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Real-Time PCR Quantification of Full-Length and Exon 11 Spliced *BRCA1* Transcripts in Human Breast Cancer Cell Lines

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Germline alterations of the *BRCA1* tumor suppressor gene have been implicated at least in half of familial breast cancers. Nevertheless, in sporadic breast cancer no mutation of this gene has been characterized to date. In sporadic breast tumors, other BRCA1 gene loss of function mechanisms, such as down-regulation of gene expression, have been suggested. In an effort to better understand the relationship between *BRCA1* expression and malignant transformation, we have adapted the new real-time quantitative PCR method based on a 5′ nuclease assay and the use of doubly labeled fluorescent TaqMan probes to quantify *BRCA1* mRNA. We have compared expression of *BRCA1* mRNA with or without exon 11 in the normal breast epithelial cell line MCF10a and in three cancer cell lines (MCF-7, MDA-MB231 and HBL100) by comparing two methods of quantification: the comparative C_T and the standard curve. We found that the full length *BRCA1* mRNA, which encodes the functional nuclear protein, was down-regulated in tumor cells when compared with MCF10a cells.

Breast cancer is one of the most common malignancies and causes of death among women in Western industrialized societies. Although the majority of these cancers are the result of somatic mutations, epidemiological data indicate that 5–10% of all breast cancers are associated with inherited mutations of tumor suppressor genes. *BRCA1* seem to be responsible for 52% of familial breast cancer (1). However, unlike the precedent of other tumor suppressor genes, where mutant forms of the gene are responsible for both the inherited and sporadic forms of the same type of cancer, *BRCA1* mutations have not yet been detected in sporadic breast cancer (2, 3).

Alterations of *BRCA1* mRNA levels have been observed in sporadic breast cancer. The transition from *in situ* carcinoma to invasive cancer is correlated with a decrease of *BRCA1* mRNA expression (4).

In addition to the 7.8 kb mRNA, at least four alternative splice variants have been described (5). The 4.6 kb form, which lacks exon 11, appears to be the most highly expressed (6, 7). It encodes a cytoplasmic protein of unknown function.

In an effort to characterize the expression pattern of the different forms of *BRCA1* mRNA in breast tumor cell lines, we have used the real-time PCR quantification, based on the cleavage of doubly labeled fluorescent TaqMan probes (8, 9). The results presented in this report confirm *BRCA1* gene expression in the studied tumor cell lines is lower than in immortalized, non-tumorigenic breast epithelial cell-line MCF10a and that expression of exon 11-containing *BRCA1* (*BRCA1*-exon11) mRNA is cell-line specific.

MATERIALS AND METHODS

Cell Lines

Breast epithelial normal cell MCF10a and breast cancer cells MCF-7, HBL100 and MDA-MB231 were purchased from American Type Culture Collection (10–13). RPMI 1640, L-15, DMEM/Ham's F-12 media, fetal bovine serum and horse serum were obtained from Gibco BRL (Life Technologies SARL). All cell lines were cultured as described previously (14).

RNA Extraction

3×10^6 cells were plated in T75 cm^2 flasks. At 80% confluence, cells were washed. Total RNA was isolated using 7 ml of Trizol (Gibco BRL) according to manufacturer's protocol. One μg of total RNA was used for the synthesis of first strand cDNA using the First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) following the manufacturer's instructions.

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Quantification of BRCA1 mRNA Using Real-Time Quantitative RT-PCR

PCR amplification. For *BRCA1* expression analysis, probes and primers (Table 1) have been designed using the Primer Express software (PE Biosystems) so that they overlapped splice junctions (exons 11–12 for *BRCA1* Ex11 probe and exons 23–24 for *BRCA1* 3′) (Fig. 1). The TaqMan universal PCR master Mix and the 18S rRNA doubly labeled TaqMan probe and primers were obtained from PE Biosystems.

Multiplex PCR was carried out in 96-well plates on cDNA equivalent to 10 ng of total RNA. A typical 25 μL reaction sample contained 12.5 μL TaqMan universal PCR Master Mix (containing 1X TaqMan buffer, 200 μM dATP, dCTP, dGTP, and 400 μM dUTP, 5 mM $MgCl_2$, 1.25 unit of AmpliTaqGold, 0.5 unit of Amperase uracil-*N*-glycolsylase (UNG), 200 nM of *BRCA1* primers and 50 nM of 18S rRNA primers, 200 nM of *BRCA1* TaqMan probes and 50 nM of 18S rRNA TaqMan probe. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed with 40 cycles at 95°C for 30 s and 60°C for 1 min. Data were collected using the ABI PRISM 7700 SDS analytical thermal cycler (PE Biosystems). Each sample was tested in triplicate.

Relative quantification of BRCA1 gene expression. It was performed using the comparative C_T method or the standard curve method.

First, the comparative C_T method (15), which consists of the normalization of the number of target gene copies to an endogenous reference gene (i.e., 18S rRNA), designated as the calibrator, in order to normalize quantity and quality of the cDNA samples.

The level of *BRCA1* mRNA expression in each tumor cell line was then normalized to the result obtained in the normal cell line MCF10a, so that *BRCA1* normalized expression ($BRCA1_N$) is given by

$$BRCA1_N = 2^{-\Delta\Delta C_T},$$

where $\Delta\Delta C_T = \Delta C_T$ cancer cells $- \Delta C_T$ normal cells and assuming that the efficiency of the PCR reaction was close to one.

The second quantification strategy is based on the use of a standard curve constructed with serial dilutions of specific PCR products. *BRCA1* Ex11 and *BRCA1* 3′ primers were used to amplify specific fragments from cDNA equivalent to 10 ng of total RNA extracted from MCF10a cells. PCR products were purified on MicroSpin S-400 HR columns (Amersham Pharmacia Biotech) and their concentration was estimated by A_{260} measurement. Aliquots were analyzed by agarose gel electrophoresis to check their quality and to ensure the correct size of the fragment. PCR products were diluted in plasmid DNA to a final DNA concentration of 10 ng/μL. The standard curve was determined using serial dilutions, ranging from 2.2×10^4 to 7×10^2 copies. Each point and each assay were done in triplicate.

RESULTS AND DISCUSSION

Validation Experiments

In previous work based on competitive RT-PCR, an end-point quantification analysis, we determined the *BRCA1* expression level in normal and cancer breast cell lines (14). But, unlike the real-time TaqMan PCR presented in this paper, competitive quantitative PCR requires the design and storage of an internal control, and the validation of its amplification efficiency is tedious.

We present herein the application of the new kinetic method developed to quantify the expression of very

TABLE 1

Summary of Oligonucleotide Primer Pairs and TaqMan Probes

Names	Forward primers	TaqMan probes[a]	Reverse primers
BRCA1 3′	5′ - ^{5566}[b]CAGAGGACAATGGCTTCCATG^{5586} - 3′	5′ - ^{5588}AATTGGGCAGATGTGTGAGGCACCTG^{5613} - 3′	5′ - ^{5623}CTACACTGTCCAACACCCACTCTC^{5646} - 3′
BRCA1 Ex11	5′ - ^{4157}AAGAGGAACGGGTTGGGA^{4175} - 3′	5′ - ^{4177}AAAATAATCAAGAAGAGCAAAGCATGGATTCAAACTTA^{4215} - 3′	5′ - ^{4217}CACACCCAGATGCTGCTTCA^{4235} - 3′
rRNA 18S	5′ - CGGCTACCACATCCAAGGAA - 3′	5′ - TGCTGGCACCAGACTTGCCCTC - 3′	5′ - GCTGGAATTACCGCGGCT - 3′

[a] The fluorescent reporter dyes FAM for *BRCA1* 3′ and *BRCA1* Ex11; and VIC rRNA 18S are covalently linked to the 5′-end of the probe oligonucleotide and their fluorescence is quenched by TAMRA located at the 3′-end.

[b] Numbers indicate the nucleotide position of oligonucleotides in the *BRCA1* cDNA sequence (GenBank Accession No. U37574).

TABLE 2

Reproducibility of the C_T Parameter with the *BRCA1* 3′ TaqMan Probe with MCF-7 Cells

	RNA extractions: A			B
	Reverse transcriptions: RTA_1		RTA_2	RTB_1
$\Delta C_T = C_T$BRCA1-C_T18s	23.47	23.30	23.00	24.37
	23.41	23.31	22.87	24.69
	23.42	23.29	23.09	24.18
Mean ± SD	23.43 ± 0.03	23.30 ± 0.01	22.09 ± 0.09	24.41 ± 0.21

Note. Four quantifications were determined with 2 independent total RNA extractions (A and B) from MCF-7. With RNA extraction A, two independent reverse transcriptions (RTA_1, and RTA_2) were done. With extraction B, one reverse transcription (RTB_1) was effected. On the first RTA_1, two quantifications were done according to the ΔC_T formula. With the reverse transcriptions (RTA_2 and RTB_1), only one quantification was performed. In addition, each assays was done in triplicate and expressed in mean ± SD.

rare mRNA such as *BRCA1:* the real-time quantitative PCR based on the cleavage of doubly labeled fluorescent TaqMan probes (8, 16).

For each cell line, we extracted total RNA on two occasions and carried out several reverse transcription assays and real-time TaqMan PCRs. For each quantification assay, samples were tested in triplicate. Comparison of C_T values showed minimal variation based on standard deviation (Table 2). Results obtained for the same cDNA sample or between two cDNA samples prepared with the same total RNA yielded similar results for *BRCA1* quantity. The highest level of variability was observed between RNA preparations. This result is a strong argument in favor of using a reference gene (i.e., 18S rRNA) to normalize the amount and the quality of cDNA.

For relative quantification of *BRCA1* gene expression analysis, we first used the comparative C_T method (15). Before using this method, we performed validation experiments to demonstrate that the efficiencies of *BRCA1* and 18S rRNA PCRs were equal. The absolute value of the slope of log input amount *versus* ΔC_T should be ≤0.1. Both slopes (0.1 and 0.05, respectively) pass this test.

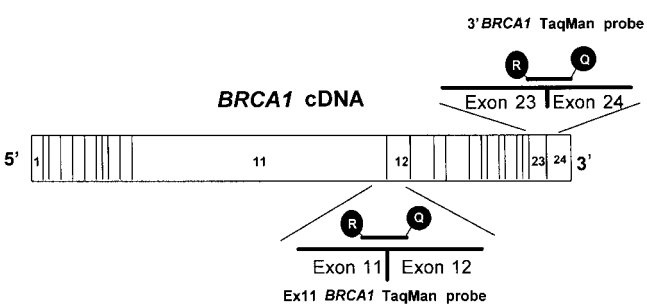

FIG. 1. Location of the two TaqMan probes in the *BRCA1* cDNA sequence. Probe *BRCA1* Ex11 is located at the exon 11–12 junction and probe *BRCA1* 3′ at the exon 23–24 junction. Exact locations of the two probes are indicated in Table 1.

Determination of Total BRCA1 Transcript Expression Level in Cancer Cell Lines

We compared the level of expression of *BRCA1* mRNA in the immortalized non tumorigenic breast epithelial cell line MCF10a (considered as normal breast cell control) and three tumor cell lines: MCF7, MDA-MB231 and HBL100.

The *BRCA1* 3′ TaqMan probe was designed to mainly quantify the expression of all *BRCA1* mRNA species together because no alternative splicing of exon 23 has been described (7) (Fig. 1 and Table 1). Expression of *BRCA1* in cancer cell lines was normalized to the *BRCA1* expression level in MCF10a cells. As shown in Fig. 2, expression of each *BRCA1* mRNA species was dependent on the cell line. In MCF-7 and MDA-MB231, the abundance of the 7.8 and 4.6 kb mRNAs was significantly lower than in MCF10a (MCF-7: 0.54 ± 0.04; MDA-MB231: 0.27 ± 0.04). However, in HBL100 cells,

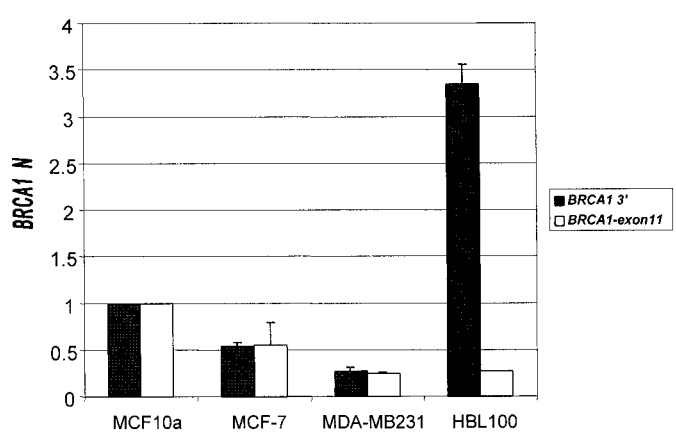

FIG. 2. Real-time PCR quantification of relative *BRCA1* mRNA expression with *BRCA1* 3′ (filled bars) or *BRCA1 Ex11* (empty bars) TaqMan probes according to comparative C_T method. Data obtained from four separate experiments was standardized by setting the value of *BRCA1* expression in MCF10a at 1.0. Those for other cell lines are expressed (mean ± SD) relative to this standard.

BRCA1 total transcript expression was up-regulated (3.34 ± 0.22), although *BRCA1*-exon 11 transcripts were down-regulated.

These results are in agreement with previous reports: quantitative analysis based on competitive PCR have shown that the *BRCA1* mRNA copy number in HBL100 cells was twofold higher than in MCF-7 (17). Rice *et al.* have shown, using an end-point quantitative analysis, that MCF-7 exhibited a three-fold decrease of *BRCA1* mRNA expression relative to normal breast epithelial cells (18). Romagnolo *et al.*, using RNase protection assay, have established that expression of the *BRCA1* gene was 10-fold higher in HBL100 than in MCF-7 cells (19). It was hypothesized that up-regulation of *BRCA1* mRNA was related to SV-40 immortalization. The decrease of *BRCA1* expression was more important in the estrogen receptor negative (ER^-) MDA-MB231 cell line than in the ER^+ cell line MCF-7. Many data have implicated ER in the up-regulation of *BRCA1* gene expression (18, 20, 21), but correlation between the expression of *BRCA1* and ER expression appeared to be specific to tumor cells, since we have shown that in the ER^- MCF10a cells, level of *BRCA1* expression was high as compared to tumor cells.

The level of expression in MCF-7 and MDA-MB231 cells relative to MCF10a was not consistent with another study by Gudas *et al.* using Northern blotting (6). According to their results, the level of expression in MCF10a was lower than in tumor cells. These results may be due to the use of a different method.

Determination of Exon 11-Containing BRCA1 mRNA (BRCA1-Exon11) Level of Expression in Breast Cancer Cell Lines

BRCA1 Ex11 TaqMan probe was used to estimated the level of *BRCA1*-exon11 mRNA. Results obtained with this probe are presented in Fig. 2. Expression of full-length *BRCA1* mRNA in breast cancer cells was about half the level of MCF10a normal cells (MCF-7, 0.55 ± 0.24; MDA-MB231, 0.26 ± 0.012).

Our studies have confirmed that in two breast tumor cell lines, the expression of *BRCA1-exon11* mRNA, which encodes the functional nuclear protein, was highly down-regulated (50% for MCF-7, 75% MDA-MB231). The mechanisms involved in decreasing *BRCA1* expression are not clearly understood, but several lines of evidence suggest promoter methylation (18, 22, 23).

For the HBL100 cell line, the expression of *BRCA1-exon11* mRNA was about fourfold lower than in MCF10a cells (HBL100, 0.27 ± 0.001). These results differ from those by Wilson *et al.* who showed that *BRCA1*-Δ*11* (*BRCA1* mRNA which lacks exon 11) and *BRCA1*-exon11 mRNA were expressed almost at the same level (7). Here we have shown that *BRCA1*-exon11 was dramatically underexpressed relative to total *BRCA1* mRNA. To confirm this result we performed absolute quantification of *BRCA1*-exon11 mRNA and all *BRCA1* transcripts in all cell lines.

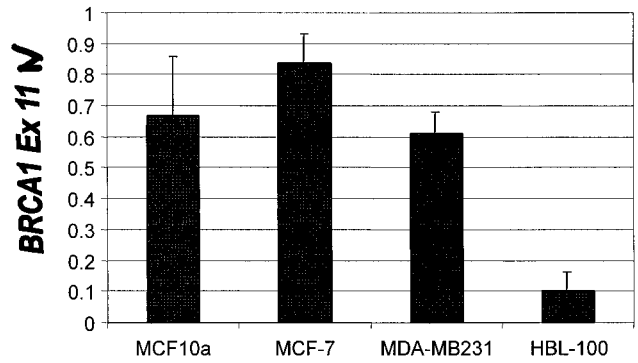

FIG. 3. Relative expression *BRCA1*-exon 11 mRNA as a fraction of all *BRCA1* messengers. The level of expression of total *BRCA1* mRNA was determined with *BRCA1* 3′ TaqMan probe owing to the specific standard curve method. The level of expression of *BRCA1*-exon 11 mRNA was determined with *BRCA1* Ex11 TaqMan probe using the specific standard curve. Data obtained from four separate experiments was standardized by setting the value of total *BRCA1* mRNA expression at 1.0. Data for *BRCA1*-exon 11 mRNA in each cell line was expressed (mean ± SD) relative to this standard.

Prevalence of BRCA1-Exon11 mRNA versus All BRCA1 Transcripts

To evaluate the relative expression of *BRCA1*-exon11 transcripts versus the total *BRCA1* mRNA species, we used the absolute standard curve method (23, 24). Two standard curves were constructed with dilutions of PCR products obtained with each specific set of primers (*BRCA1* 3′ and *BRCA1* Ex11). We performed a comparative evaluation for each cell line.

The level of *BRCA1*-exon11 mRNA was normalized to the expression of all *BRCA1* splice variant transcripts as shown in Fig. 3. For MCF10a, MCF-7 and MDA-MB231 cell lines, *BRCA1*-exon11 mRNA was the most highly expressed *BRCA1* mRNA (MCF10a, 0.67 ± 0.19; MCF7, 0.84 ± 0.09; MDA-MB231, 0.612 ± 0.07). These results were consistent with other studies which have shown that *BRCA1*-exon11 was the most abundant transcript (6, 18).

Nevertheless, our quantitative analysis of mRNA in HBL100 showed that the majority of transcripts lacked exon 11. The discrepancies between our results and those of others might come from the method used to evaluate expression of *BRCA1* (RNase protection *versus* real-time TaqMan PCR). We also cannot exclude differences between clones of HBL100 due to prolonged *in vitro* culture of the cell line leading to important genomic alterations. If we compare the ratios of *BRCA1*-exon 11 mRNA/all *BRCA1* splice variants as calculated according to the comparative C_T method

(Fig. 2) and the absolute standard curve method (Fig. 3), we notice a slight difference in the values obtained for the MCF-7 and MDA-MB231, but not for the HBL100 cell lines. This may be due to the use of a PCR-produced DNA fragment to build the standard curve. The PCR efficiency may be slightly different from the PCR efficiency of the cDNA produced by the reverse transcription.

The significance of the elimination of exon 11 is not clear. The product of this mRNA is a protein which does not include an NLS and so cannot autonomously reach the nucleus (25). The biological role of this polypeptide is not well understood but, according to Wilson *et al.*, it does not exhibit the cell toxicity apparent with overexpression of the full-length protein in transiently transfected cells (7). Cui *et al.* have established that BRCA1-Δ11 associates with CBP coactivator which is a component of the RNA polymerase II holoenzyme (26, 27). Furthermore, *BRCA1-Δ11* may encode a protein of physiologic importance since this splice variant has also been described in the mouse (28). Maintenance of a proper ratio between *BRCA1*-exon11 and *BRCA1-Δ11* could be functionally relevant. A precedent for such a situation has already been described for *WT1* gene, where a splice variant product was a more potent tumor suppressor than the full length version (29).

In conclusion, real-time quantitative TaqMan PCR is a powerful method allowing rapid and accurate quantification of rare transcripts such as *BRCA1* mRNA. We have developed a strategy to evaluate quantitative expression of the different splice variants of *BRCA1*. Our results suggest that modifications of transcription level and regulation of specific post-transcriptional events were fundamental features of *BRCA1* gene expression.

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REFERENCES

1. Ford, D., Easton, D. F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D. T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M. D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T. R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B. A., Gayther, S. A., Zelada-Hedman, M., *et al.* (1998) *Am. J. Hum. Genet.* **62,** 676–689.
2. Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, C., Haugen-Strano, A., Swensen, J., Miki, Y., Eddington, K., McClure, M., Frye, C., Weaver-Feldhaus, J., Ding, W., Gholami, Z., Soderkvist, P., Terry, L., Jhanwar, S., Berchuck, A., Iglehart, J., Marks, J., Ballinger, D., Barrett, J., Skolnick, M., Kamb, A., and Wiseman, R. (1994) *Science* **266,** 120–122.
3. Merajver, S. D., Pham, T. M., Caduff, R. F., Chen, M., Poy, E. L., Cooney, K. A., Weber, B. L., Collins, F. S., Johnston, C., and Frank, T. S. (1995) *Nat. Genet.* **9,** 439–443.
4. Thompson, M. E., Jensen, R. A., Obermiller, P. S., Page, D. L., and Holt, J. T. (1995) *Nat. Genet.* **9,** 444–450.
5. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., Mac Clure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P. K., Norris, F., Helvering, L., Morrison, P., Rosteck, P., Lai, M., Barrett, J. C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A., and Skolnick, M. H. (1994) *Science* **266,** 66–71.
6. Gudas, J. M., Li, T., Nguyen, H., Jensen, D., Rauscher, F. J., 3rd, and Cowan, K. H. (1996) *Cell Growth Differ.* **7,** 717–723.
7. Wilson, C. A., Payton, M. N., Elliott, G. S., Buaas, F. W., Cajulis, E. E., Grosshands, D., Ramos, L., Reese, D. M., Slamon, D. J., and Calzone F. J. (1997) *Oncogene* 14, 1–16.
8. Holland, P. M., Abramson, R. D., Watson, R., and Gelfand, D. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7276–7280.
9. Gibson, U. E., Heid, C. A., and Williams, P. M. (1996) *Genome Res.* **6,** 995–1001.
10. Soule, H. D., Maloney, T., Wolman, S., Peterson, W. J., Brentz, R., McGrath, C., Russo, J., Pauley, R., Jones, R., and Brooks, S. (1990) *Cancer Res.* **50,** 6075–6086.
11. Soule, H. D., Vasquez, J., Long, A., Albert, S., and Brennan, M. (1973) *J. Natl. Cancer Inst.* **51,** 1409–1416.
12. Cailleau, R., Young, R., Olive, M., and Reeves, W. J., Jr. (1974) *J. Natl. Cancer Inst.* **53,** 661–674.
13. Gaffney, E. V., Pigott, D. A., and Grimaldi, M. A. (1979) *J. Natl. Cancer Inst.* **63,** 913–918.
14. Favy, D. A., Rio, P., Maurizis, J. C., Hizel, C., Bignon, Y. J., and Bernard-Gallon, D. J. (1999) *Biochem. Biophys. Res. Commun.* **258,** 284–292.
15. Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., and Bohle, R. M. (1998) *Nat. Med.* **4,** 1329–1333.
16. Lee, L. G., Connell, C. R., and Bloch, W. (1993) *Nucleic Acids Res.* **21,** 3761–3766.
17. Ribieras, S., Magdinier, F., Leclerc, D., Lenoir, G., Frappart, L., and Dante, R. (1997) *Int. J. Cancer* **73,** 715–718.
18. Rice, J. C., Massey-Brown, K. S., and Futscher, B. W. (1998) *Oncogene* **17,** 1807–1812.
19. Romagnolo, D., Annab, L. A., Thompson, T. E., Risinger, J. I., Terry, L. A., Barrett, J. C., and Afshari, C. A. (1998) *Mol. Carcinog.* **22,** 102–109.
20. Gudas, J. M., Nguyen, H., Li, T., and Cowan, K. H. (1995) *Cancer Res.* **55,** 4561–4565.
21. Spillman, M. A., and Bowcock, A. M. (1996) *Oncogene* **13,** 1639–1654.
22. Magdinier, F., Ribieras, S., Lenoir, G. M., Frappart, L., and Dante, R. (1998) *Oncogene* **17,** 3169–3176.
23. Mancini, D. N., Rodenhiser, D. I., Ainsworth, P. J., O'Malley, F. P., Singh, S. M., Xing, W., and Archer, T. K. (1998) *Oncogene* **16,** 1161–1169.
24. Bieche, I., Olivi, M., Champeme, M. H., Vidaud, D., Lidereau, R., and Vidaud, M. (1998) *Int. J. Cancer* **78,** 661–666.
25. Thakur, S., Zhang, H. B., Peng, Y., Le, H., Carroll, B., Ward, T.,

Yao, J., Farid, L. M., Couch, F. J., Wilson, R. B., and Weber, B. L. (1997) *Mol. Cell. Biol.* **17,** 444–452.

26. Cui, J. Q., Wang, H., Reddy, E. S. P., and Rao, V. N. (1998) *Oncol. Rep.* **5,** 585–589.
27. Cui, J. Q., Shao, N., Chai, Y., Wang, H., Reddy, E. S., and Rao, V. N. (1998) *Oncol. Rep.* **5,** 591–595.
28. Hakem, R., de la Pompa, J., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmer, A., Billia, F., Firpo, E., Hui, C., Roberts, J., Rossant, J., and Mak, T. (1996) *Cell* **85,** 1009–1023.
29. Kudoh, T., Ishidate, T., Moriyama, M., Toyoshima, K., and Akiyama, T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4517–4521.