Gene expression of ThinPrep® fixed cervical cytopathology cells

Mitchell J Machiela[1], Peterson T Haak[1], Paul B Norton[1], James Resau[1], Mike Whalen[2], Robert Bladek[3], Edward Fody[3], Stephen Sorenson[2]. [1]Laboratory of Microarray Technology, Van Andel Research Institute, 333 Bostwick NE, Grand Rapids, Michigan, 49503, [2]Holland Hospital, 602 Michigan Avenue, Holland, Michigan, 49423, [3]West Michigan Pathology Associates, PO Box 1139, Holland, Michigan, 49422

Abstract

The ThinPrep® method for gynecologic cancer screening has significantly changed the practice of cytopathology. Optimal RNA is normally obtained from frozen, unfixed cells but if RNA isolation is possible from fixed samples, the ability to apply new molecular diagnostic tests will be significantly improved. We hypothesize that cervical cells from the ThinPrep® procedure, which are fixed in buffered methanol and captured in sample vials, would be amenable to gene expression characterization by cDNA microarrays. We collaborated with a pathology laboratory to obtain a series of samples for analysis. We collected 71 cases with IRB review and approval. The distribution of cases, based on diagnosis by the pathology department was: 30 normal, 11 HPV, 10 ASCUS, 10 LGISL, 9 fungi, and 1 Trichomonas. We isolated RNA using TRIzol® reagent and characterized RNA quality with an Agilent Bioanalyzer®. We were able to isolate RNA from 71 cases. We have found that TRIzol® extraction produces an adequate yield of RNA. We have evaluated methods to optimize the integrity and quality of RNA as measured by intact 18/28s ribosomal RNA bands. We will correlate the expression pattern of the samples with cytopathological diagnosis and verify results with Northern blots and quantitative PCR.

Introduction

The American Cancer Society predicted 10,370 new cases of invasive cervical cancer and 3,700 deaths from cervical cancer in 2005.[1] Researchers estimate non-invasive cervical cancer to be four times more common than invasive cervical cancer. Early pathological detection of cervical cancer during its curable stage can greatly decrease the mortality rate of cervical cancer.

The ThinPrep® method has made a significant contribution to clinical pathology. Since its approval by the FDA in May 1996, the ThinPrep® Pap Test has provided physicians and patients with a liquid-based cytology method to replace the traditional Pap smear. In this method, cells are fixed in a preservative and later uniformly spotted on a slide.[2] The ThinPrep® automated thin-layer preparation is a marked improvement over the Pap smear in its clarity and specimen adequacy and is significantly more effective in detecting cervical abnormalities.[3,4]

Clinical pathology is moving towards more automated, consistent, liquid-based diagnostic tests. The ThinPrep® method has been applied to a variety of diagnostic purposes. Applications in lung cancer, renal cell carcinoma, anterior uvea inflammations, and thyroid fine-needle aspirations have already been investigated.[5,6,7,8] Methods for DNA isolation for the detection of HPV have also been developed for the ThinPrep® method.[9,10] Our laboratory hypothesized that RNA is preserved by the ThinPrep® fixative and can be extracted for gene expression analysis of the cytopathology samples.

Methods

The ThinPrep® method, collects a cervical sample with a spatula sampling device and immediately suspends the cells in a transport vial containing PreservCyt® fixative. A representative slide of the sample is prepared utilizing Cytyc's Controlled Membrane Transfer™ technology and reviewed by a pathologist for diagnosis.[2]

To test the RNA preservation capability of the PreservCyt® ThinPrep® preservative, a reconstruction experiment was designed to closely simulate the ThinPrep® method. HeLa cervical carcinoma cells of unknown passage were grown to confluence in 10 cm treated culture dishes (Corning) with Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% heat inactivated Fetal Bovine Serum (Gibco) and 100 units/ml Penicillin (Gibco). Media was aspirated from the plates and the cells were washed with 10 mL PBS (Gibco, pH 7.4, no Ca, no Mg). 15 mL PreservCyt® (Cytyc) was added to all the plates except the control. The cells were gently scraped from the plates and the cell/preservative suspension was stored at 23ºC in 50 mL centrifuge tubes (Corning) for the tested time intervals. At time points of 0 hours, 4 hours, 1 day, 1 week, 2 weeks, 4 weeks, and 6 weeks; the cells were pelleted at 1,000 RCF and TRIzol® (Invitrogen) extraction was carried out at the indicated time points using 5, 10, and 15 mL volumes and 15 and 120 minute incubation times. A NanoDrop ND 1000 spectrophotometer and Agilent 2100 Bioanalyzer were used to quantify RNA mass and quality. RNA amplification was performed using Ambion's MessageAmp II aRNA kit. Two, 19,968 element cDNA microarrays were printed (VARI) using the Research Genetics 40k human clone set (www.microarray.vai.org) and hybridized as previously described.[11]

Figure 1. The ThinPrep® system utilizes a spatula like sampling device to collect cervical cells. The sampling device is rinsed in a vial containing PreservCyt® fixative and the sample is transported to the laboratory for processing. Cytyc's Controlled Membrane Transfer™ technology spots a thin, even cellular layer onto a slide which is later reviewed by a pathologist for diagnosis.

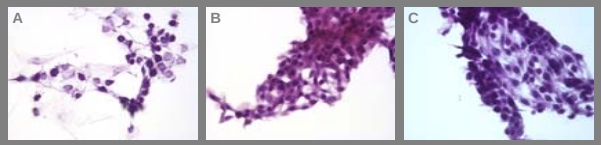

Figure 2. Hematoxylin and eosin staining of HeLa cervical carcinoma cells. The images are of the control (A) , 2 weeks in PreservCyt® fixative (B), and 6 weeks in PreservCyt® (C).

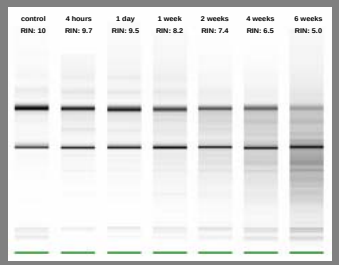

Figure 3. Bioanalyzer® pseudo-gels of total RNA from HeLa cells stored in PreservCyt® fixative over varying timepoints. A significant decrease in RNA integrity number was observed over time.

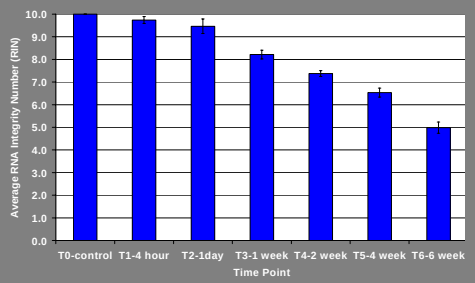

Figure 4. Graphical representation of average RNA integrity numbers from HeLa cells stored over a period of 6 weeks in PreservCyt® fixative.

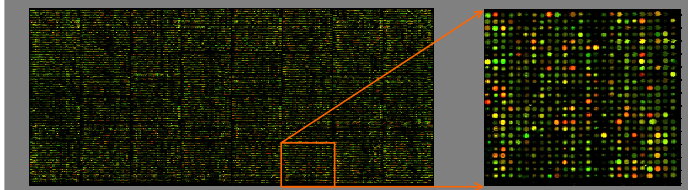

Figure 5. Hybridization of RNA harvested and amplified from HPV positive ThinPrep® preserved patient sample (Cy5, Red) vs ThinPrep® preserved HeLa cell line RNA (Cy3, Green).

Results

We were able to isolate RNA from clinical samples (e.g. normal; high grade, HPV, etc). The yield however was quite variable with Agilent RNA integrity numbers (RIN's) below 5.0. To understand the reason, we performed a reconstruction experiment using cervical HeLa cells in the PreservCyt® fixative. We determined that neither the TRIzol® volume nor TRIzol® incubation time had a significant impact on RNA quantity or quality. The amount of time HeLa cells were stored in PreservCyt® fixative was significant, with degradation of quality proportional to time in fixative (Figure 3). The number of HeLa cells at each time point was controlled and no difference in RNA yield was noted. Although the quality of the RNA from later clinical samples was sufficient (RIN>7.5), the amount without amplification is not sufficient to produce arrays. After one round of amplification, aRNA yield exceeded the 10ug aRNA required for gene expression analysis with less than 5 percent rRNA contamination. To confirm if a microarray could be generated from a ThinPrep® sample, we prepared HeLa RNA (cy3-green) and RNA from a HPV diagnosed clinical sample (cy5-red). The array is shown in Figure 5. While the focus of this hybridization was on optimizing methods, we did note that Keratin 13, Keratin 19, Retinoic Acid Induced 3, Interleukin 1 Receptor Agonist, and mal T-cell differentiation protein (MTCDP) were highly expressed in the HPV sample vs. reference in replicate arrays.

Discussion

We have demonstrated that intact RNA can be extracted from cervical cells obtained from a ThinPrep®. There is loss of RNA quality, but not quantity, over the course of six weeks in fixative. The original 71 samples we evaluated were fixed for more than 8 weeks. We hypothesized that reduced fixation time would produce increased quantity and quality of RNA. We did observe an increase in quality with reduced time in fixative. We predict and are testing a cohort of clinical cases to asses RNA yield. The amount will vary as there is considerable variation in the number of cells in a ThinPrep®. Based on the results of our HeLa reconstruction experiment, samples of similar age will have similar RIN's. Cells stored in PreservCyt® for fewer than two weeks will yield quality RNA (RIN>7.5) that is acceptable for gene expression analysis. We demonstrate that after RNA amplification, there is RNA of sufficient quality and quantity for gene expression analysis. We are planning future experiments using ThinPrep® preserved clinical samples of less than two weeks in age to build on the technique demonstrated here. Future questions to address include:

- Can total RNA gathered from all cells (both diagnostic and uninformative) in the various clinical ThinPrep® samples differentiate disease states?
- Can gene expression identify cervical cells that are precancerous?
- Is there a host-specific gene expression signature that predicts patient susceptibility to chronic HPV infection?
- What are the parameters of non-cervical, ThinPrep® preserved samples to understand in order to produce useful gene expression information?

References

1. American Cancer Socitey. *Cancer Facts and Figures 2005.* Atlanta: American Cancer Society; 2005.
2. Cytyc Corporation. www.cytyc.com. March 20, 2006.
3. Thin-layer Pap test vs. conventional Pap smear. Analysis of 400 split samples. J Reprod Med. 2002 Jan; 47(1):9-13.
4. Conventional pap smear and liquid-based cervical cytology smear: comparison from the same patient. Tumori. 2002 Jul-Aug; 88(4):288-90.
5. Early diagnosis of lung cancer. Ai Zheng. 2003 Feb;22(2):221-3.
6. Fine-needle aspiration biopsy of chromophobe renal cell carcinoma and oncocytoma: comparison of cytomorphologic features. Cancer. 1999 Jun 25;87(3):161-7.
7. The contribution of aqueous humor cytology in the differential diagnosis of anterior uvea inflammations. Ocul Immunol Inflamm. 2004 Sep;12(3):215-25.
8. Thin layer preparations in thyroid fine-needle aspiration: study of 200 cases. Rev Fac Cien Med Univ Nac Cordoba. 2003;60(2):9-22.
9. Human Papillomavirus Testing in Primary Screening for the Detection of High–Grade Cervical Lesions: A Study of 7932 Women. Brit J Cancer, 2001; 89 (12):1616–1623.
10. Management of women who test positive for high–risk types of human papillomavirus: the HART study. The Lancet 2003;362:1871–76.
11. Robust Classification of Renal Cell Carcinoma Based on Gene Expression Data and Predicted Cytogenetic Profiles, Cancer Research 64, 4117-4121, June 15, 2004.

Research funded by a generous gift from Jay and Betty Van Andel