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EORTC Receptor Study Group Report

Technical Evaluation of Thymidine Kinase Assay in Cytosols from Breast Cancers

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Pilot retrospective studies have pointed to the prognostic value of thymidine kinase (TK) in breast cancer. We studied the Prolifigen TK-REA assay (Sangtec Medical, Sweden), usually applied to serum, for TK analysis in breast cancer cytosols. Reproducibility was good, provided that small volume pipetting was performed carefully. The TK assay was not influenced by the short-term storage of cytosols in liquid nitrogen or at −80°C. However, some steps appeared critical for good laboratory practice. The TK level was affected by thawing the cytosols more than twice. Tumour storage in liquid nitrogen should be preferred over storage at −80°C. The components of the homogenisation buffer, especially sodium molybdate and KCl can have a marked influence on results. Finally, linearity problems arose with some cytosols. Thus, although assay of TK in cytosols is aparently simple, care must be taken in practice. The TK-REA kit should be standardised before widespread use in breast cancer.
Key words: breast cancer, thymidine kinase, radioenzymatic assay
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INTRODUCTION

A PROLIFERATION INDEX seems necessary in breast cancer. Evidence for a correlation between growth fraction and clinical behaviour in mammary carcinomas has been derived from thymidine labelling studies [1] and DNA flow cytometry [2]. However, measurement on cytosols prepared for steroid receptor assays would be more widely applicable and suitable for quality controls. Thymidine kinase (TK) is involved in DNA synthesis by the salvage pathway. In recent years, cytosolic TK has been associated with the S-phase fraction [3] and with a high rate of relapse in primary breast cancer [4–6]. In addition, it could be a very potent therapeutic target. The Prolifigen TK-REA kit (Sangtec Medical, Sweden) is commercially available for TK measurement in serum. Promising results were found in pilot studies using this kit on breast cancer cytosols [5, 6]. However, to date, no technical evaluation for routine measurement has been reported. A co-operative study enabled us to analyse the effect of the homogenisation procedure, the composition of the homogenisation buffer, the conditions of cytosol storage and the conditions of dilution in cytosolic TK determination. The practice of the TK-REA measurement in cytosols and the routine use of the assay in breast cancer are discussed.

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MATERIALS AND METHODS

Tumours and cytosols

The breast tumours used in this study were obtained through pathologists after confirmation of the neoplastic nature of the specimens. Tumours were obtained immediately after surgery and stored in liquid nitrogen until used. For cytosol preparation, all procedures were carried out at −2°C. Tumours were homogenised using a microdismembrator or a polytron in 10 volumes of Tris homogenisation buffer, unless otherwise indicated (10 mM Tris–HCl, 0.5 mM DTT, 1.5 mM Na_2EDTA, 10 mM sodium molybdate, 10% glycerol, pH 7.4). Cytosol was obtained by centrifugation of the homogenate for 60 min at 105 000 *g*. Cytosol protein concentrations were determined by the Lowry method [7].

TK assay procedure

The Prolifigen TK-REA assay is a radioenzymatic method optimised for the determination of the TK foetal isoenzyme. Samples are incubated with ATP and ^{125}I-labelled iododeoxyuridine as substrate, at 37°C for 4 h. The enzymatic reaction is then stopped by the addition of aluminium oxide tablets, which only bind the radioactive products. The unreacted substrate is washed off and the bound radioactivity is counted in a gamma counter. The TK activities are calculated from a calibration curve made from a TK standard. Results are expressed in mU/ml (1 U=1.2 $\times 10^{-12}$ katal).

The following modifications in the manufacturer's instructions were necessary for adaptation of the TK-REA assay to cytosolic assay: (1) a high level of TK is found in breast cancer cytosols. To obtain most values directly within the standard curve, cytosols were diluted 1:10 in the kit diluent buffer before assay. Results were converted from mU/ml diluted cytosol to

mU/mg protein. (2) Supplementary points were added to the standard curve. Low standards were prepared from the 80 mU/ml standard by dilution with the kit diluent. 100 mU/ml standard was prepared directly from 25 μl of the 80 mU/ml standard. (3) Separation was performed very carefully. Tubes were vortexed for 30 s every 10 min for 1 h. (4) Controls with low TK levels were supplied by the manufacturer. We added a supplementary control with a higher level.

Statistical methods

The least square linear regression procedure was used to compare the results of two assays.

RESULTS

Distribution of TK levels in breast cancer

TK levels were measured in 852 breast tumours collected in the same laboratory (Marseille, France) for routine assay of steroid receptors. Samples were all homogenised in Tris homogenisation buffer. TK assays were performed by the same technicians. The mean TK concentration was 150 mU/mg protein. The median, the 25th and the 75th percentiles were 75, 35, 222 mU/mg protein, respectively. Using dilution 1:10, 75% of TK levels (2–3 mg protein/ml) were directly within the standard curve.

Variability of TK assay for kit controls

TK results of the low level and the high level kit controls were compared with their expected values for 71 consecutive assays. For both kit controls, the mean coefficient of variation (CV) was 0.8%. In addition, more than 90% of the CV values were in agreement with the 20% CV predicted by the manufacturer.

Within run variability of TK assay for cytosols measured in duplicate

The TK assay was performed in duplicate for 402 cytosols. An excellent correlation was found between duplicate TK levels. The regression curve between duplicates was $y = 0.851 + 0.967x$, $r^2=0.986$. The mean CV between duplicates was 2.9%.

Intra-laboratory variability of TK assay

TK level was measured in 10 cytosols by two technicians from the same laboratory. The regression curve between TK levels obtained by the two technicians was $y = 0.604 + 0.911x$, $r^2=0.965$. The mean CV between technicians was 6.7%. However, small volume pipetting, standard reconstitution and standard dilutions must be performed very carefully. Small imprecisions are highly amplified by dilution.

Inter-laboratory variability of TK assay

TK levels were measured both in the Centre René Huguenin and in Marseille for 26 cytosols using dilution 1:10. A good correlation was found. The regression curve between TK levels obtained by the two laboratories was $y = -5.345 + 0.995x$, $r^2=0.928$. The mean CV between laboratories was 13.4%.

Effect of tumour homogenisation with polytron or microdismembrator on TK assay

Mixed fragmented tissue preparations were obtained from 10 breast cancer tumours stored at −80°C. Samples were homogenised with a microdismembrator and with a polytron. A relatively good correlation was found between cytosol TK levels for tumour homogenisation with a polytron (x) and with a microdismembrator (y): $y = -17.43 + 0.971x$, $r^2 = 0.849$.

Effect of homogenisation buffer components on TK assay

As shown by a recent survey made in French centres, many differences between laboratories still exist for homogenisation buffer composition despite standardisation efforts of receptor study groups. We, therefore, investigated the importance of homogenisation buffer composition in TK measurements.

TK levels were measured for eight breast tumours in cytosols prepared in the presence and the absence of sodium molybdate (10 mM) and KCl (0.4 M) in Tris homogenisation buffer (Figure 1). Low TK levels were always found without sodium molybdate and without KCl. High TK levels were found with both components. High TK levels were also found in some tumours without molybdate and with KCl (samples 1–4), and in other tumours with molybdate but without KCl (samples 5–8).

TK levels were measured for nine breast tumours in cytosols prepared in 10 mM K_2HPO_4 buffer (y) (containing 5 mM DTT, 1.5 mM Na_2EDTA, 10 mM sodium molybdate, 10% glycerol, pH 7.4) and in Tris homogenisation buffer (x). The regression curve between TK levels obtained with the two buffers was $y = -22.16 + 1.213x$, $r^2=0.915$.

Effect of short-term storage of cytosols in liquid nitrogen on TK assay

TK levels were measured both in fresh cytosols and in cytosols stored for 3 days in liquid nitrogen ($n = 14$). Short-term storage of cytosols in liquid nitrogen appeared to be without effect on TK levels. The regression curve between TK levels of fresh cytosols (x) and cytosols stored for 3 days in liquid nitrogen (y) was $y = 1.140 + 1.021x$, $r^2=0.974$.

Effect of short storage of cytosols at −80°C on TK assay

TK levels were measured both in fresh cytosols and in cytosols stored for 3 days at −80°C ($n = 14$). The regression curve between TK levels of fresh cytosols (x) and cytosols stored for 3 days at −80°C (y) was $y = 17.04 + 1.015x$, $r^2=0.978$.

Effect of thawing of cytosols on TK assay

The stability of the TK activity was observed by studying the resistance of the TK levels to freezing and thawing the cytosols

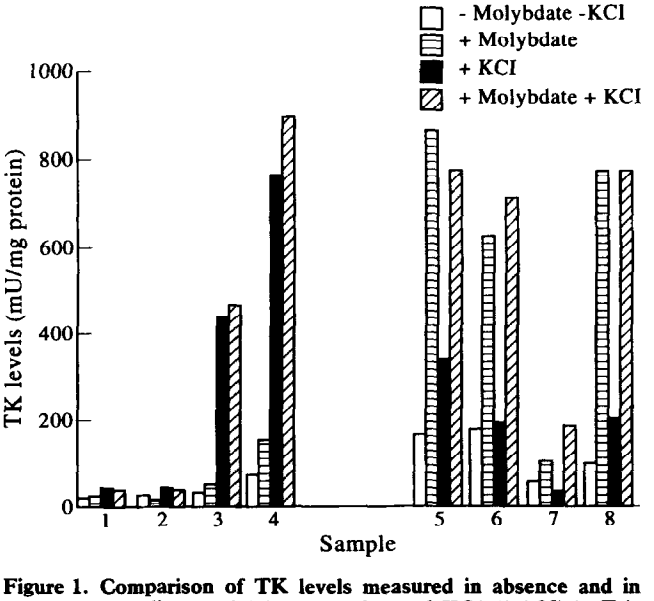

Figure 1. Comparison of TK levels measured in absence and in presence of sodium molybdate (10 mM) and KCl (0.4 M) in Tris homogenisation buffer (10 mM Tris–HCl, 0.5 mM DTT, 1.5 mM Na_2EDTA, 10% glycerol, pH 7.4) (n=8).

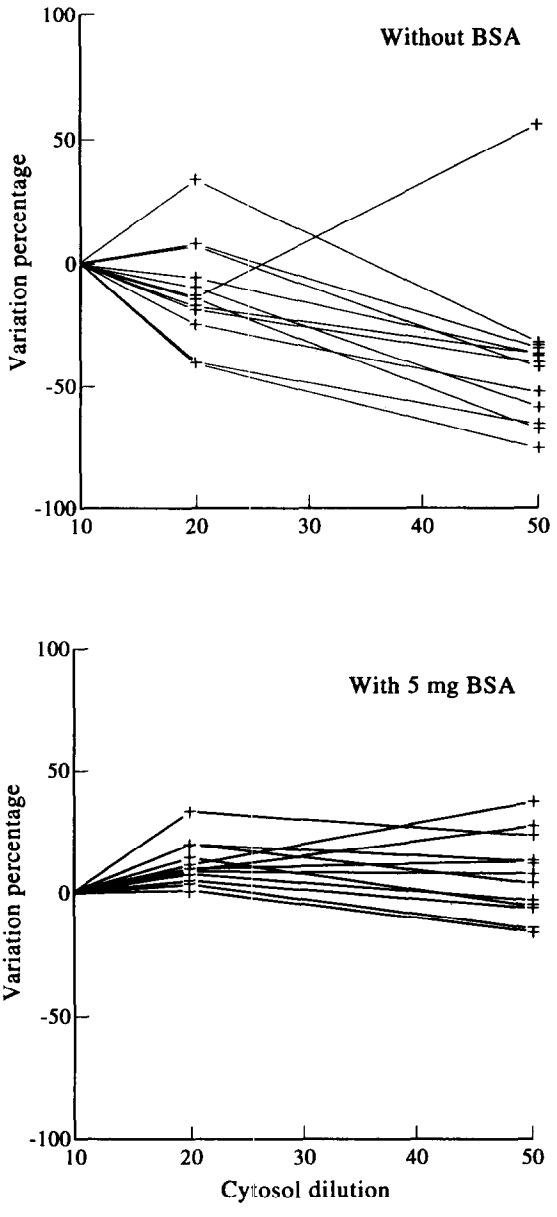

Figure 2. Effect of dilution on TK levels of cytosols, in absence and in presence of 5 mg BSA in the kit diluent (n=13). Variation percentage compared to values obtained with 1:10 dilution.

one, two and six times before assaying (n = 9). TK results after thawing twice (y) were not modified in comparison with thawing once (x), and the regression curve obtained was $y = 5.958 + 0.950x$, r^2=0.994. However, the regression curve between TK results after thawing six times (y) and after thawing once (x) was $y = 23.93 + 0.676x$, r^2=0.961.

Effect of long storage of tumours at −80°C on TK assay

TK levels were measured in cytosols prepared from 19 fresh breast tumours (x) and from the coarse remaining powders stored for 6 months at −80°C (y). The regression curve between TK levels of fresh tumours (x) and stored tumours (y) was $y = 39.16 + 1.020x$, r=0.682. Thus, large variations were found, possibly due to the effect of long-term storage and to tumour heterogeneity.

Effect of cytosol dilution on TK assay

TK levels were measured in parallel in cytosols diluted 1:10, 1:20 and 1:50 (n = 13). Dilutions 1:10 were performed in the kit diluent. Dilutions 1:20 and 1:50 were performed in the homogenisation buffer with a further 1:10 dilution in the kit diluent. A poor linearity was found for dilutions higher than 1:10 and low TK levels were generally observed with dilution 1:50. However, in most of the cytosols, addition of bovine serum albumin (BSA) (5 mg/ml) in the diluent buffer improved linearity of dilutions (Figure 2).

DISCUSSION

TK is easily measured in serum using the TK-REA radioenzymatic assay. We studied the TK-REA assay in breast cancer cytosols according to classic validation methods. The following modifications seemed of importance for assay on cytosols. Supplementary standard points and controls are first recommended. Cytosols should, in addition, be diluted before assay. Using Tris homogenisation buffer (10 mM Tris–HCl, 0.5 mM DTT, 1.5 mM Na_2EDTA, 10 mM sodium molybdate, 10% glycerol, pH 7.4) and dilution 1:10 in the kit diluent, 75% of TK levels (2–3 mg protein/ml) were directly within the standard curve.

Certain steps of TK-REA assay in cytosols are well controlled. Repeatability and reproducibility are good, provided that pipetting and standard reconstitution are performed very carefully. Tumour pulverisation may be performed with a microdismembrator or a polytron. Short storage of cytosols in liquid nitrogen or at −80°C is possible.

Other steps, however, emerge as being critical for good laboratory practice. The TK level is affected by thawing the cytosols more than twice. Tumour storage in liquid nitrogen should be preferred to storage at −80°C. Components of the homogenisation buffer, especially sodium molybdate and KCl, can have a marked influence on results. Finally, linearity problems arise with some cytosols. Addition of BSA (5 mg/ml) in the diluent buffer seems to improve the linearity of dilutions.

Promising pilot results were found using the TK-REA assay in breast cancer cytosols. However, although the assay is apparently simple, care must be taken in practice. The TK-REA kit should be standardised in collaboration with the end-user before widespread clinical application in breast cancer. This, together with a deeper understanding of patient subsets, where it may be of particular value, will be important to more fully define the importance of thymidine kinase.

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