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Hamostaseologie. 2016 November 7; 36(4): 279–283. doi:10.5482/HAMO-16-03-0010.**Inherited dysfunctional platelet P2Y₁₂ receptor mutations associated with bleeding disorders****Anna Lecchi¹, Eti A. Femia², Silvia Paoletta³, Arnaud Dupuis⁴, Philippe Ohlmann⁴, Christian Gachet⁴, Kenneth A. Jacobson³, Katharina Machura⁵, Gian M. Podda², Barbara Zieger⁵, and Marco Cattaneo¹**¹Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Ca' Granda – Ospedale Maggiore Policlinico, Milan, Italy²Divisions di Medicina 3, ASST Santi Paolo e Carlo, Dipartimento di Scienze della Salute - Università degli Studi di Milano, Milan, Italy³Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD⁴Unité Mixte de Recherche_S949, Inserm, Université de Strasbourg, Etablissement Français du Sang-Alsace-Lorraine-Champagne-Ardennes, Strasbourg, France⁵Department of Pediatrics and Adolescent Medicine, Division of Pediatric Hematology and Oncology, Medical Center – University of Freiburg, Faculty of Medicine, Germany**Summary**

The platelet adenosine 5'-diphosphate (ADP) receptor P2Y₁₂ (P2Y₁₂R) plays a critical role in platelet aggregation. The present report illustrates an update of dysfunctional platelet P2Y₁₂R mutations diagnosed with congenital lifelong bleeding problems. Described patients with heterozygous or homozygous substitution in the *P2Y₁₂R* gene and qualitative abnormalities of the platelet P2Y₁₂R are summarized. Recently, a further dysfunctional variant of P2Y₁₂R has been identified in two brothers who presented with a lifelong severe bleeding disorder. During *in vitro* aggregation studies, the patient's platelets show a markedly reduced and rapid reversible ADP-promoted aggregation. A homozygous c.561T>A substitution that changes the codon for His187 to Gln (p.His187Gln) in the *P2Y₁₂R* gene has been identified. This mutation causes no change in receptor expression but decreases the affinity of the ligand for the receptor, even at high concentrations. Structure modelling studies indicated that the p.His187Gln mutation, located in the fifth transmembrane spanning domain (TM5), impairs conformational changes of the receptor. Structural integrity of the TM5 region is necessary for agonist and antagonist binding and for correct receptor function.

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Conflict of interest

The authors state that they have no conflict of interest.

Zusammenfassung

Der thrombozytäre Adenosin-5'-diphosphat-(ADP)-Rezeptor P2Y₁₂ (P2Y₁₂R) spielt eine entscheidende Rolle bei der Thrombozytenaggregation. Im Folgenden soll ein kurzes Update über dysfunktionale thrombozytäre P2Y₁₂R-Mutationen, welche im Zusammenhang mit angeborenen, lebenslang bestehenden Blutungs-problemen diagnostiziert wurden, gegeben werden. Patienten mit bereits beschriebenen homo- und heterozygoten Substitutionen im *P2K₁₂*-Gen sowie die daraus folgenden qualitativen Abnormitäten des thrombozytären P2Y₁₂R werden hier zusammengefasst. Zudem wurde kürzlich bei einem Brüderpaar mit einer langjährigen schweren Blutungsstörung eine weitere dysfunktionale Variante des P2Y₁₂R identifiziert. In *in vitro*-Aggregationsstudien zeigen die Thrombozyten dieser beiden Patienten eine erheblich reduzierte und schnell reversible ADP-induzierte Aggregation. Hierbei wurde eine homozygote c.561T>A Substitution, die das Kodon für His187 zu Gln (p.His187Gln) im P2Y₁₂R-Gen verändert, nachgewiesen. Die Mutation verursacht dabei keine Änderung der Rezeptorexpression, erhöht aber selbst bei einer hohen Ligandenkonzentration die Affinität des Liganden zum Rezeptor. „Strukturmodellierung“-Studien zeigen, dass die in der fünften Transmembrandomäne (TM5) lokalisierte p.His187Gln Mutation die Konformationsänderung des Rezeptors beeinträchtigt, was darauf hindeutet, dass die strukturelle Integrität der TM5 Region für die Bindung von Agonisten als auch von Antagonisten notwendig ist und damit zu einer normalen Rezeptorfunktion beiträgt.

Keywords

Platelets; mutation; P2Y₁₂ receptor

Schlüsselwörter

Thrombozyten; Mutation; P2Y₁₂-Rezeptor

P2Y₁₂ is an adenosine 5'-diphosphate (ADP) receptor (P2Y₁₂R) that is mostly expressed at platelet surface and mediates the amplification of the platelet activation process necessary to achieve full and irreversible aggregation. So far, two human platelet ADP receptors have been identified, P2Y₁R and P2Y₁₂R, which belong to the seven transmembrane domain family of G protein-coupled receptors (1,2).

The P2Y₁ receptor-signaling in platelets is mediated by G_q inducing a transient increase in intracellular calcium levels by phospholipase C β (PLC β) and activation of the Rho kinases, which results in platelet shape change, and the start of reversible platelet activation (Fig. 1) (3–5). In contrast, P2Y₁₂R is coupled to G_i. The activation of the P2Y₁₂R by ADP has two main consequences: it leads to an inhibition of adenylyl cyclase (AC) and to an activation of phosphoinositide 3-kinase (PIK3), which amplifies and sustains the platelet aggregation response (2, 6, 7). The coactivation of both the G_q and G_i pathways by ADP is essential for a normal platelet aggregation.

The pathways converge to promote activation of the small G protein, Rap1b, leading to a conformational change of the integrin $\alpha_{IIb}\beta_3$ receptor from the inactive to the active form. Fibrinogen binds to the activated $\alpha_{IIb}\beta_3$ receptor and cross-links platelets (8). Some platelet

agonists promote the formation of thromboxane A₂ (TxA₂) and granule degranulation releasing ADP, adenosine 5'-triphosphate (ATP) and serotonin. ADP, serotonin and TxA₂ activate nearby circulating platelets and amplify aggregation (9–11), whereas ATP acts as an antagonist (12, 13). P2Y₁₂R plays a critical role in hemostasis and thrombosis (14, 15). This function is highlighted by the observation that patients with inherited and pharmacologically induced defects of this receptor display a bleeding diathesis of variable severity (1, 16–19). Furthermore, patients with clinical manifestations of arterial thrombosis could be protected from major adverse cardiovascular events by P2Y₁₂ receptor inhibitors (20).

As consequence P2Y₁₂R is a therapeutic target of effective antiplatelet agents (14).

Inherited P2Y₁₂R defects are autosomal recessive disorders, associated with qualitative or quantitative abnormalities of the receptor (1). While no human molecular defect of P2Y₁R function has been reported, P2Y₁ knockout mice have abnormal primary hemostasis (3).

A P2Y₁₂ receptor defect can be hypothesized when ADP, even at high concentrations (10 μmol/l), is unable to induce full and irreversible platelet aggregation, while inducing normal shape change. In addition, no fibrinogen binding is detectable after stimulation with ADP (0.25–5.0 μmol/l). Tests that evaluate the degree of inhibition of adenylyl cyclase by ADP, by measuring either the platelet levels of cyclic AMP or the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) after the exposure of platelets to prostaglandin E₁ (PGE₁) (VASP phosphorylation analysis), should be used to confirm the diagnosis (21).

So far, the following function-modifying mutations in the gene encoding P2Y₁₂R have been described (Fig. 1, Tab. 1).

One patient with inherited bleeding problems, displaying two separate heterozygous point mutations in two alleles of the gene was identified in 2003 (22). In one allele, a G to A transition changes the codon for Arg256 to Gln (p.Arg256Gln) in the sixth transmembrane domain (TM6) and, in the other one, a C to T transition changes the codon for Arg265 to Trp (p.Arg265Trp) in the third extracellular loop (EL3). Neither the p.Arg256Gln nor the p.Arg265Trp variant interfered with receptor surface expression but both blocked receptor function. The patient's platelets underwent normal ADP-induced shape change, reflecting a normal P2Y₁R function. The p.Arg256Gln and p.Arg265Trp variants supported normal binding to [³³P]2MeS-ADP, suggesting a failure in receptor function downstream of agonist interaction. The P2Y₁₂R-mediated platelet response to ADP was abnormal with reduced and reversible aggregation in response to 4 μmol/l ADP and 20 μmol/l ADP. This study shows that the structural integrity of TM6 and EL3 region of the molecule is necessary for normal receptor function (22).

Remijn et al. described a further patient with a dysfunctional mutation in EL3 of P2Y₁₂R in 2007 (23). They identified a heterozygous base pair C to A substitution, changing codon 258 from proline to threonine (p.Pro258Thr) associated with haemorrhagic diathesis. The patient showed no abnormalities regarding platelet count or standard coagulation tests (prothrombin time and activated partial thromboplastin time). However, the ADP- and collagen-induced platelet aggregation were impaired, confirming that this region of the molecule is important

for receptor function. Pro258 has been reported to be involved in conformational change of the P2Y₁₂R molecule. Since the p.Pro258Thr substitution alters the protein hydrophobicity, size, and rotational mobility, it is likely that this mutation affects the function of P2Y₁₂R to this effect (23).

Daly et al. (2009) published another patient who suffers from a mild type 1 von Willebrand disease and platelet function defect. He presented with a heterozygous mutation in the second extracellular loop of P2Y₁₂R (EL2) which is a region that is important for the receptor interaction with ADP (24). This patient showed a tyrosine to glutamate substitution (p.Lys174Glu), which is associated with impairment of platelet, aggregation in response to 1–10 μmol/l ADP. The reduced and reversible response was associated with markedly decreased binding of the ligand [³H]2MeS-ADP to P2Y₁₂R, indicating a role for Lys174 in ligand binding and involvement in conformational change of the P2Y₁₂R molecule (24).

In the same laboratory a further missense mutation, predicting a heterozygous p.Pro341Ala substitution in the postsynaptic density 95/disc large/zonula occludens-1 (PDZ)-binding motif at the carboxyl terminus of the P2Y₁₂R has been identified in 2009. The PDZ-binding domain is necessary for receptor signaling and endocytic sorting. The p.Pro341Ala receptor variant exhibits normal ability to inhibit formation of cAMP and normal platelet aggregation. However, the authors speculate that this mutation interferes with receptor recycling, indicating a role for P341 in intracellular trafficking of the P2Y₁₂R (25).

Patel et al. described a patient with a chronic bleeding disorder expressing a homozygous P2Y₁₂R mutation, predicting an arginine to cysteine (p.Arg122Cys) substitution in 2014 (26). Arg122 is located in the D(E)RY motif, which is a highly conserved region in G-protein-coupled receptors that is speculated to be important for regulation of conformational changes of the receptor and receptor activation. The patient revealed a normal platelet count and normal standard coagulation tests but reduced platelet aggregation in response to ADP in result of an impairment of P2Y₁₂R expression and signaling (26).

Recently, we identified a further platelet P2Y₁₂R defect in two brothers with an inherited bleeding disorder using functional and molecular genetic analyses (27). We sought to determine, whether a novel P2Y₁₂R mutation is the cause for their bleeding abnormalities. The index patient is a 45-year old Turkish man with a lifelong hemorrhagic diathesis. He has 11 siblings who presented with negative, mild or severe bleeding history. His parents are first cousins. The patient's bleeding manifestations are recurrent epistaxis, postoperative bleeding after tooth extraction and surgical intervention. The patient showed normal values for platelet count and standard coagulation tests and for von Willebrand factor antigen and ristocetin cofactor activity. Serum thromboxane B₂ (TxB₂) levels, the platelet contents of serotonin, ADP, adenosine 5' triphosphate (ATP), and fibrinogen were within normal limits. TxB₂ is a stable metabolite of TxA₂ used as a marker of TxA₂ generation. His 57 year old brother has the same bleeding manifestations and the same homozygous P2Y₁₂R defect (27). First, we analysed the aggregation ability and secretion in platelet-rich plasma of the index patient, using light transmission and lumi-aggregometry in Milan, Strasbourg and Freiburg. The patient's platelets exhibited a markedly reduced and rapidly reversible aggregation after stimulation with ADP compared to healthy subjects whose platelets

aggregate in an ADP-dose-dependent manner. Typically, platelets of a patient with a P2Y₁₂R defect are unable to induce full and irreversible platelet aggregation after stimulation with ADP, even at high concentrations (10 μmol/l).

The patient's platelet shape change was normal in response to ADP, being mediated by P2Y₁R, with P2Y₁₂R playing no major role (1, 17). After activation by other agonists (i.e. collagen, thrombin receptor activating peptide, or thromboxane/prostaglandin endoperoxide) the aggregation of the patient's platelets was normal. The binding ability of fibrinogen to activated platelets was measured by flow cytometry using fluorescein-conjugated fibrinogen after ADP stimulation. The binding of fibrinogen to activated platelets was severely impaired compared to healthy subjects (27). Experiments that evaluate the degree of inhibition of adenylyl cyclase by ADP were conducted to confirm the diagnosis, by measuring the platelet levels of cyclic adenosine 3',5'-monophosphate (cAMP) and the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) after exposure of platelets to prostaglandin E₁ (PGE₁) or other stimuli for adenylyl cyclase (1, 21). In the index patient no ADP-induced inhibition of cAMP generation occurred (27). Based on the observation that ADP failed to inhibit the adenylyl cyclase, it could be concluded that the function of the mutated receptor is impaired. Binding experiments performed with the radiolabeled selective P2Y₁₂ antagonist [³H]PSB-0413 allow a differentiation between quantitative and qualitative abnormalities (28–30). The number of binding sites per platelet was comparable in normal and the patient's platelets. Compared to a healthy subject, the brother of the index patient showed a reduced affinity of P2Y₁₂R to its ligand measured by competition between agonists (ADP or 2-methylthioadenosine-5'-diphosphate (2MeSADP)) and the radioligand (27). The observation that the patient's platelets had a normal number of binding sites for [³H]PSB-0413 despite a severely impaired function of the P2Y₁₂ receptor suggested that a deficient receptor was being synthesized. To identify the underlying structural changes of the receptor, we analyzed the coding sequence of the *P2Y₁₂R* gene from genomic DNA by amplifying the complete coding sequence of the *P2Y₁₂R* gene. We identified a homozygous c.561T>A substitution that changed the His187 located in the TM5 region of the receptor to a Gln (p.His187Gln) (27). Recently, it has been described that the His187 of the P2Y₁₂R forms a hydrogen bond with the 2'-OH of 2MeSADP which ultimately leads to the platelet activation process (11).

This study outlines the importance of the structural integrity of TM5 region of the P2Y₁₂R undergoing conformational changes for a normal receptor function, suggesting that this region of the molecule plays a role in signal transduction. His187 is necessary for agonist and antagonist binding, as shown by crystallography data and docking results. A homozygous mutation of His187 in the P2Y₁₂R results in an inherited severe bleeding disorder due to a disrupted P2Y₁₂R-mediated signaling and ultimately impairing platelet aggregation (27).

Conclusion

All these described P2Y₁₂R mutations affect amino acids that are essential for receptor function. The recently reported crystallographic structures of the human P2Y₁₂R in complex with the agonist 2MeS-ADP and the antagonist AZD1283 supported these results (31, 32).

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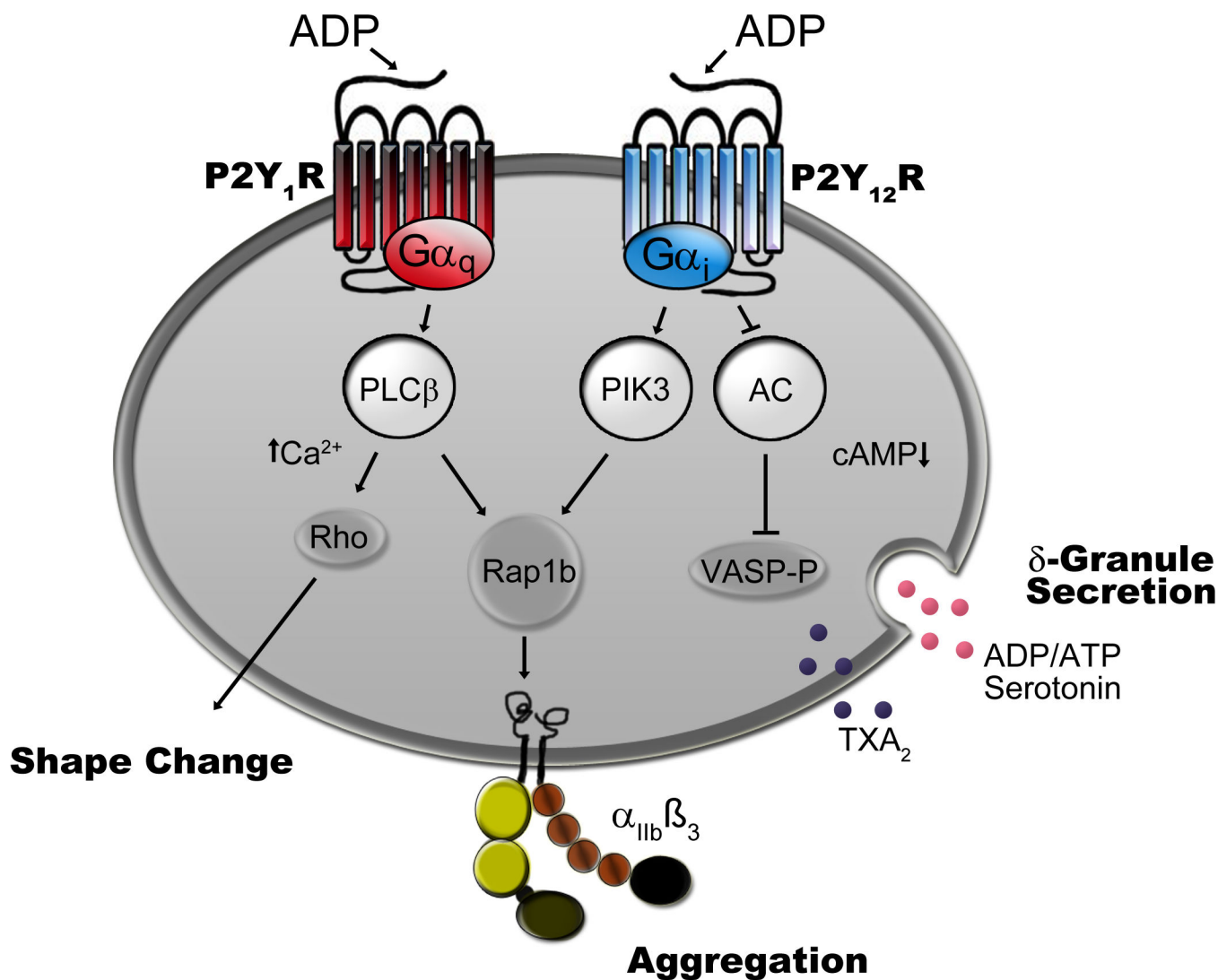


Fig. 1. Simplified receptor signaling in platelet activation: ADP acting via P2Y₁ receptor initiates platelet shape and aggregation. Activation of the P2Y₁₂ receptor results in amplified and sustained aggregation, Hence both P2Y receptors are needed for complete aggregation. ADP, adenosine 5'-diphosphate; G, G-protein; PLC, phospholipase C, adenylyl cyclase; PI3K, phosphoinositide 3-kinase; VASP-P, phosphorylated vasodilator-stimulated phosphoprotein; ATP adenosine 5'-triphosphate.

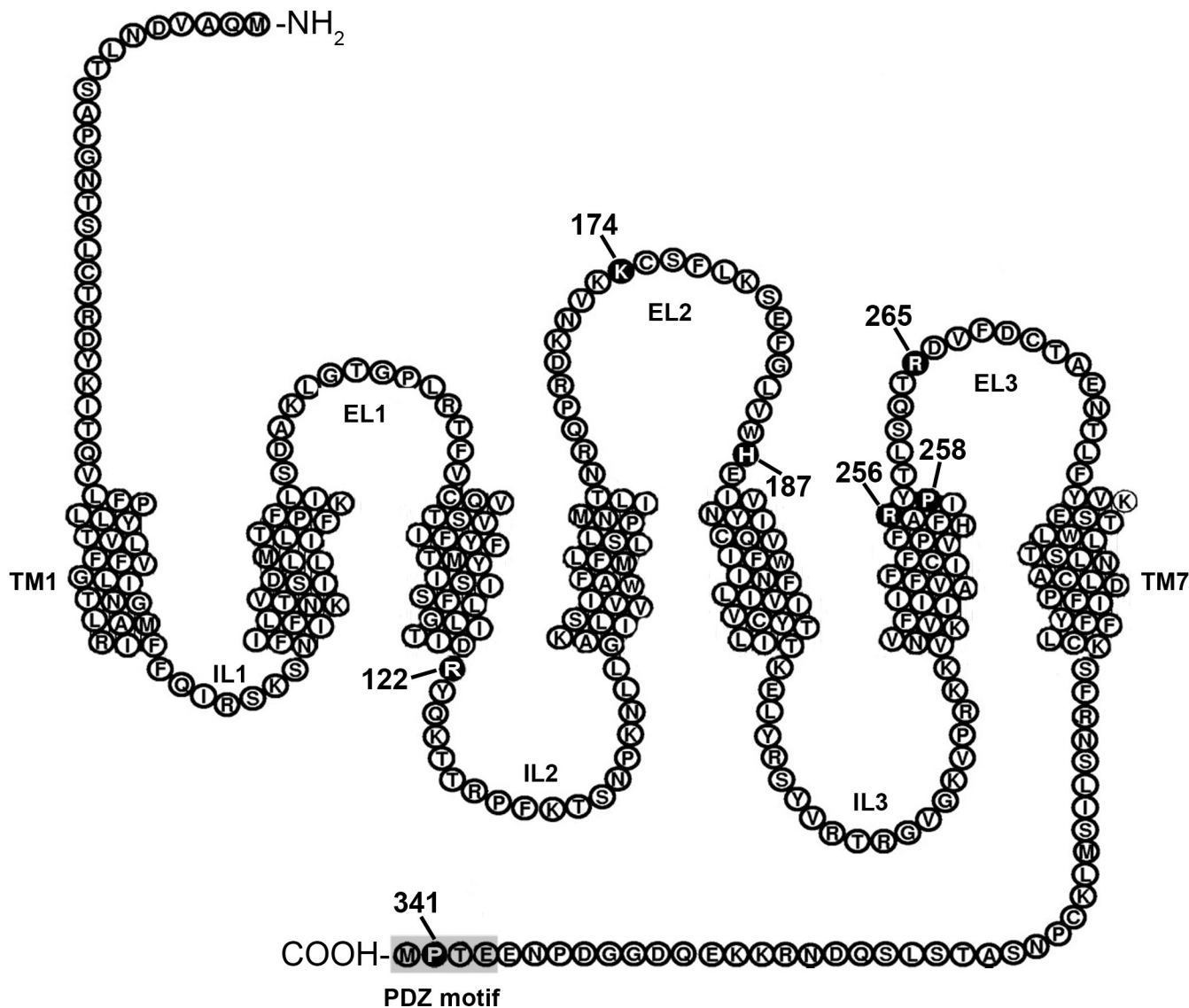


Fig. 2. Secondary structure of the human P2Y₁₂ receptor: Black circles represent P2Y₁₂ receptor mutations identified in patients. TM, transmembrane domain; EL, extracellular loop; IL intracellular loop.

P2Y₁₂ receptor mutations identified in patients. TM, transmembrane domain; EL, extracellular loop; IL, intracellular loop; Tt, heterozygous; TT, homozygous.

Tab. 1

protein position	nucleotide position/ dbSNP rs#	heredity	domain	platelet dysfunction	clinical phenotype	first author (reference)
p.Arg256Gln	NM_022788.4: c.767G>A	compound Tt	TM6			
p.Arg265Trp	rs121917885 NM_022788.4: c.793C>T rs121917886		EL3	reduced/reversible aggregation	inherited bleeding problems	Cattaneo (22)
p.Pro258Thr	c.C>A	Tt	EL3	reduced/reversible aggregation	haemorrhagic diathesis	Remijn (23)
p.Lys174Glu	c.520A>G	Tt	EL2	reduced/reversible aggregation; reduced ligand binding	bleeding disorder	Daly (24)
p.Pro341Ala	not available	Tt	PDZ motif	defective receptor trafficking	bleeding disorder	Daly (25)
p.Arg122Cys	c.364C>T rs557043245	TT	D(E)RY motif	reduced/reversible aggregation	chronic bleeding disorder	Patel (26)
p.His187Glu	NM_022788.4: c.561T>A	TT	TM5	reduced/reversible aggregation; reduced ligand binding affinity	severe bleeding disorder	Lecchi (27)