NBEAL2 is mutated in Gray Platelet Syndrome and is required for biogenesis of platelet alpha-granules

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Abstract

Gray Platelet Syndrome (GPS) is an autosomal recessive bleeding disorder with large platelets that lack α-granules. We found that mutations of NBEAL2 (neurobeachin-like 2), encoding a BEACH/ARM/WD40 domain protein, cause GPS. We demonstrated that human megakaryocytes and platelets express a unique combination of NBEAL2 transcripts. Proteomic analysis of sucrose-gradient subcellular fractions of platelets indicated that NBEAL2 localizes to the dense tubular system (endoplasmic reticulum) in platelets.

Keywords

Gray platelet syndrome; NBEAL2; neurobeachin; platelet α-granules; organelle biogenesis

Platelets are organelle-rich cells that transport granule-bound compounds to tissues throughout the body. Platelet α-granules, the most abundant platelet organelles, store large proteins that, when released, promote platelet adhesiveness, haemostasis and wound healing\textsuperscript{1,2}, while platelet dense (δ) granules contain small, non-protein molecules such as calcium, serotonin, adenosine diphosphate, adenosine triphosphate, and pyrophosphate that promote platelet aggregation\textsuperscript{1,3,4,5}. Bleeding disorders arising from defective platelet granules constitute the platelet storage pool diseases (SPD) and include isolated δ-granule deficiency (delta-SPD), combined α and δ-SPD and isolated α-granule deficiency (GPS; OMIM #139090)\textsuperscript{3,4,6}. GPS platelets are large and appear gray on light microscopy (Fig. 1a-b)\textsuperscript{5-7}; the diagnosis is confirmed by electron microscopy (EM) showing absent or markedly reduced α-granules in platelets\textsuperscript{8}(Fig. 1c-d) and in megakaryocytes\textsuperscript{9}, although both platelets and megakaryocytes have rudimentary α-granule precursors\textsuperscript{10}.

Clinical manifestations of GPS are usually mild to moderate and infrequently severe\textsuperscript{6}. GPS is also associated with myelofibrosis (Fig. 1e-f) and splenomegaly as a consequence of myelofibrosis\textsuperscript{5-7}. The basis of myelofibrosis remains unknown, but constitutive release of platelet-derived growth factor and other pro-fibrotic substances from megakaryocytes into the bone marrow may be involved\textsuperscript{5}.

Some platelet α-granule constituents are passively (e.g., immunoglobulins, albumin) or actively (e.g., fibrinogen) taken up from the plasma by receptor-mediated endocytosis; others are synthesized in megakaryocytes (e.g., platelet factor 4, β-thromboglobulin) and trafficked to the organelle\textsuperscript{3}. In GPS, proteins synthesized in megakaryocytes are markedly reduced, while endocytosed α-granule constituents are less affected\textsuperscript{3}. This suggests that GPS megakaryocytes fail to pack their endogenously synthesized secretory proteins into developing α-granules.
By genome-wide linkage analysis and homozygosity mapping of 25 GPS patients from 14 unrelated families, we previously mapped the GPS disease locus to a 9.4 megabase interval on 3p21.1-22.1 that includes 197 protein coding genes. Initial whole exome and Sanger dideoxy sequencing revealed no mutations in any of these genes, but subsequent Sanger dideoxy sequencing of exons not previously covered did reveal mutations in a single gene. In fact, 15 unrelated GPS patients exhibited NBEAL2 (ENSG00000160796) mutations, including 5 missense, 3 nonsense, 4 frameshifting and 3 consensus splice site mutations (Table 1, Fig. 1g, Supplementary Fig. 1 and 2). None of these variants was found in the 1000 Genomes Database (http://www.1000genomes.org/), which contained 629 genomes as of February 2011, or in 100 ethnically matched control individuals. The 14 affected individuals with identity by descent had homozygous mutations (Table 1) whereas those without identity by descent exhibited compound heterozygous mutations.

All missense variants alter conserved amino acids and have high pathogenicity prediction scores (Supplementary Fig. 1p). Splice donor site mutations c.1296+5G>A, c.5301+1G>A result in use of cryptic intronic donor sites demonstrated in blood mRNA, and c. 5720+5G>A is predicted to obliterate a splice donor site (Supplementary Fig. 1).

Patients from families GPS-6 and GPS-8 illustrated interfamilial variability. Although each is homozygous for splice mutation c.1296+5G>C, the patient from family GPS-6 has mild coagulopathy and the patient from family GPS-8 has severe coagulopathy. Similar findings were observed for other GPS patients; the severity of coagulopathy, myelofibrosis and splenomegaly did not correlate with the type or location of the NBEAL2 mutation (Table 1, Fig. 1g).

Human NBEAL2 is predicted to produce 15 different mRNA transcripts, of which 7 would be protein coding (Ensembl) (Supplementary Fig. 3a). We designed cDNA primers to differentially amplify all protein encoding NBEAL2 transcripts (Supplementary Fig. 3) and tested RNA from a variety of human hematopoietic cells and other tissues. A unique combination of transcripts encoding NBEAL2-001, 201/003, 203/004 and 202 was expressed in megakaryocytes and platelets (Supplementary Table 1).

Antibodies to NBEAL2 are not available. To explore the subcellular localization of NBEAL2 protein, we performed proteomic analysis on sucrose-gradient subcellular fractions from normal platelets and identified 2 tryptic peptides from the protein NBEAL2 using mass spectrometry (Fig. 1g, Supplementary Fig. 4). These peptide sequences, contained within NBEAL2 transcripts 001, 003 and 201, were found in platelet subcellular fraction 4, which contained small membrane structures on EM analysis that likely originated from the dense tubular system. In fact, fraction 4 was enriched in dense tubular system and ER markers (data not shown).

The fibrotic nature of the GPS bone marrow prevented us from obtaining sufficient bone marrow for ex-vivo expansion of GPS megakaryocytes, the only cell type that expresses the GPS phenotype of defective α-granule biogenesis. Our previous microarray data in GPS fibroblasts showed overexpression of fibronectin, essential for proplatelet formation in cultured megakaryocytes and critical for megakaryocyte-matrix interactions. Future
studies investigating GPS megakaryocytes might shed light on the pathogenesis of the myelofibrosis in GPS.

How absence of NBEAL2 function in megakaryocytes results in defective α-granule biogenesis remains unknown. However, NBEAL2 belongs to the family of proteins that contain an ARM, BEACH (beige and Chediak-Higashi syndrome) and WD40 domains, highly conserved regions that are crucial for protein-protein interactions, membrane dynamics and vesicle trafficking. Another such protein is CHS1, which is defective in Chediak-Higashi disease (CHD, OMIM #214500), a disorder of immunodeficiency, platelet dense granule defects, partial albinism, and enlarged lysosomes or lysosome-related organelles in hematopoietic cells and melanocytes. The precise cell biological defects in GPS and CHD remain unknown, but both diseases involve large proteins and impaired formation and trafficking of intracellular vesicles. NBEAL2 protein is predicted to interact with WDFY3 (WD repeat and FYVE domain containing 3), which itself interacts with CHS1, and with DLL1 and jagged 1 (http://www.sabiosciences.com/genenetwork/genenetworkcentral.php), known to have roles in hematopoiesis. DLL1 is the human homolog of the Notch Delta ligand and jagged 1 is the ligand for the receptor notch 1. These protein-protein interactions are entirely based on computational predictions; future experiments will determine their accuracy. Understanding NBEAL2 function will likely lead to the discovery of novel pathways of organelle formation and maturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Figure 1. Cellular studies of gray platelet syndrome**

(a) Light microscopy of a peripheral blood smear of the patient from family GPS-14 showing pale gray platelets (arrows), some larger than normal. (b) Normal, darkly stained platelets (arrows). (c) Transmission electron microscopy of thin sections of a platelet from GPS-13 showing absence of α-granules and abundant channels of the open canalicular system (OCS). DTS: Dense tubule system; DB: Dense body; M: Mitochondrion. (d) Normal platelet with α-granules (AG). (e) Reticulin staining of bone marrow of the patient from family GPS-4 displaying myelofibrosis (black strands). (f) Normal bone marrow without fibrosis. Scale bar indicates 10 μm (a, b) and 50 μm (e,f), (magnification x200). (g) Schematic representation of _NBEAL2_ gene (ENSG00000160796) with mutations indicated. The _NBEAL2-001_ isoform (ENST00000450053) is depicted with its BEACH, WD40 and ARM-type fold domains. Small green bars, labeled A and B, represent two NBEAL2 peptide fragments identified by mass spectrometry. A: WGSPTSLEGELGAVAIFHEALQATALR; B: AFFAEVSDGVPLVLLALVPHR.
| Family No. | Ethnicity | Severity of Bleeding | Exon/Intron | Mutation State |
|-----------|-----------|----------------------|-------------|----------------|
| GPS-1     | Muslim Bedouins | Moderate             | c.2701C>T   | p.Arg901X      | 19 Homozygous   |
| GPS-2     | Mennonite | Severe               | c.881C>G    | p.Ser294X      | 8 Homozygous    |
| GPS-3     | Caucasian (French) | Moderate         | c.1163T>C   | p.Leu388Pro    | 11 Homozygous   |
| GPS-4     | Caucasian (Turkish) | Severe            | c.5720+5G>A | Intron 35     | Homozygous      |
| GPS-5     | Caucasian (Turkish) | Mild               | c.5515C>T   | p.Arg1839Cys   | 34 Homozygous   |
| GPS-6     | Caucasian (Turkish) | Mild               | c.1296+5G>C | Intron 12      | Homozygous      |
| GPS-7     | Caucasian (German) | Severe            | c.2257_2260delGCC | p.Ala753SerfsX65 | 16 Homozygous    |
| GPS-8     | Caucasian | Severe               | c.1296+5G>C | Intron 12      | Homozygous      |
| GPS-9     | Caucasian (Turkish) | Severe            | c.3819_4174del356 | p.Val1274GlyfsX32 | 27 Homozygous    |
| GPS-10    | African American | NA                 | c.2029T>A   | p.Trp677Arg    | 14 Homozygous   |
| GPS-11    | Caucasian | Moderate             | c.7604delG   | p.Gly2535ValfsX5 | 50 Homozygous    |
| GPS-12    | Caucasian | Severe               | c.5505T>G   | p.Tyr1835X     | 34 Heterozygous |
| GPS-13    | Caucasian | Moderate             | c.2701C>T   | p.Arg901X      | 19 Compound heterozygous |
|           |           |                      | c.6787C>T   | p.His2263Tyr   | 42              |
| GPS-14    | African American | Mild               | c.2156delT  | p.Phe719SerfsX100 | 16 Compound heterozygous |
|           |           |                      | c.5497G>A   | p.Glu1833Lys   | 34              |
| GPS-15    | Hispanic (Mexican) | Mild              | c.5301+1G>A | Intron 32      | Homozygous      |

*See also Supplementary Fig. 1. Extensive clinical and mapping data on families GPS-1 to GPS-14 were previously reported\(^6\). GPS-1 to GPS-11\(^6\) and GPS-15 (Supplementary Fig. 2) exhibited identity by descent. In GPS-12, only 1 heterozygous mutation was identified and no tissue was available for NBEAL2 mRNA analysis. NA: not available.*