Advances in Brief

Expression of Nerve Growth Factor Receptors and Their Prognostic Value in Human Breast Cancer[1]

Simon Descamps, Valérie Pawlowski, Françoise Révillion, Louis Hornez, Mohamed Hebbar, Bénoni Boilly, Hubert Hondermarck, and Jean-Philippe Peyrat[2]

Laboratoire d'Oncologie Moléculaire Humaine, Centre Oscar Lambret, 59020 Lille [V. P., F. R., L. H., M. H., J-P. P.], and Equipe Facteurs de Croissance, Laboratoire de Biologie du Développement, UPRES-EA 1033 Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq [S. D., B. B., H. H.], France

Abstract

Nerve growth factor (NGF) has been shown recently to be mitogenic for human breast cancer cells. In the present study, we have assayed the expression of NGF receptors (NGFRs: TrkA and p75) mRNAs in 363 human primary breast cancers, using real-time quantitative reverse transcription-PCR. NGFRs were found in all of the tumor biopsies. TrkA and p75 were positively correlated and were respectively associated with the histoprognostic grading and the tumor type. NGFRs were both related to progesterone receptors. In univariate analyses, TrkA (>upper quartile) was associated with longer overall survival. Histoprognostic grading, tumor size, node involvement, and steroid receptors were also prognostic factors. In Cox multivariate analyses, TrkA was not a prognostic parameter. This study demonstrates the expression of NGFRs in breast cancer and points out that patients with high levels of TrkA have a more favorable overall survival prognosis.

Introduction

NGF[3] is an essential neurotrophin involved in the control of survival and differentiation of central and peripheral neuronal cells of both the sympathetic and the sensory nervous system (1). NGF interacts with its target cells through two categories of membrane binding sites. The high affinity receptor tyrosine kinase (p140^{trkA}) corresponds to the *TrkA* proto-oncogene, and a secondary receptor known as p75 neurotrophin receptor (p75) is a member of the tumor necrosis factor-α receptor superfamily and has no tyrosine kinase activity. Although it has been clearly established that p140^{trkA} tyrosine kinase activity leads to the stimulation of the mitogen-activated protein kinase cascade, the signaling mediated by p75 remains controversial (2). In addition to its neurotrophic function, several other activities of NGF have been described, including chemotactism and stimulation of proliferation. In human prostatic cells, NGF participates in tumor cell growth and invasion (3, 4). This last effect is mediated by p140^{trkA} and p75 and indicates that NGF is involved in prostatic carcinogenesis.

We and others have demonstrated recently that NGF is able to stimulate the proliferation of breast cancer cell lines (5, 6). We have evidenced, using specific antibodies, the presence of p140^{trkA} and p75 in MCF-7 and MDA-MB-231 cells. In addition, Tagliabue *et al.* (6) have shown that p140^{trkA} cooperates with p185^{Her-2} in activating growth of breast cancer cells. Altogether, these data suggest the implication of NGF in breast cancer development and progression. In previous studies, we have demonstrated that, in breast cancer biopsies, insulin-like growth factor-1 receptors, fibroblast growth factor-2 receptors as well as type I growth factor receptors are related to tumor prognosis (7–9). These results have led us to quantify the expression of TrkA and p75 mRNAs in a series of 363 breast cancer biopsies. We have shown that mRNAs for TrkA and p75 are expressed in breast cancer, and we have evaluated their prognostic significance.

Materials and Methods

Cell Culture. Cell culture reagents were purchased from BioWhittaker (France) except insulin, which was obtained from Organon (St. Denis, France). The breast cancer cell lines (MCF7, T47D, BT20, SKBR3, MDA-MB-231, MDA-MB-453, and MDA-MB-468) were obtained from the American Type Culture Collection and routinely grown as described previously (10) in monolayer cultures. The SY5Y neuroblastoma cell line was a kind gift of Dr. Luc Buée (INSERM U422, Lille, France).

Patients and Tumors. This study includes 363 patients who underwent surgery for primary breast cancer in the Center Oscar Lambret (the Anti-Cancer Center of the North of France), between May 1989 and December 1991. Tumor specimens were solely adenocarcinomas. At the time of collection, fat was removed, and samples were divided in two parts. The first part was submitted for histological studies and HPG, according to the method of Contesso *et al.* (11). The other part of the sample was immediately frozen in liquid nitrogen for receptor assays (7).

The population studied is described in Table 1. The median age of the patients was 58 years (range, 26–90 years). In prognostic studies, the median duration of follow-up of living patients was 79 months. The number of relapses (all distant relapses) was 126, and the number of patients who died from intercurrent diseases was 94.

Total RNA Isolation. Total RNA was isolated from tumor samples (40 mg weight) using the RNeasy Mini kit (Qiagen, Courtaboeuf, France), as described by Pawlowski *et al.* (9).

Production of TrkA, p75, and TBP Standards. The mRNA standards were obtained after *in vitro* transcription of a TrkA, p75, or TBP fragment cloned in pGEM-T Vector Systems (Promega, Charbonniéres, France) as described by Pawlowski *et al.* (9); the transcription was carried out using the RiboMAX Large Scale RNA Production System T7 for p75 and TBP (a component of the DNA-binding protein complex TFIID), and the SP6 Production System for TrkA (Promega, Charbonniéres, France).

TrkA, p75, and TBP PCR Primers and TaqMan Fluorogenic Probes. An amplicon of 89 bp was used for TBP, as described already (9). The PCR primers and the TaqMan fluorogenic probes were designed using the Primer Express software program (Perkin-Elmer; Demo version 1.0). TrkA sequences were: forward, 5′-CATCGTGAAGAGTGGTCTCCG-3′; reverse, 5′-GAGA-GAGACTCCAGAGCGTTGAA-3′; and probe, 5′-AGGAGTGAAATGGAA-GGCATCTGGCG-3′. p75 sequences were: forward, 5′-CCTACGGCTACT-ACCAGGATGAG-3′; reverse, 5′-TGGCCTCGTCGGAATACG-3′; and probe, 5′-CTCGGGCCTCGTGTTCTCCTGC-3′. Amplicons of 102 and 147 bp were obtained for TrkA and p75, respectively, corresponding to sequences located in the extracellular domain of each protein. The TaqMan probe carried a 5′ 6-carboxy-fluorescein reporter dye in the cases of TrkA and p75 and a 5′ VIC reporter dye in the case of TBP. Primers and probes for TrkA and p75 and

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Table 1 *Prognostic factors for patients according to TrkA and p75 status*

	TrkA			p75		
	<Upper quartile[a] (%)	≥Upper quartile[a] (%)	P	<Upper quartile[b] (%)	≥Upper quartile[b] (%)	P
Age						
<50	16.9	6.4	NS[c]	15.8	7.1	NS[c]
≥50	58.3	18.5		59	18.1	
Node						
Negative	37.8	11.4	NS	35.4	13	NS
Positive	37.2	13.6		39.7	11.9	
HPG						
I	6.5	4.7	0.034	7	4.5	NS
II	37.3	10.9		35.4	12.7	
III	32	8.7		32.2	8.3	
Tumor type						
Ductular	51.5	17.1	NS	56.6	11.8	<0.001
Lobular	8	3.3		7.3	3.9	
Others	15.4	4.7		11	9.3	
Tumor diameter						
<2 cm	6.3	2	NS	7	1.5	NS
2–5 cm	47.3	19.4		49.1	18	
>5 cm	20.8	4.3		18.3	6.1	
ER						
<10 fmol/mg	21.5	4.7	NS	19.7	5.7	NS
≥10 fmol/mg	53.1	20.7		56	18.6	
PgR						
<10 fmol/mg	22.4	5	NS	22.1	5.2	NS
≥10 fmol/mg	52.1	20.4		53.6	19.2	

[a] Upper quartile, TrkA:TBP = 0.30.
[b] Upper quartile, p75:TBP = 0.52.
[c] NS, statistically not significant.

probe for TBP were from Eurogentec (Seraing, Belgium), whereas primers for TBP were from Genset (France).

Reverse Transcription-PCR Conditions. The reverse transcription and the PCR were performed in a one-step methodology as described by Pawlowski *et al.* (9), with optimal $MgCl_2$ concentrations of 6 mM for TrkA, 3 mM for p75, and 5 mM for TBP. A template-free control was included in each experiment. The non-template controls, standard RNA dilutions, and tumor samples were assayed in duplicate.

Analysis and Expression of the Real-Time Reverse Transcription-PCR Data. The quantification of the starting quantity of mRNA in an unknown sample was performed by preparing a standard curve using dilutions of a known amount of the respective standard RNA, as detailed by Pawlowski *et al.* (9). The level of TrkA and p75 mRNA expression was expressed as a ratio between receptor expression (in copies/μg of total RNA) and TBP expression (in copies/μg of total RNA) and was referred to as normalized expression.

Statistical Analyses. All of the statistical analyses were done using SPSS (version 9.0.1). The relationships between qualitative variables were determined using the χ^2 test (with Yates correction when necessary). Correlations between parameters were assessed according to the Spearman nonparametric test. Comparison between subpopulations (PgR positive or PgR negative) were assessed according to the Mann-Whitney nonparametric test. OS and RFS were studied by Kaplan-Meier method analysis. The comparison of curves was carried out by the log-rank test. Cox's proportional hazard regression method (12) was used to assess the prognostic significance of parameters taken in association.

Results

Distribution of NGFRs in a Nonselected Series of 363 Human Primary Breast Cancers. The distribution of breast cancer biopsies according to their TrkA and p75 mRNA expression is presented in Fig. 1. The median concentration of TrkA was 0.16, ranging from 0.005 to 3.48; the lower quartile was 0.079, and the upper quartile was 0.30. The median concentration of p75 was 0.19, ranging from 0.002 to 7.94; the lower quartile was 0.075, and the upper quartile was 0.52. All of the studied breast cancer cell lines, used as controls, expressed NGFRs. TrkA expression (TrkA:TBP) ranged from 0.0013 (BT20) to 0.012 (MDA-MB-468); it was 0.002 in MCF7, T47D, and MDA-MB-231 and 0.005 in SKBR3 and MDA-MB-453. p75 expression ranged from 0.0077 (MDA-MB-468) to 6.66 (MCF-7); it was 0.01 in MDA-
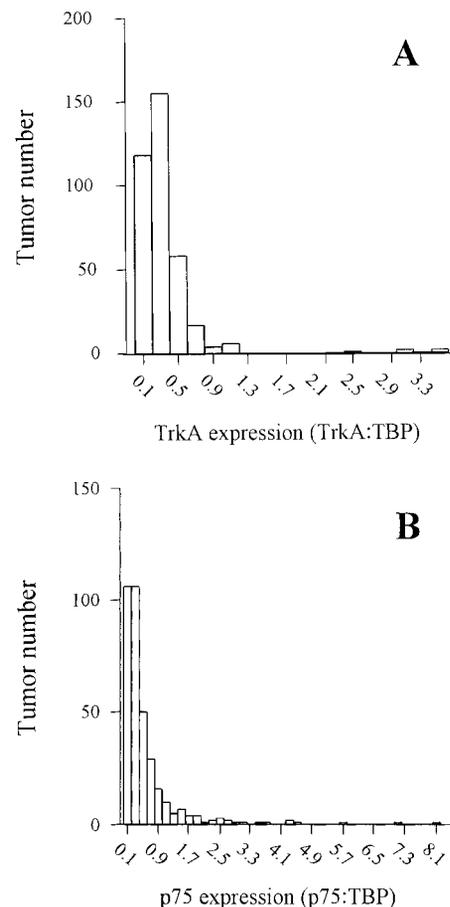

Fig. 1. *A,* distribution of TrkA mRNA expression in 363 nonselected primary breast cancers. *B,* distribution of p75 mRNA expression in 355 nonselected primary breast cancers. Tumors are distributed according to the relative expression of TrkA or p75. The TrkA or p75 expression is normalized to the TBP expression and is expressed as a ratio (TrkA or p75 expression:TBP expression).

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Table 2 *Prognostic factors in Cox univariate analyses*

The thresholds (upper quartiles) were 0.30 (TrkA:TBP ratio for TrkA and 0.52 (p75:TBP ratio) for p75.

	OS		RFS	
	P	RR[a]	*P*	RR
TrkA	0.034	0.56	NS	
p75	NS		NS	
ER (<10; ≥10 fmol/mg protein)	0.044	0.64	NS	
PgR (<10; ≥10 fmol/mg protein)	0.0042	0.54	NS	
Node involvement (0; >0)	0.034	1.56	0.0088	1.61
Tumor diameter (<2; 2–5; >5 cm)	0.0038	1.75	0.0022	1.66
HPG (I, II, III)	0.0008	1.84	NS	

[a] RR, risk ratio; NS, statistically not significant.

MB-453 and MDA-MB-231, 0.125 in T47D, 0.30 in SKBR3, and 0.5 in BT20.

Relationships between NGFRs and Other Parameters. Table 1 shows that TrkA expression levels are related to the HPG (χ^2 test), and that p75 mRNA expression levels are related to tumor type, with low levels being more frequently found in ductular type tumors. A positive correlation was found between TrkA and p75 ($P < 0.001$), and a negative correlation was found between TrkA and HPG ($P = 0.034$; Spearman test). Additionally, in the global population, we have found positive correlation between ER and PgR ($P < 0.001$), ER and age ($P < 0.001$), PgR and age ($P = 0.019$), node involvement and tumor diameter ($P < 0.001$), and tumor size and HPG ($P = 0.01$). We found negative correlations between HPG and ER ($P < 0.001$) on one hand and HPG and PgR ($P < 0.001$) on the other hand, as well as between ER and tumor diameter ($P < 0.001$).

TrkA ($P = 0.037$), as well as p75 ($P = 0.026$), were expressed to a higher level in PgR-positive tumors compared with PgR-negative tumors (Mann-Whitney nonparametric test). TrkA ($P = 0.030$) and p75 ($P = 0.054$) levels were also higher in HPG I than in HPG II or HPG III tumors. TrkA was found to be more highly expressed in small tumors (<2 cm) than in large tumors ($P = 0.088$). Finally, p75 level was higher in menopausal patients (>50 years) than in nonmenopausal patients ($P = 0.019$).

Prognostic Studies. For Cox univariate analyses (Table 2; Fig. 2), in OS studies, TrkA was a prognostic parameter, with high concentrations being associated with a good prognosis. HPG, tumor diameter, node involvement, PgR, and ER were also prognostic parameters. In RFS studies, only tumor diameter and node involvement were prognostic parameters. Fig. 2 shows OS curves for all of the population according to the expression of TrkA (threshold: upper quartile).

For Cox multivariate analyses, in OS studies, TrkA was not a prognostic factor, whereas node involvement, PgR, and HPG were prognostic factors. In RFS, node involvement, HPG, and tumor diameter were prognostic parameters.
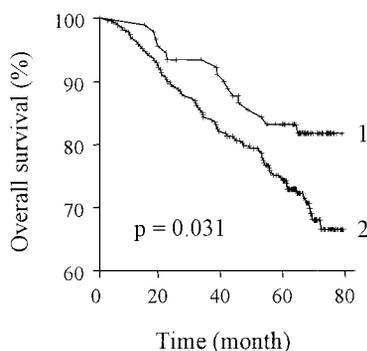

Fig. 2. OS in the whole population according to the presence of TrkA. *Curve 1,* patients with high level of TrkA expression (TrkA:TBP, >0.30); *Curve 2,* patients with low TrkA expression (TrkA:TBP, <0.30). A higher OS was found in TrkA-positive patients.

Discussion

In the present study, NGFRs were found to be expressed in all of the breast cancer cell lines and breast cancer biopsies that we have studied. We have found by immunoprecipitation and Western blotting experiments that the proteins corresponding to *TrkA* and *p75* gene expression, p140^{trkA} and p75^{NTR}, respectively, are present in breast cancer cell lines and are activated by NGF (5). Thus, NGFR mRNA expression in breast cancer cells is associated with the expression of the respective proteins. In our study, TrkA and p75 mRNAs were found to be correlated in breast cancer and to be expressed in equivalent amounts because their median concentrations and upper and lower quartiles are very similar. These results, and previous demonstrations that NGF is mitogenic for breast cancer cell lines (5, 6), strongly suggest an implication of NGF in breast cancer development. The TrkA tyrosine kinase receptor is known to mediate the NGF mitogenic effect by activating the Ras/Raf/mitogen-activated protein kinase pathway. p75 has been shown to regulate TrkA activation by NGF, and it could also have a role in the regulation of apoptosis by activation of a specific pathway involving ceramides, c-Jun NH$_2$-terminal kinase, caspases, or nuclear factor-κB (13). p75 has been shown recently to be responsible of the antiapoptotic effect of NGF on schwannoma cells (14) and could have a similar role in breast cancer cells (15). Considering the described effects of NGF, we can hypothesize that this growth factor is able to modify the equilibrium between proliferation and apoptosis and to favor tumor growth.

In breast cancers, there was a wide range of variation in TrkA (0.05–3.48) and p75 (0.002–7.94) mRNA expression (normalized to TBP expression). We demonstrate that a first source of variation is the tumor type, because TrkA level is lower in high-grade tumors and p75 expression is higher in ductular carcinomas. We found that high concentrations of TrkA were related to high concentrations of PgR. The mechanism by which TrkA is regulated in breast cancer has never been described. The observed coexpression of TrkA and steroid receptors could suggest a common regulator of these receptors; therefore, we can hypothesize that estradiol stimulates the expression of TrkA transcripts, because it has been shown for transcripts of insulin-like growth factor receptors (16). Two studies have reported the presence of NGF in milk (17) and in capsules surrounding breast implants (18), showing the presence of NGF in the mammary gland. Thus, the variation of NGFR expression that we have observed in breast cancer biopsies could be related to NGF regulation of its own receptors.

We demonstrate that a high TrkA mRNA level is associated with a good prognosis in OS univariate analyses, with a median duration of follow-up of 6.5 years. The best TrkA threshold defined was 0.30 (normalized relative to TBP), corresponding to the upper quartile. In contrast, in multiparameter Cox analyses, TrkA was not a prognostic parameter; this was not unexpected, considering its relation with HPG. It might be considered as paradoxical that a tumor containing a high level of receptors for the growth factor NGF exhibits a better prognosis than a tumor without. Such a relationship has already been reported in neuroblastoma (19). Moreover, we have reported relationships between tyrosine kinase receptors and prognosis in breast cancer for insulin-like growth factor-I receptors (7), fibroblast growth factor-2 receptors (8), and type I growth factor receptors (9). We can hypothesize that tumors with high levels of TrkA receptors have retained some physiological control of growth, which could explain the better prognosis.

In conclusion, the present study emphasizes the idea of the involvement of NGF in human breast cancer growth and points out that

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patients with a high level of TrkA receptors have a better prognosis. Then NGFRs are potential targets for new breast cancer therapies, and the recent demonstration by Tagliabue *et al.* (6) that p140^{trkA} cooperates with p185^{Her-2} in activating the growth of breast cancer cells suggests that blocking the NGFRs could improve the effect of anti-erbB2 drugs.

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