Pigment Cell & Melanoma Research

Letter to the Editor

Title: Pigmented macules in Waardenburg syndrome type 2 due to *KITLG* mutation

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Summary:

We report a patient with Waardenburg syndrome (WS) type 2 who had the unusual complication of large pigmented macules. Whole-exome sequencing revealed a previously unreported homozygous *KITLG* mutation to be the causative gene. *KITLG* defect is a rare cause of WS, with only one case having been reported previously. Interestingly, both the previously reported case and the present case had large pigmented macules (café-au-lait spots). Importantly, *KITLG* mutations are causative of another pigmentation disorder, namely familial progressive hyper- and hypopigmentation (FPHH), which may also be accompanied by pigmented macules. The *KITLG* mutation in the present case is adjacent to strikingly restricted hotspots of mutations causative of FPHH. Our thorough literature review revealed that large pigmented macules are a very rare symptom of WS, raising the possibility that WS caused by *KITLG* mutations may represent a hitherto unnoticed phenotypic subgroup. The present findings extend our knowledge of the KIT/KITLG system.

Key Words: KITLG, Waardenburg syndrome, familial progressive hyper- and hypopigmentation, hyperpigmentation, genotype-phenotype relationship

Running Title: WS2 caused by a novel *KITLG* mutation

Dear Editor,

Waardenburg syndrome (WS) is a genetic auditory-pigmentary disorder caused by a migration defect of neural crest cells. Major symptoms include congenital sensorineural hearing loss, pigmentary abnormality of the iris, and hypopigmentation of the hair and skin. WS is subcategorized into four types by the presence of additional symptoms: Dystopia canthorum is seen in WS1 (OMIM #193500) and WS3 (#128820), and limb anomalies are seen in WS3. Hirschsprung disease (HD) is a complication of WS4 (OMIM #277580, #613265 and #613266). In the absence of these additional symptoms, patients are diagnosed with WS2 (OMIM #193510, #600193, #606662, #608890 and #611584). Eight genes have been associated with WS with various degrees of genotype-phenotype correlation: *PAX3*, *SOX10*, *MITF*, *EDNRB*, *EDN3*, *KIT*, *SNAI2* and *KITLG* (Pingault et al., 2010, also summarized in Table S1). *KITLG* mutation is a rare cause of WS, with only one case having been reported (Zazo Seco et al., 2015). *KITLG* is also associated with another pigmentary disorder: familial progressive hyper- and hypopigmentation (FPHH, OMIM #145250) (Amyere et al., 2011; Cuell A et al., 2015; Wang et al., 2009). Here we report the second case of WS2 caused by a *KITLG* mutation with a rare manifestation of pigmented macules.

The patient is a 16-year-old male born to unrelated parents of Filipino origin. He was referred to our hospital because of depigmentation of his lower extremities. He had a medical history of congenital sensorineural hearing loss and psoriasis vulgaris, but no intestinal diseases. Clinical examination revealed bilateral brilliant blue eyes (Figure 1A), the absence of dystopia canthorum (W-index 1.86), and hypopigmented areas of skin with dappled pigmentation within (Figure 1B, 1C). White hairs were evident exclusively in the area of the leukoderma, but white forelock and premature graying of the hair were absent. According to the family, the hypo- and

hyper pigmentation of the skin has not changed since the patient's childhood. Notably, he had a café-au-lait spot on the trunk, and a large, well-demarcated area of hyperpigmentation on the lower abdomen (Figure 1D, 1E). Many hyperpigmented spots were found on his back and buttocks, apparently representing post-inflammatory hyperpigmentation due to psoriasis (Figure S1). None of his family members had hearing impairment, and the family history was unremarkable except that the mother had a pigmented macule similar to those in the patient. The diagnosis of WS2 was made. Whole-exome sequencing and Sanger sequencing revealed a previously unreported homozygous mutation in *KITLG* (c.94C>T, p.Arg32Cys), which was heterozygously found in the mother and the half sister (Figure 2A, 2B). The institutional review board approved this study, and written informed consent was obtained from all participants or from the parents. We were unable to contact his biological father. A quantitative PCR assay (TaqMan^{R} Copy Number Assays, QIAGEN) confirmed the diploidy of the *KITLG* gene in the patient (Figure S2) and confirmed the homozygosity of the c.94C>T mutation. The patient also had a heterozygous mutation in the *EDNRB*Δ3 isoform (c.140G>A, p.Gly47Asp); however, this mutation was also carried by the asymptomatic mother and is considered irrelevant to the occurrence of WS2 (Figure S3). SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org/) and Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) predicted the mutation as tolerated and benign, respectively. No pathogenic mutations were found in other genes associated with WS (*MITF*, *SOX10*, *PAX3*, *SNAI2*, *KIT*, and *EDN3*), nor those associated with café-au-lait spots (*NF1* and *SPRED1*). SIFT and Polyphen2 predicted the p.Arg32Cys mutation to be functionally damaging and probably damaging, respectively. The c.94C>T mutation was novel according to database searches on ExAc (the Exome Aggregation Consortium), the 1000 genomes project, and the Single Nucleotide Polymorphism database, although a rare mutation

of p.Arg32His (c.95G>A) was found on ExAc (allele frequency 2/120204, 1.664e-05). From these observations, we concluded that the homozygous *KITLG* mutation is causative of WS2 in this case. No homozygous mutations in *KITLG* have been reported for WS or FPHH.

KITLG encodes the ligand for the receptor tyrosine kinase protein KIT. The KITLG/KIT system plays a critical role in migration and survival of melanocytes, as well as in hematopoiesis and gametogenesis. The genetic evidence of the importance of KITLG/KIT system in skin melanogenesis has been long studied (Reviewed in Yoshida et al. 2001 Amyere et al. 2011 and Zazo Seco et al. 2015). Variation of *KIT* is associated with white coat color in mouse and horse, and with white spotting of pig skin. In human, single nucleotide polymorphism of *KITLG* is associated with blonde hair in Scottish and Danish population. *KITLG* mutations are causative of WS2, FPHH and non-syndromic unilateral and asymmetric hearing loss (Zhang et al., 2016), while dominant mutations of KIT are causative of piebaldism. Piebaldism is characterized by depigmented skin areas that are also seen in WS, such as white forelock and leukoderma. In addition, multiple café-au-lait spots resembling those in neurofibromatosis 1 may also appear in piebaldism.

In the skin, KITLG is expressed in keratinocytes and endothelial cells, and induces migration, development and survival of melanocytes. The role of KIT/KITLG system in melanogenesis is experimentally confirmed using animal models (reviewed in Yoshida et al. 2001 and Wang et al. 2009). In murine models, inhibition of KIT by a blocking antibody induce loss of melanocytes, whereas forced KITLG expression in epidermal keratinocytes induces ectopic proliferation and survival of melanocytes. The *KITLG* product may be either expressed as a membrane-bound form or a soluble form, decided by exclusion or inclusion of the exon 6 that contains a extracellular cleavage site, respectively (Figure 2C). KITLG transmembrane

region-defective mice have no pigmented coat color, and a study using two lines of transgenic mice, one expressing both the membrane-bound and soluble forms of KITLG, and the other expressing only the membrane-bound form, suggested that the membrane form is essential for the survival of epidermal melanocytes.

The KITLG/KIT signaling pathways and the molecular mechanism leading to melanogenesis have been well studied (reviewed in Pingault et al. 2010 and Amyere et al. 2011). The binding of KITLG to KIT induces KIT dimerization and subsequent auto-phosphorylation of its tyrosine residues in the intracellular domain, leading to activation of multiple kinase pathways including the RAS/MAPK pathway. In melanocytes, the RAS/MAPK pathway is involved in the transcriptional regulation of MITF, a key transcription factor of melanocyte development, which itself is one of WS2 causative genes. Transcription targets of MITF include other WS-causative genes such as SNAI2 and EDNRB. Importantly, gene mutations which inhibit the RAS/MAPK pathway also cause pigmentary disorders. NF1 encodes a RasGTPase and its loss-of-function results in neurofibromatosis type 1, a disease marked by multiple café-au-lait spots. Legius syndrome is also characterized by multiple café-au-lait spots, and caused by mutations of a RAS/MAPK inhibitor, namely SPRED1. All these findings altogether show that KIT/KITLG system plays a central role in skin melanogenesis.

Strikingly, in *KITLG*, all FPHH-associated mutations reside within or adjacent to the VTNN-motif, the short conserved amino-acid sequence from Val33 to Asn36, and the present mutation, p.Arg32His, is also adjacent to this motif (Figure 2C). The crystal structures of the KITLG-KIT 2:2 complex showed that this region constitutes a KITLG-KIT interaction site and that Thr34 and Asn35 bind to KIT via hydrogen bonds and salt bridges, while Arg32 and Asn36 interacts with KIT via van-der-Waals contact (Yuzawa et al., 2007). The other WS2-causing

mutation, p.Leu104Val, is also in the vicinity of this site (Figure 2D). Therefore, both the FPHH-causing mutation and the WS2-causing mutation may affect KITLG-KIT interaction at the same molecular interface leading to pigmentary diseases, although it is not known why only p.Arg32Cys and p.Leu104Val, but not neighboring mutations, cause hearing loss. In spite of the homozygous nature of the patient's mutation, the pigmentary anomaly was somewhat mild compared with those in most FPHH cases. We have no clear explanation of this discrepancy, but we suspect the van-der-Waals contact involving Arg32 may be less influential for overall KIT-KITLG interaction compared with other hydrogen bonds or van-der-Waals contact formed by the residues in VTNN motif.

Interestingly, both the present and the previously reported cases of WS2 with *KITLG* mutation manifested pigmented macules in addition to leukoderma (Zazo Seco et al., 2015). In WS, café-au-lait and large pigmented macules are rare. Our literature search of 262 genetically defined families with WS or related diseases found only three WS families with these phenotypes (Table S2): the previous case with *KITLG* mutation, a WS2 family with *MITF* mutation (Leger et al., 2012) and a WS4 patient with *SOX10* mutation (Pingault et al., 2014). Additionally, in a family with Yemenite deaf-blind hypopigmentation syndrome, which is a WS-related syndrome with severe ocular deformity, café-au-lait spots were associated with a *SOX10* mutation (Bondurand et al., 1999). The present mutation is close to the mutational hot spots of FPHH, and no causative mutation was found in *KIT*, *NF1* or *SPRED1* in the present case. The other WS2 case with *KITLG* mutation also had similar macules. Café-au-lait spots are rarely seen in WS. Taken together, these facts suggest that the present pigmented macules may represent a new genotype-phenotype correlation unique to *KITLG* mutation. Since reports of *KITLG* mutations in WS are extremely rare, this hypothesis should be tested by the

accumulation of cases.

Although the present patient fulfills the clinical criteria of WS, we cannot completely deny the idea that the patient has FPHH with concomitant hearing loss derived from genetic or non-genetic causes. However, the discoloration of irides characteristic of WS, as demonstrated in the present patient, is not usually reported in the patients with FPHH. Also considering that a pathogenic *KITLG* mutation was found in another WS2 patient, WS2 caused by *KITLG* mutation would be the most parsimonious explanation of the present condition, and we diagnosed him as having WS even if he may be within one spectrum of KIT/KITLG-associated pigmentary disorders including FPHH and piebaldism. The present patient had a novel mutation in *EDNRB*Δ3 isoform and it is known families with *EDNRB* mutations may show incomplete penetration of WS; however, *EDNRB* mutations are not usually associated with skin hyperpigmentation (Table S2). *EDNRB*Δ3 has additional 89 amino acids at the N-terminus compared with other isoforms. Among the *EDNRB* mutations reported to date, only one pathogenic mutation has been reported in this isoform-specific sequence in a patient with HD without pigmentary anomalies. Although the tissue-specific expression pattern of *EDNRB*Δ3 is yet to be elaborated, existing evidence shows it is expressed in the small intestine and the colon, implying its function in the development of enteric nervous system. Thus, we consider this mutation to be unrelated to the phenotype, although we cannot exclude the possibility completely that the *EDNRB*Δ3 mutation may have caused the disease in association with the *KITLG* mutation.

In summary, we report the second case of WS2 caused by a *KITLG* mutation. Both the present and the previously reported mutations were found in the vicinity of a VTNN-motif, a mutation hot spot of FPHH, suggesting disturbed KIT/KITLG interface as a shared mechanism in WS2

and FPHH caused by *KITLG* mutation. Considering the clinical resemblance to FPHH and the results of a literature review of WS, WS2 caused by KITLG mutation may represent a hitherto unnoticed phenotypic subgroup characterized by association with large pigmented macules, although further accumulation of case reports is warranted. The present findings expand our knowledge of the spectrum of mutations of KIT/KITLG systems by revealing a novel, structurally defined genotype-phenotype correlation.

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Figure Legends

Figure 1. Clinical features of the patient. (A) Both eyes exhibit a brilliant blue iris. Dystopia canthorum is absent. (B, C) Hypopigmented lesions with many pigmented macules inside are seen in both legs. White hairs are also found (arrow). (D) A café-au-lait spot is seen on the lateral trunk. (E) A large pigmented macule is found in the lower abdomen.

Figure 2. The homozygous *KITLG* mutation in the patient. (A) Family members of the proband are shown. The proband is shown in the black square (arrow). (B) The homozygous c.94G>A mutation is found in the patient. The heterozygous c.94G>A mutation is seen in the mother and the half-sister. (C) The structure of KITLG and the location of disease-causing mutations are shown. The *KITLG* gene produces two alternative isoforms, and the longer soluble form is illustrated. This isoform is proteolytically cleaved at the sites indicated by arrowheads and produces the soluble form of KITLG. The other shorter isoform lacks the cleavage site located proximal to the transmembrane domain and remains bound to the membrane. sKITLG, soluble KITLG; SP, signal peptide; TR, transmembrane region; CD, cytoplasmic domain; □A, □B, □C and □D, □helices; □1 and □2, □strands.(D) Close-up view of the KITLG-KIT 2:2 complex 3D structure. The main chain of KITLG is colored in cyan and KIT in green. The amino acids mutated in WS2 are labeled in yellow (Arg32) or white (Leu104). The amino acids mutated in FPHH are labeled in orange. Arg32 interacts with Ser240' of KIT via van-der-Waals contact. The red bridge shows a disulfide bond between Cys29 and Cys113 of KITLG.

Supporting Information

Figure S1. Additional clinical features of the patient.

Figure S2. Copy number assay of the KITLG gene.

Figure S3. The heterozygous *EDNRB*Δ3 isoform mutation in the patient.

Table S1. Reported cases of genetically diagnosed WS and related diseases (TS, PCWH/PCH and YDHS). (TS, Tiez syndrome; PCWH/PCH, peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, and Hirschsprung disease; YDHS, Yemenite deaf-blind hypopigmentation syndrome)

Table S2. Reported cases of genetically diagnosed WS, TS, PCWH/PCH and YDHS with hyperpigmentation of skin.

Supplementary References