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Valeria Romanelli, Julian Nevado, Mario Fraga, Alex Martín Trujillo, Maria Ángeles Mori, et al.. Constitutional Mosaic Genome-Wide Uniparental Disomy due to Diploidization: an Unusual Cancer-Predisposing Mechanism.. *Journal of Medical Genetics*, 2010, 48 (3), pp.212. 10.1136/jmg.2010.081919 . hal-00586981

HAL Id: hal-00586981

<https://hal.science/hal-00586981>

Submitted on 19 Apr 2011

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**Constitutional Mosaic Genome-Wide Uniparental Disomy due to Diploidization:
an Unusual Cancer-Predisposing Mechanism.**

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KEY WORDS: Beckwith-Wiedemann syndrome; paternal uniparental disomy; diploidization; imprinting.

Summary

Molecular studies in a patient with Beckwith-Wiedemann syndrome phenotype who developed two different tumors revealed an unexpected observation of almost complete loss of heterozygosity of all chromosomes. We demonstrate, by means of numerous molecular methods that the absence of maternal contribution in somatic cells is due to high-degree (~85%) genome-wide paternal uniparental disomy. Our observations indicate the genome-wide UPD results from diploidization, and have important implications for genetic counseling and tumor surveillance for the growing number of UPD-associated imprinting disorders.

Beckwith-Wiedemann syndrome (BWS; [MIM 130650]), one of the commonest overgrowth syndromes, is characterized by pre and postnatal overgrowth, hemihyperplasia, macroglossia and an increased frequency of embryonic tumors [1-3]. It is caused, in up to 90% of patients, by a variety of genetic or epigenetic alterations within two imprinting sub domains located on human chromosome 11p15 [4;5]. The largest molecular subgroup (~60-70% of BWS cases) is comprised of patients carrying an epigenetic error at either the *H19*DMR (imprinting control region, ICR1) or *Kv*DMR (ICR2) [6], the regions of differential DNA methylation that regulate the appropriate allelic expression of the paternal *IGF2* and maternal *CDKN1C* genes respectively. Patients may also have paternal uniparental disomy (patUPD11p15) (~10-15%), *i.e.* two paternally derived copies of chromosome 11p15 and no maternal contribution, that results in over-expression of the potent mitogen *IGF2*, and absence of the cell cycle regulator *CDKN1C* [7]. Chromosome rearrangements comprising maternal translocations/inversions or paternal duplications are rare (~2-3% of cases) as are *CDKN1C* mutations (~5-7 %).

Loss of heterozygosity (LOH) may be due either to haploinsufficiency (deletion leading to the lack of contribution of one allele) or uniparental disomy (UPD) when both copies of a chromosome pair originate from one parent. LOH is a common mechanism of tumorigenesis where it indicates the absence of a functional tumor suppressor gene in the lost region. LOH has been described in almost all cancer types [8]. Localized loss of imprinting (LOI) is a common consequence of LOH due to the lack of balanced parental contribution in specific region with imprinting either by deletion/duplications or UPD. As stated above, LOH/LOI due to patUPD11p15 is relatively common in BWS and this molecular subgroup of individuals displays one of the highest risks of cancer [9].

Diploidization means the recovery of the normal diploid human chromosome number ($2n=46$) from an initially haploid cell ($n=23$) or triploid zygote ($3n=69$). Diploidization is a relatively common mechanism in plants and non-mammalian species but it has been unfrequently observed in humans [10-13].

We here report on a woman with BWS phenotype and tumors with almost complete LOH in all chromosomes, showing a near-complete contribution of the paternal genome for the diploid status. Postzygotic diploidization due to failure of maternal DNA replication followed by paternal genome endoreduplication would be the most plausible explanation for the observed findings. Thus, present patient would represent a further example of diploidization in humans.

The diagnosis of Beckwith-Wiedemann syndrome was established at birth in a girl of healthy and non-consanguineous parents because of macrosomia, overgrowth, hypoglycaemia and hemihypertrophy. The patient is included as part of a Research Program in the Spanish Overgrowth Syndrome Registry (IRB-HULP PI446). At birth she weighed 3750 g, and showed capillary malformations in legs, hands and thorax, contracture of fingers, facial asymmetry and hemihypertrophy of left hand and leg. She continued with hypoglycaemia and needed treatment with diazoxide for years. At 16 months she had episodes of convulsions needing valproic acid treatment. She started to walk at 19 months. Language was delayed and her school performance was lower than average for age. At age 9 years she underwent pancreatic resection due to intractable, symptomatic hypoglycaemias. At age 4 years 5 months a grade II mesenchymal Wilms tumor was diagnosed and received a combination of complete nephrectomy (including right adrenalectomy) and 9 months of chemotherapy. She also had renal stones (calcium) needing close follow-up for her unique kidney. Calcium, phosphate, alkaline phosphatase, and PTH were among normal ranges. She has an average height for Spanish standards (163.5 cm; 50th centile). Skeletal surveys were normal except for the slight asymmetry due to hemihyperplasia. Her clinical outcome was then unremarkable until 20 years when an ectopic adrenocortical virilizing adenoma was suspected (and confirmed) due to hirsutism, secondary amenorrhea and the finding at MRI of an abdominal retroperitoneal mass, independent from the left adrenal gland. An excision surgery was followed by complete recovery. Six years later she remains asymptomatic, with normal adrenal function and regular menses. The patient is now 26 years old and works as administrative assistant.

Standard karyotyping along with single nucleotide polymorphism (SNP) and HumanMethylation 27 Infinium arrays, microsatellites, Methylation Specific-Multiplex Ligation dependent Probe Amplification (MS-MLPA), Methylation Sensitive-High Resolution Melting Assay (MS-HRMA), pyrosequencing, and array-CGH were performed on patient's samples (see Supplementary methods).

The diagnosis of BWS due to patUPD11p15 was suspected upon microsatellite analysis in the patient and her parents and confirmed through MS-MLPA (Fig. 1A,B). As expected, MS-HRMA and pyrosequencing of specific CpG dinucleotides in the *KvDMR* and *H19DMR*, further delineated the degree of aberrant methylation reflecting the percentage of the patUPD11p15 (Fig. 1C-F).

SNP-arrays showed an abnormal, unforeseen, unusual pattern not only at chromosome 11 but also in all chromosomes. The Log R ratio (which evaluates the genomic dosage, e.g. deletions/duplications) was between normal ranges in all chromosomes but the allele frequency (evaluation of homozygosity/heterozygosity of each SNP) disclosed an almost complete drift (~85% LOH) to homozygosity in all chromosomes (Fig. 2A and Supplementary Fig. 1). Subsequent microsatellite analyses of several chromosomes confirmed in blood, skin, urine, buccal cells and some tissues (Wilms tumor, adrenal, ceccal appendix) that almost all of the genomic contribution was paternal with a minimum, low mosaic maternal contribution (Supplementary table 1). All tissue showed a similar pattern with different degree of mosaicism. Calculation of the percentage of mosaicism was applied to some tissues and showed an average contribution of ~85% from the paternal genome and ~15% of maternal genome (Supplementary table 1). Analysis of microsatellites of X chromosome in the patient indicated that in most cells both X chromosomes came from the only paternal X chromosome with only a minimum fluorescence of maternal markers (~15%) (Supplementary Fig. 2). SRY and amelogenin amplifications were indicative of absence of Y chromosome in the patient (data not shown).

Using MLPA targeting the Prader-Willi and Angelman syndrome (PW/AS) and Pseudohypoparathyroidism (PHP) loci, and the Illumina Infinium DNA-methylation BeadArray, we were able to clearly show the lack of appropriate allelic methylation in the patient at 14 DMRs located on 7 different autosomes. These included the maternally methylated *SNPRN*, *NDN*, *GNAS XL*, *GNAS EX1A*, *GRB10*, *MEST*, *PEG10*, *PEG3*, *ZAC/PLAGL1*, *L3MBTL*, *KvDMR/KCNQ1* and *DIRAS3*, as well as the paternally methylated *NESP* and *H19* DMRs (Fig. 2B). These results confirm the methylation profiles determined by both the MS-MLPA and MS-HRM assays.

There have been scant reports of genome-wide paternal UPD in the literature [14] and as far as we know our case is the only adult patient reported to date. Three patients with proved genome-wide paternal UPD developed pediatric tumors (pheochromocytoma, hepatoblastoma and pancreatic tumor) [13;15] and another two individuals without tumors have been recently reported [11;16]. These cases presented with combined features consistent in part with known imprinting disorders associated with paternal UPD of specific chromosomes, but the most pronounced phenotypic features in all cases resembled patUPD11/BWS. Other authors have described genome-wide paternal UPD confined to specific tissues but not present in blood [12;17] or exclusively confined to placenta and not to fetal tissues [18-21]. Although, the percentage of uniparental cells has not been calculated in all reports the patient presented herein showed one of the highest mosaicism (~85% in blood, 65-87% in other tissues).

In fitting with these previous reports, our patient showed clinical features of some classical paternal UPD syndromes (Supplementary Fig.4). She showed capillary malformation, hemihyperplasia, macroglossia, Wilms tumor and adrenocortical tumor (patUPD11), linear hypopigmentation, seizures and low-average mental development (patUPD15) and hypotonia (patUPD14). The observation of BWS phenotype in this woman is in agreement with our laboratory findings since patUPD11p15 is observed in about 15-20% of individuals with BWS. Furthermore, both tumors observed in the patient, Wilms tumor at 4 years and extra-adrenal

adrenocortical tumor at age 20, are also prevalent in BWS. Somatic pat11pUPD15 has been encountered in about one third of Wilms tumors [22] and such a tumor is the commonest neoplasia observed in BWS patients encompassing about half of tumors reported in this disorder [2].

Regional or partial LOH is observed in deletions or UPDs. In the present patient, deletion was ruled-out due to normal log R ratios in the CGH-array and SNP-array. In addition, almost complete LOH in all chromosomes observed by means of SNP-arrays, microsatellite analyses, MS-MLPA and MS-HRM is consistent with a mechanism of genome-wide UPD.

Potential mechanisms of genome-wide UPD are diploidization of an initial triploid foetus or a normal fertilization followed by a failure of replication and chromosome condensation of the maternal pronuclei with normal replication and segregation of the paternal chromosome (Supplementary Fig. 5). We discarded diploidization after dispermic triploidy as a possible mechanism because the microsatellite analysis in our patient revealed the involvement of a unique X-bearing sperm. We believe that the most probable and simplest explanation for our case is normal fertilization followed by failure of maternal DNA replication and paternal genome endoreduplication, resulting in androgenetic/biparental mosaicism, with over-representation of the genome-wide paternal UPD cell lines (Supplementary Fig. 5E). This diploidization, the recovery of the normal diploid human chromosome number ($2n=46$) is common in plants and is in some ways similar to “trisomy rescue” observed in UPD, when trisomic cells (47 chromosomes) recover to normality losing the extra chromosome in the next division. The pathological consequences of UPD are identical for a single chromosome as a result of trisomy rescue or for the entire genome due diploidization.

Differences in gene expression between the allele inherited from the mother and that inherited from the father are the result of genomic imprinting, and it is the most frequent molecular aberration in BWS. Thus, BWS patients carrying a mosaic paternal UPD of chromosome 11p15 have increased risk of cancer, mainly Wilms tumor, hepatoblastoma and

others embryonal tumors [2;23]. Further, the frequency of UPD detected in neoplastic tissues varies from 3 to 100% [8]. The patient reported herein developed two different neoplasias: Wilms tumor and adrenocortical neoplasia. Consistent with this observation, chromosome 11p15 UPD is observed in 30-40% and 32% of Wilms and adrenocortical tumors, respectively [22;24].

Aberrant allelic methylation at all loci analyzed was demonstrated in the patient by means of MS-MLPA, MS-HRMA, pyrosequencing and methylation array (Fig.1B-F and 2B). Analysis of the BWS, PW/AS and PHP regions revealed a methylation level partially abnormal due to the mosaic status; such an aberrant methylation led to abnormal imprinting of these loci. Thus, our patient showed typical clinical findings of BWS but not typical features of AS, PHP or transient neonatal diabetes mellitus (TNDM), demonstrating that partial loss of imprinting is sufficient for developing BWS but not sufficient for others disorders which usually requires total LOI to manifest the clinical features of the diseases. In addition, the lack of atypical BWS phenotypic features also suggests that inappropriate methylation at the *MEST*, *L3MBTL*, *GRB10*, *PEG3*, and *DIRAS3*, as revealed by the methylation array, is not associated with obvious dysmorphologies. However, the resultant increase in expression of the paternally expressed imprinted genes, due to biallelic expression, and the loss of maternally expressed transcripts maybe involved in tumorigenesis in this patient.

Because of the patient had two different cancers and she carried genome-wide paternal UPD, she may theoretically have increased risks for additional UPD-related tumors [8]. Thus, her prospective follow-up raised some important questions because there are no published recommendations for clinical evaluation schedule and survey for these individuals. Classic recommendations for cancer surveillance in general population may or may not apply for her, since the genetic background is quite different and any cancer surveillance schedule in this patient should involve the whole body. Finally we empirically opted to evaluate this woman with regular (twice a year) clinical evaluation together with annual body MRI (including breasts),

colonoscopy and a routine laboratory work- up including WBC and serological markers for cancer.

The demonstration of genome-wide paternal UPD has important implications for medical genetics, counseling and clinical oncology. Genomic technologies such as array-CGH, high-density methylation and SNP-arrays should be incorporated in diagnostic workflows of patients with suspected imprinting/UPD disorders presenting with dysmorphic features and/or cancer. Tumor surveillance in these patients is empiric since neither guidelines are available yet nor enough experience has been accumulated for guiding the follow-up. Finally, the mechanism of diploidization should be taken into account in patients with genetic disorders and cancer because diploidization of triploid or haploid fetuses is now a recognizable, emerging mechanism with potential risk of UPD and consequently, high risk of neoplasia.

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Figure 1. Microsatellites, MS-MLPA, MS-HRM and CpG pyrograms of patient's blood sample.

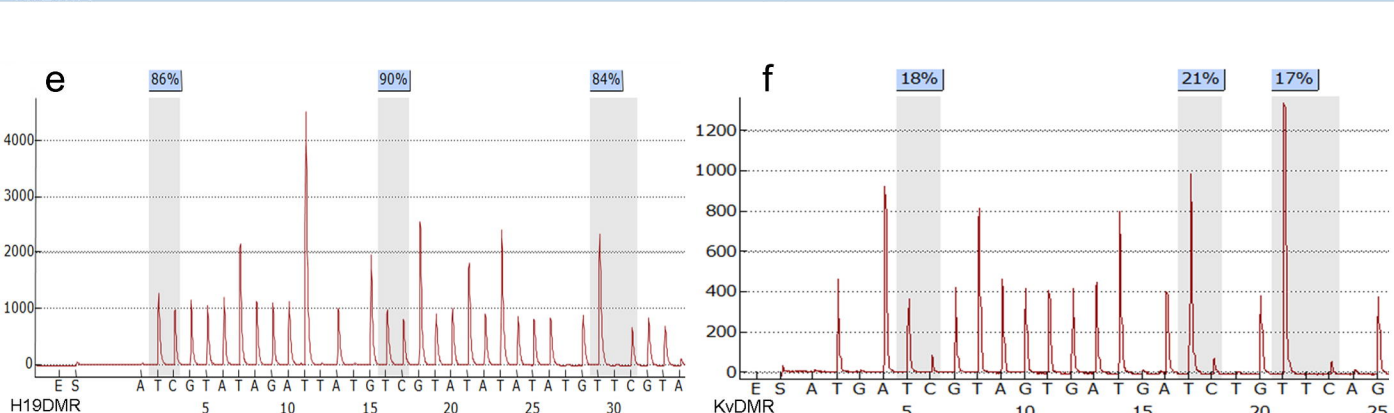
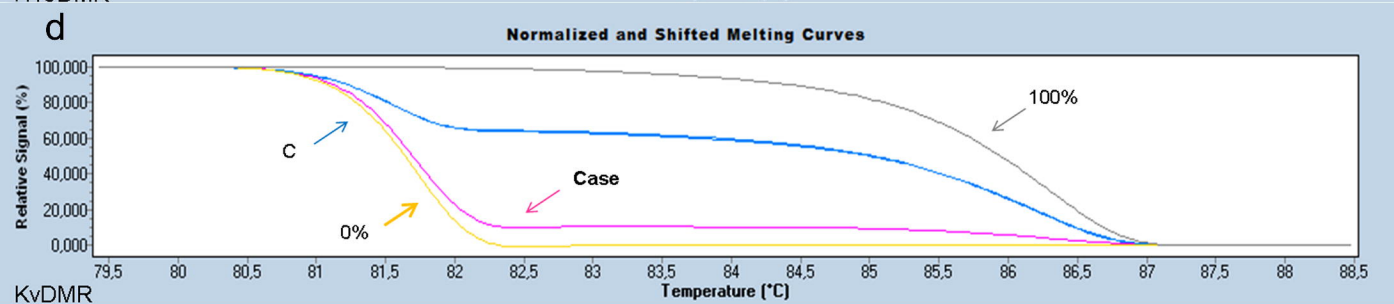
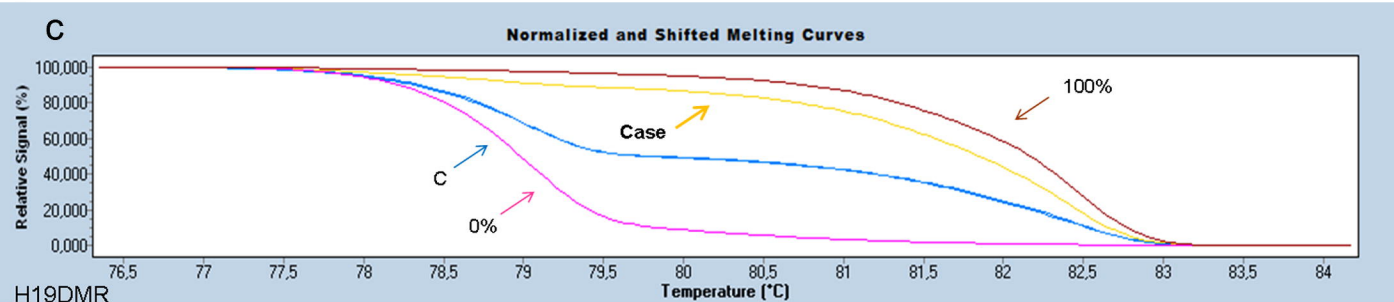
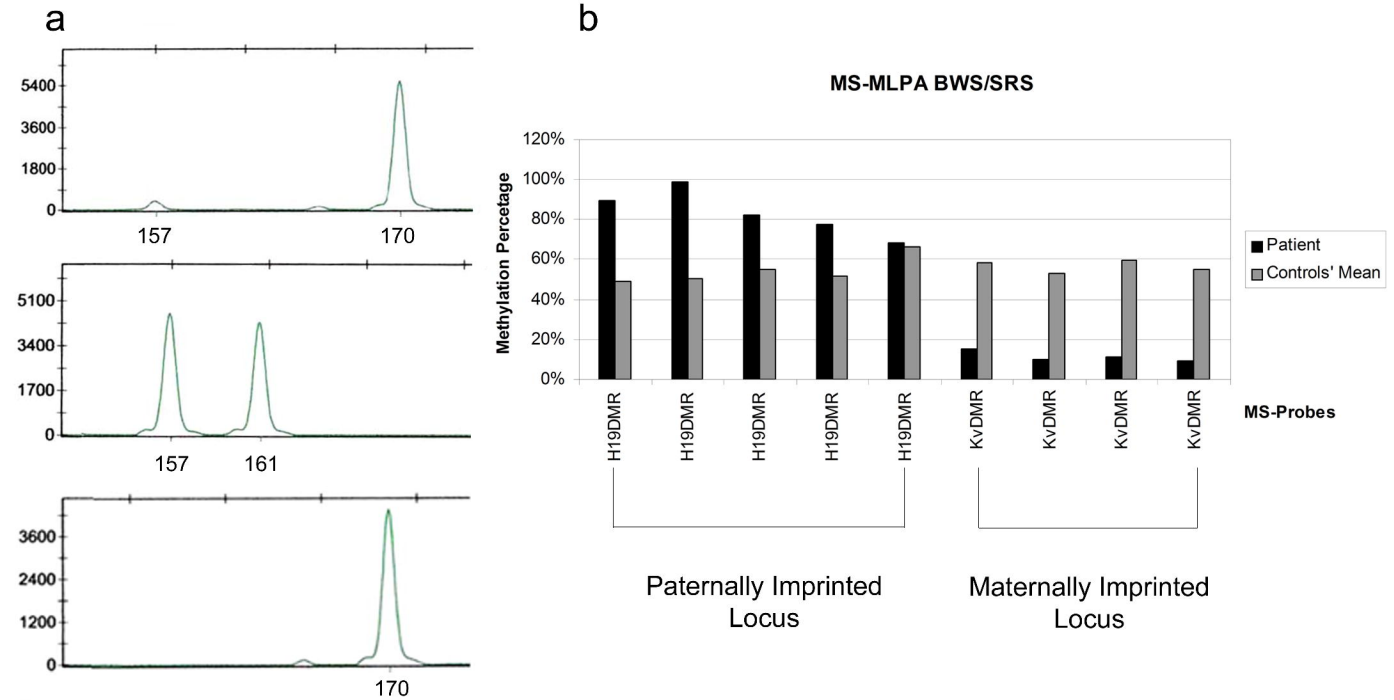
a) Genotypes of one microsatellite marker (THO1; 11p15 region), in peripheral blood from the patient, her mother and her father (from top to bottom). Note that in the patient, the maternal allele (157bp) is barely represented. b) MS-MLPA quantitative analysis of the 11p15 imprinting region in the patient. Black bars represent methylation values of the patient and grey bars represent the mean methylation values of the controls. Note hypermethylation of the 5 probes of *H19*DMR (mean methylation in controls 50.26% vs 89.68% in patient) and hypomethylation of the 4 probes of *Kv*DMR (mean methylation in controls 54.69% vs 11.43% in patient) in the patient, corresponding to patUPD1 1p15. c, d) Normalized melting curves of *H19*DMR and *Kv*DMR, respectively, with curves of controls (C), 0% and 100% methylated DNAs; e, f) CpG pyrogram of *H19*DMR and *Kv*DMR respectively, with methylation percentages calculation for each of the three CpG dinucleotides analyzed. Both analyses further demonstrate hypermethylation of the *H19*DMR and hypomethylation of the *Kv*DMR.

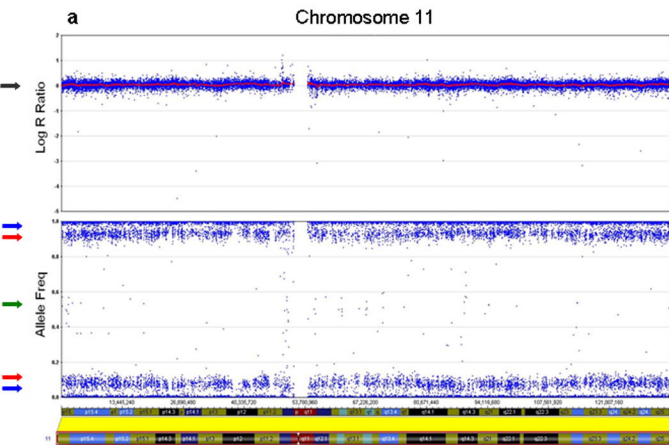
Figure 2. SNP-array of chromosome 11 and methylation profile using Illumina Bead arrays.

a) SNP-array. The Log R ratio was between normal ranges but the allele frequency showed an almost complete drift (~85%) to homozygosity (loss of heterozygosity: LOH). Upper panel: Black arrow showing no abnormal dose observed, ruling-out deletion/duplication. Lower panel: Blue arrows denote homozygous SNPs; red arrows: SNPs in mosaic state tending to homozygosity; green arrow: almost absence of heterozygosity in the sample. b) DNA Methylation profile of several imprinted genes evaluated through Illumina methylation BeadArray in lymphocytes of controls ('lymphocyte') and the patient ('pUPD'). The bar at the right grades from hypomethylation (green; 0.10) to hypermethylation (red; 0.90). Note that the majority of genes in the patient are extremely hypomethylated (green) and *H19*DMR and *NESP* are extremely hypermethylated (red). These results are in agreements with MS-MLPA and MS-HRM.

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Competing Interests: None to declare





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		Lymphocyte	pUPD	
1	DIRAS3			cg05392265
6	PLAGL1			cg25350411
7	GRB10			cg12903171
	PEG10			cg16492735
11	MEST			cg07427065
	H19			cg17769238
15	KvDMR			cg27119222
	NDN			cg13828758
19	SNURF			cg18506672
	PEG3			cg18668753
20	L3MTBL			cg02611863
	NESP			cg24975842
	GNAS XL			cg00943909
	GNAS EX1A			cg25983380

