MAP7D2 is a brain expressing X-linked maternal imprinted gene in humans

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Introductory paragraph

Increasing evidence suggests imprinted genes influence mouse and human behaviors and cognitive functions. Unlike autosomal imprinted genes, X-linked imprinted genes are expressed in a sex-dependent manner because of male hemizygosity. Therefore, these genes could directly affect sex-specific brain functions and sex-biased vulnerability to psychiatric disorders such as autism[1]. Comparing lymphoblastoid cell lines (LCL) and peripheral blood mononuclear cells (PBMC) from healthy adult male and females, we identified MAP7 domain containing 2 (*MAP7D2*) as the first human X-linked imprinted gene. Both in LCL and PBMC, *MAP7D2* expression was significantly suppressed in males by maternal imprinting. In each female LCL clone, *MAP7D2* was expressed higher in paternally derived allele and was affected by X-chromosome inactivation. In female PBMC, however, reactivation of maternal *MAP7D2* alleles was observed. *MAP7D2* was expressed specifically in the brain among human tissues with unique isoforms. These results predict a crucial role of *MAP7D2* for human sex-dependent neurobiological traits.

TEXT

Whilst early work with imprinted genes focused on their roles in feto-placental development and growth, recent studies indicate that many imprinted genes are highly expressed in the brain and affect brain functions[2]. Disorders caused by imprinted genes, i.e. Prader-Willi syndrome and Angelman syndrome,[3,4] manifest with behavioral and psychiatric abnormalities[5,6]. Also, duplications of the maternally inherited 15q11-q13 region, including the Prader-Willi/Angelman syndrome imprinted gene locus, are the most common cytogenetic abnormality observed in autism[7].

Although the allelic expression of autosomal imprinted genes is dependent on the sex of the transmitting parent, expression of these genes does not differ between males and females, and this class of genes cannot directly influence sexually dimorphic phenotypes. However, males inherit a single X chromosome invariably from their mother, and paternally expressed (maternally imprinted) X-linked genes can only be expressed in females and influence sex-specific phenotypes[8]. Supporting this idea, David Skuse and colleagues studied the parent-of-origin effect of the X chromosome on girls with Turner syndrome[9]. They revealed that 45,X^m (maternal X chromosome) subjects had impaired social cognition relative to their 45,X^p (paternal X chromosome) counterparts. In a recent mouse study, the four core genotypes (FCG) model clearly showed a sex difference of behaviors (aggression, parenting, habit formation, nociception, social interactions), gene expression (septal vasopressin), and susceptibility to disease (neural tube closure and autoimmune disease) that were determined by sex chromosome complement and not mediated by fetal gonadal hormone[10]. Several X-linked imprinted genes have been described in mice. In female extraembryonic tissue, the paternal allele of *Xist* is preferentially activated and causes nonrandom X^p inactivation[11]. *Xlr3b*, *Xlr4b* and *Xlr4c* are maternally expressed (paternally imprinted) genes that were identified by comparing the neural gene expression of 39,XO mice, in which the parental origin of the single X chromosome was different[12,13]. *Rhox5*[14] and *Fthl17*[15] are predominantly expressed from the paternal X chromosome in female pre-implantation blastocysts. However, all of these have no human

homologue or are not imprinted in human tissues[1].

To identify human X-linked imprinted genes, we used human derived samples and chose lymphoblastoid cell lines (LCL) established from normal adult males and females. To decrease individual variability, we used pooled samples. We established female LCL from five different individuals and separated each LCL into X^p active and X^m active clones by serial dilution and re-expansion. Because the early stages of established female LCLs are mixtures of X^p or X^m active clones but skew to one clone during a long culture, it is difficult to detect an X-linked imprinting gene affected by X chromosome inactivation. After determining the X chromosome active state of each clone by HUMARA analysis[16], the same amounts of these ten LCL clones from five females were pooled (**Fig. 1 top**). In addition, the same amounts of freshly established LCL clones from ten males were pooled. RNA was extracted from each pool and compared with X-linked gene expression by microarray containing 1833 sequences and 1050 genes of X chromosome (Roche diagnostics) (**Fig. 1 bottom**). From the array data, 121 genes were expressed more than 1.5 fold higher in the female LCL pool with statistical significance ($p<0.05$). Of these 121 genes, 24 were eliminated because of pseudo genes or withdrawn entries in the database (NCBI Gene; http://www.ncbi.nlm.nih.gov/gene). For the remaining 97 genes, a second screening was performed by quantitative reverse transcribed PCR (qRT-PCR). The first screening was performed between female and male pooled LCLs (data in triplicate), and eliminated ten genes with no amplification, 53 genes with average expression were higher in males, and 17 genes were expressed higher in females but less than 1.2 fold in males. For the remaining 17 genes, qRT-PCR was performed for each male and female LCL clone, and the statistical differences were analyzed between sex groups. Throughout these screenings, we found five preferentially female expression genes, *MAP7D2, MSL3, HSD17B10, RPS4X* and *KDM5C* (**Table 1**, screening data of other genes are listed in **Supplementary Table**). Of these, *HSD17B10, RPS4X, KDM5C* are previously known to escape X chromosome inactivation[17-19] and were expressed in females at twice the levels of males. *MSL3* is also suggested to escape X chromosome inactivation in LCL. In contrast, the female to male

MAP7D2 expression ratio was obviously high (7.90±2.99) (**Fig. 2a)**, and in all five female LCL clone pairs, *MAP7D2* was expressed higher in X^p active clones than X^m active clones, according to the X^m imprinting rule (**Fig. 2b**).

Expression array also indicated 99 genes that were expressed more than 1.5 fold higher in males with statistical significance ($p<0.05$). We also performed the same screening steps for these genes. However no gene fulfilled all criteria.

In the next step, to eliminate Epstein-Barr virus transformation and culture effects of LCL, we tested non-cultured peripheral blood mononuclear cells (PBMC) from normal volunteers to confirm the expression difference of these five genes between sexes. *RPS4X* and *KDM5C* showed statistically significant female dominant expression. *MAP7D2* is expressed markedly in female PBMC, 18.38±1.69 folds greater than in males (**Fig. 2a**), and this female dominancy was observed independent of age without gonadal hormone effect (**Fig. 2c**).

We used a *MAP7D2* SNP (c.662A>G, p.N182S) to examine allelic expression status. Screening five female LCLs and the blood DNA of 22 female volunteers, one LCL (LCL-F3) and a volunteer were confirmed to have this SNP. RT-PCR and direct sequencing were performed to both clones of LCL F3 and PBMCs from the SNP positive family. Only one allele is expressed in each LCL clone due to X chromosome inactivation (**Fig. 3a**). However, female PBMCs showed bi-allelic expression of *MAP7D2*, and reactivation (loss of imprinting) of *MAP7D2* on the X^m allele was demonstrated (**Fig. 3b**). Similarly, mouse X-linked imprinting genes, *Xlr3b*, *Xlr4b*, *Xlr4c* and *Rhox5*, are expressed biallelically in females at various stages and in various tissues[1].

To determine the expression profile of *MAP7D2*, we performed qRT-PCR on a human tissue RNA panel (FirstChoice® Human Total RNA Survey Panel, Ambion) (**Fig. 4 top**). *MAP7D2* showed a keen tissue specific expression pattern, with the most expression in the brain, followed by placenta, testes, kidney, thymus and female PBMC. The relative brain expression ratio was 38.9 fold to female PBMC. *MAP7D2* has several mRNA variants depending on the inclusion of exons 6 and 7 and different frames of exon 8. We determined the expression of mRNA variants in each

tissue by RT-PCR spanning exon 5 to 8 (**Fig. 4 bottom**). Interestingly, *MAP7D2* expressed two brain specific mRNA variants using new exons between exons 5 and 6. (**Supplementary Data**)

Frequently, imprinted genes make a cluster, so we examined sex specific gene expression for *MAP7D2* regional genes located around 1Mb on both sides, including *PDHA1, MAP3K15, SH3KBP1, CXorf23, LOC729609, EIF1AX, SCARNA9L, RPS6KA3* and *CNKSR2*. Also we checked another MAP7 domain containing *MAP7D3* located at Xq16.3. qRT-PCR results showed none of these genes showed significantly different expression between sexes both in LCL and PBMC. We also determined the imprinting state of mouse homologue *Mtap7d2* using 16wk Balb/c male (n=3) and female (n=3) tissues, and no difference of expression between sexes was observed in any tissues, including brain, muscle, intestine, lung, liver, spleen, kidney and heart (data not shown).

Microtubules consist of polymers of *α,* β-tubulin heterodimers whose assembly plays an essential role in adaptation and maintenance of the cytoskeleton. In nervous tissue, microtubules act in the formation and maturation of axons and dendrites, and microtubule-associated protein (MAP), *MAP1A*, *MAP1B*, *MAP2* and *MAPT* control microtubule dynamics in vivo[20]. *MAP7* was cloned by screening a HeLa cell expression library with antisera against crude microtubule-binding proteins. *MAP7* is predominantly expressed in epithelial cells and may play an important role in reorganization of microtubules during polarization and differentiation[21]. *MAP7* expression correlates to prognosis of colon cancer[22], spermatogenesis[23], and schizophrenia[24]. We checked *MAP7* and other *MAP* gene expression by qRT-PCR with a human tissue RNA panel, and *MAP4, MAP7, MAP7D1 and MAP7D3* were expressed in a relatively ubiquitous fashion in contrast to *MAP1A, MAP1B, MAP2, MAPT* and *MAP7D2,* which showed brain specific expression (**Supplementary Fig.**). These data suggest that *MAP7D2* is brain expressing MAP and its function in the brain is much more significant than *MAP7*. Furthermore, sex-dependent *MAP7D2* expression could be regulated specifically within the brain and during the developmental stage, possibly due to differences of the neural architecture of the brain between males and females. Also, PBMC data showed that maternally imprinted *MAP7D2* can be reactive. *MAP7D2* imprinting is not fixed, and

the epigenetic modifier could account for its expression level, as *MAP7* expression is affected by retinoic acid[23]. Imprinting genes affect brain phenotype including some neuro-developmental disease. For example, despite intensive studies, DNA sequence variations including single gene mutation and copy number variants are not envisioned to be a major cause of autism spectrum disorders (ASD)[25], although imbalanced genomic imprinting in brain development may be an etiology[26].

In conclusion, we identified the first human X-linked maternal imprinting gene *MAP7D2* and demonstrated its specific brain expression with unique isoforms. *MAP7D2* seemed to be a brain specific microtubule-associated protein and was implicated in sex-dependent neuro-developmental phenotypes including cognitive functions and some psychiatric disorders with sex-biased vulnerability.

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

FIGURE LEGENDS

Figure 1

LCL sample preparation and expression array result. Five LCLs established from different females were cloned and separated to paternal X active and maternal X active clone. Each clone was confirmed by HUMARA analysis (top), and equal numbers of ten clones were pooled for the female LCL sample. Array results (bottom) show good correlation of male and female expression of the majority of X chromosome linked genes. M, molecular weight marker; U, undigested by HapII; X^p, Paternal X active clone with HapII digestion; X^m, Maternal X active clone with HapII digestion.

Figure 2

MAP7D2 is a maternally imprinted X chromosomal gene and is expressed higher in females, both in LCL and PBMC. (a) q-PCR analysis between male (white bar) and female samples (gray bar). Relative gene expression ratios were expressed with male expression average of one. The upper panel shows LCL (n = 10 for males and 5 pairs of maternal X active and paternal X active LCL clones for females), and the lower panel shows PBMC (n = 22 for both males and females, for *MAP7D2*, whose scatter blot is shown in figure (c)). Data are presented as mean values ± s.e.m. and performed #*P* (Wilcoxon signed-rank test), **P* (unpaired-t test), ***P* (Welch's t test) with two-tails. (b) *MAP7D2* relative gene expression ratio of LCLs between male, maternal X (X^m) active and paternal X (X^p) active female clones. Each pair of X^m active and X^p active female LCL clones from the same individual is lined. $*P* (Tukey HSD) (c) Scatter plot of *MAP7D2* expression in PBMC showed higher expression in females (filled circle) than males (open circle) independent of age (n =22, male 0-50yr, female 1-48yr). The dashed line showed each average expression ratio based on a male average of one.

Figure 3

MAP7D2 is affected by X chromosome inactivation in LCL and reactivation of maternal allele expression in PBMC. (a) LCL-F3 has *MAP7D2* SNP c.662A>G, p.N182S. Sequence results of this SNP site showed each X^m and X^p clone expressed only one allele by the effect of X chromosome inactivation. (b) PBMC DNA and mRNA sequence of a family with N182S SNP was evaluated. In this family, the daughter inherited the G allele from the father and the A allele from the mother. The daughter PBMC expressed both alleles with reactivation of the maternal allele of *MAP7D2*. The mother, A/G heterozygous of N182S SNP, also expressed both alleles in PBMC.

Figure 4

MAP7D2 is specifically expressed in brain with unique mRNA variant. The upper panel shows *MAP7D2* relative expression ratio to female PBMC among human tissues by qRT-PCR. The lower panel shows the RT-PCR results of *MAP7D2* spanning exon 5 and 8 in each tissue. Multiple bands of each lane showed mRNA variant and the unique two variants expressed in brain tissue. Samples were run on 10% PAGE and developed by silver staining. PBMC-F, Female derived PBMC; PBMC-M, Male derived PBMC; M, 100bp ladder molecular weight marker; V1~V4, *MAP7D2* transcript variant 1 to 4; b1, b2, V5, Newly identified *MAP7D2* transcript variant brain 1, brain2 and 5.

Table 1 Preferentially female expression genes in LCL

Gene Symbol	Gene Name	Gene ID	Gene Locus on X Chromosome	Array Sequence ID	Array Call Female LCL	Array Call Male LCL	Fold change Female/Male	p.value (unpaired -*t* test)	Escape XCI
MAP7D2	MAP7 domain containing 2	256714	Xp22.12	NM_152780	558	241	2.32	0.0002	Not in LCL
MSL3	male-specific lethal 3 homolog (Drosophila)	10943	Xp22.3	NM_078628	2036	1166	1.75	0.0007	Suspected
HSD17B10	hydroxysteroid (17-beta) dehydrogenase 10	3028	Xp11.2	NM_001037811	14010	7495	1.87	0.0002	Known [17]
RPS4X	ribosomal protein S4, X-linked	6191	Xq13.1	NM_001007	369	125	2.95	0.0014	Known [18]
KDM5C	lysine (K)-specific demethylase 5C	8242	Xp11.22-p11.21	NM_004187	1865	1206	1.55	0.0003	Known [18,19]

METHODS

Human cell culture, peripheral blood mononuclear cells isolation and RNA preparation. Epstein-Barr virus–transformed lymphoblastoid cell lines (LCLs) were established from normal adults, including ten males (18 to 48 years old) and five females (17 to 45 years old), by transformed lymphocytes with B95-8 supernatant. LCLs were maintained in culture with RPMI1640 medium with 10% FCS. For female LCLs, to separate X^p active and X^m active clones, serial dilution and re-expansion was performed. DNA was extracted from each clone and the peripheral leukocytes of their parents, and X chromosome activation state was examined by HUMARA analysis[16]. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated blood by Ficoll-Hypaque gradient centrifugation. RNA was isolated by Trizol Reagents (invitrogen) according to the manufacturer's protocol. Written informed consent for molecular analysis was obtained from the volunteers after explanation of the study, which was approved by the ethics committee of Kanazawa University Graduate School of Medical Science. To determine human tissue expression profile of *MAP7D2* and other *MAP* genes, we used FirstChoice® Human Total RNA Survey Panel (Ambion).

Gene expression array analysis. Extracted RNA from male and female pooled LCLs were sent to the NimbleGen whole genome human gene expression array service (Roche diagnostics), which contains 47,633 genome-wide genes per array, eight probes per gene. Each probe is a 60mer. When a single gene had several sequences on the array, we chose the most significant data for screening purposes.

Quantitative reverse transcribed-PCR (qRT-PCR). Using RNA extracted from each LCL and PBMC or manufacturer derived human tissue RNA, single strand cDNA was synthesized by standard method with a mixture of oligo-dT primer and random primer (9 mer) (TOYOBO) and

RevaTra Ace (reverse transcriptase) (TOYOBO). qRT-PCR was performed with ABI Prism 7700 sequence-detection system (PE Applied Biosystems), Sequence Detector Software (SDS version 1.6, PE Applied Biosystems) and SYBR Premix Ex Taq II Perfect Real Time (TAKARA BIO). Data analysis was performed by $2^{-\Delta\Delta CT}$ methods[27]. To produce accurate results, the relative dose and statistical value of each gene expression between male and female groups was determined by multiple inner control method[28]. Ten inner control genes were examined, and co-efficiency was evaluated by the Best keeper program[29]. Finally, we used a geographic average of *GAPDH*, *ACTB* and *UBC* as an inner control for PBMC and human tissue RNA panel and the geographic average of *HPRT*, *HMBS* and *B2M* for LCL.

Direct sequencing. Direct sequencing of PCR products was performed by BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems) and ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems).

Statistical analysis. Gene expression array data were analyzed with NANDEMO Analysis 1.0.2 software (Roche diagnostics Japan). X-linked genes, which were expressed more than 1.5 fold higher in female pooled LCL than males, were selected. The difference of mean log ratios between two samples was analyzed by unpaired-*t* test, and $P < 0.05$ (double-tailed) was considered statistically significant. qRT-PCR data sets of each male and female LCL clone or PBMC were evaluated by F-test first, then equal variance data were analyzed by unpaired-*t* test, and unequal variance data were analyzed by Welch's t test. *MAP7D2* expression in female LCLs was not normally distributed, hence Wilcoxon signed-rank test was performed. Tukey's HSD test was performed to compare the data set between three groups.

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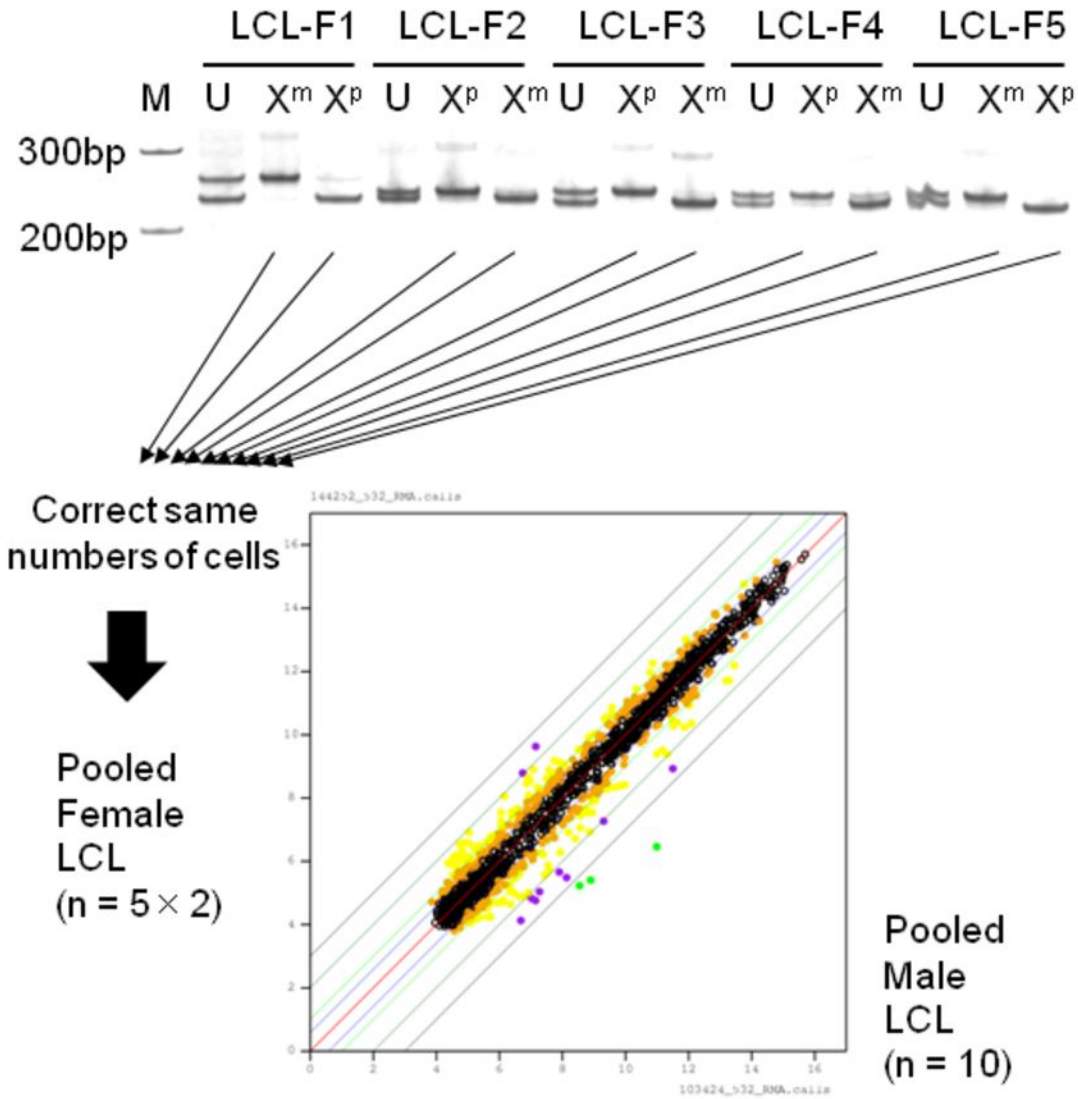

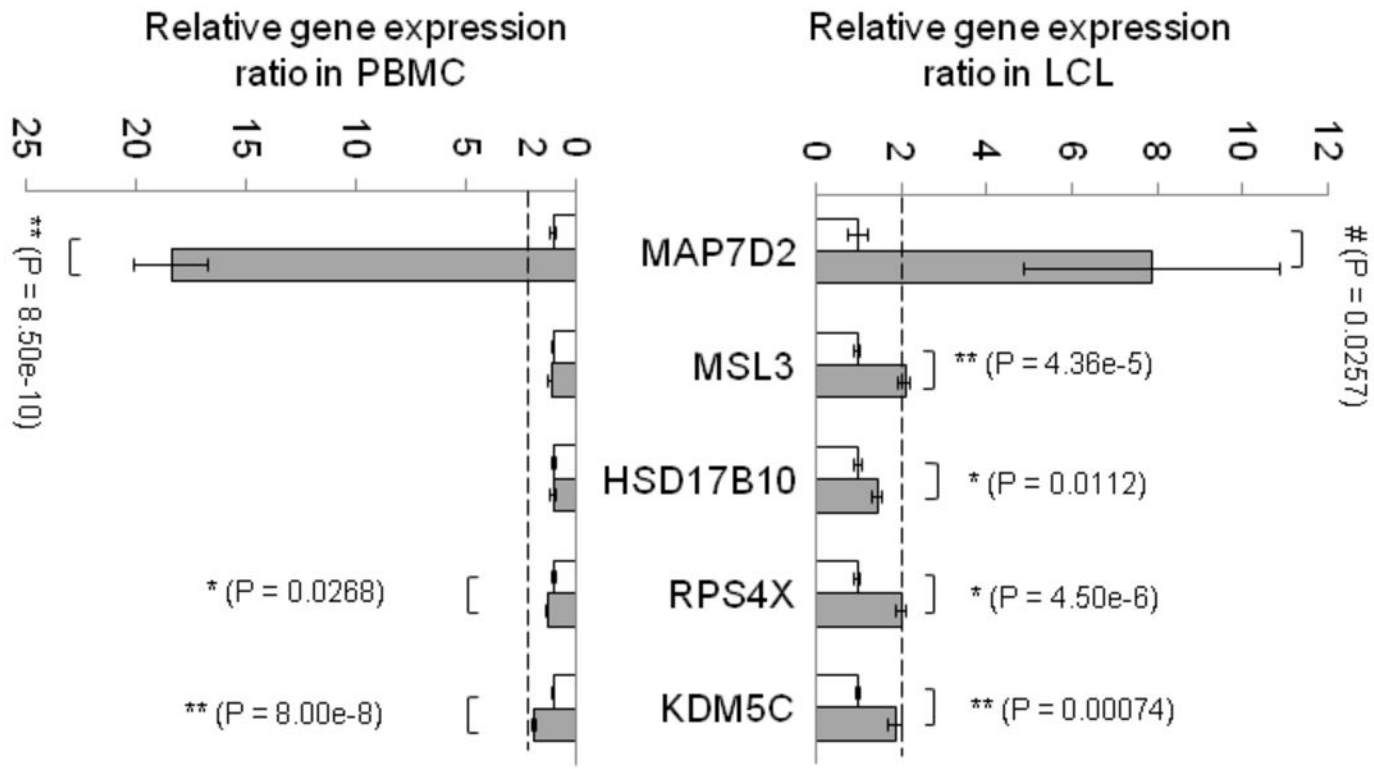
Relative gene expression ratio in PBMC
25
20
15
10
5
2
0
** (P = 8.50e-10)
* (P = 0.0268)
** (P = 8.00e-8)
Relative gene expression ratio in LCL
0
2
4
6
8
10
12
MAP7D2
(P = 0.0257)
MSL3
** (P = 4.36e-5)
HSD17B10
* (P = 0.0112)
RPS4X
* (P = 4.50e-6)
KDM5C
** (P = 0.00074)

Figure 2A

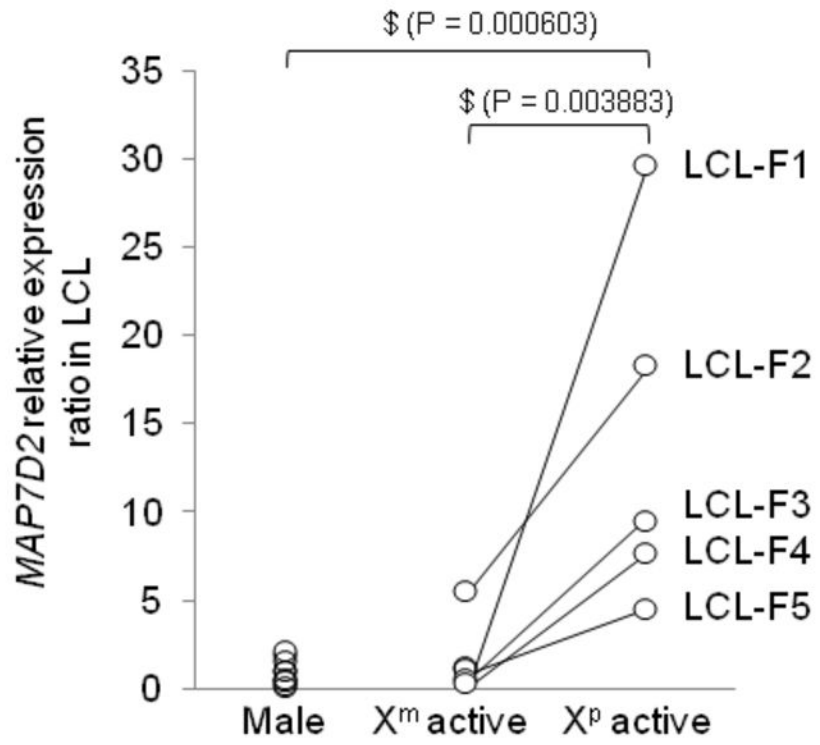

Figure 2C