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Evaluation of Allelic Expression of Imprinted Genes in Adult Human Blood

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Abstract

Background: Imprinted genes are expressed from only one allele in a parent-of-origin dependent manner. Loss of imprinted (LOI) expression can result in a variety of human disorders and is frequently reported in cancer. Biallelic expression of imprinted genes in adult blood has been suggested as a useful biomarker and is currently being investigated in colorectal cancer. In general, the expression profiles of imprinted genes are well characterised during human and mouse fetal development, but not in human adults.

Methodology/Principal Findings: We investigated quantitative expression of 36 imprinted genes in adult human peripheral blood leukocytes obtained from healthy individuals. Allelic expression was also investigated in B and T lymphocytes and myeloid cells. We found that 21 genes were essentially undetectable in adult blood. Only six genes were demonstrably monoallelic, and most importantly, we found that nine genes were either biallelic or showed variable expression in different individuals. Separated leukocyte populations showed the same expression patterns as whole blood. Differential methylation at each of the imprinting control loci analysed was maintained, including regions that contained biallelically expressed genes. This suggests in some cases methylation has become uncoupled from its role in regulating gene expression.

Conclusions/Significance: We conclude that only a limited set of imprinted genes, including IGF2 and SNRPN, may be useful for LOI cancer biomarker studies. In addition, blood is not a good tissue to use for the discovery of new imprinted genes. Finally, lymphocyte DNA methylation status in the adult may not always be a reliable indicator of monoallelic gene expression.

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Introduction

For the majority of expressed human genes there is both a maternal and a paternal contribution in the offspring. The exception to this is the small subset of imprinted genes that are expressed only from one parental allele or the other. Imprinted genes are important during human development, as demonstrated by the severe syndromes caused by a loss of imprinting (LOI), i.e., the expression of both parental alleles, or the complete loss of expression. Examples include disorders of restricted growth, as seen in Silver-Russell syndrome and over-growth in Beckwith-Wiedemann syndrome, which in most cases result from opposing imprinting disturbances on chromosome 11p15.5 [1]. Imprinting is thought to have evolved in placental mammals as an adaptive mechanism to promote or suppress fetal growth in utero [2]. LOI is also seen at high levels (30–100%) in malignant cells in at least 16 different human cancers [3]. At present, LOI is considered to be the most abundant and precocious alteration in cancer [4,5]. LOI is regarded as a potentially valuable diagnostic tool. Tests based on analysis in adult blood samples of allelic expression of the imprinted IGF2 gene in the diagnosis of colorectal cancer have become the subject of commercial development [6].

Currently there are approximately 47 protein coding and long, non-coding RNA genes known whose imprinted status in humans has been verified using parent-of-origin in vivo expression analyses. In addition, there are a large number of imprinted micro- and SNO RNA molecules (http://igc.otago.ac.nz/). Recently, several genome-wide screens have been carried out, revealing a great many more potentially imprinted transcripts, although these require validation [7–10]. To date the majority of confirmed imprinted genes are situated in the genome in clusters. This genomic organisation allows their expression to be collectively
regulated by a nearby imprinting control region (ICR) or differentially methylated region (DMR), which is methylated on one allele but not the other. This methylation is established in the germline during gonad development of the previous generation and controls the allele specific expression [11]. The imprinted expression of some genes is restricted to specific tissues, and is either: not expressed at all elsewhere e.g. PHLDA2, whose expression is limited to the placenta [12]; expressed biallelically elsewhere e.g. GNAS which in the human is ubiquitously expressed but imprinted only in the thyroid, ovary and pituitary gland [13]; or expressed from the opposite allele in different tissues e.g. GRB10 which is paternally expressed in brain but maternally expressed in the placenta [14]. For other genes, imprinted expression is found in the majority of tissues during fetal development, as in the case of IGF2, but becomes biallelic in some tissues in the adult [15]. Regulation of this temporal and tissue specificity is likely to be brought about through transcription factor binding to alternative promoters and enhancers. Once expression is enabled, imprinting can be cellular-specific mechanisms promote or prevent transcription of individual alleles. This control may include antisense transcription of non-coding RNAs, allelic histone modification, post-zygotic DNA methylation, RNA interference and the blocking of enhancers by repressors of boundary elements/transcription factors such as CTCF and YY1 [16].

Imprinted gene expression status in the healthy adult human has for obvious reasons been difficult to characterise. Peripheral blood leukocytes (PBL) are unique as they provide a practical and available resource for both genomic and epigenomic human analyses. Imprinting expression patterns and related mechanisms in the human have largely been extrapolated from experiments in the mouse, although it is clear that there are important differences between the two [11,17]. To fill this knowledge gap, particularly with the growing importance of LOI as a marker in cancer diagnostics, this study was designed to investigate genomic imprinting in adult human PBL, analysing 36 gene transcripts, including representatives from all of the known human imprinted cluster loci and several isolated imprinted genes. Expression of the 36 transcripts was quantified in adult PBL and in normal first trimester placenta, liver and brain. As three of the most important 36 transcripts was quantified in adult PBL and in normal first trimester placenta, liver and brain. As three of the most important factors such as CTCF and YY1 [16].

Non-quantitative RT-PCR
Cycle profiles for a mixed fetal tissue cDNA sample and a mixed PBL sample (n = 50) were carried out using non-quantitative (nq)RT-PCR for all genes and resolved on 1.5% agarose gels stained with ethidium bromide. Gel quantitation was performed using Imagemaster VDS 2.0 (Pharmacia Biotech, Bucks, UK) and amplicon densitometry readings vs cycle plots generated for each gene (data not shown). Mid log-linear phase cycle numbers were then used for allelic expression nqRT-PCR (Table S1).

Quantitative expression of imprinted genes in blood
Total RNA was isolated from two individual adult PBL samples, second trimester fetal brain, placenta and liver samples (each pooled from three fetuses) and adult bone marrow (from six individuals aged 38–59 years). cDNA amplification was quantitated with SYBR green dye using the Applied Biosystems 7500 Fast Real-Time PCR System (Cheshire, UK) for 40 cycles. Delta CTs (ΔCTs) were generated in triplicate of each transcript using GAPDH as an endogenous control. Primer sequences and conditions are listed in Table S2.

Allelic expression analysis
Genotypes were obtained for expressed genes in 50 individuals using a minimum of one exonic SNP for each gene either by digestion or sequencing. Sequences were interrogated using Sequencher™ v.4.6 (Gene Codes Corporation, MI) to distinguish heterozygotes (informative) and homozygotes. Informative samples were analysed for allelic expression using intron crossing primers (Table S3). Sense/antisense pairs (RCNQ1/RCNQ1OT1 and MEST/MESTI1) were analysed independently by ensuring antisense transcript amplicons lay wholly within introns of the sense genes. In addition, the samples informative for RCNQ1OT1 were analysed using strand specific RT-PCR. cDNA was prepared using only B-ACTIN and RCNQ1OT1 reverse oligonucleotides and RT-PCR carried out as before. For lineage-specific analysis, whole blood from three individuals was separated into CD19+ B-lymphocytes (5% total cell count), CD3+ T-lymphocytes (25%) and CD15+ myeloid cells (50–70%) using an autoMACS™ Separator (Miltenyi Biotech, Surrey UK).

No parental genotypes were included so imprinting is inferred from monoallelic expression in heterozygous individuals. Primer sequences and conditions for genotyping and allelic expression analysis are listed in Table S3.
Analysis of differentially methylated regions

Combined bisulphite and restriction analysis (CoBRA; Xiong and Laird, 1997) was performed using sodium bisulphite treated PBL DNA samples (n = 2) and purified using the EZ Gold Methylation Kit™ (ZYMO, Orange, CA). Bisulphite specific primers (Table S4) for each ICR were used with Hotstar Taq polymerase (Qiagen, West Sussex, UK) at 45 PCR cycles and amplicons digested using either Tαp1, Tai1, MboI or BsuUI restriction enzymes (enzymes used for each amplicon shown in Table S4; New England Biolabs). Differentially methylated products were resolved on 3–4% agarose gels and stained with ethidium bromide. For pyrosequencing analysis, each assay included three to eleven CpGs, 12 individuals were analysed. Genomic DNA was bisulphite treated using EZ DNA methylation kit (Zymo Research) and used to generate PCR amplified templates in a two round PCR. The first round used unique forward and reverse primers. The reverse primer contained an M13 sequence, which was used to tag the 5' end with biotin prior to sequencing (specific primers and PCR conditions are available on request). Pyrosequencing was carried out on PSQ HS 96 System and PyroMark MD System using Pyro Gold Reagent kits (Biotage, Uppsala, Sweden). Methylation was quantified using Pyro Q-CpG Software (Biotage, Uppsala, Sweden) that calculates the ratio of converted C's (T's) to unconverted C's at each CpG and expresses this as a percentage methylation. Average methylation across the DMR regions for all CpGs was calculated. Median and IQR methylation for different samples were analysed using Prism Graphpad software.

Results

Analysis of imprinted gene expression levels in adult PBL

In order to study imprinting in adult blood, we first investigated the mRNA expression levels of 36 gene transcripts by quantitative (q)RT-PCR (Fig. 1), which was performed in two unrelated healthy adult individuals. Expression values were based on the amplified product reaching a gene expression threshold (T) following a number of PCR cycles (C), normalised (Δ) to the expression of an endogenous control (GAPDH). The ΔCT value was then used as a measure of relative expression. Gene expression between the two individuals showed a high level of consistency, where ΔCT of each gene for the two individuals differed by two PCR cycles or less for 81% of the genes analysed. Of these, 50% had a difference of only one cycle between the two individuals (Table S1).

Non-quantitative (nq-)RT-PCR was carried out in parallel to qRT-PCR in order to analyse allelic expression on a pool of the PBL cDNA from our 50 individuals. NqRT-PCR was able to generate amplicons that could be reliably analysed by sequencing when the qRT-PCR ΔCT for that gene was below nine in the majority of cases. A ΔCT value of nine is equivalent to $2^{-\Delta CT}$ of 0.0015, and this is indicated on the graph in Fig. 1 by a dotted line. A ΔCT of above nine generally correlated with gene expression that was too low to be amplified by nqRT-PCR and sequenced.

Three genes were expressed at notably high levels: GNAS, SNRPN and IGF2R. Another nine genes, KCNQ1, KCNQ1OT1, NAP1L5, IPW, GNAS EXON 1A, SLC22A18, CDKN1C, MEST.

![Figure 1. Quantitative expression levels and allelic status of imprinted genes in the PBL of two individuals.](image-url)

(i) Allelic expression of the transcripts we could detect with non-quantitative RT-PCR is shown, aligned with the genes from bar graph (ii). Red bars represent monoallelic expression and black bars biallelic expression. Using a maximum of 45 cycles the transcripts we could amplify predominately corresponded to those which on Q-PCR had a ΔCT < 9, shown by the dotted line. The grey bars represent transcripts that could not be detected using non-quantitative RT-PCR methods so could not be assigned an allelic preference. (ii) Gene names are plotted on the x axis, against log(10) 2^-ΔCT (ΔCT = the cycle number at which transcript level reached threshold, corrected to an endogenous control) on the y, using thresholds defined by the linear phase of curves generated from the real-time amplification. ΔCTs for triplicates of each transcript were calculated using a GAPDH endogenous control, thus log(10) 2^-ΔCT of GAPDH = 1. (iii) Outline of the position of each gene within the human genome, demonstrating the extent of each imprinting cluster.

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**ISOFORM 2** and **PEG10** were expressed at a level decreased by approximately ten fold relative to the three highly expressed genes, and the remaining 24 genes decreased by at least 100 fold. In comparison to PBL, fetal placenta, liver and brain expression levels were higher both in magnitude and in representative coverage for 28 of the 36 analysed transcripts. These data indicate that overall, compared to fetal tissues, imprinted genes are not highly expressed in adult blood (Fig. S1). There were eight genes whose expression levels in PBL were comparable to or increased compared to the other tissues; **NAPIL5, PLAGL1 Isoform 2, IGF2R, SLC22A18, KCNQ1OT1, KCNQ1, IPW, SNRPN, TP73** and **GNAS** (Fig. S1).

**Analysis of allelic expression status of imprinted genes in adult PBL**

To investigate the actual imprinting status of genes in adult blood, we genotyped 50 unrelated individuals with respect to common exonic SNPs present in the 15 transcripts that were expressed at sufficient levels for detection by sequencing. This analysis revealed three groups of genes (Fig. 1 and Table 1): Group 1: **PLAGL1 Isoform 2, IGF2R, MEST Isoform 2** (polymorphic), **KCNQ1** and **GNAS** were expressed biallelically. Each of these genes are widely expressed but imprinted only in certain tissues, or are developmentally regulated, so their biallelic status in PBL was not surprising; Group 2: **PEG10, IGF2, SNRPN, NDN, IPW and GNAS** were expressed monoallelically, which was consistent with their reported status in human fetal and adult tissues; and finally Group 3: **NAPIL5, INPFSF_V2** (polymorphic), **SLC22A18** and **KCNQ1OT1** which were expressed biallelically. An apparent allelic bias was present in some of the samples for **NAPIL5** (Fig. 2), although without parental DNA we cannot establish which allele was preferentially expressed. This bias was not present upon separation of the whole blood into individual fractions. Strand-specific RT-PCR of **KCNQ1OT1** transcripts was carried out to ensure no unspliced nascent **KCNQ1** transcripts were amplified, and also showed **KCNQ1OT1** to be biallelic (Fig. S2). Group 3 is distinct from Group 1 as the biallelic expression of the genes in Group 3 was unexpected given their monoallelic expression status in other tissues. Interestingly, of the ten genes that were more highly expressed in blood than fetal tissues (Fig. S1), only **SNRPN** remained monoallelic (**IPW** and **TP73** could not be analysed as they were undetectable by nqRT-PCR).

PBL are a circulating cell population, and are therefore not in contact with a static cell niche. To evaluate changes in gene expression from the time of cell release into the circulation, we also analysed adult human bone marrow samples (n = 6), as the origin

| Locus/Cluster | Transcripts in cluster & accession | UCSC SNP | Methylated DMR* | P of O** | Mean\(\Delta C_T\) | QPCR relative exp | RT PCR | Allelic Expression |
|---------------|-----------------------------------|---------|-----------------|---------|------------------|------------------|--------|-------------------|
| 4q24 NAPIL5   | NAPIL5: NM_153757 rs710834        | Prom MAT\(^T\) | Pat 5.6 | Highest | 33 | 9/9 | B |
| 6q25 PLG1     | PLG1 Isoform 2: NM_001080951 rs9373409 | Prom MAT\(^G\) | B 12.6 | Highest | 45 | 3/3 | B |
| 6q25 IGF2R    | IGF2R: NM_000876 rs614754 | Mat 2.3 | Highest | 35 | 1/1 | B |
| 7q21 PEG10    | PEG10 NM_015068 rs13073 | Pat 9.1 | PI, Li, Br | 36 | 4/4 | M |
| 7q32 MEST     | MEST Iso 2 NM_177524 Pat 7.6 | Br 35 | 8/9 | B; 1/9 | M |
| 10q26 INPPFSF_V2 | NR_0003252 rs3188055 | Prom MAT\(^G\) | Pat 16.0 | PI, Li, Br | 40 | 8/11 | M; 3/11 | B |
| 11p15 KCNQ1   | SLC22A18 NM_183233 rs10480467 | KvDMR MAT\(^G\) | Pat 8.2 | Highest | 40 | 5/5 | B |
| 11p15 IGF2/H19 | IGF2 NM_000612 rs680 | DMR0,1,2 PAT\(^G\) | Pat 11.8 | PI, Li, Br, BM | 40 | 20/20 | M |
| 15q11 SNURF/SNRPN | NDN NM_002487 rs2192206 | Prom MAT\(^T\) | Pat 13.6 | PI, Li, Br, BM | 40 | 3/3 | M |
|               | SNRPN NM_022806 rs705 | Pat 2.4 | Highest | 40 | 3/3 | M |
|               | IPW NR_023915 rs691 | Pat 6.2 | Br, Li | 35 | 5/5 | M |
| 20q13 GNAS    | GNAS NM_000516 rs7121 | 1A Prom MAT\(^G\) | Mat 0.3 | Highest | 33 | 13/13 | B |
|               | Exon 1A X56009 | 1A Prom MAT\(^G\) | Pat 6.7 | PI | 40 | 7/7 | M |

Table 1. Summary of expression levels and allele specificity in human PBL.
of the predominant myeloid cell population in peripheral blood (Fig. S1). Although displaying the same pattern of expression where GNAS, SNRPN and IGF2R are most highly expressed, bone marrow has a lower level of expression of imprinted genes compared to PBL (Tables 1 and S1).

Analysis of allelic expression in separated leukocyte populations

Both imprinted and non-imprinted monoallelic expression can be cell-type specific within certain organs [20,21]. To ensure that a sub-population of biallelically expressing cells was not masking monoallelic expression from other blood lineages we isolated the three predominant cell types found in the PBL population. PBL samples from three informative individuals were analysed with respect to the allelic expression of NAPIL5, PLAGL1 Isoform 1, PLAGL1 Isoform 2, MEST Isoform 2, KCNQ1OT1, SLC22A18, IGF2, SNRPN and NDN in CD19+ B-lymphocytes (5%), CD3+ T-lymphocytes (25%) and CD15+ myeloid cells (50–70%). The biallelic/monoallelic expression pattern of the genes was identical for each cell type and for the total PBL population previously analysed (Fig. 2 and Table 2).

Analysis of differential methylation at imprinting control regions

Monoallelic expression of imprinted genes is controlled by differential methylation at imprinting control regions (ICRs). We therefore investigated a number of the differentially methylated regions (DMRs) known to be associated with the imprinted genes analysed in this study. Each of the 15 chosen DMRs were analysed by combined bisulphite and restriction analysis (CoBRA) in two individuals and were all found to be maintained (Fig. 3).

To quantify DMR methylation we also analysed nine DMRs, and one non-DMR, by pyrosequencing in 12 PBL samples. The 12 individuals analysed included those used for quantification of gene expression (PBLA and B) and demonstrated that neither were outliers, shown in Fig. S3. Threshold levels of methylation in these samples were set as described previously [20]. This allowed analysis of between three and 11 CpGs per region, where CoBRA permits only one or two. Average methylation across the region for the 12 samples was calculated for each DMR and is displayed in Fig. 4. Individual data for each CpG dinucleotide are shown for KvDMR (b), NESP promoter (c), H19 DMD (d) and the non-DMR (e) in Fig. 4, and the remaining DMRs in Fig. S4. In PBL DNA, each of the DMRs analysed revealed intermediate levels of methylation, ranging between 35 and 50%, which is consistent with methylation levels if only one parental allele is methylated. Methylation at a control region which is not a DMR in blood, and is hypomethylated, is shown with median methylation levels of 10%. An adult brain DNA sample was analysed as a reference tissue sample and methylation levels fell within the interquartile ranges of the methylation detected in PBL, with the exception of IGF2 DMR0 and KvDMR. The latter DMR in brain had higher methylation levels than blood, but these were still within normal levels as expected for an imprinted gene. Similarly, IGF2 DMR0 in brain was less methylated compared to PBL but still within the normal threshold levels (Fig. 4). These results together demonstrate the presence of the methylation mark as would be expected...
for imprinted monoallelic expression. However, biallelic expression is observed for two of these regions in PBL.

In silico analysis of a gene expression histone signature
To investigate the possibility that histone modifications could be responsible for the regulation of expression at the promoters of imprinted genes with maintained DMRs, we consulted the online human ChIP database to assess the levels of histone methylation and acetylation [http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.html]. For almost all of the imprinted genes that we found to be expressed in PBL, a histone signature comprising 17 modifications was present. For genes where we could not detect expression, the signature was partially or completely absent (Fig. S5). Of the imprinted genes analysed, two, MEST and PLAGL1, are known to have alternatively expressed, biallelic promoters that are active in PBL only. Expression in all other tissues is imprinted and originates from the respective DMR [22,23]. The histone modification signatures were most pronounced at the PBL-specific transcriptional start sites of MEST and PLAGL1. These PBL-specific promoters were associated with the full complement of modifications

Table 2. Expression and allele specificity in separated myeloid, T and B lymphocyte fractions.

| Gene    | n | Whole PBL | Myeloid Cells | T-Lymphocytes | B-Lymphocytes |
|---------|---|-----------|---------------|---------------|---------------|
| NAP1L5  | 1 | Biallelic | Biallelic     | Biallelic     | Biallelic     |
| PLAGL1 Iso1 | 1 | NE        | NE            | Monoallelic   | Monoallelic   |
| PLAGL1 Iso2 | 1 | Biallelic | Biallelic     | Biallelic     | Biallelic     |
| MEST Iso2 | 1 | Biallelic | Biallelic     | NE            | Biallelic     |
| KCNQ1OT1 | 2 | Biallelic | Biallelic     | Biallelic     | Biallelic     |
| SLC22A18 | 2 | Biallelic | Biallelic     | Biallelic     | Biallelic     |
| IGFB1   | 1 | Monoallelic | Monoallelic | Monoallelic   | Monoallelic   |
| SNRPN   | 1 | Monoallelic | Monoallelic | Monoallelic   | Monoallelic   |
| NDN     | 2 | Monoallelic | Monoallelic | Monoallelic   | Monoallelic   |

M, monoallelic; B, biallelic; NE, not expressed. Three informative individuals were selected, and n numbers are the number of individuals informative for SNP in each respective gene. Expression patterns varied slightly but allele specificity was identical between sub-populations.

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Figure 3. Analysis of differential methylation at imprinted loci using combined bisulphite and restriction analysis (CoBRA). Imprinting control regions at each locus were analysed in two adult PBL samples (PBLC (1) and PBLD (2)) and the presence of a DMR verified by the presence of both undigested and digested amplicons (restriction enzymes used in each case are given in Table S4). Undigested products are representative of unmethylated DNA alleles, where the restriction site is lost through C conversion to T. Digested products indicate the presence of an unmodified CpG dinucleotide in the recognition site, representative of methylated DNA. All differentially methylated regions analysed were maintained, crucially including those ICRs for genes which in our hands were expressed biallelically (b, d, e and o). The DMRs analysed represent both those which are germline (a - e, g, j, i - m, o - p) and somatic (f, h, k and n) in origin, and those methylated on the maternal (a - f, i, m, o and p) and paternal (g, h, j, k and n) alleles.

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associated with high expression, whereas the DMR associated promoters were not.

Discussion

In this study we investigate the use of adult human blood as a tissue to detect expression and imprinting status of a number of known imprinted genes. Of the 36 tested only six were detectably imprinted, with the others not proving to be reliable for LOI studies. Principally this was due to a very low level or complete lack of expression, while others were either biallelic or variably expressed in different individuals, despite maintaining differential methylation at respective imprinting control regions. These biallelic expression data were largely unexpected given the monoallelic status of these genes in the human fetus [17,24]. Previous findings describing extremely low but monoallelic IGF2 expression and robust monoallelic expression of SNRPN in PBL were supported [25,26].

In vivo loss of ICR differential methylation in mice results in a loss of imprinting [27]. In humans, hypo- or hypermethylation of individual loci results in either a loss of, or biallelic, gene expression [28,29]. However, in adult PBL, we show that a lack of imprinting does not correlate with a loss of differential ICR methylation. Discordant methylation and expected imprinted expression at the IGF2-H19 locus has been reported in many cancer studies [30–32]. Our analysis contained both germline DMRs, such as the H19 ICR and somatic DMRs which acquire methylation after fertilisation, such as IGF2 DMR0. Somatic DMRs are more variable in their methylation levels and are also likely to be tissue specific. For example, it has been shown that hypomethylation of DMR0 increases with age and does not result in loss of imprinting of IGF2 in normal individuals [26].
Methylation of the somatic DMR0 is thought to be controlled by, and indicative of, the methylation state of the H19 ICR [33].

These observations are suggestive of an uncoupling of methylation and imprinted expression, indicating that an epigenetic mechanism other than DNA methylation may be involved. This has been observed previously in stem cells [34] and in normal tissues, for example Igf2/Igf2 and H19 are biallelic in certain regions of the brain despite maintenance of the H19 DMD [35,36] and also Kingl expression in mice becomes biallelic after birth despite maintenance of the KvDMR [37]. Recently, a histone signature comprising 17 modifications has been shown to mark the transcriptional start sites of highly expressed genes in T lymphocytes [38]. This histone modification signature correlated almost universally with whether the gene was expressed in PBL or not. Of particular interest are MEST and PLAGLI, which have alternatively expressed, biallelic promoters that are active in PBL only, whereas expression in all other tissues is imprinted and originates from the respective DMR [22,23]. This is strongly associated with histone allocation since the PBL-specific transcriptional start sites of MEST and PLAGLI are associated with the full complement of modifications associated with high expression, whereas they are absent at the DMR associated promoters of these two genes.

The KCNQ1OT1 transcript is paternally expressed, and biallelic expression of this transcript is caused by a loss of maternal methylation at the KvDMR, as occurs in BWS. This usually leads to silencing of the other imprinted genes in this domain (SLC22A18, PHLDA2, CDKN1C, and KCNQ1) [39]. We find KCNQ1OT1 to be universally biallelic amongst PBL samples, however, surprisingly neither SLC22A18, CDKN1C nor KCNQ1 were silenced, and SLC22A18 and KCNQ1 were found to also be biallelic. In addition, methylation at the KvDMR was still within normal ranges for a DMR. Analysis of KCNQ1OT1 and SLC22A18 allelic expression in the separated T-lymphocyte, B-lymphocyte and myeloid cell sub-populations within PBL found the same biallelic expression pattern in each cell type. Recently the KCNQ1OT1 ncRNA is was shown to be dispensable for silencing at this locus, specifically in the mouse embryo, dependent only on DNA methylation at the KvDMR, and possibly subsequent CTCF binding [40]. We suggest that this mechanism also occurs in human adult PBL, explaining the continued expression of KCNQ1 and CDKN1C despite biallelic KCNQ1OT1.

In a highly proliferative and circulatory cell type such as leukocytes it may be favourable to down-regulate imprinted gene expression, as we observe, either through alternative promoter usage or another epigenetic process i.e. enrichment of repressive histone modifications. The disruption of imprinted gene DMRs has been linked with diseases such as cancer and syndromic imprinting disorders [6,41]. Our results do not contradict this, as we find no changes in DMR methylation in the PBL of healthy people when compared to another tissue. Subsequently, changes in methylation at multiple imprinted loci in leukocyte DNA, as observed in some cases of BWS and SRS, remain useful for diagnosis of imprinting disorders [42].

It has been suggested that biallelic expression of imprinted genes such as IGF2 may be a useful predictive marker for risk of colorectal cancer development, even in other, unafflicted tissues such as blood [6]. The monoallelic status in normal adult blood of six of the imprinted genes we analysed potentiates their use as a marker, however, of these only IGF2 has been linked to tumorigenesis. The remaining 30 imprinted genes were either biallelic or below detectable levels in adult peripheral blood leukocytes. Here we demonstrate that many imprinted genes are not highly expressed in blood, and those that are expressed are mostly biallelic. Therefore, a lack of imprinting in adult human blood is both common, normal and in most cases unlikely to provide a useful marker for disease.

**URLs**

Imprinted genes: http://igc.otago.ac.nz/  
Histone Methylation: http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.html

**Supporting Information**

**Figure S1** Comparison of imprinted gene (in alphabetical order) expression in fetal tissues, adult PBL and bone marrow. The expression of genes in PBL was quantitatively compared to that in the following tissues: (in order of graph, from left to right) adult bone marrow (very pale grey), fetal brain (pale grey), fetal liver (dark grey), fetal placenta (dark grey) and adult peripheral blood leukocyte samples A and B (PBLA/B in red). Fetal samples were a mix from two fetuses (Moore fetal tissue cohort) and the adult bone marrow was from a mix of six individuals who had died suddenly (Clontech, CA). The graphs are plotted as in Figure 1, with $y = \log2$-deltaCt. This calculation makes the expression level of GAPDH equal to 1.

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**Figure S2** Strand specific RT-PCR of KCNQ1OT1 cDNA was created as before except random primers were not included and reverse transcriptase was primed using B-ACTIN and KCNQ1OT1 reverse primers. 2 ug RNA was converted to cDNA using MMLV reverse transcriptase and RT- samples created in parallel with MMLV RT omitted. B-ACTIN RT-PCR for 30 cycles confirmed successful cDNA synthesis without DNA contamination. KCNQ1OT1 RT-PCR was carried out for samples informative for the rs231357 A/T polymorphism in KCNQ1OT1 (PBL6, PBL7 and PBL13) using 2 ul of RT+ and RT- for each sample. RT-PCR was carried out for 40 cycles and the amplicons sequenced in the forwards direction. In each case, KCNQ1OT1 was found to be biallelically expressed.

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**Figure S3** Quantitative measurement of methylation at each DMRs in the two individuals for which expression levels were analysed. The two individuals for which we analysed expression of imprinted genes were included in the 12 individuals for which pyrosequencing methylation analysis were carried out on. The max and min bars in Fig. 4 and S4 demonstrate the presence of outliers in the sample, so to check whether these two samples were those outliers - and thus not representative for expression analysis, we created box plots including these samples only (PBLA in Blue, PBLB in Red). Both samples followed the pattern of the analysis for the pool of 12, there were no large variations between the two samples. There was insufficient DNA in PBL2 to allow analysis of the PLAGLI DMR so no data point is present for this sample.

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**Figure S4** Quantitative measurement of methylation at individual CpGs of analysed DMRs A: DIRAS3; B: PLAGL1; C: IGF2 DMR0; D: H19 ICR; E: KvDMR; F: GTL2 Promoter; G: SNRPN; H: MCTS2; I: NESP Promoter J: Non DMR. To quantify DMR methylation we analysed 10 DMRs by pyrosequencing in 12 PBL samples, and compared them to fetal brain. Pyrosequencing allowed analysis of between three and 11 CpGs per region, with methylation levels shown as individual box plots.
for the PBL samples, and a black cross for the fetal brain sample. Average methylation between the 12 PBL samples is shown as a box plot. Each of the DMRs analysed revealed the expected methylation level for a DMR in PBL and in brain. The non DMR region in J was hypomethylated in PBL compared to brain.

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**Table S1** Complete list of QRT-PCR, non quantitative PCR and allelic expression analysis data for all imprinted genes analysed. Prom, promoter; MAT/PAT, maternally/paternally methylated DMR; G/S, DMR established in the germline/post fertilisation; Mat/Pat, maternal/paternal allele expressed; BT, below QPCR detection, i.e. linear amplification was not reached by Threshold, equivalent to a 2-dCt of less than 1x10-12; U, below non-quantitative RT-PCR detection up to 45 cycles; M/B, mono/biallelic; For QPCR: Pl, Placenta; Li, Liver; Br, brain; BM, bone marrow; ND, not done, ‘highest’ indicates that expression relative to the other tissues was highest in PBL. dCtB-dCtA, difference between the dCts of PBLA and PBLB. 2-dCtPBL A/B (3 s.f.), values are shown to 3 significant figures. The fractions in the ‘Allelic Expression’ column indicate the number of biallelic or monoallelic samples there were of the total that were heterozygous (i.e. informative) for each SNP. The number of informative samples represents the total found following genotyping of 50 individuals. *Methylated allele of DMR identified in previously published work, references as shown. ** Parent-of-origin of each gene may be located at http://igc.otago.ac.nz/table.html.

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**Table S2** Quantitative RT-PCR primers for analysis of gene expression levels. PCR carried out using cDNA template, i.e. post reverse transcription reaction (RT-PCR). Gene transcripts, primers and amplicon size are shown. Each reaction was carried out with a Tm of 60°C for 40 cycles. DM = primers designed by Dave Monk, JF = primers designed by Jennifer Frost.

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**Table S3** Allele specific assays for gene expression - detailing SNPs, transcripts, primers and amplicon size. Genotyping, PCR using genomic DNA template; RT, PCR using cDNA template, i.e. post reverse transcription reaction; D, direction; Tm, annealing temperature; JF, primers designed by Jennifer Frost.

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**Table S4** Bisulphite PCR primers for analysis of differential methylation at imprinted differentially methylated regions. Amplicons were subjected to Combined Bisulphite and Restriction (CoBRA) assays and bisulphite sequencing. Primers are specific for bisulphite converted DNA. Where CpG dinucleotides appear in the primer, 1:1 primer mixes are prepared with either a C or a T base in the forwards direction (VG) and a G or an A base in the reverse (CR). Bisulphite PCR was carried out using Tm of 53 °C, except for KvDMR where the Tm was 50 °C, and all for 45 Cycles. The size/enzyme column indicates the restriction endonuclease used in the CoBRA assays. DM = primers designed by Dave Monk, JF = primers designed by Jennifer Frost.

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**Author Contributions**

Conceived and designed the experiments: JMF, DM, AM, PS, GEM. Performed the experiments: JMF, DM, TSM, KW, GEM. Contributed reagents/materials/analysis tools: TSM, LSC, AM, GEM. Wrote the paper: JMF, PS, GEM.

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