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Authors

Ioannidis, Angeliki-Diotima
Khan, Sikandar G
Tamura, Deborah
et al.

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1 **Trichothiodystrophy Hair Shafts Display Distinct Ultrastructural Features**

2 Angeliki-Diotima Ioannidis[1]*, Sikandar G. Khan[2], Deborah Tamura[2], John J. DiGiovanna[2],

3 Elizabeth Rizza[2], Kenneth H. Kraemer[2], and Robert H. Rice[1]

4

5 [1]Department of Environmental Toxicology and Forensic Science Program, University of

6 California, Davis CA

7 [2]DNA Repair Section, Laboratory of Cancer Biology and Genetics, Center for Cancer Research,

8 National Cancer Institute, Bethesda, MD

9

10 Correspondence

11 Kenneth H. Kraemer, DNA Repair Section, Laboratory of Cancer Biology and Genetics, Center

12 for Cancer Research, National Cancer Institute, Bethesda, MD;

13 Robert H. Rice, Department of Environmental Toxicology and Forensic Science Program,

14 University of California, Davis CA. Emails: kraemerk@mail.nih.gov; rhrice@ucdavis.edu.

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Trichothiodystrophy Hair Shafts Display Distinct Ultrastructural Features

*Present address: Angeliki-Diotima Ioannidis, Department of Environmental Toxicology and Forensic Science Program, University of California, Davis, CA, adioannidis@g.ucla.edu

Sikandar G. Khan, DNA Repair Section, Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD, khans@mail.nih.gov

Deborah Tamura, DNA Repair Section, Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD, tamurad@mail.nih.gov

John J. DiGiovanna, DNA Repair Section, Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD, jdigiova@mail.nih.gov

Elizabeth Rizza, DNA Repair Section, Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD, elizabeth.heller@nih.gov

Kenneth H. Kraemer, DNA Repair Section, Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD, kraemerk@mail.nih.gov

Robert H. Rice, Department of Environmental Toxicology and Forensic Science Program, University of California, Davis, CA, rhrice@ucdavis.edu

Ethical statement: The three patients were studied at the NIH Clinical Center under protocol 99-C-0099 approved by the Institutional Review Board of the National Cancer Institute. The parents of the patients signed the protocol informed consent forms and the photography consent forms. The signed forms are on file at NIH. A redacted copy with the identifiers blacked out can be provided on request.

Photo consent statement: The signed forms are on file at NIH. A redacted copy with the identifiers blacked out can be provided on request. We are not able to send the form signed by the patients' family members since that would violate patient privacy regulations. Other journals have accepted our written statements that we have signed copies of the photo permission forms.

Statement of author contributions

ADI: Performed experiments, co-wrote first draft, revised manuscript

SGK: Performed experiments, revised manuscript

DT: Supervised patients, revised manuscript

JJD: Supervised patients, analyzed images, revised manuscript

ER: Analyzed images, revised manuscript

KHK: Supervised patients, analyzed images, revised manuscript

RHR: Provided original idea, supervised experiments, co-wrote first draft and revisions

Abstract

Hair shafts from three trichothiodystrophy (TTD) patients with mutations in the *ERCC2 (XPD)* gene were examined by transmission electron microscopy. TTD is a rare, recessive disorder with mutations in several genes in the DNA repair/transcription pathway, including *ERCC2.* Unlike previous studies, the hair shafts were examined after relaxation of their structure by partial disulfide bond reduction in the presence of sodium dodecyl sulfate, permitting improved visualization. Compared to hair shafts of normal phenotype, TTD cuticle cells displayed aberrant marginal bands and exocuticle layers. Clusters of cells stained differently (light versus dark) in the cortex of aberrant shafts, and the keratin macrofibrils appeared much shorter in the cytoplasm. Considerable heterogeneity in these properties was evident among samples and even along the length of single hair shafts. The results are consistent with not only a paucity of high sulfur components, such as keratin-associated proteins, but also a profound imbalance in protein content and organization.

1. Background

Trichothiodystrophy (TTD) is a rare, autosomal recessive multisystem disorder characterized by short brittle hair, failure to thrive, ichthyosis and developmental delays [1]. In addition, TTD patients have brain dysmyelination, bone and dental abnormalities, congenital cataracts, immune deficiencies, and a 20-fold increased risk of death before age 10 years [2]. TTD patients are often acutely sun sensitive and experience blistering burns after minimal exposure. TTD children are frequently born with a collodion membrane, and 70% are low birth weight (<2500 g) requiring admission to the neonatal intensive care unit (NICU). In addition, the mothers of TTD children experience a high frequency of pregnancy complications including preterm labor, pre-eclampsia, and HELLP (hemolysis, low platelets, elevated liver enzymes) syndrome [3-5].

Two diagnostic features of the hair are alternating light/dark banding visible in polarized light, which is termed 'tiger tail banding', and low cysteine content, typically about half of normal, revealed by amino acid analysis [6-8]. Visible by light microscopy, unusual morphological features are evident, including narrowing of the shaft, ribboning, trichorrhexis nodosa-like fractures and trichoschisis. With higher resolution, scanning electron microscopy reveals surface irregularities and indicates loose attachment of cuticle cells to the shaft. The degree of departure of TTD hair from normal parallels the degree of cysteine deficiency in the shaft [7]. Understanding the basis for this low cysteine content and hair fragility may help elucidate the pathogenesis of this neuroectodermal disease [2, 8].

Since a high degree of disulfide bonding occurs in the mature hair shaft, and likely contributes greatly to its cohesiveness, considerable effort has focused on explaining the brittleness of TTD hair as a consequence of its lower cysteine content. Ordinarily, cuticle cells exhibit a high overall cysteine content (estimated 17.5%) concentrated especially in the marginal band (estimated 37%) and to a lesser extent in the exocuticle [9]. However, the marginal band in TTD hair is

93   deficient in sulfur, consistent with loss of cuticle cells by weathering along the length of the

94   shaft, not only at the distal end [10]. This deficiency has been attributed to a lack of ultrahigh

95   and certain high sulfur proteins [11].

96   **2. Questions addressed**

97   A genetic basis for TTD has now been established. Causative mutations have been reported in

98   10 genes: DNA repair/transcription genes XPB, XPD and TTDA [2, 12]; a M-phase-specific

99   MLK1 interacting protein TTDN1 whose function is not clear [13-15]; GTF2E2 which has a role

100   in the initiation of RNA transcription [16, 17]; CARS1, TARS1, AARS1, MARS1, whose function

101   is to load each amino acid onto its specific cognate tRNA through aminoacylation reactions [18];

102   and RNF113A which has a role in the transcriptional process [19, 20]. Since the original work

103   characterizing the ultrastructure of TTD hair preceded the genetic findings, we have examined

104   sections of TTD hair with known defects in the *XPD* gene *(ERCC2)* by transmission electron

105   microscopy to find whether they differ from previous findings and from each other. To permit

106   improved visualization, the hairs were processed after relaxation of the structure by partial

107   disulfide bond reduction in the presence of sodium dodecyl sulfate.

108   **3. Experimental Design**

109   Hair samples and genotypes. Hair shafts were obtained with informed consent from three

110   unrelated TTD patients, a (clinically normal) parent of each patient, and an unaffected individual.

111   In all cases, the parental and unaffected individual control hair shaft samples were visibly

112   indistinguishable. The genotypes of the samples examined were all compound heterozygotes in

113   the *XPD (ERCC2)* gene as indicated below. For convenience, the samples were numbered T1-

114   T3 (NIH cell/Patient ID number in parentheses) and the parental samples were numbered P1-

115   P3, respectively.

116   Patients.

117   **T1** (TTD568BE) was a 2 year 11 month old female who has heterozygous mutations in the

118   *ERCC2* gene: one allele c.1867dupG (p.V623Gf*26) resulted in a premature stop mutation and

119   a novel second allele, c.2158 T > C (p. F720L), resulted in a missense mutation. She was born

120   at 31 weeks gestation following a pregnancy complicated by severe maternal pre-eclampsia;

121   extremely low birth weight (1452 g) and had a collodion membrane. She was admitted to the

122   NICU for five weeks. She had recurrent infections with multiple hospitalizations, poor linear

123   growth and weight gain, and delayed development. Due to feeding difficulties and poor weight

124   gain she had a gastrostomy tube placed at age 2 ½ yrs (Figure S1A and B).

125   **T2 (**TTD397BE) was a 19-month-old male who has heterozygous mutations in the *ERCC2*

126   gene: one allele, c. 2176 C > G (p.Q726E) was missense, and the other allele was an in-frame

127   large deletion (g.16603_18447del; c.1666_2190del; p.G556_R730del) expected to result in a

128   truncated XPD protein missing the amino acids encoded by exons 18 to 22. Hair sulfur content

129   was 2.30% [normal 5.0%]. He was born at 35 weeks gestation following a pregnancy

130   complicated by preterm labor and abnormal maternal serum screening values. He had low

131   birthweight (2313 g) and a collodion membrane. He was admitted to the NICU for 19 days. He

132   had 2 older siblings with TTD. A hair sample was obtained while he was hospitalized and found

133   to have tiger tail banding on polarized microscopy, and he was diagnosed with TTD at one week

134   of age. His developmental and motor milestones were delayed, and he did not begin walking

135   until age 18 months. He had multiple otitis media infections and pneumonias which did not

136   require hospitalization. He has been found to be neutropenic with mildly abnormal

137   immunoglobulins (Figure S1 C and D).

138   **T3** (TTD475BE) was a 14-month-old male who has heterozygous mutations in the *ERCC 2*

139   gene: one allele has an insertion of 'T' at the exon 15 splice donor site (c.1479+2insT) and the

140   other allele has a deletion of 'G' at exon 22 splice donor site (c.2190+1delG). Hair sulfur content

141   was 2.2% (normal 4.9%). The patient was born at 36 ½ weeks following a relatively

142   uncomplicated pregnancy; however, the mother had mildly elevated blood pressure toward the

143   end of the pregnancy. He had a collodion membrane at birth and was admitted to the NICU for 9

144   days. He was diagnosed with cryptorchidism, congenital cataracts and a patent foramen ovale

145   in the immediate neonatal period. He had feeding difficulties in the neonatal period with

146   gastrointestinal reflux. His cataracts were removed at age 6 weeks and he wears soft contact

147   lenses. He was diagnosed with TTD by assessing his hair for tiger tail banding and sulfur

148   content (Figure S1E and F).

149   **Transmission electron microscopy (TEM).** Samples were cut into 5 cm lengths and incubated

150   at room temperature for 1 hr or 3 hr in 2 ml of 0.1 M sodium phosphate buffer (pH 7.8)

151   containing 2% sodium dodecyl sulfate (SDS) and 25 mM dithiothreitol. These conditions

152   induced swelling of the hair shaft [10, 21] and better embedding for TEM [22]. The different

153   incubation times gave equivalent results, although in some cases the features of interest were

154   slightly more prominent at 3 hr. Processed by standard methods [23], samples were then

155   immersed in Karnovsky's fixative for several days, rinsed with 0.1 M phosphate buffer and post-

156   fixed for 2 hr in 1% buffered osmium tetroxide. Samples were dehydrated with graded ethanol,

157   50% propylene oxide in ethanol and finally two changes of pure propylene oxide. Samples were

158   infiltrated with Spurr's resin in three ascending concentrations in propylene oxide. Infiltration

159   was accomplished with microwave assistance (Pelco 34700 BioWave, Ted Pella Inc., Redding,

160   CA). Following three resin changes, samples were allowed to polymerize overnight in fresh

161   resin solution. Ultrathin sections (60-80 nm) of the polymerized blocks were cut on an

162   ultramicrotome (Leica Ultracut UCT, Austria) using a Diatome diamond knife (Switzerland,

163   Electron Microscopy Sciences USA distributor). Sections were placed on formvar/carbon coated

164   slotted copper grids and post-stained using uranyl acetate and lead citrate. The prepared

165   sections were viewed in a FEI Talos 120C TEM (ThermoScientific Hillsboro, OR, USA made in

166 Eindhoven, The Netherlands) and images were acquired using an integrated FEI Ceta CMOS

167 camera.

168 **Results**

169 Despite having the same average thickness, TTD cuticle cells exhibited dramatic differences in

170 appearance under electron microscopy from those in phenotypically normal hair. The fine

171 structure of cuticle cells in the normal hair shaft can be visualized readily after treatment with

172 detergent under reducing conditions, as shown in Figure 1A. Three prominent features are (a)

173 an intensely stained layer at the superficial edge of the cell (called the marginal band or A-

174 layer), (b) a layer of fine-grained, nearly homogeneous material beneath the marginal band

175 (called the exocuticle) and (c) a layer of more coarsely-grained material (called the endocuticle)

176 in which fragments or remnants of intracellular organelles frequently appear embedded.

177 Ordinarily little material appears to be extracted from the cuticle even by harsh detergent

178 treatment except for some small features in the endocuticle.

179 The cuticle seen in TTD hair shafts exhibited distinct differences from that in normal parental

180 hair (Figure 1B). First, the marginal band (A layer) often appeared incomplete or discontinuous

181 and even absent in some cells. Although mostly fine grained as in the normal samples, the

182 exocuticle in TTD hair was less uniform in width and sometimes contained large features similar

183 to those in the endocuticle. The boundary between exo- and endocuticle was often quite

184 variable, appearing disorganized along the length of the cuticle cell. Considerable variation in

185 the integrity of the marginal band and the boundaries of the exo- and endocuticle was noted

186 even in adjoining cells in the same hair shaft.

187 In the cortex, longitudinal sections frequently revealed distinctive regions of differential staining

188 intensity in TTD hair that were uncommon in the parental normal hair. As seen in Figure 2,

189 these regions at lower magnification appeared as an intermingling of small clusters of cells

190   staining light or dark (panels T1, T2), or elongated structures that were more tightly arranged

191   (panel T3). By contrast, the parental control samples were much more uniformly stained (panels

192   P1, P2, P3).

193   At higher magnification, the dark stain in the TTD cortex appeared to delineate islands of curved

194   short macrofibrils (Figure 3). By contrast, the macrofibrils in parental hair appeared to extend

195   along the length of the cells. The parental cells also exhibited more electron dense fine granules

196   among individual macrofibrils and at the edges of cortical cells (the latter also illustrated in

197   Figure S2).

198

Conclusions & Perspectives

Present findings of aberrant features in the TTD hair cuticle and cortex resemble those in previous work of unknown genotype [24]. A striking observation is the intra-individual variability in each sample. As previously reported, the marginal band can be discontinuous or absent, the exocuticle can appear disturbed, and staining of the cortex can show regional variation [10, 25]. Analogous to findings with defects in TFIIEβ [17], we hypothesize that such defects result from transcriptional deficiencies of certain components, including ultrahigh sulfur proteins that normally assist in proper intracellular protein distribution during the final stages of development in the follicle. Since the deficiencies permit near normal cell growth, deficiencies in the hair keratinocytes could occur primarily in transcription or translation of components needed in relatively high amount for terminal differentiation. If it occurred in epidermal keratinocytes, this phenomenon could help rationalize the frequent finding of an ichthyosis phenotype and onychodystrophy in epidermis and nail plate in TTD, respectively.

Lack of expression of proteins with high cysteine content participating in disulfide bonding is unlikely to be the only factor bestowing fragility on TTD hair shafts. Some TTD hair samples also display a near-total lack of isopeptide-stabilized protein in the exocuticle, allowing for resistance to detergent extraction [26]. A deficiency in such protein in the cuticle could be responsible for the absence or irregular appearance of a marginal band in present samples after extraction with detergent and reducing agent. The different alleles of XPD are known to affect the phenotype in the related condition xeroderma pigmentosum [28]. Possibly even cellular mosaicism in hair shaft formation [29] may play a role in the TTD phenotype. Moreover, the wide spectrum of clinical abnormalities observed in TTD patients [2], affecting multiple organ systems, points to transcriptional defects in numerous cell types and thus beyond those participating in keratinocyte differentiation.

Acknowledgments

229 **Conflict of Interest**

230 The authors state that they have no conflict of interest.

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Figure Legends

FIGURE 1. Transmission electron micrographs. (A) Cuticle of normal hair incubated in 2% SDS – 25 mM DTT at pH 7.8 for 6 hr. The shaft was fixed, stained, sectioned and examined by transmission electron microscopy as described [24]. The dark layer on the outer edge of each cell (*a*) is called the marginal band or A-layer. The fine-grained layer immediately beneath the marginal band is called the exocuticle (*b*). The coarse-grained layer at the bottom edge of the cell is the endocuticle (*c*), in which remnants of intracellular organelles frequently appear embedded, some of which are extracted by the harsh detergent treatment (one indicated by *). (B) Hair shaft cuticle from TTD patients (T1, T2, T3), their respective parents (P1, P2, P3). Arrows in T1 point to discontinuities in the marginal band of the outermost cell; note the clear discontinuities in the next two inner cells. Discontinuities in the exocuticle layer in the outermost cell in T1 and T3 and an inner cell in T2 are denoted by Δ. Note the disorganized appearance of the endocuticle in cells in the TTD samples. Scale bars = 0.5 µm.

FIGURE 2. Longitudinal sections of hair shafts (3 hr incubation) from TTD patients (T1, T2, T3) and their parents (P1, P2, P3). Note the variegated staining patterns in T1, T2 and T3 (black arrows), which differ from the parental samples and from each other. The dark ellipsoids seen in all the samples (white arrowhead in P2) are melanosomes (0.4-0.9 µm diameter) [27]. Scale bar 2 µm.

FIGURE 3. Sections of hair cortex from TTD patients (T1, T2, T3) and their parents (P1, P2, P3). Thick arrows in panels T1, T2 and T3 point to concentrations of darkly stained short fibrils. Thin arrows in panels P1, P2 and P3 draw attention to clusters of darkly staining particles appearing in intercellular spaces. Scale bar = 0.5 µm.

Supplementary Material

341   **FIGURE S1.** TTD patients studied and polarized light micrography of their hair. (A) Patient T1

342   (TTD568BE) a 2 year 11-month-old girl with unruly brittle hair, loss of lateral eyebrows and

343   typical TTD features as described in the text. (B) Hair from patient T1 with alternating dark and

344   light (tiger tail) bands (white arrows), trichoschisis (horizontal split in hair strand) (black arrow),

345   abrupt change in diameter of hair shaft (*) and shafts of different diameters. Overlapping hair

346   strands cause isolated bands that is not true tiger tail banding. (40x original magnification). (C)

347   Patient T2 (TTD397BE) a 19-month-old boy with short brittle hair and an outgoing smile typical

348   of TTD patients. (D) Hairs from patient T2 with tiger tail banding and irregularities of hair shaft

349   surface on the middle hair strand in vertical orientation. (100x original magnification). Note that

350   the banding may not be visible in all hairs at the same time. However, rotation of the hairs or the

351   stage will show this banding by changing the relative orientation of the hair shaft and the

352   polarized light. (E) Patient T3 (TTD475BE) a 14-month- old boy with brittle, unruly hair, sparse

353   eyebrows and typical TTD features as described in the text. (F) Hairs from patient T3 with tiger

354   tail banding and hair shafts of varying diameters. (40x original magnification).

355   **Figure S2**. Electron dense granules at the edges of cortical cells in hair from Parent 1.

14

A

a
b
c

T1   T2   T3

P1   P2   P3

B
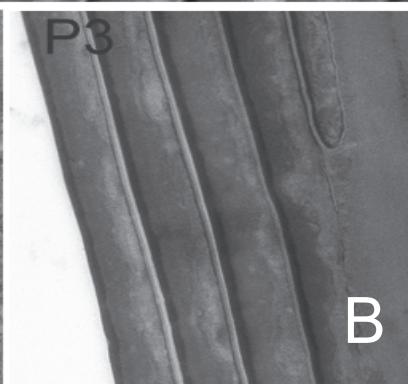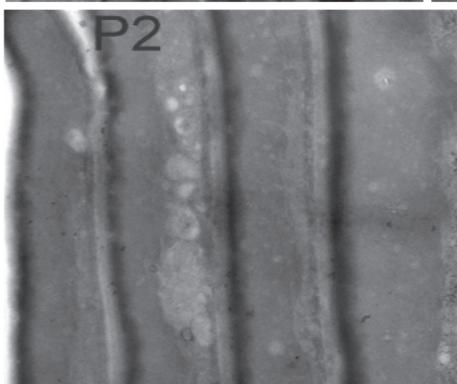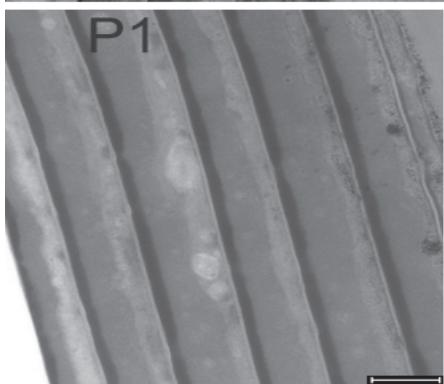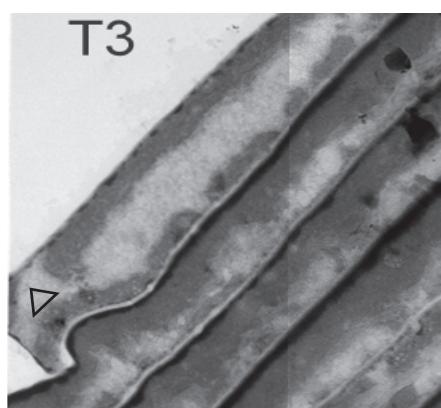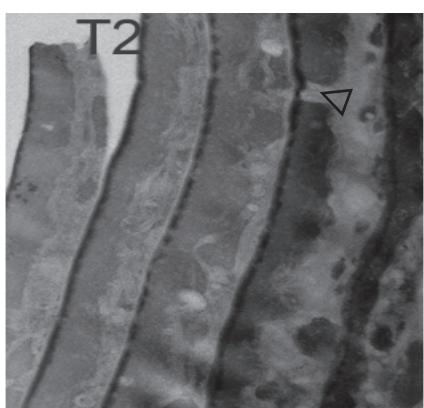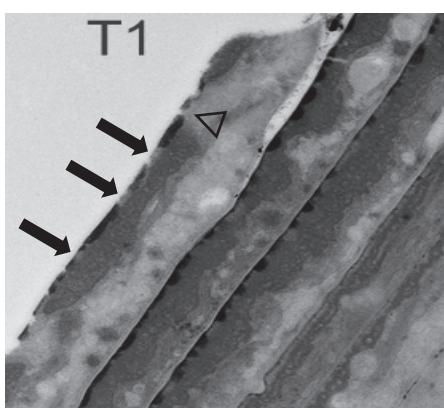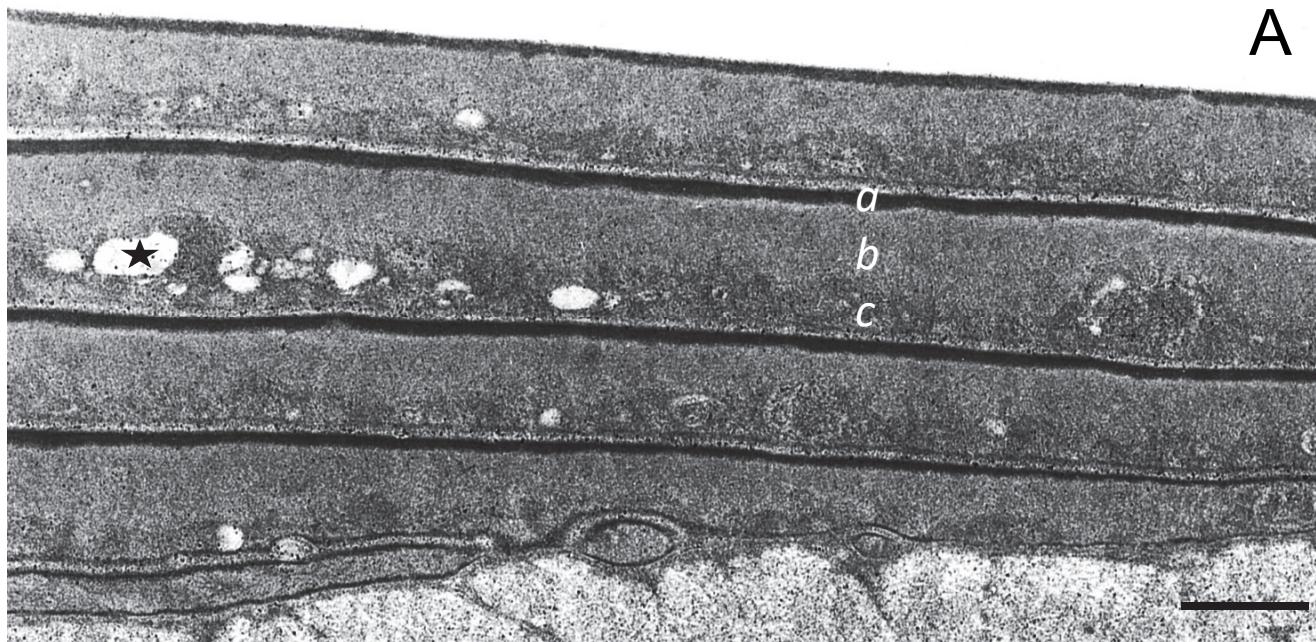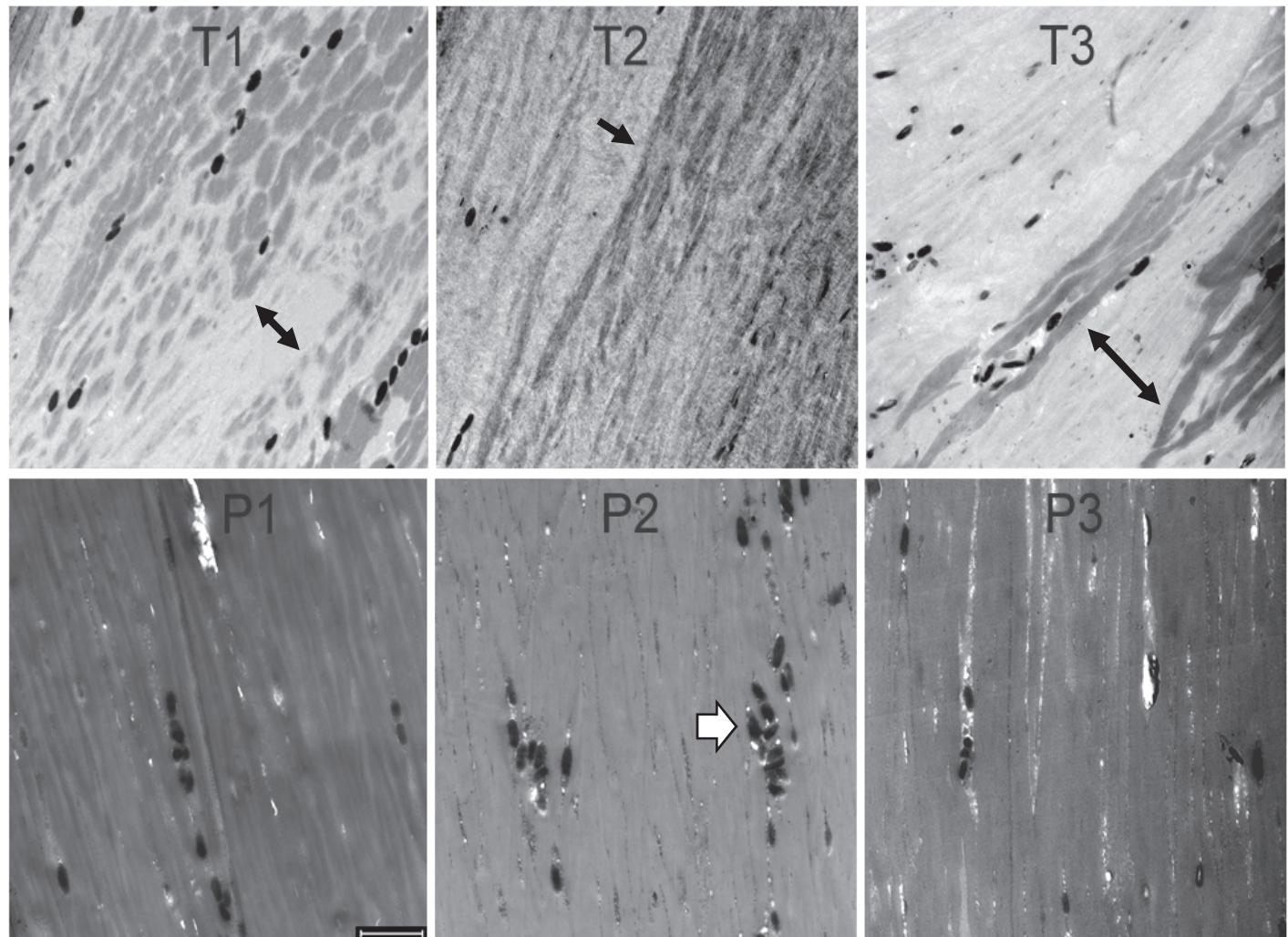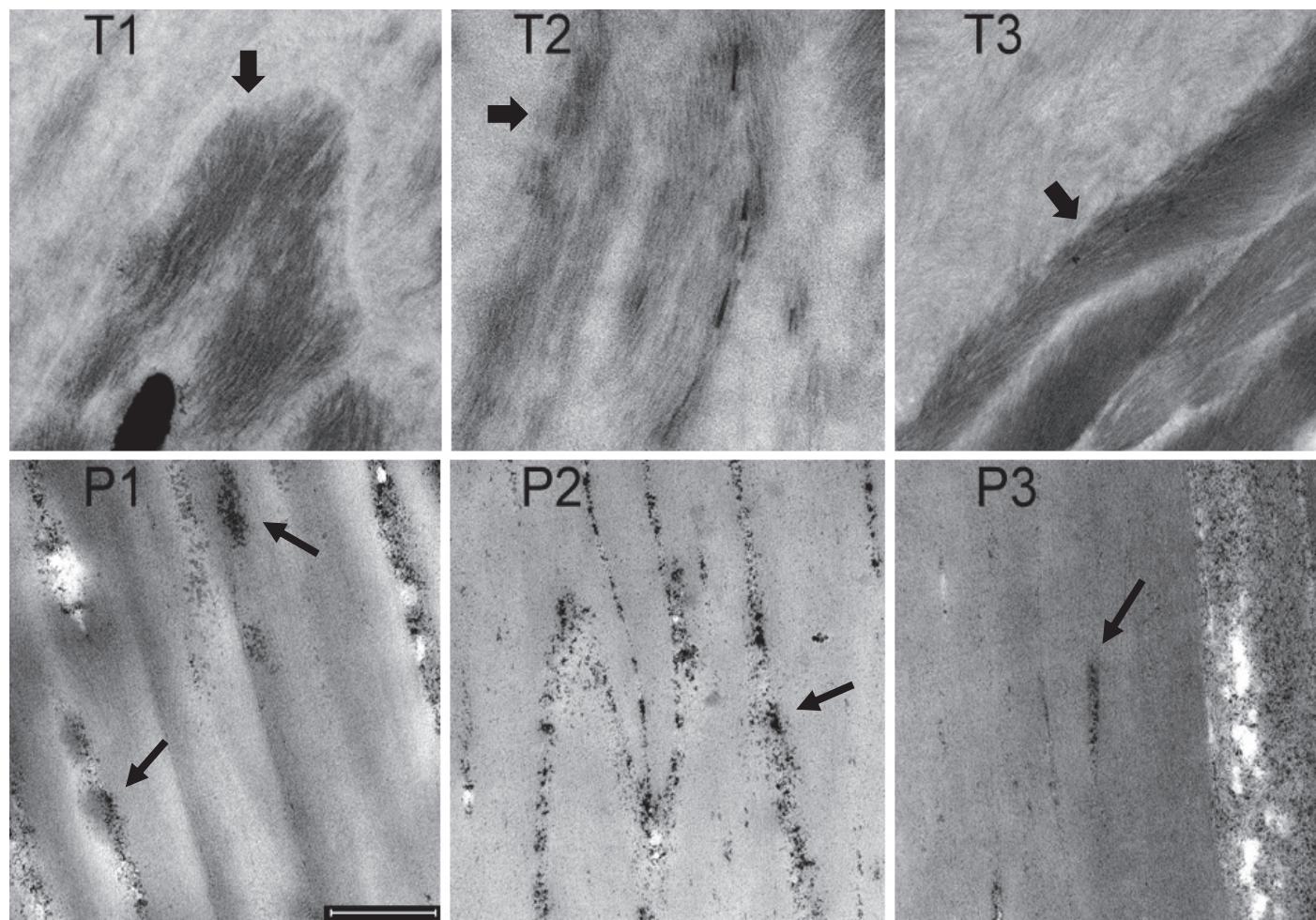
Figure 1

Figure 2

Figure 3
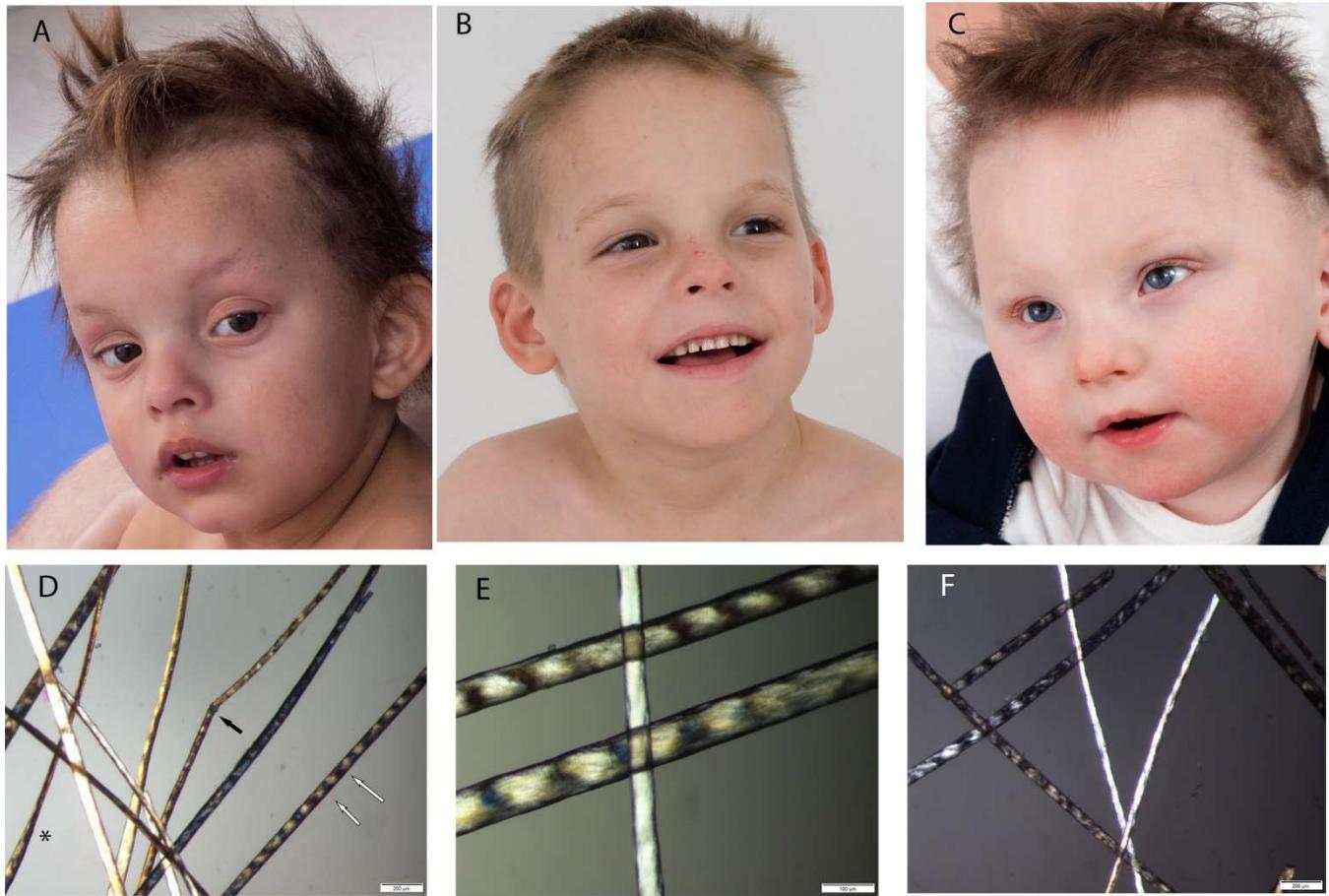
FIGURE 1. TTD patients studied and polarized light micrography of their hair. (A) Patient T1 (TTD568BE) a 2 year 11-month-old girl with unruly brittle hair, loss of lateral eyebrows and typical TTD features as described in the text. (B) Patient T2 (TTD397BE) a 19-month-old boy with short brittle hair and an outgoing smile typical of TTD patients. (C) Patient T3 (TTD475BE) a 14-month- old boy with brittle, unruly hair, sparse eyebrows and typical TTD features as described in the text. (D) Hair from patient T1 with alternating dark and light (tiger tail) bands (white arrows), trichoschisis (horizontal split in hair strand) (black arrow), abrupt change in diameter of hair shaft (*) and shafts of different diameters. Overlapping hair strands cause isolated bands that is not true tiger tail banding. (40x original magnification). (E) Hairs from patient T2 with tiger tail banding and irregularities of hair shaft surface on the middle hair strand in vertical orientation. (100x original magnification). Note that the banding may not be visible in all hairs at the same time. However, rotation of the hairs or the stage will show this banding by changing the relative orientation of the hair shaft and the polarized light. (F) Hairs from patient T3 with tiger tail banding and hair shafts of varying diameters. (40x original magnification).

Figure S2 Electron dense granules at the edges of cortical cells in hair from Parent 1.