Cervical acid phosphatase: a new biomarker of cervical dysplasia

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BACKGROUND: *Cervical acid phosphatase (CAP) has recently been described as a biomarker labeling abnormal squamous cells on Pap smears (USPTO #6,143,512). The enzyme activity is presented as a red, granular deposit on a modified Papanicolaou background. This unique property was utilized for development of a test and tools intended for cervical cancer screening.*

METHODS: *We conducted a multicenter, random assignment, assessor blinded, 2-group (test and control), and split-sample design clinical trial on 1,500 subject/specimens to assess safety and efficacy of the new test in comparison with the control for cervical cancer screening in standard Pap test environment. Safety was measured with frequency, severity and relation of adverse events. Efficacy was measured with primary endpoints (portion of positive/abnormal specimens detected, and the false negative rate), and with accuracy (sensitivity/specificity) and predictive values as secondary efficacy endpoints.*

RESULTS: *In March 2003, the recruitment was completed and the first thousand cases were evaluated. There were no serious or related adverse events in both groups. Minor, unrelated adverse events were rare and insignificantly distributed in both groups. Primary endpoints: A: Portion of positive/abnormal specimens detected: Pe (new test) = 0.17, Ps (pap test): 0.082; Ps' (American standard): 0.07. $Pe \geq Ps + \delta$, for $\delta = 0.5Ps$. B. False negative rate: $Pe = 0.05$, $Ps' = 0.10$. Test sensitivity: 0.81, specificity: 0.97, PPV: 0.83, NPV: 0.96. Chi-square between test and controls 40.69101 was greater than the critical value of 3.841 ($p<0.05$).*

CONCLUSION: *We concluded that CAP had added to visibility of Pap test and has enabled cytoscreeners to significantly improve detection of positive/abnormal specimens and reduce false negative rate.*

KEY WORDS: *Cervix Dysplasia; Colposcopy; Acid Phosphatase; Biological Markers; Cervix Neoplasms; Vaginal Smears*

INTRODUCTION

In the middle of the last century, the Pap test was introduced and promoted as a screening test for selection of women at risk for cervical cancer. Application of this test has resulted into a dramatic reduction of both mortality and morbidity of cervical cancer in the countries where this test had been made available. More than 50M Pap tests are performed annually in the U.S., with 3.5M (7%) being classified as positive/abnormal.

However, there are still 4,500 deaths and 13,000 new cervical cancer cases each year (1). Studies have shown that 20% of new cancer cases in the U.S. have never had or had a negative Pap test within 3-5 years before disease progress (2). The high false negative rate is the major obstacle of Pap test, otherwise the most successful cancer screening test available (1). This problem persists in spite of recent improvements of Pap test achieved with introduction of liquid-based Pap (LBP) technology (3) automation, HPV testing (4), and better interpretation of results (5).

Is there any alternative? Between 1960 and 1980, few articles in medical literature described the presence of an intracellular acid phosphatase activity in cervical cancer (6,7), and in vaginal secretions originating from 44 patients suffering from cervical and uterine cancer (8). This information has never reached major reference cytology books, probably because normal female genital

superficial epithelium does not contain acid phosphatase, while detecting this enzyme in vaginal fluids has been used in forensic medicine as an indicator of seminal acid phosphatase (9). There were no other data until 1997 when Markovics raised the question whether this enzyme could play a more important role for detecting cervical dysplasia on Pap smears, and could assist for reducing false-negative readings (10). In 1998, we published our pilot results in the Archive of Oncology (16).

Since the description of the new Cervical Acid Phosphatase-Papanicolaou Test (CAP-PAP Test, trademark MARKPAP™ for visualization of cervical acid phosphatase (CAP) inside abnormal cervical cells on Pap smears (9), it has become possible to explore the nature of this enzyme as a biomarker for cervical dysplasia, and as a possible surrogate endpoint for detection of cervical intraepithelial neoplasia (CIN). This article is presenting evidence (collected from a clinical laboratory trial using MARKPAP™ Technology in a second, research, arm of the routine cervical cancer screening) in favor of CAP playing more important role in cervical pathology than it was previously anticipated (11,12).

Cervical acid phosphatase as a biomarker

Normal cervical epithelium contains acid phosphatase, but the enzyme activity is gradually reduced subsequently to the maturation from basal to intermediate cells. Superficial cells are always negative. However, abnormal intraepithelial growth such as hyperplasia, dysplasia (mild and severe) and cancer are always positive. This discrepancy between enzyme activity inside normal and abnormal cells, makes cervical acid phosphatase a natural biomarker for detecting abnormal growth. Pap test is performed with the use of an L shaped spatula and endocervical brush. The specimen is collected with scrapping (under a slight pressure) a full circle of cervical epithelium, and turning the brush inside the *orificium externum cervicis*. Abrasive collection of specimen removes the superficial layer and reveals deeper layers. If CIN (cervical intraepithelial neoplasia) is present, this technique radically increases the probability that a certain number of abnormal cells will be collected with the specimen. Positive specimens must be further investigated (colposcopy, biopsy, histology).

Method for marker presentation

The MARKPAP™ Test is a single-slide, double-staining procedure for demonstration of CAP on the background of a modified Papanicolaou staining. The test is intended for demonstration of cervical acid phosphatase activity on microscopic slides. Details of the method are described elsewhere (13). The biomarker is presented as a brilliant red intracellular pigment while cell morphology is identified by Papanicolaou staining-based cytological criteria (Figure 1).
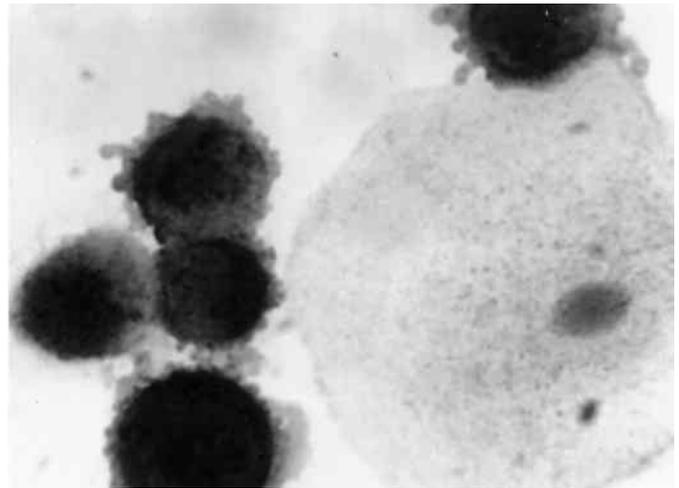
Figure 1a. MARKPAP (Test Images): COMBO control slide. HeLa cells are CAP Positive. They contain red granular deposit inside cytoplasm
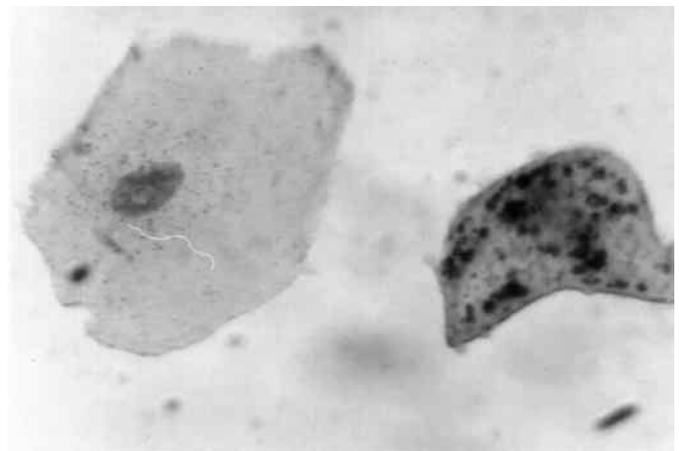
Figure 1b. MARKPAP (Test Images): CAP positive abnormal cell, CAP negative normal squamous cell

Figure 1c. MARKPAP (Test Images):HPV infected, CAP positive, abnormal squamous cell

CAP activity is absent in all normal squamous cells excoriated from superficial, intermediate of outer basal layer of cervical epithelium (Figures 1d). In the same time, CAP activity was present in all squamous cells showing morphological signs of cervical cell abnormality (Figures 1b,c). The red color "flags" abnormal

cells increasing their visibility on the slide (Figure 1a-d). It is our impression that CAP activity increases with the degree of cervical dysplasia (12,14).
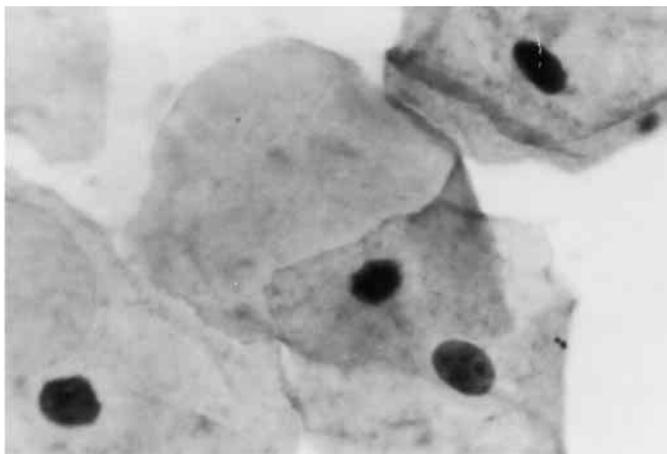
Figure 1d. MARKPAP (Test Images): CAP negative normal squamous cells

CAP activity is also present in cervical cancer cells, and in HeLa cell line cells derived from human cervical cancer (Figure1a) (12,15). Positive non-squamous epithelial cells (monocytes and endocervical cells) serve as internal quality control for adequacy of sampling and staining. Control slides made of HeLa cell line cells and buccal cells (COMBO controls) serve as external QC/QA (Figure 1a).

This data have confirmed the early works of Gross and Kinzie, Malvi and Sirsat, Panazzolo et al., (6-8). Our preliminary work on this issue became a foundation for creating the idea to utilize on CAP biomarker selective distribution in order to enhance visibility of abnormal cells on Pap smears/monolayers; thus, to help cyto-screeners to reduce false negative readings of Pap test-based cervical cancer screening. Trying to make the most of this idea, we have developed a MARKPAP™ line of products (test, kit, accessories) and we have employed them in clinical laboratory trials (15-17).

APPLICATION

The BioSciCon sponsored, and NCI-NIH-SBIR (Phase-1 and Phase-2) funded project *CAP-PAP Test for Cervical Cancer Screening* is a clinical laboratory trial with objectives to assess safety and efficacy of MARKPAP™ Test, our new biomarker-based technology, to assist cytoscreeners to improve their own sensitivity for detecting abnormal cervical cells, thus, to reduce false negative results of the Pap test (12).

Study design

Multicenter (seven clinical and two laboratory sites participating as contract research organizations), assignment per order of arrival at each site, split-sample design, assessors blinded, 2-

group study (test versus control) to assess the accuracy (sensitivity/specificity) of the new test in comparison with the control (Pap smear or ThinPrep Pap) to select abnormal/positive from normal/negative specimens obtained from 1,500 healthy women who were referred to doctor's offices for regular Pap test check-up, and who agreed to participate in this research. Interim analyses were designed to assess the trend of efficacy in comparison with historical control. Failure to maintain improving sensitivity for at least 30% (level of clinical significance) at any month since the beginning would have resulted in study closure.

The selected study design allows for threshold based, clinically relevant endpoints such as Yes/No (positive/negative) signs of epithelial cell abnormality determined at screening, and Yes/No disease determined either by adjudication of cytological results or by clinical outcomes (alternative: clinical action following diagnostic procedures such as colposcopy, biopsy and/or histology). We have also used a Decision Tree Model to plot screening data (18). This model presents our new 2-level Screening Protocol for cervical cancer screening and provides opportunity for plotting previous (historic/prior studies) probabilities. The use of this model permitted regular monitoring of the study progress via interim analyses of endpoints in independent groups (test and control before un-blinding at the end of the study) (Figure 3).

Study procedures

Conventional Pap test was used for the control of samples obtained as smears, and the ThinPrep Pap test was used for samples obtained in solution (LBP). All relevant study procedures are summarized on the Figure 2.

Study results

The results of our March 2003 Interim Analysis are summarized on the diagram below (Figure 3).

In comparison with the control Pap test, these numbers indicate:
- Doubling the portion of positive/abnormal slides referred to pathologist (27% : 13%)
- Significant reduction in the portion of false negative slides found at rescreen (5% : 9%)
- Increase of the portion of disease positive slides (true positive) identified by pathologists (17% : 8.2%)

Obviously, these three effects were related to better visibility of abnormal cells that were labeled by CAP, and to upgrading the cytological results found on the research slides.

Increasing sensitivity should be reflected with reduced specificity. However, in this case, there was only insignificant change of specificity because cytopathologists were using the same 2001 Bethesda nomenclature for interpreting control and test slides. Therefore, the MARKPAP™ Test had increased sensitivity for

Figure 2. Split-sample specimen evaluation design for study CPS for LBP

Legend: PS = primary screening (cytotechnologist); **RS** = rescreen (senior cytotechnologist); **RW** = review (cytopathologists); Numbers and percents along lines = MARKPAP Test data; Numbers in text boxes = Pap test data (either Pap smear or ThinPrep Pap test). NFS = need further screening (positive/abnormal including benign changes); NRI = no review indicated (negative/normal); = unsatisfactory smear or inadequate staining.

Figure 3. Comparison between PAP test historical control (boxes) and MARKPAP test (actual data on March 23, 2003)

detection of abnormal slides, while keeping an equivalent specificity. This result equals to conclusion that the new test is more accurate than the control.

Another advantage of using biomarker was reduction of screening time. Primary screening time was reduced from 6 to 3 min per slide, and rescreen was usually performed for slightly above 1 min per slide (19).

Data analysis

In this interim analysis we used comparison between two independent groups (March 2003, data). Data were provided from the laboratory and the clinical database without connecting paired samples. The results are summarized below (Table 1). Analysis of paired samples is deferred for the end of the study.

Table 1. Comparison of screening results between test and control group

	CPT		PAP	
	Number	Percent	Number	Percent
N (subjects)	970	100	1202	100
Positive/abnormal	132	13.6	99	8.2
Negative/normal	838	86.4	1103	91.8
Relative risk	0.158	-	0.090	-
95%CI	$0.115 - 0.158$		$0.067 - 0.098$	
95%CI-difference		$0.028 - 0.080$		
Odds Ratio		1.75		
χ^2 test	$\alpha=0.05$; df=1	Critical value: 3,841	$\Sigma(O-E)^2/E =$ 16.29	P = 0.00005409

After more than twelve hundred subjects recruited, and almost a thousand specimens completed and screened, the results in this table support our hypothesis that MARKPAP[TM] test is superior (more sensitive; equivalent specificity) to Pap alone for detecting cervical specimens with epithelial cell abnormalities. The study is ongoing.

DISCUSSION

In this report, we have shown some evidence in favor that the new marker of cervical cells abnormality combined with the conventional Papanicolaou staining, should be superior to the conventional staining-alone for early detection of conditions that may evolve into cervical cancer; consequently, for a timely removal of suspect cervical lesions. Also, this report supports our decision to sponsor a program for development of the MARKPAP[TM] technology and a line of related products. Due to the fact that this technology utilizes a selective chemical reaction (not an artwork of subjective understanding of cellular size, color, shape, inclusions, and their relations), the identification of the biomarker, and interpretation of results, resulted in a more timely, reliable and definitive clinical-decision-making than it is possible with other technologies (based on Papanicolaou staining-based cytological diagnosis) that are currently in use for cervical cancer screening. Further work is necessary to accumulate robust data because the CAP biomarker technology could be the first real challenge to the conventional Papanicolaou staining in 50 years.

CONCLUSION

This report has presented our accumulated evidence from an ongoing study in support of CAP as a biomarker for enhancing visibility of abnormal squamous cells on Pap smears and monolayers of LBP.

The MARKPAP test, which allows simultaneous visualization of the new CAP marker and cell morphology, has been found superior (more sensitive; equivalent specificity) than the Pap test-alone for detecting cervical specimens with epithelial cell abnormalities. Data supports further development of this technology toward a new in vitro diagnostic device (a system of devices and procedures) for cervical cancer screening (20,21).

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