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Overexpression of the *Tpl-2/Cot* oncogene in human breast cancer

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Tpl-2/Cot proto-oncogene encodes a serine threonine kinase and was initially cloned as a provirus insertion site in MoMuLV-induced T cell lymphomas in rats. _Tpl-2_ locus was also shown to be affected by provirus insertion in MMTV-induced mammary carcinomas in mice. The involvement of _Tpl-2_ in 35 human breast paired tumour specimens versus their corresponding adjacent normal tissue was evaluated. _Tpl-2_ was found overexpressed in 14 of the 35 breast tumours tested using a semi-quantitative RT–PCR method. Gene amplification was detected in eight out of the 14 specimens overexpressing _Tpl-2_, suggesting the increased number of copies of _Tpl-2_ gene as a possible mechanism for _Tpl-2_ overexpression. Significant association was found between the overexpression of _Tpl-2_ and stage I of the tumours, indicating that this molecular alteration may be an early event in the development of the disease. Furthermore, overexpression of _Tpl-2_ was associated with positive progesterone receptor status of the samples. This is the first report on the _Tpl-2_ oncogene linked to human breast tumours suggesting that it may be a key molecule for the study of human breast cancer.

Keywords: *Tpl-2/Cot*; gene amplification; breast cancer

Introduction

Breast cancer is the most common cancer in women in Europe, currently affecting one in 12 (Black, 1994). A series of genes has been identified linking breast cancer with inherited mutations (BRCA1, BRCA2) (Miki *et al*., 1994; Tavtigian *et al*., 1996) as well as acquired mutations or gene amplifications in a series of oncogenes including HER-*2* (Szollosi *et al*., 1995), *ras* (Miyakis *et al*., 1998), and *myc* (Visscher *et al*., 1997). These oncogenes encode proteins that induce signals leading to cell transformation and proliferation. Development of breast tumours is also known to involve secretion of growth factors and cytokines such as TGFα (Davis and Snyderwine, 1995), IGF-I (Janssen and Lamberts, 1998), TGFβ (Christeli *et al*., 1996), PDGF (de Jong *et al*., 1998), and persistent activation of these signalling pathways is considered a critical component of the mammary oncogenesis.

Tpl-2 gene encodes a serine threonine kinase, which is activated by provirus insertion in MoMuLV-induced rat T cell lymphomas and MMTV-induced mouse mammary carcinomas (Patriotis and Tsichlis, 1994; Erny *et al*., 1996). Provirus insertion occurs between exon 7 and exon 8 giving rise to a protein with truncated carboxy-terminus. The truncated protein is highly oncogenic when overexpressed in the thymus of transgenic animals inducing thymomas (Ceci *et al*., 1997) and morphological transformation in SHOK and NIH3T3 cells (Miyoshi *et al*., 1991). It is expressed in most tissues but at relatively low levels, including ovarian and mammary tissues (Ohara *et al*., 1995; Erny *et al*., 1996). The human *Tpl-2* homologue, known as *Cot*, was initially cloned by transfection of cDNA derived from the thyroid tumour cell line TCO-4 into the hamster cell line SHOK (Miyoshi *et al*., 1991). *In situ* hybridization histochemistry in a series of cell lines and tumour tissues revealed that Tpl-2/cot was overexpressed in parotid gland, gastric and colonic gland tumours as well as in the colon adenocarcinoma cell lines SW40 and WiDr (Ohara *et al*., 1995).

Tpl-2 kinase activates the MAPK and SAPK pathways (Salmeron *et al*., 1996; Patriotis *et al*., 1994; Ceci *et al*., 1997) and induces IL-2 and TNFα expression in T cell lines (Tsatsanis *et al*., 1998a; Ballester *et al*., 1998) by activating the transcription factors NFAT and NFκB (Tsatsanis *et al*., 1998b; Belich *et al*., 1999). Both MAPK and SAPK pathways play an important role in the transduction of signals generated by growth factors produced in mammary epithelial neoplasms (Cowley *et al*., 1994; Johnson *et al*., 1996). NFAT and NFκB are also key transcription factors for the activation of cytokines and growth factors (Rao *et al*., 1997; Royds *et al*., 1998). Thus, deregulation of Tpl-2 kinase may induce signals that lead to cell transformation.

In the present study we analysed a series of breast tumours for overexpression of *Tpl-2* mRNA. We identified 14 out of 35 tumours with overexpression of *Tpl-2*. Eight out of 14 specimens representing increased *Tpl-2* expression levels, exhibited gene amplification, suggesting amplification of the genomic locus of *Tpl-2* as a possible mechanism for *Tpl-2* overexpression. Overexpression of *Tpl-2* was associated with early stages of the disease, suggesting that the activation of this oncogene is an early event in the development of breast cancer.

Results and discussion

Tpl-2 is overexpressed in human breast tumours

Fourteen out of 35 breast tumours (40%) showed increased *Tpl-2* expression levels as compared to their corresponding adjacent normal tissue (Table 1; Figures

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1 and 2). *Tpl-2* mRNA was expressed at 1.9–7.4-fold higher levels in tumour samples than in normal mammary tissues.

In situ hybridization experiments demonstrated that mRNA expression of the Cot proto-oncogene was detected primarily in glandular tissues of the adult mouse (Ohara *et al.*, 1995). Since breast carcinomas contain an increased number of epithelial cells compared to normal breast tissues, we equalized for the presence of epithelial cells in our samples. Expression of cytokeratin 18 was used as a marker for epithelial cell content and the samples were equalized for its expression. β2 microglobulin was also used as control. Samples that showed overexpression of Tpl-2 mRNA compared to β2-microglobulin also showed overexpression compared to cytokeratin 18 at similar levels (Table 1). None of the specimens that indicated normal levels of Tpl-2 expression compared to β2-microglobulin showed overexpression compared to cytokeratin 18. The above observations confirm that the overexpression of Tpl-2 in breast tumours is due to molecular alterations in the cancer cells and may be an important event towards tumour formation.

Tpl-2 is a potent serine threonine kinase which, when overexpressed, activates the MAPK and SAPK pathways and the transcription factors NFAT and NFκB. Activation of these pathways contributes to cell transformation and cell proliferation. The MAPKinases ERK1/ERK2 are activated by mitogens that affect breast cancer cells, such as IGF-I (Janssen and Lamberts, 1998), and promote cell proliferation (Coutts and Murphy, 1998). ERK2 is also activated by the growth inhibiting agent TPA. Activation of ERK2 by TPA can be inhibited by the MEK1/2 inhibitor PD98059 but the same inhibitor is unable to block insulin-dependent activation of ERK1/2 (Alblas *et al.*, 1998). Similarly, activation of ERK1/2 by *Tpl-2* cannot be inhibited by PD98059 or a dominant

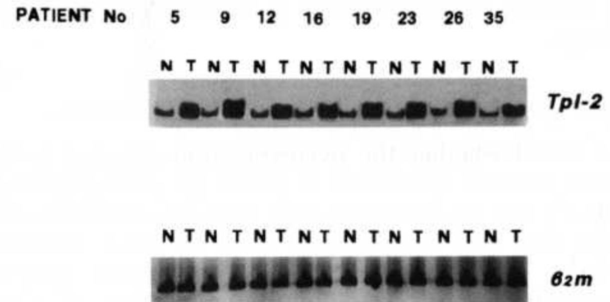


Figure 1 Representative examples of specimens exhibiting overexpression of *Tpl-2/Cot* oncogene in human breast tumour mRNA. Upper lane: PCR amplification of the *Tpl-2* mRNA, lower lane: PCR amplification of the control mRNA of *β2m*. T: tumour sample, N: normal tissue from the same patient

Table 1 Expression levels of *Tpl-2* gene using β2-microglobulin and Cytokeratin 18 as controls, amplification of the *Tpl-2* gene, and clinicopathological parameters of patients with breast cancer

Pt No.	*Expression* (β2m)	*Tpl-2 expression* (*CK18*)	*Gene amplification*	*Stage*	*Grade*	*ER*	*PR*	*Age*[a]
1	N (1.05-fold)	N (1.12-fold)	NA	II	III	–	+	39
2	N (0.99-fold)	N (1.08-fold)	NA	I	II	–	–	52
3	↑ (2.10-fold)	↑ (2.40-fold)	NA	I	II	+	+	65
4	N (1.01-fold)	N (1.14-fold)	NA	I	II	+	–	65
5	↑ (4.70-fold)	↑ (4.30-fold)	A	II	I	+	+	39
6	N (1.20-fold)	N (1.27-fold)	NA	III	II	+	–	75
7	N (1.12-fold)	N (1.03-fold)	NA	II	II	–	+	41
8	N (1.03-fold)	N (1.12-fold)	NA	II	II	–	–	55
9	↑ (7.40-fold)	↑ (7.10-fold)	A	I	II	–	–	49
10	N (1.16-fold)	N (1.20-fold)	A	III	II	+	–	50
11	N (1.00-fold)	N (1.06-fold)	NA	III	II	–	–	61
12	↑ (4.40-fold)	↑ (4.05-fold)	A	I	II	–	+	52
13	↑ (2.10-fold)	↑ (2.26-fold)	NA	I	II	–	+	48
14	↑ (2.60-fold)	↑ (2.78-fold)	NA	I	II	–	+	78
15	N (1.13-fold)	N (1.02-fold)	NA	III	II	–	–	36
16	↑ (4.20-fold)	↑ (4.53-fold)	A	II	II	–	–	38
17	↑ (2.50-fold)	↑ (2.38-fold)	NA	I	II	+	+	76
18	↑ (1.90-fold)	↑ (1.43-fold)	NA	II	II	–	+	49
19	↑ (2.70-fold)	↑ (3.02-fold)	A	I	II	–	–	67
20	N (1.12-fold)	N (1.24-fold)	NA	II	II	–	–	72
21	N (1.19-fold)	N (1.11-fold)	NA	I	II	–	+	44
22	N (1.20-fold)	N (1.12-fold)	NA	II	II	+	+	55
23	↑ (2.90-fold)	↑ (3.12-fold)	A	I	II	–	–	65
24	N (1.17-fold)	N (1.23-fold)	NA	II	II	+	–	67
25	N (1.23-fold)	N (1.28-fold)	NA	II	II	–	–	51
26	↑ (2.80-fold)	↑ (3.09-fold)	NA	III	III	–	–	80
27	N (1.16-fold)	N (1.06-fold)	NA	II	II	+	–	71
28	N (1.21-fold)	N (1.14-fold)	NA	II	II	+	–	53
29	N (1.17-fold)	N (1.26-fold)	NA	III	II	–	–	62
30	N (1.02-fold)	N (1.13-fold)	NA	II	II	–	+	40
31	N (1.11-fold)	N (1.03-fold)	NA	II	II	+	+	68
32	↑ (2.70-fold)	↑ (2.87-fold)	NA	II	II	+	+	50
33	N (1.21-fold)	N (1.13-fold)	NA	II	II	–	–	48
34	N (1.15-fold)	N (1.26-fold)	NA	II	II	–	+	62
35	↑ (3.10-fold)	↑ (3.24-fold)	A	II	II	+	+	50

↑: Overexpression; N: Normal expression; *β2m*: β2-microglobulin; CK18: Cytokeratin 18; A: Amplified; NA: Not amplified; ER: Estrogen receptor; PR: Progesterone receptor; +: Positive; –: Negative: [a]Years

negative MEK1 mutant (MEK1S621A) suggesting that *Tpl-2* activates MAPK in a MEK1/2 independent fashion (Tsatsanis *et al*., 1998b). Overexpression of *Tpl-2* may, therefore, mimic or enhance the insulin-dependent signals towards cell proliferation.

In the present study we report for the first time involvement of the *Tpl-2* oncogene in human breast tumours. Overexpression of *Tpl-2* was not significantly associated with a particular histological subtype, tumour grade or with the age of the patients. Statistically significant association was found between the overexpression of *Tpl-2* and Stage I of the tumours ($P=0.01$), indicating that overexpression of *Tpl-2* may be an early molecular event in the development of the disease.

Tpl-2 gene amplification in human breast tumours

We tested whether the overexpression detected in the breast tumour specimens is linked to gene amplification. Of the 14 specimens that showed overexpression, eight also had the genomic locus of *Tpl-2* amplified (Figure 3). We further tested for linkage between overexpression of *Tpl-2* and amplification of *Tpl-2* locus, examining the incidence of allelic imbalance in breast cancer specimens using two microsatellite markers at chromosome 10p11.2, D10S1768 and D10S1555, located upstream and downstream of the *Tpl-2* gene respectively. The detection of imbalance between the alleles in the tumour samples as compared to their corresponding normal alleles may provide evidence for the increased number of copies of this

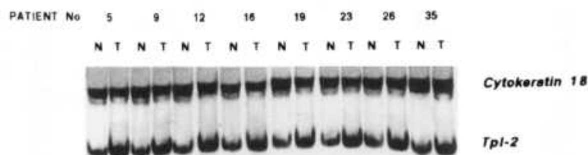


Figure 2 Representative examples of specimens exhibiting overexpression of *Tpl-2/Cot* oncogene in human breast tumour mRNA. Upper lane: PCR amplification of the *Tpl-2* mRNA, lower lane: PCR amplification of the control mRNA of Cytokeratin 18. T: tumour sample, N: normal tissue from the same patient

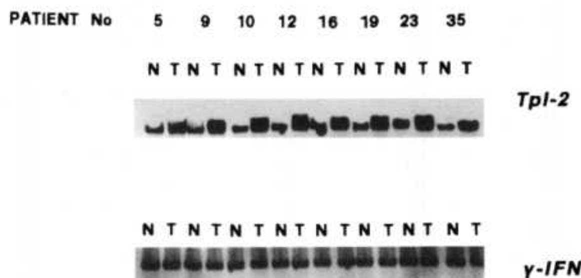


Figure 3 Representative examples of specimens exhibiting amplification of the *Tpl-2/Cot* oncogene in human breast tumours. Upper lane: PCR amplification of the genomic locus of *Tpl-2*, lower lane: PCR amplification of the control gene *γ-IFN*. T: tumour sample, N: normal tissue from the same patient

chromosomal region. Indeed, all eight samples exhibiting amplification using the first methodology displayed allelic imbalance for this microsatellite locus. The distance between the two markers is approximately 1 cm suggesting that the amplicon is larger than 1 cm. None of the samples with no amplification exhibited allelic imbalance. Representative examples of specimens exhibiting allelic imbalance are shown in Figure 4.

These results confirm our first observation showing gene amplification in some of the samples overexpressing *Tpl-2* which constitutes a possible mechanism for the elevated expression of the gene in breast cancer. Our results are consistent with previous studies, revealing increased copy numbers on chromosomal arm 10p in breast cancer (Ried *et al*., 1995; James *et al*., 1997; Murphy *et al*., 1995).

Correlation of *Tpl-2* overexpression and amplification was statistically significant ($P<0.009$) suggesting a possible mechanism for *Tpl-2* overexpression in human breast tumours. No significant correlation was found between gene amplification and any clinicopathological parameters.

Expression of estrogen receptor (ER) or progesterone receptor (PR) in breast tumours overexpressing Tpl-2

The same set of breast tumours was analysed for the presence of ER and PR. Expression of ER is often linked to progression of breast cancers from a hormone-dependent, nonmetastatic, antiestrogen-sensitive phenotype to a hormone-independent, antiestrogen- and chemotherapy-resistant phenotype with invasive and metastatic properties (Ito *et al*., 1995). One of the characteristics of the ER negative invasive breast cancer cells is constitutive high levels of NFκB activity. NFκB binding activity is normally low in the ER positive rat mammary carcinoma cell line RM22-F5 but it is significantly enhanced when it progresses to the ER negative malignant phenotype (Nakshatri *et al*., 1997). Since *Tpl-2* activates NFκB in lymphoid cells we tried to define a possible correlation between *Tpl-2* overexpression and presence of estrogen receptor. Of the 14 cases where *Tpl-2* was overexpressed, nine were found negative for the presence of ER and five were positive for ER expression (Table 1). Of the *Tpl-2* non-overexpressing tumours 13 were ER negative and eight ER positive, suggesting that there is no significant correlation between the presence of estrogen receptors and overexpression of *Tpl-2*.

The above samples were also tested for the presence of progesterone receptor. PR is known to interact with the RelA subunit of NFκB and they are mutually repressed (Kalkhoven *et al*., 1996). *Tpl-2* is a potent activator of NFκB (Tsatsanis *et al*., 1998b; Belich *et al*., 1999) and the presence of Tpl-2 kinase may overrule the inhibitory effect of the PR. Among the 14 *Tpl-2* overexpressing samples, ten were found positive for PR expression while four did not express the receptor, suggesting that there is a significant correlation between *Tpl-2* overexpression and presence of progesterone receptor ($P=0.029$). From the non *Tpl-2* overexpressing samples seven were PR positive and 14 were PR negative. These observations indicate a requirement for high levels of *Tpl-2* in the PR positive tumours. This suggests a possible mechanism for tumorigenesis in which *Tpl-2* activates NFκB and

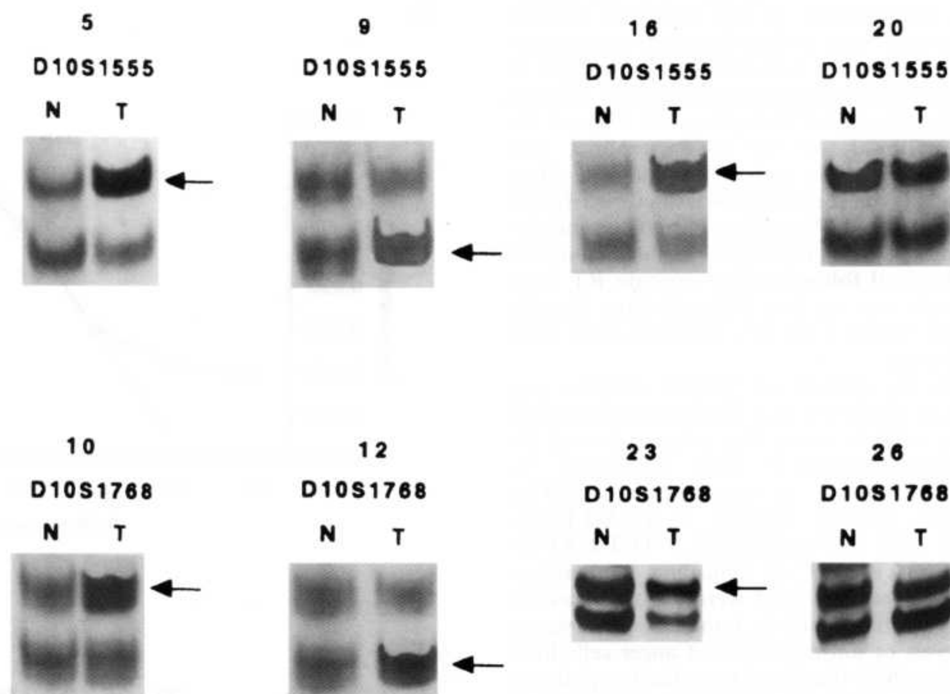


Figure 4 Representative examples of specimens exhibiting allelic imbalance. N = normal DNA; T = tumour DNA. Arrows indicate the position of the imbalanced allele. Specimens 20 and 26 are normal regarding allelic imbalance

overrules the anti-proliferative effect that may be triggered via the PR. Five of the seven tumours found positive both for ER and PR expression overexpressed *Tpl-2* (Table 1), indicating that *Tpl-2* may be an event that contributes to the tumour formation in patients that are otherwise considered of good prognosis.

In conclusion, we examined the expression of the *Tpl-2* mRNA in human breast tumours. In 14 out 35 (40%) samples *Tpl-2* was overexpressed. Overexpression was significantly linked to gene amplification. There was a significant association with stage I tumours, indicating that *Tpl-2* overexpression may be an early event in the development of breast cancer. Overexpression of *Tpl-2* may be linked to expression of PR and in particular with the simultaneous expression of both PR and ER. The molecular mechanism through which *Tpl-2* contributes to the development of breast cancer remains to be identified.

Materials and methods

Tissue specimens

Thirty-five tumour specimens from breast cancer paired with their corresponding adjacent normal tissue were surgically obtained and frozen at -70°C (Sourvinos *et al.*, 1997). All the specimens corresponded to primary tumours. Clinical data (stage, grade, histological subtype, age) were available for all the specimens tested.

RNA and DNA extraction

Total RNA was isolated from fresh tissues using Trizol (Life Technologies) following the manufacturer's instructions. RNA samples were digested with DNaseI (Gibco BRL) in order to discard genomic DNA. DNA from fresh tissues was extracted as previously described (Kiaris *et al.*, 1994) and stored at 4°C until PCR amplification.

cDNA synthesis and PCR

For first strand cDNA synthesis, 1–5 μg of total RNA was reverse transcribed in a 20 μl reaction volume containing 2 μl of 10 × PCR buffer, 50 ng random hexamers, 50 mM $MgCl_2$, 200 ng dNTPs, 0.1 M DTT and 200 U Reverse Transcriptase, (SuperScript II RT, Life Technologies) for 50 min at 42°C. PCR amplification of cDNA was performed in a 50 μl reaction volume containing 1 μg cDNA, 1 μM of each primer, 200 ng dNTPs, 5 μl of 10 × buffer (670 mM Tris.HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 μM β-mercaptoethanol and 1% (w/v) Triton X-100) and 1 U of *Taq* DNA polymerase. *Tpl-2* specific cDNA sequence was amplified using a pair of amplimers derived from different exons of the *Tpl-2* gene, separated by an intron, to prevent amplification of genomic DNA sequences that may contaminate cellular RNA preparations. The oligonucleotide primers for *Tpl-2*, derived from the *Tpl-2/Cot* sequence, were as follows: *Tpl-2* (sense) 5′-CAG TAA TCA AAA CGA TGA GCG TTC TAA-3′ and *Tpl-2* (antisense) 5′-GAA - CAT CGG AAT CTA TTT GGT AAC GTC-3′. Only a small segment (227 bp) of *Tpl-2* cDNA was amplified, so that even significantly degraded RNA could be used as template.

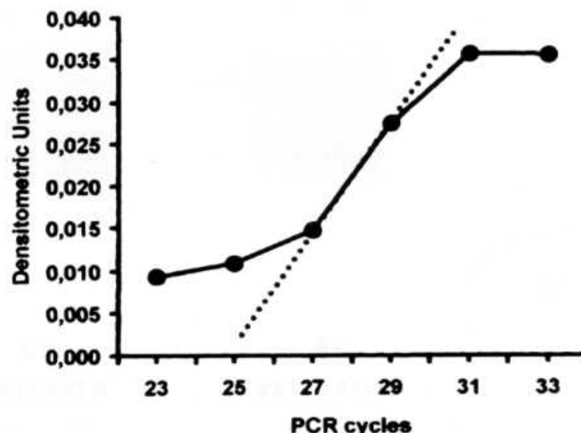
The PCR reactions were performed on a DNA thermal cycler (Perkin Elmer-Cetus Instruments, Norwalk, CT, USA). The PCR program consisted of 27 cycles as follows: denaturation at 94°C for 40 s, annealing at 57°C for 40 s and extension at 72°C for 35 s. Cycles were preceded by incubation for 5 min at 94°C to ensure full denaturation and at 72°C after the final cycle to ensure full extension of the product. Preliminary experiments had revealed the conditions in which the amplification reaction remained in the

exponential phase (data not shown) and thus the results could be used for quantitation of the template. Several negative control reactions were included in each experiment. Some of the negative controls contained water instead of cDNA and other control reaction mixtures contained aliquots of cDNA reaction mixtures that were prepared without the addition of RNA. Ten μl of the PCR product was electrophoresed through a 12% polyacrylamide gel, silver stained and the intensity of the bands was analysed by an image analysis system (Adobe Photoshop 5.0). The reproducibility of this protocol was evaluated by densitometric analysis of the PCR signal following five separate RT from the same RNA sample run on two different days. Results indicate a maximum variation of 6% between each PCR product (data not shown).

The quantity and the quality of mRNA samples was normalized after the amplification of a 120 bp fragment of β2 microglobulin (*β2m*) mRNA using the primers (sense) 5′-AAAGATGAGTATGCCTGCCG-3′ and (antisense) 5′-ACTCAATCCAAATGCGGC-3′ or cytokeratin 18 (263 bp fragment) using the primers CK18F 5′-AGA TTG C-CA GCT CTG GGT TGA C-3′ and CK18R 5′-TCT CAT G-GA GTC CAG GTC GAT CT-3′ at annealing temperature of 57°C. The mRNA levels for *Tpl-2* were expressed as the ratio of the intensity of the bands in tumour tissues versus the corresponding levels of normal tissues. Cancer cells have a cell-kinetic system in which the signal transduction pathway of oncogenes is induced. Thus, we arbitrarily considered as normal expression, levels lower than 1.5-fold and as overexpression levels higher than 1.5-fold in malignant specimens compared to corresponding normal tissues (Miyakis *et al*., 1998).

Genomic PCR analysis for detection of gene amplification

To determine whether the overexpression of *Tpl-2* was due to gene amplification or caused by other genetic events, genomic DNA was isolated from the same tissues and proceeded to PCR (Frye *et al*., 1989). Since the genomic sequence of the human *Tpl-2/cot* gene is not known, we designed primers that amplified a region at the 5′ untranslated region of exon 1 of the *Tpl-2* gene. The pair of primers used were (sense) 5′-AGA TGC AAT CTT CTT ACC GCG A-3′ and (antisense) 5′-TCA GAC TCC TGG CTT TGC ACA-3′, and amplified a fragment of 136 bp. To eliminate the possibility of mRNA contamination, the DNA preparations were treated with RNAseH (Gibco BRL) for 20 min at 37°C and ethanol precipitated and resuspended in TE. PCR analysis was carried out as described above. The PCR program consisted of 28 cycles as follows: denaturation at 94°C for 40 s, annealing at 63°C for 30 s and extension at 72°C for 30 s. Cycles were preceded by incubation for 5 min at 94°C to ensure full denaturation and 72°C after the final cycle to ensure full extension of the product. To establish conditions for amplification of the *Tpl-2* sequence at the exponential phase we performed kinetic analysis increasing the number of PCR cycles. Two-hundred ng of genomic DNA were amplified for 23, 25, 27, 29, 31 and 33 PCR cycles. PCR products were separated on a 12% polyacrylamide gel which was then silver stained, photographed and scanned. Quantitation of the bands was performed by digital imaging of the bands (Adobe Photoshop 5.0). A graph correlating densitometric units and number of PCR cycles was created to determine the exponential phase of the reaction (Figure 5). Our results revealed an exponential range between 26 and 30 PCR cycles. Thus, we carried out 28 PCR cycles in breast samples for the detection of gene amplification. The ratio between *Tpl-2* PCR products from tumour and the corresponding normal samples was determined by dividing the densitometric area of the tumour band by that of the normal band. In this procedure γ-interferon (γ-*IFN*) gene was used as a reference gene. The sequences of the oligonucleo-



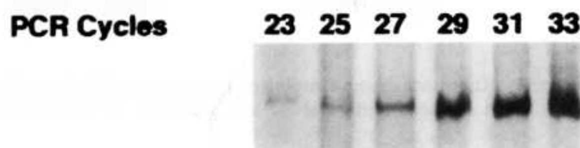


Figure 5 (**a**) Optimization of the linearity curve for analysis of gene amplification at *Tpl-2* locus in breast cancer by PCR. Two-hundred ng of genomic DNA were amplified for 23, 25, 27, 29, 31 and 33 PCR cycles, using a set of primers annealing at the 5′ untranslated region of exon 1 of the *Tpl-2* gene. Gel was silver stained, scanned and subsequently underwent densitometric analysis. Continuous line represents the kinetic analysis of PCR reaction while dotted line corresponds to the exponential phase of the reaction. (**b**) A photograph of the silver stained gel for varying number of PCR cycles is shown

tides used were γ-*INF* (sense) 5′-AGT GAT GGC T-GA ACT GTC GC-3′ and γ-*INF* (antisense) 5′-CTG GGA TGC TCT TCG ACC TC-3′ yielding a 85 bp PCR product (Neubauer *et al*., 1992). The reproducibility of the PCR in the gene amplification analysis was tested performing three separate reactions for each sample on the same day. The densitometric analysis of the silver stained gels showed deviation of no more than 7% (data not shown).

Allelic imbalance analysis The DNA samples were examined for allelic imbalance at *Tpl-2* locus, using two highly polymorphic microsatellite markers, D10S1768 and D10S1555 which are located upstream and downstream of the *Tpl-2* gene, respectively. Oligonucleotide primers for D10S1555 were sense 5′-TGT TCC CGA TCA AAC A-GA GT-3′ and anti-sense 5′-TTT CTG TGT CAA TTC T-CA GCC-3′ while for D10S1768 were sense 5′-CTG TGG GCT ATC TGG GGT CA-3′ and antisense 5′-GCC ACA CAC TGA GAA TGC CA-3′. PCR analysis was performed in a 50 μl reaction volume as described above. The reactions were denatured for 5 min at 94°C and the DNA was subsequently amplified for 28 cycles at 94°C, 55°C and 72°C each step. Ten μl of the PCR product was analysed in a 12% polyacrylamide gel and stained with silver staining. Gels were scanned and the intensity of the bands corresponding to the microsatellite alleles was quantitated by a UVP image analysis system (Adobe Photoshop 5.0). Allelic imbalance was scored as a significant difference (>40%) in the intensity of one allele

relative to the other as determined from comparison of tumour and normal DNAs.

Expression of estrogen receptor or progesterone receptor in breast tumours

The same set of breast tumours was analysed for the presence of ER and PR receptor status. Samples exhibiting ER or PR levels above 5 fmol/mg protein were considered as ER or PR positive, whereas specimens displaying ER or PR levels below 5 fmol/mg protein were considered as ER and PR negative, respectively.

Statistical analysis

Statistical analyses were performed using the χ^2-test or Fisher's exact test. One-tailed *P*-values <0.05 were considered statistically significant.

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