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Title: "*PLCD1* and pilar cysts"

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Abstract (60 words)

Trichilemmal or "pilar" cysts are commonly found on the scalp and are derived from the outer root sheath of the hair follicle. Multiple trichilemmal cysts present in an autosomal dominant pattern of inheritance, yet the genetic mechanism has remained elusive. In this issue, Hörer *et al.* highlight predisposing variants in *PLCD1* in such families and propose a monoallelic mutational mechanism that drives cyst formation.

Pullquote: "a somatic mutation *in cis* with the high-risk allele is seen in cyst tissue, supporting a model of monoallelic mutation driving cyst formation"

Clinical implications:

- Trichilemmal cysts may be inherited in an autosomal dominant manner; variants in *PLCD1*, a gene that encodes the phospholipase enzyme PLCδ1, are shown to be associated with cyst formation in these families.
- Study of cyst tissue in tandem with peripheral leukocyte DNA demonstrate an additional cyst specific variant in *PLCD1* occurs on the same allele as the predisposing variants, suggesting a monoallelic second hit genetic mechanism results in cyst formation.
- *PLCD1* variants have been previously associated with the inherited nail disorder leukonychia totalis (porcelain white nails). The novel association of *PLCD1* variants with cyst formation reveal diverse functional roles of PLCδ1 in skin homeostasis.

Trichilemmal (pilar) cysts are benign, keratin filled cysts typically seen on the scalp. Despite their frequent clinical presentation, their genetic basis has not been elucidated. This month, Hörer *et al.* (Horer 2019) highlight variants in *PLCD1* in families with multiple trichilemmal cysts and propose that the acquisition of an additional somatic *PLCD1* variant leads to cyst formation.

Trichilemmal cysts, leukonychia and PLCδ1

The hair follicle is recognised as the origin of diverse cysts. Trichilemmal cysts are thought to derive from the outer root sheath, and are distinct from epidermoid cysts, sebaceous cysts and milia. Trichilemmal cysts present as skin coloured nodules (Fig. 1a) and may warrant surgical intervention if they become symptomatic or cause diagnostic concern. Sporadic and familial trichilemmal cysts are reported. Familial trichilemmal cysts are inherited in an autosomal dominant manner, and patients typically present with multiple cysts. Careful phenotyping of these patients highlighted the absence of an association with colorectal polyposis and cancer (Leppard et al., 1977), a feature which distinguished these families from patients with epidermoid cysts in Gardner's syndrome, which arises due to *APC* variants. Trichilemmal cysts demonstrate a pattern of keratinization known as trichilemmal keratinization, that lacks a granular layer in the cyst wall (Fig 1b). Whilst most families with trichilemmal cysts develop multiple scalp cysts as the sole clinical manifestation, trichilemmal cysts can present in combination with porcelain white nails (leukonychia) in rare families, where the genetic basis has not been elucidated (Rodríguez-Lojo et al., 2011). Leukonychia is thought to arise from a defect in keratinisation in the nail plate, preventing the normal visualisation of the pink vascular nail bed. These rare families clinically link trichilemmal cysts and leukonychia, which is of interest, as familial leukonychia has been shown to be associated with variants in *PLCD1* (Kiuru et al., 2011). *PLCD1* is also a gene that lies within a candidate locus for familial trichilemmal cysts on chromosome 3 called

TRICY1, that was discovered using linkage analysis in multigenerational pedigrees with trichilemmal cysts (Eiberg et al., 2005). *PLCD1* encodes phosphoinositide-specific phospholipase C delta enzyme (PLCδ1), one of 13 members of the phospholipase C family. PLCδ1 has five domains, including a pleckstrin homology domain, three calcium binding domains and a catalytic domain (Fig 1c). PLCδ1 hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG further mediates the activation of protein kinase C (PKC), and IP3 releases calcium from intracellular stores playing a role in a variety of physiological functions. PLCδ1 is expressed in nail matrix and the hair follicle (Kiuru et al., 2011). The importance of PLCδ in keratinocyte biology is underscored by the progressive hair loss, epidermal hyperplasia and epidermoid cyst formation seen in *PLCD1* knockout mice (Nakamura et al., 2003). Notably, the development of epidermoid cysts instead of trichilemmal cysts highlights limitations of murine genetic models in recapitulating certain human hair diseases. Nonetheless, in these knockout mice, PLCδ1 loss resulted in impairment of calcium mobilisation and activation of calcineurin and NFAT, as well as reduced phosphorylation of PKC in keratinocytes. Taken together, these data support *PLCD1* as a potential candidate gene in familial trichilemmal cysts.

PLCD1 variants predispose to inherited trichilemmal cysts

In this study by Hörer *et al*, five patients with inherited trichilemmal cysts were investigated using whole exome sequencing. Two variants within *PLCD1* that co-occurred *in cis* were found in peripheral leukocyte DNA (NP_006216.2: p.(Pro301Pro) and p.(Ser460Leu) Fig. 1c). Analysis of 35 further affected individuals from a total of 12 families confirmed the presence of this haplotype, termed a “high-risk” allele (Fig. 1b). Analysis of DNA and RNA from cyst tissue highlighted the frequent presence of an additional cyst specific variant (NP_006216.2:p.(Ser745Leu) in a conserved region that had not been previously reported in

public databases. Using allele-specific PCR, the authors confirm the presence of this variant occurring *in cis* with the high-risk allele. To assess functional impact of these variants, the authors express these variants in mammalian cells, and assess the impact on TRPC4 and Kir3 channel activation, both of which are PLCδ1 dependent. In both assays, expression of the cyst specific variant was associated with reduction of channel activation. In addition, the authors measured levels of different DAG species in cells expressing the cyst specific variant. In this assay, cells expressing the cyst specific variant generated reduced DAG species 38:4 and 38:5, recognised to be specific PIP2 breakdown products of PLCδ1 (Holub et al., 1970). These data support the somatic acquisition of the cyst specific variant as damaging to normal PLCδ1 function. Dysregulated calcium signalling that ensues may be implicated in cyst formation, as seen in renal cysts (Kuo et al., 2014).

A monoallelic "two-hit" mechanism leads to trichilemmal cyst formation

To date, dominant truncating and recessive missense germline variants in *PLCD1* have been identified in patients with familial leukonychia (Kiuru et al., 2011). In this study by Hörer *et al*, a dominantly inherited haplotype with a synonymous and missense variant seen in tandem constitutes a "high-risk allele", and this genetic difference may account for the lack of leukonychia in these patients. This allele predisposes to the acquisition of *in cis* somatic variants within the C2 domain of PLCδ1, which is associated with cyst formation. It still is unclear as to why the high-risk allele predisposes to somatic acquisition of the cyst specific variant, however this is a recognised finding in other genes such as *JAK2*, where "hypermutable" and "fertile-ground" hypotheses have been posited as hypothetical models to explain how germline variants may attract additional somatic mutation events (Campbell, 2009).

The genetic study of cyst material has been central to the discovery of the proposed monoallelic model. Due to the nature of the techniques employed in this study, it remains to

be proven if there is loss of heterozygosity involving the cyst-specific variant, and this could be investigated using single cell sequencing approaches in future studies. To date, Knudson's two hit hypothesis has been frequently demonstrated in a range of skin tumour predisposition syndromes, as well as in cyst formation in other tissues such as the kidney. Recently however, evidence for monoallelic mechanisms have been emerging in renal cyst formation (Cornec-Le Gall et al., 2018), adding support to the proposed mechanism here in the skin. Cyst formation has been associated with loss of planar cell polarity and deregulated primary ciliary function, and it would be of interest to determine PLCδ1's role in these important tissue patterning mechanisms.

This work by Horer *et al.* is important as it highlights PLCδ1 as an important protein in the maintenance of human hair follicle homeostasis. In addition, the *PLCD1* risk allele identified may inform the interpretation of genetic tests in families with trichilemmal cysts. Finally, the monoallelic model of cyst formation proposed may be relevant in other genodermatoses, and highlights the importance of studying genetic changes in the skin in conjunction with peripheral leukocyte DNA.

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Figure legend

Figure 1 (a) A typical trichilemmal cyst on the scalp, and the subcutaneous appearance on surgical excision. (b) The trichilemmal cyst wall is indicated with white arrows, an absence of a granular layer is noted above, and the keratin within the cyst is indicated with a white star. Original magnification 200x, H+E. (c) A cyst specific variant within the C2 domain of *PLCD1* in combination with the "high-risk" variants is associated with cyst formation. Candidate genetic mechanisms are indicated.