Identification and Characterization of Novel Spliced Variants of Neuregulin 4 in Prostate Cancer

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Abstract Purpose: The neuregulin (NRG) 1, 2, and 3 genes undergo extensive alternative mRNA splicing, which results in variants that show structural and functional diversity. The aims of this study were to establish whether the fourth member of this family, NRG4, is expressed in prostate cancer, if it is alternatively spliced and whether any functional differences between the variants could be observed.

Experimental Design: The expression of NRG4 was determined using immunohistochemical staining of 40 cases of primary prostate cancer. Bioinformatic analysis and reverse transcription-PCR (RT-PCR) using NRG4 isotype-specific primers on a panel of normal and prostate cancer cell lines were used to identify alternatively spliced NRG4 variants. Expression of these variants was determined using isotype-specific antibodies. Transfection into Cos-7 cells of two of these green fluorescent protein-tagged variants allowed analysis of their subcellular location. Four of the variants were chemically synthesized and tested for their ability to activate the ErbB4 receptor.

Results: NRG4 was variably expressed in the cytoplasm in the majority of prostate cancer cases, and in a subset of cases in the membrane, high levels were associated with advanced disease stage. Four novel NRG4 splice variants (NRGA2, NRG4 B1-3) were characterized, where each seemed to have a different subcellular location and were also expressed in the cytoplasm of the prostate tumors. NRG4 B3 was also present in endothelial cells. In transfected cells, the A type variant (NRG4 A1) was localized to the membrane, whereas the B type variant (NRG4 B1), which lacks the predicted transmembrane region, had an intracellular localization. Only the variants with an intact epidermal growth factor-like domain activated ErbB4 signaling.

Conclusion: NRG4 overexpression is associated with advanced-stage prostate cancer. The alternative splice variants may have different roles in cell signaling, some acting as classic receptor ligands and some with as-yet unknown functions.

The epidermal growth factor (EGF) family of proteins are involved in normal development and in the pathology of many diseases, including cancer (1, 2). They can be divided into three categories: those that bind to the ErbB1 receptor only, those that bind the ErbB1 and the ErbB4 receptor, and those that bind to ErbB3 or ErbB4 (3). The latter group are termed the neuregulins (NRG) and consist of four genes, NRG1 to 4 (4).

The most notable distinguishing characteristic of this family is that unlike the other EGF family ligands, they are subject to extensive alternative splicing of their mRNA transcripts, leading to the expression of a diverse range of protein products. The NRG1 gene products are now arranged into six classes defined by their alternative promoter usage, but further variation occurs in their 3' regions because they can encode different (partial) EGF elements and may contain or lack transmembrane sequences (2, 5). The NRG2 (6) and NRG3 genes (7, 8) are also subject to similar splicing events, although they have been studied to a lesser degree of detail. Thus far, only a single gene product has been described for the NRG4 gene (9), and this has been shown to activate the ErbB4 receptor only.

NRG1 has been shown to be expressed in prostate cancer and in normal prostate stromal, basal epithelial and luminal cells, and the ErbB4 receptor in normal prostate luminal cells, but not in prostate cancer cells (10). NRG1, when added to prostate cancer cell lines, reduced their proliferation presumably acting through the ErbB3 receptor. These authors hypothesize that the loss of ErbB4 expression and apparent loss of NRG1 expression relieve an inhibitory autocrine loop contributing to cell transformation.

In this work, we show that the NRG4 protein is made in clinical prostate cancers, and that high levels of expression are
associated with advanced tumor stage. We determine the structure of the NRG4 gene and define four additional transcripts and show that these are expressed in human prostate cancer epithelial cells. Some of these have a predicted transmembrane sequence, and others do not. We show using green fluorescent protein (GFP)-tagged constructs that these are expressed in the cell membrane or as intracellular proteins, respectively. We synthesized and refolded four of the predicted proteins and tested their ability to signal via the ErbB4 receptor. We confirm here that prostate cancer cells can express the ErbB1, ErbB2, or ErbB3 receptors while lacking ErbB4; however, because NRG4 has been reported only to bind to ErbB4, this suggests that the secreted NRG4 isoforms are involved in paracrine rather than autocrine action. The function of the intracellular forms remains enigmatic, although it is clear that some isoforms of the NRG1 gene can accumulate in specific subnuclear compartments (11).

**Materials and Methods**

**Bioinformatics.** Human genomic sequence encoding NRG4 (Swisssprot:Q8WWG1) was retrieved from Ensembl (ref. 12; Ensembl gene: ENSG00000169752). To identify expressed sequence tags (EST) deriving from the NRG4 gene, the genomic sequence was submitted to the European Molecular Biology Laboratory (EMBL) Gene2EST Blast server (13). Only spliced ESTs identified by Gene2EST that contained more than three exons were considered for analysis. ESTs and cDNA sequences generated were further compared with genomic sequence using EST2GENOME in the EMBOSS package (14) and Artemis (15) to identify the exon structure of the gene. The sequences of predicted transcripts and show that these are also expressed in human prostate epithelial cells. Some of these have a predicted structure of the NRG4 gene and define four additional

**Reverse transcription-PCR of NRG4 isoforms and ErbB receptors from prostate cell lines.** Approximately 1 μg RNA (RNAeasy Mini Kit, Qiagen Ltd.) from two breast cancer cell lines (SKBR-3 and ZR-75), three normal prostate cell lines (PNT2-C2, P4E6, and PZ-HPV-7) and four prostate cancer cell lines (PC-3, DU-145, LNCaP, and Ca-HPV-10) was reverse transcribed and reverse transcription-PCR (RT-PCR) was carried out using NRG4-specific primers. NRG4 F 5'-CACATGCGAAGCAATCACAGAAGAC-3' was used as the forward primer for all isoforms; the reverse primers were for NRG4 A1, 5'-GTGGTGTCACGTACCTGGTGG-3'; NRG4 A2, 5'-CTCACCTGATGTTCAACACC-3'; NRG4 B1, 5'-GAAACCCAGCATGAAACCT-3'; NRG4 B2, 5'-TGACACATITTCGGTTCCCTCATTAATC-3'; NRG4 B3, 5'-CTTCAAGTTGGAAGCTATC-3', respectively; NRG1 to NRG3 primers (19); ErbB1 (238 bp) primers, forward 5'-CTGAGAACCAATACCAGAC-3', reverse 5'-AGACCCACTTCCATACCTG-3'; NRG4 A1/A2 (rat) DHEQPCGPRHRSFCNLINGCYVPIYPITPSIRCENIYTGARECIVEFL (b) NRG4 B1 DHEEPCGPRHSFCLNGLNCYVPIYPITPSFRCR, (c) NRG4 B2 DHEEPCGPRHSFCLNGLNCYVPIYPITPSFSCS, and (d) NRG4 B3 DHEEPCGPRHSFCLNGLNCYVPIYPITPSFCNLGSHELSENDENELDDDLLPLNE were synthesized by the fmoc strategy (23). The precipitated peptides were dissolved in water and recovered by freeze-drying. Peptide refolding was carried out (24) and reduced in 10 mmol/L DTT at room temperature before mass spectroscopy. Electrospray mass spectra were recorded on a Thermo Finnigan LCQ ion trap mass spectrometer. Samples were desalted online by reverse-phase high-performance liquid chromatography (HPLC) on a Vydac C18 column (2.1 × 250 mm) running on an Agilent 1100 HPLC system. Mass spectra were deconvoluted using Thermo Finnigans BioExplot to give intact peptide masses. One-dimensional proton nuclear magnetic resonance and two-dimensional NOESY were carried out on water dissolved in 25 mmol/L NaH2PO4 (pH, 7.4), 0.5 mol/L NaCl (PBS) fixed with 4% paraformaldehyde for 10 min, permeabilized (if required with 0.1% Triton/PBS for 5 min at 4°C and processed for immunofluorescence as described in ref. 22.

**Peptide synthesis, refolding, and nuclear magnetic resonance.** NRG4 isotype specific peptides (a) NRG4 A1/A2 (rat) DHEQPCGPRHSFCLNLNCYVPIYPITPSFRCENIYTGARECIVEFL, (b) NRG4 B1 DHEEPCGPRHSFCLNGLNCYVPIYPITPSFRCR, (c) NRG4 B2 DHEEPCGPRHSFCLNGLNCYVPIYPITPSFSCS, and (d) NRG4 B3 DHEEPCGPRHSFCLNGLNCYVPIYPITPSFCNLGSHELSENDENELDDDLLPLNE were synthesized by the fmoc strategy (23). The precipitated peptides were dissolved in water and recovered by freeze-drying. Peptide refolding was carried out (24) and reduced in 10 mmol/L DTT at room temperature before mass spectroscopy. Electrospray mass spectra were recorded on a Thermo Finnigan LCQ ion trap mass spectrometer. Samples were desalted online by reverse-phase high-performance liquid chromatography (HPLC) on a Vydac C18 column (2.1 × 250 mm) running on an Agilent 1100 HPLC system. Mass spectra were deconvoluted using Thermo Finnigans BioExplot to give intact peptide masses. One-dimensional proton nuclear magnetic resonance and two-dimensional NOESY were carried out on water dissolved in 25 mmol/L NaH2PO4 (pH, 7.4), 0.5 mol/L NaCl (PBS) fixed with 4% paraformaldehyde for 10 min, permeabilized (if required with 0.1% Triton/PBS for 5 min at 4°C and processed for immunofluorescence as described in ref. 22.

**Production of affinity-purified anti-NRG4 antibodies and immunoblotting.** Five rabbit NRG4 polyclonal antibodies were made using synthetic peptides: anti-127 (pan NRG4), PTDEEPCGPRHSFCLNLNCYVPIYPITPSFRCENIYTGARECIVEFL, anti-135 (COOH-terminal NRG4 A2 specific), KFKLRSRGNTSM; and anti-134 (COOH-terminal NRG4 B3 specific), DNNEDYLDLPLNE. The immunizing peptide for NRG4 A2 was modified to produce a soluble peptide for immunization. The anti-peptide sera were affinity purified (20), and the titer was determined by ELISA.

Isotype-specific peptides (0.1–10 μg/dot) were used to form an immunodot blot. Antibodies (1 or 2 μg/mL) were used to probe the nitrocellulose strips, and the blots were developed using enhanced chemiluminescence reagents (Amersham) as described in ref. 19.

**Clinical samples and immunohistochemistry.** A tissue microarray of 2-mm cores from formalin-fixed, paraffin wax–embedded tumors containing 40 cases of human prostate adenocarcinoma and nine matched normal pairs was obtained from TCS Cellworks Ltd.

Staining of both human prostate tissue and rat salivary glands was done using peptide antibodies with the StreptABC complex/Duet kit (DAKO). Countertaining was done using Gill’s hematoxylin (BDH). Scoring was done independently by M.B. and G.R., and any discrepancies were resolved by discussion. Scoring was done as described in ref. 21, in which both the percentage of positive cells and the intensity of staining were taken into account.

**Statistics.** Associations between the percentage of positive cells and the intensity of staining and other covariates were assessed by Spearman’s rank correlation coefficient using Genstat.

**Plasmid construction.** Full-length image clones of NRG4 A1 (4071605) and NRG4 B1 (3879188) were used to obtain both isoforms without stop codons (the primers are described above). The NRG4 A1 (345 bp) and the NRG4 B1 (106 bp) products were TA cloned into the directional expression vector pCDNA3.1/D/V5-His-TOPO (Invitrogen), then Kpn1/SacI cleavage allowed subcloning into the enhanced GFP-N1 vector (Clontech).

**Transfections and immunofluorescence.** For immunofluorescence, Cos-7 cells (which lack endogenous ErbB4 as confirmed by PCR analysis) were grown in 24-well plates to ~70% confluency and transfected with 0.2 μg DNA for each coverslip using FuGENE 6 (Roche Molecular Biochemicals). Eight hours post-transfection, the proteins and tested their ability to signal via the ErbB4 receptor. We confirm here that prostate cancer cells can express the ErbB1, ErbB2, or ErbB3 receptors while lacking ErbB4; however, because NRG4 has been reported only to bind to ErbB4, this suggests that the secreted NRG4 isoforms are involved in paracrine rather than autocrine action. The function of the intracellular forms remains enigmatic, although it is clear that some isoforms of the NRG1 gene can accumulate in specific subnuclear compartments (11).
NRG4 A1 is expressed in normal and cancer prostate tissue. NRG1 may be involved in the early progression of prostate cancer (10), but to date, none of the other NRGs have been studied in this disease. To address whether NRG4 is expressed in prostate cancer, a small immunohistochemical study on 40 prostate tumors and nine matched normal samples was carried out. An antibody to the EGF domain of NRG4, anti-123, which was previously used to detect protein expression in breast cancer (19), was used. In all tumors (including the hyperplastic glands), where present, staining was cytoplasmic and variable in intensity (Fig. 1A-D). Membrane staining was present in 13 tumors, being strong in one case (Fig. 1D) with no membrane staining observed in any of the nine normal samples. The stroma showed moderate and strong staining in smooth muscle and weak to moderate staining in stromal fibroblasts. Weak staining was noted in nerve bundles, but nuclear staining was not seen. NRG1 α/β and NRG3 have been found in the nuclei of cases of breast ductal carcinoma in situ (DCIS), but NRG4 was not (26). There was no significant difference in the levels of NRG4 expression in the small number of matched normal cases compared with the cancer cases, nor was there any association between the staining intensity or quantity and either age, Gleason grade, or prostate-specific antigen level. However, there was evidence of a positive rank correlation between stage and quantity of staining (Spearman’s ρ = 0.646; P = 0.007) and between pathological tumor-node-metastasis (pTNM) and quantity of staining (ρ = 0.644; P = 0.003) and intensity of staining (ρ = 0.466; P = 0.043).

Gene organization and identification of novel NRG4 isoforms. Previous work examining the expression of NRG4 in breast cancer cell lines (19) and the identification of a large number of other NRG isoforms in the literature prompted us to search for novel NRG4 splice variants using bioinformatic and PCR approaches. The human NRG4 gene is located on chromosome.

Fig. 1. Detection of NRG4 variants by immunohistochemical staining in human prostate cancer cases. Examples of weak (A), moderate (B), and strong (C) cytoplasmic staining using the anti-123 antibody to the NRG4 A1/A2 isoforms; intense plasma membrane staining (D). The anti-127 antibody, which reacts with all known NRG4 isoforms (E), anti-128, specific for the NRG4 A1 isoform (F), anti-134, specific for the NRG4 B3 isoform (G), and anti-135, specific for the NRG4 A2 isoform (I) all show epithelial cytoplasmic staining of the tumor with weak staining in the stroma. Anti-134 antibody also detects NRG4 B3 in the endothelial cells of blood vessels (H). Counterstaining was done using Gill’s hematoxylin. Original magnification, ×40.
To identify ESTs deriving from this gene, we compared the genomic sequence to all human ESTs in the EMBL database. This revealed substantial variation in the exon composition of NRG4 mRNA products. We designate the prototypical NRG4 protein (SwissProt:Q8WWG1) as NRG4 A1 (Fig. 2B). Three variants, B1, B2 and B3, accession numbers, BX538100, AM392365, AM392366, respectively (Fig. 2B), were identified in the open reading frames of multiple ESTs. These all lack the transmembrane segment and cytoplasmic tail of NRG4 A1, as well as the exon that encodes cysteine residues 5 and 6 of the EGF domain. We confirmed the expression of each B-type variant by RT-PCR from a normal prostate cell line (Fig. 1C).

We also analyzed PCR products amplified from full-length NRG4 cDNAs. Among these, we identified a cDNA encoding a protein shown in Fig. 2B as NRG4 A2 (accession number AM392364). This cDNA encodes the complete EGF (exons 2 and 6) and transmembrane domain (exon 6) and also contains a novel exon (exon 7) that encodes a short cytoplasmic tail. We confirmed the expression of NRG4 A2 mRNA by PCR with primers to exons 1 and 7 and sequencing of the product. No ESTs were found in the EMBL database for NRG4 A2, and thus, it represents a previously unidentified splice variant.

All five NRG4 isoforms identified here have the same translation initiation site at the start of exon 1 and contain exon 2, which encodes two thirds of the EGF-like domain (i.e., cysteine residues 1 to 4; Fig. 2B). The individual NRG4 isoforms are produced by alternative splicing between exons 3 and 9. NRG4 B1, B2, and B3 do not contain exon 6, which indicates that these are not likely to be membrane-associated proteins. NRG4 A2 has a predicted class I PDZ binding motif, which is represented by the three COOH-terminal amino acids, TCM (27). NRG4 B1 is spliced from exon 2 into exon 5, which encodes a single lysine, and NRG4 B2 is spliced from exon 2 into exon 4, which encodes a single serine before stop codons. NRG4 B3 is spliced from exon 2 into exon 3, which encodes an acidic 21-amino region, which contains a predicted nonreceptor tyrosine kinase phosphorylation site (NDNNED-LYDDLPLN).

Detection of novel NRG4 mRNAs in a human normal prostate cell line. The anti-123 antibody detects the COOH-terminal region of the EGF domain and so does not discriminate between NRG4 A1 and A2. To investigate which individual NRG4 splice variants were present in normal prostate, RT-PCR analysis using full-length isotype-specific primers for each NRG4 variant was carried out on an immortalized normal prostate cell line PNT2-C2. Four PCR products [NRG4 A1 (345 bp), NRG4 B1 (108 bp), B2 (105 bp) and B3 (171 bp)] were obtained, cloned, and sequenced. NRG4 A2 could not be detected in this cell line, but it was present in a breast cancer cell line, ZR75 (276 bp; Fig. 2C).

Expression of ErbB growth factor receptors, NRGs, and NRG4 splice variants in prostate cell lines. NRG4 activates ErbB4 but does not bind to the other ErbB receptors (ErbB1-3; ref. 28). Activated ErbB4 may, however, undergo heterodimeric interactions with other receptor family members. To explore if this could take place, a complete receptor profile of a panel of human prostate cell lines was obtained (Fig. 3A). RT-PCR using primers specific for the receptors ErbB1-4 revealed that ErbB1-3 were present in all the cell lines. Neither splice variants of ErbB4 (ErbB4Ct and ErbB4Ctβ) were present in any of the prostate cell lines (in agreement with ref. 29). RT-PCR using NRG1-3–specific primers showed that (a) the predicted 69-bp NRG1 product was present only in the Ca HPV-7 cell line, and a smaller 50-bp product, which may represent a different splice variant of NRG1, is present in the...
PC-3 cell line; (b) NRG2 (53 bp) was found in all the cell lines examined; and (c) NRG3 was only present in DU-145, LNCaP, and CaHPV-7.

RT-PCR using primers that detect all the NRG4 splice variants showed that all variants could be detected in the panel of prostate cell lines. These cell lines represent different stages of prostate cancer progression; the PNT2-C2 and P4E6 cell lines are derived from normal prostate, Ca-HPV-7 is derived from a prostatic adenocarcinoma, and DU-145, LNCaP, and PC-3 are derived from metastatic prostate cancer. Interestingly, NRG4 A2 is not detectable in the PNT2-C2, P4E6, and Ca-HPV-7 cell lines, i.e., it is only found in those cell lines derived from metastatic tumors (Fig. 3B).

Expression and specific subcellular localization of NRG4 isotypes in rat salivary gland and prostate cancer. To show that the mRNA is translated into protein in epithelial tissue, where possible, isotype-specific anti-peptide antibodies were made to discriminate between the NRG4 variants. It was not possible to make specific antibodies to NRG4 B1 and B2 because the only unique sequence in both isotypes is a single COOH-terminal amino acid. The specificity of each affinity-purified antibody was confirmed by ELISA, Western blotting, and by immunoblotting against all five of the peptides used for immunization (Fig. 4, top left).

It has been reported (by Northern blot analysis) that NRG4 is expressed in human muscle and pancreas (9). We carried out a comprehensive immunohistochemical study on a large range of rat tissues using the anti-123 antibody (data not shown). Rat salivary gland showed good staining with anti-123, and this tissue was used to look for protein expression of the NRG4 variants (Fig. 4). Anti-127 detected NRG expression in the cytoplasm; this antibody will detect all NRG4 splice variants that are present. Anti-123 (which detects both NRG4 A1 and NRG4 A2) showed strong uniform epithelial immunoreactivity. In contrast, there was intense nuclear and cytoplasmic immunoreactivity observed with anti-128 (NRG4 A1 specific). Both the NRG4 A2 (anti-135) and the NRG B3 (anti-134)–specific antibodies seem to detect proteins that show a polarized apical distribution in the epithelial cells.

Immunohistochemical staining with the anti-127 (Fig. 1E), anti-128 (Fig. 1F), anti-134 (Fig. 1G), and anti-135 (Fig. 1I) antibodies on the prostate cancer tissue array showed positive

Fig. 3. ErbB1-4 and NRG1-4 expression in human prostate cancer cell lines. RT-PCR was carried out on each cell. A., lane 1, SKBR3; lane 2, ZR75; lane 3, DU-145; lane 4, LNCaP; lane 5, PC-3; lane 6, Ca-HPV-7; lane 7, P2-HPV-10; lane 8, PNT2-C2; and lane 9, P4E6 either using primers to ErbB1-4 or NRG1-3. B., lane 1, ZR75; lane 2, DU-145; lane 3, LNCaP; lane 4, PC-3; lane 5, Ca-HPV-7; lane 6, PNT2-C2; and lane 7, P4E6 using primers to NRG4 A1, A2, B1, and B3 specific primers. Note that PCR using primers to NRG4 B1 also allows the synthesis of NRG4 B3.

Fig. 4. Specificity of isoform-specific peptide NRG4 antibodies and subcellular localization of NRG4 isotypes in rat salivary gland. Peptide antibodies to the amino acids within the boxed regions were raised to the NRG4 isotypes; anti-127 (NRG4 antibody to the common NH2-terminal region), anti-123 (COOH-terminal region of the EGF-like domain which detects NRG4 A1 and NRG4 A2), anti-128 (unique COOH-terminal of NRG4 A1), anti-135 (unique COOH-terminal of NRG4 A2), and anti-134 (unique COOH-terminal of NRG4 B3). An immunodot blot (top left) shows the specificity of each antibody against each of the peptides used for immunization. The immunohistochemical staining shows the reactivity of each antibody on formalin-fixed normal rat salivary gland. Epithelial staining is observed with each anti-NRG4 antibody. A uniform epithelial distribution is seen with anti-127 and anti-123. Nuclear staining is seen with the anti-128 antibody, and a polarized distribution at the apical side of the epithelium is observed with anti-134 and anti-135. Counterstaining was done using Gill’s hematoxylin. Original magnification, ×40.
staining in the cytoplasm of the tumors with all the antibodies, indicating that all five NRG4 isotypes are present in prostate cancer. Moderate staining was observed in smooth muscle with all the antibodies. The NRG4 B3-specific antibody was strongly positive in the endothelial cells of blood vessels both in the tumor (Fig. 1H) and in normal tissue. Membrane staining was only observed with the anti-123 (the A type specific) antibody.

A and B type NRG4 variants have different subcellular locations. In the light of the different subcellular locations observed in the rat tissue, we next determined whether the A and B type of NRG4 variants have different subcellular localizations. Cos-7 cells were transfected with a COOH-terminal GFP-tagged NRG4 A1 or B1 and counterstained with the anti-127 antibody. The cells were either permeabilized to detect intracellular proteins or left unpermeabilized to detect membrane-associated proteins (Fig. 5). NRG4 A1GFP was localized to membrane ruffles at the cell surface, and the anti-127 antibody confirms this. In addition, in permeabilized cells, red intracellular vesicles (i.e., vesicles that are associated with NRG4 A1, which have an intact NH2-terminal domain but lack the COOH-terminal GFP) are observed. This indicates that processing of this variant occurs. There was no red signal observed in unpermeabilized cells, which indicated that NRG4 B1 has an intracellular location. These results confirm the predictions that NRG4 A1, which possesses a putative TM sequence, is localized to the membrane, and that NRG4 B1, which lacks this sequence, is intracellular.

**Activation of the ErbB-4 tyrosine kinase and stimulation of neurite outgrowth by the NRG4 isoforms.** To assess whether the different isoforms were capable of activating ErbB4 and stimulating a biological response, we synthesized the EGF-like domain shared by the A1 and A2 variants and the complete B1, B2, and B3 sequences. These were subject to refolding using a redox system (24). The A1/A2 EGF domain lost six mass units after refolding, consistent with the formation of three disulfide bonds (Fig. 6A and B). One- and two-dimensional nuclear magnetic resonance spectra obtained on the refolded form suggested that a single conformational species was present containing a significant proportion of beta sheet structure as expected (Fig. 6C and D). Equal concentrations of each peptide were added to PC12 Her4 cells (PC 12 cells stably transfected with the ErbB4 receptor, ref. 25). Only the A1/A2 isoform stimulated neurite outgrowth (Fig. 6E). We next assessed their ability to stimulate phosphorylation on tyrosine residues of the ErbB4 receptor. NIH3T3 Her4 cells expressing ~10⁶ ErbB4 receptors per cell were separately treated with each refolded peptide. Consistent with the biological assay, only the A1/A2 forms elicited ErbB-4 phosphorylation.

**Discussion**

The NRGs are a complex family of ligands that directly activate ErbB 3 and ErbB4 receptor tyrosine kinases. There are four genes NRG1-4 (4), which each encode an EGF-like domain. NRG1, the best characterized NRG, is essential for normal development in several organs, including the nervous system, heart, and breast (30, 31). NRG2 is predominantly expressed in the adult central nervous system (32). Extensive alternative mRNA splicing and differential promoter usage of NRG1 allows the transcription of six classes of protein isoforms (2, 4). NRG2 is also alternatively spliced to produce α and β variants (6), and recently, isoforms of NRG3 have also been described (7, 8).

Evidence that NRG1 is involved in the pathogenesis of cancer has come from breast cancer cell lines where, in the absence of ErbB2 expression, NRG1 promotes tumor formation (33, 34) and in animal models where overexpression of the extracellular domain of NRG1 produces tumors in mouse mammary glands (35). In primary human tumors, NRG1 has been found in endometrial cancers (36, 37) ovarian (38) and prostate cancers (10).

The structure of the NRG2, NRG3, and NRG4 genes and their corresponding protein products have been less well studied in normal and cancer tissues. NRG1-4 have, however, been reported to be present in DCIS of the breast (26) and in invasive breast cancers (19). There have been no previous reports of NRG4 in prostate cancer.

We first looked to see if NRG4 protein was expressed in clinical prostate cancers. Immunohistochemical staining using an antibody to the EGF domain of NRG4 revealed that one or both of the A forms of NRG4 were expressed at variable levels.
in both the cytoplasm and membrane of prostate cancer cases (Fig. 1A-D). In a single sample in this pilot study of 40 cases, strong uniform membrane staining was observed. No membrane staining was seen in any of the matched normal prostate tissues. Statistical analysis suggested an association between expression levels and pTNM and stage, but as the numbers of cases were small, a larger study is required to investigate this further.

![Figure 6](image-url)

**Fig. 6.** Chemically refolded NRG4 A type peptides promote neurite outgrowth in PC12 Her4 cells and stimulate phosphorylation of ErbB4. NRG4 A1/A2 peptide was synthesized, refolded, and subject to oxidation (A) and reduction (B) and one-dimensional proton nuclear magnetic resonance (C) and two-dimensional NOESY (D). Refolded isotype-specific peptides were incubated with PC-12 Her4 cells (E) and NIH3T3 Her4 cells (F); lane 1, NRG4 A1/A2; lane 2, NRG4 B1; lane 3, NRG4 B2; lane 4, NRG4 B3; and lane 5, negative control.
The search for novel NRG4 spliced products identified five variants, each having a unique COOH terminus (Fig. 2B). All share exons 1 and 2; two variants (NRG4 A1 and NRG4 A2) have an intact EGF-like domain, which is the minimum requirement for the stimulation of ErbB receptor tyrosine kinases, and possess a putative transmembrane sequence in exon 6. Because the B forms lack exon 6, (a) they are predicted to be soluble and possibly intracellular, and (b) they possess a partial EGF domain lacking the final two cysteines and, therefore, are very unlikely to interact with the extracellular ligand-binding site in ErbB4 (10, 39). Another NRG, HRG-γ, is a truncated splice variant of NRG1 (4), which also lacks an intact EGF domain. As yet, a function has not been ascribed to this variant, but it is interesting that the EGF domain is not required for intranuclear localization of NRG1-β3 (11, 40).

NRG4 B1 and B2 are distinguished from the other NRG variants by a single COOH-terminal amino acid (present in different exons), which encodes either lysine or serine, respectively, followed by a stop codon. NRG4 B3 has a novel acidic COOH-terminal sequence encoded by exon 3, within which there is a predicted nonreceptor tyrosine phosphorylation site (NDNEDYDDLLPLN). Cytosolic protein tyrosine kinases have a preference for sites that have an acidic residue at the Y + 1 position and a hydrophobic residue at the Y + 3 position (41); however, we have not as yet explored if this is phosphorylated in vitro or in vivo.

The different subcellular localization of these alternatively spliced NRG4 variants is indicative of potentially different functional properties (Fig. 4) as is the case for NRG1 in the nervous system (42). Iso type-specific antibodies for the COOH terminus of NRG4 A1 show cytoplasmic and nuclear localization for this variant. Recently, it has been shown that both NRG1α (40) and NRG1β (11) are localized to the nucleus in breast cancer and in DCIS of the breast (26). The significance of the polarized apical localization (in epithelium) of both NRG4 A2 (Fig. 4) and NRG4 B3 (Fig. 4) remains to be determined.

In agreement with other studies, we have found that ErbB4 is absent from the normal prostate and prostate cancer cell lines investigated in this work. However, in prostate cancer, there are conflicting reports as to whether the ErbB4 protein is expressed (43) or not (10) in primary cancers. It has been suggested that NRG1 is able to inhibit prostate cell growth and division (10), induce apoptosis (44) and promote differentiation (45). At this time, it is therefore difficult to suggest hypotheses regarding the role of the NRGs in this disease, but a minimal requirement is to know the components of the system so that experiments that are able to explore this system can be designed.

A particularly interesting finding has been that three of the isoforms (designated the B variants), which are expressed at the mRNA and protein levels in normal tissues, in prostate cancer cell lines and primary tumors do not possess a full EGF domain. We therefore tested whether they could activate the ErbB4 receptor directly in a biochemical assay or indirectly in a biological assay. Only the A1/A2 full EGF-like domain gave a positive response. It has been reported that the NRG1 gene also encodes a similar product called the γ isoform (4). It will be important to determine whether these types of structures are involved in modulating cellular processes.

In addition to suggesting a role for the NRG4 gene and its products in prostate cancer, these findings contribute to the increasing amount of evidence, which indicate that some NRG variants are not prototypical ligands to cognate membrane-associated receptor tyrosine kinases, but may have other, possibly intracellular targets. There may be two classes of NRGs, those with transmembrane sequences, which undergo proteolytic cleavage to produce secreted and intracellular (nuclear) biologically active species, and those like NRG4 B3 without a transmembrane region or intact EGF domain, which are cytosolic and in which mechanism of action remains to be discovered.

References

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