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Keratitis-Ichthyosis-Deafness Syndrome Caused by *GJB2* Maternal Mosaicism

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TO THE EDITOR

Keratitis, ichthyosis, and deafness (KID) syndrome (OMIM #148210) is a rare autosomal disorder associating vascularizing keratitis with hyperkeratotic skin lesions and profound hearing loss. It is caused by dominant mutations in the gene *GJB2* (OMIM *121011) encoding connexin-26 or, more rarely, in *GJB6* (OMIM *604418) encoding connexin-30 (Jan *et al*, 2004). Connexin-26 is a protein of gap junctions in epithelia, notably of the corneal limbus, the epidermis, sweat glands and ducts, hair follicles, and the cochlea. Most cases are sporadic and arise from a recurrent missense mutation, c.148G>A (p.Asp50Asn) (Richard, 2005). We report a

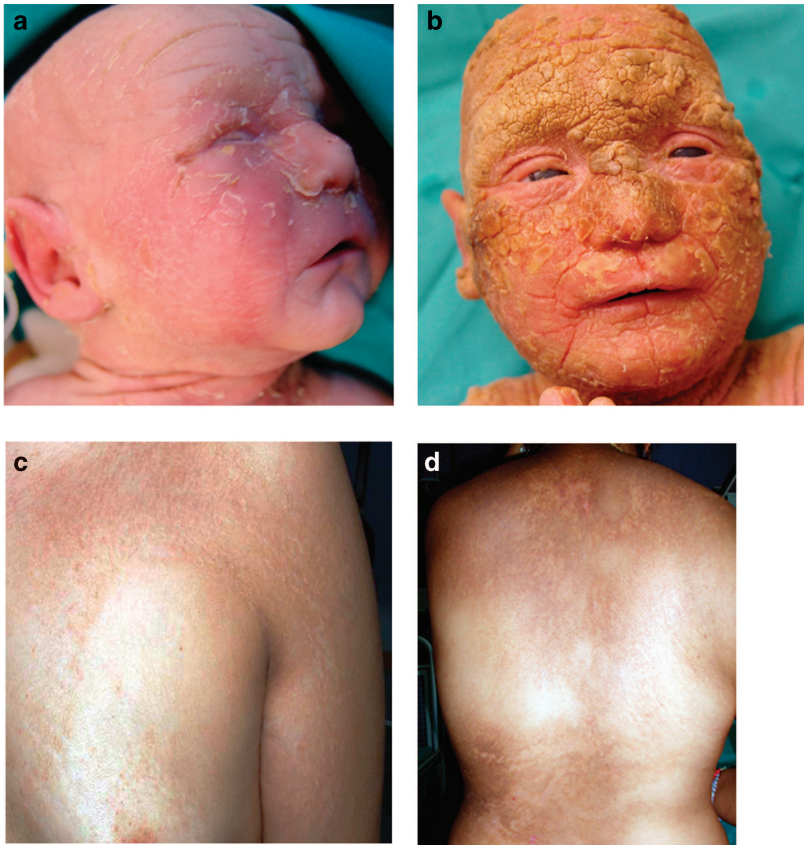

Figure 1. Clinical presentation of the patient and his mother. (**a**) The proband at birth, showing generalized erythema and scaling, and the absence of scalp hair, eyebrows, and eyelashes. (**b**) The proband aged 27 months, showing opaque corneas, total alopecia, and generalized verrucous plaques especially prominent on the scalp and face. (**c** and **d**) Bilateral hyperkeratotic and hyperpigmented lesions along the Blaschko lines on the mother's chest, shoulders, and back.

Abbreviations: KID, Keratitis-Ichthyosis-Deafness; OMIM, Online Mendelian Inheritance in Man

severe case of KID syndrome in a child who inherited this *GJB2* mutation from his mother, who displayed a segmental form of the disease.

CLINICAL FINDINGS AND MOLECULAR DIAGNOSIS

This study was approved by the CPP Toulouse I medical ethics committee, and conducted according to the principles of the Declaration of Helsinki. All clinical samples were obtained with informed consent.

The proband was a Portuguese boy, the firstborn of nonconsanguineous parents, who presented at birth with generalized erythema, ichthyosiform scaling, dystrophic nails, and absent scalp hair, eyebrows, and eyelashes (Figure 1a). A skin biopsy showed orthokeratotic hyperkeratosis and acanthosis. At age 12 months, bilateral corneal stromal keratitis with vascularization and deafness were noted. At age 18 months, he displayed generalized keratotic plaques more prominent on the scalp and face, where they had a verrucous appearance (Figure 1b), deep peribuccal skin grooves, thickening of the skin, and palmoplantar hyperkeratosis. Total alopecia, dystrophic nails, and enamel defects were noted. Corneal opacification and photophobia were also observed. The observed severe global developmental delay was attributed to early deafness and near-blindness. Cerebellar vermis hypoplasia was revealed by nuclear magnetic resonance imaging, associated with nystagmus, generalized hypertonia, and spastic tetraparesia. The patient suffered from frequent infections of the skin and oral mucosa and developed severe malnutrition by age 27 months. Humoral and cellular immunological functions were normal.

The father was clinically healthy but the mother showed low stature and bilateral, hyperkeratotic, hyperpigmented, linear cutaneous lesions on the chest, shoulders, and back along the Blaschko lines (Figure 1c and d), suggestive of segmental manifestations of KID syndrome. Her vision and hearing were normal. PCR amplification and direct sequencing of *GJB2* revealed heterozygosity for the c.148G>A recurrent causative mutation of KID syndrome in the proband's leukocyte DNA (Figure 2a), though not in his parents' (Figure 2b and c), confirming the clinical diagnosis of severe KID syndrome. Cerebellar and neuromuscular defects, as observed here, have been reported only in few cases of this syndrome previously (Caceres-Rios *et al*, 1996).

STUDY OF MATERNAL MOSAICISM

The c.148G>A mutation was barely detectable on the sequence electrophoregram in DNA from a lesional skin biopsy of the mother (Figure 2d), suggesting its presence in a minority of cutaneous cells. The mutation was corroborated by plasmid cloning of the *GJB2* cutaneous PCR amplimer and sequencing of several clones (Figure 2e and f). This finding and the mother's circumscribed lesions were indicative of mosaicism. Lastly, we developed an allele-specific PCR protocol that selectively amplified a fragment of the c.148G>A allele of *GJB2* from the proband's blood and the maternal lesional skin DNA (Figure 2g and Supplementary Materials and Methods), and showed a difference in signal intensity between the proband

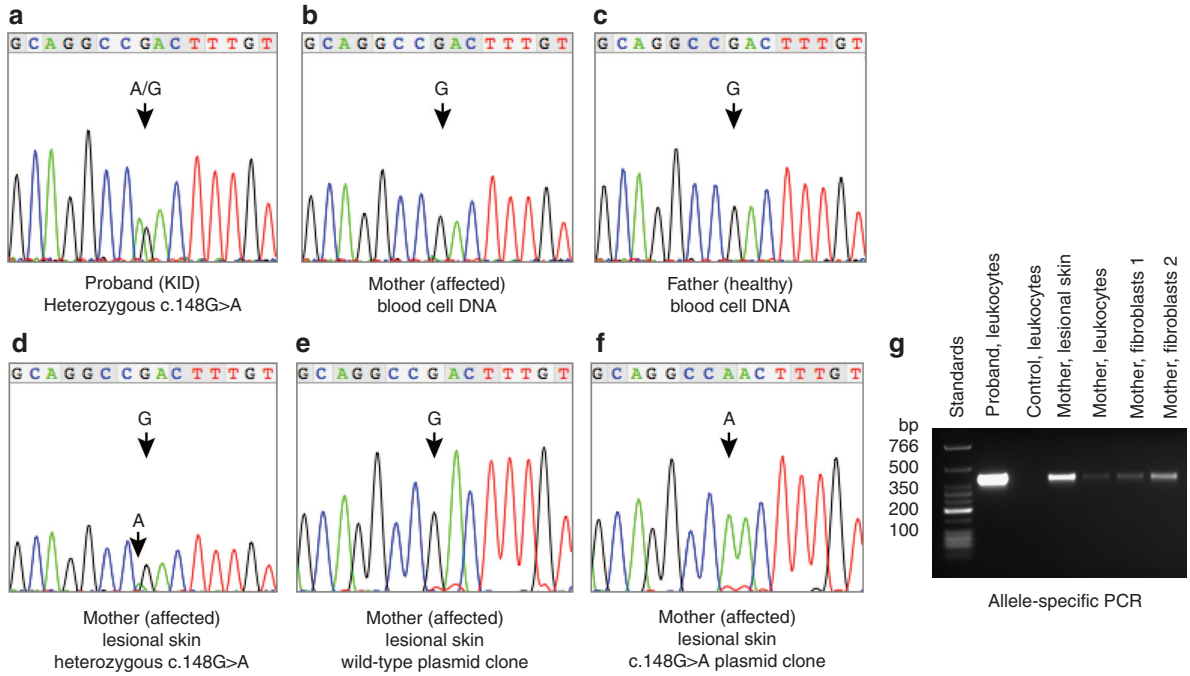

Figure 2. Identification of the *GJB2* mutation. Direct sequence analysis of *GJB2* was performed on DNA from peripheral leukocytes of the index patient (**a**) and his parents (**b** and **c**). The patient's c.148G>A mutation (arrow) was not observed in his parents' leukocytes, but was detected on lesional skin DNA from the mother as a discreet peak on the electrophoregram (**d**). Individual plasmid clones of the relevant *GJB2* amplimer from maternal lesional skin showed either the wild type (**e**) or the c.148G>A mutant sequence (**f**). A PCR specific for the c.148G>A allele amplified a 387 bp *GJB2* fragment from the proband's blood DNA and maternal DNA from lesional skin, leukocytes, and two primocultures of dermal fibroblasts, but not from the DNA of a healthy control (**g**).

and his mother consistent with the maternal mosaicism detected by DNA sequencing. Although direct sequencing failed to detect the mutation in fibroblast primocultures from maternal lesional skin, this allele-specific amplification test did detect the mutant allele in DNA from these cells and from maternal leukocytes (Figure 2g).

This is the first example of KID syndrome inheritance from a proven mosaic parent displaying lesions consistent with type 1 segmental manifestations (Happle, 1997). The localized epidermal involvement and the presence of the *GJB2* mutation in the skin indicated maternal mosaicism. We found the mutation to be present also in two mesodermal cell types, leukocytes and dermal fibroblasts. Although the proportion of mutated germ cells is unknown, this result established a risk for the mother to bear further children with KID syndrome. DNA-based prenatal diagnosis was therefore recommended during future pregnancies.

Molecular evidence of mosaicism has been provided in the context of several cutaneous disorders with type 1 or 2 segmental involvement (Happle, 1997). These include epidermolytic hyperkeratosis (Paller *et al*, 1994; Chassaing *et al*, 2006), neurofibromatosis type 1 (Tinschert *et al*, 2000), Darier disease (Sakuntabhai *et al*, 2000), Hailey-Hailey disease (Poblete-Gutierrez *et al*, 2004), and Apert syndrome (Munro and Wilkie, 1998). Restano *et al*. (1999) proposed parental gonadal mosaicism to explain KID syndrome in sibling pairs born to clinically unaffected parents. Recently, we described brother and sister cases of KID syndrome with healthy parents (Mazereeuw-Hautier *et al*, 2007), and Jonard *et al*. (2008) reported a family with dizygotic twins suffering from a lethal form of KID. These reports suggested parental mosaicism but this could not be formally confirmed.

This case study argues for meticulous clinical assessment of the progenitors to address the origin of a dominant causative mutation in a patient, and therefore the need for prenatal diagnosis in later pregnancies. Formal diagnosis of mosaicism may be difficult if the mutant allele is near or below the sensitivity threshold of direct DNA sequencing in the affected tissue, as observed here. A mutation identified in the index case, however, may be addressed with higher sensitivity in the progenitors by allele-specific PCR amplification (Bottema *et al*, 1993). In KID syndrome, where the recurrent mutation c.148G>A accounts for 80% of cases (Richard, 2005), the allele-specific amplification we have designed will be useful to detect this mutation in parental DNA if mosaicism is suspected. To address the low abundance of the mutant allele, sensitivity could be further improved by culturing the keratinocytes and fibroblasts from lesion biopsies, and quantitative real-time PCR as used by Maertens *et al*. (2007) for neurofibromatosis type 1 may be considered.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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Matthias Titeux[1,2,12], Vanessa Mendonça[3,12], Audrey Décha[1,2], Elisabete Moreira[4], Sofia Magina[4,5], Ana Maia[3], Laetitia Lacaze-Buzy[6,7], José Enrique Mejía[1,2], Luís Torrão[8,9], Filipa Carvalho[10], Júlia Eça-Guimarães[3,11] and Alain Hovnanian[1,2,6,7]

[1]INSERM, U563, Toulouse, France; [2]Université Toulouse III Paul Sabatier, Toulouse, France; [3]Department of Pediatrics, Hospital de São João do Porto, Porto, Portugal; [4]Department of Dermatology and Venereology, Hospital de São João do Porto, Porto, Portugal; [5]Institute of Pharmacology and Therapeutics, University of Porto School of Medicine, Porto, Portugal; [6]Reference Centre for Rare Skin Diseases, Toulouse, France; [7]CHU Toulouse, Hôpital Purpan, Département de Génétique Médicale, Toulouse, France; [8]Department of Ophthalmology, Hospital de São João do Porto, Porto, Portugal; [9]Department of Ophthalmology, University of Porto School of Medicine, Porto, Portugal; [10]Department of Genetics, University of Porto School of Medicine, Porto, Portugal and [11]Department of Pediatrics, University of Porto School of Medicine, Porto, Portugal
E-mail: alain.hovnanian@inserm.fr
[12]These authors contributed equally to this work.

SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Figure S1. Selective amplification of the c.148G>A (p.Asp50Asn) allele of *GJB2*.

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Hypothesis Letter: The Reason Sentinel and Lymph Node Dissections Do Not Improve Melanoma Mortality

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TO THE EDITOR

Despite decades of effort and improved accuracy of sentinel lymph node detection, the removal of draining lymph nodes repeatedly fails to improve melanoma patient mortality (Morton *et al.*, 2006). The literature is rich with pro and con arguments regarding the role of sentinel lymph node biopsy and lymph node chain removal. The responses to the article of Morton *et al.* (A'Hern, 2007; Grichnik, 2007; Kanzler *et al.*, 2007; Lipsker, 2007; Retsas, 2007; Thomas, 2007), a commentary by Gonzales (Gonzalez, 2007) and responses to that commentary (Cochran and Thompson, 2008; Morton and Elashoff, 2008) serve as a window into this active debate. It is possible that a different study design or prolonged follow up will eventually reveal a survival advantage. It is important to note the patients with positive sentinel lymph nodes often receive complete lymphadenectomy that has significant morbidity (Guggenheim *et al.*, 2008). It is also important to note that lymphadenectomy has thus far failed to improve overall survival in solid tumors (Gervasoni *et al.*, 2007). However, a review of these arguments is not the intent of this paper. Instead the purpose is to present a hypothesis that may provide insight into the lack of an overall survival benefit to lymph node removal in melanoma patients.

The premise behind sentinel and lymph node dissections is based on the assumption that the metastatic cells enter the lymph system and the lymph nodes then act as a net capturing the melanoma cells first in the sentinel node(s). When the sentinel node fails to retain all the melanoma cells, then it is thought that the next node in the chain will net and hold the released cancer cells. Certainly an element of this process is supported by the fact that with disease progression more lymph nodes can be found to be involved with melanoma cells (Morton *et al.*, 2006).

If the premise is correct that lymph nodes act as a net retaining malignant melanoma cells, then removal of the primary and secondary nodes draining a tumor site should result in a cure as long as tumor is not found at the end of the lymph node chain. Unfortunately, melanoma patients with lymph node removal still go on to suffer metastatic disease and die in a similar time frame to those not undergoing lymph node removal.

One possible hypothesis is that the malignant cells merely bypass the lymph system all together by directly entering the blood circulatory network. This is certainly possible given studies demonstrating direct tumor entry into the vessels (Warren *et al.*, 1978) and a correlation of thin melanoma tumor risk with vascularity (Graham *et al.*, 1994).

However, if the lymph system is the major mechanism through which tumor cells leave the primary, then understanding the lack of overall survival is more difficult. Maybe it is due to a fundamental flaw in our concept of (1) how lymph nodes function and (2) how tumors behave.

Hypothesis: A virulent subpopulation of melanoma cells freely traverse lymph nodes and are subsequently rapidly disseminated through the vascular system.

HOW DO LYMPH NODES FUNCTION?

Lymph nodes are perfused by two fluid channel systems. The first one is a customary blood circulatory network with entering arterial and exiting venous flows. The second is a lymph fluid channel system entering the node through afferent vessels and exiting via efferent vessels (Ohtani *et al.*, 2003).

The blood circulatory network, in addition to providing for the metabolic needs of the lymph node, delivers T and B cells. These cells enter through the high endothelial vessels, and traverse nodal tissue (Ohtani *et al.*, 2003). Most of these cells fail to find an activating antigen, but rather than leaving the lymph node via the blood stream they exit via the efferent lymph system. Cells that encounter an antigenic target may locally proliferate but then also leave via the efferent lymph system. The number of cells transported back to the circulatory system from the lymph nodes has been calculated in the canine system to be about 245,000 cells per minute per kilogram of weight (Minnebaev *et al.*, 1981).

Afferent lymph is derived from tissue fluid and includes essentially any cells, dissolved or particulate matter that can be washed into the lymph channel system. Immune cells that have traveled into tissue also use the lymph channel system to return to the circulation. The afferent lymph vessels enter the lymph

Keratitis-Ichthyosis-Deafness Syndrome Caused by GJB2 Maternal Mosaicism

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Supplementary Materials and Methods

Allele-specific PCR amplification. A fragment of the c.148G>A (p.Asp50Asn) allele of *GJB2* was amplified from genomic DNA by two successive PCR reactions. Allele specificity was achieved by matching the 3'-terminal base in the reverse PCR primer to the c.148G>A mutation. Stage II (reamplification) reactions involved a nested forward primer and the same reverse primer. The amplimers were 430 and 387 base pairs in size, respectively.

Forward primer, first reaction	GJB2_intron_1F	CAGGTGAACAAGCTAC
Forward primer, second reaction	GJB2_intron_3F	GTTCAAGAGGGTTTGG
Reverse primer	GJB2_148A_1R	TGTTGCAGACAAAGTT

The PCR reactions were carried out in the presence of 1.5 mM Mg^{2+} and 1 M betaine, using the buffer from the DNA polymerase supplier (Promega, Charbonnières, France) and a 5 M betaine stock solution. The 50-µl reactions involved 10 pmol of each primer, 0.2 mM dNTPs, and 1.25 U Go Taq DNA polymerase. Stage I reactions, which included a negative, wild-type control, were seeded with 100 ng of genomic DNA and subjected to 35 thermal cycles. Stage II reactions were seeded with 1 µl of the respective stage I reaction and subjected to 20 thermal cycles. Following an initial DNA denaturation step (1 minute, 94°C), thermal cycles consisted of denaturation at 94°C for 30 seconds, primer annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was carried out for 10 minutes at 72°C. 20-µl samples of the PCR reactions were run on a 1.8% agarose gel in standard Tris-acetate-EDTA (TAE) buffer, containing 0.125 µg/ml ethidium bromide (Figure S1).

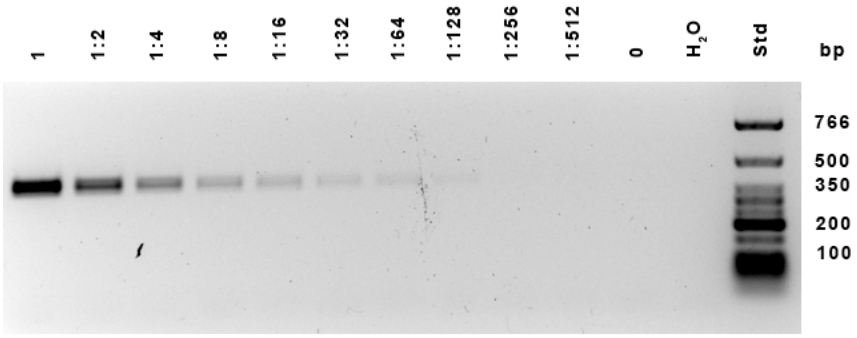

Figure S1. Selective amplification of the c.148G>A (p.Asp50Asn) allele of GJB2. Two successive PCR reactions were carried out on a dilution series of proband genomic DNA into the DNA of a healthy control, and the products of the second reaction were analyzed by agarose gel electrophoresis. **1**, non-diluted proband DNA ; **0**, control DNA alone ; **H_2O**, no-DNA control reaction ; **Std**, DNA size standards.