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Programmable Bio-Nano-Chip based Cytologic Testing of Oral Potentially Malignant Disorders in Fanconi Anemia

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Abstract

Fanconi anemia (**FA**) is caused by mutations of DNA repair genes. The risk of oral squamous cell carcinoma (OSCC) among FA patients is 800-folds higher than in the general population. Early detection of OSCC, preferably at it precursor stage is critical in FA patients to improve their survival. In an ongoing clinical trial, we are evaluating the effectiveness of the programmable bio-nano-chip (**p-BNC**)-based oral cytology test in diagnosing oral potentially malignant disorders (OPMD) in non-FA patients. We used this test to compare the cytomorphometric and molecular biomarkers in OSCC cell lines derived from FA patients and non-FA patients and brush biopsy samples of a FA patient's OPMD and normal mucosa of healthy volunteers. Our data showed that expression patterns of molecular biomarkers were not notably different between sporadic and FA OSCC cell lines. The p-BNC assay revealed significant differences in cytometric parameters and biomarker MCM2 expression between cytobrush samples of the FA patient and cytobrush samples of normal oral mucosa obtained from healthy volunteers. Microscopic examination of the FA patient's OPMD confirmed the presence of dysplasia. Our pilot data suggests that p-BNC brush biopsy test recognizes dysplastic oral epithelial cells in a brush biopsy sample of a FA patient.

Keywords

Fanconi anemia; Oral squamous cell carcinoma; Oral potentially malignant disorders; Brush biopsy; Bio-nano-chip; Oral epithelial dysplasia

Introduction

Oral and oropharyngeal squamous cell carcinomas (OSCC) represent the sixth most commonly diagnosed cancer, with an estimated 500,000 new cases diagnosed annually

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worldwide and accounting for approximately 3% of new cancer cases in the United States (Ferlay et al., 2010). Despite advances in diagnostic and therapeutic modalities, five-year relative survival rate has remained low (60%) for the past three decades, possibly secondary to presentation of patients at late stages of disease (Vigneswaran & Williams, 2014). Both incidence and mortality rates of OSCC vary considerably depending on the anatomic location of the tumor (Vigneswaran & Williams, 2014). The tongue is the most common site for OSCC among both European and US populations and tongue cancers carry a less favorable prognosis compared to other oral anatomic sub-sites (Chen & Myers, 2001, Warnakulasuriya, 2009, Vigneswaran & Williams, 2014). The locoregional relapse rates remain high for tongue SCC and as a result the prognosis of a patient diagnosed with tongue cancer is poor, particularly when the disease is diagnosed in its advanced stages. Hence, early detection of tongue SCC is critical to improve survival rates. Furthermore, patients treated for OSCC are at risk of developing secondary primary tumors at a rate of 3.7% per year, accounting for one-fourth of all OSCC-related deaths, because of “field cancerization” (Day & Blot, 1992). Therefore, close surveillance of patients who were successfully treated for oral cancer is critical for early detection of recurrences and/or second primaries.

The majority of patients diagnosed with OSCC reside in low- and middle-income countries (Warnakulasuriya, 2009). In the Indian subcontinent and neighboring countries, OSCC ranks first in prevalence among all cancers in males and third in females (Warnakulasuriya, 2009). Owing to their limited health care resources, developing countries cannot afford the model used for screening and diagnosis of OSCC that is used in developed countries. Therefore, OSCC patients from these countries are diagnosed at a late stage when treatment is more expensive and less successful than when patients are diagnosed at earlier stages. Hence, development of a simple, reliable and non-invasive test for diagnosis of OSCC at an early stage, ideally during its precursor stage will certainly improve the chances for curative treatment.

Similar to common solid malignancies, OSCC development is a multistep process often preceded by precursors, which are commonly known as oral potentially malignant disorders (OPMD). These include premalignant lesions and conditions that have an increased risk for malignant transformation (Warnakulasuriya et al., 2007). Premalignant lesions are defined as morphologically altered oral mucosae in which OSCC is more likely to develop (i.e. Leukoplakia and Erythroplakia) (Warnakulasuriya et al., 2007, van der Eb et al., 1993). Premalignant conditions are defined as intraoral conditions that are associated with a substantially increased risk for OSCC and can include oral lichen planus, oral graft versus host diseases (GVHD), and oral submucous fibrosis (Warnakulasuriya et al., 2007).

Individuals, who smoke cigarettes, use smokeless tobacco and/or areca nut, consume excessive alcohol and have persistent oral infections with high-risk oncogenic human papilloma virus (HPV) are at a higher risk for developing sporadic OSCC (Vigneswaran & Williams, 2014). In addition, patients with Fanconi anemia (FA; MIM 227650) exhibit inherited risk for developing OSCC even in the absence of the above mentioned risk factors. FA is a chromosomal instability disorder inherited as an autosomal- or X-chromosomal recessive trait. It is caused by germline mutations in one of 15 FA genes involved in the DNA repair pathway, resulting in increased risks for bone marrow failure, leukemia and

solid malignancies (Levitus et al., 2006). The risk of OSCC among FA patients is 800-times higher than in the general population, occurring at younger ages (median age: 27-years) (Kutler et al., 2003a, Masserot et al., 2008). Further, OSCC in FA patients is highly aggressive with poor prognosis (Bachoura & Vigneswaran, 2014). Early intervention and aggressive surgical management are needed because radiation and chemotherapy are contraindicated due to significant adverse risks in these patients (Scheckenbach et al., 2012). Moreover, FA-OSCC patients have a higher risk for developing multiple primary tumors than the general population due to the presence of multifocal pre-malignant disease. Hence, semi-annual oral cancer screening is recommended for FA patients beginning at a young age (Singh, 2008).

Visual and tactile examination of the oral cavity by a trained oral health care provider under white light remains to be the gold standard for oral cancer screening (Lingen et al., 2008). Unlike cervical cancer were Papanicolaou (PAP) smears are instrumental in cancer screening, cytology for oral cancer screening has not been proven effective (Dolens Eda et al., 2013, Folsom et al., 1972). Histopathologic examination of the tissue biopsy is the gold standard for diagnosing OED or invasive carcinoma (Lingen et al., 2008). Although tissue biopsy is ideal for diagnosing and monitoring the progression of OPMD towards OSCC, frequent re-biopsy of OPMD in FA patients is impractical, expensive, discomforting and associated with significant complications. Moreover, scalpel biopsy has significant sampling limitation in FA patients because of diffuse and multifocal involvement of OPMD. Hence, regular and meticulous clinical examination by an expert clinician remains the only option currently available for detection and monitoring of OPMD in FA patients. However, such expert clinical services are not usually available in the community dental and medical clinics where most FA patients seek care. Hence, there is a desperate need for an objective non-invasive test for surveillance and monitoring for OPMD in FA patients that can be performed in a community dentist's or physician's office.

In an ongoing clinical trial, we are evaluating the effectiveness of a programmable bionanochip (p-BNC)-based oral cytology test in diagnosing OED and OSCC compared to the standard tissue biopsy diagnosis. The p-BNC assay integrates multiple laboratory processes within a microfluidic platform in three primary steps consisting of: (i) cell separation/capture on the membrane filter, (ii) biomarker immunolabeling and cytochemical staining, and (iii) computer assisted image and data analysis. The p-BNC assay is used to measure a combination of cytomorphometric features and biomarker expression levels in cell samples collected using a transepithelial brush biopsy to discriminate benign, dysplastic and malignant oral epithelial cells. Six antibodies against biomarkers are used in the p-BNC assay which label either the cell surface (EGFR: Epidermal Growth Factor Receptor, CD147/EMMPRIN: Extracellular Matrix MetalloPRoteinase Inducer and αvβ6 integrin) or the nucleus (MCM2: MiniChromosome Maintenance protein 2, geminin and Ki67). These biomarkers were chosen based on previous studies reporting their utility in distinguishing benign, dysplastic and malignant oral epithelial cells (Torres-Rendon et al., 2009, Vigneswaran et al., 2006, Weigum et al., 2010). In this report, we present the data from a pilot study that we conducted to evaluate the value of the p-BNC based oral cytology test as an adjunct to the standard oral cancer screening for the diagnosis of OPMD in FA.

Materials and methods

Cell lines

The human FA-OSCC cell lines VU-1365 and VU-1131were obtained from Dr. Ruud H. Brakenhoff (Vrije University Medical Center, The Netherlands) and OHSU-974 was a gift from Dr. Laura Hayes (Oregon Health and Science University, Oregon, USA)). The human sporadic OSCC cell lines UMSCC-22A and UMSCC-101A were obtained from Dr. Thomas E. Carey, University of Michigan, and MDA-686Tu was a gift from Dr. Peter G. Sacks (NYU College of Dentistry). Molecular phenotypes of these cell lines have been defined in published reports and are shown in Table 1 (Brenner et al., 2010, van Zeeburg et al., 2005). These cell lines were grown in adherent conditions using the recommended culture medium and used for p-BNC assay as described previously.

Patient information

This study was conducted as part of a large non-interventional clinical trial which evaluated the usefulness of a p-BNC brush test in the diagnosis and management of patients presenting with OPMD. This study was reviewed, approved and monitored by the Institutional Review Boards of the University of Texas Health Science Center at Houston and Rice University, Houston, Texas. The FA patients for this study were recruited at the Meeting for Adults with FA, held in Baltimore, Maryland in March 2014. Twenty-six patients attending this camp voluntarily consented to participate in an oral cancer screening and to undergo a brush biopsy if an OPMD was detected during screening. After informed and written consent, participants were subjected to a conventional oral examination (COE) under white light, as well as a tissue autofluorescence examination using VELscope. During screening, a 34-year old male FA patient presented with a large white plaque (leukoplakia) in the right lateral and ventral surfaces of the tongue. Brush biopsy sample was taken from this lesion using the Rovers® Orcellex® oral cytology brush (Rovers Medical Devices B.V., Oss, The Netherlands). For healthy volunteers (n=10), a brush biopsy was taken from the normal appearing mucosa on the lateral surface of the tongue. The brush was applied directly to the lesion and normal mucosa using mild pressure and rotated 360° for 15 times in the same direction to collect the cellular samples. Cells were recovered by vortexing the brush tip in brush biopsy transport/storage media (CytoLyt® or MEM culture media). The cells stored in the MEM culture media were washed, re-suspended in a cryo-preservative (FBS with 10% DMSO) and stored at −80°C until processing for p-BNC assay. Cells stored in CytoLyt® media did not require further processing.

p-BNC assay sample processing

Cultured OSCC cells and cytobrush cells samples collected from the FA patient and normal tongue mucosa of healthy volunteers (n=10) were processed for p-BNC assay. Prior to sample processing on the p-BNC platform, cell samples were fixed in 0.5% formaldehyde for one hour, washed twice in PBS, and resuspended in 150 μL of PBS with 0.1% BSA. After priming the fluidic tubing and p-BNC device with PBS, the cell suspension was loaded into an injection port and delivered over the membrane for two minutes at a flow rate of 1.5mL/min followed by a PBS wash step for 2.5 minutes at 1mL/min. Primary antibody solutions against EGFR, CD147, αvβ6, MCM2, geminin and Ki67 in PBSAT (0.1% PBS +

0.1% BSA + 0.1%Tween-20) were then delivered over the captured cells for 2.5 min at a flow rate of 250 μL/min, followed by a second PBS wash step. The final staining cocktail consisting of AlexaFluor®488-labelled secondary antibodies (green), a nuclear stain 4′,6-diamidino-2-phenylindole (DAPI; blue) and a cytoplasmic stain phalloidin-AlexaFluor®647 (red) in PBSAT was delivered over the captured cells in the same fashion as the primary antibody. After a final PBS wash step, a custom imaging sequence automatically recorded 25 fields of view at three different z-slices for a total imaging area of 20mm^2.

Image analysis

Enhanced depth-of-field was achieved by utilizing a custom focusing algorithm that combined the three different z-slices at each field of view. Individual cell outlines were identified by applying adaptive thresholds to DAPI and Phalloidin channels for each image (Cell Profiler, www.cellprofiler.com) (Carpenter et al., 2006) and used to create regions of interest (ROIs) specific to each cell. These ROIs were then analyzed through automated custom macros (ImageJ, Matlab) to obtain cytomorphometric and molecular data at a single-cell level.

Data analysis

The normal control group consisted of 10 lesion-free healthy volunteers who provided brush biopsy samples of their tongue and buccal mucosa. To maintain site-specific consistency, only brush biopsies of the tongue from the same clinical site were included in the analysis. Lone nuclei and cellular debris were identified and flagged through the use of several filters based on criteria for different morphometric parameters. These objects were removed from analysis in order to concentrate on whole, intact cells. After data filtering, there were a total of 104 individual cells present in the Fanconi-Anemia subject and 18,140 individual cells combined between the 10 healthy volunteers in the database. For each morphometric and molecular parameter, a one-way analysis of variance (ANOVA) test (significance level of $\alpha = 0.05$) was performed to identify significant differences between the two populations.

Results

p-BNC assay of FA and sporadic OSCC cell lines

During this automated assay procedure, a cell suspension is delivered to the disposable bio-nanochip (BNC) using a pressure-driven flow, whereupon cells larger than the membrane pore size are retained on the membrane surface (Figure 1). The captured cells are stained with fluorescent dyes and primary and secondary immuno-reagents. Cellular cytoplasm counterstained with phalloidin/AlexaFluor®-647 appears red and its nucleus counter stained with DAPI appears blue (Figure 2). The expression levels and patterns of molecular biomarkers are detected by green fluorescence (AlexaFluor®488). Fluorescence microscopy is performed in an X, Y, Z scan of the membrane surface and is followed by automated image analysis for quantitative intensity standardization and cell/nuclear contouring from the red/blue/green channels (Figures 1 & 2). Sporadic (UM-SCC-22A and MDA-686-Tu) and FA-OSCC cell lines (VU-1365, VU-1131 and OHSU-974) did not show any significant differences in the expression patterns of these biomarkers (Figure 3). Except for geminin, expression all five biomarkers are detected in both sporadic and FA OSCC cells (Figure 3);

however, the expression patterns of these biomarkers are heterogenous within each cell line. Nuclear abnormalities such as bi-nucleated tumor cells and tumor cells with micronucleus are frequently noted in FA-OSCC cells compared with sporadic OSCC cells (Figure 4).

p-BNC assay of brush biopsy samples

The p-BNC data of the brush biopsy sample obtained from the FA patient's OPMD was compared with brush biopsies of the ten healthy volunteers. We limited our biomarker selection to MCM2, which allows a better discrimination between benign, dysplastic and malignant oral epithelial cells, based on unpublished preliminary studies. Results from the ANOVA tests for each parameter are summarized in Table 1. Overall, it was found that the brushed cells from the FA patient's tongue lesion expressed a significantly higher level of the nuclear biomarker MCM2 than the healthy controls and also exhibited distinct morphological characteristics such as increased cell area, increased nuclear area, increased cell circularity, and increased nuclear-to-cytoplasm ratio. In this study, nuclear-to-cytoplasm ratio was defined as the total nuclear area divided by the whole cell area; and circularity was defined as 4pi (area/perimeter^2), resulting in a value between 0 and 1 where a value of 1 indicates a perfect circle. Common cell morphologies are presented for both brushed cells from the tongues of normal volunteers (Figure 5A) and the OPMD of the FA patient (Figure 5B). In addition to the fewer number of cells observed across different imaging fields for the FA subject, the cells also exhibit a higher degree of circularity as well as enlarged nuclei. While no significant difference was seen in cell area by analyzing the combined cell populations, small sub-populations of cells with unique morphologic features can be extracted from density plots, such as seen in Figure 6.

Excisional biopsy of the erythroleukoplakia in the FA patient

The FA patient presented with a diffuse area of mixed red and white plaque involving the right lateral and ventral surface of the tongue which measured approximately 2.8 × 2.1 cm (Figure 7). Tissue autofluorescence examination with VELscope showed loss of fluorescence of this OPMD. The patient was advised to consult his head and neck surgeon for further diagnostic evaluation and treatment. Subsequently, this lesion was excised and submitted for histopathologic examination. Microscopic examination of the excisional biopsy revealed moderate epithelial dysplasia and no evidence of an invasive cancer (Figure 8). The surgical margins were free of dysplasia.

Discussion

In this case report, we present the pilot data to demonstrate the feasibility of a programmable bio-nanochip (p-BNC) brush biopsy test as a non-invasive method for early detection of OSCC at its precursor stage of OED. This exploratory study is aimed to evaluate the effectiveness of using a p-BNC brush biopsy test for surveillance of OSCC and its precursors in the high risk FA patient population.

Fanconi anemia is an inherited cancer syndrome with an increased risk for head and neck squamous cell carcinomas (HNSCC) (Kutler et al., 2003a, Masserot et al., 2008, Scheckenbach et al., 2012). It is a rare autosomal recessive DNA disorder caused by

mutations in at least one of the 15 genes in the FA pathway which regulates cell cycle check point and DNA repair (Levitus et al., 2006). Clinically, FA is characterized by various congenital malformations, increased risk for malignancies and progressive bone marrow failure (Kutler et al., 2003b). It is estimated that malignancies are 10,000–15,000-fold higher in FA patients than in the general population (Deeg et al., 1996). Hematopoietic stem cell transplantation (HSCT), which greatly extends the life-expectancy in FA patients, appears to increase the risk of malignancies in these patients. The cumulative incidence of head and neck cancers in bone marrow transplanted FA patients is estimated to be 100% by 45-years of age, whereas the risks in FA patients who have not had HSCT is 50%. (Rosenberg et al., 2005). Development of chronic oral graft-versus-host disease (GVHD) in FA patients who have undergone HSCT increases their risk for OSCC. Furthermore, prolonged immunosuppressive treatment for chronic oral GVHD also increases the risk for OSCC in these patients. More than two thirds of the HNSCC in FA patients are OSCC, and the tongue is the most common subsite for OSCC (Kutler et al., 2003a, Masserot et al., 2008, Scheckenbach et al., 2012). The condition of OSCC in FA patients occur at a younger age than the general population (median age: 27-years) and have a worse prognosis (Birkeland et al., 2011, Scheckenbach et al., 2012, Bachoura & Vigneswaran, 2014). One of the most significant clinical challenges in the management of FA patients with OSCC is that most of these patients present with a locally advanced stage of this disease (Singh, 2008). Management of locally advanced OSCC in FA patients is more difficult than in the general population because chemo- and radiation therapies are not recommended for managing solid cancers in FA patients. These patients have increased sensitivity to DNA-cross linkers and susceptibility to radiation-related toxicity in non-cancerous tissues due to inherent defects in their DNA repair pathways. Currently, surgical resection is the only treatment option available for FA patients with OSCC. Surgery is effective in FA tongue cancer only if it is detected at an early stage, preferably during the precancer stage (Singh, 2008). OSCC in FA patients frequently occurs as a multifocal disease and is often preceded by premalignant lesions manifesting as leukoplakias (Singh, 2008). Therefore, it is strongly recommended that FA patients undergo semi-annual oral cancer screenings by conventional oral examination, beginning by the age of 10–12 years (Singh, 2008). High-risk FA patients with oral leukoplakias or chronic GHVD and those who have a history of total-body radiation are recommended to undergo oral cancer screening every three months. Distinguishing OPMD from a benign keratosis (i.e. frictional keratosis) and inflammatory oral mucosal lesion by conventional oral examination can be challenging and is dependent upon the skill of the examiner (Lingen et al., 2008). Even experienced practitioners will have difficulty in recognizing subtle clinical findings indicative of malignancy in FA patients because of the high prevalence of chronic mucositis related to GVHD, xerostomia and opportunistic candidiasis. To date, the diagnosis and management of OPMD in the general population is based on visual inspection by COE with scalpel biopsy of lesions deemed clinically suspicious. Although COE has been shown to have a high discriminatory ability in distinguishing normal and abnormal oral mucosal patterns, it has poor sensitivity and specificity in discriminating oral premalignant and malignant lesions from benign reactive and inflammatory oral mucosal lesions (Lingen et al., 2008, Shin et al., 2010). Poor specificity of oral cancer screening by COE frequently leads to unnecessary biopsies. As a surgical procedure, a biopsy is invasive and the selection of a representative biopsy site is

always problematic in OPMD with diffuse or multifocal involvement, as is the case of OPMDs in FA patients. Usually, diagnosis of OPMD in FA patients requires referral to a specialist and surgical biopsy of suspicious lesions is often delayed due to various reasons. Many clinicians in the community are reluctant to perform invasive biopsies delaying the diagnosis. Therefore, an effective non-invasive surveillance method that can reliably aid the clinician in identifying OPMDs most likely to progress to OSCC in FA patients is desperately needed. Development of a simple and non-invasive diagnostic test, which can be administrated in resource-limited settings, will make it possible to identify OSCC in FA patients in earlier stages, thus reducing the number of unnecessary biopsies.

There are a number of commercially available adjuncts for COE that have been developed and marketed to oral health providers for the detection and diagnosis of OPMD. These adjunctive techniques include imaging of oral mucosa using a hand held tissue reflectance or autofluorescence based devices and oral exfoliative cytology. Efficacy and limitations of these tests in detecting and diagnosing OPMD are discussed elsewhere (Lingen et al., 2008, Shin et al., 2010).

Oral exfoliative cytology is a diagnostic test which consists of microscopic examination of epithelial cell samples scraped from the surface of the mucosal lesions. Contrasting the cervical Papanicolaou (PAP) smear, a well-established test for screening cervical cancer, oral exfoliative cytology is not advocated for screening of OSCC and its precursors. Most clinicians feel that the sensitivity of oral exfoliative cytology, with a reported false negative rate as high as 31%, is not sufficient to warrant its widespread use in the diagnostic evaluations of OPMD(Dolens Eda et al., 2013, Folsom et al., 1972). This is due to the fact that exfoliative cells obtained by scraping the lesional surface are often devoid of basal and parabasal cells and therefore is not representative of the whole thickness of the epithelium. A renewed interest in oral cytology started after the introduction of brush cytology test known as OralCDx (OralCDx Laboratories Inc., Suffern, NY) (Sciubba, 1999). This technology utilizes a small wire brush, similar to the one used in the current study, to obtain cells representing the whole thickness of the epithelium (transepithelial). This brush biopsy test reports the results as “negative/benign” or “positive” or “atypical” and the samples yielding positive or atypical test results will need scalpel biopsy for a definitive diagnosis. The major limitation of the brush cytology test is its poor positive predictive values, as low as 38%, which leads to many unnecessary biopsies (Lingen et al., 2008). To improve the positive predictive value, the p-BNC brush biopsy test uses quantitative measurements to characterize multiple cytometric and molecular biomarkers within the same cell samples. In contrast to the p-BNC brush test, the conventional brush cytopathologic diagnosis relies on the qualitative assessment of the cellular and nuclear morphologies and is associated with a high degree of inter-and intraobserver disagreement, depending on the experience of the pathologist.

In an ongoing non-interventional clinical trial, we used the p-BNC assay to measure the cytometric and molecular biomarkers on brush biopsy samples of 775 non-FA patients with OPMD who needed scalpel biopsy for the diagnosis. The p-BNC brush biopsy data base, consisting of key cytometric and molecular biomarker signatures of these patients’ samples, is being used to develop a diagnostic model for classifying OPMDs into benign, dysplastic

and malignant. The data from this clinical trial will be published elsewhere. The p-BNC assay provides an objective and reproducible quantitative data, independent of pathologist's fatigue, experience, and inter- and intraobserver variability which are the critical drawbacks of conventional cytopathology.

In our preliminary studies, we used the p-BNC assay to examine the cytometric and molecular biomarker signatures of established cell lines derived from sporadic and FA OSCC. Our data showed that expression patterns of molecular biomarkers CD147, EGFR, Ki67, McM2, Geminin, αvβ6 integrin were not notably different between sporadic and FA OSCC cell lines. These findings are in line with the previously published reports showing that molecular phenotypes of FA and sporadic oral cancer cells are similar except for their sensitivity to cisplatin (van Zeeburg et al., 2005).

Next we tested whether the p-BNC brush test is capable of detecting dysplastic oral epithelial cells in a brush biopsy from a FA patient who presented with clinically high-risk OPMD. The data from the assay revealed significant differences in cytometric parameters (nuclear area, cell circularity) and biomarker MCM2 expression between cytobrush samples of the FA patient and site matched healthy volunteers (Table 1). Based on the clinical and p-BNC assay findings, the patient was advised to consult his head and neck surgeon for further evaluation. Subsequently, the lesion was excised and the presence of oral epithelial dysplasia in the surgical specimen was confirmed by histopathologic examination. In this study, we compared the brush sample of the lesion with brush biopsies from the normal mucosae of healthy volunteers, because of the lack of clinically normal appearing mucosa on the contralateral side of the patient's tongue. Obtaining brush biopsy samples from clinically normal oral mucosa for comparison can be difficult in FA patients because of increased prevalence of chronic oral mucositis and multifocal nature of precancers in these patients. The need for brush samples of normal mucosa will not be necessary after the diagnostic model to interpret the p-BNC data is developed. FA OSCC cell lines were associated with elevated number of bi-nucleated tumor cells and tumor cells with micronuclei reflecting their DNA repair defects. However, similar cells were not detected in the patient's brush biopsy sample and therefore more FA patients' brush biopsy samples need to be tested before determining their diagnostic utility and biologic significance of nuclear aberrations and micronuclei in OPMD's of FA patients.

In conclusion, this non-invasive p-BNC brush biopsy test distinguishes dysplastic oral epithelial cells in a brush biopsy sample of a FA patient's leukoplakia from normal oral brush biopsy samples of healthy volunteers. We want to acknowledge that the findings of this study are limited to a single FA patient and cannot be generalized to all FA patients with OPMD. Additional studies involving more FA patients with OPMD are needed to substantiate these findings. Considering FA is a rare disorder, it will take a longer time to recruit sufficient sample size to determine the sensitivity and specificity of the p-BNC based cytology compared to scalpel biopsy in diagnosing oral epithelial dysplasia and invasive carcinoma.

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Abbreviations

COE	Conventional oral examination
FA	Fanconi anemia
HNSCC	Head and neck squamous cell carcinoma
OED	Oral epithelial dysplasia
OSCC	Oral squamous cell carcinoma
OPMD	Oral potentially malignant disorders
p-BNC	Programmable bionanochip

References

Bachoura A, Vigneswaran N. Oral and maxillofacial pathology case of the month. Squamous cell carcinoma of the tongue in a patient with Fanconi Anemia. Texas Dental Journal. 2014; 131 In press.

Birkeland AC, Auerbach AD, Sanborn E, Parashar B, Kuhel WI, Chandrasekharappa SC, Smogorzewska A, Kutler DI. Postoperative clinical radiosensitivity in patients with fanconi anemia and head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2011; 137:930–4. [PubMed: 21930984]

Brenner JC, Graham MP, Kumar B, Saunders LM, Kupfer R, Lyons RH, Bradford CR, Carey TE. Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. Head Neck. 2010; 32:417–26. [PubMed: 19760794]

Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 2006; 7:R100. [PubMed: 17076895]

Chen AY, Myers JN. Cancer of the oral cavity. Dis Mon. 2001; 47:275–361. [PubMed: 11477373]

Day GL, Blot WJ. Second primary tumors in patients with oral cancer. Cancer. 1992; 70:14–9. [PubMed: 1606536]

Deeg HJ, Socie G, Schoch G, Henry-Amar M, Witherspoon RP, Devergie A, Sullivan KM, Gluckman E, Storb R. Malignancies after marrow transplantation for aplastic anemia and fanconi anemia: a joint Seattle and Paris analysis of results in 700 patients. Blood. 1996; 87:386–92. [PubMed: 8547667]

da Dolens ES, Nakai FV, Santos Parizi JL, Alborghetti Nai G. Cytopathology: a useful technique for diagnosing oral lesions?: a systematic literature review. Diagnostic cytopathology. 2013; 41:505–14. [PubMed: 22645047]

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010; 127:2893–917. [PubMed: 21351269]

Folsom TC, White CP, Bromer L, Canby HF, Garrington GE. Oral exfoliative study. Review of the literature and report of a three-year study. Oral Surg Oral Med Oral Pathol. 1972; 33:61–74. [PubMed: 4550162]

Kutler DI, Auerbach AD, Satagopan J, Giampietro PF, Batish SD, Huvos AG, Goberdhan A, Shah JP, Singh B. High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. Arch Otolaryngol Head Neck Surg. 2003a; 129:106–12. [PubMed: 12525204]

Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, Giampietro PF, Hanenberg H, Auerbach AD. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood. 2003b; 101:1249–56. [PubMed: 12393516]

Levitus M, Joenje H, de Winter JP. The Fanconi anemia pathway of genomic maintenance. Cell Oncol. 2006; 28:3–29. [PubMed: 16675878]

Lingen MW, Kalmar JR, Karrison T, Speight PM. Critical evaluation of diagnostic aids for the detection of oral cancer. Oral Oncol. 2008; 44:10–22. [PubMed: 17825602]

Masserot C, Peffault de Latour R, Rocha V, Leblanc T, Rigolet A, Pascal F, Janin A, Soulier J, Gluckman E, Socie G. Head and neck squamous cell carcinoma in 13 patients with Fanconi anemia after hematopoietic stem cell transplantation. Cancer. 2008; 113:3315–22. [PubMed: 18831513]

Rosenberg PS, Socie G, Alter BP, Gluckman E. Risk of head and neck squamous cell cancer and death in patients with Fanconi anemia who did and did not receive transplants. Blood. 2005; 105:67–73. [PubMed: 15331448]

Scheckenbach K, Wagenmann M, Freund M, Schipper J, Hanenberg H. Squamous cell carcinomas of the head and neck in Fanconi anemia: risk, prevention, therapy, and the need for guidelines. Klin Padiatr. 2012; 224:132–8. [PubMed: 22504776]

Sciubba JJ. Improving detection of precancerous and cancerous oral lesions. Computer-assisted analysis of the oral brush biopsy. U.S. Collaborative OralCDx Study Group. J Am Dent Assoc. 1999; 130:1445–57. [PubMed: 10570588]

Shin D, Vigneswaran N, Gillenwater A, Richards-Kortum R. Advances in fluorescence imaging techniques to detect oral cancer and its precursors. Future Oncol. 2010; 6:1143–54. [PubMed: 20624126]

Singh, B. Head and neck squamous cell carcinoma in Fanconi anemia patients. In: Eiler, M.; Frohnmayer, D.; Frohnmayer, L.; Larsen, K.; Owen, J., editors. Fanconi Anemia: Guidelines for Diagnosis and Management. 3. Fanconi Anemia Research Fund, Inc; Eugene, Oregon 97401: 2008. p. 250-263.

Torres-Rendon A, Roy S, Craig GT, Speight PM. Expression of Mcm2, geminin and Ki67 in normal oral mucosa, oral epithelial dysplasias and their corresponding squamous-cell carcinomas. Br J Cancer. 2009; 100:1128–34. [PubMed: 19293805]

van der Eb MM, Leyten EM, Gavarasana S, Vandenbroucke JP, Kahn PM, Cleton FJ. Reverse smoking as a risk factor for palatal cancer: a cross-sectional study in rural Andhra Pradesh, India. Int J Cancer. 1993; 54:754–8. [PubMed: 8325705]

van Zeeburg HJ, Snijders PJ, Pals G, Hermsen MA, Rooimans MA, Bagby G, Soulier J, Gluckman E, Wennerberg J, Leemans CR, Joenje H, Brakenhoff RH. Generation and molecular characterization of head and neck squamous cell lines of fanconi anemia patients. Cancer Res. 2005; 65:1271–6. [PubMed: 15735012]

Vigneswaran N, Beckers S, Waigel S, Mensah J, Wu J, Mo J, Fleisher KE, Bouquot J, Sacks PG, Zacharias W. Increased EMMPRIN (CD 147) expression during oral carcinogenesis. Exp Mol Pathol. 2006; 80:147–59. [PubMed: 16310185]

Vigneswaran N, Williams MD. Epidemiologic trends in head and neck cancer and aids in diagnosis. Oral and maxillofacial surgery clinics of North America. 2014; 26:123–41. [PubMed: 24794262]

Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol. 2009; 45:309–16. [PubMed: 18804401]

Warnakulasuriya S, Johnson NW, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med. 2007; 36:575–80. [PubMed: 17944749]

Weigum SE, Floriano PN, Redding SW, Yeh CK, Westbrook SD, McGuff HS, Lin A, Miller FR, Villarreal F, Rowan SD, Vigneswaran N, Williams MD, McDevitt JT. Nano-bio-chip sensor platform for examination of oral exfoliative cytology. Cancer Prev Res (Phila). 2010; 3:518–28. [PubMed: 20332305]

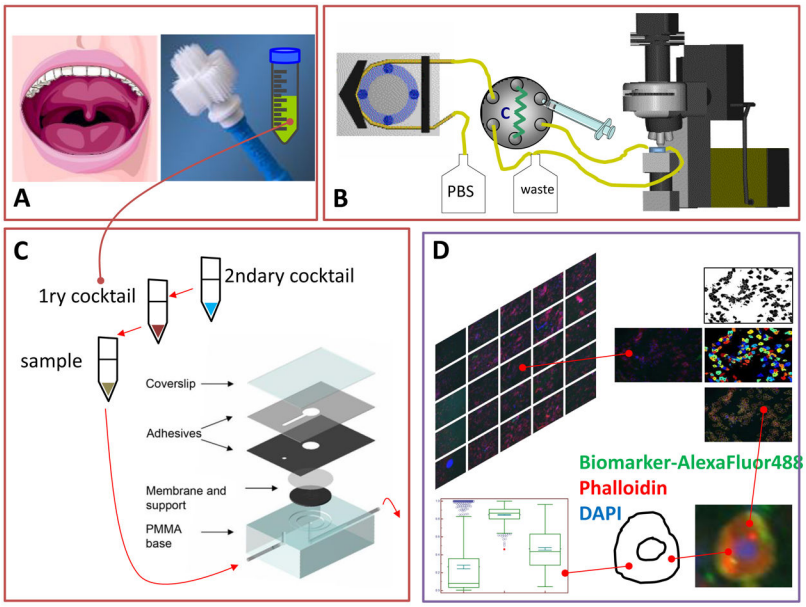

Figure 1.

Programmable Bio-Nano-Chip Assay (p-BNC) Instrumentation and Work Flow. Fluorescence-microscopy images of cells stained with a cocktail of fluorescent dyes and antibody- based reagents such that the cytoplasm appears red (Phalloidin-AlexaFluor-647), the nucleus blue (4′-6-diamidino-2-phenylindole: DAPI), and biomarker is green (AlexaFluor-488). PMMA: Poly (methyl methacrylate).

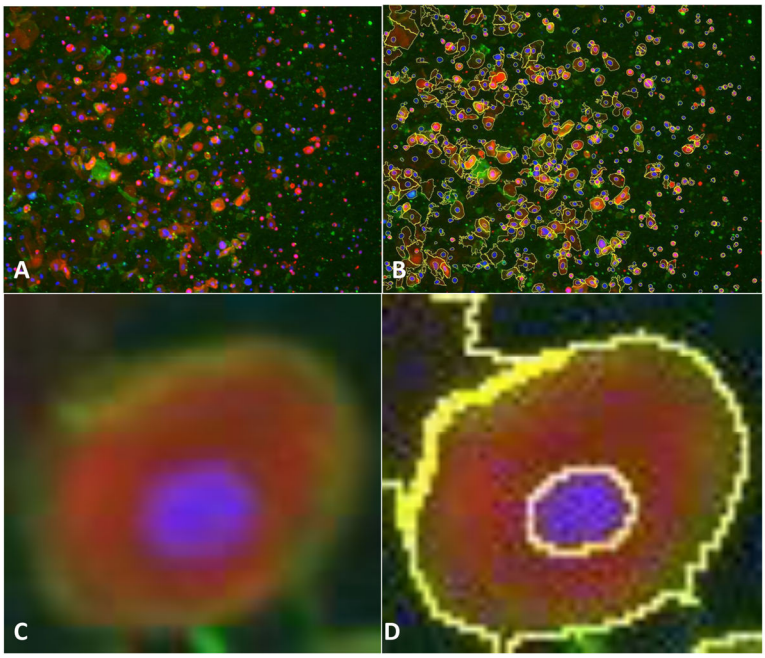

Figure 2.

Automated single cell measurement capability of p-BNC assay. In the raw image (A), individual cell outlines were identified by applying adaptive thresholds to DAPI and Phalloidin channels for each image (B) and used to create regions of interest (ROIs) specific to each cell (C &D). These ROIs were then analyzed through automated custom macros (ImageJ, Matlab) to obtain cytomorphometric and molecular data at a single-cell level. Measurements up to 20 parameters (i.e. area, mean value, standard deviation, modal value, centroid, center of mass, perimeter, bounding rectangle, fit ellipse, circularity, Feret's diameter, median, integrated density) are made for each color (Red, Green, Blue) separately for the cytoplasm and the nucleus.

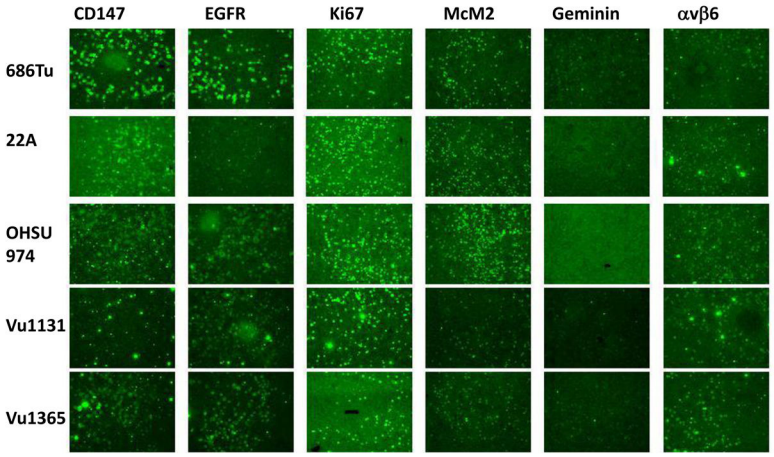

Figure 3.

Biomarker expression patterns in sporadic (686TU & 22A) and FA (VU1365, VU1131 & OHSU974) OSCC cell lines. Expression levels of these biomarkers in tumor cells are heterogenous among different cell lines as well as within the same cell line. However, there were no notable differences in the expression patterns of these biomarkers between FA and sporadic OSCC cell lines.

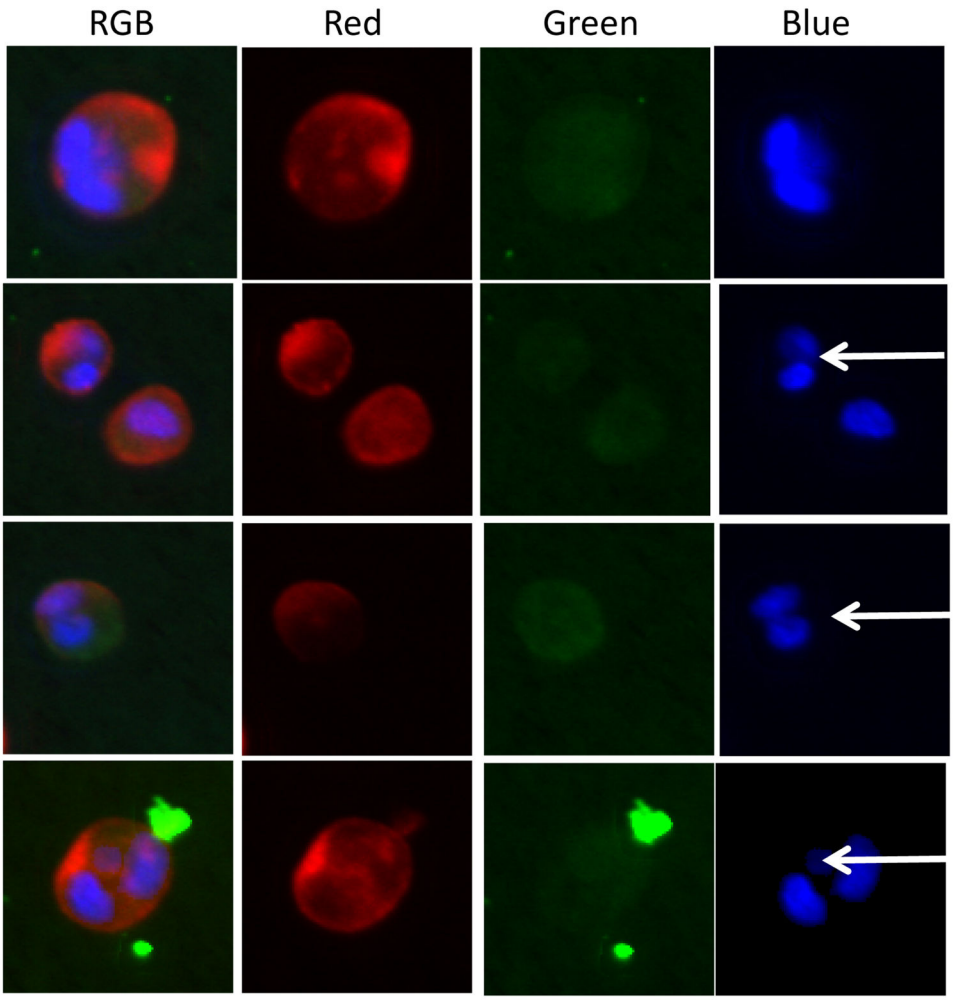

Figure 4.
Representative images illustrate the increased occurrence of binucleated and micronucleus in FA OSCC cell lines.

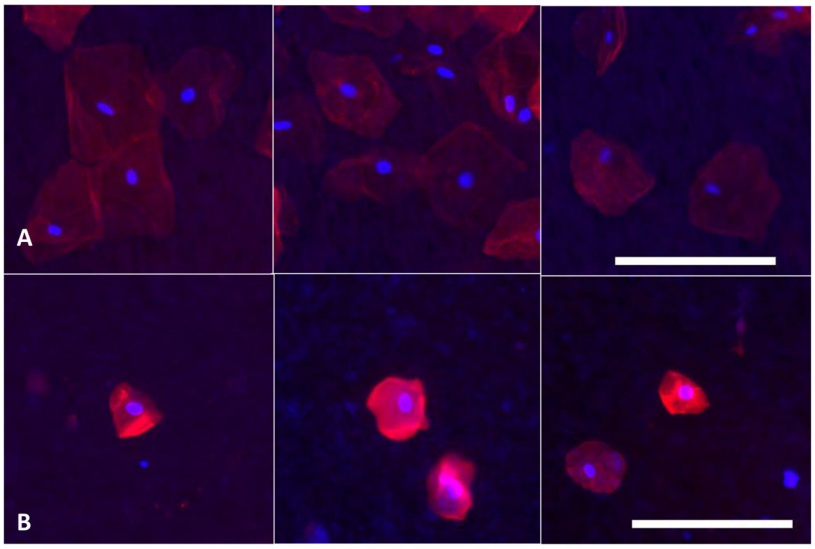

Figure 5.

Representative images of brush biopsy samples processed by p-BNC device. Each image is a false-color merged monochrome fluorescent image where the phalloidin dye is seen in red to discern the cytoplasm and DAPI is seen in blue to identify the nuclei. Row A includes randomly selected cells from the normal volunteer group and Row B includes randomly identified **cells** from the FA patient. The scale is the same between each image and the scale bar represents 200μm.

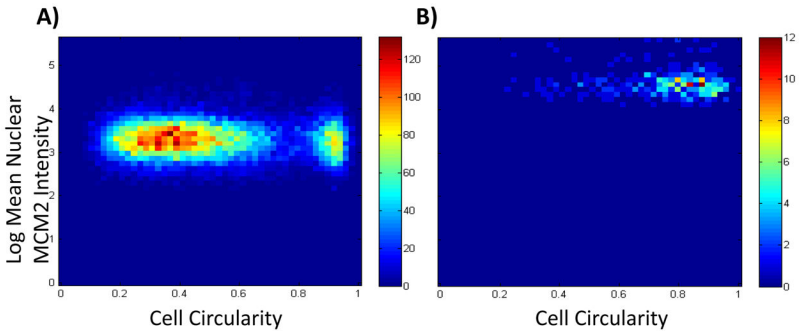

Figure 6.

Density scatter plots depicting the log mean nuclear MCM2 intensity (Y-axis) as a function of cell circularity (X-axis). A) Response for normal volunteer population across approximately 20,000 cells. B) Response for FA subject across approximately 100 cells. The color bars depict locations of maximum clustering as red, but are scaled to different values based on the total number of cells.

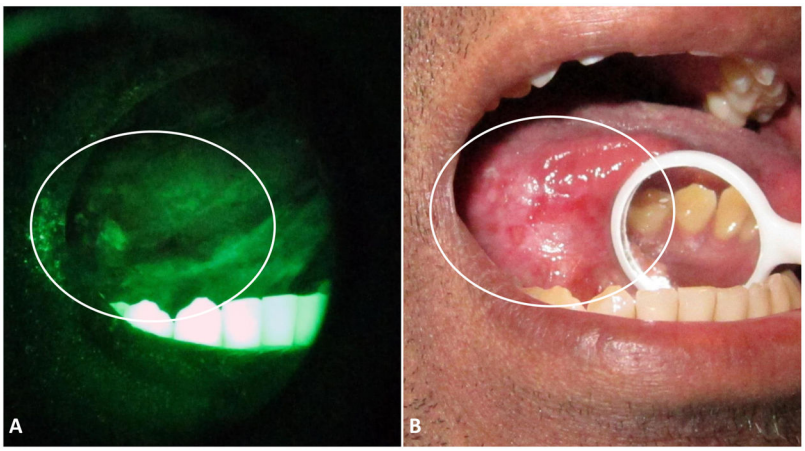

Figure 7.

Tissue autofluorescence (A) and clinical (B) images of the leukoplakia in the FA patient. This leukoplakia was considered as a high-risk lesion based on the clinical appearance and due to the loss of fluorescence when examined with the VELscope (A).

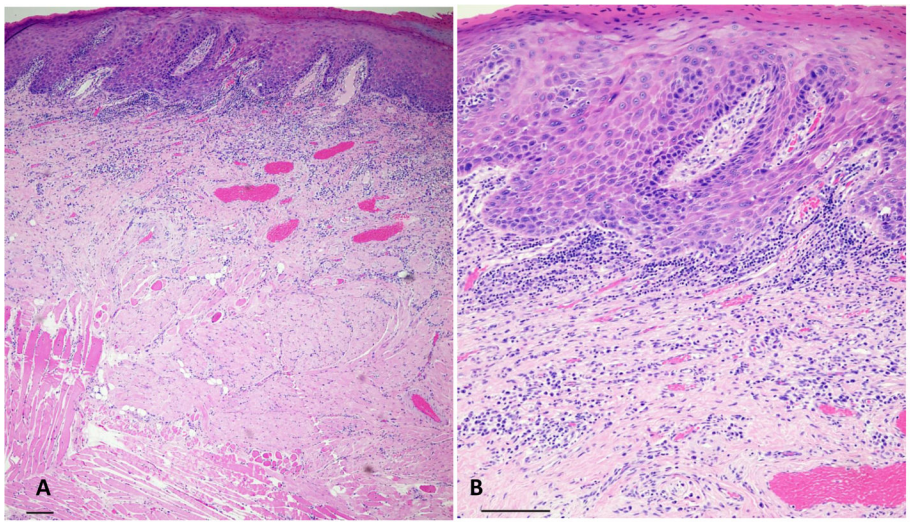

Figure 8.

Low (A) and high (B) power microscopic images of the excisional biopsy of the leukoplakia revealed epithelial hyperplasia with moderate dysplasia. The scale bar represents 100 μM.

Table 1

Results from ANOVA test for brush biopsy samples

Parameter	Subject Group	Median Value	p-value
Cytometric Biomarkers			
N-C Ratio	FA	0.12	p = 0.613
	Normal	0.11	
Nuclear Area (px)	FA	393.00	p< 0.001
	Normal	247.70	
Cell Circularity	FA	0.59	p< 0.001
	Normal	0.40	
Cell Area (px^2)	FA	2916.00	p = 0.219
	Normal	2312.00	
Molecular Biomarkers			
Nuclear MCM2 (Mean) (afu)	FA	86.32	p< 0.001
	Normal	28.61	
Nuclear StDev MCM2 (afu)	FA	7.15	p = 0.014
	Normal	4.12	