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Hidradenitis suppurativa: Haploinsufficiency of gamma-secretase components does not affect gamma-secretase enzyme activity *in vitro*

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Hidradenitis suppurativa (HS) is a chronic inflammatory dermatosis that presents with nodules, cysts, abscesses and sinus tracts in apocrine gland-bearing areas. It is thought to occur secondary to follicular occlusion, has been associated with smoking and obesity and may be inherited in an autosomal dominant manner in a minority of cases[1]. Heterozygous mutations in the gamma-

secretase genes *PSENEN*, *PSEN1* and *NCSTN* underlie 0-7% of cases[2,3]. Gamma-secretase is an intra-membranous protease complex comprised of NCSTN, Presenilin, PEN-2 and APH1 involved in regulated intra-membrane proteolysis and the subsequent clearance of substrates from cell membranes (endopeptidase and carboxypeptidase activity). The functional effects of gamma-secretase gene mutations in HS are yet to be elicited. It was hypothesised that two previously reported mutations (*NCSTN* c.1125+1 G>A, *PSENEN* c.66_67insG)[3] would affect corresponding RNA and protein expression/ function and affect gamma-secretase enzyme activity.

This study was approved by the London Stanmore Research Ethics Committee (11/LO/0966) and conducted in accordance with Declaration of Helsinki principles. Diagnostic criteria are detailed in the supplementary methods. Two 6mm axillary skin biopsies (affected and unaffected skin) were taken from two mutation-positive individuals and three healthy volunteers for immunohistochemistry and cell culture. DNA, RNA and protein was extracted from primary human fibroblasts and analysed via agarose electrophoresis, Sanger sequencing, gene expression assays and immunoblotting . Gamma-secretase enzyme activity assays were performed using a well established *in vitro* enzyme activity assay (supplementary methods)[4]. Statistical analyses were performed as detailed in supplementary methods.

NCSTN and PSENEN co-localised to the hair follicle, sebaceous gland, apocrine gland, epidermis and inflammatory infiltrates in healthy and mutation-positive human skin (Figs 1, S1, S2). There was no difference in NCSTN or PEN-2 epidermal staining intensity in mutation-positive versus healthy volunteer skin. Antibody specificity was verified using grade IV invasive breast cancer tissue (NCSTN expression is upregulated in invasive ductal breast cancer [5]) and by showing co-localisation of NCSTN and PEN-2 in healthy skin (replicated using a second NCSTN antibody, Fig. S3).

Agarose electrophoresis and sequencing of the full length NCSTN transcript revealed that *NCSTN* exon 9 is missing in the NCSTN c.1125+1G>A mutant allele (Fig. S4). NCSTN transcript expression was significantly reduced in mutant versus control primary human fibroblasts (Fig. S5A, S5B). An incremental increase in transcript abundance was observed in cycloheximide treated cells over a 24 hour period (Fig. S5C), suggesting that the mutant transcript is subject to decay. Immunoblotting revealed a significant corresponding reduction in mature nicastrin expression in mutant versus control fibroblasts ($p<0.001$, Fig. S6), with no additional protein bands to support the presence of a mutant protein (specificity of band validated by siRNA knockdown, Fig. S7).

Amplification and direct sequencing of the PSENEN transcript from *PSENEN* mutant fibroblasts revealed the c.66_67insG insertion (Fig. S8). This is predicted to alter the reading frame and result in an altered and elongated PEN-2 protein product (p.Phe23ValfsX98, Fig. S9). Transcript and corresponding PEN-2 protein expression was significantly reduced in mutant versus control fibroblasts ($p<0.001$, Figs. S10, S11). No additional protein bands were identified to support the presence of a mutant protein.

Gamma-secretase enzyme activity assays revealed no difference in the total number of mature gamma-secretase complexes present (assessed by measuring PSEN1-CTF), complex maturity (as measured by the PSEN1-CTF/ PEN-2 ratio), endopeptidase enzyme activity per complex (as measured by the AICD-Flag/ PSEN1-CTF ratio) or carboxypeptidase activity (as measured by the Aβ42:Aβ40 ratio) in *NCSTN* and *PSENEN* mutant fibroblasts versus controls (Fig 2). S20 cells (over-express gamma-secretase components) acted as a positive control and the gamma-secretase inhibitor L458 acted as a negative control (Fig S12)[6]. NCSTN and PEN-2 blot densities were calculated from solubilised membrane preparations generated for the enzyme activity assays. In contrast to total cell fractions, there was no significant difference in NCSTN and PEN-2 expression in solubilised membrane fractions harvested from mutant versus control fibroblasts (Fig. S13, Fig. S14).

These data demonstrate that the gamma-secretase components NCSTN and PEN-2 are expressed in cutaneous structures that are commonly distorted or altered in HS[7,8]. The two mutations studied share a downstream pathogenic mechanism of haploinsufficiency (consistent with other globally reported gamma-secretase gene mutations reportedly reducing transcript abundance [9-11]). The subsequent total cell reduction in respective protein expression was not, however, replicated in cell membrane preparations. Gamma-secretase protein expression is very tightly regulated [12], less than 5% of assembled complexes reach the cell membrane. 50% wild type protein may therefore be sufficient to support complex assembly and function. Notch 1, 2 and 3 knockdown in mice results in follicular keratinisation [13], potentially implicating gamma-secretase-Notch signalling in HS. However, only one of four missense mutations so far reported in HS was found to affect Notch processing [14], and that effect was negated with heterozygous expression of the mutant allele, again supporting the notion that 50% wild-type protein can maintain enzyme function. It may therefore be postulated that heterozygous mutations in the gamma-secretase genes are not sufficient to cause HS alone. It may be that partial reductions in protein expression only become functionally detrimental in situations of cellular stress. The unusual and characteristic cutaneous distribution of HS might implicate intrinsic and extrinsic factors such as temperature, sweat, friction, glandular secretion and microbes in disease pathogenesis. Temperature, pH and salt concentrations have all been shown to affect endopeptidase and carboxypeptidase enzyme activity *in vitro*[6] and these factors require further investigation in this context.

Limitations in this study include the *in vitro* nature of the experiments, the efficiency of protein solubilisation and the accuracy with which one can measure the concentration of solubilised cell membrane protein when working with protein complexes. The immunohistochemistry data would imply that keratinocytes may be a relevant cell type around which to focus future study.

The nature of the globally reported gamma-secretase gene mutations in HS, these data demonstrating haploinsufficiency, and *in vivo* data showing that NCSTN or PSEN1 knockdown results in the development of follicular keratinisation, follicular atrophy and the formation of epidermal cysts [13,15,16], suggest that a reduction in respective gene product may be involved in the pathogenesis of some cases of HS. Ongoing efforts to elicit the functional consequences of these mutations on gamma-secretase activity and downstream signalling pathways have the potential to identify novel therapeutic targets in this debilitating condition.

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FIGURE LEGENDS

Figure 1. NCSTN and PEN-2 expression in healthy volunteer axillary skin. NCSTN and PEN-2 staining co-localised in the epidermis, hair follicle, sebaceous gland and apocrine glands. This represents skin harvested from one healthy volunteer, but the distribution of staining was the same in all individuals. Scale bar = 50μm.

Figure 2. Gamma-secretase enzyme activity assay results. A) PSEN1-CTF expression in solubilised membrane preparations harvested from mutant and control fibroblasts. There was no significant difference in PSEN1-CTF expression in the *NCSTN* or *PSENEN* mutant fibroblasts versus controls (error bars = standard error (SE)). B) PSEN1 CTF/ PEN-2 ratio for each sample expressed as a percentage of the ratio observed in the positive control S20 cells (representative of gamma-secretase complex maturity). There was no discernible difference in mutant fibroblasts versus control subjects (error bars = SE). C) Gamma-secretase enzyme activity per complex expressed as a percentage of the positive control (S20 cell line). There was no significant difference in enzyme activity per complex between mutant fibroblasts and controls (error bars = SE). D) Aβ42: Aβ40 ratios

expressed as a percentage of the ratio derived from S20 cells. There was no significant difference in the ratio derived in assays run with samples harvested from mutant fibroblasts versus control fibroblasts (error bars = SE).