# Relationship between Variant Forms of Estrogen Receptor RNA and an Apoptosis-Related RNA, TRPM-2, with Survival in Patients with Breast Cancer

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***Background.*** **Although smaller variant forms of estrogen receptor (ER) messenger RNA (mRNA) have been detected in breast tumors, neither their prevalence nor their prognostic significance have been evaluated. Similarly, TRPM-2 mRNA, the product of a gene induced principally during the onset of apoptosis, is present in mouse and human breast cancer cell lines, but whether it also occurs in primary breast tumors and is related to disease outcome is unknown.**

***Methods.*** **The relative expression and transcript size of ER mRNA and TRPM-2 mRNA in 126 primary breast tumors were measured by Northern analysis and compared with tumor grade, hormone receptor status, extent of tumor necrosis, and survival.**

***Results.*** **In ER-positive tumors, 64% of the tumors had only the normal 6.5 kb ER mRNA, an additional 9% had the normal plus smaller ER mRNA, and 2% had variant forms. Only 8% of ER-negative tumors had ER mRNA transcripts. There were significant relationships between the occurrence of ER mRNA and low tumor grade, ER-positive receptor status, and better survival. In contrast, TRPM-2 mRNA was found in only 17% of breast tumors, none of which could be grouped with respect to grade, hormone receptor status, or survival.**

***Conclusions.*** **The presence of smaller variant forms of ER mRNA either alone or in association with the normal ER transcript is not indicative of an unfavorable prognosis, whereas TRPM-2 mRNA occurs in many primary breast tumors, but has no apparent relationship to survival.** ***Cancer*** **1993; 72:3648–54.**

**Key words: breast cancer, variant estrogen receptor mRNA, TRPM-2, prognostic markers.**

More than 80% of human breast tumors contain measurable estrogen receptor (ER), but only about two thirds of these cancers respond to estrogen ablation.[1,2] The failure of the remaining 20% of ER positive tumors to respond to hormonal therapy is generally attributed to either tumor cell heterogeneity or a defect in the pathway involving ER activation of gene transcription. A mutation in the ER gene itself could yield ER forms that are active in the absence of estrogens (dominant-positive) or that are totally inactive and noninducible (dominant-negative).[3] Variant forms of ER messenger RNA (mRNA) have been detected in human breast tumors, although their contribution to endocrine unresponsive phenotype has not been established.[3–5] The variants may arise as a consequence of a genetic mutation or, more often, as the result of alternative splicing of the ER RNA transcript.[4] Regardless of their origins, in transient expression systems, the proteins encoded by the variant ER mRNA are usually unable to activate gene transcription or to interfere with normal ER function.[6]

The prognostic implications of the presence of ER mRNA variants in breast tumors have not been investigated. In studies where the occurrence of ER mRNA and the binding of estrogens to ER proteins were measured, there was a good correlation between both parameters.[7,8] Furthermore, in situ hybridizations and immunohistochemical analyses revealed that ER mRNA is

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Accepted for publication July 26, 1993.

located in the same cells that are immunoreactive with ER antibodies.[7] Regarding ER proteins, there are several reports that patients with ER-positive tumors have longer disease-free intervals and overall survival time.[9,10] Whether analysis of ER mRNA provides a more sensitive or an equivalent predictor of favorable disease outcome is not known.

A prognostic indicator of the relative aggressiveness of a breast cancer is necrotic cell death. Fisher et al.[11] reported that the presence of tumor necrosis was commonly associated with treatment failure and was independent of nodal status, tumor size, and histologic grade. More recently, a model using ER concentration, nodal status, and histopathologic assessment of tumor necrosis was found to best predict survival after disease recurrence.[12] Unfortunately in the absence of a molecular marker for tumor necrosis, the subjective nature of pathologic scoring for this condition reduces its use as a prognostic indicator.

Objective assessment of apoptotic or programmed cell death can be done through measurement of the level of expression of the TRPM-2 gene.[13–15] This gene codes for a protein that shares significant homology with a complement cytolysis inhibitor and, by inference, may act to restrict cytolytic attack on cell membranes.[16] In the Shionogi mouse mammary carcinoma[14] and in MCF-7 human breast cancer cells,[15] induction of apoptosis after hormonal ablation is accompanied by increased expression on TRPM-2. However, it is unclear whether the expression of TRPM-2, as a marker of programmed cell death rather than necrotic cell death, has any prognostic significance in evaluating disease outcome.

In the current investigation, we measured the expression and transcript size of ER mRNA and TRPM-2 mRNA in primary breast tumors from 126 patients. These results were compared with those obtained with the standard ligand-protein assays for ER and progesterone receptor (PgR) and with histopathologic evaluations of tumor necrosis. The relative value of these biologic markers in predicting survival time in these patients with breast cancer was determined.

## Patients and Methods

### *Patient Group*

RNA analyses were performed and evaluated on tumor biopsy specimens from 126 unselected patients who were referred to the British Columbia Cancer Agency with primary breast cancer between 1978 and 1990. The average age of the patients (mean ± standard deviation) at time of diagnosis was 64 ± 14.4 years. The median and mean follow-up times were 2.4 and 2.7 years, respectively, at which point 38 patients had died. The distribution of tumor grades was as follows: well differentiated, 5.7%; moderate, 57.5%; poor, 23.6%; and lobular, 13.2%. The same pathologist evaluated a section adjacent to each of the tumor specimens used for RNA extractions and hormone receptor determinations for (1) histologic type of the dominant growth pattern, (2) degree of differentiation (well, moderate, and poor), and (3) amount of confluent tumor necrosis (0–60%). All the biopsy specimens had been stored previously at −70°C in a breast tumor bank maintained in the Hormone Receptor Laboratory of the Vancouver General Hospital, Vancouver, Canada.

### *RNA Analyses*

RNA was prepared from frozen tumor tissue by the acid guanidinium thiocyanate/phenol-chloroform extraction method.[17] Poly A plus RNA was purified by oligo deoxythymidylate chromatography, quantified by spectrophotometry, separated by agarose gel electrophoresis, and blotted on Nytran (Schleicher and Schuell, Inc., Keene, NH) membranes exactly as described previously.[14,18] Northern analyses (prehybridizations and hybridizations with $^{32}$P-labeled probe) were performed as before.[19] The filters were washed with two cycles of shaking for 15 minutes at room temperature in 2 times SSC (1 times SSC is 15 mM sodium citrate, 150 mM sodium chloride, pH 8.0), 1.0% sodium dodecyl sulfate and two cycles for 30 minutes at 60°C with 0.1 times SSC, 0.1% SDS. Autoradiography was done using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) with intensifier screens at −70°C.

The cDNA probes (specific activity approximately 1 times $10^9$ dpm/$\mu$g DNA) were labeled with alpha-$^{32}$P deoxycytidine triphosphate using a random priming kit from Pharmacia (Pharmacia Fine Chemicals, Montreal, Quebec, Canada) and purified using Geneclean kits (Bio 101, La Jolla, CA). The plasmid pHE15[20], containing the 900 bp N-terminal half of the human ER, was provided by Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France); TRPM-2 cDNA[13] was provided by Dr. M. Tenniswood (University of Ottawa, Ottawa, Ontario, Canada); and the PA-1 plasmid,[21] containing a 900 bp region of chicken beta-actin, was obtained from Dr. D. Mager (British Columbia Cancer Agency, Vancouver, British Columbia, Canada). MCF-7 RNA and mouse kidney RNA were used as positive controls for ER and TRPM-2 transcripts, respectively. Every filter was rehybridized with the beta-actin cDNA to ensure that the RNA was of sufficient quantity and integrity for evaluation with the ER and TRPM-2 probes.

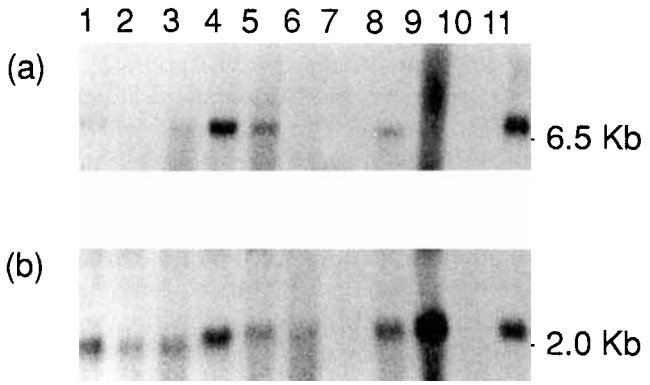


Figure 1. Northern Analysis of ER mRNA. RNA from 10 breast tumors was blotted and probed with radioactive (a) ER cDNA or (b) beta-actin cDNA (internal control). Lane 11 shows the positive control using RNA from MCF-7 cells.

### *Hormone Receptor Determinations*

ER and PgR measurements were performed by radioactive ligand methods as described previously[10,22] and were corrected for serum protein contamination as per the European Organization for Research on Treatment of Cancer recommendation.[23,24] Although the lowest amount that could be detected above background was 1 fmol/mg protein,[12] samples were considered receptor-positive only if the concentration was greater than or equal to 10 fmol/mg protein.

## Results

### *Expression of Normal and Variant ER mRNA in Breast Tumor Biopsy Specimens*

Total RNA was extracted from frozen breast tumor biopsy specimens and examined for the presence of ER mRNA by Northern analysis. When sufficient quantities of total RNA were available, then the sample was also processed for poly A + RNA. The results obtained when total RNA (25 ug aliquots) from 10 different breast tumors (lanes 1–10) were hybridized with the ER cDNA probe are shown in Figure 1a. Compared with the positive control using RNA from MCF-7 cells (lane 11), there is considerable variation in the amount of the normal 6.5 kilobases (kb) form of ER mRNA detected. No ER mRNA is observed in the samples run in lanes 2, 6, 7, and 10; small amounts are present in samples shown as lanes 1, 3, and 8; and moderate to large concentrations of ER mRNA are detected in those samples run in lanes 4 and 5. In lane 9 there is a broad smear pattern that made evaluation difficult. Subsequent purification of poly A + RNA from this sample revealed the presence of a 6.5 kb ER mRNA (data not shown).

To assess the relative quantity and integrity of the RNA samples, the filters were stored over a sufficient period to allow for radioactive decay of the bound $^{32}$P-ER cDNA and then rehybridized with radioactive beta-actin cDNA probe (Fig. 1b). Those samples with a 2.0 kb band corresponding to the beta-actin transcript were deemed to have acceptable quality mRNA, whereas those where the band was absent or appeared as a smear were rejected. It is evident that as a result of degradation or poor extraction, mRNA in the samples in lanes 7 and 10 are of too low a concentration for further evaluation. Using the integrity of the beta-actin internal control as the criterion for acceptance, 126

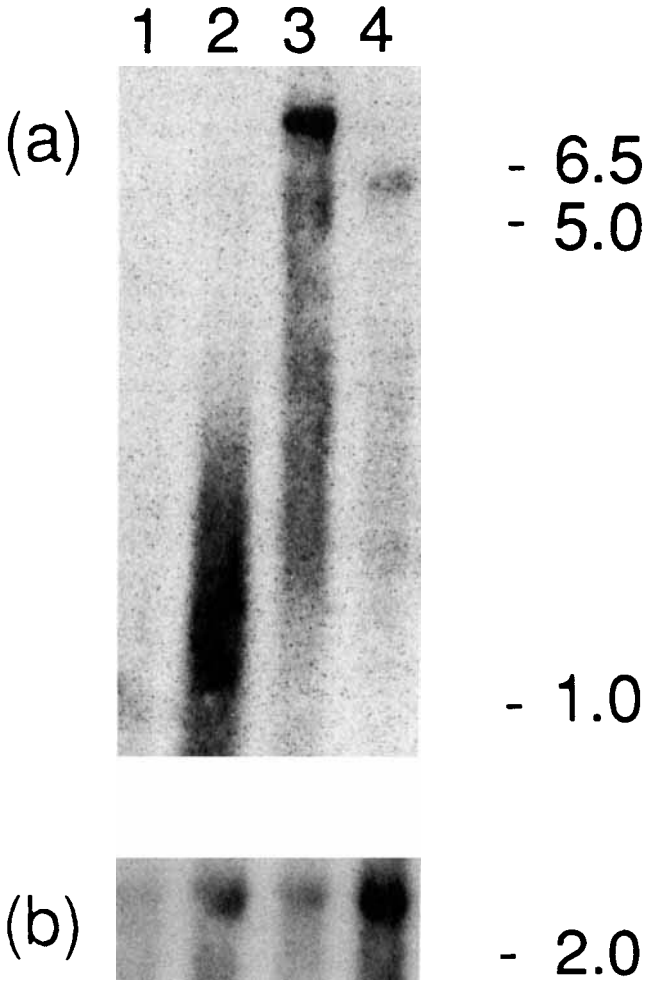


Figure 2. Examples of variant forms of ER mRNA. (a) Tumors with RNA containing no ER mRNA (lane 1), variant forms of ER mRNA (lanes 2 and 4), and normal ER mRNA (lane 3) are shown and compared with results for (b) beta-actin mRNA (internal control).

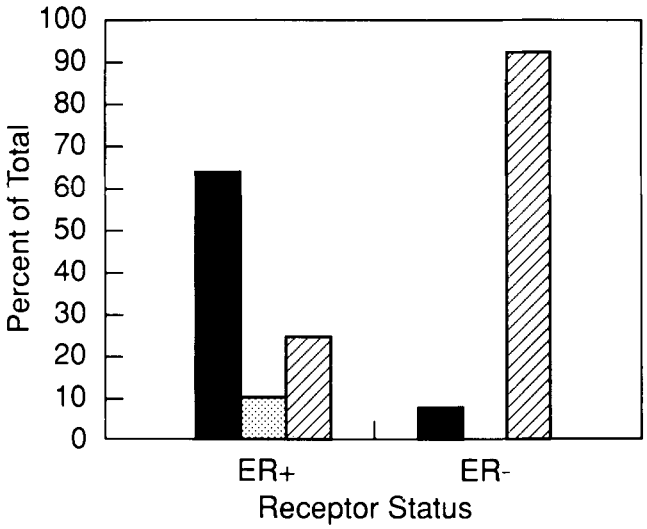


Figure 3. Distribution of normal and variant forms of ER mRNA in ER-positive and ER-negative tumors. The percent of ER-positive and ER-negative tumors with normal ER mRNA alone (solid bars), variant ER mRNA (shaded bars), or no ER mRNA (hatched bars) is shown.

breast tumors were found to have adequate amounts of intact mRNA for analysis.

Different sizes of ER mRNA transcripts ranging from 1.0 to 5.0 kb were found in only 11 of the 126 tumors with nondegraded mRNA; most of these (9 tumors) also contained the normal 6.5 kb transcript. The two preparations with a single yet abnormal form of ER mRNA are shown in Figure 2a. A normal beta-actin transcript was found in each of the samples (Fig. 2b). No ER mRNA was found in the tumor RNA in lane 1, and a normal 6.5 kb form of the ER mRNA was present in the sample in lane 3. By comparison, the tumor RNA in lane 2 that hybridized with ER cDNA had a single broad band with a size of approximately 1–2.5 kb, and that in lane 4 had an ER mRNA species of 5.0 kb. The origin of these variants is unknown, but the integrity of the beta-actin transcript implies that they are not simply the product of nonspecific ribonuclease digestion.

### *Relationship of ER mRNA Expression With Hormone Receptor Binding Activity*

ER mRNA transcripts were found in 75 of the 100 tumors that were ER positive (i.e., ER concentration ≥ 10 fmol) in the estrogen-binding assay. As indicated in Figure 3, approximately 11% of the ER-positive tumors had variant forms of ER mRNA, whereas most tumors in this category (64%) had the 6.5 kb transcript only. No ER mRNA was detected in 25% of ER-positive tumors and in most of the ER-negative tumors (92%; n = 23). There was a highly significant relationship (Pearson chi-square, $P < 0.001$) between the occurrence of ER mRNA and ER status. Similarly, there was a significant association with tumor grade ($P = 0.002$).

No variant forms of ER mRNA were found in any ER-negative tumors, suggesting that the occurrence of abnormal mRNA is not associated with loss of estrogen-binding activity. In two of the ER-negative tumors, a normal ER mRNA transcript was observed. These may be representative of the small number of ER-negative tumors in which there is a response to endocrine therapy.[2]

In specimens where PgR was also measured (n = 116), ER mRNA was present in approximately 75% of PgR-positive tumors (PgR concentration ≥ 10 fmol) and absent in 70% of PgR-negative tumors. As with ER, the association of ER mRNA with PgR status was highly significant ($P < 0.001$).

### *Expression of a Marker of Apoptosis*

To determine the frequency of occurrence of apoptotic cells in primary, untreated breast tumors, the RNA samples used for ER mRNA measurement were analyzed for the presence of TRPM-2 mRNA, a marker of apoptosis.[13–15] The Northern blot shown in Figure 4 illustrates the spectrum of TRPM-2 expression in the tumors. In most of the tumors (83%; n = 105), no TRPM-2 mRNA is detected (lane 1). In those where expression of

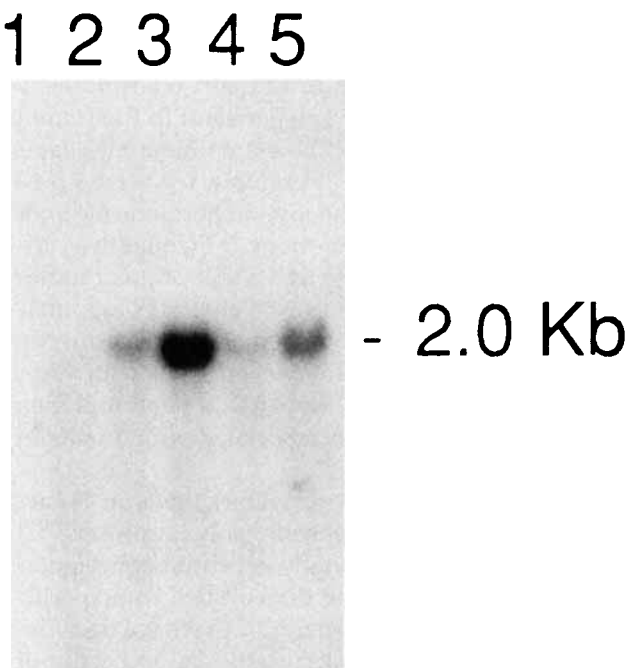


Figure 4. Northern analysis of TRPM-2 mRNA. Poly A + RNA form 5 tumors was blotted and probed with radioactive TRPM-2 cDNA.

this gene is evident (17%; n = 21), the relative intensity of the 2.0 kb transcript varies from low (lanes 2 and 4) to high (lanes 3 and 5).

In comparing TRPM-2 expression with the presence of either ER mRNA or ER and PgR binding activity, no correlations were found (Pearson chi-square, $P = 1.00$ and $P = 0.58$, respectively). Similarly, there was no statistically significant relationship between TRPM-2 and tumor grade ($P = 0.45$) or pathologic tumor necrosis ($P = 0.15$).

### *Relationship Between the Presence of ER and TRPM-2 mRNA With Survival*

It is well established that the level of ER binding activity is an excellent prognostic marker in breast cancer that allows prediction of the likelihood of a response to endocrine therapy as well as survival outcome.[9,10] As shown in the Kaplan–Meier survival plot[25] (Fig. 5, top), the occurrence of ER mRNA in a tumor was also related to survival time (log-rank chi-square, $P = 0.005$). Although those patients whose tumors lacked measurable ER mRNA had poorer survival times, those with variant ER mRNA did not differ from the group with only normal ER mRNA in their tumors. As anticipated, there was a similar relationship with ER binding activity (Fig. 5, middle). In contrast, the presence of TRPM-2 mRNA in the breast tumors was of no prognostic value in predicting survival outcome (Fig. 5, bottom).

## Discussion

Interest in the occurrence of variant forms of steroid receptors is based in part on their potential to supplant the normal receptor with a form that is constitutively active and hence hormone independent. In the steroid receptor family of proteins, there are numerous examples of this phenomenon. For example, the v-*erb*A protein is a truncated form of the thyroid hormone receptor that cannot bind thyroid hormone.[26] Frequently, variant receptors appear to arise as a result of point mutations rather than truncations or deletions. Point mutations in the androgen receptor gene have been directly linked to androgen insensitivity syndromes with varying degrees of severity.[27,28] Similarly, a point mutation in the vitamin D receptor is associated with a vitamin D–resistant form of rickets.[29]

Other than nucleotide variations that can be accounted for by normal genetic polymorphisms,[30,31] there are no reports of naturally occurring point mutations in the ER that can be directly tied to a specific phenotype. Rather, most variant ER that have been detected have a reduced RNA transcript size, indicative of a truncation or deletion.[4] A truncated ER that inhibits the transcriptional activation of the normal ER has been

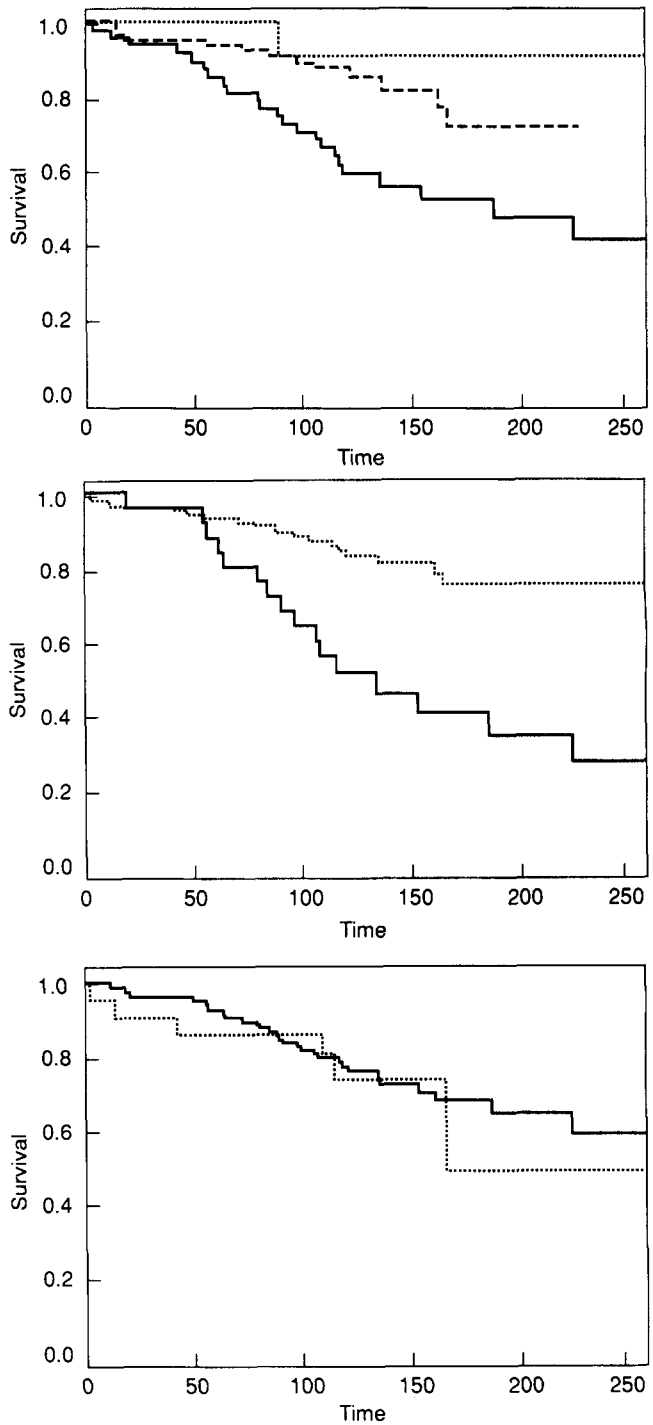


Figure 5. Survival curves. Survival curves were constructed on the bases of (top) ER mRNA (dashed line, normal ER mRNA; dotted line, variant ER mRNA; solid line, no ER mRNA), (middle) ER status (dotted line, ER-positive; solid line, ER-negative) and (bottom) TRPM-2 mRNA (dotted line, present; solid line, absent).

cloned from an ER-positive/PgR-negative breast tumor,[32] and a deletion of exon 5 in the hormone-binding domain of the ER gene has been found in an ER-nega-

tive/PgR-positive breast tumor.[33] However, in most instances there has been no linkage between the occurrence of smaller forms of ER mRNA and a particular ER/PgR profile or growth pattern. Only one of the tumors containing a variant ER mRNA was PgR-negative.

In examining the RNA from 126 breast tumors, we found that in the ER-positive group (n = 101), approximately 75% had ER mRNA, of which 64% had only the normal 6.5 kb ER mRNA, 9% had the normal plus a smaller form, and 2% had only a smaller ER mRNA transcript (Figs. 1–3). The relative frequency of occurrence of the normal ER mRNA alone is similar to that seen by Murphy and Dotzlaw.[5] However, the authors did not report what percentage of tumors was characterized by variant forms; when an abnormal ER mRNA was present, it always coexisted with the normal transcript. Furthermore, the proteins encoded by the variant ER mRNA were unable to activate transcription or modulate the activity of the wild-type ER in transient transfection experiments.[6] This suggested that even if variant ER mRNA is translated in vivo, it may be nonfunctional. Although we did not test biologic activity, our results regarding survival (Fig. 5, top) suggest that the presence of abnormal ER mRNA is not a negative indicator. Rather, it is of similar prognostic significance as the presence of normal ER mRNA, which is associated with a favorable outcome. However, the finding cannot be considered conclusive because of the small proportion of persons with the variant forms.

We found no evidence that analysis of ER mRNA is superior to the ligand-binding assay for ER for assessing prognosis (Fig. 5, middle). Nevertheless, measurement of ER mRNA could potentially identify subgroups within the ER-positive and ER-negative tumors with differential sensitivity to endocrine therapy. For example, there may be a relationship between the 30–40% of ER-positive tumors that fail to respond to antiestrogen treatments and the 25% of these tumors in which ER mRNA is undetectable.[1,2] Also, the two (8%) ER-negative tumors that have ER mRNA may be representatives of the small percentage (<10%) of ER-negative tumors that respond to endocrine therapy.[2] However, the finding that both of the ER mRNA-positive tumors are PgR negative is contrary to this hypothesis, because it implies that little or no active ER protein is being produced from this RNA. Additional studies are necessary to establish whether ER mRNA analysis can discriminate between responders and nonresponders.

The enhanced expression of the TRPM-2 gene is usually associated with programmed or apoptotic cell death.[13–15] Because of its nucleotide sequence homology with SGP-2, a complement inhibitor in the male genital tract, it is likely that TRPM-2 plays a protective role in apoptosis rather than acting as a mediator of the destructive processes.[16] In rodent and human breast tumor lines, TRPM-2 expression increases rapidly after hormone withdrawal and persists during all the stages of apoptosis.[14,15] There is some evidence that deregulation of TRPM-2 is linked to the emergence of the hormone-independent phenotype.[34]

In the current study, expression of TRPM-2 in primary breast tumors varied considerably in terms of its relative intensity and prevalence (Fig. 4). With respect to the latter, TRPM-2 mRNA was found in approximately 17% of the tumors. However, the presence (or absence) was not related to other parameters, such as hormone-receptor status, tumor grade, or the degree of tumor necrosis. Similarly, there was no relationship between the occurrence of TRPM-2 and survival outcome (Fig. 5, bottom). On the bases of these results, it would seem that TRPM-2 expression has no apparent use as a prognostic marker in breast cancer.

In conclusion, we found that although the expression of TRPM-2 is not related to any of the parameters examined, the presence of ER mRNA correlates well with hormone receptor status and with survival outcome. Furthermore, variant forms of ER mRNA, whether accompanied by the normal transcript or not, are not associated with an unfavorable prognosis. Patients with variant forms of ER mRNA in their tumors had a survival pattern more similar to those with normal ER mRNA than those without. However, the biologic significance of the occasional occurrence of ER mRNA in ER-negative tumors or the absence of ER mRNA in many ER-positive tumors is unknown but may be related to clinical subgroups.

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