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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.04.007.

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Postzygotic Mutations in Beta-Actin Are Associated with Becker's Nevus and Becker's Nevus Syndrome

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TO THE EDITOR

Becker's nevus is a cutaneous hamartoma affecting approximately 1 in 200 individuals (Tymen et al., 1981). Becker's nevus appears in childhood as a unilateral tan patch, increasing in thickness, pigmentation, and hair growth during adolescence (Becker, 1949; Glinick et al., 1983) (Figure 1a). Histologically, epidermal acanthosis is accompanied by irregularly dispersed ectopic smooth muscle bundles and increased terminal hair follicles (Figure 1b). In rare cases, termed Becker's nevus syndrome, nevi can be associated with musculoskeletal abnormalities, unilateral breast hypoplasia, mental retardation, developmental delay, and cardiomyopathy (Danarti et al., 2004; Dasegowda et al., 2014; Happle and Koopman, 1997). Although most Becker's nevi are innocuous, rapid onset during adolescence presents significant cosmetic distress. Despite prevalence of Becker's nevi, the pathogenesis remains unknown.

A 13-year-old girl presented with hyperpigmented patches overlying her arms, legs, torso and back, and unilateral left breast and pectoralis muscle hypoplasia (Figure 1c). Lesional skin biopsy revealed epidermal acanthosis with flat-tipped rete ridges, basilar hyperpigmentation, and smooth muscle hypertrophy (Figure 1d). Her presentation was characteristic of Becker's nevus syndrome. To identify genetic alterations underlying her condition, biopsies from a pigmented patch and adjacent nonlesional skin were subjected to exome-sequencing, along with seven independent biopsies of nonsyndromic Becker's nevi, and an additional adjacent nonlesional skin (Supplementary Materials and Methods online; Supplementary Tables S1–S4 online). This study was approved by the institutional review board of Stanford University. Patients and parents of minors signed consent for skin biopsies performed for this study, and provided verbal consent for publication of photos. For research conducted on preexisting paraffin-embedded samples, signed consent was waived by Stanford institutional review board.

Our analysis identified an *ACTB* point mutation (c.C439T, p.R147C) in the index case absent from adjacent normal skin. An additional five of seven nonsyndromic Becker's nevi contained a point mutation affecting the same codon resulting in *ACTB* c.C439T, p.R147C or c.C439A, p.R147S (Figure 2a, Supplementary Table S1). These variants

Abbreviation: Hh, Hedgehog

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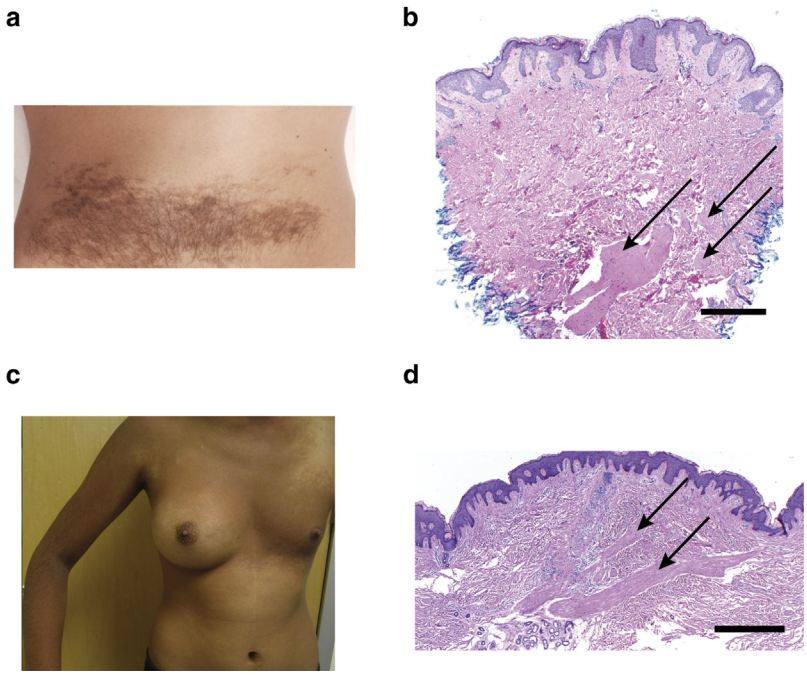

Figure 1. Features of Becker's syndrome and histology. (**a**) Photograph of classic Becker's nevus. (**b**) Histopathologic image of a Becker's nevus depicting elongated rete ridges of the epidermis and disordered bundles of ectopic smooth muscle proliferation. Scale bar = 0.5 mm. (**c**) Photograph of the index case. Note the large size of Becker's nevus and associated left breast hypoplasia. (**d**) Lesional skin biopsy of the index case showing elongated, flat-tipped rete ridges, basilar pigmentation, and smooth muscle hypertrophy. Scale bar = 0.5 mm.

are absent from the COSMIC, ExAC, and EVS databases, and predicted damaging with Polyphen scores 0.998 and 0.959, respectively. *ACTB* p.R147 is highly conserved across primate species, supporting likely deleterious effects of these mutations.

Sanger sequencing was conducted on 15 additional formalin-fixed, paraffin-embedded Becker's nevi. To increase sensitivity for detecting low-frequency hotspot *ACTB* mutations, we used a restriction enzyme/PCR screening assay, identifying *ACTB* hotspot point mutations in 6 of 15 lesional samples (Supplementary Materials and Methods, Supplementary Figures S1–S3 online). We detected mutations in two additional samples with deep amplicon sequencing of mutation-negative Becker's nevi (MGH DNA Core). In total we identified *ACTB* mutations in 14 of 23 (61%) Becker's nevi (Supplementary Table S1).

To determine the cell lineage containing the *ACTB* mutation, laser capture microdissection was performed to isolate DNA from epidermis, stroma, and pilar muscles in one syndromic and two nonsyndromic Becker's nevi (Supplementary Materials and Methods). In all three cases, the *ACTB* mutation was found only in pilar muscle with an average allele frequency 0.22, consistent with the *ACTB* mutation in muscle lineage (Figure 2b). We speculate that the mutation may also be present in rare mesenchymal-derived cells such as dermal papilla, but this could not be assessed because of the limited number of dermal papilla cells.

A study of a patient with Baraitser-Winter syndrome found that *ACTB* p.E117K conferred differing cytoskeletal size and hyperstability to F-actin in yeast (Johnston et al., 2013). To explore molecular effects of our *ACTB* mutations, we expressed FLAG-tagged wild-type and *ACTB* p.R147C and p.R147S mutant proteins in the C2C12 myoblast cell line, and performed phalloidin immunofluorescence staining (Supplementary Materials and Methods; Supplementary Figure S4a online). No major differences in F-actin cytoskeletal staining were appreciated between wild-type versus mutant cells, suggesting no gross disruption of actin organization. However, further experiments are needed to determine if these mutants have subtle effects on actin cytoskeleton.

Next, we evaluated the effect of the *ACTB* mutations on major signaling pathways. Previous studies linked *ACTB* mutations to altered mitogen-activated protein kinase signaling (Moustakas and Stournaras, 1999). To assay Ras/mitogen-activated protein kinase signaling, we evaluated phosphorylated extracellular signal-regulated kinase in wild-type and mutant *ACTB*-expressing C2C12 cells that were stimulated with epidermal growth factor (Supplementary Figure S4b). Quantitative immunoblot demonstrated equivalent induction of phosphorylated extracellular signal-regulated kinase, suggesting no major perturbations to mitogen-activated protein kinase signaling.

Finally, we assessed the effect of the *ACTB* mutations on Hedgehog (Hh) pathway signaling. Becker's nevi display abnormalities in hair growth and muscle development, two processes closely connected to the Hh pathway (Paladini et al., 2005). We found myoblast cells transfected with C439A and C439T *ACTB* mutants compared with wild-type did not have a statistically significant change in Gli1 expression in basal conditions (Figure 2c). However, a trend toward increased Hh signaling was noted for *ACTB* mutants when cells were stimulated with smoothened agonist. Increased Gli1 expression for C439T variant was significant compared with wild-type *ACTB* ($P = 0.03$) (Figure 2d). Because transfection efficiency was not 100%, these results may underestimate the full biological effect of these mutations on Hh signaling.

We identified recurrent *ACTB* p.R147C and p.R147S hotspot mutations in 61% of Becker's nevi. *ACTB* encodes for beta-actin, an intracellular cytoskeletal molecule, ubiquitously expressed with functions in cell migration, proliferation, signaling, and gene expression (Bunnell et al., 2011). Mutations in *ACTB* p.T120I, p.G74S, and p.R196C were previously identified in Baraitser-Winter syndrome, a developmental disorder with hypertelorism, ptosis, impaired neuronal migration, and mental disability (Di Donato et al., 2014; Rivière et al., 2012). *ACTB* p.R183W and p.E364K are also associated with juvenile-onset dystonia (Gearing et al., 2002) and neutrophil dysfunction

a

Sample	*ACTB* Site Change	AA Change	Allele Fraction (V^2, R^3)
1[1]	c.C439T	R147C	0.02 (4, 220)
2	c.C439T	R147C	0.13 (23, 156)
3	c.C439T	R147C	0.05 (18, 336)
4	c.C439T	R147C	0.15 (54, 308)
5	c.C439T	R147C	0.10 (37, 337)
6	c.C439A	R147S	0.06 (20, 320)
7	WT	WT	0 (0, 332)
8	WT	WT	0 (0, 332)

[1]The index case (Becker's nevus syndrome) is indicated as Sample 1.
[2]V = Number of variant reads
[3]R= Number of reference reads

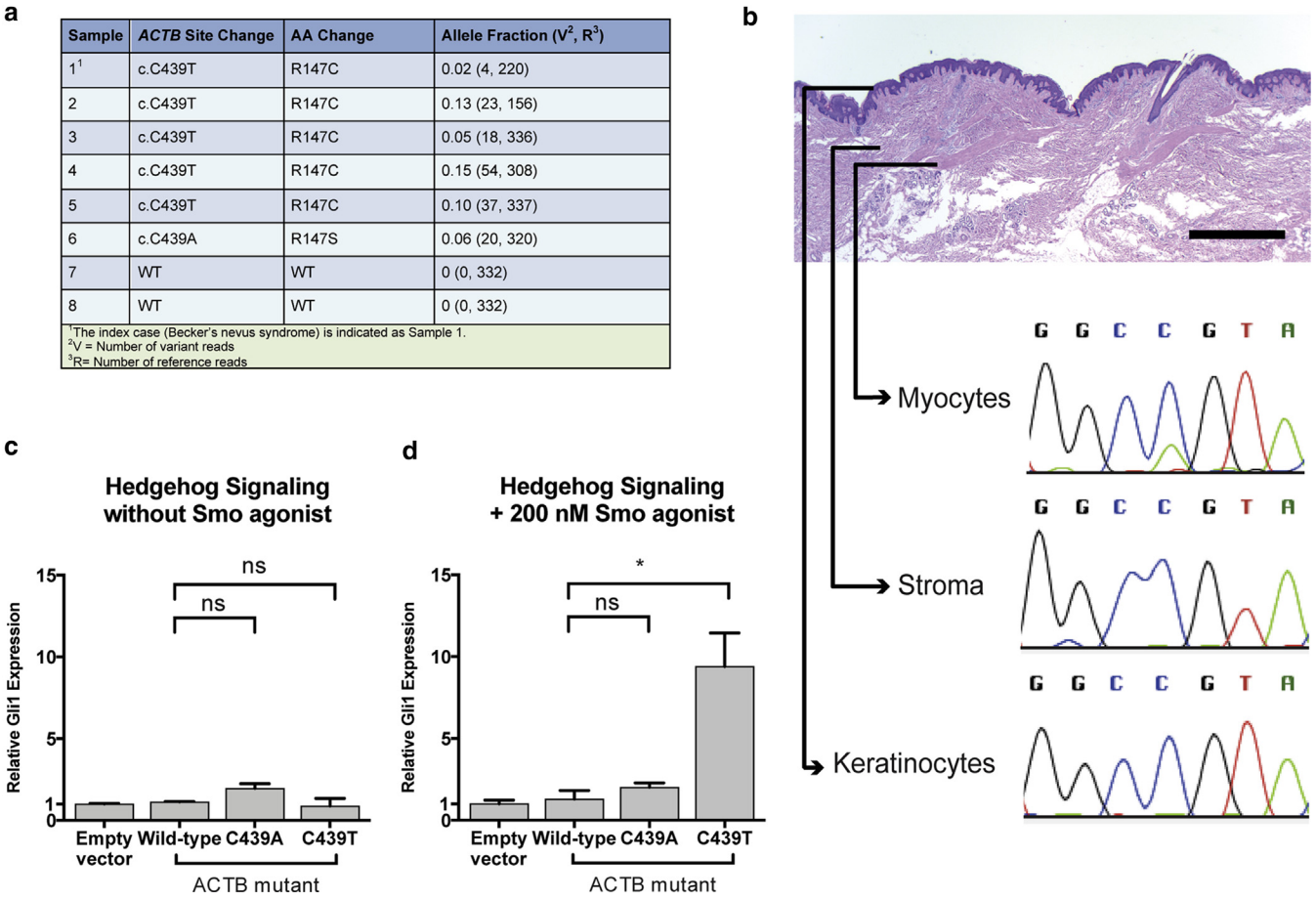

Figure 2. *ACTB* exome sequencing, laser capture microdissection, and functional study results. (**a**) Identification of hotspot *ACTB* mutation in Becker's nevus syndrome and Becker's nevi. Exome sequencing reveals *ACTB* p.R147C or p.R147S in six of eight Becker's nevi, including one case of Becker's nevus syndrome. (**b**) Laser capture microdissection (LCM) reveals *ACTB* mutations in myocyte lineage. Sanger sequencing of LCM samples from an exome sequencing mutation positive Becker's nevus confirms the presence of the mutation p.R147S (c.C439A) exclusively in myocytes. Sequencing of keratinocytes and stroma in the same nevus did not show the mutation. Deep sequencing of two unrelated mutation positive Becker's nevus confirms the presence of *ACTB* p.R147C only in myocytes. Scale bar = 1 mm. (**c**) Hedgehog pathway signaling in beta-actin mutants under unstimulated conditions and (**d**) after smoothened agonist (SAG) stimulation. Quantitative real-time reverse transcriptase-PCR of Gli1 expression in myocytes transfected with empty vector, wild-type (WT) and mutant beta-actin constructs are shown. Gli1 expression in WT versus C439A and C439T *ACTB* mutants did not differ significantly in unstimulated state ($P > 0.05$). After SAG stimulation, increased Gli1 expression was observed in *ACTB* mutants, with statistically significant elevated Gli1 expression in C439T mutant versus WT ($P = 0.03$). Error bars = mean ± SD, n = 2. Two-tailed *t*-test performed between WT and each mutant beta-actin construct. *$P < 0.05$. ns, not significant; SD, standard deviation.

(Nunoi et al., 1999). Becker's nevi have not been reported in these syndromes, although musculoskeletal abnormalities similar to Becker's nevus syndrome have been described.

We hypothesize that *ACTB* mutations associated with Becker's nevi act in a non-cell autonomous manner, as the mutations were identified only in pilar muscle but clinical phenotype involves hyperplasia of epidermis and hair follicles. Becker's nevus syndrome may reflect a mutation earlier in development, affecting multiple cell lineages compared with isolated Becker's nevus. In conclusion, our results suggest that *ACTB* p.R147C and p.R147S in mesenchymal lineage underlie the development of Becker's nevi. Our findings of *ACTB* mutations in Becker's nevi support the hypothesis that *ACTB* p.R147 mutations potentiate Hh signaling, thereby disrupting hair follicle and pilar muscle development. Future studies will further elucidate pathways affected by these *ACTB* mutations.

ACCESSION NUMBER

The raw data have been deposited to the NCBI Sequence Read Archive (PRJNA359805).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Erdr1 Attenuates *Dermatophagoides farina* Body Extract-Induced Atopic Dermatitis in NC/Nga Mice

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TO THE EDITOR

Atopic dermatitis (AD) is a multifactorial inflammatory skin disease characterized by a defective skin barrier and dysregulation of the immune system in terms of T helper type 1 (Th1) and Th2 cell imbalance (Peng and Novak, 2015). Th2 cytokines, such as IL-4, IL-13, and thymic stromal lymphopoietin, disrupt skin barrier and immune system function and induce inflammation in the lesional skin. Thymic stromal lymphopoietin is significantly increased and activates dendritic cells to produce Th2 chemoattractants, including chemokine (C-C motif) ligand 17 (CCL17) and CCL22, resulting in an increased Th2-skewed inflammatory responses in the lesional skin (Soumelis et al., 2002).

Previously, erythroid differentiation regulator 1 (Erdr1) was shown to have a negative correlation with IL-18, which is closely associated with AD severity (Kim et al., 2016a; Trzeciak et al., 2011). Erdr1 is first discovered in WEHI-3 cell line and released under stress conditions, modulating growth and survival of cells (Dormer et al., 2004a, 2004b). Although functions and mechanisms of action remain unclear, recent studies show that Erdr1 displays anti-inflammatory and anti-cancer properties on various skin diseases, including psoriasis, rosacea, and melanoma (Jung et al., 2011; Kim et al., 2015, 2016a, 2016b). Therefore, we hypothesized that Erdr1 would exert preventive effects on AD through its anti-inflammatory functions, similar to its effects on psoriasis and rosacea.

Here, we compared the expression level of Erdr1 between AD lesional skin and normal skin from humans and mice. To determine Erdr1 expression in AD lesional skin from human subjects, skin tissues were collected from normal control (n = 5) and patients with AD (n = 11). This experiment using human subjects was approved by the ethical committee of the Catholic University of Korea, and all human subjects provided written informed consent. Also, all experimental procedures with mice were approved by the Institutional Animal Care and Use Committee of Sookmyung Women's University (SM-IACUC-2013-0726-022). As shown in Supplementary Figure S1a and b online, Erdr1 expression was significantly lower in AD lesional skin than normal skin, and effective treatments restored expression, indicating that Erdr1 may play a critical role in AD. To investigate the role of Erdr1 in AD, *Dermatophagoides farina* body ointment was topically applied to the ears of NC/Nga mice to induce AD-like skin inflammation. Then, 10 μg/kg of recombinant Erdr1 (rErdr1) or 2 mg/kg of dexamethasone as a positive control was administered by intraperitoneal injection. As shown in Figure 1a, rErdr1 significantly improved the external symptoms of AD-like skin inflammation compared with the group injected with the vehicle, phosphate buffered saline. Histological analysis also showed that rErdr1 administration resulted in decreased inflammatory cell infiltration, such as eosinophils and mast cells, and hyperproliferation in the epidermis.

To determine the severity of AD, symptoms of redness, edema, scaling, and excoriation were scored, and the overall score was evaluated by the sum of each category. The scores were

Abbreviations: AD, atopic dermatitis; CCL17, chemokine (C-C motif) ligand 17; CCL22, chemokine (C-C motif) ligand 22; Erdr1, erythroid differentiation regulator 1; rErdr1, recombinant Erdr1; Th, T helper

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