**SLUG (SNAI2) deletions in patients with Waardenburg disease**

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Waardenburg syndrome (WS; deafness with pigmentary abnormalities) is a congenital disorder caused by defective function of the embryonic neural crest. Depending on additional symptoms, WS is classified into four types: WS1, WS2, WS3 and WS4. WS1 and WS3 are caused by mutations in *PAX3* whereas WS2 is heterogeneous, being caused by mutations in the *microphthalmia (MITF)* gene in some but not all affected families. The identification of *Slug*, a zinc-finger transcription factor expressed in migratory neural crest cells, as the gene responsible for pigmentary disturbances in mice prompted us to analyse the role of its human homologue *SLUG* in neural crest defects. Here we show that two unrelated patients with WS2 have homozygous deletions in *SLUG* which result in absence of the *SLUG* product. We further show that Mitf is present in Slug-deficient cells and transactivates the SLUG promoter, and that Slugh and Kit genetically interact *in vivo*. Our findings further define the locus heterogeneity of WS2 and point to an essential role of SLUG in the development of neural crest-derived human cell lineages: its absence causes the auditory–pigmentary symptoms in at least some individuals with WS2.

**INTRODUCTION**

Waardenburg syndrome is a rare (1/40 000) disorder with pigmentary abnormalities and sensorineural deafness, usually inherited in an autosomal dominant manner (1). The main subtypes of WS are type 1 (WS1), associated with dystopia canthorum and type 2 (WS2), without dystopia. WS is a disorder of the neural crest or its derivatives and molecular dissection of WS provides an avenue for investigating neural crest differentiation. WS1 (and the rare type 3 and type 4 variants WS3 and WS4) represent failures of several neural crest derivatives, whereas WS2 appears to be a specific failure of melanocytes. WS1 and WS3 are caused by mutations in *PAX3* (2–4), whereas WS2 is heterogenous (5). Some WS2 families have mutations in the *microphthalmia (MITF)* gene and occasional cases are caused by mutations in endothelin 3 or its receptor *EDNRB* (these are more usually associated with Hirschsprung disease in the rare WS4 variant), but for many cases the cause is unidentified. *SLUG* is one possible candidate (6). This zinc finger transcription factor is a marker of neural crest cells in *Xenopus*, zebrafish and chick embryos and probably has a functional role in formation of premigratory neural crest (reviewed in 7). In the mouse, the corresponding gene, *Slugh*, is expressed in migratory but not premigratory neural crest cells and is not essential for neural crest development (7). We recently showed that mice homozygous for loss-of-function mutations of *Slugh* often have a white forehead blaze and areas of depigmentation on the ventral body, tail and feet (6), indicating that the function of Slug is necessary for normal development of melanocytes. Because of the phenotypic similarity of these mice to patients with WS2, we set out to investigate a possible role of the human *SLUG (SNAI2)* gene in Waardenburg syndrome.

**RESULTS AND DISCUSSION**

An initial series of DNAs from 38 unrelated patients, all with features of Waardenburg syndrome [but not necessarily fulfilling the formal diagnostic criteria of Liu et al. (8)] and negative for *MITF* mutations, were screened by Southern blotting for *SLUG* gene rearrangements or deletions using a full-length *SLUG* cDNA probe (6). As shown in Figure 1, two subjects showed deletions of *SLUG* spanning the entire *SLUG* coding region. As a control for differences in DNA loading, the blot was stripped and rehybridized with a probe for the *KIT* gene (6), which maps to chromosome segment 4q11–q12. No significant differences in DNA loading were observed with this control probe (Fig. 1C). We tested the extent of the *SLUG*
deletions by analysis of two closely flanking markers, STSG29942 and STSG54511. Both of these markers, which flank the SLUG locus, were not homozygously deleted in the two subjects, indicating that the deletions in these patients are relatively short. To confirm these data and accurately determine SLUG copy number, we developed a real-time quantitative PCR assay for part of exon 1 of the gene. As shown in Table 1, the target sequence was not detected in subjects B and D, or in Slugh+/− mice. These results thus confirm SLUG deletions in subjects B and D.

Figure 1. Southern blot hybridization of SLUG and KIT probes to DNAs from individuals with WS2. Schematic representation of the genomic organization of the human SLUG gene with the three exons (A). DNAs were cleaved with NheI and EcoRI. The filter was sequentially hybridized to probes for SLUG (B) and KIT (C). Size markers are labelled in kb.

Table 1. SLUG (SNAI2) copy number quantitation by real-time PCR. The Ct is defined as the PCR cycle number at which the fluorescence passes a fixed threshold, which is inversely proportional to the number of target copies present in the sample.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Subjects</th>
<th>Slug+/+</th>
<th>Slug+/−</th>
<th>Slug−/−</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLUG</td>
<td>A</td>
<td>23.63±0.1</td>
<td>27.63±0.1</td>
<td>ND</td>
<td>23.29±0.51</td>
<td>ND</td>
<td>23.54±0.36</td>
<td>ND</td>
<td>23.64±0.25</td>
</tr>
<tr>
<td>β-Actin</td>
<td>E</td>
<td>23.44±0.21</td>
<td>23.35±0.9</td>
<td>23.35±0.29</td>
<td>23.59±0.43</td>
<td>23.61±0.23</td>
<td>23.44±0.27</td>
<td>23.29±0.10</td>
<td>23.19±0.24</td>
</tr>
</tbody>
</table>

Ct values ± SEM of triplicate assays are shown. ND, fluorescence not detected.
We next searched for point mutations in the SLUG gene in the remaining 36 WS2 patients where we did not detect homozygous deletion of the SLUG gene. Primers were designed to amplify each coding exon with flanking intron sequence from genomic DNA, and the PCR products were sequenced. We failed to detect any SLUG mutations in DNA from the 36 non-deleted patients. Because we have not examined the non-coding part of the gene, it is possible that a more extensive analysis of the entire gene might detect additional mutations in our panel of patients. The possibility of a population polymorphism was excluded as we detect no SLUG deletions in 150 controls studied.

Patients were not selected for any particular mode of inheritance. Of the 38, 13 had a clearly dominant family history, 12 (including the two deletion cases) had pedigrees fully compatible with recessive inheritance and 13 were classified as possible recessive. The ‘possibly recessive’ category included patients where a parent or more distant relative was reported to have some minor pigmentary change such as early graying or a white forelock. In some cases patients with similar histories have turned out to be homozygous for connexin 26 mutations (A.P.R., unpublished), showing that such pedigrees can be compatible with recessive inheritance. Connexin 26 mutations were excluded in our patients.

Subject B was a 15-year-old girl of Bangladeshi origin. She had profound bilateral sensorineural hearing loss, reported to be congenital and heterochromia irides, but no other dysmorphic features or pigmentary changes. Her non-consanguinous parents were normal, as were four siblings. As a child she had suffered fits of unknown origin; CT scan revealed no abnormality. In this regard, W" heterozygotes can show audiogenic seizures, which may be related to the fits described.

Subject D was a 3-year-old Dutch boy who had a 60 dB hearing loss and unilateral heterochromia. All other investigations (ophthalmology, thyroid function, ECG) were normal. He was the only child of normal unrelated parents, born after an uneventful pregnancy and delivery. There was no recorded family history of hearing loss or pigmentary abnormalities.

Mice (Fig. 2) and humans lacking Slugh/SLUG show neural crest dysfunction. In the human cases this manifests as heterochromia irides and hearing loss, both attributable to patchy absence of melanocytes (1). Hearing loss has not been assessed in the Slugh"/" mice, but we have observed hyperactivity and circling in some mutant animals, a behaviour suggestive of hearing impairment. In the Slugh"/" mice, the phenotypes are influenced by genetic background (6, unpublished observations). Mapping and identifying modifiers of the phenotype may help identify phenotypic determinants in human disease.

The molecular mechanism by which SLUG regulates embryonic development is not known. Thus, we next examined the expression of Pax3, mi, Sox10 and Slugh in the Slugh"/" mice model. As shown in Figure 3, Pax3, mi and Sox10 mRNA were present in Slug-deficient cells. These results are congruent with the observations that, in mouse embryos, mi is expressed earlier than Slugh in the neural crest where melanocytes
functions upstream of mi Kit development and survival of the melanocyte lineage. melanocytes (10) exhibit striking coat colour phenotypes due to loss of viable Waardenburg syndrome. containing the transfected into NIH-3T3 cells along with the reporter vector MITF expression vector containing an failure to activate SLUG responded to MITF activation (Fig. 4). It seems likely that start. When this E box was mutated, the promoter no longer sequence CA[C/T]GTG 1396 bp upstream of the transciptional promoter region of the human SLUG gene were cloned from DNA sequences present in the SLUG promoter, an expression vector containing an MITF cDNA was co-transfected into NIH-3T3 cells along with the reporter vector containing the SLUG promoter. Co-expression of MITF resulted in an approximately three-fold increased in luciferase activity compared with the activity with the empty vector (Fig. 4). These results strongly suggest that MITF interacts with a sequence in the SLUG promoter. Indeed, there is a potential MITF binding site, CATGTG, that fits the E box consensus sequence CA[C/T]GTG 1396 bp upstream of the transcriptional start. When this E box was mutated, the promoter no longer responded to MITF activation (Fig. 4). It seems likely that failure to activate SLUG explains why MITF mutations cause Waardenburg syndrome.

Like mi mutants, Kit and Scf (stem cell factor) mutant mice exhibit striking coat colour phenotypes due to loss of viable melanocytes (10–12). These genes co-ordinately modulate the development and survival of the melanocyte lineage. Kit functions upstream of mi (13) and it has been demonstrated that Kit and mi interact in vivo, resulting in profound melanocyte loss in compound heterozygous mice (14). Thus if Slug mediates the effect of mi on melanocyte survival, Kit and Slug should genetically interact in vivo similarly to Kit and mi. To examine genetic interactions between Kit and Slug in vivo, we generated double heterozygotes for a Slug null allele and the W' mutation of Kit. Complete absence of functional Kit produces mice devoid of melanocytes from birth (Fig. 5D) but heterozygous W'/+ mice are born black with occasional white spots (Fig. 5A). Similarly, homozygous mice with the targeted mutation of the Slug gene are born black with occasional white spots (Fig. 2). In the context of W'/+ heterozygosity, additional loss of a single Slug allele produced extensive white spotting from birth (Fig. 5B and C). Similar to the genetic cooperativity between Kit and mi, these observations demonstrate genetic cooperativity between Kit and Slug for melanocytes.

In summary, we have shown that mice lacking Slug have patchy deficiency of melanocytes, a phenotype similar to human Waardenburg syndrome. We showed that some human patients with Waardenburg syndrome carry homozygous deletions of SLUG as their only detected genetic abnormality, thus defining a recessive form of type 2 WS. Preliminary investigations of the role of SLUG in melanocyte development show that it is a downstream target of mi MITF, which acts on an E-box sequence in the SLUG promoter and that Slugh Kit double heterozygous mice show similar melanocyte defects to mi Kit double heterozygotes, suggesting that SLUG may be the main effector of mi/MITF action in melanoblasts. The frequency and phenotypic range of humans deficient for SLUG remain to be determined.

MATERIALS AND METHODS

Subjects

DNAs from 38 unrelated individuals with WS2 were studied. All patients lack detectable MITF gene mutations. The two affected have unaffected parents and are sporadic cases. None of these patients’ parents are consanguinous.

Southern blot analysis

Blood was obtained with informed consent and genomic DNAs were prepared from peripheral blood lymphocytes. DNAs were digested with restriction endonucleases as described (15), separated by electrophoresis in 0.8% agarose, transferred to Hybond-N (Amersham). Filters were UV cross-linked and hybridized to a [32P]-radiolabelled full-length SLUG cDNA probe. DNA loading was monitored by reprobing the filters with a full-length mouse Kit cDNA.

Real-time PCR quantification

Real-time quantitative PCR (16) was developed and carried out for the detection and quantitation of both mouse and human SLUG genes. The exon 1 primers SLUGF (5’-ATGCCCGGCTCCTTCTCT-3’) and SLUGB (5’-TGTTGCCTCAGTTGC-3’) were used in conjunction with a fluorogenic probe SLUGP (5’-(FAM)-CATTTCAAGCCCTCAGCCTAAGCC-(TAMRA)-3’). The fluorogenic probe contained a 3’ blocking phosphate group to prevent probe extension during
PCR. Fluorogenic PCRs were set up in a reaction volume of 50 μl using the TaqMan PCR Core Reagent kit (PE Biosystems). Fluorogenic probes were custom-synthesized by PE Biosystems. PCR primers were synthesized by Isogen. Each reaction contained 5 μl of 10× buffer; 300 nM of each amplification primer; 25 nM fluorogenic probe; 200 μM of each dNTP; 1.25 U AmpliTaq Gold, 2 mM MgCl2 and 10 ng DNA. DNA amplifications were carried out in a 96-well reaction plate format in a PE Applied Biosystems 5700 Sequence Detector.

Thermal cycling was initiated with a denaturation step of 10 min at 95°C followed by 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 1 min. Multiple negative water blanks were tested and a calibration curve determined in parallel with each analysis. DNAs (10 ng each) from Slug+/+, Slug−/−, and Slug+/− mice were used for constructing the calibration curve. The β-actin endogenous control (PE Biosystem) was included to relate both mouse and human SLUG samples to total genomic DNA in each sample.

Cycle threshold (Ct) readings from Slug+/+, Slug−/−, and Slug−/− mice were utilized to establish standard curves for each reaction series. The Ct was computed for each sample using Sequence Detection Software (Applied Biosystems) and these settings were automatically used for further computations. Standard curves were generated by plotting the mean of triplicate Ct values versus the log of the Slug copy number. The copy numbers for unknown samples were determined by applying the mean Ct value of triplicates to the standard curve. Although equal amounts of DNA were used (10 ng each), a

Figure 5. Genetic interaction between Kit and Slug in vivo. Mice of the indicated genotypes were crossed and their offspring genotyped. Note that a Wv+/; Slug+/- mouse has an almost fully pigmented coat on the dorsal side whereas a 4-week-old Wv+/; Slug+/- and a 4-week-old Wv−/−; Slug−/− mouse show extensive white spotting from birth. The Wv−/− mouse, which is a compound heterozygote carrying two different mutant alleles of the same gene, is white from birth.
β-actin endogenous control was included to relate both mouse and human Slug/SLUG samples to total genomic DNA in each sample.

Analysis of genomic DNA

The three exons of the human SLUG gene plus adjacent non-coding and flanking sequences were amplified in duplicate from DNA of patients without SLUG deletions by PCR. The nucleotide sequences of at least six independent clones of each were determined.

Mouse crosses

Animals were housed under non-stereile conditions in a conventional animal facility. Mice heterozygous and homozygotes for the SlugA1 mutation generated by removing the genomic sequences of the entire Slug protein-coding region (SlugA1 mutant mice) have been described previously (7). W/Wv and breeding pairs were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Heterozygous SlugA/- mice were bred to Wv mice to generate compound heterozygotes. F1 animals were crossed to obtain null SlugA/- mice heterozygous for Wv deficiency.

Plasmid constructs and luciferase assays

The ~1777 bp upstream promoter sequence of Slug was isolated from a P1 clone containing the SLUG gene (Genome Systems) and cloned into the luciferase reporter plasmid pGL3-basic (Promega) and termed pSLUG-1777. The pSLUG-1777mut is identical to pSLUG-1777 except that the E-box sequence (CATGTTG) in position −1396 is substituted with the mutant sequence gAgGTG (mutated nucleotides in lower case). Human MITF cDNA was generated by RT–PCR of total RNA from human melanocytes. Nucleotide sequence was verified by sequencing and the cDNA was cloned into the expression vector. N. Engl. J. Med., 334, 28–33.

REFERENCES