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Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) has been widely used as a control RNA in Northern blotting and in reverse transcriptase–polymerase chain reaction (RT–PCR) analyses. We investigated the expression of *GAPDH* in a large series of primary breast cancers and in MCF7 human mammary epithelial breast cancer cells treated with oestradiol. The expression of *GAPDH* was quantified by a real-time one-step RT–PCR assay, based upon the 5′ nuclease activity of *Taq* polymerase using an Abi Prism 7700 Sequence Detector System (Perkin Elmer, France). Using the Spearman test, *GAPDH* expression was found to correlate inversely with the age of the patients at diagnosis ($P = 0.003$; $r = -0.147$), oestradiol receptors (ER) ($P < 0.0001$; $r = -0.327$) and progesterone receptors (PgR) ($P < 0.0001$; $r = -0.206$). A positive correlation was observed between *GAPDH* expression and the histo-prognostic grading (HPG) ($P < 0.0001$; $r = 0.344$). Moreover, the overall survival (OS) and the relapse-free survival (RFS) were significantly reduced in patients whose tumours showed an enhanced level of *GAPDH* expression (OS, $P = 0.046$; RFS, $P = 0.021$). Multivariate analyses demonstrated that GAPDH was not an independent prognostic factor. Finally, in MCF7 cells treated with oestradiol, a statistically significant dose-dependent increase in *GAPDH* expression was observed. These results show that *GAPDH* expression is associated with breast cancer cell proliferation and with the aggressiveness of tumours. The present study demonstrates that, in cancer, the use of *GAPDH* gene expression should not be used as a control RNA.

Keywords: *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*; Gene expression normalisation; Human breast cancer; Cell proliferation; Tumour aggressiveness; Survival; Reverse transcription–polymerase chain reaction (RT–PCR); Real-time RT–PCR; 5′ Nuclease assay

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key regulatory enzyme of glycolysis, catalysing the formation of 1,3-bisphosphoglycerate, and has been commonly considered as a constitutive housekeeping gene. Therefore, it has been widely used as a control RNA for gene expression analyses by Northern blot and by reverse transcription–polymerase chain reaction (RT–PCR), on the basis that its level of expression is unaffected by the experimental or physiological conditions used.

However, several lines of evidence indicate that GAPDH is involved in various biological processes such as endocytosis, control of gene expression, DNA replication and repair and neuronal apoptosis [1]. Moreover, it has been demonstrated that *GAPDH* expression is substantially increased in human cancers of various origins such as the lung [2], pancreas [3] and cervix [4].

In this study, we investigated the expression of *GAPDH* in a series of 404 unselected primary breast cancers, and analysed the correlations between *GAPDH* expression and the clinical, pathological and biological parameters. We also tested the effect of oestradiol on *GAPDH* expression in MCF7 human breast cancer cells. The expression of *GAPDH* was quantified by a real-time one-step RT–PCR assay, based upon the 5′ nuclease activity of the *Taq* polymerase using an Abi Prism 7700 Sequence Detector System (Perkin Elmer, France).

2. Patients, materials and methods

2.1. Cells

The cell line MCF7 was a gift from G. Leclercq (Brussels, Belgium). It was routinely grown in 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin

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in minimum essential medium (supplemented MEM) containing 10% fetal calf serum (FCS). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

In regulation studies, the cells were seeded at 10 000/cm^2 and were grown for 48 h in supplemented MEM without phenol red containing 10% FCS. They were then incubated for 24 h with phenol red-free supplemented MEM, containing 30 μg/ml transferrin and 2 μg/ml fibronectin. They were finally grown for 72 h in fresh medium containing oestradiol (Steraloïds Inc, Pawling, NJ, USA) and/or 4-hydroxytamoxifen (ICI Pharmaceuticals, Macclesfield, UK). For each concentration, three flasks were set up.

2.2. Patients

This study involves 404 unselected breast tumour samples from patients undergoing surgery for locoregional disease in the Center Oscar Lambret (the Anticancer Center of the North of France) between May 1989 and December 1991. The mean age of the patients was 58 years (range: 26–90). Patients' treatment have been previously described [5]. The median duration follow-up of living patients was 82 months. The number of deaths was 132 (33%) and the number of relapses was 146 (36%).

2.3. ER and PgR assay

Both oestradiol receptors (ER) and progesterone receptors (PgR) were determined by the dextran-coated charcoal method, as previously described [6]. Our laboratory is affiliated to the European Organization for Research and Treatment of Cancer Receptor Study Group, which undertook the quality control of the assays [7].

2.4. Total RNA isolation

Total RNA was isolated from tumour samples (40 mg) and from each flask of MCF7 cells using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France). The disruption and homogenisation of the tumour samples were performed using a Rotor-Stator Homogeniser (Ribolyzer, Hybaid, Paris, France). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was checked by measuring the ratio of the absorbance at 260 and 280 nm, where a ratio ranging from 1.6 to 1.9 was taken to be pure. The absence of degradation of the RNA was confirmed by electrophoresis of the RNA on a 1.5% agarose gel containing ethidium bromide.

2.5. Real-time RT–PCR

GAPDH expression was quantified using the primers, probe and standard provided in the TaqMan GAPDH Control Reagents Kit (Perkin Elmer, Courtaboeuf, France).

The reverse transcription and the polymerase chain reaction were performed in a one-step methodology. The reaction mixture (50 μl final volume) contained 100 ng of total RNA from the cell lines or tumour samples. RT–PCR conditions for *GAPDH* were as follows: 5.5 mM $MgCl_2$, 12.5 units of MuLV reverse transcriptase, 1.25 units AmpliTaq Gold DNA polymerase, 100 nM forward and reverse primers and 100 nM probe. RT–PCR was performed as follows: 48°C for 30 min, 10 min at 95°C and then 15 s at 95°C and 90 s at 60°C for 40 cycles.

A non-template control was included in each experiment. The non-template controls, standard dilutions, tumour samples and cells samples were assayed in duplicate.

The quantification of the PCR products was based upon the TaqMan 5′ nuclease assay [8,9] using a 7700 ABI PRISM sequence detector system (Perkin Elmer–Applied Biosystems, Courtaboeuf, France). Quantification of the starting quantity of a specific mRNA in an unknown sample was performed by preparing a standard curve using known dilutions of the standard RNA. The standard curve was generated on the basis of the linear relationship existing between the Ct value (corresponding to the cycle number at which a significant increase in the fluorescence signal was first detected) and the logarithm of the starting quantity [10]. Quantification in the unknown samples was performed by the software of the 7700 ABI PRISM sequence detector system, which calculated the Ct value for each sample and then determined the initial quantity of target using the standard curve.

2.6. Statistical analyses

All the statistical analyses were done using SPSS (Version 8.0.1F). The correlations between parameters were assessed according to the Spearman non-parametric test. Overall survival (OS) and relapse-free survival (RFS) were studied by the Kaplan–Meier method. The comparison between curves was carried out by the log-rank test. The proportional hazard regression method of Cox [11] was used to assess the prognostic significance of parameters taken in association. A level of $P < 0.05$ was taken to be significant.

3. Results

3.1. Expression of GAPDH in human breast cancer samples

The distribution of *GAPDH* mRNA expression in the tumour samples was not normal and exhibited a wide

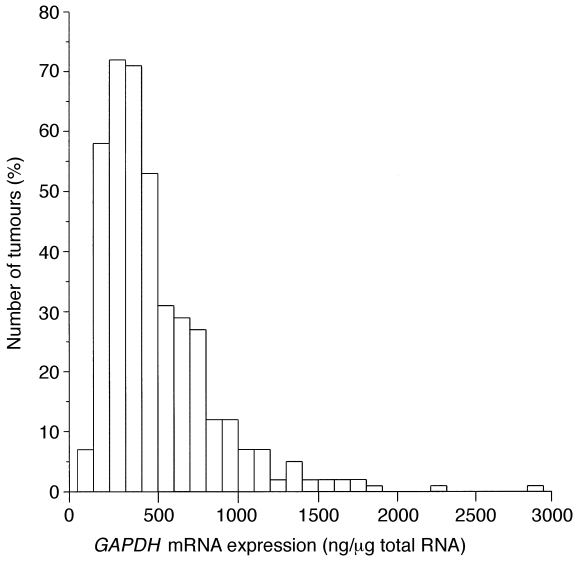

Fig. 1. Distribution of *GAPDH* mRNA expression in 404 breast cancers.

range of values from 0.4 to 2800 ng/μg total RNA (median 337 ng/μg total RNA) (Fig. 1).

3.2. Correlations with biological, clinical, and anatomopathological parameters

Using the Spearman test, *GAPDH* mRNA expression was found to be inversely correlated to the age of the patients at diagnosis of less than 40 years ($P=0.003$; $r=-0.147$), to ER ($P<0.0001$; $r=-0.327$) and to PgR ($P<0.0001$; $r=-0.206$). Furthermore, a positive correlation was observed between *GAPDH* mRNA expression and the histo-prognostic grading ($P<0.0001$; $r=0.344$).

In this population, 71.5% of the tumours were ER-positive and 70.5% were PgR-positive. The classical correlations between ER and PgR ($P<0.0001$; $r=0.65$), ER and age ($P<0.0001$; $r=0.27$), and PgR and age ($P=0.019$; $r=0.113$) were also observed [5,12,13].

3.3. Relationships with survival

With respect to *GAPDH* mRNA expression, the population of patients was stratified into four different classes corresponding to the first, second, third and fourth quartiles. The survival curves corresponding to each sub-group of patients were referred to as numbers 1 to 4 respectively in Fig. 2 and Fig. 3. The OS was significantly reduced in patients who presented tumours with increased *GAPDH* mRNA expression ($P=0.046$, Fig. 2). Similarly, a shorter RFS was observed in patients with increased *GAPDH* expressing tumours ($P=0.021$, Fig. 3).

The *GAPDH* mRNA median value allowed us to distinguish two populations of different prognosis (OS, $P=0.023$; RFS, $P=0.003$). Nodal involvement (OS, $P=0.0001$; RFS, $P=0.0004$), histo-prognostic grading (OS, $P=0.0001$; RFS, $P=0.01$), PgR (OS, $P=0.0004$; RFS, $P=0.01$), ER (OS, $P=0.005$; RFS, $P=0.006$) and tumour size (OS, $P=0.002$; RFS, $P=0.006$) were also prognostic factors. In multivariate analyses, when combining the parameters that had a prognostic value in univariate analyses, GAPDH did not retain its clinical prognostic significance; only nodal involvement (OS, $P=0.03$; RFS, $P=0.04$), tumour size (OS, $P=0.05$; RFS, $P=0.01$), PgR (OS, $P=0.005$; RFS, $P=0.03$) and

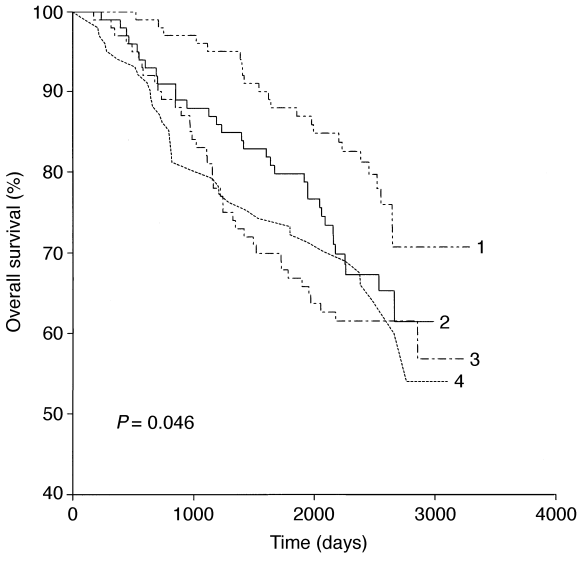

Fig. 2. *GAPDH* mRNA expression and overall survival in the population of patients stratified in quartiles.

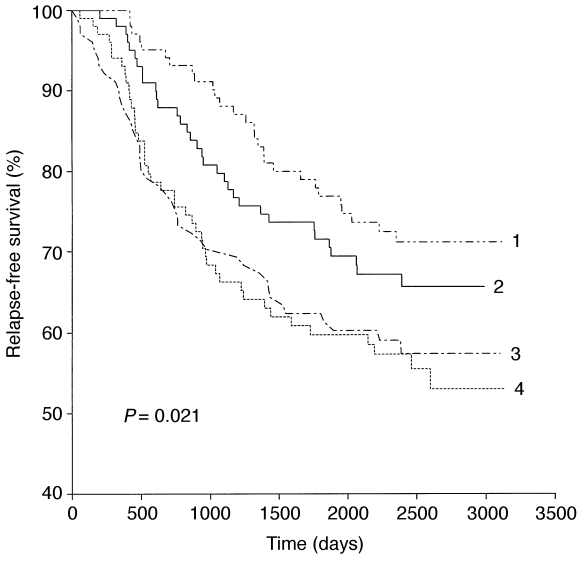

Fig. 3. *GAPDH* mRNA expression and relapse-free survival (RFS) in the population of patients stratified in quartiles.

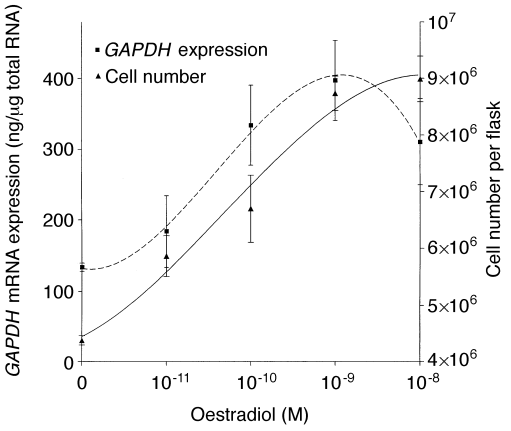

Fig. 4. Effect of oestradiol on *GAPDH* mRNA expression in MCF7 cells.

histo-prognostic grading (OS, $P = 0.005$) were independent prognostic parameters.

3.4. *Expression of* GAPDH *in oestradiol-stimulated MCF7 cells*

The effect of increased concentrations of oestradiol on *GAPDH* mRNA expression is shown in Fig. 4. The MCF-7 cells were treated for 72 h with various concentrations (10^{-11} M–10^{-8} M) of oestradiol prior to the measurement of *GAPDH* expression. The data represent the mean±S.D. of the determinations from three separate flasks. A statistically significant dose-dependent increase in *GAPDH* was observed. The maximum of stimulation was reached at 10^{-9} M oestradiol. At this concentration, the level of GAPDH was increased 3-fold over the control reading at 0 M of oestradiol. Concomitantly, the total number of cells per flask increased until a concentration of 10^{-9} M oestradiol was reached (Fig. 4). The stimulating effect of oestradiol on *GAPDH* mRNA expression was completely abolished by 4-hydroxytamoxifen (10^{-7} M) (data not shown). A statistically significant increase in *GAPDH* expression was also observed when MCF7 cells were stimulated with insulin growth factor I (IGFI) (glycosylated form, 10 ng/ml) and basic fibroblast growth factor (bFGF) (0.5 ng/ml) resulting in a 1.3-fold and 1.4-fold increase, respectively (data not shown).

4. Discussion

In the present study, we have quantified the expression of the *GAPDH* gene in a large series of unselected primary breast cancers, and in MCF7 cells cultured with oestradiol.

In the series of primary breast cancers, our results demonstrated that *GAPDH* gene expression was statistically inversely correlated to oestradiol and progesterone receptor concentrations, and age at diagnosis of less than 40 years and statistically positively correlated to histoprognostic grading. Therefore, the mRNA expression of *GAPDH* seems to reflect the aggressiveness of the tumour. Corroborating this finding, we observed that an increase in the expression of the *GAPDH* gene was also associated with a reduced overall survival and RFS in patients with primary breast cancer. However, in multivariate analyses, GAPDH did not preserve its prognostic value, demonstrating that it is not useful as a prognostic factor.

Several studies have already demonstrated an increased expression of *GAPDH* in lung, pancreatic and cervical carcinomas compared with normal tissues [2–4]. This has been attributed to the increased glycolysis generally found in tumour cells [14]. Moreover, an association between *GAPDH* expression and cell motility and metastatic potential has been observed in Dunning rat prostatic adenocarcinoma cells [15].

In MCF7 human breast cancer cells, we have demonstrated that oestradiol induced a statistical increase in *GAPDH* expression. The effects of oestradiol were dose-dependent and observed as early as 10^{-11} M, and were maximal at 10^{-9} M. It is noteworthy that, in our hands, a statistically significant stimulating effect on *GAPDH* mRNA expression was also observed in MCF7 cells when their proliferation was stimulated with IGF1 and bFGF. This is in agreement with the increased *GAPDH* expression observed in rat fibroblasts in response to epidermal growth factor (EGF) or serum [16] and also described in pre-adipocytes and hepatoma cells treated by insulin [17]. These observations are related to the fact that aerobic glycolysis is stimulated by increasing the proliferation rate of the cells [18]. Therefore, the increase in *GAPDH* expression induced by oestradiol, IGF1 and bFGF reflects the accelerated rate of proliferation, rather than a direct effect of these various agents in regulating *GAPDH* expression.

In conclusion, this study shows that the expression of *GAPDH* is associated with breast cancer cell proliferation and with tumour aggressiveness. Consequently, the use of *GAPDH* as a control RNA is particularly irrelevant in studies investigating markers for proliferation and prognosis.

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