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Quality Control of Cathepsin-D Measurement by the EORTC Receptor Study Group

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Cathepsin-D is a lysosomal protease that can serve as an important prognostic cytosolic factor in breast cancer. To evaluate the performance of a commercially available immunoradiometric assay kit, 15 lyophilised cytosol preparations containing various levels of cathepsin-D have been sent to 13 participating EORTC laboratories for quality control purposes. The between-laboratory variation, when expressed as coefficient of variation, did not exceed 24%. The within-laboratory variation was assessed by analysing five samples of the 15 specimens containing lyophilised material from a homogeneous cytosol pool. The mean within-laboratory coefficient of variation was 7%. Analysis of human breast tumour cytosols in one of the participating EORTC laboratories resulted in a median within-assay variation between duplicate measurements of 2.1% (n=408), with a between-assay variation of a pooled human breast tumour cytosol of 4.6% (n=11).
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INTRODUCTION

MUCH EFFORT has been put into research on cell biological prognostic factors which may predict tumour cell behaviour in breast cancer. Oestrogen and progesterone receptors were the first factors of which the presence in the primary tumour was found to be associated with a longer relapse-free and overall survival of the patient. In addition, the steroid receptor status of the primary tumour appeared to be helpful in assisting the physician in his choice of treatment for metastatic disease [1, 2]. The oestrogen and progesterone receptor contents of breast tumour biopsies are now routinely measured worldwide, and quality assessment of the assays in large scale multicentre trials are common practice in Europe and in the USA [3–6].

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Although very useful, knowledge of the steroid receptor status in combination with patient (age, lymph node status, menopausal status) and tumour characteristics (size and differentiation grade of the tumour) does not always give sufficient information for accurate prediction of survival/response for any individual patient. For this reason several new, potentially independent, biological prognostic factors have, therefore, been proposed [7]. One of these factors is cathepsin-D, a lysosomal protein which belongs to the family of proteases [8]. It has recently been described as an important prognostic factor in breast cancer cytosol. High levels of cathepsin-D appeared to be associated with a worse prognosis, also in the subgroup of patients with no lymph nodes involved [9–11].

In view of the potential importance of the knowledge of the cathepsin-D status of the primary breast tumour, a quality control trial involving measurement of cytosolic cathepsin-D values has been organized by the EORTC Receptor Study Group. Results of this trial, employing a commercially available radiometric immunoassay used in 13 participating EORTC laboratories, are reported in the present paper.

MATERIALS AND METHODS

Preparation of lyophilised tissue material

Human normal uterine and human breast tumour tissues, after storage at −70°C, were used to prepare quality control material. Tissue specimens were pulverised in the frozen state (liquid nitrogen) to fine homogenous powders by means of a Microdismembrator (Braun, Melsungen, Germany) [12]. Calf non-endocrine muscle tissue was processed additionally and powders were mixed with the human tissue powders to enable adjustment to adequate protein levels in diluted human cytosol preparations. Cytosols were prepared by stirring the obtained tissue powders with phosphate buffer (20 mmol/l K_2HPO_4/KH_2PO_4, 1.5 mmol/l EDTA, 3.0 mmol/l NaN_3, 10 mmol/l $NaMoO_4$; pH 7.5, w/v: 1/7) for 30 min at 4°C, and subsequent centrifugation of the homogenates at 32 000 g for 30 min at 2°C. 5 ml aliquots of the cytosols were lyophilised, and glass vials containing the lyophilised tissue powders were sealed

under vacuum and stored at 4°C until shipment by regular surface mail.

Source of quality control samples

The sources of the samples provided for the quality control trial (QC-trial) of the EORTC Receptor Study Group are presented in Table 1. For an assessment of the within-laboratory variation the shipment contained five vials, i.e. numbers 1, 2, 6, 9 and 12, from a homogeneous cytosol pool.

Distribution and reconstitution of quality control samples

Vials were mailed from the Sint Radboud Hospital in Nijmegen exclusively to laboratories which are members of the EORTC Receptor Study Group. No prior information was given to the participants with respect to the composition of the samples. The participants were instructed to reconstitute the lyophilised material in 5.0 ml of deionized water containing 10% (v/v) glycerol. Protein values of the reconstituted cytosols were in the range of 3–4 mg/ml.

Cathepsin-D assay

Cathepsin-D was assayed by an immunoradiometric kit (ELSA Cath-D kit, CIS Bio international, Gif-sur-Yvette, France) according to the protocol provided by the company in 1/40 and in 1/80 dilutions of the reconstituted cytosols, both in duplicate. All procedures used by the participants were exactly as described in the kit-protocol. The assay procedure involves the binding action of two monoclonal antibodies [13, 14] recognising the 34 kD chain of the protein (allowing measurement of precursor 52 kD form, and the processed 48 kD, and 34 kD mature forms [8] of the protein). The first antibody (D7E3) is coated on the ELSA solid phase and the other one (M1G8) is radiolabeled with iodine-125.

All 13 participants used kits with the same lot number.

Statistics

One-way analysis of variance (ANOVA) was applied to evaluate within- and "true" between-laboratory variation from the data of the five identical samples, after performance of tests for normal distribution according to David [15], and homogeneity of variance, Cochrane test [15]. A value was considered as an outlier when it exceeded the 99.9% confidence limits of the *t*-distribution.

Table 1. *Source of vials for quality control trials*

Vial number	Source
1=2=6=9=12	Pool of human breast tumour biopsy specimens
3	A human uterus
4	Pool of human breast tumour biopsy specimens
5	Pool of human breast tumour biopsy specimens
7	Pool of human breast tumour biopsy specimens
8	Pool of human breast tumour biopsy specimens
10	A human myoma
11	A human uterus
13	Pool of human uteri
14	Pool of human breast tumour biopsy specimens
15	Pool of human uteri

Table 2. Between-laboratory variation

	Cathepsin-D		
Vial number	Mean (pmol/ml)	S.D.	CV (%)
1	136	17	13
6	130	15	11
2	130	16	13
9	129	17	13
12	128	11	9
14	69	9	13
4	68	6	10
8	66	9	13
5	64	8	12
7	55	7	13
13	31	4	13
3	19	4	24
11	18	4	24
15	17	3	19
10	undetectable		

The cathepsin-D values presented are the means, standard deviations (S.D.) and coefficients of variation (CV%) calculated from the data obtained in the 13 participating laboratories analysing 15 vials. Vial numbers correspond to the numbers given in Table 1.

RESULTS

Between-laboratory variation

The mean cathepsin-D values and the between-laboratory variations calculated from the data obtained in the 13 laboratories analysing the 15 vials of the quality control trial are shown in Table 2. In one of the samples (no. 10), the cytosol of a myoma, no cathepsin-D was detectable. The Table shows that the between-laboratory coefficients of variation (CV) ranged from 9 to 24% (CV). At the higher cathepsin-D concentrations (>30 pmol/ml, Table 2) the CV did not exceed 13%. Below that level the CVs amounted to 24%.

Within-laboratory variation

Five samples of the 15 specimens listed in Table 1 contained lyophilised material from a homogeneous cytosol pool. These five "identical" vials enabled estimation of the within-laboratory variation. The cathepsin-D values and the within-laboratory variations are shown in Table 3. The mean values obtained by the 13 participating laboratories ranged from 113 to 152 pmol/ml cytosol [131(12) pmol/ml cytosol; mean (S.D.)] without detectable evidence of non-normal distribution. The within-laboratory CVs ranged between 2 and 18%. The within-laboratory variance of lab M was significantly elevated ($P < 0.01$, Cochrane test) as compared to the others. This could not be ascribed to the presence of an outlier.

"Gross" between-laboratory variation, expressed as the coefficient of variation (CV) of the results obtained by each laboratory for the same sample, comprises both within- and "true" between-laboratory variation. ANOVA was applied to the data from the remaining 12 laboratories for evaluation of overall within- and "true" between-lab variation. The overall within-laboratory CV was 6.8%. A significant "true" between-laboratory variation ($P < 0.001$) with a CV of 7.4% was detected. The "gross" overall between-lab CV was 11.0%.

Table 3. Within- and between-laboratory variations

Laboratory	Cathepsin-D (pmol/ml cytosol) Vial 1	Vial 2	Vial 6	Vial 9	Vial 12	Mean	S.D.	CV %
A	137	134	139	126	128	133	6	4
B	121	122	125	151	143	131	13	10
C	140	127	154	135	150	141	11	8
D	130	128	119	117	117	122	7	5
E	130	134	127	122	121	126	6	5
F	135	131	136	139	132	135	3	2
G	145	145	128	117	130	133	12	9
H	123	110	119	112	120	117	6	5
I	129	136	126	144	123	131	8	6
J	117	117	116	101	115	113	7	6
K	128	105	109	113	128	117	11	9
L	146	134	160	152	144	147	10	7
M	185	171	138	150	117	152	27	18

The cathepsin-D values presented are the means obtained in the participating laboratories analysing five "identical vials" (numbers 1, 2, 6, 9 and 12). The right row in the Table represents the within-laboratory variation (CV %).

Within-assay and between-assay variation

For the assessment of the within-assay variation of cathepsin-D, assays were performed on a series of 408 human breast tumour cytosols in one of the laboratories of the EORTC Receptor Study Group. Eleven kits with the same lot number were used and the assays were performed in duplicate on cytosols which were prepared exactly as recommended by the EORTC Breast Cancer Cooperative Group [16] for assays of the estradiol and progesterone receptor. Protein concentrations were adjusted to 1.0 mg/ml prior to a further 1/80 dilution during the assay. The CVs of 408 duplicates ranged from 0 to 34%. The median CV was 2.1% and 10th and 90th percentiles were 0.4% and 5.2%, respectively).

The between-assay variation was assessed in the same laboratory by assaying in duplicate a pooled human breast tumour cytosol sample for cathepsin-D levels in 11 consecutive runs with kits with identical lot numbers. A coefficient of variation of 4.6% was observed for the pooled cytosol sample containing 93.2 (4.2) pmol cathepsin-D/ml [mean (S.D.)] when assayed in a 1/80 dilution. For the control vial included in the kit, which vial was stated to contain 1.50 pmol cathepsin-D/ml, a between-assay variation of 5.2% [1.54(0.08) pmol/ml]; was obtained (sample assayed without further dilution).

DISCUSSION

Multi-centre studies on any bio-marker are only meaningful if all the participating laboratories are assessed through a common external quality control program [3–6]. Laboratories organising themselves in this way can contribute greatly to the assessment and improvement of assay performance [17]. Moreover, measurement of precision is a fundamental process in the assessment of the quality of an analytical procedure. This applies the more for the assay of newly introduced cell biological factors which may have clinical significance. One of these promising factors is cathepsin-D, which is a lysosomal protease [8]. The 52 kD pro-cathepsin-D protein is a phosphoglycoprotein synthesised by all cells but its concentration is elevated in breast cancer cells. Pro-cathepsin-D is the precursor for different mature cathepsin-D forms of 48, 34 and 14 kD molecular weight forms [20]. It is induced by oestrogens and secreted in excess by ER-positive human breast cancer cells [21]. In hormone-independent breast cancers, cathepsin-D is produced and secreted in a constitutive way [22]. It has been shown to be a marker of tumour invasiveness [23], and its measurement in cytosolic extracts of primary human breast tumours may particularly be of prognostic use for breast cancers without any detectable node invasiveness [9–11]. Hence, in case of elevated levels, adjuvant therapy might be considered. Before initiation of multicentre clinical trials, based on either cathepsin-D or any other new cell biological factor which may have clinical significance, it is essential that the data produced in one laboratory is strictly comparable with that in another participating laboratory. This can only be achieved if all laboratories agree to participate in an external quality assessment scheme.

The primary goal of the present study was to evaluate the performance of a commercially available kit for the assay of cathepsin-D. As in earlier quality control trials for steroid receptor analysis we have chosen to use lyophilised cytosol preparations [18, 19]. The between-laboratory variation did not exceed 13% for samples with cathepsin-D concentrations higher than 30 pmol/ml (Table 2). Since the protein concentrations of the reconstituted cytosols analysed were in the range of 3–4 mg/ml, it can be concluded that the assay performed very well for samples containing cathepsin-D concentrations even far below the reported clinically significant cut-off level of 24–78 pmol/mg protein [9, 10]. The within-laboratory variation as assessed by analysing five identical vials did not exceed 10%, except for one laboratory (18% for laboratory M; Table 3). For these five vials the between laboratory variation was higher than the within-laboratory variation. Without results from this latter lab, the overall within-lab variation was less than 7%. This does not completely account for the variation in the results from all laboratories for the same sample, i.e. the "gross" between-assay variation, being 11% overall. A significant "true" between-laboratory effect with CV of 7.4% was detected. In addition to the observed low between- and within-laboratory variations, the reliable performance of the cathepsin-D kit was furthermore substantiated by the observed very low within- and between-assay variations (< 5%).

Provided that quality of the assay remains with forthcoming batches and between batch variation is low, it may be concluded that the commercially available cathepsin-D kit which has been used in these studies is very reliable with respect to its performance. The precision and between-laboratory comparability of this convenient two-site radiometric immunoassay should further facilitate the clinical evaluation of this new marker in oncology.

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A Dose-escalation Study of Recombinant Interferon-alpha in Patients with a Metastatic Carcinoid Tumour

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The efficacy of interferon alpha-2b in doses up to 12 × 10^6 IU three times weekly was studied in 21 patients with a metastatic carcinoid tumour. Of these 21 patients, 19 were evaluable for response. Patients were treated with escalating dosages of interferon alpha-2b: 3 × 10^6 IU, 6 × 10^6 IU and 12 × 10^6 IU. The escalation was performed every 8 weeks when no objective tumour regression was observed. Patients were also evaluated for biochemical response and symptomatic improvement. One objective tumour regression was observed. Of the 15 patients with elevated 5-hydroxyindole acetic acid (5-HIAA) excretion, 5 (33%) had a more than 50% decrease in 5-HIAA excretion. Relief of symptoms occurred in 11 patients (58%). This improvement was already apparent during the initial 8 weeks of treatment. Increasing the dose to 6 or 12 × 10^6 IU interferon alpha-2b did not result in further symptomatic improvement. In contrast toxicity was considerable with the higher dosages of interferon alpha-2b. It is concluded that low dose interferon alpha-2b (3 × 10^6 IU) three times weekly is as effective as higher dosages of interferon alpha-2b at ameliorating symptoms of the carcinoid syndrome.
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INTRODUCTION

CARCINOID TUMOURS are uncommon neoplasms thought to arise from basogranular argentaffin cells in the base of the intestinal crypts [1]. Carcinoids of truly malignant behaviour most commonly originate in the small bowel [2, 3]. These tumours most commonly metastasise to the liver, with bone and lung being the next most frequent sites [4]. Carcinoids are often slow growing tumours and even in the presence of metastatic disease survival can be several years without treatment [5].

Treatment options for metastatic carcinoid should always include surgical procedures [1, 2], hepatic artery occlusion [6], or chemotherapy [7–9]. Chemotherapy has produced only minimal evidence of benefit. Response rates with the most favourable regimens are in the 20–30% range, and these