Supplementary Information

Materials and Methods

Isolation of total cellular DNA from normal liver and liver tumor tissues

Mouse liver cellular DNA was isolated from frozen liver tissue collected from a naive C57Bl6 mouse. The patient's normal liver and liver tumor cellular DNA was isolated from the frozen tissues collected at autopsy (Raper *et al.,* 2002; Wilson *et al.,* 2012). The frozen liver and tumor tissue was homogenized in lysis buffer using TissueLyser II (Qiagen, Valencia, CA), and total cellular DNA was extracted by the Qiagen DNEasy kit (Qiagen).

Isolation of total cellular DNAs from paraffin-embedded colon tumor tissue

The mouse liver cellular DNA was also isolated from liver tissue collected from a naive C57Bl6 mouse and paraffin embedded. The patient's colon tumor cellular DNA was isolated from the tissue collected at biopsy and paraffin embedded (Raper *et al.*, 2002). Before DNA isolation, the tissue was treated with xylenes to remove paraffin. Five to seven sections from the paraffin-embedded tissue were treated with 1.5 mL of xylenes at 37°C for 10 min, followed by centrifugation at 13,800 × g for 5 min to remove the supernatants. After repeating this procedure three times, the tissue sections were treated with 100% ethanol under the same conditions twice more followed by drying to powder at 90°C for 10 min. The total cellular DNA was then extracted by the Qiagen QiAamp DNA FFPE tissue kit (Qiagen).

Development, optimization, and validation of nested PCR for amplification of rAd and wtAd sequences in cellular DNA

For optimization and validation of the PCR recovery assay, we started by using the recombinant adenovirus (rAd) vector (rH5.001CB-hOTC) DNA or plasmid DNA containing wt adenovirus genomes (pH5wt) as the templates. We optimized the nested PCR conditions with the rAd vector or plasmid DNA template, ranging from 1 to 10^4 copies per reaction for amplification of rAd or wtAd in the presence of 200 ng of naive mouse liver gDNA. The first-round primary PCR products were diluted 1:100 in nuclease-free H_2O followed by a second-round of nested PCR using the primers presented in Supplementary Fig. S1 and Supplementary Table S1. The PCR products were detected by 1.5% agarose gel electrophoresis with ethidium bromide staining and the sensitivities of the PCR assays were determined. The PCR products were also validated by TOPO-cloning followed by conventional sequencing. TAKARA LA *Taq* polymerase (Fisher Scientific, Pittsburgh, PA) was used in the PCR amplifications.

Once the nested PCR-based recovery of rAd and wtAd sequences was optimized and validated, we attempted to amplify the rAd or wtAd sequences using total cellular DNA samples isolated from normal liver, liver tumor, and colon tumor tissues of the two subjects who had previously received AdOTC gene transfer. Two hundred nanograms each of cellular DNA was subjected to nested PCR. PCR amplification of the Ad sequence in 200 ng each of cellular DNA from naive mouse liver tissue was spiked with 1 GC of the isolated rH5.001CB-*hOTC* vector; wtAd plasmid (pH5wt) DNA was used as a positive control. Each sample was tested in triplicate. PCR products were detected by 1.5% agarose gel electrophoresis and subjected to TOPO-cloning followed by conventional sequencing.

Development, optimization, and validation of nested PCR for verification of the mutated OTC genotype of the subjects who had participated in the gene therapy trial

Primers were designed and PCR conditions were optimized to amplify endogenous mutant human *OTC* sequences in Exon 3, Exon 2, and Intron 9 (IVS9) as already described. Using optimized primers and PCR conditions, 100 ng each of the cellular DNA from liver and colon tissue of the patients were subjected to nested PCR for amplifications of endogenous mutant human *OTC* sequences. PCR products were detected by 1.5% agarose gel electrophoresis and subjected to TOPO-cloning followed by conventional sequencing.

TOPO cloning and conventional sequencing analysis of rAd and wtAd sequences

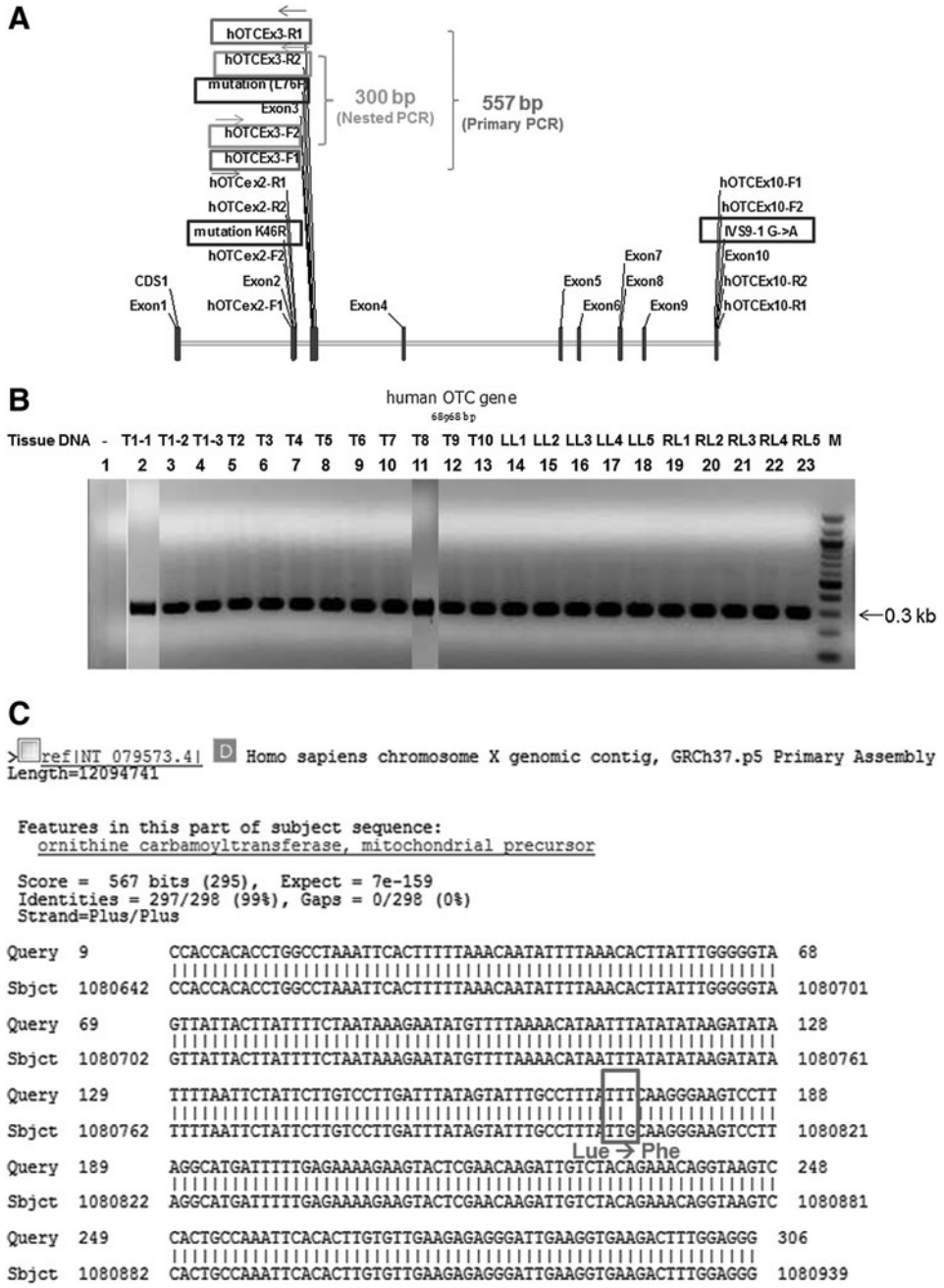
To examine the quality and representation of the PCR amplicons of rAd and wtAd sequences, the second-round PCR products were cloned into TOPO cloning vector pCR4-TOPO using the TOPO cloning kit following manufacturer's instructions (Invitrogen, Carlsbad, CA) and then subjected to conventional sequencing by Eurofins MWG Operon (Huntsville, AL).The sequencing data were analyzed using the alignment tool in the Vector NTI software (Invitrogen) against the original sequences in rAd or wt Ad genome or the NCBI blast tool against *hOTC* gene (www.ncbi.nlm .nih.gov).

Quality Assurance

This is a research based study and there was no formal quality assurance (QA) program.

Results

For an internal control to demonstrate that our PCR assay could readily amplify a single copy gene from the same cellular DNA sample as well as for molecular identification of the patient samples, we also designed primers and amplified the mutant loci in both normal tissue and liver-derived genomic DNA to genotype the patient specific *OTC* mutations (Supplementary Fig. S1A, S1B). The *hOTC* mutant alleles L76F in exon 3 (patient 1) (Supplementary Fig. S1B) and K46R in exon 2 combined with IVS9-1 G→A (patient 2) (data not shown) were sequence verified (Fig. S1C).



SUPPLEMENTARY FIG. S1. Verification of the mutated *OTC* genotype of an AdOTC gene therapy trial patient. **(A)** Location of the primary and nested PCR primers used for amplification of endogenous mutant human *OTC* sequences in Exon 3 (L76F). Exon 2 (K46R) and intron 9 (IVS9-1 G→A) are schematically presented. **(B)** With optimized primers and PCR conditions, 100 ng each of cellular DNA from liver tissue of the second OTC deficiency patients who received AdOTC gene therapy >10 years ago were subjected to nested PCR for amplifications of endogenous mutant human *OTC* sequences in Exon3. PCR products were detected by 1.5% agarose gel electrophoresis. **(C)** The *hOTC* mutant allele (L76F) in the PCR product was verified by sequencing and blast against the gene bank database on the NCBI website. T, tumor; LL, left adjacent normal liver; RL, right adjacent normal liver; −, no DNA template control; M, molecular weight ladder.

References

Raper, S.E., Yudkoff, M., Chirmule, N., *et al.* (2002). A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency. Hum. Gene Ther. 13, 163–175.

Wilson, J.M., Shchelochkov, O.A., Gallagher, R.C., and Batshaw, M.L. (2012). Hepatocellular carcinoma in a research subject with ornithine transcarbamylase deficiency. Mol. Genet. Metab. 105, 263–265.

Supplementary Table S1. Sequences of Oligonucleotides Used for Nested PCR to Amplify rAd and wtAd Sequences and the Mutated hOTC Sequence in Cellular DNA from Tissues of AdOTC Gene Therapy Trial Patients

Sequence for amplification	*Primers*	*Sequences*
Ad DNA polymerase gene	AdpolF1	5′-TGATGCGTTTCTTACCTCTGGTTTCCATGAG
	AdpolR1	5′-AGTTCTACATGCTAGGCTCTTACCG
	AdpolF2	5′-GTGACGAAAAGGCTGTCCGTGTCCCCGTA
	AdpolR2	5′-TCACGTGGCCTACACCTACAAACCAATCAC
hOTC transgene/remnant E1 gene junction	hOTCF1	5′-ACCAAGCTGTTGCTGACAAA
	AdE1R1	5′-AGCCCATCACATTCTGACGC
	hOTCF2	5′-AAGTGGATGATGAAGTCTTT
	AdE1R2	5′-CCTAATATGTTTACCGCCAC
Native Ad E1 sequence	E1F1	5′-AAATTATGGGCAGTGGGTGA
	E1R1	5′-AGCAGGCGATTCTTGTGTCT
	E1F2	5′-GTCTGAACCTGAGCCTGAGC
	E1R2	5′-TCTTCCAAACACTCCCAAGC
hOTC gene across Exon 3 and intron	hOTCEx3F1	5′-AGTGCTGGGATTAAGGCATG
	hOTCEx3R1	5′-CACGTGAGTTCAAGGCCTGA
	hOTCEx3F1	5′-AACCACCACACCTGGCCTAAATTC
	hOTCEx3R1	5′-CCCTCCAAAGTCTTCACCTTCA