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A monoclonal antibody driven biodiagnostic system for the quantitative screening of breast cancer

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Abstract

A prospective clinical parametric study comprising women afflicted by breast cancer and otherwise healthy participants was undertaken. The mean plasmatic concentration of putative leucine amino peptidase and nucleoside diphosphate phosphotransferase enzymatic complex in breast cancer cases was significantly elevated [43.9 ± 2.8 μg ml^{-1} ($n = 9$)] when compared to those found in otherwise healthy women [8.07 ± 0.14 μg ml^{-1} ($n = 8$)]. Women without images compatible with any tumours ($n = 13$) had a mean concentration of 10.77 ± 1.49 μg ml^{-1}. The mean value obtained in women with fibroadenomas was 10.15 ± 0.81 μg ml^{-1} ($n = 6$) and with cystic fibrosis mastopathy 8.75 ± 0.28 μg ml^{-1} ($n = 7$). The efficacy of a tandem quantitative biodiagnostic system as a parametric screening tool for the early detection of breast cancer is underlined, raising the possibility of increasing the cost effectiveness of current imaging non-parametric technologies.

Introduction

Despite all efforts made to reduce its incidence and mortality, breast cancer remains a public health priority affecting women of all races and geographical location, and, in particular, those from industrialized and developing nations (Parkin 2001). In these regards, a prime biotechnology application within the Health Sector both in the fields of biodiagnostics and biotherapeutics, remains the isolation and characterization of biomarkers with the assistance of monoclonal antibodies. Acknowledging that early detection of breast cancer enables a more efficacious treatment and disease management, various health institutions and governments have encouraged the widespread use of breast self examinations and screening mammography, particularly amongst women at risk. Nonetheless, the efficacy of the aforementioned screening methods has been recently debated (Larkin 2001, Elmore *et al.* 1998, Fletcher & Elmore 2003). From a genetic perspective, the estimated relatively low frequency of mutations on genetic markers, such as BRCA1 and BRCA2 (Wooster *et al.* 1995, Newman *et al.* 1998), equally restrains the usefulness of genetic biotechnology testing systems to less than 10% of the general population. Amid all suspected risks factors, oestrogens and oestrogen-stimulated cell cycle regulators remain the most direct and important determinants related to the early onset and progression of breast carcinogenesis (Shekhar *et al.* 1997). Re-

cent population-based studies have shown mounting evidence in support of an increased risk of developing breast cancer, particularly of invasive lobular tumours, through the sustained use of hormone replacement therapy (HRT) (Chlebowski *et al.* 2003, Beral 2003).

It is within this context that we have proceeded, along with others, to study changes in the distribution and circulating levels of both leucine amino peptidase (LAPase, EC3.4.11.1) and nucleoside diphosphate phosphotransferase (NDP-kinase, EC 2.7.4.6) in response to sustained cell mediated stimulation by 17-β-estradiol (Pulido-Cejudo 1994, 2001, 2003, Pulido-Cejudo *et al.* 1994, 1999, Lacombe *et al.* 2000, Roymans *et al.* 2002). Considering their enzymatic stability, steroid dependency and their role in the immediate early events of cellular proliferation within the cell cycle and cell spreading, NDP-kinase and LAPase could be valuable quantitative proteomic indicators of early tumour formation. Using a quantitative first order assay (Pulido-Cejudo *et al.* 1994) and specific monoclonal antibodies against both NDP-kinase and LAPase respectively, it was shown that these enzymes are elevated in women affected by breast cancer (Pulido-Cejudo *et al.* 1999). This work has led to the subsequent development of a quantitative blood-based tandem immuno-assay for breast cancer screening (Pulido-Cejudo 2003). In this communication we summarize the results of a first report pertaining to both the activity profile of this monoclonal antibody driven biodiagnostic screening test system as well as of its estimated accuracy in the early detection of breast cancer.

Materials and methods

Blood sample collection

Blood samples were collected in glass, vacuum tubes containing 1.5 ml anticoagulant solution (136 mM dextrose in 42 mM citrate buffer, pH 5) and sent, by air courier, to The Canadian Breast Cancer Laboratories, Canbreal, in Ottawa, Canada for analysis. Each sample was identified by a unique bar code, which was recorded by the clinicians at the time of blood withdrawal as the reference sample ID. This ID ensured that all patient information remained confidential and blind during the entire analytical process. The analysis was performed through a capturing tandem immuno-assay with the aid of two monoclonal antibodies against NDP-Kinase and LAPase respectively (Pulido-Cejudo 2003). Separation of blood cells from plasma and cell debris was achieved through two subsequent centrifugations at 4 °C. The first centrifugation of the collected blood samples was performed at 450 × *g* for 20 min, followed by a second centrifugation at 4900 × *g* for 10 min.

Table 1. Volumetric measurements required for the preparation of the reference standard curve.

	Std[a]0	Std1	Std2	Std3	Std4
RAS[b] (μl)	0	5.5	11	27.5	55
RB[c] (μl)	200	194.5	189.5	172.5	145

[a]Standard.
[b]Reference Antigenic Standard.
[c]Reaction Buffer.
Each standard curve was prepared and analysed in duplicate.

Plasma analysis

Clear plasma samples resulting from the second centrifugation were analyzed using an Interston-based immuno-assay system. Each assay system consists of: Interstron I, Interstron II, reference antigenic standard (RAS), stabilizer detection buffer (SDB), detection buffer (DB) and reaction buffer (RB). Prior to the commencement of the assay, three additional buffers were prepared: (i) coating buffer (50 mM Na_2CO_3/$NaHCO_3$, pH 9.6); (ii) 1× PBS (phosphate buffered saline, pH 7.4); and (iii) blocking buffer (3% w/v BSA in 1× PBS). Each of the buffer components (RB, DB, and SDB) and RAS were diluted with reverse osmosis water to their corresponding 1× solution concentrations. Interstron I was diluted 1:20 with coating buffer and the plasma samples were diluted 1:200 with 1× RB. Plates included in the system aforementioned were coated overnight at 4 °C with 200 μl of the diluted Interstron I. After coating, the plate was washed three times with 1× PBS and blocked with 200 μl of blocking buffer for 1 h at room temperature. Samples for the reference standard curve were prepared according to Table 1. Once the standard solutions were prepared, 80 μl of each standard concentration was transferred to the designated reference area on the plate. Blank controls were made from 1:1 (v/v) RB and DB. The diluted plasma samples were transferred to the plate with 80 μl in each well at quadruplet repeats. Then, after 4 μl of Interstron II was added to each plasma sample, standard curve wells and blank controls. Finally, while operating under indirect light exposure, 20 μl 1× SDB was quickly added to all wells. The plate was then incub-

Table 2. Differential comparison between plasmatic concentrations of NDP-Kinase/LAPase complex against histopathological and/or mammographic analyses. Plasmatic concentrations of NDP-Kinase/LAPase complex were determined in diluted plasma samples using Interston-based immuno-assay system

Sample type	Mean (μg ml^{-1})	SD (μg ml^{-1})	Median (μg ml^{-1})	95% CI* median (μg ml^{-1})	Range (μg ml^{-1})
Negative biopsy for breast cancer ($n = 8$)	8.07	0.14	1.73	1.3–38.4	1.3–52.6
Positive biopsy for breast cancer ($n = 9$)	43.94	2.85	46.76	22.2–59.7	1.2–70.4
Images compatible with any tumour (benign or malign) ($n = 13$)	10.77	1.49	1.96	0.5–36.9	0.3-46.8
Fibrocystic mastophatyc ($n = 7$)	8.75	0.28	2.11	0.5–49.9	0.6–49.9
Fibroadenoma ($n = 6$)	10.15	0.81	1.7	–	1.3–52.6

*Confidence Interval was determined at 95% according to mean value $\pm$ two standard errors of the mean value (Wassertheil-Smoller 2004).

ated at 37 °C for 30 min following which, fluorometric readings were obtained at an excitation wavelength of 340 nm and an emission wavelength of 400 nm, respectively.

Using a systematic calibration analysis according to lot numbers of each of the quantitative capturing tandem biodiagnostic testing systems employed, plate readings together with plate arrangements and sample bar code information were entered into a database to compute the NDP-Kinase/LAPase levels (expressed in μg ml^{-1}). Inter and intra plate variations were monitored based on the plating of RAS on three plates with 8 sets of curves per plate and duplicated measurements for each set of curve. Overall, the sensitivity of the analysis varies from 1% over the detection range (or 14% CV) at the nearest zero point (Std1) to 5% over the detection range (or 5% CV) at the far end of the detection range (Std4).

Evaluation of clinical data

Clinical parametric values were determined based on the available pathophysiological reports and were established using means, median, standard deviations and 95% confidence intervals for the median. Based on these parametric values, the accuracy of diagnosis was also determined using the complete and accurate reporting procedure as per outlined in the standards for reporting of diagnostic accuracy (STARD) (Bossuyt *et al.* 2003). Ethical guidelines from The World Medical Association in Medical Research involving human subjects were followed during the entire study.

Results and discussion

Patient characteristics

From the 47 women that were invited to participate in the prospective clinical parametric study, 36 women between the ages of 35 and 70 years old were selected. Eleven females were excluded because of pregnancy, status involving lactation, exposure to chemotherapy, radiation treatment or to cytotoxic agents such as anti-oestrogen or selective oestrogen-receptor modulators. Mammograms were performed on each of the participants with 17 women having images compatible with tumours and 19 women showing no tumour evidence. Biopsies were performed on those 17 women whose images were compatible with tumour presence. Nine (53%) were confirmed as breast cancer, 8 women (47%) were confirmed with benign tumours (6 fribroadenoma, 1 phyllodes tumour, 1 cystic fibrosis mastopathy). The non-parametric analyses of mammograms of the remaining 19 women were: 6 (31.5%) with cystic fibrosis mastopathy and 13 (68%) without any images suggestive of the presence of tumours.

Establishment of the thresholds and diagnostic accuracy

The results of plasmatic concentration values of NDP-Kinase and LAPase in relation to phenotype of samples analyzed according to histopathology and mammography are summarized in Table 2. There were 3 women (false positives) with values ranging between 36 and 52.6 μg ml^{-1} and one woman (false negative) with breast cancer confirmed by biopsy with 1.24 μg ml^{-1}. The diagnostic accuracy reveals a sensitivity of 90% and a specificity of 82% (Figure 1). The corresponding estimated positive predictive value of

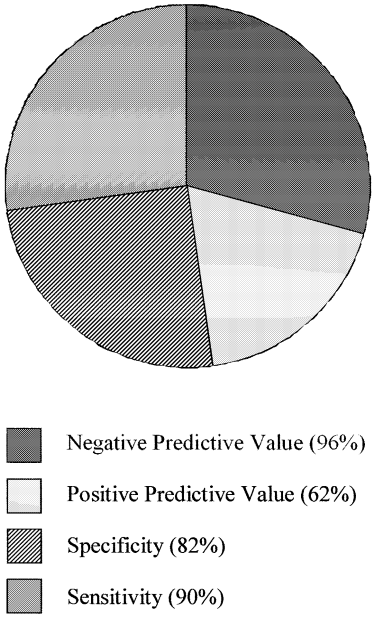

Fig. 1. Estimated screening parameters of the quantitative biodiagnostic system. The sensitivity and specificity of the monoclonal antibody driven biodiagnostic screening test system was determined considering as true positive the patients with mammography-based diagnosis further confirmed by histopathological analysis of the corresponding tumour biopsies.

the capturing tandem immuno-assay was 62% and the negative predictive value was estimated at 96%.

Conclusions

The results compile within this study, show that the monoclonal antibody-driven tandem biodiagnostic system used as a parametric assay, has the ability to function as an accurate, non-invasive blood-based surrogate test for screening open populations. This biodiagnostic system has both the potential and ability to enhance the cost effectiveness of the early detection of breast cancer in women older than 35 years old due to its rapid sampling and quantitative analysis. This is also due primarily to the test sensitivity, negative predictive value as well as the significant differences detected between the mean plasmatic concentrations among confirmed patients with breast cancer compared to those found in the remaining groups tested. Additionally, concentration values were similar for fibroadenoma, cystic fibrosis mastopathy and women without any image compatible with tumours respectively. In terms of the 3 women considered as false positives, the presence of other pathological processes involved with oestrogen dependency was detected. The false negative case was a canalicular cancer. Taken together, these results strongly suggest that this innovative test could be useful as a potential screening biodiagnostic parametric system for an early detection of breast cancer in combination with subsequent confirmatory image and or histopathological non-parametric diagnostic procedures.

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References

Beral V, Million Women Study Collaborators (2003) Breast cancer and hormone-replacement therapy in the million women study. *Lancet* **362**: 419–427.

Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Lijmer JG, Moher D, Rennie D, de Vet HC (2003) Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. *Clin. Chem.* **49**: 1–6.

Chlebowski RT, Hendrix SL, Langer RD, Stefanick ML, Gass M, Lane D, Rodabough RJ, Gilligan MA, Cyr MG, Thomson CA, Khandekar J, Petrovitch H, McTiernan A (2003) Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women. *JAMA* **289**: 3243–3253.

Elmore JG, Barton MB, Moceri VM, Polk S, Arena PJ, Fletcher SW (1998) Ten-year risk of false positive screening mammograms and clinical breast examinations. *N. Engl. J. Med.* **338**: 1089–1096.

Fletcher SW, Elmore JG (2003) Mammographic screening for breast cancer. *N. Engl. J. Med.* **348**: 1672–1680.

Lacombe ML, Milon L, Munier A, Mehus JG, Lambeth DO (2000) The human Nm23/nucleoside diphosphate kinases. *J. Bioenerg. Biomembr.* **32**: 247–258.

Larkin M (2001) Breast self examination does more harm than good, says task force. *Lancet* **357**: 2109.

Newman B, Mu H, Butler LM, Millikan RC, Moorman PG, King MC (1998) Frequency of breast cancer attributable to BRCA1 in a population-based series of American women. *JAMA* **279**: 915–921.

Parkin M (2001) Global cancer statistics in the year 2000. *Lancet Oncol.* **2**: 533–542.

Pulido-Cejudo G (1994) Monoclonal antibodies directed against NDP-Kinase. Canadian Patent 2,108,533, Ministry of Health Canada.

Pulido-Cejudo G (2001) A monoclonal antibody against estrogen stimulated leucine aminopeptidase. Canadian Patent 2,303,505, Ministry of Health Canada.

Pulido-Cejudo G (2003) Tandem immnuo-assay for cancer. US Patent 6,521,415 B1, Ministry of Health Canada.

Pulido-Cejudo G, Gagnon J, Leclerc JM, Jamison K, Gordon J, Campione-Piccardo J (1994) Measurement of nucleoside diphosphate kinase-Nm23 activity by anion-exchange high-performance liquid chromatography. *J. Chromatogr. B, Biomed. Appl.* **660**: 37–47.

Pulido-Cejudo G, Jamison K, Alderdice S, Burke K, Pulido A (1999) Critical interdependency: from the role of estrogen on breast cancer to the susceptibility of women towards HIV infection. In: Skouteris GG, Nicolson GL, eds. *Intermolecular Cross-Talk in Tumour Metastasis*. Vol. 311, 1st edn. Amsterdam: IOS Press, pp. 123–136.

Roymans D, Willems R, Van Blockstaele DR, Slegers H (2002) Nucleoside diphosphate kinase (NDPK/NM23) and the waltz with the multiple partners: possible consequences in tumour metastasis. *Clin. Exp. Metastasis* **19**: 465–476.

Shekhar PVM, Werdel J, Basrur VS (1997) Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. *J. Natl. Cancer Inst.* **89**: 1774–1782.

Wassertheil-Smoller S (2004) *Biostatistics and Epidemiology*, 3rd edn. New York: Springer.

Wooster R, Bignell G, Lancaster JM, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G (1995) Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**: 789–792.