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Variability of cytokine gene expression in intestinal tissue and the impact of normalization with the use of reference genes

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ABSTRACT

Objective: To determine the intra- and inter-subject variability of mucosal cytokine gene expression in rectal biopsies from healthy volunteers and to screen cytokine and chemokine mRNA as potential biomarkers of mucosal inflammation.

Design and methods: Rectal biopsies were collected from 8 participants (3 biopsies per participant) and 1 additional participant (10 biopsies). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to quantify IL-1β, IL-6, IL-12p40, IL-8, IFN-γ, MIP-1α, MIP-1β, RANTES, and TNF-α gene expression in the rectal tissue. The intra-assay, inter-biopsy and inter-subject variance was measured in the eight participants. Bootstrap re-sampling of the biopsy measurements was performed to determine the accuracy of gene expression data obtained for 10 biopsies obtained from one participant. Cytokines were both non-normalized and normalized using four reference genes (GAPDH, β-actin, β2 microglobulin, and CD45).

Results: Cytokine measurement accuracy was increased with the number of biopsy samples, per person; four biopsies were typically needed to produce a mean result within a 95% confidence interval of the subject's cytokine level approximately 80% of the time. Intra-assay precision (% geometric standard deviation) ranged between 8.2 and 96.9 with high variance between patients and even between different biopsies from the same patient. Variability was not greatly reduced with the use of reference genes to normalize data.

Conclusions: The number of biopsy samples required to provide an accurate result varied by target although 4 biopsy samples per subject and timepoint, provided for >77% accuracy across all targets tested. Biopsies within the same subjects and between subjects had similar levels of variance while variance within a biopsy (intra-assay) was generally lower. Normalization of inflammatory cytokines against reference genes failed to consistently reduce variance. The accuracy and reliability of mRNA expression of inflammatory cytokines will set a ceiling on the ability of these measures to predict mucosal inflammation. Techniques to reduce variability should be developed within a larger cohort of individuals before normative reference values can be validated.

1. Introduction

The use of molecular biological techniques such as quantitative reverse transcription PCR (RT-qPCR), microarrays, and most recently droplet digital PCR has revolutionized the ability to detect and quantify gene expression in mucosal biopsy specimens. In the field of inflammatory bowel disease, pro-inflammatory cytokines have been measured, quantified, and compared in endoscopic biopsies of normal and inflamed colonic mucosa patients [1]. Within human immunodeficiency virus (HIV) research, elevated mucosal viral loads have been found to correlate with an increase in inflammatory cytokine gene expression [2] which remains elevated compared to HIV-negative controls in colonic mucosa even when peripheral blood viral loads are undetectable from antiretroviral suppression [3]. Quantifying the amount of pro-inflammatory cytokines in colonic mucosa, as a biomarker of product related mucosal damage, is also of interest in HIV microbicide development [4].

The majority of studies evaluating gene expression in mucosal tissue use RNA isolated from a single biopsy although it is

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uncertain how representative these data are in characterizing mucosal disease or in defining the presence or absence of changes in gene expression at mucosal sites. By definition, a tissue biopsy contains a heterogeneous collection of epithelial, stromal, and lymphoid tissue cellular elements. It is conceivable, if not probable, that there might be variation in gene expression in biopsies collected from the same individual or from the same region but collected from different individuals. Regional heterogeneity in colorectal gene expression has been documented in two studies that characterized cytokine and chemokine gene expression in biopsies collected in the rectum and sigmoid colon [4,5]. In contrast, it is routine to collect multiple mucosal biopsies to diagnose or exclude gastroenterological conditions such as celiac disease [6], Barrett's esophagus [7], and ulcerative colitis associated dysplasia [8].

RT-qPCR is the method of choice for the characterization of cytokine gene expression. Gene expression is often normalized against reference genes that are considered to be constitutively expressed at all times and invariable. However, controversy also exists over the use of reference genes because of the variable ranges of expression noted in different types of tissue [9–11].

The purpose of this study was to determine intra- and inter-subject variability in biopsy mucosal gene expression in a healthy population as a first step in attempting to define normal ranges for cytokine gene expression in mucosal biopsies. We also explored whether normalization using reference genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, β2 microglobulin (β2M), or CD45 reduced the variability in gene expression within and among participants. The final goal of the study was to define assay validation criteria for a putative cytokine biomarker and determine which of the cytokines/chemokines evaluated best met these criteria. A biomarker that had a geometric standard deviation (GSD) less than 20%, accuracy >80%, was normally distributed without outliers, and had a range that spanned less than two logarithms was defined as a potential biomarker of mucosal inflammation.

2. Materials and methods

2.1. Biological samples and participant characteristics

Rectal biopsies were obtained from healthy volunteers ($N = 9$). Exclusion criteria included a history of inflammatory bowel disease or other chronic gastrointestinal disease, immune disorders, rectal gonorrhea, chlamydia, or herpes simplex virus infections, bleeding disorder or anticoagulation use. Participants were confirmed HIV negative within the last 6 months or had a rapid HIV test performed on site. Consent was obtained for endoscopic biopsy by flexible sigmoidoscopy or anoscopy. Biopsies were obtained by trained staff, and patients agreed to abstain from any aspirin-containing medications or other non-steroidal anti-inflammatory drugs for at least 3 days prior to biopsy. In this study, 8 patients had 3 biopsies taken while 1 patient had 10 biopsies taken (see Table 1 for participant characteristics). The study was approved by the University of Pittsburgh Institutional Review Board (IRB# PRO08030370).

2.2. RNA preparation

Following collection, tissue biopsies were immediately placed in RNA*later*™ (Applied Biosystems/Ambion, Austin, TX, USA) solution and kept at −4 °C for at least 4 h prior to final storage at −80 °C. In preparation for extraction of total RNA, the samples were thawed on ice. Biopsies were removed from the RNA*later*™ and placed in guanidinium lysis buffer supplied with the RNAqueous®-4PCR Kit (Applied Biosystems/Ambion, Foster City, CA, USA). Samples were homogenized in the presence of 0.5 mm RNase-free zirconium beads with the aid of the Bullet Blender homogenizer (Next Advance Inc., Averil Park, NY, USA). RNA was then purified on columns according to the RNAqueous®-4PCR Kit instructions (Applied Biosystems/Ambion, Foster City, CA, USA). The extracted total RNA was eluted in a volume of 100 μL and DNase-1 treated. DNase-1 was inactivated before RNA was used in cDNA preparation.

Table 1
Study participant characteristics.

Study number	No of biopsies	Age	Sex	Race	HIV status
APT 036.2	3	21	Male	White	Negative
APT 042.2	3	22	Female	White	Negative
APT 049.2	3	24	Female	Black	Negative
APT 048.1	3	23	Male	White	Negative
APT 050.1	3	55	Male	White	Negative
APT 054.2	3	28	Female	Black	Negative
APT 057.2	3	28	Female	Black	Negative
APT 065.2	3	51	Male	White	Negative
APT 099.1	10	47	Female	Asian	Negative

All DNAse-1-treated RNA samples were assessed for quantity and quality on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA, USA). Quantitative measurements were determined and recorded in ng/μl. Quality measurement were assessed by the 18S/28S ribosomal RNA peak ratios. Based on these measurements, RNA Integrity Numbers (RIN) were calculated automatically by the Agilent 2100 Bioanalyzer software. The absence of genomic DNA contamination was assessed by visualization of low baselines between the 18S and 28S ribosomal RNA peaks from the electropherogram image. RNA quality control limits were set within the laboratory as nucleic acid concentrations >25 ng/μL, 18S/28S ratios of 2.0 ± 0.5, and RIN $\geqslant$ 6.0.

2.3. Quantitative RT-PCR

Real-Time quantitative reverse transcription PCR (RT-qPCR) was used to quantify mucosal mRNA expression. Equal quantities (1000 ng) of total RNA from biopsy samples were converted to cDNA using MultiScribe™ reverse transcriptase and TaqMan® Reverse Transcription reagents (Applied Biosystems, Roche Molecular Systems, Inc., Branchburg, NJ, USA). Oligo(dT)$_{20}$ (Invitrogen, Grand Island, NY, USA) was used to prime the reverse transcription (RT) reaction and reactions were run on the Applied Biosystems Veriti™ Dx Thermal Cycler (Life Technologies Corporation, Carlsbad, CA, USA). Identical quantities of RNA and reagents were used in the no reverse transcriptase (NRT) reaction which contained all components with the exception of reverse transcriptase.

Rectal cDNA was used as a template for the PCR amplification. The three reference genes used to normalize cytokine gene expression were GAPDH, β-Actin and β2 Microglobulin (β2M) (Table 2). All PCR reactions were performed using the Bio-Rad CFX96 RT-PCR System (Bio-Rad, Hercules, CA, USA). Probes and/or primers were designed to span the intron–exon boundaries to ensure amplification from cDNA rather than from genomic DNA. The master mix and primer/probe mixes for the reference genes (GAPDH, β-Actin, and β2M) were obtained from Solaris QPCR Gene Expression Assays (Thermo Fisher Scientific Inc., Waltham, MA, USA). One μL of the cDNA was added to 5 μL of Solaris qPCR Master Mix (2X), 0.5 μL of Primer/Probe Set (20X), and 3.5 μL of nuclease free water for each reaction replicate. Solaris master mix assays utilized standard TaqMan cycling conditions of 95 °C denaturation for 15 min followed by 40 cycles of 95 °C denaturation for 15 s and 60 °C annealing for 1 min. Serial 1:10 dilutions of plasmid DNA