation of mitochondrial anti-apoptotic BCL2 family members, such as BCL2 at the transcriptional and the translational levels and BCL2L1, BCL2L2 and MCL1 transcripts, together with downstream mitochondria XIAP transcripts down regulation by microarray strongly support mitochondrial pathway associated delayed apoptosis in the GDM placentas. Collectively our results suggested death receptor and mitochondrial pathways down regulation in the GDM placentas. As caspase-3 is activated universally during apoptosis, regardless of the specific death-initiating stimulus, and this protease generally coordinates the demolition phase of apoptosis by cleaving a diverse array of protein substrates^{55,56} including PARP, it is conceivable that decreased

cleaved caspases-3 and PARP expression reflected an important placental cell survival mechanism in which both FAS receptor and mitochondrial pathways were inhibited in trophoblast from GDM placentas. However, further investigations are warranted to identify other components that will further clarify the role of each pathway to decreased apoptosis in the GDM placentas.

In addition to their role in apoptosis, a subfamily of caspases such as caspase-4 (CASP4) and caspase-5 (CASP5) play a major role in the activation of inflammatory cytokines, and the formation of inflammasome to stimulate inflammatory processes.^{57–59} Thus it is possible that in our study the significant increase in caspase-4 by 1.7-fold in the GDM placentas may promote activation of CASP4 leading to induction of inflammatory response. In support of our hypothesis elevated circulating concentration of inflammatory cytokines and inflammation-related genes were observed in GDM placentas.³ However, the exact pathophysiological relevance of a role of CASP4 in placental response under GDM insult merits further exploration. On the other hand, the decrease in CASP5 transcript by -2.2-fold post GDM insult makes it unlikely to play a role in GDM induced placental inflammation, but it may associate with placental resistance to apoptosis via a mechanism that remains to be discovered.

Tumor necrosis factor (TNF) (also called TNF alpha or TNF α) is a multifunctional cytokine, and a central regulator of inflammation,⁶⁰ although its death-inducing ability is weaker than other family members such as Fas. Modulation of serum cytokines in GDM patients have been reported.⁶¹ Further TNF α transcript has been detected in human placentas at both early gestation and at term, with more TNF α present at later rather than earlier stages of pregnancy.⁶² Our data showed that TNF α transcript was significantly increased in placentas from women with GDM at term, compared to non-diabetic Control women. The mechanism responsible for the increase of TNF α expression is not known, but it appears that TNF α can activate docosahexaenoic acid, an essential ω -3 polyunsaturated fatty acid³ that accumulates in placenta of offspring from mothers with GDM causing increased adiposity in newborns.⁶³ We suggest that the present results may represent a mechanism linking local TNF α placental-mediated inflammatory responses with increased lipid substrate availability for fetal fat deposition³ and subsequent increase adiposity at birth.

TNF α also participates in the endocrine mechanism of pregnancy-induced insulin resistance⁶⁴ by adding a placental component to the insulin resistance developing in the mother.³ Hence elevated levels of TNF α transcript in the GDM placentas being likely to associate with decreased insulin signaling is consistent with the strikingly decreased insulin sensitivity observed in GDM pregnancies.⁶⁵ Increased TNF α -induced insulin resistance in GDM placentas may also originate from decreased peroxisome proliferator-activated receptor (PPAR- γ),⁶⁶ and insulin receptor substrate (IRS)⁶⁷ both of which contribute to reduced insulin suppression of lipolysis. However, to what extent placental TNF α may contribute to the pathogenesis of GDM is yet to be established.

Many pro-inflammatory genes are regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) transcription factor, and an abnormal NF κ B activity may underline a number of inflammation-related disorders.⁶⁸ Although NF κ B transcript expression was

unchanged by GDM insult, several NF κ B pathway activators including death-associated protein kinase 1 (DAK1), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NF κ BIZ), nucleotide-binding oligomerization domain containing 1 (NOD1), tumor necrosis factor receptor superfamily member 25 (TNFRSF25), and inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (I κ BE) were upregulated in the GDM placentas. We postulate that modulation of these transcripts may occur through NF κ B independent mechanisms to promote greater placental inflammation and enhanced placental mass or both following GDM insult although further research at the translational level is needed to understand the potential significant role these genes play in GDM pathogenesis.

In spite of maternal glycemic control, increased placental weight was paralleled by significantly elevated newborn weight in GDM patients compared to non-diabetic Controls. The elevated fetal growth may result from intrinsic placental properties (mass, glucose transport expression) as observed in diabetic rats,⁶⁹ or from placental transcriptome and translational signaling dysregulation. Although it is not possible to determine exactly if placental overweight is the cause, or the consequence of fetal overweight, ultrasounds demonstrated that fetuses of GDM pregnancies weighed significantly more than non-diabetic controls at the same gestational age.⁷⁰ We are well aware of the small number of specimens in the present study, however, the extend of the differences and the statistical significance strongly corroborates validity of the results. In support of our finding, In support of our finding, a prospective analysis of 28,358 mother-infant pairs who enrolled in the National Collaborative Perinatal Project between 1959 and 1965 demonstrated that compared to their non-diabetic counterparts, mothers with GDM gave birth to offspring that had higher weights at birth.⁷¹ More recently, Grissa et al.⁷² reported increased weight in 30 newborns of GDM women, compared to their 30 newborn matched controls.

CONCLUSION

Our results indicate that GDM-associated increased placental and newborn weights may be triggered, in part, by reduced trophoblast apoptosis and increased inflammation following altered placental transcripts/proteins implicated in key apoptotic as well as inflammatory functions as summarized in Figure 4. Altered placental morphology subsequent to lower apoptosis and higher inflammation in the trophoblast area may account for functional adaptations in the GDM placentas to optimize fetal growth in the existing conditions *in utero*. Detrimental changes in the amount of nutrient supplied to the fetus as a result of altered placental phenotype and genotype are anticipated to have long-term impact on adult health and morbidity.⁷³ Studies that further dissect the role of apoptotic and inflammatory processes in GDM placentas will ultimately lead to innovative therapeutic approaches against mechanisms that induce excessive placental and fetal growth in GDM pregnancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Weights of newborns (A) and placentas (B) from women with GDM (black bar) and non-diabetic control (white bar). Asterisks indicate a significant difference between GDM (black bars) vs. non-diabetic Controls (open bars) at $P < 0.05$. GDM, gestational diabetes mellitus.

Figure 2.

Representative TUNEL-positive nuclei in trophoblasts from women with GDM (A) and non-diabetic controls (B). TUNEL-positive nuclei in the trophoblast (red arrows). Negative controls (C–D) with no immunostaining. Quantitative analysis of TUNEL-positive nuclei in placental trophoblasts from women with GDM and non-diabetic Controls (E) Asterisk indicates significant difference between % TUNEL-positive nuclei in placental trophoblasts from women with GDM (black bars) vs. non-diabetic Controls (white bars) at $P < 0.05$. Scale bars 50 μm . GDM, gestational diabetes mellitus; TUNEL, terminal deoxynucleotidyl transferase dUTP- mediated nick-end labeling.

Figure 3. Effect of GDM insult on levels of placental proteins from the FAS receptor and the mitochondrial apoptotic pathways. Representative immunoblots of FAS (A), FASL (B),

BCL2 (D), BAK (E), caspase-3 (G), and PARP (H) protein expression. Densitometric analysis of FAS and FASL (D), BCL2 and BAK1 (F), caspase-3 and PARP (I) protein expression. P-value indicates a significant difference between GDM (black bars) v. non-diabetic Controls (open bars). GDM, gestational diabetes mellitus; PARP, poly(ADP-ribose) polymerase. An asterisk indicates a significant difference ($P < 0.05$) between protein expression from GDM vs. non-diabetic Controls.

Figure 4.

Diagram of gene and protein profile alterations in FAS and TNF death receptors, and mitochondrial pathways post GDM insult at term gestation in humans. Decrease (↓), increased (↑), or unchanged (↔) full or dotted arrows indicate modulation of transcripts as detected by microarray and/or RT-PCR (green), proteins as observed by Western blotting (blue), or likely change in transcripts or proteins (black). Question mark in black (?) represents hypothetical pathway. A Cross in red denotes the inhibition of apoptosis.

Table 1

Primer sequences for qPCR

Gene	Accession number	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
BAK1	NM_001188	CATTCTACCTGAGGCCAGGA	CCCATCTCTTAGGGTGCTGA
BCL2	NM_000633	TTGTTCAAACGGGATTACACA	ACAAAACCCACAGCAAAAG
BCL2L2	NM_001199839	TTTGGTTCGGCTTTATCAGG	GCAAACAGTGTGGCTTCAAA
CSP8P	NM_004346.3	TTTGAGCCTGAGCAGAGACA	CGTATGGAGAAATGGGCTGT
DAPK1	NM_000043.4	GCCTGGGCTTTGGTGAGGCG	ACTGTCCACTGCCAAGTTCCT
FAS	NM_000043.4	CAAGGGATTGGAATTGAGGA	ACCTGGAGGACAGGGCTTAT
FASLG	NM_000639.1	GGCCTGTGTCTCCTTGTGAT	TGCCAGCTCCTTCTGTAGGT
NOD1	NM_006092.2	GTTGAGGTTGGGATGTGGGGCA	AGTGGTCAGGCTGGGGTGGT
TNFRSF1B	NM_001066.2	AGGAGTGGTGAAGTGTGTCAT	TTATCGGCAGGCAAGTGAGG
18S	M11188	ATTCCGATAACGAACGAGACT	AGCTTATGACCCGCACTTACT

Table 2

Antibodies used in Western blot analysis

Primary and horse radish peroxidase (HRP)-linked secondary antibodies and their commercial source	Antibody dilution
BAK1 (sc-832; Santa Cruz, USA)	1:500
BCL2 (sc-7382, Santa Cruz)	1:700
Caspase-3 (sc-7148, Santa Cruz)	1:700
FAS (sc-74540; Santa Cruz)	1:300
FASL (sc-834; Santa Cruz)	1:500
PARP (BML-SA253-0025; BioMol Res. Lab., USA)	1:2000
β -actin (A544; Sigma, USA)	1:10,000
Anti-Mouse IgG (H+L) HRP Conjugate (172-1011; BioRad, USA)	1:10,000
Anti-Rabbit IgG (H+L) HRP Conjugate (170-6515; BioRad)	1:1,500-3,000

Table 3

Maternal and infant demographics

	Non-diabetic Controls	GDM	P-value
Maternal age (years)	26.3±1.3	34.3±2.6	0.02
Maternal weight (kg)	73.0±9.0	90.0±13.0	0.004
Maternal blood glucose (mg/dL)	Non-fasting glucose: 80.5±11.7	Fasting glucose: 94.3±7.3 2h OGTT: 152.3±34.3	-
Therapeutics	-	Diet (N=4) and metformin (N=1)	-
Mode of delivery	Caesarean section	Caesarean section	-
Newborn gender	3 females and 2 males	3 females and 2 males	-
Gestational age at delivery (weeks)	39.1±0.4	39.0±0.2	0.81
Neonatal weight (kg)	3.36.0±0.3	4.02±0.2	0.05
Neonatal body length (cm)	49.7±1.9	51.3±1.1	0.09
Placental weight (kg)	0.45±0.04	0.57±0.1	0.04

-: Not applicable

Table 4

Summary of significantly modulated genes at the threshold of 1.5 absolute value

Symbol	Name	Mean Control $\pm$ SD	Mean GDM $\pm$ SD	Log2 Ratio	P-value
BCL2	B-cell CLL/lymphoma 2	5.7E-06 $\pm$ 1.9E-07	3.6E-05 $\pm$ 5.3E-06	2.7	0.03
BCL2L1	BCL2-like 1 or BCL-XL/S	2.1E-06 $\pm$ 9.7E-07	9.9E-06 $\pm$ 2.8E-07	2.3	0.05
BCL10	BCL2-like 10	1.6E-05 $\pm$ 1.1E-06	3.0E-06 $\pm$ 5.6E-07	-2.3	0.001
BCL2L11	BCL2-like 11	5.8E-06 $\pm$ 1.9E-07	5.1E-05 $\pm$ 2.4E-06	3.1	0.005
BIRC6	Baculoviral IAP repeat-containing 6	0.0002 $\pm$ 2.9E-05	3.2E-05 $\pm$ 9.4E-06	-2.3	0.02
BIRC7	Baculoviral IAP repeat-containing 7	1.9E-06 $\pm$ 1.9E-08	6.2E-07 $\pm$ 3.4E-08	-1.7	0.06
CASP4	Caspase 4	3.6E-05 $\pm$ 1.4E-06	1.7E-04 $\pm$ 5.9E-06	2.2	0.01
CASP5	Caspase 5	9.0E-07 $\pm$ 5E-08	2.3E-07 $\pm$ 8.4E-08	-1.7	0.02
CASP8AP2	Caspase 8	1.5E-05 $\pm$ 4.4E-06	3.9E-06 $\pm$ 1.1E-07	-1.7	0.02
CFLAR	FLIC-like inhibitory protein (FLIP)	3.9E-05 $\pm$	1.3E-04 $\pm$ 1.6E-05	1.7	0.05
DAPK1	Death-associated protein kinase 1	9.7E-05 $\pm$ 1.9E-06	2.7E-04 $\pm$ 6.0E-05	1.5	0.02
FAS	Tumor necrosis factor receptor superfamily, member 6	1.7E-05 $\pm$ 1.5E-06	6.6E-06 $\pm$ 2.7E-06	-1.6	0.03
IkBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon.	7.5E-07 $\pm$ 2.5E-08	2.2E-06 $\pm$ 2.2E-07	1.5	0.05
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	1.5E-05 $\pm$ 7.4E-08	6.0E-05 $\pm$ 1.1E-06	2.0	0.05
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	7.7E-06 $\pm$ 1.0E-08	3.6E-05 $\pm$ 5.8E-06	2.2	0.006
NOD1	CARD4, Nucleotide-binding oligomerization domain-containing protein 1	4.9E-06 $\pm$ 1.5E-07	1.6E-05 $\pm$ 3.3E-06	1.7	0.02
PEA15	FLIC-like inhibitory protein (FLIP)	4.6E-05 $\pm$ 1.5E-05	2.2E-04 $\pm$ 6.3E-06	2.2	0.04
TNF	Tumor necrosis factor alpha (TNF superfamily, member)	1.5E-06 $\pm$ 3.9E-07	5.7E-06 $\pm$ 0.0001	1.9	0.01
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	1.8E-06 $\pm$ 6.3E-07	6.1E-06 $\pm$ 1.5E-07	1.8	0.02
XIAP	X-linked anti-apoptosis	4.5E-06 $\pm$ 1.7E-07	1.5E-05 $\pm$ 2.7E-06	1.7	0.04

Table 5

Gene expression fold change by PCR microarrays and qPCR

Gene symbol	PCR microarrays		RT-PCR	
	Fold change	P-value	^a Fold change	P-value
BCL2	6.3	0.03	2.9	0.03
BCL2L2	4.6	0.06	2.2	0.33
BAK1	3.0	0.05	4.0	0.05
CASP8P	0.5	0.05	0.4	0.05
Caspase 3	0.8	0.86	3.0	0.05
DAPK1	2.8	0.02	3.2	0.02
FAS	0.3	0.03	0.4	0.02
FASLG	1.3	0.75	4.0	0.45
NOD1	3.2	0.02	2.8	0.04
TNFRSF1B1	2.4	0.01	3.8	0.05

^aFold change = 2^{-CT}.

Table 6

Ontological summary of differentially expressed gene expression by biological function category

Biological process	Number of genes per group	Gene symbol	Function	P-value
GO:0042592	8	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,FAS,MCL1,XIAP	Homeostatic process	1.30E-02
GO:0048872	5	BAK1,BCL10,BCL2,BCL2L11,FAS		9.36E-04
GO:0001776	5	BAK1,BCL10,BCL2,BCL2L11,FAS		1.68E-06
GO:0002260	5	BAK1,BCL10,BCL2,BCL2L11,FAS	Lymphocyte homeostasis	6.80E-07
GO:0001782	4	BAK1,BCL10,BCL2,BCL2L11	B cell homeostasis	5.72E-06
GO:0043029	3	BCL2,BCL2L11,FAS	T cell homeostasis	2.53E-03
GO:0050789	18	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,BIRC6,CASP4,CA SP5,CASP8AP2,CFLAR,DAPK1,FAS,MCL1,NFKBIZ,NOD1,PEA15,TNFRSF25,XIAP	Regulation of biological process	8.52E-04
GO:0048518	13	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,BIRC6,CASP4,CFLAR,DAPK1,FAS,NOD1,TNFRSF25,XIAP		2.34E-03
GO:0065009	10	BAK1,BCL10,BCL2,BCL2L11,CASP8AP2,CFLAR,DAPK1,FAS,NOD1,XIAP		2.34E-03
GO:0048583	10	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CFLAR,DAPK1,MCL1,NOD1,XIAP	Regulation of response to stimulus	4.54E-02
GO:0044093	8	BAK1,BCL10,BCL2,BCL2L11,CASP8AP2,CFLAR,FAS,NOD1	Positive regulation of molecular function	5.22E-03
GO:0009628	7	BAK1,BCL10,BCL2,BCL2L1,CASP5,CASP8AP2,FAS	Response to abiotic stimulus	9.49E-03
GO:0050896	16	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CASP5,CASP8AP2,CFLAR,DAPK1,FAS,MCL1,NFKBIZ,NOD1,PEA15,TNFRSF25,XIAP	Response to stimulus	1.40E-02
GO:0051716	14	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CASP5,CASP8AP2,CFLAR,DAPK1,FAS,MCL1,NOD1,TNFRSF25,XIAP		2.59E-02
GO:0009605	8	BAK1,BCL10,BCL2,CASP5,CASP8AP2,FAS,NOD1,XIAP		2.81E-02
GO:0051704	8	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CFLAR,FAS,NOD1	Multi-organism process	4.94E-02
GO:0051707	7	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,FAS,NOD1	Response to other organism	1.76E-03
GO:0009607	7	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,FAS,NOD1	Response to biotic stimulus	2.54E-03
GO:0071214	6	BAK1,BCL10,BCL2L1,CASP5,CASP8AP2,FAS	Cellular response to abiotic Stimulus	4.73E-06
GO:0071496	5	BAK1,BCL10,CASP5,CASP8AP2,FAS	Cellular response to external stimulus	3.55E-03
GO:0009612	5	BAK1,BCL10,CASP5,CASP8AP2,FAS	Response to mechanical stimulus	2.40E-04
GO:0071260	5	BAK1,BCL10,CASP5,CASP8AP2,FAS	Cellular response to mechanical stimulus	1.68E-06
GO:0008219	17	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,BIRC6,CASP4,CA SP5,CASP8AP2,CFLAR,DAPK1,FAS,MCL1,NOD1,PEA15,TNFRSF25,XIAP	Programmed cells death	7.29E-14
GO:0097194	8	BAK1,BCL2,BCL2L1,BCL2L11,CASP8AP2,CFLAR,FAS,MCL1		4.19E-08
GO:0097285	6	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,FAS		1.58E-04
GO:0071887	4	BAK1,BCL10,BCL2L11,FAS	Leukocyte apoptotic process	2.88E-04

Biological process	Number of genes per group	Gene symbol	Function	P-value
<u>GO:0070227</u>	<u>4</u>	BAK1,BCL10,BCL2L11,FAS	Lymphocyte apoptotic process	6.48E-05
<u>GO:0001783</u>	<u>3</u>	BAK1,BCL10,BCL2L11	B cell apoptotic process	7.10E-04
<u>GO:0043276</u>	<u>3</u>	BCL2,BCL2L1,MCL1	Anoikis	9.91E-04
<u>GO:0050794</u>	<u>18</u>	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,BIRC6,CASP4,CA SP5,CASP8AP2,CFLAR,DAPK1,FAS,MCL1,NFKBIZ,NOD1,PEA15,TNFRSF25,XIAP	Regulation of cellular process	3.01E-04
<u>GO:0048522</u>	<u>13</u>	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,BIRC6,CASP4,CFLAR,DAPK1,FAS,NOD1,TNFRSF25,XIAP	Positive regulation of cellular process	6.50E-04
<u>GO:1900117</u>	<u>8</u>	BAK1,BCL2,BCL2L1,BCL2L11,CASP8AP2,CFLAR,FAS,MCL1	Regulation of execution phase of apoptosis	4.25E-09
<u>GO:0010942</u>	<u>9</u>	BAK1,BCL10,BCL2L1,BCL2L11,CASP4,DAPK1,FAS,NOD1,TNFRSF25	Positive regulation of cell death	2.26E-07
<u>GO:0043069</u>	<u>9</u>	BCL10,BCL2,BCL2L1,BIRC6,CFLAR,FAS,MCL1,PEA15,XIAP	Negative regulation of programmed cell death	1.66E-06
<u>GO:0006917</u>	<u>7</u>	BCL10,BCL2L11,CASP4,DAPK1,FAS,NOD1,TNFRSF25	Induction of apoptosis	1.02E-06
<u>GO:2000811</u>	<u>3</u>	BCL2,BCL2L1,MCL1	Negative regulation of anoikis	3.17E-04
<u>GO:0044068</u>	<u>2</u>	BCL2L1,BCL2L11	Modulation by symbiont of host cellular process	4.96E-02
<u>GO:0019054</u>	<u>2</u>	BCL2L1,BCL2L11	Modulation by virus of host cellular process	3.09E-02
<u>GO:0023051</u>	<u>10</u>	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CFLAR,DAPK1,MCL1,NOD1,XIAP	Regulation of signaling	7.14E-03
<u>GO:0010646</u>	<u>10</u>	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CFLAR,DAPK1,MCL1,NOD1,XIAP	Regulation of cell communication	7.38E-03
<u>GO:0050790</u>	<u>8</u>	BAK1,BCL2,BCL2L11,CASP8AP2,CFLAR,FAS,NOD1,XIAP	Regulation of catalytic activity	4.17E-02
<u>GO:0043281</u>	<u>6</u>	BAK1,BCL2L11,CASP8AP2,FAS,NOD1,XIAP	Regulation of cysteine-type endopeptidase activity involved in apoptotic process	1.87E-05
<u>GO:0007165</u>	<u>13</u>	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CASP8AP2,CFLAR,DAPK1,FAS,MCL1,NOD1,TNFRSF25,XIAP	Signal transduction	2.13E-02
<u>GO:0007166</u>	<u>11</u>	BCL10,BCL2,BCL2L11,CASP8AP2,CFLAR,DAPK1,FAS,MCL1,NOD1,TNFRSF25,XIAP	Cell surface receptor signaling pathway	3.79E-03
<u>GO:0009966</u>	<u>10</u>	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CFLAR,DAPK1,MCL1,NOD1,XIAP	Regulation of signal transduction	2.07E-03
<u>GO:0035556</u>	<u>9</u>	BAK1,BCL10,BCL2,BCL2L1,BCL2L11,CFLAR,DAPK1,NOD1,XIAP	Intracellular signal transduction	4.05E-02
<u>GO:0097190</u>	<u>8</u>	BAK1,BCL2,BCL2L1,BCL2L11,CASP8AP2,CFLAR,FAS,MCL1	Apoptotic signaling pathway	4.25E-09
<u>GO:0010950</u>	<u>5</u>	BAK1,BCL2L11,CASP8AP2,FAS,NOD1	Positive regulation of endopeptidase activity	1.41E-04
<u>GO:0043280</u>	<u>5</u>	BAK1,BCL2L11,CASP8AP2,FAS,NOD1	Positive regulation of cysteine- type endopeptidase activity involved in apoptotic process	1.08E-04
<u>GO:0006919</u>	<u>5</u>	BAK1,BCL2L11,CASP8AP2,FAS,NOD1	Activation of cysteine-type endopeptidase activity involved in apoptotic process	2.50E-05
<u>GO:0005741</u>	<u>5</u>	BAK1,BCL2,BCL2L1,BCL2L11,MCL1	Mitochondrial outer membrane	1.04E-04
<u>GO:0051400</u>	<u>4</u>	BAK1,BCL2,BCL2L1,MCL1	BH domain binding	4.56E-08
<u>GO:0097193</u>	<u>4</u>	BAK1,BCL2,BCL2L1,BCL2L11	Intrinsic apoptotic signaling pathway	4.70E-03

Biological process	Number of genes per group	Gene symbol	Function	P-value
<u>GO:0097191</u>	<u>4</u>	CASP8AP2,CFLAR,FAS,MCL1	Extrinsic apoptotic signaling pathway	2.88E-04
<u>GO:0001836</u>	<u>4</u>	BAK1,BCL2,BCL2L1,BCL2L11	Release of cytochrome c from mitochondria	1.24E-04
<u>GO:0008633</u>	<u>4</u>	BAK1,BCL2,BCL2L11,FAS	Activation of pro-apoptotic gene products	2.02E-05
<u>GO:2001234</u>	<u>4</u>	BCL2,BCL2L1,CFLAR,MCL1	Negative regulation of apoptotic signaling pathway	3.13E-06
<u>GO:0035872</u>	<u>4</u>	BCL2,BCL2L1,NOD1,XIAP	Nucleotide-binding domain, leucine rich repeat containing receptor signaling pathway	1.72E-04
<u>GO:0051881</u>	<u>3</u>	BAK1,BCL2,BCL2L1	Regulation of mitochondrial membrane potential	2.53E-03
<u>GO:0010821</u>	<u>3</u>	BAK1,BCL2L1,BCL2L11	Regulation of mitochondrion organization	9.86E-03
<u>GO:0046902</u>	<u>3</u>	BAK1,BCL2,BCL2L1	Regulation of mitochondrial membrane permeability	3.17E-04
<u>GO:0002020</u>	<u>3</u>	BCL10,BCL2,CFLAR	Protease binding	4.24E-02
<u>GO:0070513</u>	<u>3</u>	BCL2,BCL2L1,MCL1	Death domain binding	4.90E-05
<u>GO:0010506</u>	<u>3</u>	BCL2,BCL2L1,DAPK1	Regulation of autophagy	2.63E-02
<u>GO:0010332</u>	<u>3</u>	BAK1,BCL2,BCL2L1	Response to gamma radiation	1.06E-02

Table 7

Functional annotation charts using KEGG

KEGG Number	Gene number	Pathway	P-value
04210	6	Apoptosis	5.6E-4
04210	5	NFκB signaling pathway	1.46E-04
04064	5	Pathways in cancer	2.13E-02
04621	3	NOD-like receptor signaling pathway	1.5E-1
05222	3	Lymphadenopathy	3.93E-02
05200	3	Small cell lung cancer	3.71E-02
04940	2	Type 1 diabetes mellitus	2.31E-02