Increased expression profile of NCSTN, Notch and PI3K/AKT3 in hidradenitis suppurativa

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Abstract

Background In a small number of kindreds with familial hidradenitis suppurativa (HS) different mutations of NCSTN (nicastrin) have been identified. Blocking of NCSTN leads to impairment of the Notch and PI3K/AKT signalling pathway, which is assumed to play a pathogenic role in HS. However, very limited data are available concerning expression levels of these pathway components in HS skin.

Objectives To analyse the mRNA and protein expression of NCSTN, Notch1–3, PIK3R3 and AKT3 in HS.

Methods Skin samples from healthy controls, lesional and perilesional skin of HS patients with and without a positive family history were analysed by quantitative real-time RT-PCR and immunohistochemistry. Univariate statistical analyses were conducted regarding association between expression levels and patient’s characteristics.

Results Expression levels of all investigated genes showed significantly higher levels in lesional HS skin compared with healthy controls. Univariate analysis showed no association between a positive family history and mRNA expression levels. Perilesional HS skin of patients with mild disease severity (Hurley I) showed significant higher mRNA expression levels of the investigated pathway components compared to moderate (Hurley II) and severe disease (Hurley III).

Conclusion We found no evidence for diminished expression levels of the Notch signalling. In contrast, the NCSTN, Notch and PI3K/AKT signalling components are overexpressed in HS. Future research is needed to investigate a possible pathogenetic role or to reveal a coactivation of these overexpressed components during inflammatory response in HS.

Introduction

Hidradenitis suppurativa (HS) is a chronic inflammatory skin disease that results in recurrent painful nodules, abscesses, sinus tracts and scarring.1 Hyperplasia of the hair follicle epithelium, follicular hyperkeratosis and interfollicular epidermal hyperplasia are considered to be the early events in HS accompanied by a dysregulated immune-mediated inflammatory response.2,3

Approximately 30–40% of patients with HS report a positive family history with a proposed autosomal dominant inheritance pattern.4 Thus, genetic predisposition is frequently discussed as an important risk factor.5 Predominantly loss-of-function mutations have been reported in genes of the γ-secretase complex, namely NCSTN, PSEN1, PSENEN and PSTPIP1.6–8 To date, the highest number of reported mutations was found in the NCSTN gene encoding nicastrin, which is important for the regulation of the γ-secretase activity.9,10

γ-secretase is a transmembrane multiprotein complex, which functions as a protease mediating the cleavage of various membrane proteins, such as Notch.11 After cleavage, the Notch intracellular domain (NICD) is released from the plasma membrane and translocate into the nucleus, where it forms transcriptional-activator complexes to activate transcription of target genes.12

Notch signalling is involved in immune cell development, normal epidermal cell differentiation and proliferation and
Materials and methods

In this prospective study, punch biopsies from 60 HS patients were taken intraoperatively from a representative inflammatory HS lesion. For an intra-individual control group, additional skin samples were taken from adjacent (1 cm from the lesional skin border) healthy-appearing skin. Thirty-eight (63.3%) were female and 22 (36.7%) were male with a mean age of 37.9 ± 11.4 years. Eleven (18.3%) patients were in Hurley stage I, 32 (53.3%) in Hurley stage II and 17 (28.3%) in Hurley stage III. A positive family history (first- or second-degree relatives with HS symptoms) was reported by 19 (35.8%) patients. Six patients could not provide information about family history. In total, 39 (65%) HS patients were current smokers. The mean body mass index (BMI) was 28.9 ± 6.1 kg/m². None of the patients had received systemic antibiotics, immunosuppressive or biological medications 4 weeks before inclusion. Nineteen healthy subjects [11 (57.9%) females and 8 (42.1%) males] with a mean age of 49.9 ± 9.3 years represented the control group.

Skin samples were immediately placed in RNAlater solution (Qiagen, Hilden, Germany) and stored at −80°C. This study was approved by the Ethical Review Board of the Ruhr-University Bochum, Germany (registration no.: 5076-14), which was conducted according to the Declaration of Helsinki. All study subjects signed informed consent.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from skin samples by means of the RNeasy Lipid Tissue Kit (Qiagen, Chatsworth, CA, USA). The mRNA expression levels were quantified by real-time real-time polymerase chain reaction (RT-PCR) in accordance with MIQE Guidelines using the PowerSYBR Green PCR Master Mix (Cobas 480 Analyzer; Roche, Mannheim, Germany). Three widely used reference genes, namely GAPDH, β-2-microglobulin, and RPL-38 were tested. The latter gene exhibited the most stable expression levels, and this gene was used as the housekeeping gene. Target mRNA expression results were normalized to corresponding RPL38 mRNA transcript levels (2−△△CT method).

The primer pairs for NCSTN, Notch1, Notch2, Notch3, PIK3R3, AKT3 and RPL-38 were designed using Primer Express software (PE Applied Biosystems, Foster City, CA, USA) and were shown in Table 1.

Immunohistochemistry and scoring

Immunostaining for nicastrin, Notch1, Notch2, Notch3, PIK3R3 and AKT3 was performed in skin samples of the lesional HS group (n = 37), of corresponding healthy-appearing perilesional HS group (n = 37) and of the healthy control group (n = 20). Skin biopsy specimens were cut into 7 μm sections, and immunohistochemical staining was performed by using the following commercially available antibodies: rabbit polyclonal anti-NCSTN antibody (HPA054846, Atlas Antibodies, Stockholm, Sweden), rabbit polyclonal anti-Notch1 antibody-ChIP Grade (ab27526; Abcam, Cambridge, UK), rabbit polyclonal anti-Notch2 antibody (HPA048743, Atlas Antibodies), rabbit polyclonal anti-Notch3 antibody (ab23426; Abcam), rabbit polyclonal anti-PIK3R3 (HPA071988; Atlas Antibodies) and rabbit polyclonal AKT3 antibody (ab152157, Abcam) at a 1:500 dilution. After deparaffinization, heat-induced antigen retrieval was performed using Target Retrieval Solution Low pH (Code K8005, Dako, Glostrup, Denmark) for anti-NCSTN, anti-Notch1–3 and anti- PIK3Kinase or Target Retrieval Solution High pH (Code K8004; Dako) for anti-AKT3. Before staining the sections were pretreated with Dual Endogenous Enzyme Block (Code S2003; Dako) for 30 min. The primary antibodies were incubated 1 h at room temperature or overnight at 4°C. After a washing step, the staining was continued with Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse, Code K5005 according to manufacturer’s procedure, including the haematoxylin counterstaining. No staining was shown when omitting the primary antibodies (negative controls).

In each section, four fields were chosen at random and the entire epidermis was evaluated at 40× magnification with a light microscope by two investigators. In accordance to the H-score system, intensity of immunostaining was scored (0, negative; 1, weak staining; 2, intermediate staining; 3, strong staining) and multiplied by the percentage (0–100) of keratinocytes, which
Table 1 Median (interquartile range; IQR) quantitative real-time RT-PCR data of NCSTN, Notch1, Notch2, Notch3, PIK3R3 and AKT3 in skin samples from healthy controls (HC), healthy-appearing perilesional skin (PeriLS) and lesional skin (LS) of hidradenitis suppurativa patients.

|       | HC (n = 19) | PeriLS (n = 60) | LS (n = 60) | P-values |
|-------|-------------|-----------------|-------------|----------|
| NCSTN | 2.63 (1.97–4.26) | 6.14 (4.61–7.64) | 11.92 (6.57–15.67) | HC vs. PeriLS: P < 0.0001 |
|        |             |                 |             | HC vs. LS: P < 0.0001 |
|        |             |                 |             | PeriLS vs. LS: P < 0.0001 |
| Notch1 | 0.74 (0.53–1.1) | 1.99 (0.91–2.57) | 3.49 (1.54–5.42) | HC vs. PeriLS: P = 0.0017 |
|        |             |                 |             | HC vs. LS: P = 0.0006 |
|        |             |                 |             | PeriLS vs. LS: P < 0.0001 |
| Notch2 | 2.52 (1.66–3.53) | 5.79 (4.89–6.91) | 5.26 (3.33–7.01) | HC vs. PeriLS: P = 0.0001 |
|        |             |                 |             | HC vs. LS: P = 0.0001 |
|        |             |                 |             | PeriLS vs. LS: P = 0.0001 |
| Notch3 | 4.67 (3.04–6.48) | 9.41 (6.02–14.36) | 10.84 (5.44–19.36) | HC vs. PeriLS: P = 0.0001 |
|        |             |                 |             | HC vs. LS: P = 0.00003 |
|        |             |                 |             | PeriLS vs. LS: P = 0.0039 |
| PIK3R3 | 1.48 (0.86–2.04) | 1.75 (1.1–2.68) | 3.69 (1.99–7.16) | HC vs. PeriLS: P = 0.0022 |
|        |             |                 |             | HC vs. LS: P = 0.0001 |
|        |             |                 |             | PeriLS vs. LS: P < 0.0001 |
| AKT3  | 0.94 (0.51–1.39) | 2.3 (1.29–3.14) | 5.02 (1.91–9.82) | HC vs. PeriLS: P < 0.0001 |
|        |             |                 |             | HC vs. LS: P < 0.0001 |
|        |             |                 |             | PeriLS vs. LS: P < 0.0001 |

IQR, interquartile range. †Mann– Whitney U-test; ‡Wilcoxon-test; §Unpaired two-sided t-test. Additionally, the primer sequences used in this study are described.

indicated staining intensity: 0 (cells with negative staining) + 1 (cells with weak staining) + 2 (cells with intermediate staining) + 3 (cells with strong staining).

The average scores of the two investigators were used for statistical analysis.

Statistical analysis
MedCalc software version 19.1.7 (MedCalc, Ostende, Belgium) was used for statistical analysis. To analyse distribution of data, the Shapiro–Wilk test was used. Differences between two means were tested by the paired and unpaired two-sided t-test. For non-normally distributed variables the Wilcoxon-test (dependent samples) and the Mann–Whitney U-test (independent samples) were used. Correlations were calculated using Spearman’s rank correlation coefficient. Differences among groups (n > 2) were analysed using the Kruskal–Wallis ANOVA, including the Conover post hoc test for pairwise comparisons. P < 0.05 was considered significant.

Results
The mRNA expression levels of NCSTN, Notch1–3, PIK3R3 and AKT3 were significantly higher in lesional HS skin compared with the healthy control group (Fig. 1 and Table 1).

Furthermore, mRNA expression levels of NCSTN, Notch1, Notch3, PIK3R3 and AKT3 were significantly increased in lesional HS skin compared with perilesional HS skin.

mRNA expression levels of NCSTN, Notch1–3 and AKT3 were significantly higher in perilesional HS skin compared with healthy controls.

Next, we examined whether the disease severity, as assessed by the Hurley classification system, was associated with the expression levels of the investigated variables. Interestingly, there was a significant difference between the three Hurley stages in perilesional skin (Fig. 2). mRNA expression levels of all investigated variables were significantly higher in skin samples of Hurley stage I patients compared with Hurley stage II and III patients. There was no difference between the Hurley II and Hurley III group. Regarding lesional HS skin, there was no statistically significant difference in the mRNA expression of the investigated variables between the three Hurley stages.

Regarding smoking status and family history, univariate analysis revealed no significant association with the expression levels of the investigated variables (Table 2). Correlation analysis revealed a significant but weak positive correlation between Notch2 and Notch3 and BMI in perilesional HS skin (Table 2). 23
Figure 1 Median (interquartile range) relative mRNA expression of (a) NCSTN, (b) Notch1–3, (c) PIK3R3 and (d) AKT3 in skin samples from healthy controls (HC), healthy-appearing perilesional skin (PeriLS) and lesional skin (LS) of hidradenitis suppurativa patients measured by quantitative real-time RT-PCR. *P < 0.05, **P < 0.001, ***P < 0.0001.

Figure 2 Multiple-comparison box-and-whisker plots showing median (interquartile range) relative mRNA expression levels between the three Hurley groups in healthy-appearing perilesional skin (PeriLS) and lesional skin (LS) of hidradenitis suppurativa (HS) patients. (a) In perilesional HS skin median expression levels of NCSTN ($P = 0.017008$), Notch1 ($P = 0.024321$), Notch2 ($P = 0.040038$), Notch3 ($P = 0.004163$), PIK3R3 ($P = 0.006706$) and AKT3 ($P = 0.006515$) were significantly higher in skin of patients with Hurley stage 1 compared to Hurley stage 2 and Hurley stage 3 (Kruskal–Wallis test). However, not for Hurley stage 2 compared with Hurley stage 3 group. (b) In contrast, in lesional HS skin median expression level of the investigated variables showed no significant difference between the three Hurley groups.
Table 2 Univariate association between patient characteristics (smoking status and positive family history) and median (interquartile range: IQR) quantitative real-time RT-PCR data of NCSTN, Notch1, Notch2, Notch3, PIK3R3 and AKT3 in perilesional and lesional skin of hidradenitis suppurativa patients

| Smoking status | Yes (n = 21) | P-values | Yes (n = 39) | P-values | BMI (n = 60) |
|----------------|-------------|----------|-------------|----------|-------------|
|                | No (n = 34) |          |             |          |             |
| Perilesional   |             |          |             |          |             |
| NCSTN          | 6.52 (5.75–9) | 0.0518 | 6.21 (4.51–8.48) | 0.5653 | 0.16 (0.2214 |
| Notch1         | 2.02 (1.2–4) | 0.9259 | 2.11 (1.12–2.57) | 0.4638 | 0.193 (0.1406 |
| Notch2         | 6.04 (5.56–7.04) | 0.1409 | 5.79 (5.04–7.08) | 0.6832 | 0.271 (0.036 |
| Notch3         | 10.08 (6.02–13.26) | 0.5611 | 8.75 (6.89–11.42) | 0.5263 | 0.318 (0.0132 |
| PIK3R3         | 1.92 (1.12–2.79) | 0.5302 | 1.64 (1.08–3.28) | 0.6832 | –0.089 (0.4981 |
| AKT3           | 2.09 (1.46–3.06) | 0.6364 | 2.32 (1.9–3.1) | 0.61 | –0.017 (0.8979 |
| Lesional       |             |          |             |          |             |
| NCSTN          | 10.58 (6.05–15.56) | 0.5404 | 11.92 (8.48–15.18) | 0.6901 | –0.087 (0.5075 |
| Notch1         | 4.04 (0.53–5.94) | 0.7862 | 3.85 (1.39–5.87) | 0.5162 | 0.124 (0.3442 |
| Notch2         | 5.11 (3.28–6.57) | 0.2358 | 6.06 (3.74–7.03) | 0.3489 | –0.142 (0.2801 |
| Notch3         | 9.28 (2.52–16.38) | 0.2546 | 10.84 (6.7–18.3) | 0.9926 | 0.014 (0.9162 |
| PIK3R3         | 3.19 (2.7–14) | 0.5875 | 3.69 (2.03–7.38) | 0.4305 | –0.013 (0.9187 |
| AKT3           | 3.93 (1.69–9.03) | 0.3366 | 6.56 (2.32–10.88) | 0.2618 | 0.028 (0.8292 |

P-values from Mann–Whitney U-test. Six patients could not provide information about family history. Correlation analysis (Spearman’s rho) between mRNA expression of NCSTN, Notch1, Notch2, Notch3, PIK3R3 and AKT3 with body mass index (BMI) in perilesional and lesional skin of hidradenitis suppurativa patients.

Results of immunohistochemical analysis for nicastrin, Notch1, Notch2, Notch3, PIK3R3 and AKT3 are shown in Table 3. Consistent with the mRNA expression data, staining intensity increased significantly from healthy controls to lesional HS skin. Immunoreactivity was found mainly in cytoplasm of keratinocytes (Fig. 3). For all investigated components, immunopositive cells were observed in the full thickness of the epidermis.

Table 3 Protein expression of nicastrin, Notch1, Notch2, Notch3, PIK3R3 and AKT3 as assessed by immunohistochemistry and quantified by the median (interquartile range; IQR) H-score in skin samples from healthy controls (HC), healthy-appearing perilesional skin (PeriLS) and lesional skin (LS) of hidradenitis suppurativa patients

|                | HC (n = 20) | PeriLS (n = 37) | LS (n = 37) | P-values |
|----------------|-------------|-----------------|-------------|----------|
| Nicastrin      | 7.5 (2.5–15) | 62.2 (36.48–83.73) | 87.5 (45.39–142.29) | HC vs. PeriLS: P = 0.0001† |
|                |             | 162.9 (143.63–183.55) |             | HC vs. LS: P = 0.0001† |
| Notch1         | 38.65 (23–54) | 102.2 (91.5–131.23) | 162.9 (143.63–183.55) | PeriLS vs. LS: P = 0.0029† |
| Notch2         | 47.8 (28.5–60) | 108.8 (88.75–170.63) | 172.9 (138–194.75) | HC vs. PeriLS: P = 0.0011† |
|                |             | 172.9 (138–194.75) |             | HC vs. LS: P < 0.0011† |
| Notch3         | 71.3 (65–85.65) | 105 (100–120) | 140.6 (118.75–153.18) | PeriLS vs. LS: P = 0.0001† |
| PIK3R3         | 100 (75–186.65) | 115 (74.6–162.9) | 195 (141.23–243.58) | HC vs. PeriLS: P = 0.0489† |
| AKT3           | 160 (137.5–192.5) | 165 (134.2–208.3) | 248.5 (235.8–261.03) | HC vs. PeriLS: P < 0.0011† |
|                |             | 195 (141.23–243.58) |             | HC vs. LS: P < 0.0011† |

IQR, interquartile range.
†Mann–Whitney U-test. †Wilcoxon-test. †Unpaired two-sided t-test. †Paired two-sided t-test.
Discussion

Apart from results derived from cell cultures and mouse models, there is scarce data available concerning expression of NCSTN (nicastrin) and Notch in HS skin. Xiao et al.\(^\text{14}\) found in lesional skin of one affected family member with a non-sense mutation of NCSTN reduced mRNA and protein expression of nicastrin and Notch1–3.\(^\text{14}\) A recent analysis of gene expression data of Notch 1–4 obtained from publicly available genomic data from HS and other inflammatory skin diseases showed no significant differential expression in lesional HS skin compared with healthy controls.\(^\text{19}\)

In contrast, we found a significantly increased mRNA and protein expression of NCSTN, Notch1–3, PIK3R3 and AKT3 in lesional HS skin compared to healthy controls in a large sample size. Neither a positive family history nor smoking status influenced the mRNA expression levels of the investigated genes. A positive correlation was found between BMI and Notch2 and Notch3 mRNA expression in perilesional HS skin. Though, the correlation coefficient indicated only a weak relationship.\(^\text{23}\)

There was also an increase in the mRNA expression levels of NCSTN, Notch1, Notch3, PIK3R3 and AKT3 from perilesional to lesional HS skin revealing a possible stepwise increase. In contrast to our data, mRNA microarray analysis of lesional vs. clinically healthy skin of 13 HS patients (patient characteristics were not described) revealed no different expression of NCSTN.\(^\text{24}\) However, further analysis of these publicly available microarray data described significantly increased expression of genes in lesional HS skin, which reside on chromosomal cytoband 1q21–1q25 and are linked to γ-secretase-Notch signalling pathway.\(^\text{25}\)

Notch signalling promotes keratinocyte proliferation and lead to the activation of the innate immunity by regulating immune cell development and function.\(^\text{13,26–28}\) In HS, assumed aspects of pathogenesis include a dysregulated inflammatory response leading to a profound skin inflammation and keratinocyte hyperproliferation with a strong tendency to sinus tract formation and hypertrophic scars.\(^\text{30–32}\) In turn, this inflammatory microenvironment can induce epidermal hyperproliferation.\(^\text{3,33}\) So, it can be suggested that Notch signalling may be a major contributor to this inflammatory vicious circle in HS. In accordance, previous studies revealed that Notch1–3 and the Notch pathway components (e.g. the NICD) are expressed in the interfollicular epidermis and within the hair follicle. Within these skin compartments, the Notch pathway is primarily active in cells undergoing or initiating terminal differentiation.\(^\text{12}\)

![Figure 3](image_url) Immunohistochemistry staining for (a, b) NCSTN, (c, d) Notch1, (e, f) Notch2, (g, h) Notch3, (i, j) PIK3R3 and (k, l) AKT3 in skin samples from healthy controls (a, c, e, g, i, k) and lesional skin (b, d, f, h, j, l) of hidradenitis suppurativa patients (original magnification 400×). Representative examples are shown.

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Against the background of the available data, the herein reported results allow the following assumptions. The observed dysregulated expression of the pathway components is either primarily responsible for the dysregulated inflammatory response in HS. Or they are secondarily co-activated very early during the skin inflammation and the inflammatory-mediated epidermal hyperproliferation.34

Due to the published IHC and RT-PCR data by Xiao et al.,14 which demonstrated altered expression of nicastrin and Notch, the possibility that different pathways are involved in European vs. East Asian cohorts should be acknowledged.

In respect to inflammation, it was shown that activation of the Notch signalling pathway can enhance the expression of the proinflammatory cytokine IL-17 and is critical for the differentiation of Th17 cells.26,27 In a psoriatic mouse model reduction of Notch1 mRNA expression by injections of the γ-secretase inhibitor, DAPT resulted in downregulation of IL-17, Th17 cell-specific transcription factor RORγt and IL-17A expression with diminished skin inflammation.27 The authors postulated that DAPT could be a potential therapeutic candidate.

Our results show significantly increased expression levels of PI3K3R3 and AKT3, two downstream signalling pathway components of NCSTN and Notch in lesional HS skin.14 In mice models activation of the PI3K/AKT pathway induced progenitor cell proliferation in hair follicles and interfollicular epidermis leading to epidermal and follicular hyperplasia.35

Regarding DNA damage responses and genome stability, there is evidence that AKT has regulatory effects on the DNA damage checkpoint ATR/CHK1 pathway.36 A recent study, which characterized hair follicle stem cells (HFSCs) isolated from HS patients, showed spontaneous activation of the ATR/CHK1 signalling in HS-ORSCs due to perturbation of cell cycle pathways with spontaneous replication stress leading to an increased number of proliferating ORSCs in HS patients. These data may indicate that replication stress in HFSCs is involved in the inflammatory pathogenesis of HS.37

In addition, the PI3K/AKT pathway and AKT3 can promote conversion of fibroblasts to functional keratinocyte-like cells by inducing fibroblast activation and differentiation.15 In turn, increasing research revealed the interaction between fibroblasts and keratinocytes and their contribution to fistula formation and scarring during chronic inflammation.38 Thus, these findings allow to suggest an association between an overactive PI3K/AKT signalling and dysregulated fibroblasts and the observed fistula formation and hypertrophic scarring in HS skin.

Interestingly, in perilesional and lesional skin of HS patients diagnosed as Hurley stage I mRNA expression levels of NCSTN, Notch1–3, PI3K3R3 and AKT3 were higher compared with Hurley stage II and III patients. In perilesional skin the differences in the expression levels were significant. This may be explained by the fact that in our study skin biopsies from Hurley stage I patients were derived solely from cases, in which small excision or deroofing had been performed to reveal acute inflammation and pain. A difference in histomorphology and composition of inflammatory cells in early HS lesions vs. chronic lesions could already be shown in HS.39 Thus, it is very likely, that the skin samples in the Hurley I group predominantly represent aspects of an acute inflammation compared to Hurley II and Hurley III skin samples. The latter representing a more chronic inflammation, in which secondary events are more likely to be present. This supports the hypothesis that the investigated pathway components are involved in the very early stage of the inflammatory response.

The herein presented results challenge the assumed importance of loss-of-function mutations in genes of the γ-secretase complex. However, it should be noted that there is evidence for a non-canonical, γ-secretase independent Notch signalling.40 This could explain the immunohistochemical staining pattern found in this study with predominant cytoplasmic staining.

In summary, we demonstrated a significant overexpression of NCSTN and members of the Notch and PI3K/AKT pathway in perilesional and lesional HS skin. Our findings point towards an important function during the inflammatory HS pathogenesis. Future functional studies are warranted to investigate a possible pathogenetic role or to reveal a coactivation of these overexpressed components during the inflammatory response in HS.

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