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Molecular analysis of coronal perisutural tissues in a craniosynostotic rabbit model using polymerase chain reaction-suppression subtractive hybridization (PCR SSH)

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Abstract

Background—In the United States the incidence of craniosynostosis (premature fusion of the sutures of the cranial vault) is 1 in 2,000-3,000 live births. The condition can cause increased intracranial pressure, severely altered head shape, and mental retardation. We have previously described a colony of rabbits with heritable coronal suture synostosis. This model has been instrumental in describing the post-surgical craniofacial growth associated with craniosynostosis. The molecular analysis of this model has been limited by the lack of molecular tools for use in rabbits. In order to understand the pathogenesis of craniosynostosis, we compared gene expression in perisutural tissues between wild-type (WT) and craniosynostotic (CS) rabbits using polymerase chain reaction-suppression subtractive hybridization (PCR SSH).

Methods—PCR SSH was performed on RNA derived from pooled samples of calvariae from 10-day old WT (n=3) and CS (n=3) rabbits to obtain cDNA clones that are either enriched in WT tissues (underexpressed in CS tissue) or enriched in CS tissues (overexpressed in CS compared to WT).

Results—Differential expression was identified for approximately 140 recovered cDNA clones upregulated in CS tissues and 130 recovered clones for WT tissues. Of these, four genes were confirmed by quantitative reverse-transcriptase (RT)-PCR as being overexpressed in CS sutural

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tissue: β -globin, osteopontin (SPP1), SPARC, and cathepsin K (CTSK). Two genes were confirmed to be underexpressed in the CS samples: COL3A1 and RNF12.

Conclusions—The differential expression of these gene products in our naturally occurring CS model appears to be the result of differences in the normal bone formation/resorption pathway.

Keywords

craniosynostosis; rabbit; gene expression; molecular tools; osteogenesis; differential expression

Introduction

Craniosynostosis is defined as the premature fusion of one or more of the fibrous joints of the skull, termed cranial sutures. This disorder results from an overgrowth of bone at the osteogenic fronts of the affected suture. In the United States the incidence of craniosynostosis is 1 in every 2,000-3,000 live births (1-8). Afflicted individuals demonstrate a continuum of severity ranging from subclinical phenotypes to severe cases involving multiple sutures and noticeable cranial malformation. This phenotypic variability is thought to be a result of an interaction between genetic and epigenetic/environmental factors (2-4, 6). In the more severe cases, surgical intervention and cranial reconstructions are necessary. Surgical complications can include infection, encephalocele, hydrocephalus, dura mater compromise, hematoma, cerebrospinal fluid leaks, and post-operative resynostosis. Risk of each of these complications increases with multiple surgeries, which are often necessary in severe cases (9-15).

Genetic mutations have been identified for several syndromes that involve craniosynostosis. Disease-producing genetic aberrations have been linked to fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3) (2, 3, 16-25), TWIST, msh homeobox 2 (MSX2) (2, 3, 26, 27), and the transforming growth factor-beta receptors (TGF β R1, TGF β R2) (28-31). However, the genetic basis is unknown for 85% of craniosynostosis cases. This subset of craniosynostoses are classified as nonsyndromic, meaning they are not associated with any other clinical diagnosis or known etiology (2, 3, 32). Underlying genetic mutations most likely lead to these cases of nonsyndromic craniosynostosis by affecting either gene interaction or gene-environmental interactions (2, 3, 33-37). A better understanding of the molecular control of bone overgrowth in nonsyndromic craniosynostosis can benefit from relevant animal models.

A rabbit model with congenital nonsyndromic craniosynostosis of the coronal suture has been described (38-43). Similar to humans, this colony of New Zealand White rabbits demonstrates autosomal dominant transmission with variable phenotypic expression (38). The model presents with a broad range of phenotypic expression for the isolated coronal suture synostosis pathology (including unilaterally affected animals, animals with delayed-onset suture synostosis, and animals with complete bilateral fusion) (41-43). These affected rabbits over-express Msx2 at the suture site (44) as well as TGF β 2 (45), suggesting that the same gene(s) or pathways may be involved in this pathogenesis as in human syndromes (27, 46, 47).

The molecular description of the model has suffered from the lack of a complete genomic sequence available for rabbit similar to that described for human and mouse. In addition, very few commercially available molecular probes, primers, or antibodies are available for use in rabbit. Here we describe the use of PCR suppression subtractive hybridization (PCR SSH) to identify gene products that are differentially expressed between fused sutures derived from our craniosynostotic rabbit model versus non-fused sutures from wild-type control rabbits. PCR SSH is a transcriptomic approach that allows for discovery of genes

that are relatively over-expressed or under-expressed between two compared conditions (here a pathologically fused suture versus a patent normal suture) (48-50). Because PCR SSH allows the isolation of differentially expressed cDNA fragments without previous knowledge of their sequence, it is particularly useful for the study of differential expression in rabbit, where the genome has not been fully characterized. Once identified by PCR SSH, the validity of the recovered sequences can be confirmed using quantitative reverse-transcriptase PCR (qPCR).

This study was designed to test the hypothesis that tissues from rabbits with craniosynostosis exhibit a differential pattern of gene expression compared to control tissues from wild-type rabbits.

Materials and Methods

Animals

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Bone tissues were harvested from wild-type (n=3) rabbits and rabbits from the breeding colony with bilateral coronal suture synostosis (n=3) housed at the University of Pittsburgh. Craniosynostosis was diagnosed by the presence of a completely ossified and obliterated coronal suture at 10 days of age. Following diagnosis, the region of the skull that contained the fused coronal suture (synostotic suture bone) or the normal patent coronal suture (wild-type suture bone) was removed using a small periosteal elevator and a 0.5mm dental bur, fixed to a low-speed hand engine. Overlying periosteum and underlying dura mater were removed. Excised bone was cut into small pieces and placed into RNA later (QIAGEN) and stored at -80 C.

RNA extraction / purification of samples

For all tissue RNA purifications, total RNA was purified using the RNeasy Mini Kit (Qiagen Inc. USA, Valencia, CA) following manufacturer protocols after homogenization using a homogenizer and an on-column DNase treatment step. The quality of all RNA extracted from tissue was determined by capillary electrophoresis using an Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Palo Alto, CA) and the quantity determined using the OD₂₆₀/OD₂₈₀ ratio measured using a ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE).

Polymerase Chain Reaction-Suppression Subtractive Hybridization screen

The PCR SSH screen was completed using the SMARTer PCR cDNA synthesis kit (Clontech, Palo Alto, CA; 634926), Advantage Polymerase mix (Clontech, Palo Alto, CA; 639101), and the PCR Select cDNA subtraction kit (Clontech, Palo Alto, CA; 637401). Briefly, WT and CS sample total RNA was used to generate cDNA libraries using the SMARTer PCR cDNA synthesis kit, following manufacturer instructions for preparation with PCR SSH use. The WT and CS cDNA libraries generated were digested with Rsa I to create smaller cDNA fragments, and the subtraction hybridizations were performed as per the manufacturer's instructions. Both WT and CS cDNAs have been used as the "tester" population to identify genes overexpressed in CS (CS as tester) or underexpressed in CS (WT as tester).

The resulting subtracted cDNA populations were re-amplified by PCR and subcloned into the pCR4 vector with TOPO-mediated cloning (Invitrogen Corporation; cat # K4580-01). Ligations were transformed into TOP10 cells by electroporation following manufacturer protocols. Colonies were picked for each population and grown in Terrific Broth (BD, Franklin Lakes, NJ) containing ampicillin (100 mg/mL); minipreps of plasmid DNA were

then prepared using Nucleospin-96 kits following manufacturer's protocol (Macherey-Nagel). Plasmids were sequenced using M13 forward and M13 reverse primers on an Applied Biosystems 3730xl DNA Analyzer using standard methods. All sequences were compared and analyzed for similarity to the GenBank databases using the basic local alignment search tool "BLAST" program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Quantitative real time RT-PCR

The rabbit Taqman real time PCR primer sets for beta-globin (HBB2), osteonectin (SPARC), and cathepsin K (CTSK) were commercially available (Applied Biosystems; Table 1). The Taqman primer sets for osteopontin (SPP1) and collagen 3, alpha 1 (COL3A1) were derived from rabbit sequences obtained from the PCR SSH screen and custom designed and created by Applied Biosystems (Foster City, CA; Table 1). The Taqman primers/probes for RNF12 were designed using the Primer Express software (Applied Biosystems; Table 1). Primer sequences for the rabbit GAPDH (used as the endogenous control) were previously published [48]. Custom reverse primers for use in the reserve transcription reactions were designed using VectorNTI (Invitrogen). All reverse primers, RNF12 primers and probe, and GAPDH-specific primers were purchased from Integrated DNA Technologies (Coralville, IA).

90 ng of total RNA from samples were used for reverse transcriptase (RT) reactions using a gene specific reverse primer, 10 µl total volume, and MMLV reverse transcriptase (Invitrogen) as per manufacturer protocols. For real time PCR, assays were performed in triplicate using 1.5 µL of RT reaction combined with a mixture of Taqman Universal Master Mix (Applied Biosystems, 4304437) and gene-specific primer mix in a total volume of 15 µl. For all Applied Biosystems Taqman primer mixes, primers were diluted to 1X final concentration while GAPDH and RNF12 primer mixes were prepared using 800 nM of each primer and 160 nM of a probe (final concentrations) in each reaction. The remaining protocol for RT reaction and real time PCR was followed as previously described [48]. Using the comparative critical cycle (Ct) method and using GAPDH as the endogenous control, the expression levels of the target genes were normalized using a 95 % confidence interval. Results shown are representative of three independent experiments. Upon completion, real time PCR reactions were separated on an agarose gel to confirm that a single band was obtained.

Results

PCR SSH using both CS and WT tissues as the "tester" population yielded approximately 140 recovered cDNA clones for CS tissues and 130 recovered clones for WT tissues. Sequencing and BLAST alignment of these clones revealed 44 discrete gene products putatively over-expressed in CS tissues and 59 discrete gene products that are putatively over-expressed in WT tissue (under-represented in CS tissue). Of the approximately 140 CS enriched sequence clones, 46 matched to various regions of beta-globin. Other cDNAs obtained multiple times from the screen include osteopontin (SPP1- 6 times), SPARC (7 times), and cathepsin K (CTSK- 3 times). Other cDNAs of interest that were detected as enriched in the CS samples included osteonectin (BGLAP), CCT3, and KLF3 (Table 2).

Fifty-nine discrete gene products were recovered from the approximately 130 WT enriched sequence clones and numerous sequences appeared multiple times, including collagen, type III, alpha 1 (COL3A1- 9 times), periostin (twice) and CTHRC1 (twice). Many other cDNAs were detected as enriched in the WT samples (Table 3). Interestingly, in both PCR SSH sample sets, sequences with no similarity to any known genes were also identified.

To validate the results obtained from our PCR SSH screen, we selected a subset of target gene products identified in the screen (Figures 1a and 1b) and used quantitative RT-PCR assays for these targets to interrogate pooled RNA samples representing WT and CS samples. Using GAPDH as an endogenous control, beta-globin (HBB), CTSK, SPARC, and SPP1 mRNA levels were determined to be 5.3-fold, 1.95-fold, 1.65-fold, and 2.16-fold, respectively over-expressed in CS samples relative to WT samples (Figure 1A). In WT samples, COL3A1 and RNF12 mRNA levels were observed to be 3.8-fold and 1.3-fold over-expressed relative to CS samples, corresponding to a 74 % reduction of COL3A1 and a 22 % reduction of RNF12 in CS samples compared to WT samples (Figure 1B).

Discussion

PCR SSH was successfully able to distinguish between the fused and wild-type phenotypes at the expressomic level, yielding multiple putative differentially expressed gene products, six of which have been validated. Experimental confirmation with qPCR and the multiple appearances of the same genes in the initial screen support the assertion that the identified differences in expression reflect meaningful biological differences between the conditions examined.

Our analysis identified numerous annotated gene products in which no mutation has been specifically implicated as contributory to the pathobiology of craniosynostosis. Many of these gene products are members of the bone growth and development, maintenance, and turnover pathways. The finding that beta-globin is over-expressed in CS tissues is also likely related to bone physiology: beta-globin expression would be expected to be higher in bone marrow where its expression is intrinsically linked to erythropoiesis (51). It is possible that beta-globin upregulation in our CS was due to the thicker bony table of the skull (43), which may contain more diploic bone marrow compared to WT controls.

SPP1 (osteopontin) expression is important in the early differentiation of membranous bone, remodeling of bone, and osteoclastic activity (52). In normal mouse fusing suture models, SPP1 has been shown to be expressed at the osteogenic fronts (53). In a craniosynostotic murine model of Apert syndrome, SPP1 has been shown to be over-expressed in pre-osteoblasts of the calvaria (54) and the limbs (55) compared to normal controls. In humans with diagnosed Apert Syndrome, digit-derived bone was shown to over-express SPP1 compared to polydactyly patients with no known craniosynostotic symptoms (56). SPP1 has also shown increased mRNA expression in murine calvarial derived osteoblasts after stimulation with dihydrotestosterone (57) and retinoic acid (58). Stimulation of a murine suture with FGF was shown to increase SPP1 expression, which was subsequently modulated by blocking the ERK pathway (59). Most recently it was shown that viral transfection of murine cells to express either an Apert or Crouzon mutation of the FGFR2 gene demonstrated increased SPP1 expression when co-culturing Crouzon bone cells with Apert derived dura cells (60). Our data reinforce these findings and support a role for SPP1 in our model, consistent with the increased bone deposition seen in the fused sutures of craniosynostotic rabbits.

SPARC (osteonectin), another gene product recovered in our screen, is important in bone formation, bone cell differentiation and mineralization, as well as extracellular matrix formation (61). It has been suggested that osteonectin expression appears down-regulated when osteopontin is up-regulated (62). This was not the case here, possibly indicating over-expression of multiple factors in the osteoblast pathway.

CTSK (Cathepsin K) is important for osteoclast expression and bone resorption. It has been suggested, however, that CTSK is not necessary for osteoclastogenesis (63). Aberrant

expression of CTSK has been linked to osteoporosis (64, 65). The over-expression observed in our CS model may be indicative of the presence of mature bone. Mature bone has a distinctive bone physiology of cyclical bone deposition and bone resorption. An active growth site is more likely to have greater expression of osteoblastic growth factors. Thus, the over-expression of a growth factor related to osteoclastogenesis is not surprising and rather consistent with the larger volume of bone within the extirpated region compared to WT tissue.

COL3A1 (collagen type III, alpha I) is an extracellular matrix protein found in fibrillar collagen in bone, but more abundant in skin, muscle and blood tissue. Studies have shown COL3A1 to be expressed at higher levels in juvenile mouse dura mater compared with adult mouse controls (66). Here we report under-expression for our CS model, suggesting a potential relationship between COL3A1 presence and suture patency. RNF12 (Ring Finger Protein 12, RLIM) is an E3 ubiquitin protein ligase that acts as a negative coregulator of LIM homeodomain transcription factors. The function of RNF12 in suture formation is still unknown.

Expression of periostin has been linked to TWIST1 expression. Of interest is that haploinsufficiency of TWIST1 (with presumable decreased expression of periostin as well) results in the rare craniosynostosis disorder Saethre Chotzen (67). However, it is also possible that the under-expression in our CS tissues is due to comparative lack of osteoblast activity at the time of harvest (after suture fusion and mature bone formation) than any reflection on the pathogenesis of the condition. CTHRC1 (collagen triple helix repeat containing 1) is specifically expressed in vascular calcifications of carotid artery lesions and may contribute to vascular remodeling of injured arteries (68). The under-expression of CTHRC1 observed for our CS rabbit may be related to the lack of ongoing calcification in the already-fused suture of this model, whereas we may expect more active bone growth and calcification in the suture of the WT control.

The differential expression of our candidate gene products identified in our screen in our naturally occurring CS model appears to be at least in part the result of differences in the normal bone formation/resorption pathway. Some of the genes found to be over-expressed after PCR/SSH analysis in the CS model are associated with osteoclast activity. Some of the under-expressed genes in the CS model are associated with either the osteoblast pathway or calcification proper. With exception of SPP1, none of these gene expression data have been specifically addressed in the literature dealing with molecular expression in human craniosynostosis.

Some studies have begun to elucidate those genes differentially regulated in children presenting with nonsyndromic craniosynostosis. Interestingly, these analyses have revealed candidate genes that are outside the FGF-TGF β -TWIST-MSX paradigm. These candidate genes have included Ephrin A4 (EFNA4), homeobox protein aristaless-like 4 (ALX4), runt related transcription factor (RUNX2), and protein kinase C-binding protein (NELL1) (32). In addition, molecular studies have begun to clarify those genes that may be important for suture patency, including retinol binding protein (RBP4), glypican 3 (GPC3) and complement C1q tumor necrosis factor-related protein 3 (C1QTNF3), and those that appear to be upregulated in fused and fusing sutures including Wnt inhibitory factor 1 (WIF1), annexin A3 (ANXA3) and cytoplasmic FMR1-interacting protein 2 (CYFIP2), from data collected using tissue derived from craniosynostotic children (69). Here in our report, we also identify candidate genes involved with the craniosynostosis pathogenesis in our rabbit model outside the FGF-TGF β -TWIST-MSX paradigm.

The technique we employed in this study, PCR SSH, does have technical limitations and should not be expected to recover every potential differentially expressed transcript (50). It is interesting to note that, in both directions of the PCR SSH, sequences with no clear homology to known gene products were recovered. These may represent truly novel mammalian gene products, rabbit-specific gene products, or simply portions of longer transcripts that are actually recognized and annotated but are unrecognizable from the limited span of the sequence that was cloned. Also, the PCR SSH protocol calls for cDNA construction from the 3' end of transcripts and 3' untranslated sequences are therefore more likely to be recovered. There is a distinct possibility that these 3' untranslated sequences would not be subject to the stronger evolutionary pressure that acts upon coding sequences, and may thus show greater variation (with greater opacity to BLAST alignment). While admittedly imperfect, PCR SSH is able to successfully tease out interesting and valid differences in the expressomes of craniosynostotic versus wild-type tissues, and remains a potentially valuable tool to further investigate the molecular control of bone formation and the molecular pathobiology in this rabbit model system. Once a more complete understanding of these processes is obtained, it may be possible to better relate them to the pathobiology of children with nonsyndromic craniosynostosis.

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Figure 1. Quantitative PCR Results

A) Enriched Craniosynostotic Gene Products confirmed as overexpressed

B) Enriched Wild-Type Gene Products confirmed as overexpressed

Table 1

Quantitative PCR Primer Data

Gene	Primer	Primer Sequence	Taqman Probe	Custom rabbit reverse primer
ABI Pre-made Rabbit probes beta-hemoglobin (HBB2) osteonectin (SPARC) cathepsin K (CTSK)		Oc03823433_s1		GTGGTATTTGTGAGCCAGGGCAATTG
		Oc03395840_m1		ATCACGAGATCCTTGTGCGATA
		Oc03398667_m1		CACAGGGGTTGTTCTTATTCCGAG
Custom Rabbit primers / probes osteopontin (SPP1) collagen 3, alpha 1 (COL3A1) RNF12 GAPDH (43)	F	TGGCTAAACCCCTGACCCATCT	FAM-CAGCCTTTTGAAATTC	AGTGACTTCATCAGACTCATCAGA
	R	CCTTTTCTTCTGAGGACATAGGATTCT		
	F	CAGAACATCACCTATCACTGCAAGA	FAM-CCCACCTGGCCTGATCC	AAACAGGGCCAACGTCCGCACCAA
	R	TCAGCTTCAGGGCTTCTTTACATT		
	F	ATAATGGCTGCCTGAGCAA	6'FAM-CTCTAACCCAGCGCCTGCTCG-TAMRA	TCCGATGAGTCTCTGGTGGAGA
	R	TCCGATGAGTCTCTGGTGGAGA		
	F	CGCCTGGAGAAAGCTGCTAA	6'FAM-AAGCAGGCATCCGAGGGCCCC-TAMRA	CCTCGGTGTAGCCCCAGGAT
	R	CCTCGGTGTAGCCCCAGGAT		

Table 2**Genes Overexpressed in Craniosynostotic Rabbit Samples**

a disintegrin and metalloprotease domain 10 (ADAM10)	lysyl oxidase (LOX)
ADP-ribosylarginine hydrolase (ADPRH)	mortality factor 4 like 1 (MORF4L1)
ADP-ribosylation factor 4 (ARF4)	myocyte enhancer factor 2C (MEF2C)
ATP Synthase, subunit F6 (ATP5J)	nucleoporin 54 (NUP54)
ATP synthase, subunit G (ATP5L)	Osteocalcin (BGLAP)
calnexin (CANX)	osteonectin (SPARC)
carbonic anhydrase II (CA2)	osteopontin (SPP1)
cathepsin K (CTSK)	protein inhibitor of activated STAT 2 (PIAS2)
CD24 molecule (CD24)	protocadherin, beta 2 (PCDB2)
cell cycle progression 1 (CCPG1)	ribosomal protein L24 (RPL24)
ceruloplasmin (CP)	ribosomal protein L26 (RPL26)
chaperonin subunit 3 (gamma) (CCT3)	ribosomal protein S8 (RPS8)
citrate synthase (CS)	SH3 domain binding glu rich protein like (SH3BGRL)
G protein-coupled receptor 89 (GPR89A)	signal sequence receptor, gamma (SSR3)
geranylgeranyltransferase 1, beta (GGT1B)	small EDRK-rich factor 2 (SERF2)
hemoglobin, beta (HBB)	targeting protein for Xk1p2, homolog (TPX2)
homeodomain interacting protein kinase 1 (HIPK1)	tetratricopeptide repeat domain 3 (TTC3)
interferon-induced protein like (IFIT1L)	thioredoxin interacting protein (TXNIP)
kelch-like 24 homolog (KLHL24)	thymosin, beta 10 (TMSB10)
KIAA1432	trafficking protein, kinesin binding 2 (TRAK2)
Kruppel-like factor 3 (KLF3)	WD repeat domain 92 (WDR92)
lysozyme (LYZ)	zinc finger protein 277 (ZNF277)

Table 3**Genes Overexpressed in Wild Type Rabbit Samples**

actin-like 6A (ACTL6A)	myristoylated alanine-rich protein kinase C substrate (MARCKS)
asporin (ASPN)	non-metastatic cells 7, protein expressed in (NME7)
beta-2 microglobulin (B2M)	periostin, osteoblast specific factor (POSTN)
cell cycle associated protein 1 (CAPRIN1)	peroxidasin homolog (PXDN)
cell division cycle 26 (Cdc26) homolog (CDC26)	phospholipase D1 (PLD1)
collagen triple helix repeat containing 1 (CTHRC1)	platelet-derived growth factor receptor-like (PDGFRL)
collagen, type III, alpha 1 (COL3A1)	procollagen, type XI, alpha 1 (COL11A1)
collagen, type V, alpha 2 (COL5A2)	prolyl4-hydroxylase, alpha polypeptide II (P4HA2)
cytochrome oxidase subunit IV isoform 1 (COX4I1)	ring finger protein 12 (RNF12) (RLIM)
damage specific DNA binding protein 1 (DDB1)	SEC61 gamma (SEC61G)
DEAD box polypeptide 5 (DDX5)	sirtuin homolog 1 (SIRT1)
decorin (DCN)	SMT3 suppressor of mit two 3 homolog 2 (SUMO2)
dihydrolipoamide dehydrogenase (DLD)	SNW domain containing 1 (SNW1)
dihydropyrimidinase-like 2 (DPYSL2)	solute carrier family 25, member 4 (SLC25A4)
ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2)	S-phase kinase-associated protein 2 (p45) (SKP2)
eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	stress-associated endoplasmic reticulum protein 1, SERP1
Ewing sarcoma breakpoint region 1 (EWSR1)	suppressor of cytokine signaling 6 (SOCS6)
Forkhead box protein N3 (FOXN3)	tissue specific transplantation antigen P35B (TSTA3)
forty-two-three domain containing 1 (FYTTD1)	translocated promoter region (TPR)
golgi transport 1 homolog B (S. cerevisiae) (GOLT1B)	tubulin, beta (TUBB)
HERPUD family member 2 (HERPUD2)	Twinfilin homolog 1 (TWF1)
immunoglobulin superfamily, DCC subclass, member 4 (IGDCC4)	tyrosyl-tRNA synthetase (YARS)
karyopherin (importin) beta 1 (KPNB1)	ubiquinol-cytochrome c reductase binding protein (UQCRB)
Lectin, mannose-binding, 1 (LMAN1)	ubiquitin-conjugating enzyme E2 variant 2 (UBE2V2)
member RAS oncogene family (RAB14)	UDP-glucose dehydrogenase (UGDH)
mitochondrial ribosomal protein L11 (MRPL11)	voltage-dependent anion channel 1 (VDAC1)
mitochondrial ribosomal protein L33 (MRPL33)	YME1-like 1 homolog (YME1L1)
mitochondrial ribosomal protein L47 (MRPL47)	zinc finger E-box binding homeobox 2 (ZEB2)
moesin (MSN)	zinc finger, C3H1-type containing (ZFC3H1)
mult inositol polyphosphate histidine phosphatase, 1 (MINPP1)	zinc finger, DHHC-type containing 6 (ZDHHC6)