

# Nerve growth factor and its receptor tyrosine kinase TrkA are overexpressed in cervical squamous cell carcinoma

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## Abstract

Nerve growth factor (NGF) and its receptors are increasingly implicated in cancer progression, but their expression in cervical cancer is unclear. The objective of this study was to define the protein expression of NGF, its precursor (proNGF), as well as their receptors, the tyrosine kinase receptor TrkA, the common neurotrophin receptor p75<sup>NTR</sup> and the pro-neurotrophin receptor sortilin in cervical cancer. Immunohistochemistry was performed in a cohort of cervical cancers (n = 287), including the two major subtypes of the disease: squamous cell carcinomas (SCC) and adenocarcinomas (AC). Normal cervical tissues (n = 28) were also analyzed. Protein expression was determined by computer-based digital quantification of staining intensity and comparative statistical analyses were made with clinicopathological parameters including histological subtype, age, grade, tumor size, lymph node invasion, and stage. The expression of NGF, proNGF, TrkA, p75<sup>NTR</sup>, and sortilin was higher in cervical cancer compared to normal cervical tissues. NGF and TrkA were found overexpressed in SCC compared to AC ( $P = .0006$  and  $P < .0001$ , respectively). The expression of NGF ( $P = .0053$ ), proNGF ( $P = .0022$ ), and p75<sup>NTR</sup> ( $P = .0002$ ), but not that of TrkA or sortilin, was associated with increasing grade in SCC. In addition, nerve infiltration into the tumor microenvironment was assessed using the pan-neuronal marker PGP9.5. Infiltrating nerves were detected in 27% of cervical tumors and expressed TrkA. Functional investigations using the HELA cervical cancer cell line indicated that the Trk tyrosine kinase inhibitor GNF-5837 reduced cell viability through decreased ERK1/2 activation. Together, these data reveal the overexpression of NGF and TrkA in cervical SCC, suggesting a potential therapeutic value of targeting the NGF-TrkA signaling pathway in this subtype of cervical cancer.

## KEYWORDS

cervical cancer, nerves, NGF, p75<sup>NTR</sup>, proNGF, sortilin

**Abbreviations:** AC, adenocarcinoma; NGF, nerve growth factor; *NTRK1*, neurotrophin receptor tyrosine kinase 1; p75<sup>NTR</sup>, p75 neurotrophin receptor; proNGF, NGF precursor; SCC, squamous cell carcinoma; TMA, tissue microarray.

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## 1 | INTRODUCTION

Cervical cancer represents 7% of female cancers worldwide.<sup>1</sup> Histological subtypes are divided into the predominant squamous cell carcinoma (SCC) and adenocarcinoma/adenosquamous carcinoma (AC/ASC), accounting for about 75% and 25% of all cases, respectively.<sup>2</sup> Despite a decrease in incidence and disease-specific mortality worldwide, 50% of cervical cancer patients in developing countries and over 10% in developed countries are diagnosed with late stage disease.<sup>3,4</sup> The presence of distant metastases indicates a worse prognosis (median survival of 8-13 months),<sup>5</sup> and the development of novel diagnostic, prognostic, and therapeutic strategies for clinically advanced cervical cancers is warranted.

Nerve growth factor (NGF) is well described for its role in the development of the nervous system.<sup>6</sup> NGF drives neuronal outgrowth (axonogenesis) during development by activating the receptor tyrosine kinase TrkA (also called neurotrophin receptor tyrosine kinase 1, NTRK1) and the p75<sup>NTR</sup> common neurotrophin receptor, a member of the tumor necrosis factor receptor family.<sup>7</sup> The precursor for NGF (proNGF) also binds to the membrane protein sortilin, a member of the vacuolar protein sorting 10 protein (Vps10p) family of transmembrane receptors, to induce neuronal differentiation or apoptosis, depending on neuronal subtypes.<sup>7</sup> More recently, the expression of NGF and its receptors has been shown in cancer, where they participate in cancer progression.<sup>8</sup> For instance, NGF, proNGF and their receptors promote breast cancer cell survival, proliferation, and invasion.<sup>9</sup> Recent preclinical studies have also shown a participation of NGF and TrkA in driving the infiltration of human tumors by nerves, which contributes to cancer progression.<sup>10-13</sup>

In cervical cancer, NGF has been linked to perineural invasion,<sup>14</sup> a process whereby cancer cells invade nerves, but the extent of NGF expression, and that of its receptors, as well as the clinical significance have not been investigated. In addition, the expression of proNGF and sortilin has not been reported in cervical cancer. The present study aimed to elucidate the immunohistochemical profile and clinicopathological significance of proNGF, NGF, TrkA, p75<sup>NTR</sup>, and sortilin in cervical cancers. We report a significant increase in proNGF, NGF, p75<sup>NTR</sup>, and sortilin in cervical tumors as well as an association with higher grade for proNGF, NGF, and p75<sup>NTR</sup> in SCC. Interestingly, NGF and TrkA were distinctly overexpressed in SCC, compared to AC and normal cervical tissue. Furthermore, *in vitro* experiments revealed that targeting TrkA with the Trk tyrosine kinase inhibitor GNF-5837 resulted in a decreased viability of cervical cancer cells, suggesting a potential utility of TrkA as a therapeutic target.

## 2 | MATERIALS AND METHODS

### 2.1 | Cervical tissue samples

High-density tissue microarrays (TMA, catalogue number CR6161) were obtained from US Biomax Inc and included 294 cases of cervical carcinomas (257 SCC, 30 AC, and 7 unspecified histopathological subtypes) with a combined 28 adjacent normal and normal cervical tissues. The following clinicopathologic information was available for analysis: patient age, histopathological diagnosis, classification of malignant tumors (TNM), stage, and grade. US Biomax Inc quality controls are described as follows. Each single tissue spot on every array slide is individually examined by pathologists certified according to WHO published standardizations of diagnosis, classification, and pathological grade. Each specimen collected was consented to by both hospital and individual. Discrete legal consent was obtained and the rights to hold research uses for any purpose or further commercialized uses were waived. The study was conducted in accordance with the Declaration of Helsinki, and the protocol approved by the Human Research Ethics Committee of the University of Newcastle, Australia (HREC reference: H-2012-0063; March 26, 2019).

### 2.2 | Immunohistochemistry and digital quantification

Immunohistochemistry was performed as previously described by our laboratory.<sup>15</sup> Antibodies against proNGF (1:300 dilution; catalogue number ab9040, Merck Millipore), NGF (1:500 dilution; catalogue number ab52918, Abcam), TrkA (1:200 dilution; catalogue number 2508, Cell Signaling Technology), p75<sup>NTR</sup> (1:400 dilution; catalogue number 4201, Cell Signaling Technology), and sortilin (0.8 µg/mL; catalogue number ANT-009, Alomone Labs) and the pan-neuronal marker PGP9.5 (1:200 dilution; catalogue number ab15503, Abcam) were applied. Negative control testing was also performed using the following antibodies: rabbit IgG, purified serum nonimmune, isotype control (1:200 dilution; catalogue number 20009, Alpha Diagnostic International) or rabbit (DA1E) monoclonal antibody IgG Isotype Control (0.8 µg/mL; catalogue number 3900, Cell Signaling Technology). TMAs were digitized using the Aperio AT2 scanner (Leica Biosystems) at 400× absolute resolution. The HALO™ Image Analysis Platform (Indica Labs) was used to objectively and digitally quantify each TMA core stained by immunohistochemistry. Pixel intensity values were used to determine a h-score (0-300) for each tissue core (index calculated as the sum of 3x% of pixels with strong staining +2x% of pixels with intermediate staining +1x% pixels with weak staining).

## 2.3 | Statistical analysis of immunohistochemistry quantification

H-scores were analyzed as continuous variables, with summary statistics presented as group level medians and interquartile ranges (IQR). H-score distributions were compared using the Wilcoxon RankSum (dichotomous) or Kruskal Wallis (multiple comparisons) tests. To assess the primary hypothesis (difference in neurotrophin and receptor expression between benign and malignant samples), a two-sided alpha of 0.05 was used. To avoid a type 1 error when comparing h-scores between prespecified demographic and clinicopathological parameters (Table 1), we adjudicated significance using an adjusted alpha for multiple pairwise comparisons ( $0.05/6 = 0.008$ ). Analyses were based on complete cases and performed using Stata (version 14.1, Statacorp), with additional figures created using Prism (version 8.2.0, GraphPad Software).

## 2.4 | Impact of GNF-5837 on cervical cancer cell growth and TrkA signaling

### 2.4.1 | Cell culture

HELA cervical cancer cells (catalogue number CCL-2, American Type Culture Collection) were maintained in DMEM supplemented with 10% (v/v) of fetal bovine serum (FBS) (catalogue number SFBS-F, Bovogen Biologicals) and 2 mmol/L GlutaMAX supplement (catalogue number 35050061, Thermo Fisher Scientific) in a humidified incubator at 37°C with 5% (v/v) of CO<sub>2</sub>. Routine mycoplasma testing was performed using the MycoAlert Mycoplasma Detection Kit (catalogue number LT07-118; Lonza). Cells were not maintained in culture for longer than 3 months to ensure passage number remained fit for purpose.

### 2.4.2 | Protein extraction and western blotting

Protein extraction from cell lines and western blotting experiments were performed as previously described.<sup>16</sup> Complete mini-protease inhibitor cocktail tablets (catalogue number 4693124001, Roche) and PhosSTOP phosphatase inhibitor tablets (catalogue number 4906837001, Roche) were also used. An anti-TrkA antibody (catalogue number ANT018, Alomone Labs) was used at a dilution of 1:500 and  $\beta$ -actin detection (catalogue number A2066, Sigma-Aldrich) was used at a 1:5000 dilution as the equal loading control. The following antibodies from Cell Signaling Technology were all used at a dilution of 1:500 to assess cellular signaling pathways: anti-phospho-TrkA (anti-p-TrkA) (Tyr490, catalogue

number 9141), anti-p44/42 MAPK (Erk1/2) (catalogue number 9107), anti-phospho-p44/42 MAPK (p-Erk1/2) (Thr202/Tyr204, catalogue number 4370), anti-Src (catalogue number 2123), anti-phospho-Src (anti-p-Src) (Tyr416, catalogue number 2101), anti-Akt (catalogue number 2920), and anti-phospho-Akt (anti-p-Akt) (Ser473, catalogue number 4060).

### 2.4.3 | GNF-5837 treatment

The effect of treatment with GNF-5837, an oxindole inhibitor of Trk tyrosine kinase activity,<sup>17</sup> on HELA cell viability was investigated using the CellTiter-Blue® Cell Viability Assay (catalogue number G8081, Promega). Briefly,  $1 \times 10^4$  HELA cells/well were seeded within 100  $\mu$ L DMEM growth media in 96-well plates and incubated overnight. Cells were then treated with 100  $\mu$ L GNF-5837 (catalogue number SML0844, Sigma-Aldrich) to a final concentration of 0-100  $\mu$ mol/L as well as 0.1% of DMSO (catalogue number D2650, Sigma-Aldrich) as the negative control and incubated for 48 hours. About 40  $\mu$ L of CellTiter-Blue® Reagent was added to each well and the fluorescent intensity was recorded after 4 hours using the FLUOstar Optima Microplate Reader with a 560(20)<sub>Ex</sub>/590(10)<sub>Em</sub> filter set (BMG Labtech). Each treatment condition was performed in triplicate and data are presented as mean  $\pm$  SD from three independent experiments. The sensitivity of GNF-5837 treatment was calculated using half-maximal inhibitory concentration (IC<sub>50</sub>) estimated from the plot of the cell viability (percentage of control) vs increasing log concentrations of GNF-5837 using Prism (version 8.4.2, GraphPad Software).

## 3 | RESULTS

Cervical cancers and normal cervical tissues were analyzed by immunohistochemistry (IHC). Representative IHC staining for NGF, proNGF, TrkA, p75<sup>NTR</sup>, sortilin, and nerves are illustrated in Figures 1-6, respectively. Digital quantification of staining intensities, presented as median h-scores, are reported in Table 1 and Table S1. The assessment of nerve infiltration is presented in Table 2. The response of the HELA cervical cancer cell line to the Trk inhibitor GNF-5837 is shown in Figure 7.

### 3.1 | NGF is overexpressed and associated with increased grade in cervical SCC

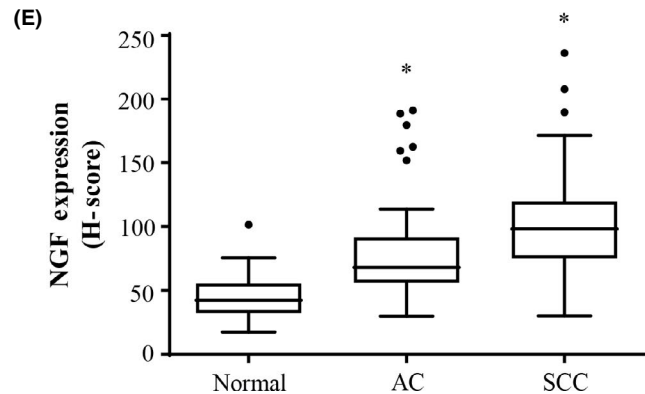
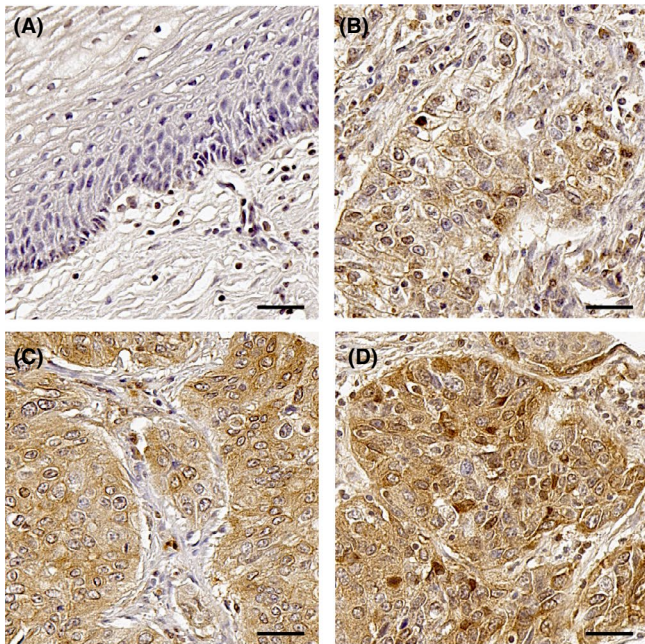
NGF immunohistochemical staining appeared low in normal cervical tissues (Figure 1A) while increased in both AC (Figure 1B) and SCC (Figure 1C,D). Digital quantification revealed a low NGF expression in normal cervical tissue

**TABLE 1** Associations between NGF, proNGF, TrkA, p75<sup>NTR</sup> and sortilin expression with clinicopathological parameters in SCC

Parameter	NGF <sup>a</sup> intensity			proNGF intensity			TrkA <sup>b</sup> intensity			p75 <sup>NTRc</sup> intensity			Sortilin intensity		
	Median h-score	IQR	P value	Median h-score	IQR	P value	Median h-score	IQR	P value	Median h-score	IQR	P value	Median h-score	IQR	P value
Age (y)															
<50 (n = 154)	100	78-121	.17	152	137-165	.17	73	52-106	.15	159	127-183	.11	61	51-73	.69
≥50 (n = 103)	93	69-117		144	128-164		63	42-106		151	119-170		61	49-73	
Grade															
1 (n = 23)	75	58-106	.0053	128	117-151	.0022	80	61-133	.03	131	112-156	.0002	54	41-62	.01
2 (n = 158)	100	78-119		156	139-166		73	52-111		152	126-178		62	52-73	
3 (n = 56)	111	80-133		150	136-169		63	46-83		170	149-190		65	53-73	
Tumor size (T)															
T1 (n = 226)	98	75-120	.96	152	134-166	.38	70	49-104	.32	154	127-179	.9	61	49-73	.65
T2-T4 (n = 29)	102	76-115		147	139-157		84	49-136		160	124-176		63	58-71	
Lymph node (N)															
Negative (n = 238)	99	75-120	.61	151	134-166	.29	71	49-106	.73	155	126-179	.19	61	49-72	.85
Positive (n = 18)	93	70-111		142	132-158		60	44-105		139	123-168		59	50-75	
Stage															
I + II (n = 229)	99	76-120	.68	152	134-166	.08	71	49-105	.97	155	127-179	.09	61	50-73	.73
III + IV (n = 26)	94	70-115		141	132-157		64	44-130		139	114-168		59	47-70	

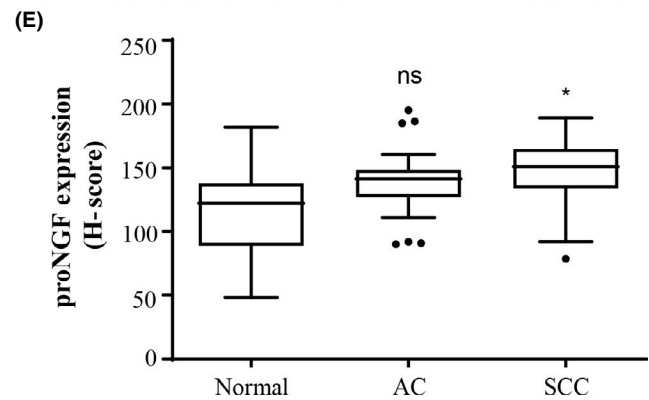
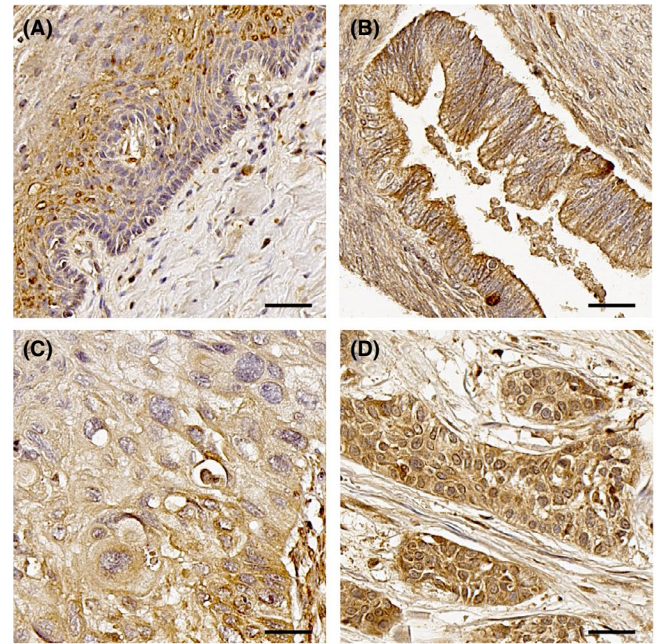
Note: Immunohistochemical staining were quantified and h-scores were used to compare protein expression levels. Group-levels medians (interquartile range, IQR) for h-score staining intensities are presented. Family-wise alpha significance (P value) is .05/6 = .008 using the Mann-Whitney test (pairwise) or Kruskal-Wallis test (multiple comparisons, as in the case of "Grade," where the P value is assessing an overall within-group difference). P values are presented on the same line as the heading to which they refer. Statistically significant P values are shown in bold. Cases missing clinicopathological parameters were not presented in the table or included in statistical analyses.

<sup>a</sup>Nerve growth factor.  
<sup>b</sup>Tyrosine kinase receptor A.  
<sup>c</sup>p75 neurotrophin receptor.



**FIGURE 1** NGF expression in cervical cancers and normal cervical tissue. Immunohistochemical staining of NGF in normal cervical tissues (A), adenocarcinomas (AC) (B) and squamous cell carcinomas (SCC) (C, D). Scale bars: 50  $\mu$ m (A-D). (E) Digital quantification of NGF expression was performed and h-scores were calculated. Data are expressed as medians (horizontal line in the center of the box) and box limits indicate the interquartile range (IQR) with the whiskers extending 1.5 times the IQR from the 25th and 75th percentiles; outliers are represented by dots. \* $P < .005$  compared to normal tissue expression, assessed using the Kruskal-Wallis test with adjustment for multiple comparisons

(h-score = 42, IQR 32-55) as compared to both AC (h-score = 68, IQR 52-84,  $P < .0001$ ) and SCC (h-score = 98, IQR 75-120,  $P < .0001$ ) (Figure 1E; Table S1). In SCC, there was a significant association observed between increased NGF ( $P = .0053$ ) and increasing grade (Table 1). ProNGF (Figure 2A-D) was expressed in the normal cervical epithelium (h-score = 122, IQR 90-137), with similar levels found in AC (h-score = 141, IQR 123-150) and SCC (h-score = 151, IQR 134-164) (Figure 2E; Table S1). The high level of proNGF staining compared to low level of NGF

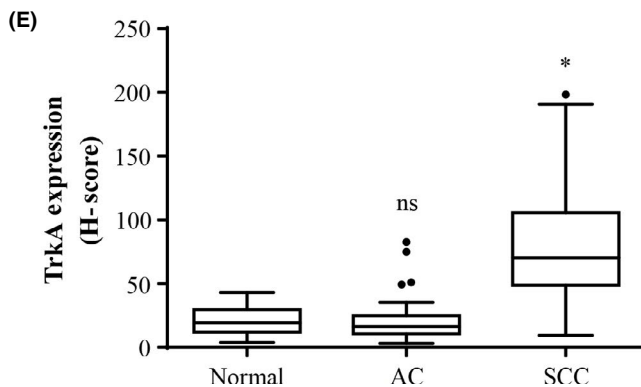
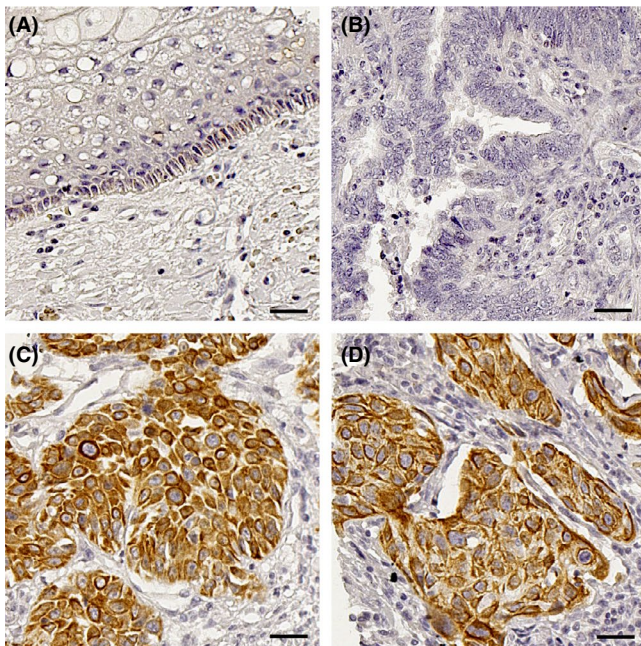


**FIGURE 2** ProNGF expression in cervical cancers and normal cervical tissue. Immunohistochemical staining of proNGF in normal cervical tissues (A), adenocarcinomas (AC) (B) and squamous cell carcinomas (SCC) (C, D). Scale bars: 50  $\mu$ m (A-D). (E) Digital quantification of proNGF expression was performed and h-scores were calculated. Data are expressed as medians (horizontal line in the center of the box) and box limits indicate the interquartile range (IQR) with the whiskers extending 1.5 times the IQR from the 25th and 75th percentiles; outliers are represented by dots. \* $P < .005$  compared to normal tissue expression, assessed using the Kruskal-Wallis test with adjustment for multiple comparisons

in the normal cervix suggests that proNGF is not processed into NGF in the normal epithelium. In SCC, there was a significant association between proNGF ( $P = .0022$ ) expression and grade (Table 1).

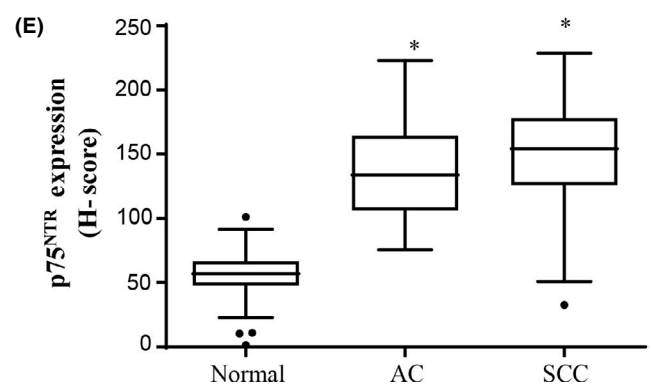
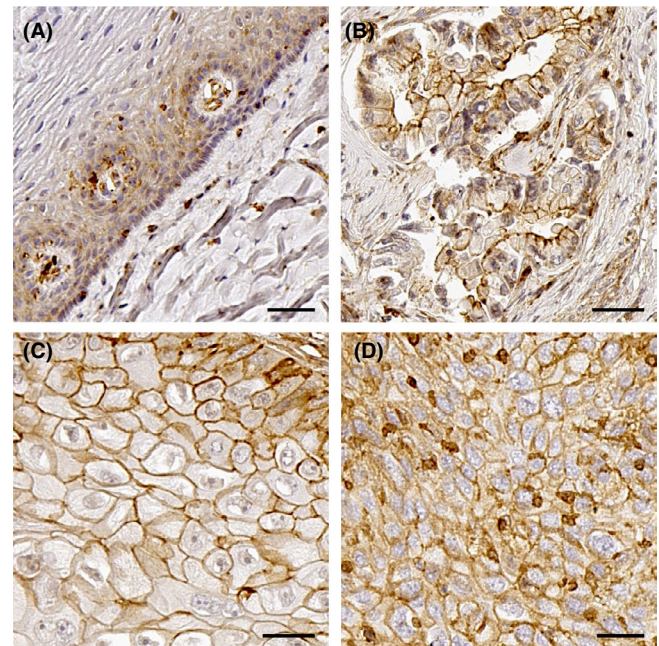
### 3.2 | TrkA is overexpressed in cervical SCC

In the normal cervical epithelium, TrkA IHC staining was detected only in the parabasal/basal layer (Figure 3A); whereas the upper layers of normal epithelium and



**FIGURE 3** TrkA expression in cervical cancers and normal cervical tissue. Immunohistochemical staining of TrkA in normal cervical tissues (A), adenocarcinomas (AC) (B) and squamous cell carcinomas (SCC) (C, D). Scale bars: 50  $\mu$ m (A-D). (E) Digital quantification of TrkA expression was performed and h-scores were calculated. Data are expressed as medians (horizontal line in the center of the box) and box limits indicate the interquartile range (IQR) with the whiskers extending 1.5 times the IQR from the 25th and 75th percentiles, with outliers represented by dots (\* $P < .005$  compared to normal tissue expression, assessed using the Kruskal-Wallis test with adjustment for multiple comparisons)

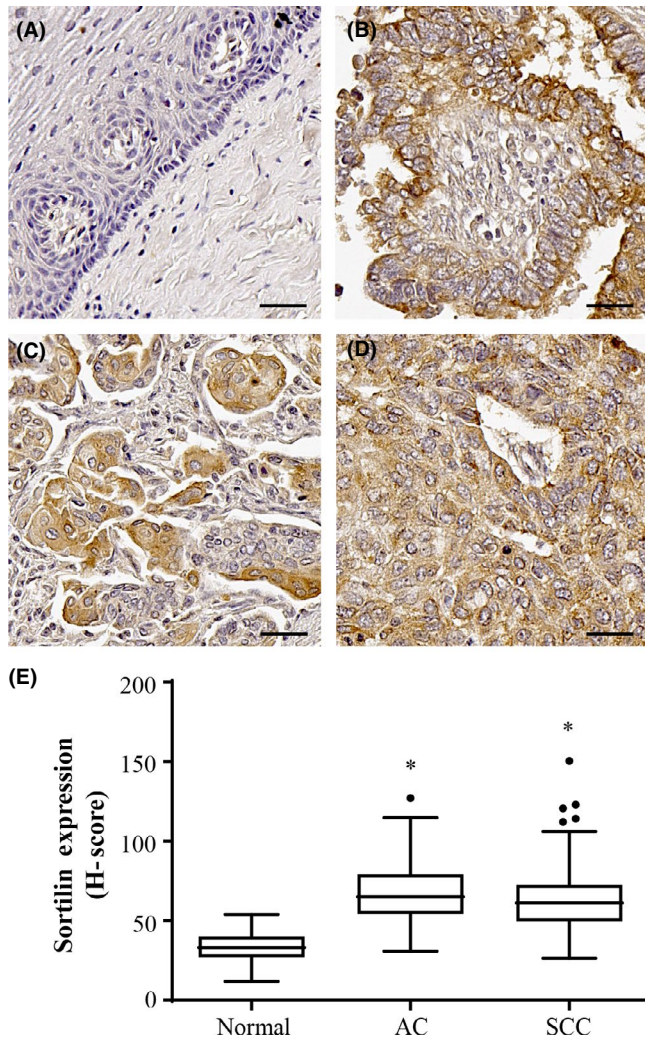
AC (Figure 3B) were negative. TrkA was strongly expressed in all SCC cells (Figure 3C,D). Digital quantification confirmed that TrkA immunoreactivity was low in normal cervical tissue (h-score = 22, IQR 15-29) and AC (h-score = 15, IQR 10-25) and high in SCC (h-score = 70, IQR 49-106,  $P < .0001$ ) (Figure 3E; Table S1). No associations were found between TrkA staining and any of the available clinicopathological parameters: age, grade, tumor size, lymph node status or stage (Table 1).



**FIGURE 4** p75<sup>NTR</sup> expression in cervical cancers and normal cervical tissue. Immunohistochemical detection of p75<sup>NTR</sup> in normal cervical tissues (A), adenocarcinomas (AC) (B) and squamous cell carcinomas (SCC) (C, D). Scale bars: 50  $\mu$ m (A-D). (E) Digital quantification of p75<sup>NTR</sup> expression was performed and h-scores were calculated. Data are expressed as medians (horizontal line in the center of the box) and box limits indicate the interquartile range (IQR) with the whiskers extending 1.5 times the IQR from the 25th and 75th percentiles; outliers are represented by dots. \* $P < .005$  compared to normal tissue expression, assessed using the Kruskal-Wallis test with adjustment for multiple comparisons

### 3.3 | p75<sup>NTR</sup> is associated with increasing grade in cervical SCC

IHC staining for p75<sup>NTR</sup> was observed in the parabasal/basal layer of normal cervical tissue (Figure 4A) and in stromal and cancer cells of AC (Figure 4B) and SCC (Figure 4C,D). However, p75<sup>NTR</sup> staining intensity was significantly higher in cervical cancer (median h-score = 152, IQR 125-178) compared to normal cervical tissue (h-score = 57, IQR 49-65,  $P < .0001$ ) (Figure 4E; Table S1). The increase in p75<sup>NTR</sup> staining intensity was observed across



**FIGURE 5** Sortilin expression in cervical cancers and normal cervical tissue. Immunohistochemical detection of sortilin in normal cervical tissues (A), adenocarcinomas (AC) (B) and squamous cell carcinomas (SCC) (C, D). Scale bars: 50  $\mu\text{m}$  (A-D). (E) Digital quantification of sortilin expression was performed and h-scores were calculated. Data are expressed as medians (horizontal line in the center of the box) and box limits indicate the interquartile range (IQR) with the whiskers extending 1.5 times the IQR from the 25th and 75th percentiles; outliers are represented by dots. \* $P < .005$  compared to normal tissue expression, assessed using the Kruskal-Wallis test with adjustment for multiple comparisons

both histopathological subtypes (Figure 4E; Table S1). Interestingly, there was a significant association between increased p75<sup>NTR</sup> protein expression and increasing grade in SCC ( $P = .0002$ , Table 1).

### 3.4 | Sortilin is increased in both AC and SCC of the cervix

Sortilin immunohistochemical staining was not detected in most normal cervical tissues (Figure 5A). In cervical cancers,

sortilin was detected in cancer cells and not in the stroma of both AC (Figure 5B) and SCC (Figure 5C,D). Digital quantification of immunoreactivity indicated that sortilin expression was higher in cervical cancers (h-score = 61, IQR 50-73) as compared to normal cervical tissues (h-score = 31, IQR 27-40,  $P < .0001$ ), with both AC (h-score = 62, IQR 51-78) and SCC (h-score = 61, IQR 50-72) expressing comparable levels (Figure 5E; Table S1). However, no associations were found between sortilin protein expression and the available clinicopathological parameters: age, grade, tumor size, lymph node status or stage (Table 1).

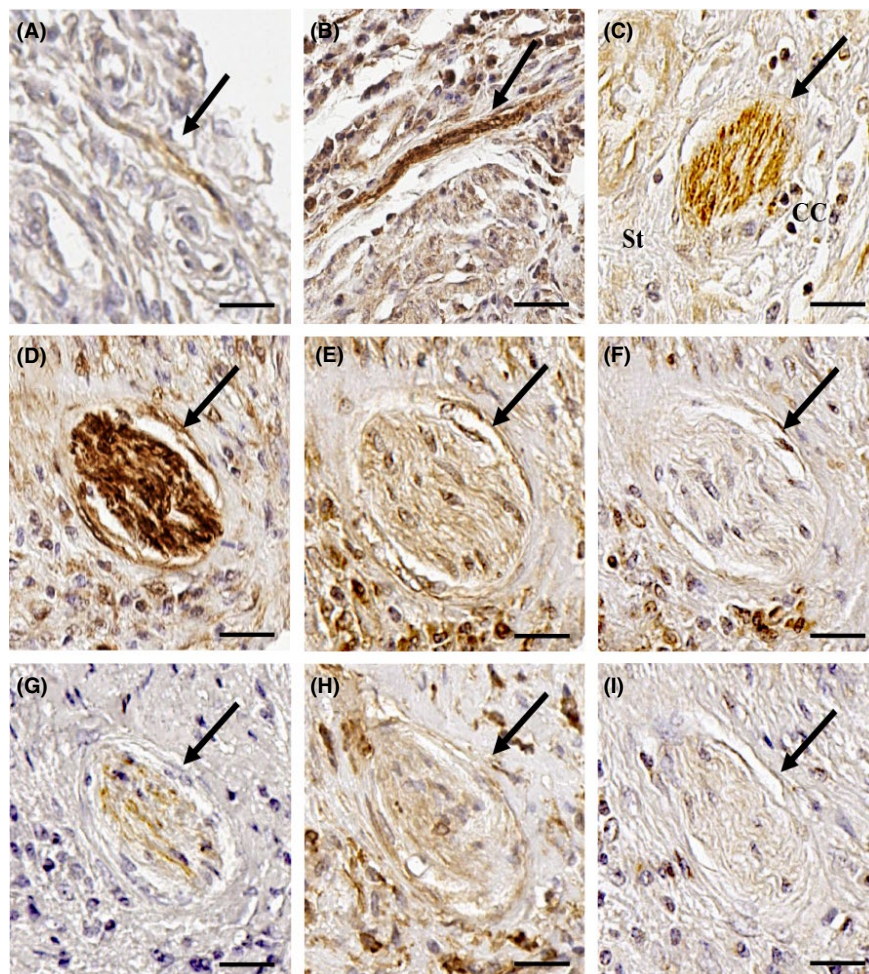
### 3.5 | Nerves infiltrate the tumor microenvironment of cervical cancers and express TrkA

To investigate the presence of nerve infiltration in cervical cancer, immunohistochemical staining using the neuronal marker PGP9.5 was performed. Typical morphologies of nerves were observed (Figure 6). Nerves were localized within the tumor microenvironment, adjacent to cancer epithelial cells (Figure 6A-C). Cervical carcinomas were classified as either negative or positive for the presence of nerves, and comparisons were made with clinicopathological parameters of the patient cohort. Nerves were detected in 27% of cervical cancers, however, cases with nerves present did not show different clinical or pathological parameters compared to cases with nerves (Table 2). Interestingly, large nerves within the tumor microenvironment (Figure 6D) were found to also express TrkA (Figure 6G), whereas proNGF (Figure 6E), NGF (Figure 6F), p75<sup>NTR</sup> (Figure 6H), and sortilin (Figure 6I) were not detected in nerves.

### 3.6 | Cytotoxic effect of the Trk inhibitor GNF-5837 in cervical cancer cells

To define the impact of TrkA signaling in cervical cancer cells, we treated HELA cells with the Trk inhibitor GNF-5837 and assessed drug sensitivity, impact on viability and signaling (Figure 7A,B). The data show that HELA cells are sensitive to GNF-5837 and that viability is reduced in a dose dependent manner, with an IC<sub>50</sub> of 12.45  $\mu\text{mol/L}$  (Figure 7A,B). TrkA phosphorylation and various downstream tumorigenic and metastatic-related pathways such as Erk, Src, and Akt were also examined following treatment with 0-20  $\mu\text{mol/L}$  of GNF-5837 (Figure 7C). Phosphorylated TrkA (Tyr490) and p-Erk1/2 (Thr202/Tyr204) were markedly decreased in response to GNF-5837, in a dose dependent manner, whereas downstream Src and Akt signaling were not altered (Figure 7C). These results suggest that targeting TrkA signaling can decrease survival of cervical cancer cells.

**FIGURE 6** Detection of nerves in the microenvironment of cervical cancer. Immunohistochemical detection of nerves was performed using the pan-neuronal marker PGP9.5 in a cohort of 287 cervical cancers and 30 normal cervical tissue samples. (A-D) Nerves stained for PGP9.5 adjacent to stroma (St) and cancer cells (CC). Immunohistochemical detection of proNGF (E), NGF (F), TrkA (G), p75<sup>NTR</sup> (H) and sortilin (I) was performed in serial sections containing the same nerve shown in (D). Scale bars: 50  $\mu$ m



## 4 | DISCUSSION

This study has clarified the protein expression of NGF and its receptors TrkA and p75<sup>NTR</sup> in cervical cancer. In addition, we describe for the first time the protein expression of proNGF and its receptor sortilin in cervical cancer. Prior to immunohistochemical profiling, cBioportal platform<sup>18</sup> was used to interrogate 275 complete cases of cervical cancer found within The Cancer Genome Atlas (TCGA).<sup>19,20</sup> The following degree of genetic alterations (amplifications, deletions, and missense mutations) were reported: 1.1% for *NGF*, 2.5% for *NTRK1*, 0% for *NGFR* (p75<sup>NTR</sup>) and 1.8% for *SORT1* (sortilin). Despite these relatively low frequencies, it is now well established in the literature that the level of mRNA does not directly correspond to the observed protein abundance in tumors, as demonstrated in colorectal cancer.<sup>21</sup> Multiple global transcriptomic and proteomic analyses have shown that only 30%-60% of changes in protein levels are directly related to changes in mRNA.<sup>22,23</sup> Genetic alterations and miRNA regulations are crucial to the synthesis and posttranslational modification of proteins and are all key factors that can contribute to

the discrepancies observed.<sup>23</sup> This emphasizes the need to look for cancer biomarkers at the protein level and we have performed IHC profiling of proNGF, NGF, TrkA, p75<sup>NTR</sup>, and sortilin on a cohort of cervical cancers.

The present study demonstrates that proNGF, NGF, TrkA, p75<sup>NTR</sup>, and sortilin are all overexpressed in cervical cancer compared to normal cervical tissues. Similar immunohistochemical profiles for NGF, TrkA, p75<sup>NTR</sup>, and sortilin have previously been described in breast,<sup>24-27</sup> prostate,<sup>28</sup> thyroid,<sup>15,29</sup> and lung cancers,<sup>30</sup> suggesting that the upregulation of this family of growth factors and receptors is a common phenotypical feature across a variety of human tumor types. In developing neurons, the activation of TrkA upon NGF binding results in the stimulation of various signaling pathways leading to axonogenesis.<sup>6</sup> In the adult, TrkA acts as a pain receptor in sensory neurons and contributes to the transmission of pain signals to the central nervous system.<sup>31</sup> Outside of the nervous system, there is accumulating evidence for TrkA expression and tumor-promotive signaling in several human cancers, including those of the breast,<sup>26</sup> oral cavity,<sup>32</sup> thyroid<sup>29</sup> and lung.<sup>30,33</sup> Our data demonstrate that TrkA is expressed in cervical cancer and most particularly in SCC. Similar to our findings, a recent study has demonstrated



**TABLE 2** Association between the presence of nerves and clinicopathological parameters in cervical carcinomas

Parameter	Nerve negative		Nerve positive		<i>P</i> value
Total cases					
Cancer (n = 287)	208	<b>(73%)</b>	79	<b>(27%)</b>	
Cancer histological subtype					.7135
Adenocarcinoma (n = 30)	21	<b>(70%)</b>	9	<b>(30%)</b>	
Squamous (n = 257)	187	<b>(73%)</b>	70	<b>(27%)</b>	
Age (years)					.7446
<50 (n = 171)	124	<b>(73%)</b>	47	<b>(27%)</b>	
≥50 (n = 116)	84	<b>(72%)</b>	32	<b>(28%)</b>	
Tumor size (T)					.5498
T1 (n = 254)	185	<b>(73%)</b>	69	<b>(27%)</b>	
T2-T4 (n = 31)	21	<b>(68%)</b>	10	<b>(32%)</b>	
Missing (n = 2)					
Lymph node status (N)					.4211
Negative (n = 266)	195	<b>(73%)</b>	71	<b>(27%)</b>	
Positive (n = 20)	13	<b>(65%)</b>	7	<b>(35%)</b>	
Missing (n = 1)					
Stage					.0529
I + II (n = 257)	191	<b>(75%)</b>	66	<b>(25%)</b>	
III + IV (n = 28)	16	<b>(57%)</b>	12	<b>(43%)</b>	
Missing (n = 2)					
Grade					.4570
1 (n = 29)	23	<b>(79%)</b>	6	<b>(21%)</b>	
2 (n = 168)	125	<b>(74%)</b>	43	<b>(26%)</b>	
3 (n = 62)	47	<b>(76%)</b>	15	<b>(24%)</b>	
Missing (n = 28)					

Note: Immunohistochemical staining for the pan-neuronal marker PGP9.5 was performed on a cohort of normal cervical tissue and cervical carcinomas. Cases were classified as nerve negative vs nerve positive and comparisons were made with clinicopathological parameters. No differences between groups were observed (assessed using the Chi-square test with a *P* value < .008). The bold values represent the % of cases.

that TrkA is also overexpressed in SCC of the lung<sup>30</sup> and TrkA fusion proteins have also been implicated in lung cancer progression.<sup>33</sup> Importantly, various Trk pharmacological inhibitors are currently undergoing clinical trials in various cancers<sup>34-36</sup> and the present study suggests that these Trk targeting drugs should also be tested in cervical SCC.

The concomitant and increased expression of NGF with TrkA in SCC is suggestive of an NGF-mediated autocrine loop, potentially involving the secretion of NGF from cervical cancer cells and subsequent signaling through both TrkA and p75<sup>NTR</sup>. An autocrine loop of NGF stimulation in cancer cells involving TrkA has already been demonstrated in breast cancer.<sup>37</sup> We also report that the expression of proNGF, NGF

and p75<sup>NTR</sup> was associated with increasing grade in SCC, thus, highlighting potential prognostic value as markers of tumor aggressiveness. No additional associations were found with any of the other clinicopathological parameters of the patients, whereas there could be a dual signaling of NGF, via both TrkA and p75<sup>NTR</sup>, in cervical cancer cells, which warrants further investigation.

In recent years, nerves have emerged as promoters of tumor initiation and progression.<sup>10,38</sup> Nerves infiltrated in the tumor microenvironment are releasing growth promoting factors, such as neurotransmitters, that stimulate cancer growth and metastasis.<sup>10</sup> In prostate,<sup>39</sup> gastric<sup>40</sup> and pancreatic<sup>41</sup> cancers, tumor denervation can block tumor progression and similarly to angiogenesis, axonogenesis in the tumor microenvironment is, therefore, increasingly viewed as a new target for innovative therapies.<sup>10</sup> Innervation of the tumor microenvironment in cervical cancer has recently been described.<sup>42</sup> Nerves infiltrating cervical tumors are of sensory origin and axonogenesis appears to be mediated by cancer-derived exosomes.<sup>42</sup> Our results show that TrkA is expressed in nerves infiltrated in cervical cancer and suggest that similarly to gastric<sup>11</sup> and pancreatic<sup>43</sup> cancer, NGF and TrkA may be involved in driving nerve infiltration in the tumor microenvironment of cervical cancer. However, further investigations are warranted to explore the molecular mechanism of cervical cancer innervation and the potential role played by NGF and its receptors.

Together, the findings of this study outline the clinicopathological significance of proNGF, NGF and their receptors in cervical cancer. In particular, the overexpression profile of NGF and TrkA in SCC suggests that targeting NGF and TrkA may constitute a targeted therapeutic strategy in this subtype of cervical cancer and further preclinical and clinical investigations are warranted to confirm this hypothesis.

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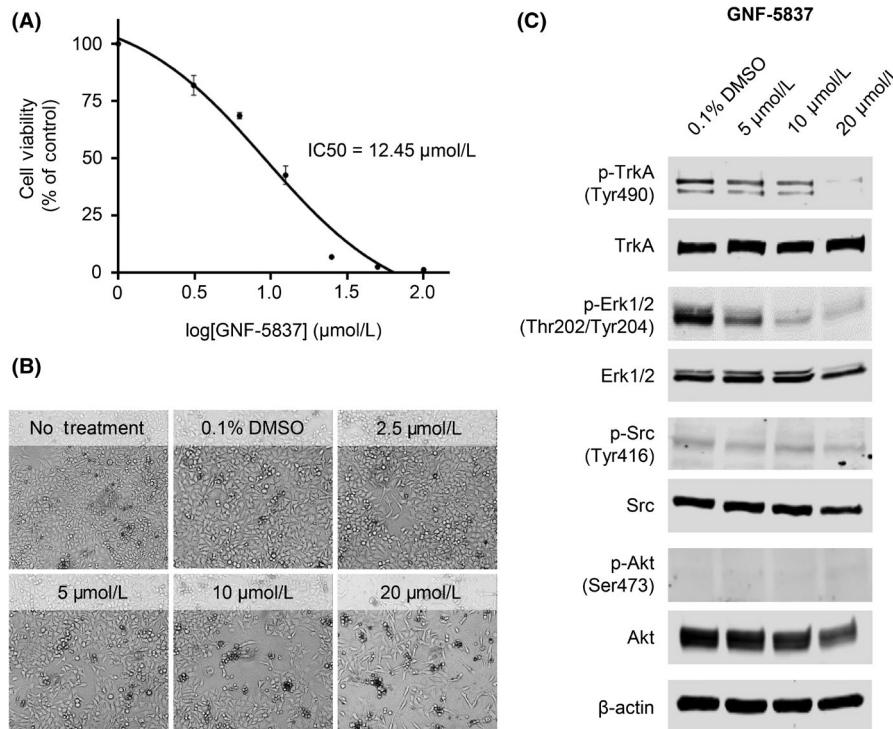
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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

S. Faulkner: conceptualization, methodology, software, formal analysis, investigation, data curation, writing (original draft preparation), writing (review and editing), visualization and project administration. N. Griffin: validation, data curation and writing (review and editing). C. W. Rowe: formal



**FIGURE 7** Impact of the GNF-5837 Trk inhibitor in the HELA cervical cancer cell line. (A) HELA cells were treated with 0–100  $\mu\text{mol/L}$  grade concentrations of the TrkA inhibitor GNF-5837 in media containing 10% FBS for 48 hours and a dose response curve was generated to determine an  $\text{IC}_{50}$  of 12.45  $\mu\text{mol/L}$ . Data are presented as mean  $\pm$  SD of samples from three independent experiments. (B) Representative images of HELA cells treated with 0–20  $\mu\text{mol/L}$  grade concentrations of GNF-5837 for 48 hours. (C) Effect of 0–20  $\mu\text{mol/L}$  GNF-5837 on TrkA phosphorylation and downstream cellular signaling. Western blot analysis was performed 48 hours posttreatment with GNF-5837. The level of phospho-TrkA (p-TrkA) and phospho-Erk1/2 (p-Erk1/2) both decreased in a dose dependent manner. A 0.1% of DMSO was used as a negative control and  $\beta$ -actin protein expression was used as an equal loading control

analysis and writing (review and editing). P. Jobling: writing (review and editing). J. M. Lombard: writing (review and editing). S. M. Oliveira: writing (review and editing). M. M. Walker: validation and writing (review and editing). H. Hondermarck: conceptualization, validation, resources, writing (original draft preparation), writing (review and editing), visualization, supervision, project administration, and funding acquisition.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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