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General Article

GENERAL ARTICLE

Temperature-dependent autoactivation associated with clinical variability of *PDGFRB* Asn666 substitutions

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Abstract

Ocular pterygium-digital keloid dysplasia (OPDKD) presents in childhood with ingrowth of vascularized connective tissue on the cornea leading to severely reduced vision. Later the patients develop keloids on digits but are otherwise healthy. The overgrowth in OPDKD affects body parts that typically have lower temperature than 37°C. We present evidence that OPDKD is associated with a temperature sensitive, activating substitution, p.(Asn666Tyr), in *PDGFRB*. Phosphorylation levels of PDGFRB and downstream targets were higher in OPDKD fibroblasts at 37°C but were further greatly increased at the average corneal temperature of 32°C. This suggests that the substitution cause significant constitutive autoactivation mainly at lower temperature. In contrast, a different substitution in the same codon, p.(Asn666Ser), is associated with Penttinen type of premature aging syndrome. This devastating condition is characterized by widespread tissue degeneration, including pronounced chronic ulcers and osteolytic resorption in distal limbs. In Penttinen syndrome fibroblasts, equal and high levels of phosphorylated PDGFRB was present at both 32°C and 37°C. This indicates that this substitution causes severe

Nomenclature: Ocular pterygium-digital keloid dysplasia (OPDKD): Chr5(GRCh37):g.149503840 T > A, NM_002609.3(PDGFRB):c.1996A > T, p.(Asn666Tyr). Penttinen type of premature aging syndrome: Chr5(GRCh37):g.149503839 T > C, NM_002609.3(PDGFRB):c.1997A > G, p.(Asn666Ser).

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constitutive autoactivation of PDGFRB regardless of temperature. In line with this, most downstream targets were not affected by lower temperature. However, STAT1, important for tissue wasting, did show further increased phosphorylation at 32°C. Temperature-dependent autoactivation offers an explanation to the strikingly different clinical outcomes of substitutions in the Asn666 codon of *PDGFRB*.

Introduction

It is well known that substitutions in different protein domains of a specific gene can result in diverse phenotypes. However, it can be more challenging to explain how different missense changes within the same codon of a gene can give rise to distinctly different diseases. Ocular pterygium digital keloid dysplasia (OPDKD) is characterized by extensive corneal vascularization in early childhood. Typically, the patients develop keloids on fingers and toes later in life but are otherwise healthy. In contrast, Penttinen type of premature aging syndrome (Penttinen syndrome: OMIM#601812) is a devastating syndrome with involvement of multiple organs. The condition is caused by activating substitutions in the receptor tyrosine kinase (RTK) domain of *PDGFRB* (NM_002609.3(PDGFRB): c.1994T > C, p.(Val665Ala), NM_002609.3(PDGFRB): c.1997A > G, p.(Asn666Ser) (1,2). Patients with the p.(Asn666Ser) substitution have widespread destruction of connective tissue causing severe disfigurement. They also have extensive skin ulceration and degeneration, particularly at the feet and hands. In early adulthood they develop corneal vascularization. Thus, except for the severe corneal changes early in life, OPDKD is in all other aspects a much less severe condition than Penttinen syndrome (3,4).

In this report we describe a dominant activating substitution in *PDGFRB*, NM_002609.3(PDGFRB):c.1996A > T, p.(Asn666Tyr), in a family with OPDKD, thus affecting the same codon as reported for Penttinen syndrome. In contrast to Penttinen syndrome, tissues affected by OPDKD are restricted to body parts (cornea and digits) with lower and more variable temperature than the core temperature (5,6). We examined the role of this physiological temperature difference in respect to the activation of PDGFRB in OPDKD and Penttinen syndromes. We observed that these two *PDGFRB* missense variants differ both in their levels of constitutive autoactivation as well as in their response to small, physiological temperature differences. While the Penttinen substitution causes a constitutively autoactivated PDGFRB receptor independent of temperature, the OPDKD substitution is highly activated only at 32°C. This can explain why these two substitutions within the same codon of PDGFRB lead to so distinctly different conditions.

Results

Case report

The clinical features of the OPDKD patients have previously been described (individuals I-1, II-1 and II-2 in ref. 3) (3,4). Briefly, the proband (I-1) and her oldest son (II-1) developed ingrowth of vascularized tissue over the cornea in the first 2 years of life. The lesions progressed rapidly leading to loss of vision. The youngest son (II-2) developed similar corneal changes at 6 (right eye) and 20 (left eye) years of age. Later, from around 20 years of age, progressive keloids appeared on fingers and subsequently, a decade later, on their toes. Over time, they developed multiple fibromas and enlarged, hard auricles. Individual II-2 was treated with imatinib (Novartis, Germany). Imatinib inhibits PDGFRB and was, *in vitro,* found to reduce levels of phosphorylated PDGFRB and AKT in patient fibroblasts, as described below. The medication was well tolerated but discontinued after 10 months as skin lesions continued to grow and also recurred after surgery. Patient III-1 had a corneal nodule in one eye from the age of 3 years. Despite treatment with oral and topical cyclosporine and steroid eye drops, this has progressed and become vascularized (Fig. 1A).

The patient with Penttinen syndrome caused by the p.(Asn666Ser) substitution in *PDGFRB* has previously been described (2). The phenotype is characterized by a prematurely aged appearance and severe tissue destruction such as chronic ulcers, lipodystrophy, acro-osteolysis and pronounced connective tissue destruction. Some features of increased growth, such as tall stature and congenital haemangiomas, are also present. Although affecting the whole body, the tissue destruction is most severe on feet and hands (Fig. 1B).

A *PDGFRB* variant is associated with OPDKD

Exome sequencing and haplotype investigations revealed a *PDGFRB* variant NM_002609.3: c.1996A > T, p.(Asn666Tyr) that co-segregated with the disease in the family. Microsatellite marker analysis around the *PDGFRB* locus demonstrated that the variant occurred as a *de novo* mutation in individual I-1 as two other unaffected family members shared the affected haplotype but not the mutation (Supplementary Material, Fig. S1, Supplementary Material, Tables S1 and S2). This missense variant changes an amino acid located in the RTK class III signature motif of PDGFRB, an essential part of the autoinhibitory domain. This motif is highly conserved, both at the nucleotide and the amino acid level, and non-synonymous variants within this codon have previously been associated with both Penttinen syndrome and somatic infantile myofibromatosis (2,7–9).

The PDGFRB variant in OPDKD is temperature sensitive

We studied the effect of the physiological temperature differences (Supplementary Material, Fig. S2) on PDGFRB autophosphorylation and phosphorylation of selected downstream proteins (Supplementary Material, Fig. S3) in fibroblasts and transduced HeLa cells (5,10,11). At 37°C, increased levels of phosphorylated PDGFRB and downstream proteins, P-AKT, P-STAT1 and P-PLCγ1, were present in OPDKD fibroblasts compared to controls. However, at 32°C (average corneal temperature), both autophosphorylation and phosphorylation of downstream signalling proteins were greatly increased (Fig. 2). This was particularly pronounced for P-AKT and P-PLCγ1 (5). This indicates that the p.(Asn666Tyr) substitution causes ligand-independent autoactivation of PDGFRB and that the process is temperature sensitive.

In contrast, in Penttinen patients' fibroblasts, equally high levels of phosphorylated PDGFRB were present in cells cultured at both 32°C and 37°C (Fig. 2). There were also increased levels of downstream signalling proteins P-STAT1, P-AKT and P-PLCγ1 at both temperatures. Interestingly, a further increase in P-STAT1 was observed in cells cultured at 32°C (Fig. 2B). In transduced HeLa cells similar findings were made (Supplementary Material, Fig. S4).

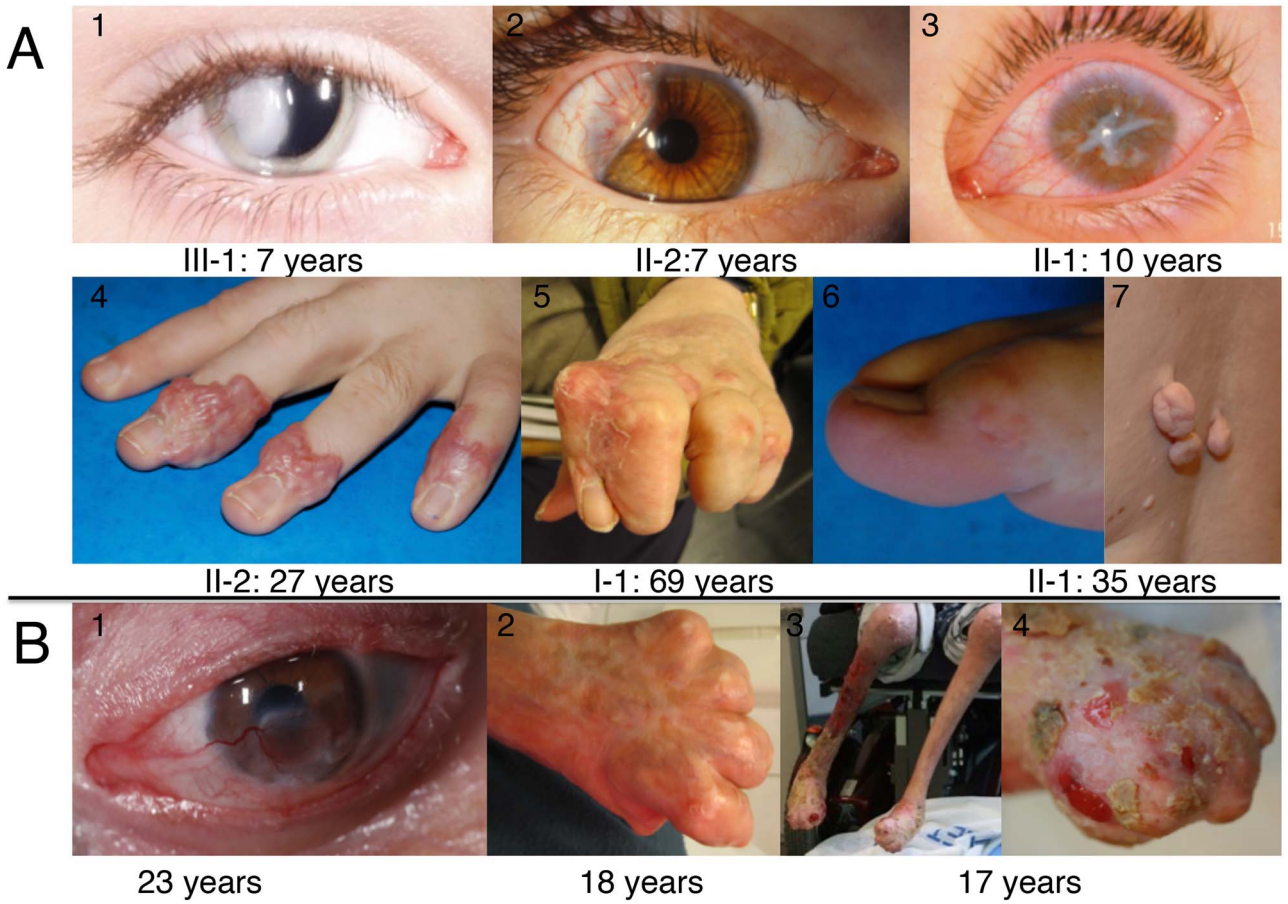

Figure 1. Clinical pictures of patients with activating Asn666 variants. Panel A: OPDKD and panel B: Penttinen syndrome. A1–3: corneal vascularization and tissue ingrowth of OPDKD individuals III-1, II-2 and II-1. The lesions presented early in life and progressed rapidly. A4–7: skin lesions with keloid formation on fingers starting from around 20 years of age. Later on, multiple fibromas and keloids on toes developed. B1–4 illustrates corneal neovascularization, malformed fingers, lipodystrophy and chronic ulcers on the feet of a patient with severe Penttinen syndrome.

When examining known phosphorylation sites, using the Human Phospho-Kinase Array, increased levels of GSK-3α/β, AKT 1/2/3, PRAS40 and WNK1 phosphorylation were seen in OPDKD fibroblasts at 32°C compared to 37°C. No temperature differences were found in healthy control fibroblasts (Fig. 3).

The PDGFRB variant in OPDKD is less sensitive to imatinib

In Penttinen fibroblasts, imatinib greatly reduced autophosphorylation of PDGFRB. In contrast, OPDKD fibroblasts were less sensitive to imatinib with significantly reduced levels of phosphorylated PDGFRB only at 32°C (Fig. 2A). Still, levels of the downstream target P-AKT, and to a lesser extent P-PLCγ1, were significantly reduced in these fibroblasts, both at 37°C and 32°C (Fig. 2B).

Discussion

Signalling through PDGFRB typically occurs after dimerization upon ligand binding followed by autophosphorylation of tyrosine residues in the intracellular tyrosine kinase domain. This activates downstream signalling pathways such as PI3K, PLCγ, STAT and MAPKs (Supplementary Material, Fig. S3) (7,12). Recently, several distinct conditions with overlapping features have been associated with missense mutations that lead to ligand-independent activation of PDGFRB: infantile myofibromatosis (OMIM#228550), Kosaki overgrowth syndrome (OMIM#616592) and Penttinen syndrome (reviewed in (2)).

We found a novel substitution in *PDGFRB*, p.(Asn666Tyr), to be associated with OPDKD. Activating variants in this codon have been linked to Penttinen syndrome p.(Asn666Ser), a related phenotype p.(Asn666His) and a somatic mutation in infantile myofibromatosis p.(Asn666Lys) (2,7–9,13). Although increased levels of phosphorylated PDGFRB were found in both OPDKD and Penttinen fibroblasts incubated at 37°C, this was significantly higher in the latter (Fig. 2B). In transduced Hela cells at 37°C, increased PDGFRB autophosphorylation was present only in cells with the p.(Asn666Ser) substitution (Supplementary Material, Fig. S4A). This indicates that the p.(Asn666Ser) variant functionally affects the autoinhibitory domain more than the p.(Asn666Tyr) substitution. Although this could explain the difference in severity between Penttinen syndrome and OPDKD, it remained puzzling why the corneal changes were much more pronounced in OPDKD (Fig. 1).

In OPDKD, the affected areas (corneas, fingers and toes) are all exposed to lower and variable temperatures (10). In typical room temperature (20–22°C) corneal temperatures lie around 32–34° and fall to around 30°C as the air temperature reaches 0°C (5). Human skin also has variable temperatures with low temperatures found on the hands and feet (Supplementary Material, Fig. S2) (11). Further increased phosphorylation of PDGFRB was observed when temperature

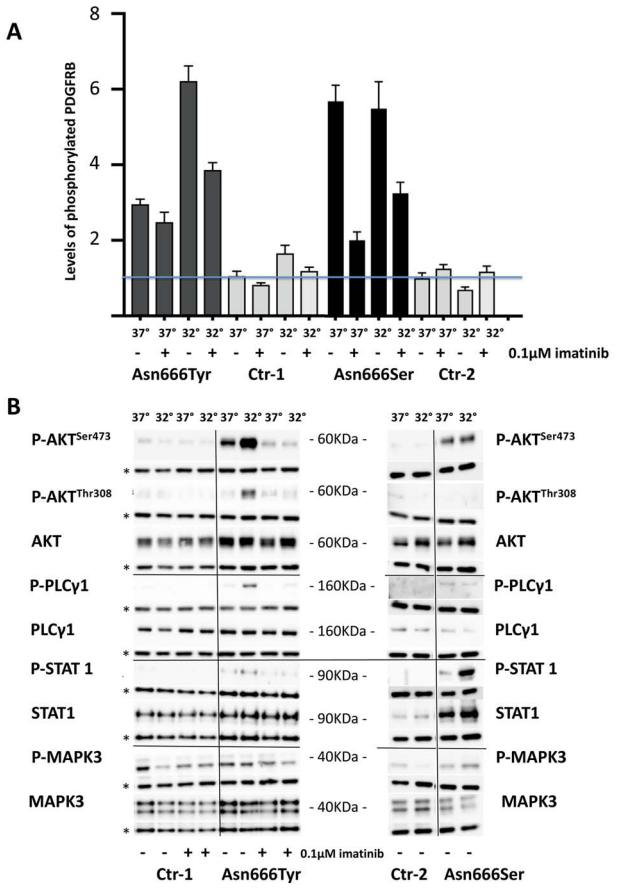

Figure 2. Effect of temperature and imatinib treatment on PDGFRB and downstream signalling. Panel A: levels of phosphorylated PDGFRB measured by ELISA analysis. In p.(Asn666Tyr) and p.(Asn666Ser) fibroblasts, increased levels of phosphorylated PDGFRB were present at 37°C. Only p.(Asn666Tyr) fibroblasts were temperature sensitive, with increasing levels after incubation at 32°C overnight. Panel B: increased levels of downstream ligands, P-AKT^{Ser473}, P-PLCγ1 and P-STAT1, at 37°C in p.(Asn666Tyr) fibroblasts. Incubation at 32°C led to higher levels of P-AKT (P-AKT^{Ser473} and P-AKT^{Thr308}) and P-PLCγ1, but not P-STAT1. Imatinib treatment of p.(Asn666Tyr) fibroblasts reduced levels of P-AKT (P-AKT^{Ser473} and P-AKT^{Thr308}) and P-PLCγ1, but not P-STAT1. In p.(Asn666Ser) fibroblasts increased levels of P-AKT^{Ser473}, P-PLCγ1 and P-STAT1 and STAT1 were present at 37°C, but only P-STAT1 had further greatly increased levels at 32°C. There was no difference on P-MAPK3 signalling.

was lowered to 32°C in OPDKD. No such temperature effect was observed in control or Penttinen cells (Fig. 2A and Supplementary Material, Fig. S4A). We therefore concluded that the p.(Asn666Tyr) is temperature sensitive with increased autoactivation of the kinase at 32°C, and that this is not the case for the p.(Asn666Ser). In line with this, increased phosphorylation of downstream signalling partners, particularly P-AKT and P-PLCγ1, was present in OPDKD fibroblasts at reduced temperatures (Fig. 2B). Possibly the slightly increased autoactivation observed at 37°C in OPDKD is compensated by negative feedback mechanisms explaining why the patients do not have manifestations at higher temperature locations. Interestingly, in Penttinen syndrome, a strong temperature sensitivity of downstream signalling protein P-STAT1 was seen. In contrast, only a slight temperature effect of P-STAT1 was observed in OPDKD (Fig. 2B). This indicates a temperature-sensitive effect also on downstream signalling, either directly or by influencing RTK substrate binding.

Activating *PDGFRB* variants can cause both tissue wasting and overgrowth (2,12,14). OPDKD patients have increased tissue growth, whereas individuals with the p.(Asn666Ser) variant primarily have tissue destruction. Increased PDGFRB-STAT1 signalling has been linked to tissue wasting, whereas in the absence of STAT1, tissue overgrowth is seen (12). This suggests that STAT1 activation is responsible for the degenerative features. Patients with severe Penttinen syndrome have disabling chronic ulcers and wasting of the lower limbs. This could be explained by increased phosphorylation of STAT1 at these lower temperature locations. In contrast, in OPDKD increased signalling in the AKT-PI3K pathway was found (P-AKT, PRAS40 and GSK-3α/β), particularly in cells incubated at 32°C. Increased AKT signalling is associated with cell survival and growth. This could explain why tissue growth (corneal vascularization and keloid formation) is seen primarily in body parts with lower temperature (15). These patients had enlarged, hard auricles and some nasal tip fibromas but no symptoms from the scrotum, also a lower temperature location (16).

Taken together, temperature sensitivity seems to affect autoinhibition of PDGFRB and ligand-independent activation in OPDKD and, to some extent, Penttinen syndrome. Together with differences in basal autoinhibition of PDGFRB this convincingly explains why conditions caused by different activating substitutions of the same *PDGFRB* codon can be so strikingly different.

Effects of physiological temperature changes are seen in some hereditary diseases. Examples include familial cold autoinflammatory syndromes (FCAS1 #120100, FACS2 #611762, FCAS3 #614468 and FCAS4 #616115). Patients with FACS3 get a rash on the nose, ears and fingers upon small decreases in temperature. The condition is associated with heterozygous variants in *PLCG2* (#600220) with increased activation of PLCγ2 at lower temperatures (17,18). In oculocutaneous albinism type IB (#606952), the degree of loss of tyrosinase activity is temperature sensitive. Thus, hair in warmer areas is white, whereas hair in cooler areas (extremities) remains pigmented (19). Regarding the eye, it has been suggested that the high growth rate for amoebas around 32°C is the reason most amoebic eye infections are confined to the cornea (20). Similarly, in leprosy corneal changes are common probably as *Mycobacterium leprae* proliferate between 27°C and 30°C (21,22). We are not aware of temperature being directly linked to corneal angiogenesis or of any temperature-sensitive mutation in RTK associated with human disease.

In summary, we suggest that altered signalling caused by physiological temperature differences is an important reason for the localization and the organ specific manifestations of Asn666 PDGFRB-related conditions.

Materials and Methods

Detailed experimental methods are included in the Supplementary Material. Briefly, DNA from affected and unaffected family members was subjected to exome sequencing using NimbleGen v3 exome capture on Illumina HiSeq. Upon identification of a *PDGFRB* variant that co-segregated with the disease in the family, haplotype analysis with microsatellite markers around the *PDGFRB* locus was performed. Fibroblasts from affected individuals and healthy controls were obtained. Transgenic HeLa cells were transduced with a murine retroviral vector containing the *PDGFRB* (NM_002609.3) c.wt, c.1996A > T, p.(Asn666Tyr) and c.1997A > G, p.(Asn666Ser) variants.

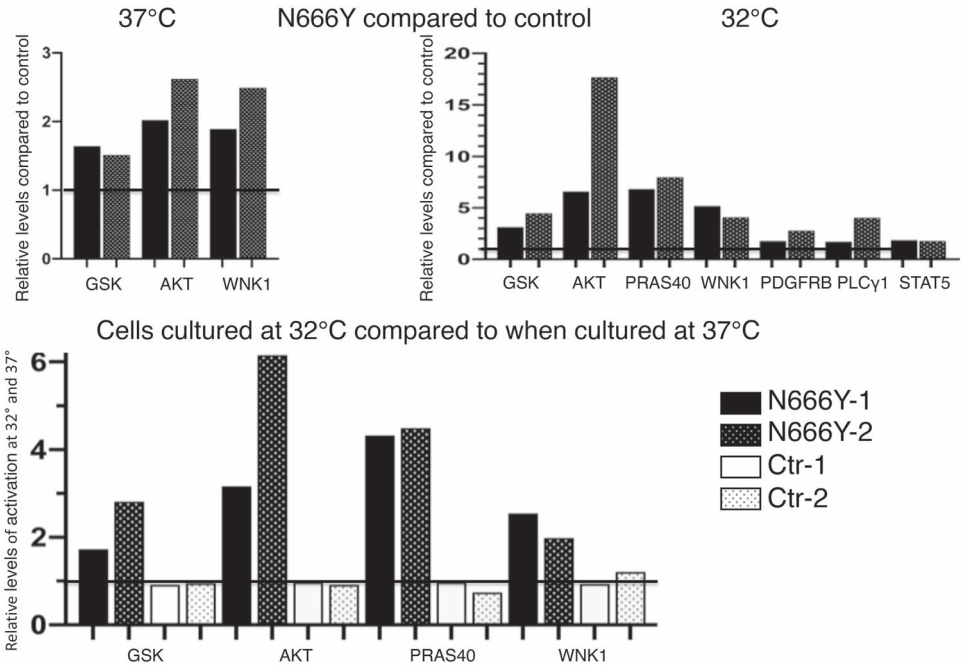

Figure 3. Kinase phosphorylation measured by Human Phospho-Kinase Array. Two different OPDKD and control fibroblasts, incubated for 6 h at 32°C, were compared to cells kept at 37°C. We defined a significant difference to be of >50% increased phosphorylation measured by chemiluminescence. Top left: p.(Asn666Tyr) fibroblasts compared to control at 37°C showed increased levels of GSK-3α/β, AKT 1/2/3 and WNK1. Top right: At 32°C, PRAS40, PDGFRB, STAT5 α/β and PLCγ1 were also significantly different. PDGFRB, STAT5α/β had weak bands, whereas the band representing PLCγ was barely visible. Bottom: When fibroblasts harvested at 32°C were compared to cells harvested at 37°C, increased levels were found for GSK-3α/β (with 1.73/2.81 increase), AKT 1/2/3 (3.16/6.15), PRAS40 (4.32/4.49) and WNK1 (2.54/1.98) in both p.(Asn666Tyr) cell lines. No differences were found in either of the control fibroblasts. We found that in one of the p.(Asn666Tyr) cell lines there were more proteins with increased activation. This, and its control fibroblast cell line, were early passage (P3) cells with a rapid growth pattern, in contrast to the other p.(Asn666Tyr) cell line that was P5 and growing slightly slower. P-STAT1 is not part of the Human Phospho-Kinase Array.

To study the effect of reduced temperature, cells were either kept at 37°C or incubated overnight at average corneal temperature (32°C) (5). Cells were then left untreated or treated with 0.1 μM imatinib (STI571, Selleckchem) for 6 h. ELISA analysis of phosphorylated PDGFRB was performed (DuoSet® IC Phospho-PDGFRB, R&D systems). Cell lysates were also subjected to immunoblot analysis with primary antibodies against phospho-Ser473-AKT, phospho-Thr308-AKT, phospho-Tyr70-STAT1, STAT1, phospho-Tyr783-PLCγ1, PLCγ1, phospho-Thr202/Tyr204-MAPK3/ERK1 and MAPK3/ERK1 (all Cell Signaling Technology). Relative levels of phosphorylation of various kinase phosphorylation sites were examined using the Human Phospho-Kinase Array Kit (R&D Systems) and quantified using chemiluminescence.

The study was approved by the Regional Committee for Medical and Research Ethics, Western Norway (IRB# 00001872) (ref. no. 2010/3337 and 2014/59). The patients and their families were invited to participate in the study and were included after informed written consent.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement: The data in the paper are subject to a patent application filed 19 February 2020.

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