Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Barnes AM, Chang W, Morello R, et al. Deficiency of cartilage-associated protein in recessive lethal osteogenesis imperfecta. N Engl J Med 2006;355:2757-64.

APPENDIX

Study Population

Proband dermal fibroblasts were collected under an IRB-approved protocol at the NICHD, NIH. These had been previously determined to synthesize type I collagen with overmodification along the full length of the alpha chains. The full-length cDNA for both type I collagen chains had been sequenced and no collagen structural mutation had been identified.

Cell Culture

Human dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Conditioned media for 3-hydroxylation, differential scanning calorimetry and amino acid chromatography studies were prepared by incubating confluent fibroblasts for 24 hr in DMEM supplemented by 0.1% FBS, 50 μg/ml ascorbic acid, L-glutamine, penicillin and streptomycin, and collecting with protease inhibitors.

Mutation Detection

Genomic DNA (gDNA) for CRTAP exons 2-7 and surrounding intronic sequences was amplified by standard polymerase chain reaction (Invitrogen); exon 1 was amplified with 2 primer pairs using a GC-rich PCR system (Roche, Basel, Switzerland). Mutations identified by sequencing[26] were confirmed in patient and parental fibroblast gDNA by restriction enzyme digestion.

mRNA Levels and Alternatively Spliced Transcripts

Total RNA from fibroblasts was harvested using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH).[27] For Real-Time RT-PCR (Assays-on-Demand, Applied Biosystems, Foster City, CA), standard curves for CRTAP and GAPDH were established using total RNA from a normal fibroblast control line (ATCC2127). Relative quantities of CRTAP and GAPDH were determined in triplicate control and patient assays using the standard curves; these values were used to calculate the

ratio of CRTAP:GAPDH for each sample. We normalized the CRTAP:GAPDH ratio of the patients to that of control cells. For patients, we assayed two independent total RNA samples in triplicate. The cultured fibroblasts of Infant 1 were treated with with 100 μg/ml cycloheximide, prior to RT-PCR amplification of CRTAP mRNA using Titan One Tube RT-PCR Kit (Roche) with primers complementary to exons 1 and 7, respectively.

***In vitro* Biochemical Studies**

For CRTAP protein analysis, fibroblasts were lysed in modified Radioimmunoprecipitation buffer (RIPA; 50mM Tris, pH 7.4, 1% NP-40, 150mM NaCl, 0.25% Na-deoxycholate) supplemented with a protease inhibitor cocktail. SDS-polyacrylamide gels of fractionated lysates were electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and blocked with 5% non-fat powdered milk prior to antibody probing. To study collagen modification, confluent fibroblasts were labeled overnight in serum-free media containing 260 μCi/ml [^{3}H]-proline. Procollagen was precipitated from both media and cell lysates prior to pepsin digestion and electrophoresis[24]. Differential scanning calorimetry was performed from 10-50 ºC at 0.125 and 1 ºC/minute heating rates in a NanoII DSC instrument (Calorimetry Sciences Corporation, Lindon, UT). Amino acid chromatography was performed by high pressure liquid chromatography (Commonwealth Biotechnologies, Inc., Richmond, VA).

For determination of prolyl 3-hydroxylation, media from stimulated cultures were acidified and digested with pepsin at 4 °C for 24 hrs. Collagens were precipitated at 1.0 M NaCl and resolved by SDS-PAGE. The α1(I) band was excised from the gel and subjected to in-gel trypsin digestion.[28] Electrospray mass spectrometry was performed on the tryptic peptides using an LCQ Deca XP ion-trap mass spectrometer equipped with in-line liquid chromatography (ThermoFinnigan, Waltham,

MA), using a C8 capillary column (300 μm x 150 mm; Grace Vydac 208MS5.315) eluted at 4.5 μl per min.

FIGURE LEGENDS

Supplementary Figure 1. *CRTAP* mutations in Recessive Lethal Osteogenesis Imperfecta

Panel A1 shows a homozygous splice site defect in Infant 1. Restriction digestion with MwoI confirms that the parents (C, control; F, father; P, proband; M, mother) are heterozygous for the mutation, as evidenced by presence of 74 and 42 bp fragments from mutant allele as well as a 116 bp fragment from the normal allele. Panel A2 shows the structure of 4 alternatively spliced forms of the *CRTAP* transcript in Infant 1, determined by sequencing of subcloned RT-PCR products from total RNA of cycloheximide-treated cells. The premature stop codon resulting from abnormal splicing is underlined. Panel B shows a homozygous point mutation in Infant 2, and confirmation of the HpyCH4 III restriction site eliminated by the mutation. Panel C displays both parental alleles in I nfant 3. C1 shows heterozygosity for the start codon mutation and confirms its presence in child and father by NlaIV digestion. C2 shows sequences of subclones separating normal exon 1 and the allele containing the 16 nt duplication. Digestion with HpaII confirms the maternal origin of the duplication.

Supplementary Figure 2. Biochemical Studies of Type I Collagen.

Panel A shows a tracing of the fragmentation spectra of the peptide containing Pro986. The b and y ions correspond to the N-terminal (b) and C-terminal (y) daughter ions. Daughter ion y6 localizes the extra 16 mass units to the C-terminal 6 amino acids containing the known Pro986 site of 3-hydroxylation. Panel B contains the thermograms of collagen melting by differential scanning calorimetry. The T_m of each infant's collagen is increased about 1 °C because of overmodification, but there is no lower stability peak indicative of a collagen structural defect.

REFERENCES

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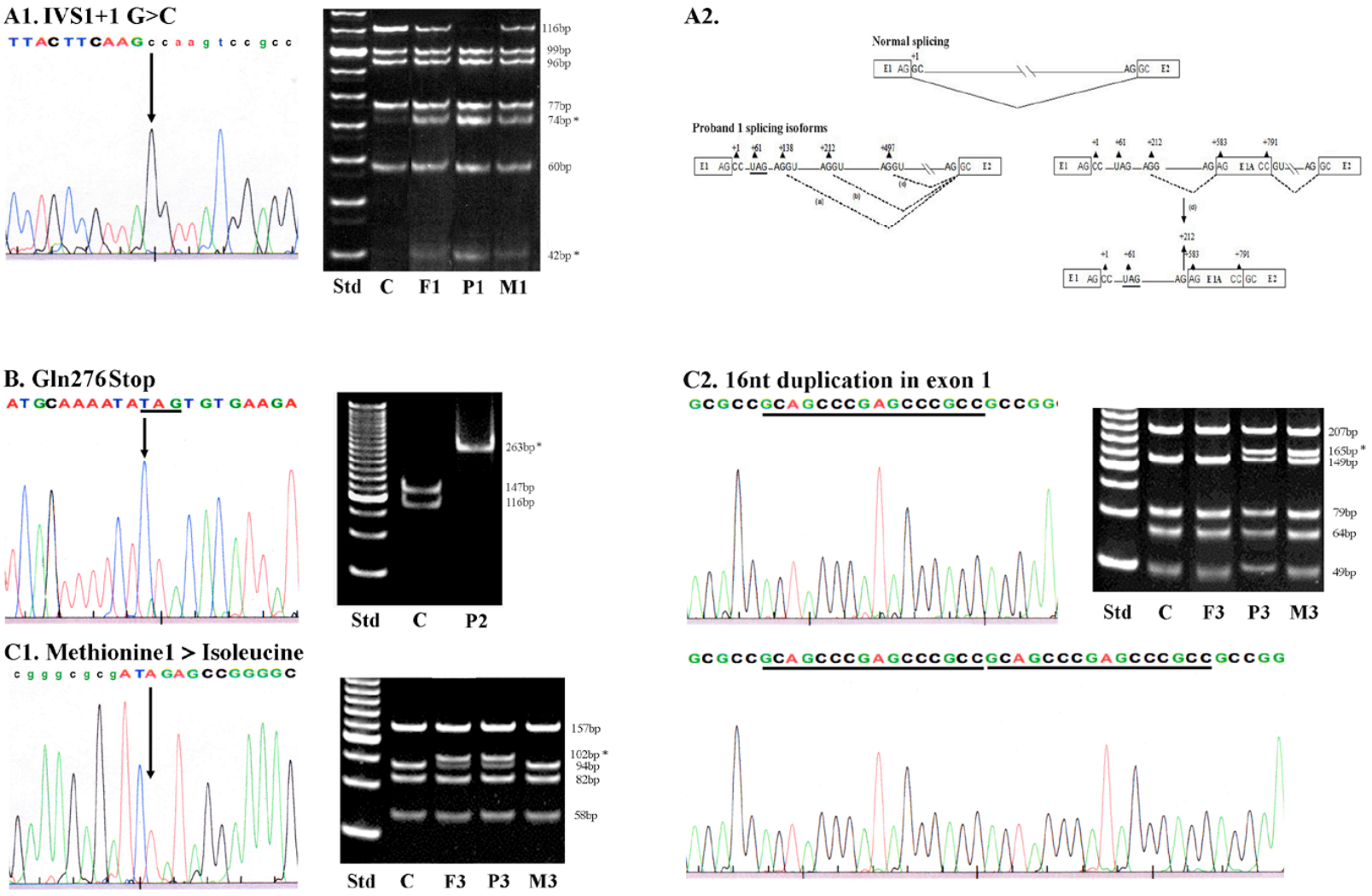
Supplementary Table 1: Modified Sillence classification of Osteogenesis Imperfecta

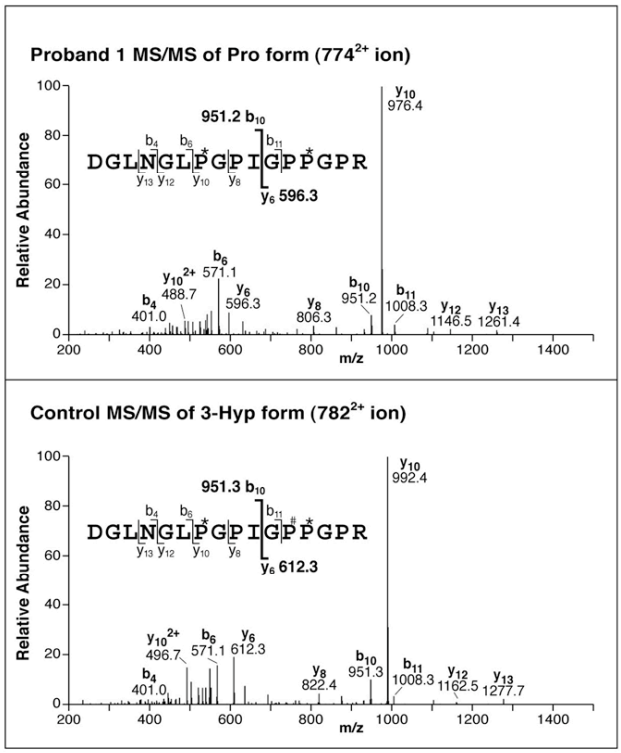
Type	Severity	Clinical Features	Growth Impairment	Blue Sclera	Dentinogenesis Imperfecta	Deafness	Inheritance
I	Mild	Few fractures, little bowing of long bones	Little to none	Present	A: DI absent B: DI present	Some	AD
II	Perinatal Lethal	Many perinatal fractures, severe bowing of long bones, undermineralization	—	Present	—	—	AD, parental mosaicism
III	Progressive Deforming	Moderate to severe limb bowing, many fractures	Severe	Variable	Common	Common	AD
IV	Moderate	Mild to moderate bowing of long bones, fractures	Moderate	Present or absent	A: DI absent B: DI present	Some	AD

Modified from the original Sillence classification[2]



Supplementary Figure 1

A.



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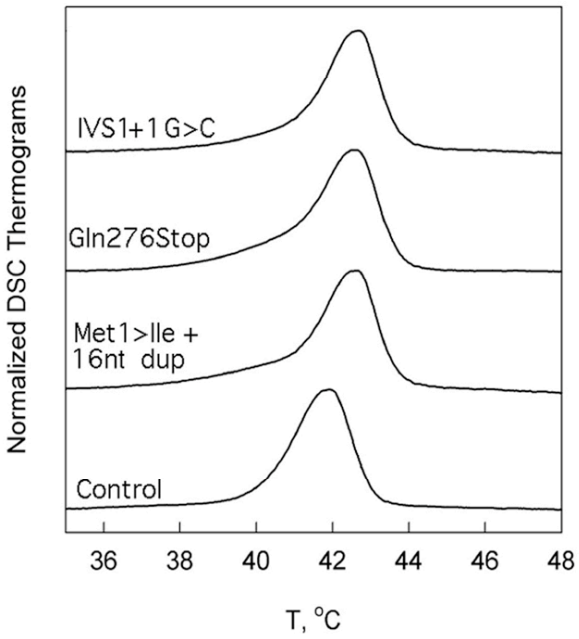

Supplementary Figure 2