Differential expression and alternatively spliced variants of NRP-1 and CC3/TIP30 in breast cancer-derived cell lines.

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ABSTRACT

NRP-1 and CC3/TIP30 expression was assessed in the Sk-Br-3 and T-47D breast cancer cell lines. The expression of NRP-1 and CC3/TIP30 was determined by RT-PCR. RT-PCR results indicate that NRP-1 is expressed greater in the Sk-Br-3 cell line compared to the T-47D cell line. RT-PCR results also indicate CC3/TIP30 is expressed greater in the T-47D cell line compared to the Sk-Br-3 cell line. RT-PCR of NRP-1 transcripts yielded an alternatively spliced variant of the NRP-1 gene. This alternatively spliced variant was equally expressed in the Sk-Br-3 and T-47D cell lines.

INTRODUCTION

Breast carcinoma is the most frequent malignancy occurring in women of Western developed countries (Bray et al. 2004). There are numerous genes that have been demonstrated to have a role in breast cancer. However, more genes need to be assessed in order to determine which genes have the greatest impact on this cancer.

The genes neuropilin-1 (NRP-1) and CC3/TIP30 were examined in this study to see if their expression levels varied between the breast cancer cell lines Sk-Br-3 and T-

47D. NRP-1 is a 130 to 140 kDa single spanning transmembrane glycoprotein originally described as a receptor for both semaphorin and vascular endothelial growth factor (Fujiswasa et al. 1995). NRP-1 is up-regulated in a variety of aggressive human cancers including pancreatic (Parirkh et al. 2003) and colon cancer (Parirkh et al. 2004). Therefore, NRP-1 may also be up-regulated in aggressive invasive breast cancer cell lines. While there are some reports of NRP-1 expression in breast cancer, those results differ in the pattern of its expression and have only been tested in a few breast cancer cell lines (Baba et al. 2007).

The human CC3/TIP30 gene is a putative metastasis suppressor gene, based on the results of experimental studies using lung and colon cancer cell lines (Shtivelman 1997). There are at least two mechanisms by which CC3/TIP30 exerts this metastasis suppressing effect. One mechanism that as been shown is that CC3/TIP30 induces apoptosis in v-SCLC cells deprived of growth factors (Shtivelman 1997). Secondly, CC3/TIP30 has been shown to have an angiogenesis-inhibiting effect, since it reduces the expression of certain angiogenic modulators in various tumor cell lines (NicAmhlaoibh and Shtivelman 2001). Due to the ability of CC3/TIP30 to act as a metastasis suppressor it may have a role in the invasiveness of certain cancers, especially breast cancer. However, there are a only a few reports of CC3/TIP30 expression in breast cancer cells (Zhang et al. 2003a, Zhang et al. 2003b), and these studies assess a limited number of breast cancer cell lines.

In this study, I sought to investigate the expression pattern of NRP-1 and CC3/TIP30 in an invasive and noninvasive breast cancer cell line. The two cell lines used in this study were Sk-Br-3 and T-47D. Sk-Br-3 is an invasive breast cancer cell line

(Trempe and Fogh 1973) and T-47D is a noninvasive breast cancer cell line. (Keydar et al. 1979). The goal of this study was to examine gene expression of NRP-1 and CC3/TIP30 in the Sk-Br-3 and T-47D cell lines to determine if expression patterns correlated with a particular cell line.

MATERIALS AND METHODS

Cell Lines

Sk-Br-3 and T-47D, cell lines were kindly provided by Dr. Masaaki Hamaguchi. Both cell lines were maintained in a mixture of 45% Eagle's Essential Medium with no-essential amino acids, 45% Ham's Nutrient Mixture F12 (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum without antibiotics.

Total RNA Extraction

Sk-Br-3 and T-47D cells in logarithmic growth phase were washed with PBS, detached from the substrate with Accutase (Innovative Cell Technologies, San Diego, CA) and pelleted by centrifugation in a tabletop centrifuge for 5 min. Pelleted cells were frozen by liquid nitrogen and stored at -80 °C until used. Total RNA was extracted from these pelleted cells using the RNeasy® kit (Qiagen. Valencia, CA) according to the manufacturer's protocol. The RNA concentration was quantified using a

spectrophotometer at 260 nm. The RNA was then serially diluted with RNAse free diH_2O and stored at -80 °C.

Primer Design and RT-PCR

Two sets of primers were designed for both the NRP-1 transcript and CC3/TIP30 transcript. One set of primers was designed for the GAPDH transcript as a control (Table 1, Figure1). Specifically, forward and reverse primers used to amplify mRNA corresponding to NRP-1, CC3/TIP30, and GAPDH were as follows: NRP-1 primer 1 forward, 5′-ACGATGAATGTGGCGATACT-3′; NRP-1 primer 1 reverse, 5′-AGTGCA-TTCAAGGCTGTTGG-3′; NRP-1 primer 2 forward, 5′-CAACGATAAATGTGGCG-ATACT-3′; NRP-1 primer 2 reverse, 5′-TATACTGGGAAGAAGCTGTGAT-3′; CC3/TIP30 primer 1 forward, 5′-G- GCGGAGGGATTTGTTCGTGTT-3′; CC3/TIP30 primer 1 reverse, 5′-CATGCGCTTTCCCCAGGTCA-3′; CC3/TIP30 primer 2 forward, 5′-TCACCTTCGACGAGGAAGCT-3′; CC3/TIP30 primer 2 reverse, 5′-TCAGCAT-TGCTCTAACCACG-3′; GAPDH forward, 5′-GAAGGTGAAGGTCGGAGT-3′; and GAPDH reverse, 5′-GAAGATGGTGATGGGATTTC-3′. All primers were designed such that there are intervening intronic sequences between primer locations so that mRNA amplification could be distinguished from genomic DNA amplification. These primer locations and corresponding NCBI accession numbers are shown in Figure 1 and Table1, respectively.

RT-PCR was performed on total RNA samples from Sk-Br-3 and T-47D cell lines using the Qiagen (Valencia, CA) OneStep RT-PCR kit as per the manufacturer's

protocol. For each reaction, the following was used: 5 µL of 5x RT buffer, 1 µL of dNTPs, 1 µL RT-PCR mix, 0.5 µL of appropriate forward primer, 0.5 µL of appropriate reverse primer, 20 ng of isolated RNA, and enough RNAse free diH_2O to bring the final reaction volume to 25 µL. RT-PCR reactions for NRP-1 and CC3/TIP30 were carried out in a standard thermal cycler as follows: 50 °C for 30 min., 95 °C for 15 min, 40 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 10 min. RT-PCR reactions for GAPDH were performed the same as above expect reactions were subjected to 35 cycles of amplification. RT-PCR products were analyzed on a 1% agarose gel.

RT-PCR Product Purification and Sequencing

RT-PCR products were excised of agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according the manufacturer's protocol. The purified RT-PCR products were then quantified using a spectrophotometer at 260 nm and 80 ng of each RT-PCR product and 8 pM of corresponding primer for each product was sent to Genewiz Inc.(South Plainfield, NJ) for sequencing. Sequence identities were then determined using the Blast function on the NCBI website (Table 2).

RT-PCR purification, PCR amplification, and sequencing of the smaller NRP-1 RT-PCR product

The second band generated by RT-PCR for NRP-1 using primer set 1 (Figure 2a) was excised of out the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according the manufacturer's protocol. The purified RT-PCR product was then quantified using a spectrophotometer and serially diluted (1:10, 1:100, and 1:1000). PCR was performed to amplify the serially diluted RT-PCR product using following reaction volumes: 25 µL GoTaq® Green Master Mix (Promega, Madison, WI), 1 µL of NRP-1 forward primer 1, 1 µL of NRP-1 reverse primer 1, 2 µL of serially diluted template, and 21 µL of diH_2O to bring the final volume to 50 µL. The PCR reaction was performed in a standard thermal cycler for the following reaction times: 94 °C for 5 min., 50 cycles of amplification (94 °C for 30 s, 55 °C for 30s, and 72 °C for 30s), and 72 °C for 10 min. PCR products were analyzed on a 1% agarose gel. These PCR products were excised and purified from the agarose gel the same way as described above and 80 ng of purified PCR product and 8 pM of corresponding primer were sent to Genewiz Inc. (Plainfield, NJ) for sequencing. Sequence identities were then determined using the Blast function on the NCBI website (Table 3).

RESULTS

RT-PCR results indicated that NRP-1 transcript is expressed more in the SK-BR-3 cell line compared to the T-47D cell line (Figure 2a). Both NRP-1 primer set 1 and primer set 2 confirm this result. GAPDH transcript expression is shown as a control for this result indicating that the amount of mRNA used for all NRP-1 reactions was the same for both the Sk-Br-3 and T-47D cell lines (Figure 2a). All of the RT-PCR products

produced by NRP-1 primers (1 and 2) and GAPDH primers were sequenced and those sequences were Blasted on the NCBI website confirming their identity (Table 2). Analysis of the banding pattern produced by NRP-1 primer set 1 indicates a second smaller band (Figure 2a). This smaller band was amplified by PCR and sequenced (Figure 3). The sequence was Blasted on the NCBI website and was found to be NRP-1 but was missing Exon 3 (Table 3). Accordingly, this lower band represents an alternatively spliced variant of the NRP-1 transcript. The alternative splicing site is shown in figure 4. The intensity of banding for this alternatively spliced variant indicates that it is equally expressed in both the Sk-Br-3 and T-47D cell lines (Figure 2a).

RT-PCR results also indicate that CC3/TIP30 transcript is expressed more in the T-47D cell line compared to the SK-BR-3 cell line (Figure 2b). Both CC3/TIP30 primer set 1 and primer set 2 confirm this result. GAPDH transcript expression is shown as a control for this result indicating that the amount of mRNA used for all CC3/TIP30 reactions was the same for both the Sk-Br-3 and T-47D cell lines (Figure 2b). All of the RT-PCR products produced by CC3/TIP30 primers (1 and 2) and GAPDH primers were sequenced and those sequences were Blasted on the NCBI website confirming their identity (Table 2).

DISCUSSION

The results for this study indicate that there is differential expression of NRP-1 and CC3/TIP30 transcripts in the Sk-Br-3 and T-47D cell lines. This is especially interesting because it sheds light of the potential role of these two genes in the

invasiveness of breast cancer. Sk-Br-3 is an invasive breast cancer cell line (Trempe and Fogh 1973) and T-47D is a noninvasive breast cancer cell line. (Keydar et al. 1979). NRP-1 may play a role in leading to invasive status in breast cancer as indicated by its expression pattern in this study. The high level of NRP-1 expression in this study correlates with the more invasive Sk-Br-3 cell line. On the other hand, CC3/TIP30, which has been demonstrated as a metastasis suppressor gene (Shtivelman 1997), has a much different expression pattern. CC3/TIP30 is expressed greater in the T-47D cell line, which is less invasive than the Sk-Br-3 cell lines. While these results are only preliminary they do suggest that NRP-1 and CC3/TIP30 may have a role in the invasiveness of breast cancer.

Furthermore, the alternatively spliced variant of NRP-1 discovered in this study warrants further investigation. Interestingly, the expression of this NRP-1 variant is equal in the Sk-Br-3 and T-47D cell lines despite the marked variation between the expression pattern of the normal NRP-1 transcripts in these cell lines. There may be some mechanism involved in breast cancer invasiveness that leads to this alternative splicing of the NRP-1.

ACKNOWLEDGMENTS

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Figure 1

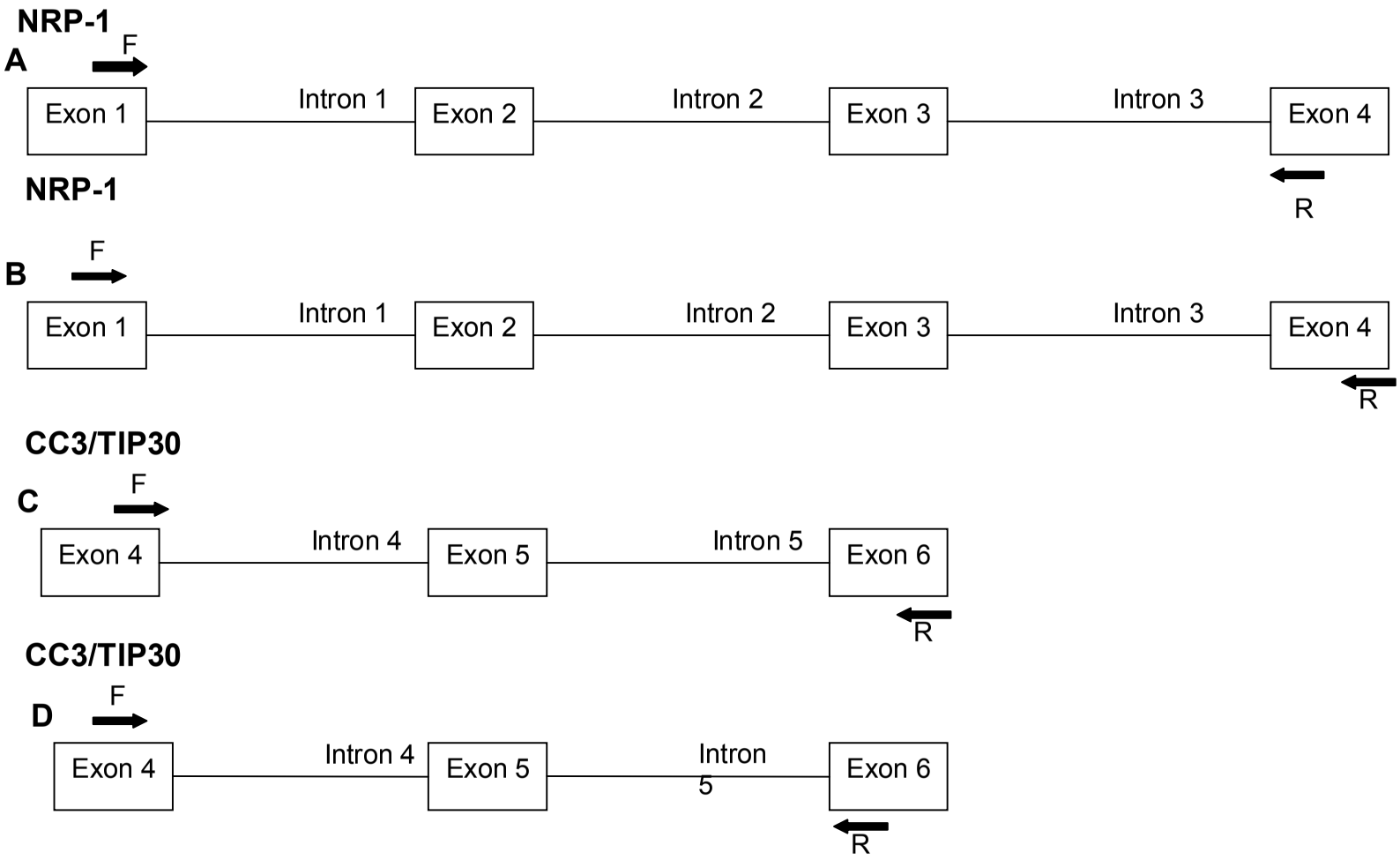

Figure 1

(**A**) Location of forward (F) and reverse (R) primer set 1 on NRP-1, (**B**) Location of forward (F) and reverse (R) primer set 2 on NRP-1, (**C**) Location of forward (F) and reverse (R) primer set 1 on CC3/TIP30, and (**D**) Location of forward (F) and reverse (R) primer set 2 on CC3/TIP30.

Figure 2a

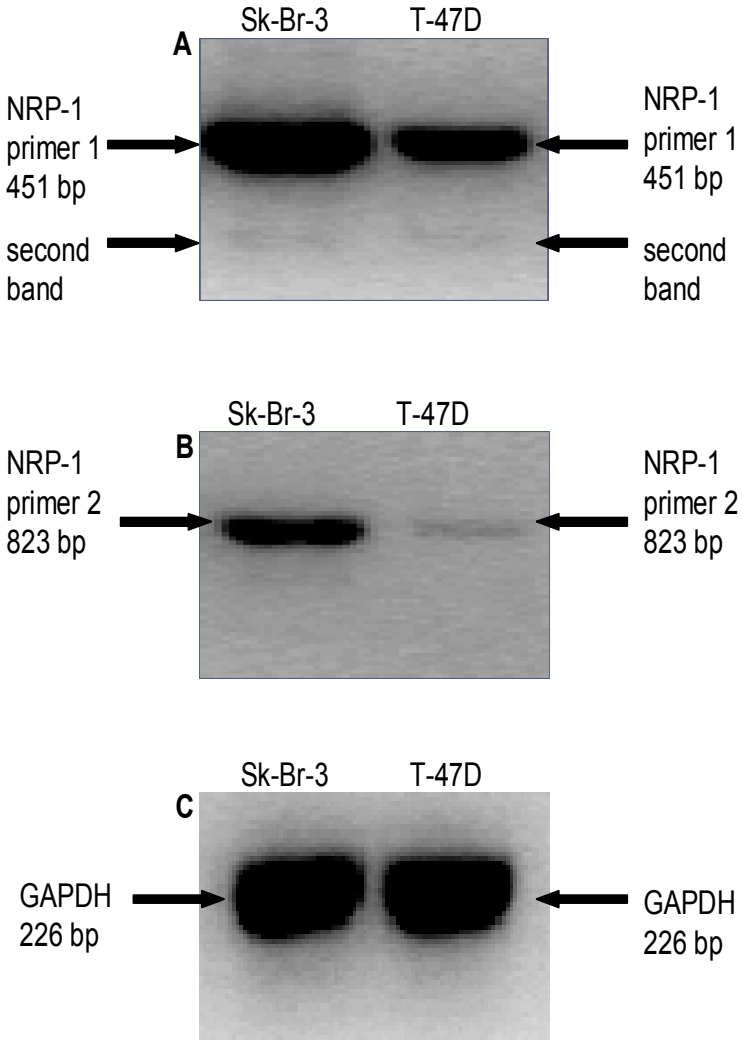

Figure 2a
Expression of mRNA in cell lines Sk-Br-3 and T-47D for **(A)** NRP-1, 40 cycles of RT-PCR using primer set 1 (**B**) NRP-1, 40 cycles of RT-PCR using primer set 2 (**C**) GAPDH, 35 cycles of RT-PCR

Figure 2b

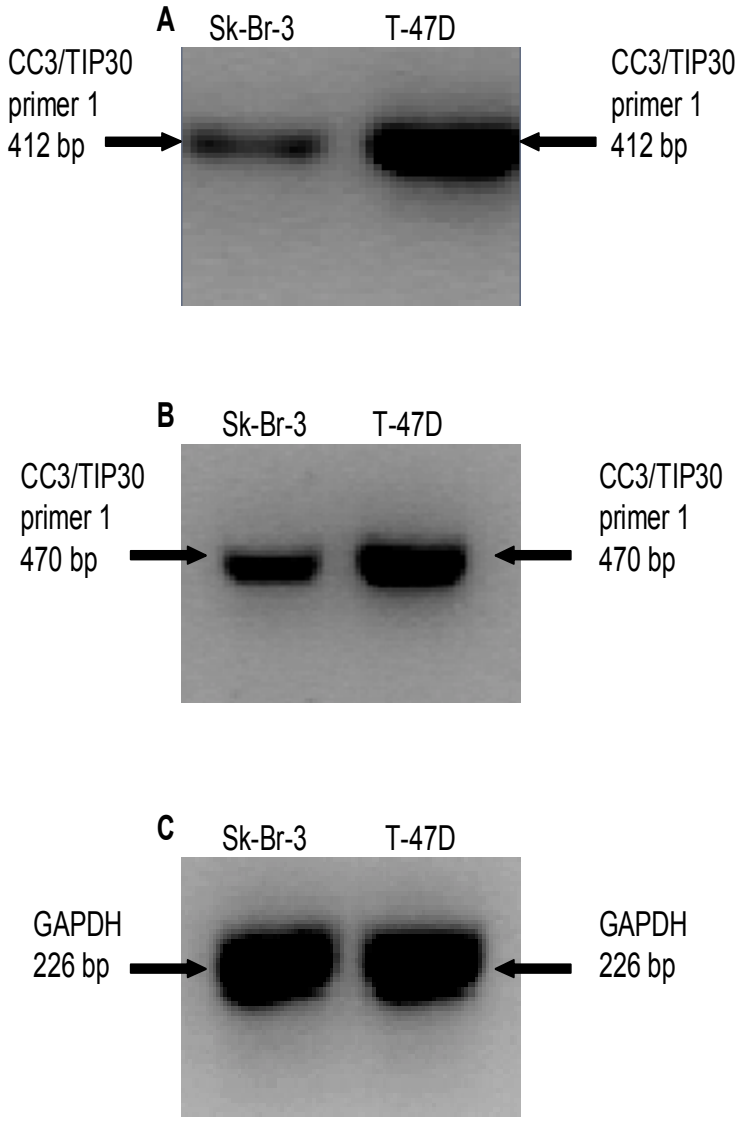

Figure 2b
Expression of mRNA in cell lines Sk-Br-3 and T-47D for **(A)** CC3/TIP30, 40 cycles of RT-PCR using primer set 1 (**B**) CC3/TIP30, 40 cycles of RT-PCR using primer set 2 (**C**) GAPDH, 35 cycles of RT-PCR

Figure 3

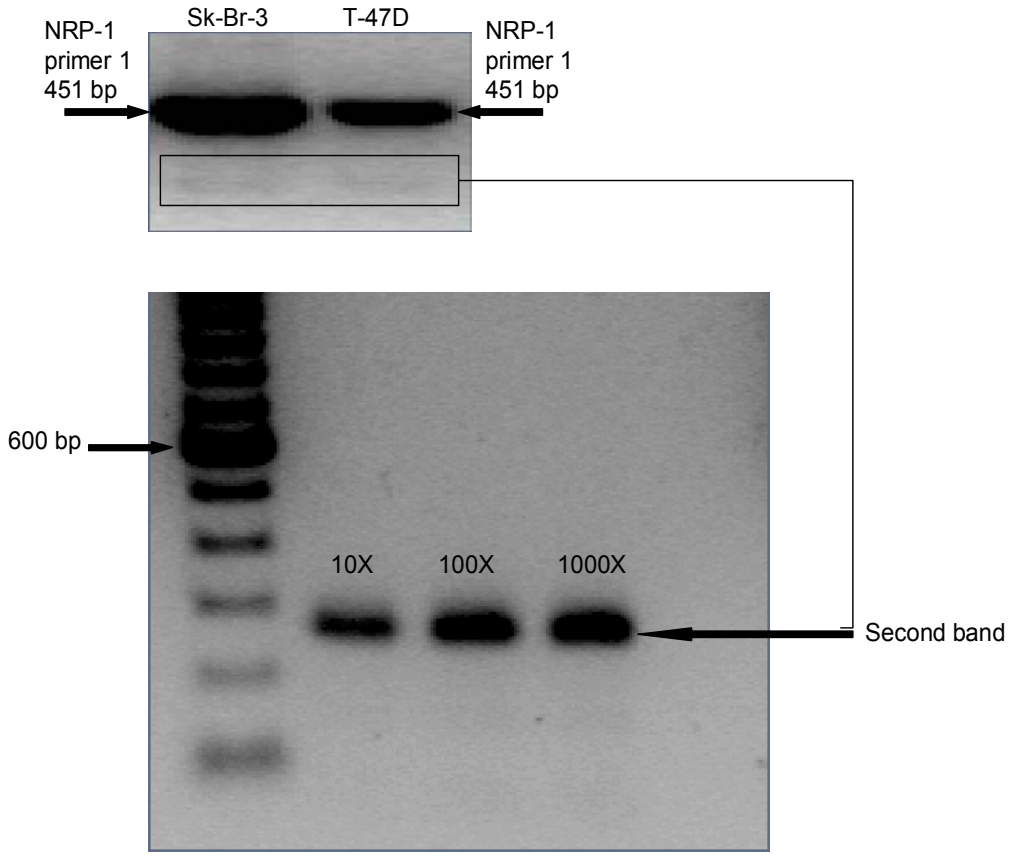

Figure 3
PCR of second band from NRP-1 primer set 1. Dilutions of 1:10, 1:100, and 1:1000.

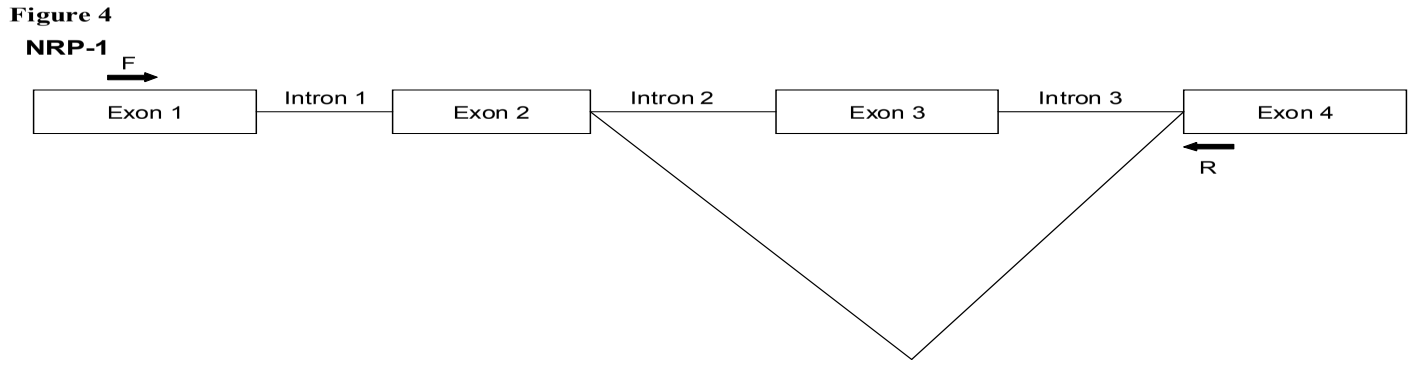

Figure 4
Location of forward (F) and reverse (R) primer set 1 on NRP-1 and alternative splicing sites for NRP-1 using primer set 1, indicating the removal of Exon 3.

Table 1

PRIMER		Sequence 5' → 3'	Position in mRNA	Predicted Size	Introns and sizes within corresponding DNA sequence	NCBI Accession # of mRNA
NRP-1 Primer Set #1	Forward	ACGATGAATGTGGCGATACT	211-230	451 bp	1) 3427 bp 2) 59851 bp 3) 6801 bp	NM_001024629.1
	Reverse	AGTGCATTCAAGGCTGTTGG	662-643			
NRP-1 Primer Set #2	Forward	CAACGATAAATGTGGCGATACT	209-230	823 bp	1) 3427 bp 2) 59851 bp 3) 6801 bp	NM_001024629.1
	Reverse	TATACTGGGAAGAAGCTGTGAT	1032-1011			
CC3/TIP30 Primer Set #1	Forward	GGCGGAGGGATTTGTTCGTGTT	412-433	412 bp	1) 5460 bp 2) 740 bp	NM_006410.3
	Reverse	CATGCGCTTTCCCCAGGTCA	824-805			
CC3/TIP30 Primer Set #2	Forward	TCACCTTCGACGAGGAAGCT	276-295	470 bp	1) 5460 bp 2) 740 bp	NM_006410.3
	Reverse	TCAGCATTGCTCTAACCACG	746-727			
GAPDH Primer Set	Forward	GAAGGTGAAGGTCGGAGT	108-125	226 bp	1) 1632 2) 90 bp	NM_002046.3
	Reverse	GAAGATGGTGATGGGATTTC	333-314			

Table 1
Primers used in this experiment including sequence, mRNA position, NCBI accession number, intervening introns, and predicted sized.

Table 2

A

```
Forward Primer 1, NRP-1
Homo sapiens neuropilin 1 (NRP1), transcript variant 1, mRNA
Length=5889

GENE ID: 8829 NRP1 | neuropilin 1

Score =  217 bits (117),  Expect = 3e-54
Identities = 132/138 (95%), Gaps = 5/138 (3%)


Query  1    CCCGGGGT-CCTT-CATCCTCCTGGGTTATCCTCATTCTTATCACCCAAGGTGAAAAATG  58
            ||| |||| |||| ||| ||||| |||||||||||||||||||||||| ||||||||||
Sbjct  628  CCCCGGGTACCTTACAT-CTCCT-GGTTATCCTCATTCTTATCACCCAA-GTGAAAAATG  684

Query  59   CGAATGGCTGATTCAGGCTCCGGACCCATACCAGAGAATTATGATCAACTTCAACCCTCA  118
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  685  CGAATGGCTGATTCAGGCTCCGGACCCATACCAGAGAATTATGATCAACTTCAACCCTCA  744

Query  119  CTTCGATTTGGAGGACAG  136
            |||||||||||||||||
Sbjct  745  CTTCGATTTGGAGGACAG  762
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B

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Reverse Primer 2, NRP-1
Homo sapiens neuropilin 1 (NRP1), transcript variant 1, mRNA
Length=5889
GENE ID: 8829 NRP1 | neuropilin 1 [Homo sapiens]


 Score =  193 bits (104),  Expect = 4e-47
 Identities = 107/110 (97%), Gaps = 0/110 (0%)

Query  21    CCAGAGCTTCCATACATTTGAAATCTTCTGAGACACTGCTCTGCANGANACTGTAGTTTG  80
            ||||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  1361  CCAGAGCTTCCATACATTTGAAATCTTCTGAGACACTGCTCTGCAAGACACTGTAGTTTG  1302

Query  81    CTGAGAAACCTTCTTTTGCTATCGCGCTGTCGGTGTAAAAAACCNTGGAG  130
            |||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  1301  CTGAGAAACCTTCTTTTGCTATCGCGCTGTCGGTGTAAAAAACCATGGAG  1252
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C

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Reverse Primer 1, CC3/TIP30
Homo sapiens HIV-1 Tat interactive protein 2, 30kDa (HTATIP2),
transcript variant 4, mRNA

 GENE ID: 10553 HTATIP2 |CC3/TIP30, 30kDa [Homo sapiens]
Score =  246 bits (133),  Expect = 3e-63
Identities = 135/136 (99%), Gaps = 0/136 (0%)


Query  1    CACAGAATGCCCCCTGGCCCAAGAGTCTGGTAAGGAGCCAAAGAACTTTCTAACCAGCCA  60
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  984  CACAGAATGCCCACTGGCCCAAGAGTCTGGTAAGGAGCCAAAGAACTTTCTAACCAGCCA  925

Query  61   TTCACCTGGGCGAGATTCTTGCCTATCACATAACAGAACTCCAGGCCTAAATACAGAGTA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  924  TTCACCTGGGCGAGATTCTTGCCTATCACATAACAGAACTCCAGGCCTAAATACAGAGTA  865

Query  121  ACGATCAAATTTTAAT  136
            ||||||||||||||||
Sbjct  864  ACGATCAAATTTTAAT  849
```

Table 2 Cont.

D

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Reverse Primer 2, CC3/TIP30
Homo sapiens CC3/TIP30, 30kDa (HTATIP2),
transcript variant 4, mRNA
GENE ID: 10553 HTATIP2 | CC3/TIP30, 30kDa [Homo sapiens]

 Score =  226 bits (122),  Expect = 4e-57
 Identities = 132/136 (97%), Gaps = 4/136 (2%)
 Strand=Plus/Minus

Query  3    CTGG-CC-AGAGTCTGGGTAAGGGAGCCAAAGAACTTTCTAACCAGCCATTCACCTGGGC  60
            |||| || ||||||| ||||| ||||||||||||||||||||||||||||||||||||
Sbjct  971  CTGGCCCAAGAGTCT-GGTAA-GGAGCCAAAGAACTTTCTAACCAGCCATTCACCTGGGC  914

Query  61   GAGATTCTTGCCTATCACATAACAGAACTCCAGGCCTAAATACAGAGTAACGATCAAATT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  913  GAGATTCTTGCCTATCACATAACAGAACTCCAGGCCTAAATACAGAGTAACGATCAAATT  854

Query  121  TTAATTCTTCAACCTT  136
            ||||||||||||||||
Sbjct  853  TTAATTCTTCAACCTT  838
```

Table 2

BLAST results from NCBI website (www.ncbi.nlm.nih.gov/blast) confirming the identity of each band obtained in RT-PCR results (Figures 2a and 2b) and PCR results (Figure 3). (**A**) NRP-1, amplified by RT-PCT with primer 1, (**B**) NRP-1, amplified by RT-PCR with primer 2, (**C**) CC3/TIP30, amplified by RT-PCR with primer 1, and (**D**) CC3/TIP30 amplified by RT-PCR with primer 2.

Table 3

```
Homo sapiens neuropilin 1 (NRP1)
Length=5889
GENE ID: 8829 NRP1 | neuropilin 1 [Homo sapiens]

 Score =  250 bits (135),  Expect = 6e-64
 Identities = 141/144 (97%), Gaps = 2/144 (1%)
 Strand=Plus/Plus

Query  3    CCGGGT-NCTTACATCTCCTGGTT-TCCTCATTCTTATCACCCAAGTGAAAAATGCGAAT  60
            ||||||  |||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  630  CCGGGTACCTTACATCTCCTGGTTATCCTCATTCTTATCACCCAAGTGAAAAATGCGAAT  689

Query  61   GGCTGATTCAGGCTCCGGACCCATACCAGAGAATTATGATCAACTTCAACCCTCACTTCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  690  GGCTGATTCAGGCTCCGGACCCATACCAGAGAATTATGATCAACTTCAACCCTCACTTCG  749

Query  121  ATTTGGAGGACAGAGACTGCAAGT  144
            |||||||||||||||||||||||
Sbjct  750  ATTTGGAGGACAGAGACTGCAAGT  773
Score =  159 bits (86),  Expect = 1e-36
Identities = 91/93 (97%), Gaps = 2/93 (2%)
Strand=Plus/Plus

Query  143   GTCCTGAATGTTCCCAGAACTACACAACACCTAGTGGAGTGATAAAGTCCCCCGGATTCC  202
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  954   GTCCTGAATGTTCCCAGAACTACACAACACCTAGTGGAGTGATAAAGTCCCCCGGATTCC  1013

Query  203   CTGAAAAATATCCCAACAGC-TTGGAATGCACT  234
             |||||||||||||||||||| ||| ||||||||
Sbjct  1014  CTGAAAAATATCCCAACAGCCTTG-AATGCACT  1045
```

Table 3

BLAST results from NCBI website (www.ncbi.nlm.nih.gov/blast) confirming the identity of PCR bands from NRP-1, second bands, amplified by RT-PCR with primer 1. Blast results for this second band indicate an alternatively spliced form of NRP-1 missing Exon 3