Model building and quantitative analysis of a tandem immuno-capturing assay as a screening tool for breast cancer

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Received May 10, 2004; Accepted July 6, 2004

Abstract. The onset of breast cancer appears to occur, on average, a decade earlier in Mexican women in comparison to American or European women. Early detection and prevention of breast cancer are of crucial importance to increase survival and improve quality of life. Based on the molecular elucidation of critical events leading to breast carcinogenesis, a tandem immuno-capturing blood test was developed as a quantitative population screening assay in view of providing a cost-effective and non-invasive alternative to population screening. Clinical analysis of 63 Mexican women within an age group of 35-70, revealed that Interstron activity increases from 800±65 IU JPA (Interstron Units) in the asymptomatic normal women to 994±110 IU JPA in the symptomatic/benign group, reaching 1289±81 IU JPA in the cancerous group. Accordingly, activity thresholds were established at 800 and 1200 IU JPA respectively, encompassing three risk groups: (i) Healthy Otherwise Normal (<800 IU JPA); (ii) Grey Risk Area (>800 and <1200 IU JPA), and (iii) At Risk group (>1200 IU JPA). Taking into account both baseline and clinical case reports, the Healthy Otherwise Normal group and the At Risk group were mostly homogeneous in nature, comprising a population of normal and cancer patients respectively. The Grey Risk group is heterogeneous, likely reflecting a transitional nature towards a potential early stage of breast disease development. Based on these results, a screening algorithm was developed as the underlining principle for population surveillance encompassing over 30,000 Mexican women. The current screening results have enabled us to objectively prioritize medical attention to approximately 1 in 8 women out of the general population mapped within the At Risk group. Overall, our findings suggest that monitoring Interstron activity units provides a valuable quantitative screening analysis as to selectively streamline the population of women in need of early medical counseling and/or mammography, thereby enhancing both the quality and cost-effectiveness of preventative population surveillance programs targeting breast cancer.

Introduction

Early detection of breast cancer enables more efficacious treatment and disease management (1). Although current guidelines for breast cancer screening include both breast examination and mammography (2), monthly breast self-examination is currently being reassessed in view of recent findings underscoring its limitations (3). Mammography remains the primary and the most acceptable screening tool (4). Nonetheless, the benefits of screening mammography may be limited among women <50 years of age since such population tend to have higher breast density, making false-positives mammograms more likely (5,6). In Mexican women this presents a dilemma since the average onset of breast cancer is about a decade earlier than the American or European women, consequently general guidelines on mammography would not cover 50% of Mexican women (7,8). From a genetic perspective, the estimated relatively low frequency of mutations on genetic markers such as BRCA1 and BRCA2 restricts the usefulness of genetic testing to <10% of the general population (9-11). Furthermore, adaptation of genetic tests or mammography as a generic screening procedure may encounter serious logistical and economical implications. For instance, according to 2002 census, there are an estimated 33.7 million women currently living in the United States with age ranging between 45 and 64 (U.S. Census, 2002). Assuming a typical minimal cost of $100 per mammogram (National Cancer Institute, 2002), if this population of women had subjected themselves to annual

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Key words: breast cancer, prevention, quantitative immuno-capturing blood test
mammograms, the cost would have exceeded $3 billion. This would have represented approximately half of the actual 2002 reported cost for medical treatment ($6 billion) of breast cancer in the United States (American Cancer Society, 2002). The necessity for efficient disease control and cost reduction entails the articulation of a more effective population surveillance program to promote prevention and early detection of breast cancer.

Amongst all suspected risk factors, estrogen and estrogen-stimulated cell cycle regulators remain the most direct and important determinants in relation to the onset and progression of breast carcinogenesis (12-18). Recent population based studies have revealed strong evidence indicating that sustained use of HRT (Hormone Replacement Therapy) is associated with an increased risk of breast cancer, particularly of invasive lobular tumors hence hampering the efficiency of mammography (18-22). Furthermore, enhanced estrogen-driven mammary epithelial proliferative capacity can be partially explained by lack of expression of functional BRCA1 peptide (23), hence providing a link between life style and environmental factors to genetic risk factors believed to be involved in the early onset of breast cancer. In brief, the identification of quantifiable estrogenic risk factors that are indicative of lifestyle and environmental influences would be an intuitive logistic behind an optimal population surveillance program.

It is within this context that we have introduced the concept of monitoring the molecular onset of breast tumour formation and metastasis through critical estrogen-interdependent cellular markers (24). Changes in the distribution and circulating levels of both the Leucine Amino Peptidase (LAPase, EC 3.4.11.1) and the Nucleoside Diphosphatase Phosphotransferase (NDP-K, EC 2.7.4.6) in response to sustained cell mediated stimulation of 17β-Estradiol have been amply documented (24-30). In view of their enzymatic stability, steroid dependency, and crucial role in the immediate early events of cellular proliferation within the cell cycle and cell spreading, NDP-K and LAPase are valuable quantitative cellular predictors of early tumour formation. Using a quantitative first order assay and specific monoclonal antibodies against both NDP-Kinase and LAPase respectively, it was shown that these enzymes were elevated in women affected by breast cancer (24,26-28).

This pioneering work has led to the development of a blood-based tandem immuno-capturing assay for breast cancer screening (29,30). This report summarizes the results from a clinical analysis performed in Mexico with the assistance of the Department of Oncology of the Mexican General Hospital. Apart from confirming the efficacy of the immuno-capturing assay aforementioned as found in previous studies, this clinical study was intended to establish empirical thresholds for a putative population screening algorithm. Cross-matching of the reported pathology classification with the estimated plasma activity levels of Interstron activities has led to the definition of three distinctive groups: Healthy Otherwise Normal Women (<800 IU mL⁻¹), Grey Risk Area (800 to 1200 IU mL⁻¹) and At Risk group (>1200 IU mL⁻¹). Based on the established clinical thresholds, a population surveillance algorithm was developed and subsequently deployed to perform a mega field analysis on the general population of Mexican women. The feasibility of adopting the tandem immuno-capturing blood test as a pre-filter for prioritizing medical attention to the At Risk group is discussed in terms of its efficiency and efficacy.

Materials and methods

Study design

Objective setting. Results from two studies are summarized in the Results and Discussion sections. The first study was the clinical trial that established the analysis model for the Interstron/Biodecan assay. The main objective of this study was to quantify the difference between normal and cancerous subjects in terms of Interstron activities. Following the determination of segregation thresholds, a population analysis was subsequently carried out to evaluate the efficiency and efficacy of Interstron/Biodecan as a quantitative screening tool for breast cancer.

Sample size estimation. For clinical analysis the response variable was Interstron activity, which was a continuous parameter. Therefore, z-statistics or t-statistics was expected to be used to evaluate the significance of the difference exhibited between the groups of subjects. Sample size formula from z and t-statistics based on two-tailed tests are shown in Equation 1 and 2 below, where α is the type I error, B is the type II error, σ denotes the difference in population, and σ denotes standard deviation. Equation 1: \( n = \frac{Z_{\alpha/2}^2 + Z_{\beta}^2 \cdot B^2}{\sigma^2} \) and Equation 2: \( n = \left( \frac{Z_{\alpha}^2 \cdot \sigma^2}{\sigma^2} + \frac{Z_{\beta}^2 \cdot \sigma^2}{\sigma^2} \right) \).

The above sample size formulas can be reduced to a function of effective size \( \Delta \), which is the ratio of group difference to the standard deviation (31). In Fig. 1, sample size \( n \) was plotted as a function of \( \Delta \) at a confidence level of 0.95, and power level of 0.80 and 0.90. Our previous clinical data suggested that \( \Delta = 1 \). Therefore, according to Fig. 1 the minimum sample size is around 16-17 per group at a power of 0.80 and 22-23 at a power of 0.90. The chosen target sample size for the clinical trial was 30 per group, which brings the total expected enrollment to 60. The actual enrollment was 63, with 32 and 31 women in the case and control groups, respectively.
Inclusion/exclusion criteria. Since Mexican women are known to have early onset of breast cancer (7,8), the age group of interest was set at 35 to 70, including younger women than the typical target group for mammography. For the clinical study, healthy and Mexican women with cancer in this age group were selected at random, with the exclusion of pregnant women, nursing mothers during the first 4 months of lactation, and patients undergoing chemotherapy, radiation treatment or taking cytotoxic agents, anti-estrogens or selective estrogen receptor modulators. For the population surveillance program, all women of the same age group were randomly enrolled based on voluntary basis. The only exclusion criterion was women with serious contagious diseases.

Case/control criteria. During the initial patient enrollment of the clinical study, case control was assigned based on the available medical diagnosis of the patient which comprised of medical examination, mammography, ultrasound, and biopsy analyses. A case was defined to be a woman with confirmed or suspected malignant breast cancer and control was defined to be randomly recruited women under the same age group with no confirmed breast cancer. Later, the case/control criteria were re-evaluated after full medical examination and analysis of all patients by the Mexican General Hospital. Based on the biopsy reports, 18 out of the 32 women in the case group were confirmed with malignant stage I to IV breast cancer whereby these women were considered true cases. Among the 31 women in the control groups, 15 had no breast cancer related symptoms, while 17 had one or more symptoms such as pain in the breast or nipple secretion. Overall, this information was taken into consideration in the statistical analysis presented in the Results and Discussion sections.

Masking. Patient information was blinded to the investigators who conducted the Interstron activity analysis. Case reports were made available only after all activity readings were determined.

Ethical considerations. Ethical Guidelines (WMA, 2000) from The World Medical Association in Medical Research involving human subjects were followed throughout the entire study (34). Members of ICT reviewed the design of the clinical trial and approved the protocol. An interview was conducted prior to formal enrollment of participating women to determine baseline patient information and to extend an invitation to enter the study on a strictly voluntary basis without affecting the ongoing diagnosis and treatment of each participant. Information collected from each patient included physical condition, examination of mammary glands, reproductive history, clinical history, family history, life style and dietary habits. All collected information was entered into a coded database, strictly maintaining the identity and confidentiality of each participant. Prior to patient recruitment, clinical personnel involved in the study were trained in Good Clinical Practices, including the adequate completion of all related and proper documentation relevant to the study.

Coordination and monitoring. Recruitment for the clinical cohort was coordinated by the Mammography Clinic of the Oncology Service in collaboration with the General Hospital of Mexico in early 2002. The overall quality control and monitoring of the clinical trial were conducted by the Investigation Science and Technology (ICT, S.A. de C.V.) in Mexico. The population analysis program was initiated in April 2003 under the supervision of the Secretary of Health of Mexico. As of July 18, 2003, the program had completed Interstron/Biodecan analyses on 32,958 Mexican women.

Medical profiling of patients enrolled in the clinical study was performed at the General Hospital of Mexico. Mammographic images were obtained by using a Cenovision Mod 2230491 General Electric equipment. Mammograms were interpreted by the Imaging Service in coordination with the Oncology Service at the General Hospital. Mammary tumor biopsies were practiced on selective tissue samples.
Laboratory on plasma proteinase MMP-9, which is usually
Based on Interstron activity values, women within this group
chemotherapy or medication may also exert adverse effects
Nonetheless, it is conceivable that other factors such as
group has reported that plasma proteinase MMP-9
elevated levels in cancer patients (47). This
consistent with the recent findings from Brookhaven National
cancer treatment such as radiation therapy can suppress the
Interstron activity (unpublished data). This observation is
Women within the Grey Risk Group constitute a
heterogeneous population of normal, benign and malignant
cases with complex activity varying between 800 and 1200
IU_{ANA} (Fig. 7). Interestingly, this group possesses all the
characteristics of a true transitional population with a
differential pathophysiological and clinical distribution (49).
Based on Interstron activity values, women within this group
may represent the potential conversion of both asymptomatic
women with yet undetected maladies as well as of
symptomatic benign lesions to potentially malignant breast
carcinomas.

The Grey Risk group can be further streamlined into a
Tumor Risk group with the assistance of clinical information.
Taking into account the presence of both abnormal mammary
glands and presence of symptoms clinically reported during
enrollment, this population can be depurated revealing a
predominant tumor-bearing population. About half of these
tumors are of benign pathology and the other half were
identified as malignant. More interestingly, most of the
malignant cases are of early stage (I-II) carcinomas, reaffirming
the transitional nature of the Grey Risk group.

Similar to the Healthy Otherwise Normal group, the
At Risk group (>1200 IU_{ANA}) is relatively homogeneous in its
composition (Fig. 7). Women within this group had clear
presence of symptoms, abnormal mammary glands and poor
histopathological outcome of tumour biopsies (malignant
tumours in Stage I-II or III-IV respectively). There were 3
individuals in the At Risk group that were classified as
pathologically normal. Two out of these three cases had
either presence of breast cancer symptoms or abnormalities
in the mammary glands, the nature of which was to be
determined by follow-up examinations. Therefore, only one
patient was considered a true false positive. Compared to
the tumor risk group streamlined from the Grey Risk Area,
women in the At Risk group appeared to have higher
Interstron activity readings and were in advanced stages of
breast cancer.

**Quantitative interpretation of risk factors.** The available
participant reports on the clinical cohort enable us to gain
some quantitative insights into the common risk factors for
breast cancer (Table II). Overall, the average activity for the
asymptomatic normal group is 800±65 IU_{ANA} with the breast
cancer patient population averaging 1289±81 IU_{ANA}. A similar
difference is observed when the cohort is subdivided into age
groups of >50 years and <50 years. Consistent with the current
age dynamic trend of breast cancer incidence the older group
(>50) has higher Interstron activities than the younger group,
correlating with the more incidence of advanced breast
cancer in this group (40-43,50). It is noteworthy that the
average of the asymptomatic normal group is about the same
for both the older and younger populations, indicating that
the baseline Interstron activity is age-independent.

Due to the limited availability of clinical reports, it is
difficult to make conclusive correlations between Interstron
activities and detailed population characteristics at this stage.
However, we did observe a higher average activity in patients
with BMI >30 (1169.7 IU_{ANA}) versus patients with BMI <25
(931.3 IU_{ANA}), consistent with other literature findings indicating
that breast cancer is associated with obesity (45,46). The age
of women at first child birth also seems to place a risk factor
in breast cancer development with the older first time mothers
(1162.3 IU_{ANA}) having higher Interstron activities than the
younger mothers (514.4 IU_{ANA}). Overall these observations are
consistent with past and present risk factors associated to
the early pathophysiological events leading to breast cancer
(50-52).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Avg. activity (IU_{ANA})</th>
<th>p-value (T &lt; t)</th>
<th>DF</th>
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<tr>
<td>Age</td>
<td></td>
<td></td>
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<tr>
<td>≥50</td>
<td>1156.1</td>
<td>0.43</td>
<td>54</td>
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<tr>
<td>&lt;50</td>
<td>967.2</td>
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<tr>
<td>&lt;50</td>
<td>1091.7</td>
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<tr>
<td>AN</td>
<td>1486.6</td>
<td>0.05</td>
<td>13</td>
</tr>
<tr>
<td>≥50</td>
<td>785.9</td>
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<td></td>
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<tr>
<td>Age at first birth</td>
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<tr>
<td>≥30</td>
<td>1162.3</td>
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<td>6</td>
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<tr>
<td>&lt;18</td>
<td>851.4</td>
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<tr>
<td>BMI</td>
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<tr>
<td>≥30</td>
<td>1169.7</td>
<td>0.36</td>
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<tr>
<td>&lt;25</td>
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<td>Tumor type</td>
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<tr>
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</tr>
<tr>
<td>Benign</td>
<td>993.9</td>
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<tr>
<td>Breast cancer</td>
<td>1289.1</td>
<td>0.009</td>
<td>25</td>
</tr>
<tr>
<td>AN</td>
<td>800.0</td>
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</table>

AN, Asymptomatic normal; BMI, body mass index calculated as kg/m^{2}. The p-values cited are the results from two-tailed heteroscedastic t-test examining the significance of the hypothesized mean difference (Δ), and DF represents the degree of freedom.
Integration of population surveillance algorithm and advance in methodology. Following the delineation of the Interstron activity thresholds and classification of risk groups, a systematic screening algorithm has been proposed (Fig. 8). During initial testing, women are grouped into the Healthy Otherwise Normal, Grey Risk Area, and At Risk categories pending on their Interstron activity levels. It is suggested that the Healthy Otherwise Normal group be examined every year. If the test result reveals an intermediate level of activity, women should be recalled for a physical examination and confirmatory test 6 months after the initial testing. If the confirmatory test assesses the woman in the same risk category, she will be assigned to a physician to closely monitor Interstron activity every 6 months. At the same time, women can be given professional advice on lifestyle and dietary habits in order to reduce potential cancer risk. Priority is given to the At Risk group (≥1200 IU/ml), which has the highest potential to develop malignant tumors. It is advised that these women undergo mammography, ultrasound or other techniques to detect and verify the presence of potential tumor growth. After prognosis, the Interstron/Biodecan test could still play an active role as to evaluate treatment efficacy and predict potential relapses by monitoring overall trend changes in the three main group distribution described according to their corresponding putative thresholds.

A similar algorithm has been adopted in the population analysis in Mexico involving more than 33,000 women. Preliminary population analysis reveals that ~12.7% of women are within the At Risk group (Fig. 9). Currently, these women are given prioritized medical attention in terms of follow-up examinations and mammography, whereby Interstron/Biodecan may function as a quantitative surveillance tool as to early detection of breast cancer. In this regard, taking into account the actual analytical rate and logistics of the Interstron/Biodecan assay, equating to 10,000 participants screened per month at a single testing centre, the target population for a more comprehensive evaluation was considerably reduced to 1 in 8 women. This proportion singles out the effectiveness of Interstron/Biodecan as a viable quantitative screening tool to assist mammography and other image technologies to selectively focus resources within the At Risk Population group.

In light of the current demand for a greater efficiency in population-based breast cancer screening, an automated multi-tasking Interstron Processing Analytical system Nova Integratum AMT/3000 is in development. The robotic system is designed to further reduce labor intensity and increase the level of systematic standardization though centralized data handling. In the near future, more field testing might unfold the presence of yet additional risk factors as to provide additional quantifiable correlations between overall exposure to both endogenous and exogenous estrogen (reflected as an estrogenic window during a woman’s lifespan) and Interstron activity levels.

Acknowledgements

This study was conducted and supported in part by the Canadian Biotechnology Strategy Grant # BT001032, the Canadian Breast Cancer Laboratories, Canbreal Theragnostics International Inc. and Columbia Laboratories. The authors also acknowledge support from Health Canada and the National Research Council of Canada. The outstanding coordination efforts from the General Hospital of Mexico under the directorship of Dr F. Higuera (Dr E. Arana, Dr H. Miranda-Hernández, Dr. J. Martin and Dr G. Perez-Palacios), as well as members from the Mexican National Cancer Institute (Dr J. de la Garza, Dr T. Ramirez and Dr Villaseñor) and members from the Columbia Laboratories in Mexico (Dr H. Hidalgo, Ms. L. Molina, Mr. O. Lopez, and Mr. A. Arias) are gratefully appreciated. Assistance from other past and present members of Canbreal Theragnostics International Inc. and Columbia Laboratories are equally valued. In addition, the authors would like to express gratitude toward women who participated in the clinical and population studies. In memory of Dr Javier Pulido Alor, Medical Practitioner and Scientist (1924-1988).

References
