Addendum: Germline epimutation of *MLH1* in individuals with multiple cancers

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***Nat. Genet.* 36, 497–501 (2004).**

In the original report, methylation of the *MLH1* promoter was detected in a small proportion of the FACS-sorted spermatozoa from male individual TT, who harbored a soma-wide *MLH1* epimutation. To determine whether the *MLH1* methylation detected was, in fact, due to residual somatically derived DNA, we reassessed the original spermatozoa sample using two methodologies, but this time, we included methylation analysis of the imprinted control gene *SNRPN*, which is unmethylated in spermatozoa cells[1].

We employed the same colony hybridization technique used in the original report for the detection of methylation at both the *MLH1* and *SNRPN* promoters. Primers (**Supplementary Table 1** online) were designed to amplify both the methylated and the unmethylated bisulfite-converted DNA templates with equal efficiency. Amplicons were cloned using the pGEMTeasy vector system (Promega), and 500 colonies for each gene were arrayed onto gridded filters. These were then hybridized sequentially with γ-^{32}P-end-labeled probes (**Supplementary Table 1**) complementary to methylated and unmethylated templates of the respective genes. For *MLH1*, 4 of 423 (0.95%) clones were derived from methylated alleles (4 had a positive hybridization signal with the methylation-specific probe and 419 with the unmethylated probe), a finding consistent with the original report of 5 of 526 (0.95%) methylated colonies. However, for *SNRPN*, 5 of 434 clones containing target inserts also gave a positive signal with the methylation-specific probe, indicating that 1.15% *SNRPN* alleles in the sample were methylated. These findings were confirmed using fluorescence-based real-time methylation-specific PCR (f-MSP) of the *MLH1* promoter[2] as well as the *SNURF-SNRPN* promoter[3], using the *MYOD1* gene to control for DNA input[4]. The levels of methylation were measured with reference to an *in vitro* 100% methylated human DNA control (CpGenome Universal Methylated DNA, Chemicon) to obtain a percentage methylation reference (PMR) value, as previously described[5]. The peripheral blood of individual TT showed PMR values consistent with monoallelic methylation of *SNRPN* and *MLH1*, as expected (**Fig. 1**). However, the PMR values of the DNA from his spermatozoa were 0.32% ± 0.25 for *MLH1* and 0.66% ± 0.09 for *SNRPN* (**Fig. 1**). In addition, we analyzed a new sample of motile spermatozoa from individual TT by f-MSP, which was isolated by 'swim-up' followed by FACS. This sample was devoid of *MLH1* methylation, with a trace of *SNRPN* methylation (0.16% ± 0.16). The spermatozoa samples from the normal control male, whether isolated by FACS or swim-up plus FACS, showed low levels of *SNRPN* methylation alone (0.35–1%), illustrating the difficulty in acquiring a pure sample of spermatozoa from semen.

Irrespective of molecular technique, the level of allelic methylation of *SNRPN* marginally exceeded that of *MLH1* in the original spermatozoa sample from individual TT. These new data indicate that the *MLH1* methylation previously reported in the spermatozoa of subject TT is most likely an artifact, attributable to a low level of contamination of the sample with either somatic cells or free DNA derived from somatic cells. In a second spermatozoa sample, no *MLH1* methylation was detected at all. These new data alter the original interpretation that incomplete resetting of the epigenetic mark on *MLH1* had occurred in a proportion of TT's spermatozoa and suggest instead that reversal is complete in the actual gametes.

Editor's note: These new data are reported by Megan P. Hitchins and Robyn L. Ward. Authors David I.K. Martin and Catherine M. Suter do not agree with the content of this Addendum.

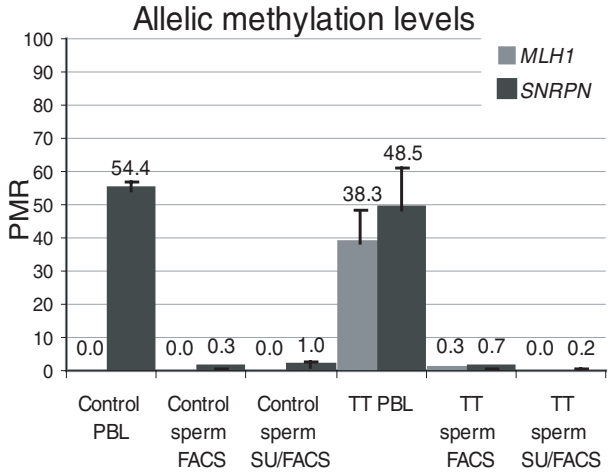

Figure 1 Equivalent levels of *MLH1* and *SNRPN* methylation in spermatozoa as determined by fluorescence real-time methylation specific PCR. Bar chart shows the percentage methylation reference (PMR) values for peripheral blood lymphocytes (PBL) and sperm from individual TT and a normal control following methylation-specific PCR of *MLH1* and the *SNURF-SNRPN* locus. The mean PMR values are provided numerically above each bar. Spermatozoa were isolated from diploid cells either by FACS or by 'swim-up' (SU) followed by FACS, as labeled. Error bars, s.d.

Note: Supplementary information is available on the Nature Genetics website.

1. El-Maarri, O. *et al.* Maternal methylation imprints on human chromosome 15 are established during or after fertilization. *Nat. Genet.* **27**, 341–344 (2001).
2. Hitchins, M.P. *et al.* Inheritance of a cancer-associated MLH1 germ-line epimutation. *N. Engl. J. Med.* **356**, 697–705 (2007).
3. Kubota, T. *et al.* Methylation-specific PCR simplifies imprinting analysis. *Nat. Genet.* **16**, 16–17 (1997).
4. Eads, C.A. *et al.* MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* **28**, E32 (2000).
5. Trinh, B.N., Long, T.I. & Laird, P.W. DNA methylation analysis by MethyLight technology. *Methods* **25**, 456–462 (2001).