ABUNDANCE OF BRCA1 TRANSCRIPTS IN HUMAN CANCER AND LYMPHOBLASTOID CELL LINES CARRYING BRCA1 GERM-LINE ALTERATIONS

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A competitive polymerase chain reaction has been developed for quantitation of *BRCA1* mRNA. In human cancer cell lines, the amount of *BRCA1* mRNA is relatively low, ranging from 6 to 38 copies per cell. The decay rate of these transcripts in actinomycin-treated cells indicates that the half-life of these molecules is about 4 hr, suggesting that the low concentration of *BRCA1* messages is not due to molecular unstability. In human lymphoblastoid cell lines derived from patients carrying germ-line alterations of *BRCA1,* the amount of *BRCA1* mRNA per cell is lowered only in cell lines exhibiting alterations leading to specific loss of transcripts from the mutated allele. These data indicate that the amount of *BRCA1* available in these cells can be related directly to the number of "active" allele.

Breast cancer is the most common malignancy affecting women, and 5 to 10% of cases are estimated to be familial. Segregation analyses of families with multiple affected individuals led to the identification of the *BRCA1* gene (Miki *et al.,* 1994). Germline alterations (frameshift, nonsense, splice mutations) of this gene appear to account for about one half of inherited breast cancers (Friedman *et al.,* 1994). Although mutations of *BRCA1* were not detected in sporadic human breast cancers, and ovarian tumors exhibit *BRCA1* mutations only exceptionally (Futreal *et al.,* 1994; Mejraver *et al.,* 1995), several experiments have correlated the level of *BRCA1* expression and cell proliferation.

Analysis of the pattern of *BRCA1* expression in mice indicated that *BRCA1* is expressed in rapidly proliferating cell types undergoing differentiation, and suggested that this gene might be implicated in the processes of proliferation and differentiation in several tissues (Marquis *et al.,* 1995). An essential role of *BRCA1* in normal mammalian development has also been shown, since homozygous deletion results, in mice, in embryonic lethality (Gowen *et al.,* 1996; Hakem *et al.,* 1996). Modulations of *BRCA1* expression are also observed in human tissues, a decreased *BRCA1* mRNA level was observed during sporadic breast-cancer progression (Thompson *et al.,* 1995) and, in cell lines, steroid hormones may affect the level of this transcript by altering cellular proliferation (Gudas *et al.,* 1995).

The involvement of *BRCA1* in cellular proliferation has been also addressed in human cell lines, using anti-sense oligonucleotides (Thompson *et al.,* 1995) and in mouse cell lines, using anti-sense RNA (Rao *et al.,* 1996). All these experiments indicate that *BRCA1* negatively regulates cellular proliferation and may therefore act as a suppressor gene in human cancers. Taken together, these data strongly suggest that the level of *BRCA1* mRNA may play an important role in human breast carcinogenesis.

Hybridization methods have been widely used for the detection of *BRCA1* transcripts in many cell types or human tumors. These studies have shown that the *BRCA1*-mRNA content in these samples can be modulated by several factors, including the presence of estradiol for estrogen-receptor-rich cell lines (Gudas *et al.,* 1995) and the tumor types for sporadic human breast cancers (Thompson *et al.,* 1995). However, all the methods used (Northern blots or RNase protection assays) give relative values, so that it is difficult to compare data between experiments. The competitive RT-PCR method appears to be an alternative to these more traditional methods, and has been successfully used to precisely quantitate low amount of RNA (Ramakrishnan *et al.,* 1994; Laghami *et al.,* 1997). This PCR method measures the absolute amount of mRNA in an RNA sample; furthermore, this quantitation is not related to the amount of an another "control" mRNA, which may vary between samples or cellular types. Data obtained from different cell lines or samples can thus be compared directly with each other. In addition, for some experiments (stability, transcription rate) direct quantitation of the amount of mRNA is needed.

We have therefore developed a competitive RT-PCR assay for the measurement of *BRCA1* transcripts. *BRCA1* mRNA molecules have been quantitated in several cell lines, and the effect of actinomycin D, an inhibitor of transcription, on the half-life of *BRCA1* mRNA has been monitored in cell lines using this method.

MATERIAL AND METHODS

RT PCR

A cDNA fragment of *BRCA1* (from position 390 to position 647) was amplified by RT-PCR using C3 primers: 5′ TGT GCT TTT CAG CTT GAC ACA GG 3′ and 5′ CGT CTT TTG AGG TTG TAT CCG CTG 3′ (Friedman *et al.,* 1994). After cloning in a pGEM-T vector (Promega, Lyon, France), the corresponding insert was cut with Van91 I at position 112 and a blunt-end oligonucleotide derived from pS2 sequence, nt −412 to nt −365 (Jeltsch *et al.,* 1983) was ligated at this site and cloned again in the same pGEM-T vector. Then the insert was characterized by sequencing. Since this vector contains a promoter site for T7 RNA polymerase the corresponding RNA was synthesized using the corresponding RNA polymerase according to the instructions of the manufacturer (Boehringer Mannheim, Meylan, France). After purification (DNaseI digestion and phenol-chloroform extraction), this competitor RNA was quantitated by spectrophotometry, and aliquots were diluted in presence of yeast tRNA as a carrier.

After extraction (Dante *et al.,* 1994), RNA was quantitated by spectrophotometry and by electrophoresis on agarose gels of serial dilutions in comparison with known amounts of standard RNA (Boehringer Mannheim). Detection of transcripts were done as described (Dante *et al.,* 1994) with some modifications. Briefly, reactions were performed with 0.3 or 0.6 µg of RNA in 100 µl containing 10 mM Tris-Hcl (pH 8.3), 3.0 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 200 µM each of the 4 deoxyribonucleoside triphosphates. After initial denaturation at 92°C for 2 min, 6 units of M-Mulv reverse transcriptase (Boehringer Mannheim) were added to the reaction mixture and incubated for 35 min at 42°C. Reverse transcriptase was then inactivated by heating (94°C, 3 minutes) and after cooling to 0°C, PCR amplification of the cDNA product was accomplished by adding 0.6 units of Taq DNA polymerase (Boehringer Mannheim). The PCR amplification was accomplished using 35 cycles in a Eppendorf thermocycler in the following conditions: 1 min denaturation at 94°C, annealing at 55°C for 2 min and extension for 3 min at 72°C. In these conditions, heterodimers between PCR products derived from

Contract grant sponsors: Ligue Nationale pour la Recherche contre le Cancer; Association pour la Recherche contre le Cancer.

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Received 25 April 1997; Revised 26 July 1997

mRNA and the competitor were not detected. In addition, control experiments for each RNA sample were performed, omitting reverse transcriptase, to confirm that the signal was the result of RNA-specific and not DNA-specific amplification. Aliquots (17 µl) were then analyzed on 2% agarose gel containing 0.1 µg/ml of ethidium bromide. Photographs of the gels were scanned (UMAX) and the intensity of the 2 bands corresponding to PCR products were determined using image analyzer software (Wayne Rasband, NIH).

Cell lines

Lymphoblastoid cell lines were established from *BRCA1*-mutation carriers, and controls have been described (Serova *et al.,* 1996). HeLa, Lovo clone C5, BT-20 and HBL-100 cell lines were grown as described (ATCC, Rockville, MD; Remy *et al.,* 1993). MCF-7 cells were grown in Dulbecco's modified Eagle's medium (Gibco, Cergy-Pontoise, France) supplemented with SVF 10%, insulin 0.6 µg/ml and estradiol 10^{-8} M. Cells were then seeded in an estrogen-free medium (phenol-red-free medium supplemented with charcoal-treated serum); actinomycin D (4×10^{-6} M) was added 48 hr after seeding and for the experiments in presence of the hormone, estradiol (10^{-8} M) was added at same time. C5 cells were grown in standard medium and actinomycin D was added 48 hr after seeding.

RESULTS

Validation of the RT-PCR method

To determine the amount of *BRCA1* mRNA a competitive RT-PCR assay was developed, using a chimeric *BRCA1* RNA as a competitor. In familial breast cancer, genetic alterations are more frequently localized in the 3′ one third of the gene (Gayther *et al.,* 1995), and only one case of alterations at exons 6 and 8 has been reported (Breast Cancer Information Core). Therefore, in order to amplify *BRCA1* transcripts in a large panel of samples or cell lines, primers were chosen in these 2 different exons (exon 6 and exon 8). A 48-bp double-stranded oligonucleotide was inserted into a cloned RT-PCR fragment of *BRCA1* mRNA (see "Material and Methods"). After cloning, the corresponding chimeric RNA was synthesized using T7 RNA polymerase, and after quantitation it was used as a competitor in the RT-PCR experiments.

In order to evaluate the sensitivity of the RT-PCR, serial dilutions of the competitor were amplified; the amount of the corresponding PCR product was then determined by densitometry on ethidium-bromide-stained agarose gels and plotted against the initial concentration of synthetic RNA. Data obtained indicate that the signal was proportional to the log of the amount of synthetic RNA within a wide range of concentrations, from 3×10^3 to 10^6 copies per assay (Fig. 1*a*,*b*). Moreover, the efficiency of PCR amplification for the cDNA corresponding to the *BRCA1* mRNA fragment and to the synthetic competitor RNA was also compared using serial dilutions of plasmids containing these inserts. Ethidium-bromide staining of the agarose gels indicates that both fragments are amplified at the same rate (data not shown).

When 0.075 µg RNA extracted from HeLa cells is mixed with 0.23 µg of RNA from CHO cell line, the RT-PCR assay gives a value of 28×10^3 copies. Since the primers used in this assay did not amplify the *BRCA1* mRNA of CHO cells, this value is very close to the value obtained (120×10^3, Table I) with 0.3 µg of RNA extracted from HeLa cells. Therefore, as expected from the titration curves obtained with the synthetic competitor (Fig. 1*a*,*b*), the quantitation of *BRCA1* mRNA/µg of total RNA is not modified by the amount of total RNA analyzed.

Quantitation of BRCA1 *mRNA in human cancer cell lines*

After these preliminary experiments, the amount of *BRCA1* mRNA was determined in several cell lines from 0.3 µg of total RNA using the RT-PCR method, as described above. RT-PCR assays were performed from dilution series of the competitor added to a constant amount of sample RNA. The amount of PCR products corresponding to *BRCA1* mRNA and to synthetic RNA was plotted against the initial number of synthetic RNA added to the test tubes (see Fig. 1*c*,*d*). The abscissa at the intersection of the curves

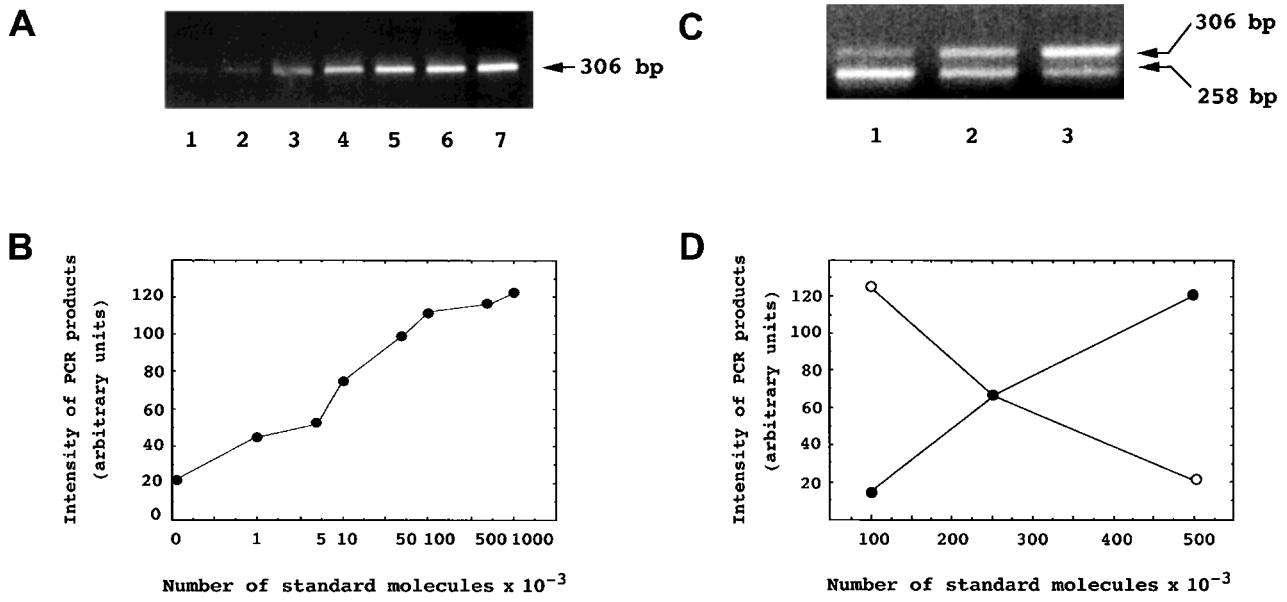

FIGURE 1 – RT-PCR assay of *BRCA1* mRNA. *(a)* 15 µl of the RT-PCR reaction (total volume 100 µl) performed from serial dilutions of chimeric *BRCA1* RNA were analyzed on ethidium-bromide-stained 2% agarose gels; the 306-bp band corresponds to the expected size of the PCR product. Initial concentrations of these standard molecules were: lane 1, 1×10^3; lane 2, 5×10^3; lane 3, 10×10^3; lane 4, 50×10^3; lane 5, 100×10^3; lane 6, 500×10^3; lane 7, 1000×10^3 molecules. *(b)* Diagrammatic representation of the above data; the intensity of the bands corresponding to the PCR product were plotted against the initial number of standard molecules. *(c)* 15 µl of the RT-PCR reaction (total volume 100 µl) performed from 0.3 µg of total RNA of MCF-7 cells mixed with various amount of chimeric *BRCA1* RNA as a competitor; 306-bp band, chimeric *BRCA1* RNA and 258-bp band, wild-type *BRCA1* RNA. Amount of standard molecules: lane 1, 100×10^3; lane 2, 250×10^3; lane 3, 500×10^3. *(d)* Diagrammatic representation of the above data; the intensity of PCR products was plotted against the initial number of standard molecules; ○, 258-bp band (WT *BRCA1* RNA); ●, 306-bp band (chimeric competitor *BRCA1* RNA).

TABLE I – *BRCA1* EXPRESSION IN HUMAN CANCER CELL LINES

	Number of *BRCA1* mRNA copies/0.3 µg ARN	Estimated number of *BRCA1* mRNA copies/cell
MCF-7	250×10^3	21
HBL-100	450×10^3	38
BT-20	68×10^3	6
LoVo clone C5	205×10^3	17
HeLa	120×10^3	10

The amount of *BRCA1* mRNA was determined using a competitive RT-PCR method from 0.3 µg of total RNA. Data obtained for each point (2 to 4 independent assays) indicated that the variations were inferior to 15%. The number of copies/cell was estimated taking 25 pg as the amount of total RNA per cell.

represents an estimation of the equivalence point between the initial amount of the competitor molecules and the number of copies of *BRCA1* mRNA in 0.3 µg of sample RNA. Results (number of mRNA molecules per 0.3 µg of total RNA) obtained with RNA extracted from MCF-7, BT-20, HBL-100, LoVo clone C5 and Hela cell lines are shown in Table I. The abundance of *BRCA1* mRNA appears relatively low, since, if we assume that 1 cell contains 25 pg of total RNA (Wilson *et al.,* 1997), the number of *BRCA1* copies per cell range from 6 copies (BT-20) to 38 copies (HBL100) (Table I). In addition, this low copy number does not seem to be dependent on cell type, since the breast-cancer cell lines exhibit approximately the same level of *BRCA1* mRNA (Table I) than the cervix-cancer cell line (Hela) and the colon-cancer cell line (LoVo clone C5). Analysis of several human cancer cell lines shows that *BRCA1* is expressed in all the cell lines studied at a relatively low rate (6 to 38 copies per cell). These low amounts of *BRCA1* transcripts suggest a relatively low rate of transcription and/or very fast turnover of these molecules.

Turnover rates of BRCA1 *mRNA*

Several methods for half-live determination have been described, including pulse labelling, and short-term promoter activation (Ross, 1995). In our case, none of these methods are easy to handle, since pulse-labelling methods are usually used for mRNA expressed at relatively high level, and little is known about transcription factors involved in *BRCA1* regulation. Therefore, despite the inherent limitations of the use of chemical inhibitor of transcription, determination of the stability of *BRCA1* mRNA was monitored in MCF-7 cells treated with the transcription inhibitor actinomycin D. Although this method does not give a very accurate measure of the half-lives of mRNA, this approach has been widely used to determine an order of magnitude (Ross, 1995).

To determine whether this low copy number is due to very fast turnover of *BRCA1* mRNA, the stability of this transcript was investigated in 2 human cancer cell lines, MCF-7 cells (breast cancer) and LoVo clone C5 cells (colon cancer) during incubation in the presence of the transcription inhibitor actinomycin D. It had been shown, in estrogen-receptor-rich cells, that estradiol (E_2) may modulate the half-life of some hormone-regulated transcripts in actinomycin-treated cells (Ross, 1995). Since MCF-7 cells are estrogen-responsive (Carr *et al.,* 1995), we took this opportunity to investigate the effect of this hormone on the stability of *BRCA1* mRNA. At time 0, MCF-7 cells were treated with actinomycin D, in the absence or the presence of estradiol. At several intervals, RNA samples were prepared and the amount of *BRCA1* mRNA was determined, using the competitive RT-PCR method described above.

These transcripts appear to be relatively stable, since 4 hr after addition of actinomycin D, MCF-7 cells still contain about one half of the initial *BRCA1* mRNA concentration (Fig. 2). The same decay rate was observed in the absence of estradiol (Fig. 2), suggesting that, in these conditions, the stability of *BRCA1* mRNA is not hormone-dependent. In agreement with this observation, it is suggested that estradiol stimulation of *BRCA1* transcription is not due to a direct effect of this hormone. In addition, in actinomycin-treated colon-cancer cells (LoVo clone C5), the level of *BRCA1* mRNA is similar to that observed in the breast-cancer cell line (Fig. 2), suggesting that the stability of *BRCA1* mRNA is not cell-type-specific.

Taken together, these data suggest that the low amount of *BRCA1* transcripts observed in the cell lines analyzed is not a consequence of fast turnover of these molecules. However, *in situ* hybridization methods have shown that *BRCA1*-linked breast tumors can be distinguished from sporadic breast tumors by their *BRCA1* mRNA content (Kainu *et al.,* 1996). We have, therefore, investigated *BRCA1* expression in lymphoblastoid cell lines exhibiting *BRCA1* alterations and in matched controls.

The amount of BRCA1 *mRNA is lowered only in lymphoblastoid cell lines carrying* BRCA1 *germ-line mutations leading to loss of transcripts*

BRCA1 transcripts were assayed in 6 lymphoblastoid cell lines exhibiting different allele-specific *BRCA1* alterations, and in 7 control lymphoblastoid cell lines (Table II). Alteration of *BRCA1* transcripts leads to allele-specific polymorphisms, and cDNA analysis indicated that, in some of these cell lines (IARC 1506, 1514 and 1947), the mutated *BRCA1* mRNA was undetectable (Serova *et al.,* 1996; Puget *et al.,* 1997). In these cell lines we found a reduced level of *BRCA1* mRNA (160×10^3 to 230×10^3 copies/µg of RNA) when compared with lymphoblastoid cell lines expressing one normal allele and one mutated allele (433×10^3 to 513×10^3 copies/µg of RNA) or to cell lines exhibiting no *BRCA1* alterations (400×10^3 to 772×10^3 copies/µg of RNA). These data strongly suggest that the amount of *BRCA1* mRNA in lymphoblastoid cell lines is directly related to the number of ''active'' alleles.

DISCUSSION

Values obtained with our RT-PCR assay are very similar to those obtained with a more classical method. Using a RNase protection assay, other authors (Wilson *et al.,* 1997) have found in the HBL100 cell line 50 copies of *BRCA1* mRNA/cell, while the RT-PCR assay gives 38 copies/cell. However, in the other cell lines analyzed the concentrations of *BRCA1* transcripts are significantly lower than these values, since we found, for example, only 6 copies of *BRCA1* message in the breast-cancer cell line BT-20. This low level of expression does not seem to be associated with instability of the mRNA. In cancer cell lines, *BRCA1* transcripts remain stable several hours after inhibition of the transcription by actinomycin D, and in lymphoblastoid cell lines the level of transcripts was not modified by *BRCA1* mutations. In addition, in lymphoblastoid cell lines carrying *BRCA1* alterations leading to loss of transcripts, the level of the remaining allele is about one half of the level observed in cell lines expressing both alleles.

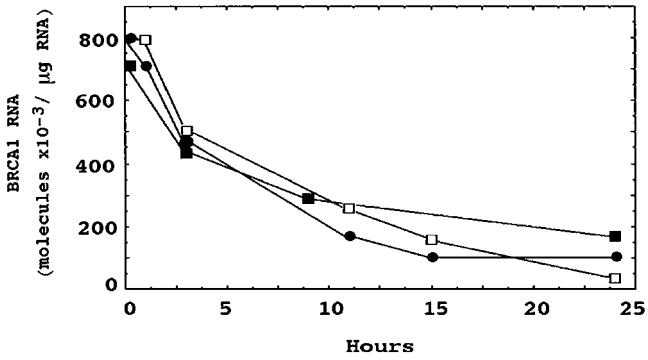

FIGURE 2 – Stability of *BRCA1* mRNA in human cancer cell lines. Actinomycin D was added to the culture medium 48 hr after seeding (see ''Material and Methods''), and *BRCA1* mRNA was assayed at a number of intervals. □, MCF-7 cells in absence of estradiol; ■, MCF-7 cells in presence of estradiol; ●, LoVo clone C5 cells.

TABLE II – *BRCA1* EXPRESSION IN HUMAN LYMPHOBLASTOID CELL LINES CARRYING *BRCA1* MUTATIONS AND IN MATCHED CONTROLS

Cell lines	*BRCA1* mRNA copies/cell	Mutation[1]	Loss of transcript[1]
IARC-1506	6	deletion exon 19	Yes
IARC-1514	5	deletion exon 19	Yes
IARC-1555	13	Cys-Gly (T-G)	No
IARC-1162	11	Arg-Ter	Not done
IARC-1947	4	deletion exon 17	Yes
IARC-1155	13	exon 5 missing in transcript	No
IARC-1468	13	Control cell line	
IARC-1235	10	Control cell line	
IARC-1475	13	Control cell line	
IARC-1625	10	Control cell line	
IARC-1220	10	Control cell line	
IARC-1220	15	Control cell line	
IARC-1102	20	Control cell line	

[1]Mutation and transcript analyses are from Serova *et al.* (1996) and Puget *et al.* (1997). The amount of *BRCA1* mRNA was determined as in Table I. Data obtained for each point (2 to 4 independent assays) indicated that variations were inferior to 15%.

These data, therefore, show that low-level *BRCA1* mRNA is not specifically associated with a cell type, but is observed in all cell lines analyzed. However, it has been reported, from *in situ* hybridization analysis, that *BRCA1*-linked breast-cancer tissues contain fewer *BRCA1* transcripts than sporadic tumors (Kainu *et al.,* 1996), and a decrease in *BRCA1*-mRNA level was observed during sporadic breast-cancer progression (Thompson *et al.,* 1995). Taken together, these findings suggest that some specific events involved in the regulation of *BRCA1* expression are induced in human breast cancers. In line with this hypothesis, it has been reported that the relative proportion between an alternatively spliced message in which the majority of exon 11 has been deleted and the full-length BRCA1 mRNA is modified in some breast- and ovarian-cancer cell lines (Wilson *et al.,* 1997).

The competitive RT-PCR method, developed for determining the level of *BRCA1* mRNA, needs only very small amounts of cells or tissues. This quantitation, therefore, can be performed from fine-needle aspirates, since typically 1 µg or less of RNA can be extracted from such samples. In contrast to traditional protocols, this method does not require the use of radioisotopes or the development or purchase of specific antibodies. This method could be a very useful tool, since it has been suggested that *BRCA1* expression might be linked to tumor progression. However, given the heterogeneity of cell types in fine-needle aspirates, it remains to established whether such assays parallel *BRCA1* expression in normal or tumoral mammary glands.

In conclusion, our results indicate that this technique is feasible for evaluating *BRCA1* transcript levels, and suggest that, in patients with germline alterations in *BRCA1,* the total level of *BRCA1* transcripts is related to the stability of the transcript carrying the alteration.

ACKNOWLEDGEMENTS

S.R. is the recipient of a fellowship from the Ligue Nationale contre le Cancer, Comité de la Saône et Loire, and F.M. is the recipient of a fellowship from the Ligue Nationale contre le Cancer, Comité de la Drôme. The present work was supported by the Ligue Nationale pour la Recherche contre le Cancer and the Association pour la Recherche contre le Cancer.

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