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Discovery of Epigenetic Biomarkers for the Non-Invasive Diagnosis of Fetal Disease

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Abstract

Objectives—The primary goal of this study was to identify CpG sites in the human genome that are differentially methylated in DNA obtained from chorionic villus samples (CVS) and gestational age-matched maternal blood cell (MBC) samples.

Methods—We used the HumanMethylation27 DNA Analysis BeadChip to characterize DNA methylation in samples of CVS and MBC. We then selected a subset of differentially methylated CpG sites on chromsome 13 and subjected them to analysis by mass spectrometry using the Epityper platform.

Results—We identified 718 tissue-specific differentially methylated regions (DMRs) between MBC and CVS. 563 of these were hypermethylated in MBC and hypomethylated in CVS whereas 155 sites were hypomethylated in MBC and hypermethylated in CVS. Further analysis of 13 DMRs on chromosome 13 by Epityper confirmed the microarray data and provided us with additional data about the methylation patterns of surrounding CpG sites.

Conclusions—Analysis of the resulting data identified a large number of CpGs that are potential biomarkers for the selective amplification of fetal DNA from maternal plasma and the subsequent non-invasive detection of trisomy 13.

Keywords

Fetal; DNA; Plasma; DNA Methylation; Aneuploidy; NIPD

Introduction

Every year in the USA, thousands of procedures are carried out for the prenatal diagnosis of fetal aneuploidy. These conventional methods require the direct collection of fetal materials through invasive procedures such as amniocentesis and chorionic villus sampling (CVS). Unfortunately these procedures are associated with a risk of miscarriage that has been reported to be somewhere between 0.1–1.0% (Mujezinovic and Alfirevic, 2007, Tabor and

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Alfirevic, Tabor et al., 1986, Odibo et al., 2008). Not surprisingly these methods are also a source of considerable parental stress and anxiety (Hewison et al., 2007, Hewison et al., 2006, Hertling-Schaal et al., 2001). Several screening methods have been developed to stratify pregnant women according to their risk of carrying a fetus affected by chromosomal aneuploidy. These include various combinations of ultrasonography and biochemical screening of maternal serum (Malone et al., 2005). However, these methods are targeted at epiphenomena associated with the chromosomal aneuploidy rather than the underlying core molecular abnormalities. Significantly they also have limited accuracy, with strictly defined gestational age windows that must be used for specific tests. For example the standard first trimester screen, involving measurement of pregnancy-associated plasma protein (PAPP-A) and free beta subunit of human chorionic gonadotropin (hCG) combined with ultrasound measurement of nuchal translucency, has a detection rate for trisomy 21 of between 87–82% between 10–13 weeks gestation with a false positive rate of 5% (Malone et al., 2005).

Developments in the non-invasive analysis of fetal nucleic acids have begun to transform the clinical practice of prenatal care. For example, methods for the detection of paternally inherited fetal alleles in maternal plasma are so robust that they are now widely used for the prediction of fetal Rhesus D blood group status (Chiu et al., 2005, Lo, 2000, Lo, 1999) and have also been used for the diagnosis of paternally inherited thalassemia and achondroplasia (Li et al., 2006, Li et al., 2007, Li et al., 2005, Saito et al., 2000). Not surprisingly there is considerable interest in the development of similar methods for the non-invasive detection of fetal aneuploidy and recessive monogenic diseases. However, the most significant obstacle preventing progress towards this goal is the fact that maternally inherited fetal alleles are identical in primary sequence to their endogenous maternal counterparts. To overcome this, recent advances in the field involve the use of high throughput shotgun DNA sequencing for the non-invasive detection of aneuploidy (Fan et al., 2008, Chiu et al., 2008, Chu et al., 2009a). Although these methods show great promise, they require expensive and complex instrumentation and are relatively low in throughput (Tong et al., 2010). Therefore there is still considerable interest in the development of specific biomarkers for the non-invasive detection of aneuploidy that may interface effectively with established analytical platforms such as real time PCR or mass spectrometry for the non-invasive prenatal detection of aneuploidy.

One strategy to identify such biomarkers for non-invasive detection of aneuploidy relies upon the observation that maternally-derived nucleic acids that are present in plasma originate largely from maternal leukocytes whereas maternally circulating fetal DNA and RNA are derived from syncytiotrophoblastic microparticles or other trophoblast-derived apoptotic bodies (Hasselmann et al., 2001, Gupta et al., 2004). It has been shown that differences in DNA methylation patterns between samples of placental villus and paired maternal leukocytes can be used to identify uniquely placental methylation marks which, if subsequently identified in maternal plasma, can be assumed to be of placental origin (Chan et al., 2006, Tong et al., 2006, Lo et al., 2007, Papageorgiou et al., 2009, Chu et al., 2009b, Old et al., 2007). These unique features of placental nucleic acids can then be used for the selective recovery of trophoblast-derived nucleic acid and subsequent fetal genetic analysis. Selective recovery may be achieved, for example, via methylation specific PCR following bisulphate conversion (Tong et al., 2006, Della Ragione et al., 2010, Tong et al., 2009) or

methylation sensitive restriction digestion (Brown et al., 2010, Tong et al., 2010, Della Ragione et al., 2010) or via immunoprecipitation of methylated fetal DNA (Papageorgiou et al., 2009, Papageorgiou et al., 2011). Aneuploidy can then be confirmed or excluded by quantifying levels of locus specific markers on chromosomes of interest, for example, via the analysis of allelic ratios at heterozygous fetal polymorphisms (Tong et al., 2006), locus specific quantitative PCR (Papageorgiou et al., 2011, Tong et al., 2009, Della Ragione et al., 2010, Tong et al., 2010) or via microarray based analysis (Brown et al., 2010).

In the current study we have utilized a commercially available microarray based platform to identify cytosine-guanine dinucleotides (CpGs) that are differentially methylated between maternal blood cells (MBCs) and chorionic villus tissue. These loci have potential as biomarkers for selective amplification of fetal DNA in maternal plasma and subsequent detection of aneuploidy (Tong et al., 2006, Tsui et al., 2011).

Materials and Methods

Tissue Handling and DNA Extraction

All samples used in this study were discarded de-identified tissues. CVS were obtained between gestational weeks 11 and 13 from the Magee Womens Hospital Cytogenetic Screening Laboratory. All samples were confirmed to have normal euploid karyotypes using standard cytogenetic techniques. Samples were dissected under a microscope and separated from any decidual tissue or flecks of blood. The culture media was removed and the tissue was stored in 1.5ml centrifuge tubes at −80°C until use. To extract DNA, one 5mm stainless steel bead and 180μL buffer ATL (from Qiagen's DNeasy Blood and Tissue kit, Qiagen, Valencia, CA) were added to each CVS sample. The samples were placed in the TissueLyser (Qiagen) Adaptor set 2 × 24, and the TissueLyser was operated for 20 seconds at 30Hz. The DNA was then purified using the DNeasy Blood and Tissue kit as per the manufacturer's protocol. MBCs were obtained between gestational weeks 11 and 13 from the Magee Womens Hospital Prenatal Screening lab. DNA was extracted from the MBC's using a modified protocol previously described by Iovannisci, *et al*., 2006 (Iovannisci et al., 2006), using reagents from the MasturePure DNA Purification Kit (Epincentre Technologies, Madison, WI, Cat. No. MCD85201). Briefly, clotted blood (approximately 1mL) was mixed with an equal volume (1mL) of 2X Tissue and Cell Lysis Solution, vortexed for 10s and combined with 2mL Tissue and Cell Lysis Solution (MasturePure kit) containing 25ng/μL proteinase K. 2mL of MPC Protein Precipitation Reagent was added to the total volume (4mL) of the lysed sample and vortex vigorously for 10–15 sec, after which samples were cooled on ice for ≥ 1 hour. Cell debris were then pelleted by centrifugation (×2) for at least 30 min at ≥ 2000g and supernatants transferred to a new 50mL conical tube. DNA was precipitated in 2 volumes of isopropanol, purified by phenol/chloroform extraction and resuspended in 50μL DNAse/RNAse free water.

Infinium Microarray Analysis

The HumanMethylation27 DNA Analysis BeadChip (Illumina) allows interrogation of 27,578 CpG sites based on the NCBI CCDS database (Genome Build 36) and also targets the promoter regions of 110 miRNA genes. Bisulfite conversion of DNA was carried out

using the EZ DNA Methylation™ Kit (Zymo Research Corp., Irvine, CA) to convert unmethylated cytosine nucleotides to uracil. Following denaturation with 0.1N NaOH, converted DNA samples were amplified by incubation at 37°C for 20 hours in a proprietary amplification reaction mix. Amplified DNA was fragmented using vendor-supplied reagents by incubation for 1 hour at 37°C. Fragmented DNA samples were precipitated and resuspended in hybridization buffer. Infinium BeadChips were cleaned and activated by washing with ethanol, formamide and vendor supplied pre-hybridization buffers. DNA samples were denatured, applied to the Infinium arrays and hybridized 16–24 hours with rocking at 48°C. The BeadChips were placed into a flow-through chamber, unhybridized and non-specifically hybridized DNA were washed away, and single base extension was performed on bound primers with labeled nucleotides. Hybridized DNA samples were removed by washing using proprietary buffers. Staining steps were performed to attach fluorescent dyes to the labeled nucleotides and the array surface was sealed to protect the dyes from atmospheric degradation. The final array was scanned using an Illumina BeadArray Reader and the data analyzed using Bead Studio 2.0.

Determination of the methylation status of CpG sites using Infinium Array Data

On an Infinium array, each targeted CpG site was interrogated by 2 probes: probe A to determine hypomethylation level and probe B to determine hypermethylation level. The A probe signals and B probe signals were normalized separately, using the cyclic loess algorithm (Wu). We then computed the log ratio of probe B to probe A, log(B/A), as well as the beta value, which was defined as approximately B/(A+B+100), assuming A, B≥0. Both beta and log(B/A) can be used as a measurement of the methylation level of a CpG site. In particular, a CpG site was hypomethylated if the log (B/A) value of that site was significantly lower than 0. It was hypermethylated if log(B/A) is significantly higher than 0. Student's t tests were used to test if a CpG site was methylated in a group of samples, or if two groups of samples had identical methylation rates at a given CpG site. Empirical Bayesian method proposed in Smyth (2004) was used to estimate the within group variance. P values were adjusted using Benjamini and Hochberg's method to control the false discovery rate (FDR) at 5%.

Sequenom Epityper/Quantitative Methylation Analysis

Quantitative Methylation Analysis was performed using the MassARRAY Compact system (Sequenom, San Diego, CA). 1ug genomic DNA was bisulfite converted using the EZ DNA Methylation (Zymo Research) as per manufacturer's instructions. PCR primers were designed by MethPrimer (www.urogene.org/methprimer) to flank the CpG regions of interest and were purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences are presented in Table 1. Amplicons for use with the MassARRAY system are typically 200–600 bp in length and are independent of the methylation state of the genomic DNA, meaning they bind to both methylated and non-methylated template (as opposed to methylation-specific primers). Amplification of 1 μL bisulfite treated DNA (20 ng/μL) was performed using HotStar Taq Polymerase (Qiagen) and primers at a final concentration of 200nM each in a 5 μL reaction volume using a 384 well plate. PCR amplification was performed in a PTC225 thermal cycler (MJ Research, Inc.) with the following parameters: 94°C for 15 min hot start, followed by denaturing at 94°C for 20 sec, annealing at 62°C for

30 sec, extension at 72°C for 1 min for 45 cycles, and final incubation at 72°C for 3 min. The PCR products were then treated with shrimp alkaline phosphatase (SAP), *in vitro* transcribed and analyzed according to the manufacturer's instructions (Sequenom).

Results

We performed a genome wide analysis of DNA methylation in first trimester CVS samples and gestational age matched MBCs using the Infinium "humanmethylation27" platform marketed by Illumina. This platform targets 27,578 CpGs mostly contained within CpG islands and well characterized promoter sequences that are spread throughout the genome. Using this method we analyzed DNA samples obtained from 12 CVS samples and 12 MBC samples.

Of the 27,578 CpG sites targeted by the Infinium array, we identified 563 that were hypermethylated in MBC and hypomethylated in CVS versus 155 sites that were hypomethylated in MBC and hypermethylated in CVS (Supplementary Tables 1A and 1B, respectively). These can be considered to be tissue-specific differentially methylated regions (DMRs).

Because the Infinium microarray contains probes for CpG loci on all human chromosomes, we filtered the data to identify DMRs that are located on chromosomes 13, 18, 21 and X. CpG sites on these chromsomes that were found to be hypermethylated in MBC and hypomethylated in CVS and visa versa are shown in Table 2A and 2B respectively.

To further explore these DMRs we selected a subset of differentially methylated CpG sites on chromsome 13 and subjected them to analysis by mass spectrometry using the Epityper platform (Sequenom) (Supplementary Table 2). These loci were chosen for this analysis because of the relative lack of information in the literature relating to potential DMR biomarkers of aneuploidy on chromosome 13. As shown in Supplementary Table 3 and Figure 1, DMRs identified by Infinium analysis were confirmed by the Epityper approach in most cases. Furthermore, considerable information about the methylation state of flanking CpG sites was provided by the Epityper analysis (Figure 1).

Discussion

The primary goal of this study was to identify CpG sites in the human genome that are differentially methylated in DNA obtained from chorionic villus samples (CVS) and gestational age-matched maternal blood cell (MBC) samples. As demonstrated previously, CpG sites identified in this way are potential biomarkers for the selective amplification of fetal DNA from maternal plasma and subsequent detection of fetal trisomy (Tsui et al., 2009, Tong et al., 2009, Tong et al., 2006, Papageorgiou et al., 2011, Della Ragione et al., 2010). When analyzing our data we focused our efforts on identifying differentially methylated CpG sites located on chromsomes 13, 18, 21, X and Y because these are most commonly found aneuploidies in human populations. Therefore biomarkers on these chromosomes have the greatest potential clinical utility. The resulting data are provided for the research community as a resource with which to develop novel biomarkers for the future diagnosis of fetal genetic disease and complex placental dysfunction during early gestation.

Such biomarkers could be potentially be assayed in a clinical context by a variety of previously demonstrated methods, which involve the selective recovery of fetal DNA either via methylation specific PCR after bisulphate treatment (Tong et al., 2006, Della Ragione et al., 2010, Tong et al., 2009) or methylation sensitive restriction digestion (Brown et al., 2010, Tong et al., 2010, Della Ragione et al., 2010) or via immunoprecipitation (Papageorgiou et al., 2009, Papageorgiou et al., 2011). These approaches could then be followed by one of a number of methods for the detection or exclusion of aneuploidy. Previously published examples include the detection of allelic ratio changes at heterozygous biallelic fetal loci (Tong et al., 2006), quantitative PCR in which a marker or multiple markers on chromosomes of interest are quantified relative to a control sequence(s) (Papageorgiou et al., 2011, Tong et al., 2009, Della Ragione et al., 2010, Tong et al., 2010) or microarray based analysis (Brown et al., 2010). Work is currently underway in our laboratory to explore some of these, and other, approaches.

One negative aspect of our study design is the relatively small number of loci (n = 27,578) interrogated by the Infinium microarray. Unlike a gene expression microarray, in which reasonably comprehensive genome wide data can be obtained with a small number of probes representing each gene, methylation analysis ideally requires much higher resolution so that methylation status of many individual CpGs per gene can be determined. Recent developments in high-throughput DNA sequencing of bisulfite converted genomic DNA promise significant developments in this area (Cokus et al., 2008). Unfortunately the Infinium platform contains very few (<4) CpG-targeted probes per target gene and these are located primarily in promoters and/or CpG islands. However, whereas this limitation might be problematic in the context of a functional genomics study, in which the goal is to investigate the relationship between DNA methylation and gene expression at high resolution, it is less of an issue in the context of biomarker discovery in which the goal is merely to identify DMRs.

A second negative aspect of our study is the fact that some of the “sentinal” CpGs (that is, those identified as being differentially methylated by the microarray) were not assayable by our Sequenom assay. We believe this to be a minor drawback, however, because in all these instances we identified flanking CpG sites that were differentially methylated. In only one instance we found that the sentinal CpG was not identified as differentially methylated by the Sequenom assay (cg11679069). In the case of most CpGs that were assayed we found that there is potential for their further development as biomarkers for fetal chromosome 13-specific enrichment. Such potential is based upon the fact that, with the exception of cg2584155, the tissue specific difference in methylation is large with, ideally, one or both tissues displaying a methylation rate of or approaching 100% or 0%. In one instance (cg22398616) we found hypermethylation in CVS versus hypomethylation in MBC and the exact opposite pattern (hypermethylation in MBC versus hypomethylation in CVS) at adjacent CpG sites. This locus may have potential for development of an internally controlled biomarker in which maternal and fetal alleles could be targeted in parallel.

Previous studies have identified a number of potentially significant DMRs in the context of non-invasive fetal diagnosis (Tsui et al., 2010, Della Ragione et al., 2010, Brown et al., 2010, Lo, 2009, Chim et al., 2008, Tong et al., 2006, Chu et al., 2009b, Papageorgiou et al.,

2009). The study presented here is the first to describe the identification of DMRs between CVS and MBC in a genome wide fashion and, to our knowledge, is the first to confirm CpG-specific differential DNA methylation using targeted single locus assays directed towards chromosome 13 markers using samples from multiple pregnancies.

In summary, we have undertaken a genome wide analysis of DNA methylation in CVS and MBC samples obtained from pregnant women between gestational weeks 10 and 13. Analysis of the resulting data identifies a large number of CpGs that are potential biomarkers for the selective amplification of fetal DNA from maternal plasma and the subsequent non-invasive detection of trisomy 13.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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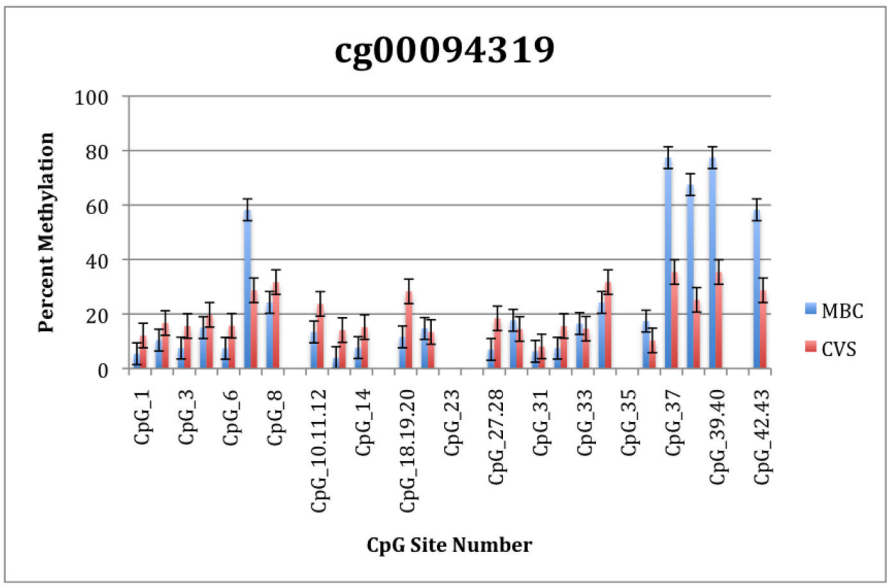
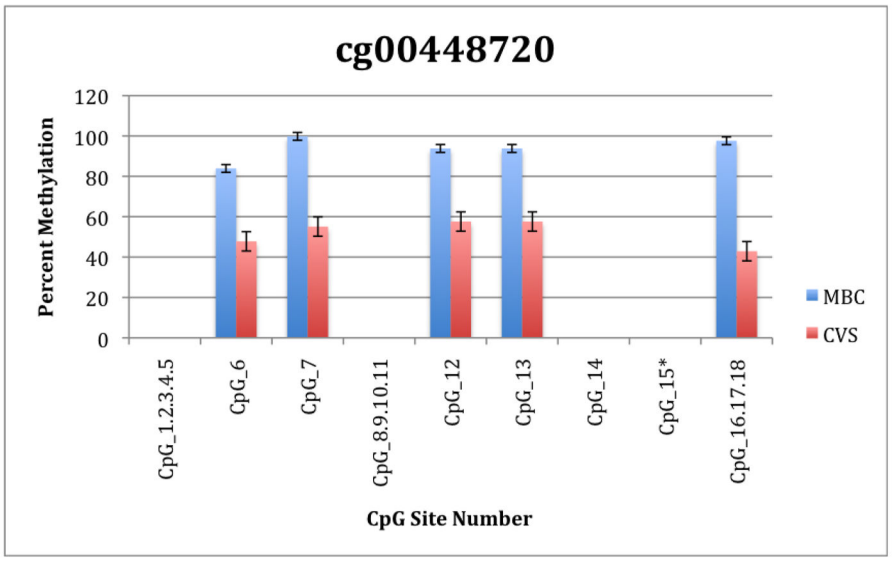
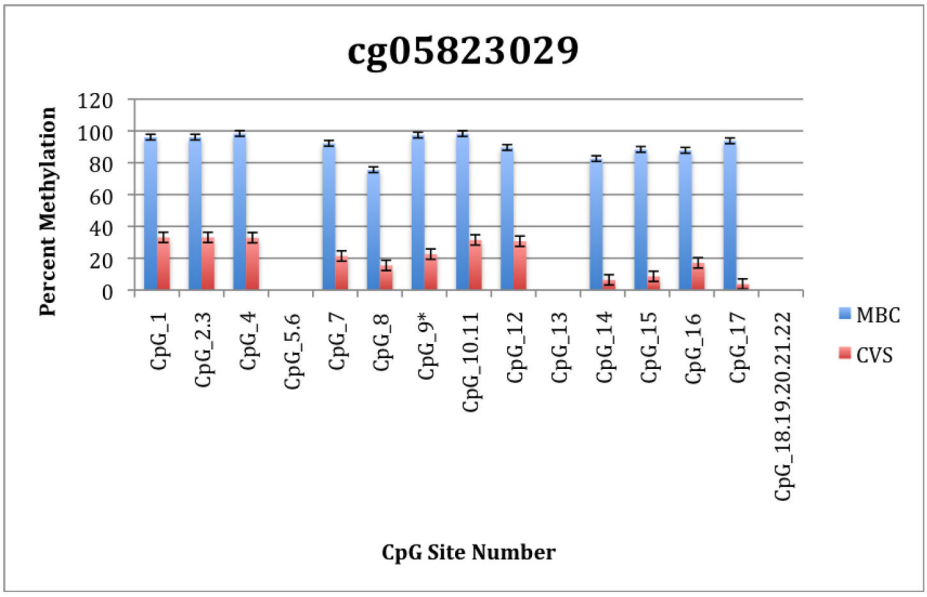
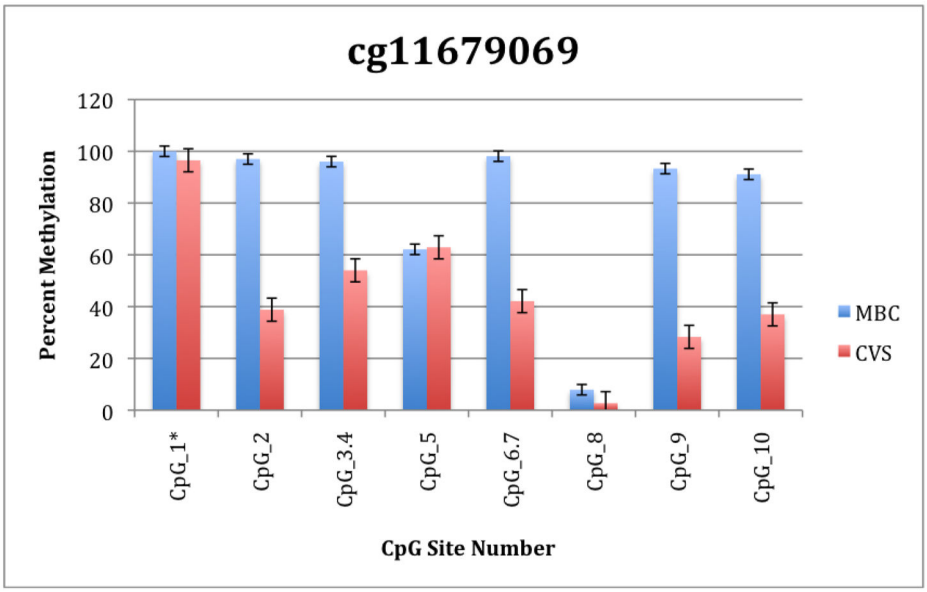
What is already known/what does this study add?

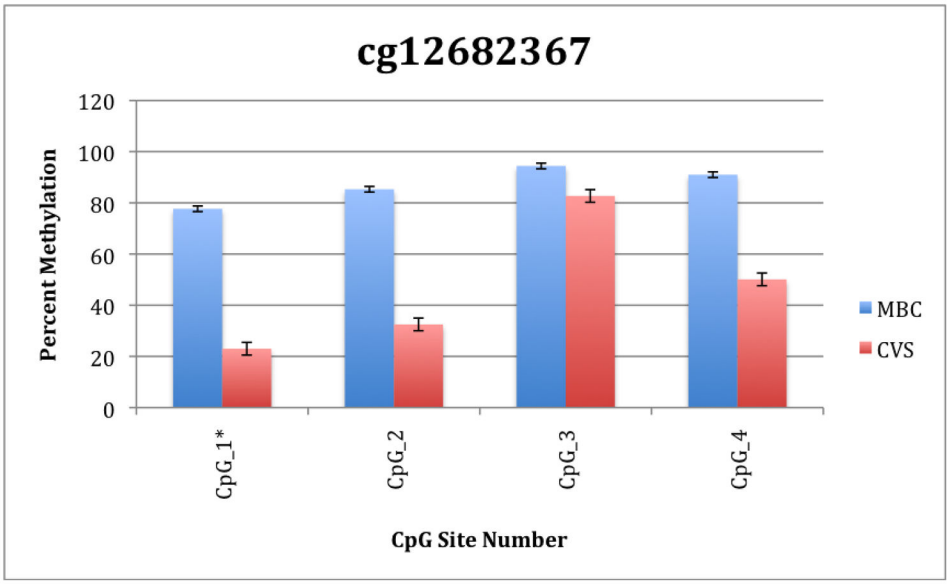
1. Differential DNA methylation between placenta and maternal leukocytes has been exploited for the specific amplification of fetal DNA from maternal plasma.
2. Previous efforts to identify differentially methylated loci have largely focused on chromosomes 21, 18, 13 and X. In previous studies, follow up analysis using targeted approaches for single loci have generally not focused on chromosome 13 markers.
3. We present a systematic analysis of DNA methylation in a genome wide fashion, with follow up of specific markers on chromosome 13.
4. The currently described discovery platform has not previously been used in this context

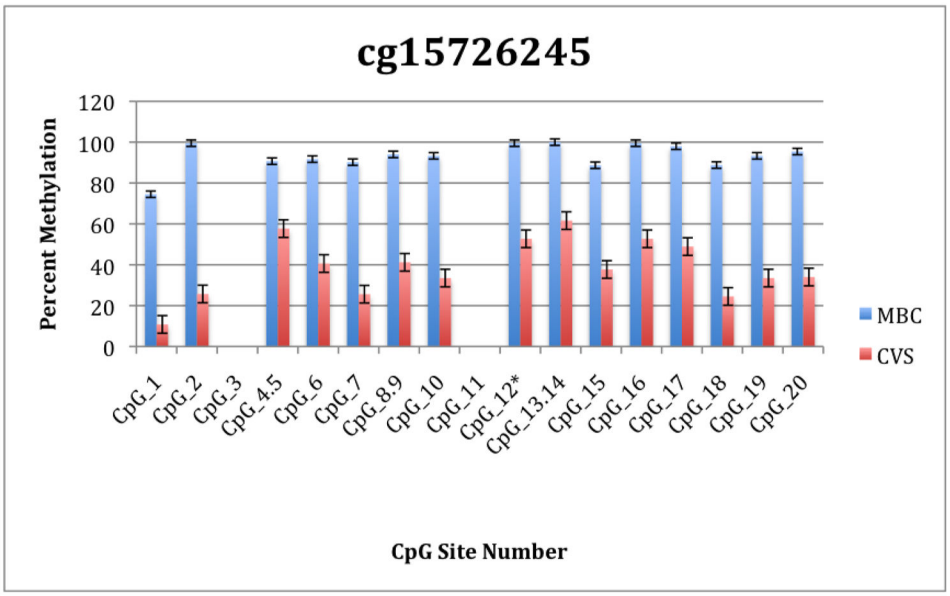


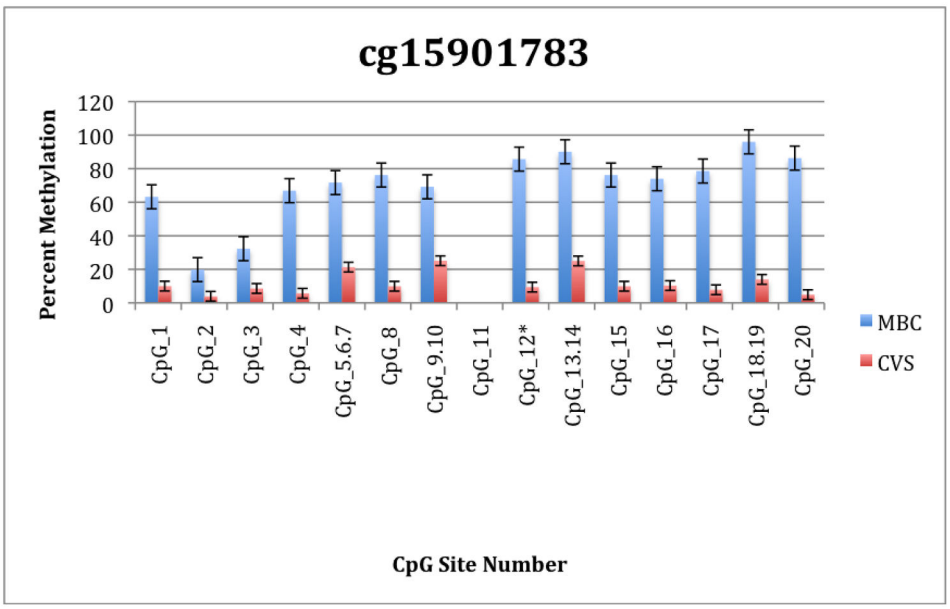


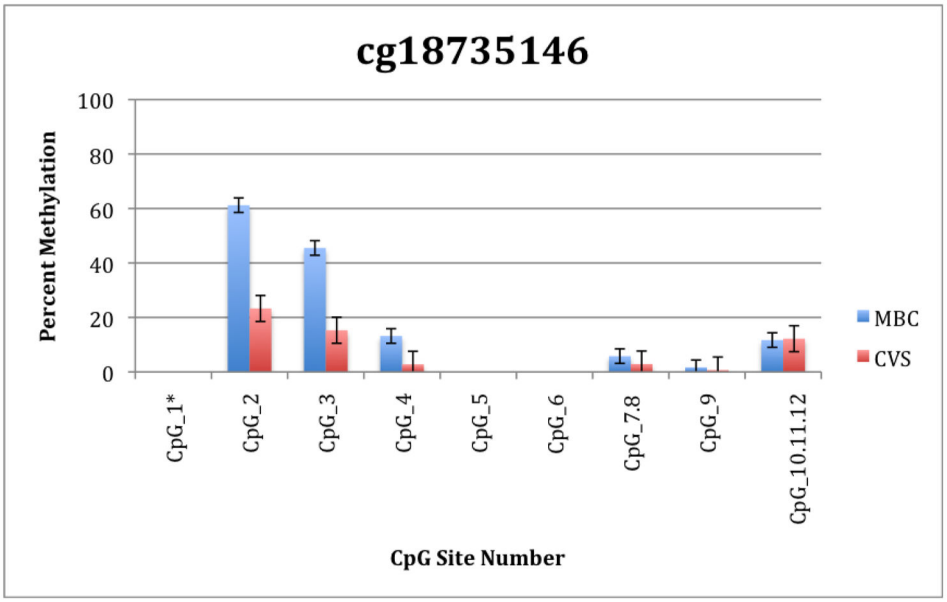


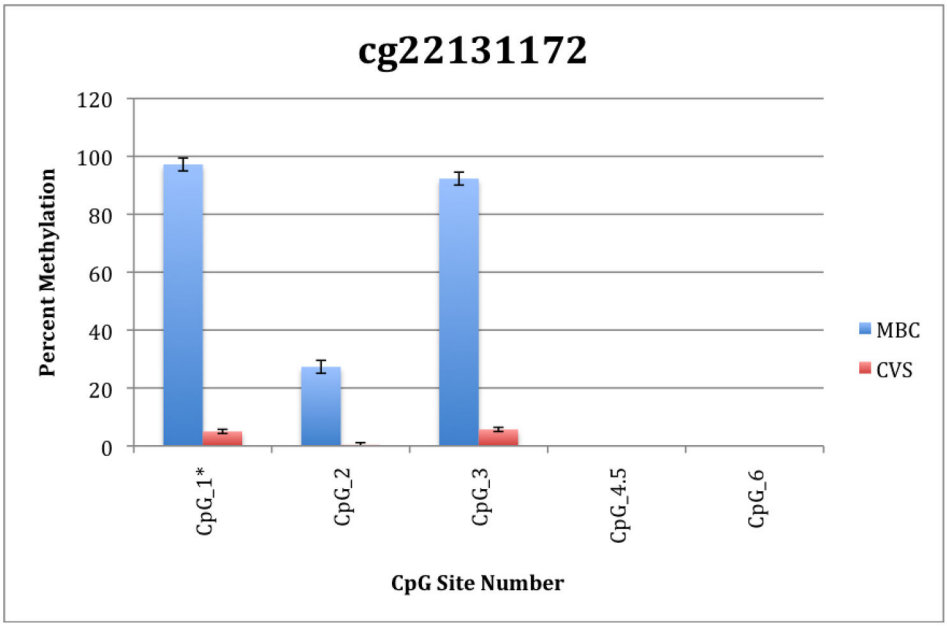


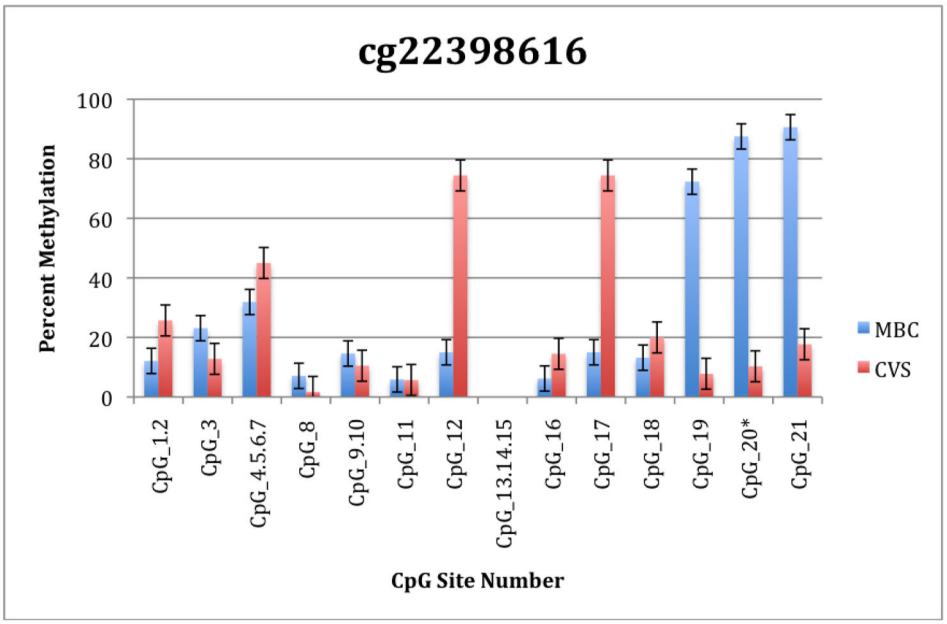


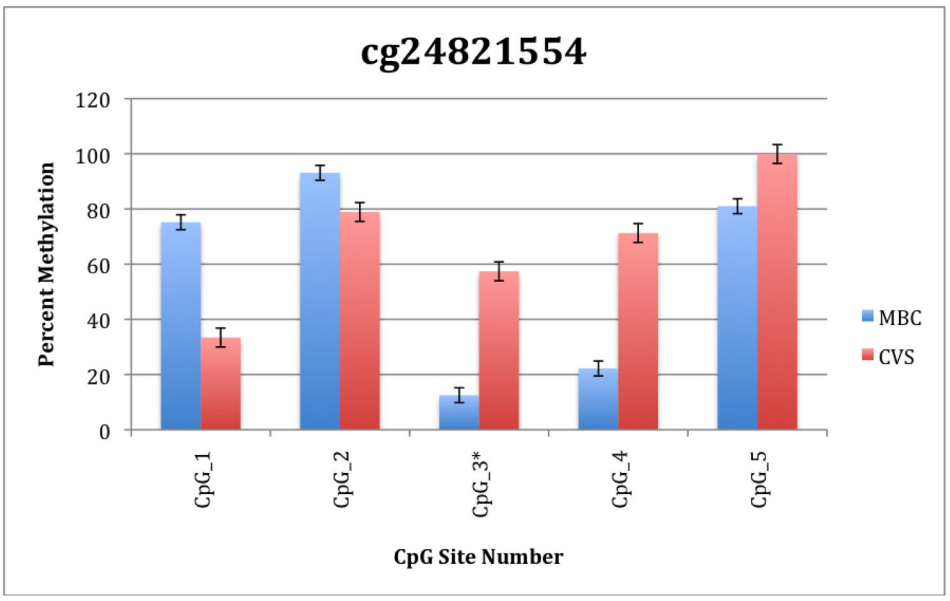


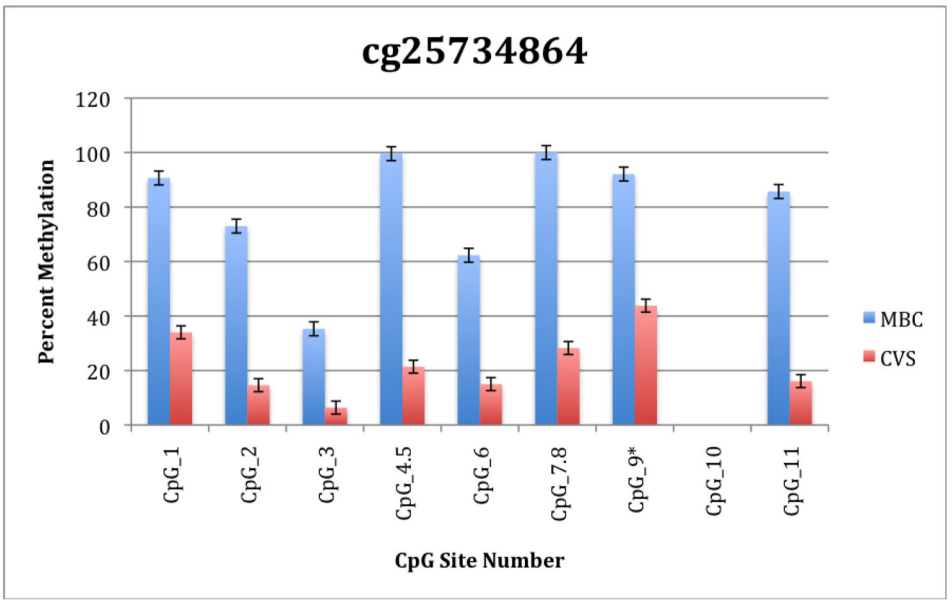












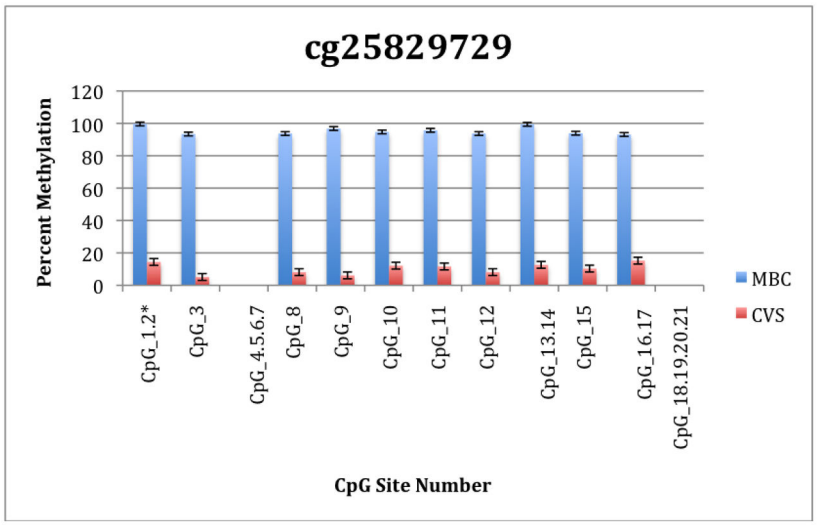


Figure 1. Epityper Analysis of MBC and CVS

DNA from 10 CVS samples and 10 MBC samples was isolated, bisulfite converted and assayed for methylation by Epityper at 13 different loci on chromosome 13. Each graph represents a different locus. For each locus, the number of CpG sites analyzed varies based on successful assay design. Multiple CpGs in a short span of the genome were read as one value. The sentinel CpG that was assayed by the microarray analysis is marked by an asterisk (*). CpG site numbers that have no data indicates that these sites were present but not assayed for by epityper. The percent methylation is the average for each of the 10 samples per sample group, and the error bars are the deviation between samples within the group. Chart names (eg “cg00094319”) correspond to CpG sites listed in Tables 2A and 2B.

Table 1

Primer Sequences for Epityper Analysis

Primer Name	Primer Sequence
Pet_cg22131172_F	AGGAAGAGAGTTTGTTTTGGGTATGGAAAATGTT
Pet_cg22131172_R	CAGTAATACGACTCACTATAGGGAGAAGGCTAAACCAAAATCATACCACTACACTCC
Pet_cg25734864_F	AGGAAGAGAGTTAGGGTTTATTTTATTTTTATTGATTA
Pet_cg25734864_R	CAGTAATACGACTCACTATAGGGAGAAGGCTAATACCTACCTAAAAACTCCACAC
Pet_cg05823029_F	AGGAAGAGAGGAAGGATTTTAGGGAGAGGTTAGG
Pet_cg05823029_R	CAGTAATACGACTCACTATAGGGAGAAGGCTCCTACCAACCCAACTCCAACTAC
Pet_cg25829729_F	AGGAAGAGAGTGGTAGATAATTGAGGAAGTTATGGT
Pet_cg25829729_R	CAGTAATACGACTCACTATAGGGAGAAGGCTCCTAAAAACCCCCAAAACAACTTA
Pet_cg00448720_F	AGGAAGAGAGGGTTTTTGTTTTTGTTTTGTG
Pet_cg00448720_R	CAGTAATACGACTCACTATAGGGAGAAGGCTAAATTCAATATCCATTCCCCTCTC
Pet_cg15726245_F	AGGAAGAGAGGTGGTTAGGAAGTGGTGTTGTAGG
Pet_cg15726245_R	CAGTAATACGACTCACTATAGGGAGAAGGCTTCAATCAAAAACCACCAAACTAATC
Pet_cg15901783_F	AGGAAGAGAGTTGGTTGTAGAGATAAAATGAGATGAAG
Pet_cg15901783_R	CAGTAATACGACTCACTATAGGGAGAAGGCTACCAAAAAAAACTTCCAAAAAAAA
Pet_cg22398616_F	AGGAAGAGAGTTTTTTGGGTATTTTGGGATTTGT
Pet_cg22398616_R	CAGTAATACGACTCACTATAGGGAGAAGGCTAACCTCCAACCAATTATCACAACA
Pet_cg00094319_F	AGGAAGAGAGAGAGATTAAGGTTTTTTTGTTGTT
Pet_cg00094319_R	CAGTAATACGACTCACTATAGGGAGAAGGCTATTCATAATATCCAAAACATACTC
Pet_cg11679069_F	AGGAAGAGAGTTTTTTTTGTTAGTTGATTTTGTAAAGG
Pet_cg11679069_R	CAGTAATACGACTCACTATAGGGAGAAGGCTAACTCACTACACCCTCTACCTCCC
Pet_cg12682367_F	AGGAAGAGAGGATTTTGTTTGGATGAGGGGTATT
Pet_cg12682367_R	CAGTAATACGACTCACTATAGGGAGAAGGCTACAAAAACAACTCCCCAACCTTAT
Pet_cg18735146_F	AGGAAGAGAGATGATTTAGGTTTTAGGTGGTTGG
Pet_cg18735146_R	CAGTAATACGACTCACTATAGGGAGAAGGCTAAATACCCAAAAAATAATAATAAACTTA
Pet_cg24821554_F	AGGAAGAGAGTGTGATTTGTAGTTGTGTAGTTTTGG
Pet_cg24821554_R	CAGTAATACGACTCACTATAGGGAGAAGGCTTCAACACCCTTAACCCTAACTTTC

Each forward primer (F) is tagged with: aggaagagag

Each reverse primer (R) is tagged with: cagtaatacgactcactatagggagaaggct

Table 2A

SYMBOL	ACCESSION	CHROMOSOME	MAPINFO	cg#	PRODUCT
STS	NM_000351.3	X	7147332	cg00596686	steryl-sulfatase precursor
RAI2	NM_021785.2	X	17731018	cg20154346	retinoic acid induced 2
NLGN3	NM_018977.2	X	70281268	cg02345317	neuroligin 3
MAGEA9	NM_005365.4	X	148670955	cg09607232	melanoma antigen family A; 9
AR	NM_000044.2	X	66680170	cg14807303	androgen receptor isoform 1
TMPRSS3	NM_024022.1	21	42689424	cg25608949	transmembrane protease; serine 3 isoform 1
TPTE	NM_013315.2	21	10013225	cg02148834	transmembrane phosphatase with tensin homology isoform alpha
KCNJ15	NM_002243.3	21	38550993	cg18248112	potassium inwardly-rectifying channel J15
KRTAP19-5	NM_181611.1	21	30797319	cg07374637	keratin associated protein 19-5
KRTAP15-1	NM_181623.1	21	30734946	cg16812893	keratin associated protein 15-1
KRTAP13-4	NM_181600.1	21	30724700	cg14062083	keratin associated protein 13-4
KRTAP11-1	NM_175858.2	21	31175631	cg07014174	keratin associated protein 11-1
C21orf70	NM_058190.2	21	45183130	cg20884362	hypothetical protein LOC85395
SETD4	NM_017438.2	21	36373719	cg08632701	hypothetical protein LOC54093 isoform a
KIAA0179	NM_015056.1	21	43902770	cg05965188	hypothetical protein LOC23076
C21orf6	NM_016940.1	21	29314090	cg05406101	hypothetical protein LOC10069
DNMT3L	NM_013369.2	21	44507956	cg27076046	cytosine-5-methyltransferase 3-like protein isoform 1
CXADR	NM_001338.3	21	17805938	cg00744433	coxsackie virus and adenovirus receptor precursor
BACH1	NM_001186.2	21	29592287	cg09143663	BTB and CNC homology 1 isoform a
TCEB3C	NM_145653.2	18	42811633	cg02432101	transcription elongation factor B polypeptide 3C
TCEB3C	NM_145653.2	18	42811405	cg16907024	transcription elongation factor B polypeptide 3C
SERPINB5	NT_025028.13	18	59308458	cg07331725	serine (or cysteine) proteinase inhibitor; clade B (ovalbumin); member 5
SERPINB5	NM_002639.2	18	59295157	cg20837735	serine (or cysteine) proteinase inhibitor; clade B (ovalbumin); member 5
SERPINB5	NM_002639.2	18	59295230	cg08411049	serine (or cysteine) proteinase inhibitor; clade B (ovalbumin); member 5
SERPINB5	NT_025028.13	18	59308595	cg06682185	serine (or cysteine) proteinase inhibitor; clade B (ovalbumin); member 5
ENOSF1	NM_017512.2	18	703200	cg16112050	rTS beta protein
KCTD1	NM_198991.1	18	22474735	cg10539808	potassium channel tetramerisation domain containing 1
METTL4	NM_022840.2	18	2562965	cg06454226	methyltransferase like 4
C18orf16	NM_153010.3	18	22699132	cg00729275	hypothetical protein LOC147429
DSC1	NM_024421.1	18	26996631	cg18356799	desmocollin 1 isoform Dsc1a preproprotein

SYMBOL	ACCESSION	CHROMOSOME	MAPINFO	cg#	PRODUCT
CTAGE1	NM_022663.1	18	18251801	cg02847216	cutaneous T-cell lymphoma-associated antigen 1 isoform 2
APCDD1	NM_153000.3	18	10444085	cg19264571	adenomatosis polyposis coli down-regulated 1
TTC10	NM_006531.2	13	20038606	cg18735146	Tg737 protein isoform 2
PROZ	NM_003891.1	13	112860987	cg25734864	protein Z; vitamin K-dependent plasma glycoprotein
KCTD12	NM_138444.2	13	76359427	cg15901783	potassium channel tetramerisation domain containing 12
PABPC3	NM_030979.2	13	24568530	cg00094319	poly(A) binding protein; cytoplasmic 3
LECT1	NM_007015.2	13	52212204	cg22398616	leukocyte cell derived chemotaxin 1 isoform 1 precursor
TEB1	NM_017826.1	13	35686667	cg25829729	hypothetical protein LOC54937
FLJ46358	NM_207439.2	13	23421698	cg12682367	hypothetical protein LOC400110
MGC48915	NM_178540.3	13	23781014	cg00448720	hypothetical protein LOC338872
LOC283487	NM_178514.3	13	110320866	cg22131172	hypothetical protein LOC283487
GPR12	NM_005288.1	13	26231506	cg15726245	G protein-coupled receptor 12
DNAJC15	NM_013238.2	13	42494577	cg11679069	DNAJ domain-containing
ATP4B	NM_000705.2	13	113360759	cg05823029	ATPase; H+/K+ exchanging; beta polypeptide

Table 2b

SYMBOL	ACCESSION	CHROMOSOME	MAPINFO	cg#	PRODUCT
KCNE1	NM_000219.2	21	34806378	cg03801286	potassium voltage-gated channel; Isk-related family; member 1
ONECUT2	NM_004852.1	18	53254351	cg02250594	one cut domain; family member 2
GUCY1B2	NM_004129.2	13	50537954	cg24821554	guanylate cyclase 1; soluble; beta 2