American Journal of Pathology, Vol. 148, No. 4, April 1996

Technical Advance

Triple Primer Polymerase Chain Reaction

A New Way to Quantify Truncated mRNA Expression

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The most practical method to quantify mRNA expression within small tumor samples is reverse transcription (RT) followed by quantitative polymerase chain reaction (PCR). One approach, known as "competitive RT-PCR" allows absolute quantitation by reference to synthetic RNA standards but is time-consuming and requires multiple manipulations that limit its usefulness as a screening assay. We describe here a new approach to quantify truncated type mRNAs relative to the wild-type transcripts in small amounts of tissue. This technique, called RT-triple primer-PCR, consists of coamplification of wild-type and truncated cDNAs using three primers in the PCR. To validate this approach, a truncated estrogen receptor variant (clone 4) was quantified relative to the wild-type estrogen receptor using plasmid preparations. The ratio of triple primer-PCR products obtained was directly related to the initial ratio of input cDNAs. RT-triple primer-PCR was then used to compare the relative expression of clone 4 mRNA in frozen sections of normal human breast tissue and human breast tumors with characteristics of good prognosis. The statistically significant difference (P = 0.03) observed between normal and tumor tissues suggests that elevated expression of the clone 4 variant may be associated with early steps of tumorigenesis. This technique provides a useful alternative to already described quantitative RT-PCR techniques for the quantification of truncated mRNA within small amounts of biological material. (Am J Pathol 1996, 148:1097–1103)

One manifestation of altered gene expression in many human diseases is the production of truncated or modified mRNAs that may be translated into modified proteins that act abnormally. Such mRNAs are involved in diseases as different as Glanzmann thrombasthenia,[1] the most common inherited disorder of platelets; aspartylblucosaminuria,[2] an inherited lysosomal storage disorder; Duchenne and Becker muscular dystrophies;[3] or cancer progression.[4,5] Several estrogen receptor (ER) variant mRNAs have also been identified in human breast cancer biopsies.[6–9] The knowledge of the relative proportion of these modified mRNAs to the wild-type mRNA can provide a useful tool in diagnosis, prognosis, or survey of the disease. The accurate quantification of such mRNAs is often difficult, and the more commonly used quantitative techniques such as Northern blot or RNAse protection assay are not sensitive enough to allow assessment of expression within small pathologically defined regions of tissue or small cell numbers. Reverse transcription associated with polymerase chain reaction (RT-PCR) is

Supported by grants from the Canadian Breast Cancer Research Initiative and the US Army Medical Research and Development Command. The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada and the Terry Fox Foundation. P. H. W. is a Medical Research Council of Canada (MRC) clinician-scientist, LCM is an MRC Scientist, and E. L. is a recipient of a University of Manitoba Faculty of Medicine Postdoctoral Fellowship.

Accepted for publication December 6, 1995.

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often the only method that can be used to qualitatively or quantitatively determine mRNAs when sample size and/or abundance are limiting factors.[10–12] "Comparative RT-PCR," which involves assessment by reference to a "housekeeping" gene, is often used but suffers from the assumption that the reference point is stable.[13] An alternative is "competitive RT-PCR," which involves the coamplification of a synthetic standard that is distinguishable from the cDNA to be quantified by the presence of an introduced restriction site or by differences in length.[14,15] This approach is based on the principle that the two targets will compete for the annealing of the two primers. Therefore, an equivalent signal after amplification means that the control DNA and the cDNA to be quantified are present in the same proportion. Techniques based on this approach have been successfully used by many laboratories. Theoretically, this approach will allow absolute quantitation, but this can be at the cost of multiple manipulations and large quantities of RNA, and therefore precious tissue samples. For these reasons we concluded that comparative and competitive RT-PCR techniques were not optimal for rapidly quantitating the relative expression of truncated mRNAs to wild-type mRNA in multiple small breast tissue samples. We therefore tested a new approach called RT-triple primer-PCR (RT-TP-PCR) to assess ER variant expression in microdissected human breast specimens.

To validate this assay, we chose to measure the relative expression of a truncated ER mRNA variant (clone 4) to the wild-type ER (WT-ER) mRNA. This truncated ER mRNA variant was initially characterized by sequencing a 2333 bp cDNA isolated from a human breast tumor cDNA library,[16] and was shown to present significantly elevated expression relative to the WT-ER transcript in tumors with parameters of poor prognosis and endocrine insensitivity.[17] As shown in Figure 1, clone 4 cDNA consists of sequences identical to exons 1 and 2 of the human ER, followed by sequences that are unrelated to those found in human ER cDNA. To perform TP-PCR, three primers are used. The upstream primer (E2U) recognizes both the truncated variant and the wild-type cDNAs. The two downstream primers (E3L, C4L) are specific for the WT cDNA and ER-clone 4 cDNA, respectively. Since the upstream primer can anneal to both cDNAs, TP-PCR leads to a competitive amplification of truncated and wild-type cDNAs, the final ratio between the coamplified products being related to the initial input cDNA ratio.

After validating this technique, it was used to compare the relative expression of clone 4 mRNA to WT-ER mRNA in normal human breast tissue and human breast tumors that displayed characteristics of good prognosis.

Materials and Methods

Human Breast Tissues and Cell Lines

Normal breast tissues were obtained from reduction mammoplasty specimens collected at the laboratory of F. Kuttenn of the Necker Hospital (four cases) and at the Manitoba Breast Tumor Bank (four cases). Human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank (10 cases). All specimens had been rapidly frozen at −70°C as soon as possible after surgical removal. A portion of the frozen tissue from each case (normal and tumor) was processed to create formalin-fixed and paraffin-embedded tissue blocks, matched, and oriented relative to the frozen tissue. This allows high quality histological sections to be assessed and pathological interpretation of the corresponding frozen sections from the immediately adjacent frozen tissue block. The presence of normal ducts and lobules was confirmed in all normal tissue specimens, as well as the absence of any atypical lesion. The 10 primary ductal carcinomas were selected from the Tumor Bank on the basis of a set of several parameters that are indicative of a good prognosis. All tumors were well differentiated (Nottingham grade 4 or 5), ER- and progesterone receptor (PR)-positive as determined by ligand binding assay (ER >3 fmol/mg protein, PR >15 fmol/mg protein), and axillary node-negative. Specific frozen tissue blocks were chosen in each case on the basis of several further criteria as assessed in histological sections. These tissue criteria included: a cellular content of >30% invasive tumor cells with minimal normal lobular or ductal epithelial components, good histological preservation, and absence of necrosis.

Ishikawa cells, an endometrial adenocarcinoma cell line initially established by Dr. H. Iwasaki (Tsukuba, Japan), were provided by Dr. E. Gurpide (Mount Sinai School of Medicine, New York, NY). These cells are known to express different ER variant mRNAs (L. C. Murphy, unpublished data) and have therefore been used as positive controls. Cos-1 cells (American Type Culture Collection, Rockville, MD) do not express ER mRNA and were used as a negative control in our experiments. Cells were grown and harvested to obtain cell pellets, which were stored at −70°C, as previously described.[18]

Plasmids

The pHEGO plasmid contains the previously cloned and sequenced WT-ER cDNA and was kindly provided by P. Chambon.[19] Clone 4 plasmid contains the previously cloned and sequenced clone 4 truncated ER variant cDNA.[16] These two plasmids were mixed to obtain solutions in which the clone 4/WT-ER cDNA proportions ranged from 1/1000 to 1/1, while maintaining a total constant plasmid concentration of 0.1 ng/μl.

Extraction of mRNA and RT

Total RNA was extracted from histologically defined regions within 20 μm cryostat sections of frozen normal and tumor tissue using a small scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH) according to the manufacturer's instructions. The yield was quantitated by spectrophotometer in a 50 μl microcuvette. The average yield of total RNA per 20 μm section was 4 μg/cm^2 for tumor and 0.6 μg/cm^2 for normal tissues ($\pm$20% variation with cellularity) with minimal contamination by DNA (optical density$^{260/280} > 1.8$).

RT was performed in a final volume of 15 μl. RNA (600 ng) was reverse transcribed in the presence of 1 mmol/L deoxyadenosine-5′-triphosphate (dATP), 1 mmol/L deoxycytidine-5′-triphosphate (dCTP), 1 mmol/L deoxyguanosine-5′-triphosphate (dGTP), 1 mmol/L deoxythymidine-5′-triphosphate (dTTP), 5 mmol/L dithiothreitol (GIBCO-BRL, Grand Island, NY), 1 unit/μl ribonuclease inhibitor (Promega, Madison, WI), 20 μmol/L random primers, 50 mmol/L Tris-HCl (pH 7.5), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, and 5 units/μl Moloney Murine Leukemia Virus reverse transcriptase (GIBCO-BRL) for 10 minutes at 22°C and 1 hour at 37°C. After 5 minutes at 95°C, 1 μl of the reaction mixture was taken for subsequent amplification using PCR.

Primers and TP-PCR Conditions

Three primers were used in this study (Figure 1). E2U (5′-AGGGTGGCAGAGAAAGAT-3′, sense, located in WT-ER exon 2; 708–725) and E3L (5′-TCATCATTCCCACTTCGT-3′, antisense, located in WT-ER exon 3; 969–986) allowed amplification of a 281 bp fragment corresponding to WT-ER mRNA. E2U and C4L (5′-GGCTCTGTTCTGTTCCATT-3′, antisense; 941–959) allowed amplification of a 249 bp fragment corresponding specifically to clone 4 truncated ER variant mRNA. Positions given correspond to published sequences of ER cDNA[20] for E2U and E3L and of clone 4 cDNA[16] for C4L primer.

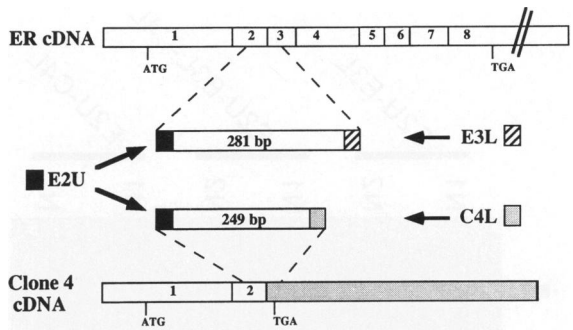

Figure 1. *Schematic representation of TP-PCR. Three primers are used simultaneously during the PCR. The upper primer (E2U) is able to recognize both WT-ER cDNA and truncated clone 4 cDNA. The lower primers (E3L and C4L) are specific for each cDNA. Competitive amplification of a 281 bp and a 249 bp fragment occurs, giving a final PCR-product ratio related to the initial input cDNA ratio.*

PCR amplifications were performed using 1 μl of RT mixture or plasmid solution in a final volume of 10 μl, in the presence of 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L dATP, 0.2 mmol/L dTTP, 0.2 mmol/L dGTP, 0.2 mmol/L dCTP, 4 ng/μl of each primer (E2U, E3L, and C4L), 1 unit of Taq DNA polymerase (GIBCO-BRL), and 10 nmol/L of dCTP [α-^{32}P] (ICN Pharmaceuticals Inc., Irvine, CA). Each PCR consisted of 40 cycles (1 minute at 60°C, 1 minute at 72°C, and 1 minute at 94°C) using a Thermocycler (Perkin-Elmer Cetus, Norwalk, CT). Three μl of the reaction was then denaturated in 80% formamide buffer and subjected to polyacrylamide gel electrophoresis (PAGE) on 6% gels containing 7 mol/L urea. After electrophoresis, the gels were dried and exposed to Kodak XAR Film at −70°C with two intensifying screens for 2 hours.

Quantification and Statistical Analysis

Autoradiographs were analyzed with a densitometry system based on a charge-coupled device camera (DAGE 72) and MCID M4 software (Imaging Research Inc., Sainte Catherines, ON). The signal corresponding to clone 4 was expressed as a percentage of the corresponding WT-ER signal; a value of 100% means that the clone 4 corresponding signal is equivalent to the wild-type corresponding signal.

For each sample, at least three independent measures of the clone 4 relative expression were performed and the mean determined. Means obtained from the eight normal breast samples were then compared with that found in the 10 tumor tissue samples using the Mann-Whitney rank sum test (two-sided).

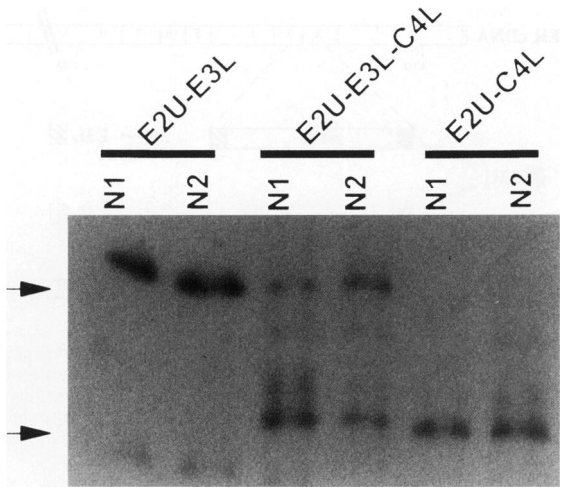

Figure 2. *Amplification of normal breast tissue cDNA using two or three primers in PCR. RNA from two normal breast tissue samples (N1 and N2) was reverse transcribed and amplified by PCR using E2U-E3L, E2U-E3L-C4L, or E2U-C4L primers. PCR products were separated by PAGE and analyzed as described in Materials and Methods. Upper and lower arrows show wild-type (281 bp) and clone 4 (249 bp) corresponding signals, respectively.*

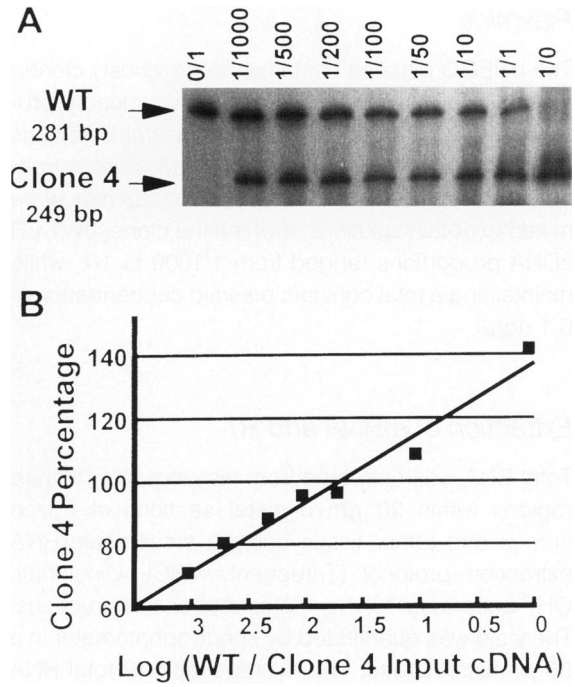

Figure 3. *Validation of TP-PCR technique using different plasmid preparations.* **A:** *Different plasmid preparations (0.1 ng) were amplified by TP-PCR: plasmid containing WT-ER cDNA alone (0/1), a mix of plasmids containing WT and truncated cDNA in varying ratios of clone 4/wild type ranging from 1/1000 to 1/1, or plasmid containing clone 4 alone (1/0). PCR products were separated by PAGE and analyzed as described in Materials and Methods.* **B:** *A mix of plasmids containing WT-ER and truncated clone 4 ER variant in varying proportions were analyzed by TP-PCR as described above. The percentage of clone 4 signal relative to the wild-type signal is expressed as a function of the log of WT/clone 4 input cDNA ratio.*

Results

Coamplification of WT-ER and Clone 4 Truncated ER Variant cDNAs Using TP-PCR

The ability of WT-ER and clone 4 cDNAs to be amplified simultaneously in a PCR reaction using three primers (TP-PCR) was initially determined. Total RNA from two normal breast tissue samples was analyzed by RT-PCR using E2U-E2L, E2U-C4L, or E2U-E3L-C4L primers (Figure 2). Using E2U and E3L, a band of 281 bp, corresponding to WT-ER cDNA was obtained. E2U and C4L primers allowed amplification of a 249 bp band, corresponding to clone 4 cDNA. When the three primers were present during the PCR reaction, both bands were obtained.

Ratio of the Clone 4 Signal Relative to Wild-Type Signal Is Constant and Proportional to the Initial Wild-Type/Clone 4 cDNA Ratio

The maintenance of a constant ratio of clone 4/WT-ER signals after RT-TP-PCR was examined under varying PCR conditions. To address this issue, RNA from Ishikawa cells, known to express clone 4 truncated ER mRNA (L. C. Murphy, unpublished data) was reverse transcribed and amplified using E2U-E3L-C4L primers for a varying number of cycles ranging from 20 to 45. Quantification of signals showed that the ratio clone 4/WT signals did not vary by more than 20% (data not shown). Similarly, using a constant number of PCR cycles (40 cycles), variation of input cDNA ranging from 50 to 0.1 ng did not affect this ratio by more than 20% (data not shown).

The relationship between the input ratio of clone 4/WT-ER cDNA and the clone 4/WT-ER signal ratio after TP-PCR was determined next. pHEGO and clone 4 plasmids, containing WT-ER cDNA and clone 4 cDNA, respectively, were mixed in varying proportion, ranging from 1000/1 to 1/1. TP-PCR was performed on these samples. Autoradiography showed that the intensities of the two PCR products are directly related to the initial ratio of cDNAs added (Figure 3A). Quantification of signals revealed a linear relationship between the final percentage of clone 4 and the log of the initial WT/clone 4 ratio (Figure 3B).

Comparison of Clone 4 Truncated ER Variant mRNA Expression in Normal Tissues and Tumor Tissues with Characteristics of Good Prognosis

The relative level of clone 4 variant ER mRNA expression was then measured by RT-TP-PCR in 8

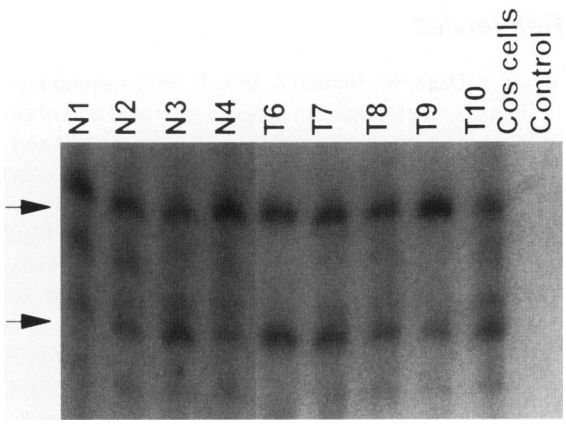

Figure 4. *Measurement of the relative expression of clone 4 variant ER mRNA in normal and neoplastic human breast tissues. RNA extracted from normal (N1–N4) or neoplastic (T6–T10) breast tissues was analyzed by RT-TP-PCR. PCR products were analyzed as described above. Negative controls consisted of RNAs from Cos-1 cells analyzed simultaneously (Cos cells), or no added cDNA in TP-PCR reaction (control). Upper and lower arrows show wild-type and clone 4 corresponding signals, respectively.*

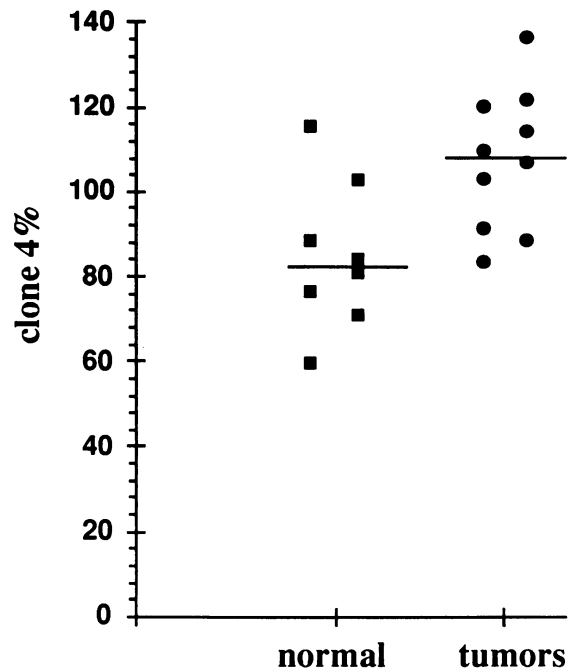

Figure 5. *Quantitative comparison of the relative expression of clone 4 variant ER mRNA in normal human breast tissue and human breast tumors with characteristics of good prognosis. RNAs extracted from 8 normal breast tissue samples and 10 tumors with characteristics of good prognosis were analyzed by RT-TP-PCR as described in Materials and Methods. For each sample, the mean of three independent measures of the clone 4 relative expression, expressed as a percentage of the corresponding wild-type ER signal, was determined (□, means of normal breast samples; ○, means of tumor breast samples). Bars represent medians.*

normal breast tissues and 10 breast tumors with characteristics of good prognosis (Figure 4). For each sample, the mean of three independent measures of the clone 4 relative expression, expressed as a percentage of the corresponding WT-ER signal, was determined (Figure 5). Using the Mann-Whitney rank sum test, the relative expression of clone 4 truncated variant ER mRNA to WT-ER mRNA was found to be significantly (P = 0.03) lower in normal breast (median = 82.5%) *versus* neoplastic breast tissues (median = 107.5%).

Discussion

In this manuscript, we provide strong evidence that TP-PCR is a reliable quantitative technique to determine relative expression of truncated transcripts, the percentage of signals measured after TP-PCR being directly correlated to the initial input ratio. This new PCR-based quantification provides several advantages over existing techniques. There is no need for synthetic controls, the internal control for both RT and PCR amplification being provided by wild-type mRNA. Moreover, serial dilutions are not necessary, and the requirement for only one tube decreases the risk of contamination and degradation as well as decreasing variability associated with multiple pipettings. This technique is optimal for the study of multiple tissue samples where the quantity of material is limited. During the preparation of this manuscript, a similar approach was used to coamplify wild-type glucocorticoid receptor and a truncated variant in myeloma patients.[21] However, in this study, the technique was used to compare relative expression, and the quantitative nature of this PCR approach was not tested.

TP-PCR was applied to assess the clone 4 truncated ER variant mRNA expression in normal and neoplastic human breast tissue. Several ER variants have previously been identified in breast cancer biopsies and cell lines.[6–8] Some of the ER-like proteins encoded by these variant mRNAs lack some ER functional domains, and have been shown to exhibit altered functions or interfere with WT-ER function.[8,22,23] Therefore, it has been speculated that these ER variants may be involved in progression from hormone dependence to independence in breast cancer.[24] Many of these variant ER mRNAs have now also been detected in normal breast tissue.[25,26] This suggests that variant ER-like proteins may play a role in the normal ER signaling pathway and that any change in the relative proportion of these variants could therefore lead to deregulation of this pathway, which may contribute to tumorigenesis. Some support for such an hypothesis had been ob-

tained previously when we used an RT-PCR approach to show that the relative expression of exon 5-deleted ER mRNA to wild-type transcript was significantly higher in breast tumors compared with normal breast tissue.[25] Furthermore, we had previously shown, using an RNAse protection assay, that clone 4 truncated mRNA was significantly elevated relative to WT-ER mRNA in those breast tumors which had characteristics of poor prognosis and hormone independence.[17] Using RT-TP-PCR, we report here that the expression of clone 4 truncated ER mRNA is significantly lower in normal tissues compared with human breast tumors with characteristics of good prognosis (ER^+, PR^+, node-negative). This result, together with our previous data, strongly supports the hypothesis that deregulation of ER variant mRNA expression occurs at relatively early steps in human breast tumorigenesis, and may indeed have a role in this process.

RT-TP-PCR, like any gel-based technique for RNA assessment (Northern blot, RNAse protection assay or RT-PCR) does not give information about the cell to cell pattern of expression. The determination of the cellular origin of expression requires the combined use of an *in situ* type technique such as *in situ* hybridization or immunohistochemistry. This technique provides, however, a useful preliminary screening method for evaluating the variant mRNAs expression. TP-PCR can be adapted to the study of numerous biological problems involving variant mRNA containing unique sequences linked to the sequences shared with wild-type transcripts. Apoptosis involving several partners, including *Bcl-2*, *Bcl-x* and bax, provides an example where relative expression of such variant mRNAs could be explored using TP-PCR.[27,28] Baxβ mRNA effectively contains unspliced sequences absent from baxα mRNA. The short form of the orphan receptor FTZ-F1 that was recently found to regulate the wild-type protein activity[29] is coded by a variant mRNA, the expression of which could also be explored by this technique. TP-PCR can also be adapted to DNA studies. Quantification of translocated DNA regions relatively to the wild-type DNA could allow the estimation of an abnormal cell population relative to the normal one in leukemia patients. TP-PCR can therefore provide a useful tool in diagnosis, prognosis, or survey of numerous diseases.

Acknowledgments

The laboratory of Pr. F. Kuttenn is gratefully acknowledged for providing some of the normal breast tissue samples.

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