Whole blood ACTB, B2M and GAPDH expression reflects activity of inflammatory bowel disease, advancement of colorectal cancer, and correlates with circulating inflammatory and angiogenic factors: Relevance for real-time quantitative PCR

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Abstract

Background. The effect of bowel inflammation and cancer on the expression of the most prevalent internal controls: ACTB, GAPDH and B2M in whole blood is unknown, although at least GAPDH occurred to be tightly regulated and suspected of supporting cancer growth, challenging its suitability as a reference.

Objectives. To evaluate the effect of colorectal cancer (CRC) and active inflammatory bowel disease (IBD) on the stability of ACTB, B2M, GAPDH, HPRT1, SDHA, and TBP leukocyte expression.

Material and methods. Gene expression in controls and CRC and IBD patients (n = 21/18/25) was evaluated in real-time quantitative polymerase chain reaction (RT-qPCR) using NormFinder, geNorm, BestKeeper, and comparative ΔCt method, and validated by comparison with absolute quantification of interleukin 1β (IL-1β) and CCL4.

Results. HPRT1, SDHA and TBP were superior normalizers in CRC and IBD. The highest expression variability was noted in active IBD. B2M was significantly lower in CRC but higher in IBD. GAPDH was higher in CRC and IBD. ACTB and GAPDH corresponded with CRC advancement (p = 0.52 and p = 0.53) and with clinical activity in Crohn’s disease (p = 0.44 and p = 0.57) and ulcerative colitis (GAPDH: p = 0.72). ACTB, B2M and GAPDH correlated with circulating inflammatory/angiogenic indices, differently in IBD and CRC.

Conclusions. Leukocyte GAPDH, ACTB, and B2M expression is affected by bowel inflammation and cancer, rendering them unsuitable as a reference in CRC and IBD.

Key words: geNorm, NormFinder, BestKeeper, whole blood transcriptome, expression stability
Introduction

Real-time quantitative polymerase chain reaction (RT-qPCR) is a powerful tool used to detect even subtle alterations in gene expression in order to unravel pathomechanisms of diseases and to aid research on new biomarkers and/or therapeutic targets. Normalization of target gene expression against reference gene(s), assumed to be uniformly expressed and hence referred to as housekeeping genes (HKG), is a common method of accounting for non-biological variation introduced during sample handling and attributed to differences in amount of loaded templates, transcription efficiencies, contamination with inhibitors, etc. Considering the high sensitivity of the method, the choice of appropriate reference genes is a prerequisite for obtaining valid and reproducible results in studies employing RT-qPCR. However, there is now solid evidence that HKG expression is in fact regulated and might not only differ between cells or tissues but also change in response to endo- or exogenous factors. Particularly, the stability of the most popular reference genes, GAPDH, ACTB and B2M, has recently been challenged, both in cancer disease and in inflammation. Consequently, a need for validating candidate normalizers prior to their application as internal control in order to find these minimally regulated under given experimental conditions has been repeatedly stressed. Inflammatory bowel disease (IBD) and colorectal cancer (CRC) evoke a response from circulating immune cells, which is why analyzing gene expression patterns in whole blood, owing to its availability, might be advantageous over more relevant but less accessible bowel tissues. Accordingly, profiling gene expression in blood is gaining interest. Recently, it has been proved useful in evaluating various pathological conditions and immune responses, including identifying new biomarkers in IBD and CRC. We have previously shown that both bowel inflammation and cancer affect tissue expression of common reference genes. However, their potential effect in whole blood has not been investigated yet. Hence, we designed our present study to find and validate optimal reference genes for whole blood transcriptome studies involving patients with CRC and IBD, active and non-active, and to examine and compare the effect, if any, bowel cancer and inflammation might have on the expression of popular reference genes in whole blood. We showed that the most popular genes used for whole blood transcriptome analysis are not suitable for CRC and IBD patients due to the high level of variation in their expression. Moreover, we showed that this variation was directional; namely, it reflected the advancement/severity of diseases and the expression of genes associated with inflammatory and angiogenic responses.

Material and methods

Study population

Sixty-four individuals were enrolled in the current study: 18 CRC patients with histologically confirmed adenocarcinoma, 25 patients with active IBD (13 with Crohn’s disease (CD) and 12 with ulcerative colitis (UC)), and 21 controls recruited from patients with adenomas (n = 4), colonic diverticulosis (n = 1) or IBD in remission (n = 16). There were 3 patients with T2N0M0, 1 with T2N × M0, 2 with T3N0M0, 5 with T3N1M0, 2 with T4N0M0, 3 with T4N1M0, 1 with T4N2M1, and 1 with T4N1M1 in cancer group. For the assessment of CD activity, the Crohn’s Disease Activity Index (CDAI) combining the evaluation of vital parameters, clinical findings and medical history was applied, and CDAI ≥ 150 was indicative of active disease. For the assessment of UC activity, the Rachmilewitz Index (RI; clinical activity index – CAI), encompassing stool frequency, number of stools with blood, general wellbeing, abdominal pain/cramp, fever, extraintestinal manifestations, and laboratory tests (erythrocyte sedimentation rate and hemoglobin concentration) was applied and RI ≥ 6 was indicative of active disease.

Female to male ratio in control/CRC/IBD groups was as follows: 7/14, 3/15 and 10/15, p = 0.257 and age distribution was as follows: median 46 years (range: 20–78), 62 years (46–77) and 34.8 years (19–60), p < 0.001. None of HKG correlated with age.

Ethical considerations

The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University (approval No. KB-575/2011 from 10 November 2011) and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983, and informed consent was obtained from all patients.

Analytical methods

Sample selection and RNA isolation, quantification and quality assessment

Whole blood samples (3 mL) were collected into PAXgene Blood RNA Tubes prior to any treatment and stored at –80°C. RNA was isolated and purified with complementary PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions, then quantified in duplicates with NanoDrop 2000 (Thermo Fisher Scientific, Waltham, USA) and its purity assessed by calculating ratios of absorbance at 260 nm, 280 nm, and 230 nm. RNA integrity was evaluated with RNA quality indicator (RQI; from 1 – degraded to 10 – intact RNA) using the Experion platform incorporating LabChip microfluidic technology and Experion RNA StdSens analysis kits (BioRad,
Hercules, USA). Only RNA isolates with RQI ≥ 7 were used for RT-qPCR. Possible presence of inhibitors in each RNA isolate was tested by calculating RT-qPCR efficiencies from standard curves prepared by serial dilutions of respective cDNA samples (five-fold dilutions, 6 point-curve, conducted in duplicates using SG qPCR Master Mix from EURx, Gdańsk, Poland). Working dilution of cDNA 1:5 was found to effectively dilute reaction inhibitors and assure near 100% qPCR efficiencies.

Reverse transcription

To assure the same load of RNA template, for all samples 0.25 µg of whole blood RNA per reaction (20 µL), previously quantified in duplicates with NanoDrop 2000 and verified in Experion electrophoresis, was reversely transcribed in C1000 termocycler (BioRad) using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) according to the protocol suggested by manufacturer. All samples were accompanied by matching negative transcription (“no-RT”) controls, devoid of reverse transcriptase, subsequently tested to assure lack of contamination with genomic DNA.

Quantitative real-time PCR

Quantitative real-time PCR (qPCRs) were conducted with CFX96 Real-Time PCR system (BioRad) using SsoFast EvaGreen® Supermix (BioRad) and the following cycling conditions: 30 s activation at 95°C, 5 s denaturation at 95°C, annealing/extension for 5 s at 61°C, 40 cycles, followed by melting step (60–95°C with fluorescent reading every 0.5°C). Reaction mixture contained 2 µL of cDNA (diluted 1:5), 10 µL of ×2 SsoFast EvaGreen® Supermix 1 µL of each 10 nM forward and reverse target-specific primers, and water up to 20 µL. Sequences and specificities (evaluated with RT-qPCR and calculated from a four-fold dilution series with 6 measuring points in triplicates, plotted as Cq vs logarithms of dilution values of the DNA templates) of optimized and validated primers spanning at least 1 intron are presented in Table 1. Primers were synthesized by Generi Biotech (Hradec Králové, Czech Republic). A mixture of all cDNA samples investigated was used as a template for calculating primers’ specificities. Samples were assessed in 3 technical replicates and accompanied by “no template” control.

Absolute quantification

For absolute quantification, plasmids with IL-1β and CCL4 inserts were prepared in the following manner: PCR products (10 ng) were cloned into pET1.2/blunt cloning vector following manufacturer’s protocol (Fermentas UAB, Vilnius, Lithuania) and subsequently used to transform NovaBlue GigaSingles™ Competent Cells-Novagen (Sigma-Aldrich, St. Louis, USA). Amplified constructs with IL-1β and CCL4 inserts were isolated using Plasmid DNA Purification Kit (Macherey-Nagel, Düren, Germany) and quantified using NanoDrop 2000. Mean concentrations of IL-1β and CCL4 plasmids were 4.47 µg/µL and 5.89 µg/µL, respectively, and 260/280 and 260/230 ratios were 2.03 and 2.4 for IL-1β and 1.99 and 2.36 for CCL4. Ten-fold dilution series was prepared for each plasmid (E = 102.1%, R² = 2.0 for IL-1β and E = 100.7% and R² = 0.999 for CCL4) and used for constructing standard curves, in which Cq values were plotted against log of copy

### Table 1. Sequences and performance characteristics of primers used in the current study

| Symbol | Gene name; function of encoded protein | Accession No. | Primer sequence 5’→3’ (forward/reverse) | Amp. size | E [%] |
|--------|----------------------------------------|---------------|------------------------------------------|-----------|------|
| ACTB¹  | actin, β; cytoskeletal structural protein | NM_001101.3   | F: caccattgcgaatagcgtt R: aagcttggaggtgctcagt | 135 bp    | 100.9 |
| B2M¹   | β₂-microglobulin; β-chain of MHC class I molecules | NM_004048.2  | F: ccaactgaaagatgtagtgcct R: ccaactccaatgcggcatctca | 126 bp | 104.3 |
| GAPDH² | glyceraldehyde-3-phosphate dehydrogenase; glycolytic enzyme | NM_002046.4  | F: gttcccttcgtactcaacagcg R: acaccccttctgtgacctggca | 131 bp | 105.4 |
| HPRT1  | hypoxanthine phosphoribosyl-transferase, purine metabolism | NM_000194.2  | F: tgcactgcaaagatagatgcct R: ccaactccaatgcggcatctca | 94 bp | 103.2 |
| SDHA²  | succinate dehydrogenase subunit A; subunit of respiratory chain complex | NM_005168.2 | F: aggcgaccaaggaaggtcgac R: cccacctgttgctctcagtagg | 267 bp | 94.8  |
| TBP     | TATA-box-binding protein; general transcription factor | NM_003194.4  | F: tataacctcagcaaggttctg R: cttgaccttaactcaattgttgg | 283 bp | 109.7 |
| IL-1β²  | interleukin (IL)-1β | NM_000576.2 | F: ccaagacctcctcaggaagagt R: gtgcagtctctgtcgtacagg | 131 bp | 100.1 |
| CCL4²  | macrophage inflammatory protein (MIP)-1 β | NM_002984.2 | F: gtgcatacaagtctctgtcag R: gttccctgcacatgttgtag | 140 bp | 103.5 |

Amp – amplicon; E – efficiency; ¹ primer sequences were as proposed by Origene (www.origene.com). Remaining primers were designed using Beacon Designer Probe/Primer Design Software (BioRad) as previously described (manuscript submitted). Forward and reverse primer sequences are denoted by “F” and “R”, respectively.
number. The qPCR were run in triplicates using SsoFast EvaGreen® Supermix (BioRad) and conditions described in the above section.

**Circulating cytokines and growth factors**

For the purpose of correlation analysis, data on circulating cytokines and growth factors: IL1-β, IL-4, IL-6, IL-8, IL-12, G-CSF, GM-CSF, FGF2, MCP-1 (CCL2), macrophage inflammatory protein (MIP)-1α (CCL3), PDGF-BB, and tumor necrosis factor α (TNF-α), were retrieved from our earlier study and were available for 26 patients (16 with CRC and 10 with IBD). Cytokines/growth factors were measured by means of flow cytometry-based method incorporating Luminex xMAP® technology, which utilizes magnetic microspheres conjugated with monoclonal antibodies. Measurements were conducted on BioPlex 200 platform with HRF (BioRad) using custom-made multiplexes validated by the manufacturer (BioRad).

Data on C-reactive protein (CRP) levels at the time of blood sample collection for current study was retrieved from patients’ medical records.

**Statistical analysis**

Technical replicates were averaged prior to any analyses and differences in primer efficiencies (Table 1) were taken into account by calculating efficiency (E)-corrected Cq values (CqE) using the following formula: CqE = \log(E^{-1/Cq}) base 2, where \( E = 10^{-1/slope~of~standard~curve} \).

Expression stability was evaluated using the 4 most popular algorithms: geNorm utility in qbasePLUS v. 2.4 software (Biogazelle BE, Ghent, Belgium), calculating stability M value described as the average pair-wise variation of a specific gene as compared with other candidate genes, NormFinder software v. 0.953 (available as MS Excel Add-in at www.mdl.dk/publicationsnormfinder.htm), calculating intra- and inter-group variability combined into stability value, BestKeeper, using pair-wise correlation analysis of candidate genes yielding BestKeeper index, and comparative ΔCt method, comparing relative expression of pairs of genes within each sample and calculating mean standard deviation (SD) for each gene. RefFinder comprehensive tool (available at www.leonxie.com) was used to create the final ranking integrating the results obtained with geNorm, NormFinder, BestKeeper, and comparative ΔCt method by assigning an appropriate weight to an individual gene, calculating the geometric mean of their weights and re-ranking the candidate genes accordingly. The smaller geometric mean, the more stably expressed reference gene.

Number of genes sufficient as normalizers was determined with geNorm algorithm. Remaining analyses were conducted using MedCalc Statistical Software v. 12.7.7 (MedCalc Software bvba, Ostend, Belgium) on absolute or normalized relative quantities (NRQ) calculated with qbasePLUS. Normalized relative quantities is a Cq of a given sample referred to the average Cq across all samples for specific gene, with differences in PCR amplification efficiencies taken into account, and normalized against geometric mean Cq of a set of genes selected as reference. If not otherwise stated, data was presented as geometric means with 95% confidence interval (95% CI). Between-group differences were analyzed using one-way analysis of variance (ANOVA) with post-hoc analysis with the Tukey–Kramer test (multigroup comparisons) or using t-test for independent samples with Welch correction when necessary (two-group comparisons). Correlation analysis was conducted using Pearson or Spearman rank correlation tests. Data distribution was tested using \( \chi^2 \) test and the homogeneity of variances was tested using Levene’s test. Frequency analysis was conducted using \( \chi^2 \) test. All calculated probabilities were two-tailed and p-values ≤0.05 were considered statistically significant.

**Results**

**Finding optimal reference genes for studying whole blood transcriptome from IBD and CRC patients**

The expression stability of 6 common reference genes, namely ACTB, B2M, GAPDH, HPRT1, SDHA, and TBP, was...
For studies on whole blood transcriptome in mixed cohorts of CRC and IBD patients or for studies on IBD patients, respective V value dropped below 0.15 (threshold) when normalization factors based on the 3 or 4 most stable reference genes were compared, indicating that normalization of target gene expression should be based on 3 most stable reference genes (Fig. 2), that is, SDHA, TBP and HPRT1. For studies on CRC patients, normalization against SDHA and TBP was found sufficient.

**Validation of selected normalizers**

The performance of reference genes was tested on 2 target genes, CCL4 and IL-1β, known to be differently expressed in CRC and IBD. Results obtained using relative quantification method in which target gene expression was normalized either against geometric mean of SDHA, TBP and HPRT1 or against individual popular normalizers – ACTB, B2M or GAPDH, were compared with these yielded by absolute quantification method with transcript copy number.

CCL4 was significantly overexpressed in CRC as compared to IBD and insignificantly downregulated in IBD as compared to controls (Fig. 3A). While results obtained using relative quantification with SDHA, TBP and HPRT1 as normalizers were concordant with those obtained using absolute quantification, normalization against B2M and GAPDH substantially overestimated between-group differences in CCL4 expression.

IL-1β was significantly underexpressed in CRC as compared to IBD, while the slight upregulation in IBD as compared to controls was not significant (Fig. 3B). Again, the results obtained with the absolute method and using the proposed panel of reference genes were concordant, while those obtained using either B2M or GAPDH underestimated CRC-IBD difference and wrongly implied the downregulation of IL-1β in IBD as compared to controls. For both target genes, of the most popular reference genes, normalization against ACTB yielded the closest results to those obtained with absolute quantification or by using a panel of normalizers.

**Effect of inflammation and cancer on GAPDH, ACTB and B2M expression in whole blood**

Using the panel of normalizers validated here, we compared the effect, if any, of bowel inflammation and cancer on the whole blood expression of ACTB, B2M and GAPDH. As depicted in Table 3, both B2M and GAPDH expressions differed significantly between groups. GAPDH expression was regulated in active IBD as compared to controls by 1.7-fold (95% CI = 1.3–2.1; p < 0.001). It was also upregulated as compared to CRC – by 1.8-fold (1.3–2.5; p < 0.001). B2M expression in active IBD was upregulated by 1.5-fold (1.1–2; p = 0.009) and by 1.5-fold (1.1–1.9;
While GAPDH in CRC was significantly higher than in controls, the expression of B2M was significantly lower. Between-group differences in ACTB expression did not reach statistical significance when analyzed with ANOVA and Tukey–Kramer post-hoc test, but when the controls and IBD patients were compared using t-test for independent samples, the difference was significant (p = 0.043).

In CRC, ACTB and GAPDH expressions increased along with the disease advancement, being associated with the metastatic potential rather than local progression. In IBD, gene expression was not affected by the disease phenotype (CD vs UC), but ACTB positively correlated with clinical activity of CD and GAPDH also with the activity of UC.

Table 3. Relationship between the whole blood expression of ACTB, B2M and GAPDH, and the disease characteristics

| Characteristics | n  | ACTB               | B2M               | GAPDH              |
|-----------------|----|--------------------|-------------------|--------------------|
| Cohort:         |    |                    |                   |                    |
| controls        | 21 | p = 0.127          | p = 0.001         | p < 0.001          |
| CRC             | 18 | 0.87 (0.78–0.98)   | 0.89 (0.73–1.1)   | 0.79 (0.69–0.91)   |
| active IBD      | 25 | 1.01 (0.85–1.21)   | 0.75 (0.6–0.94)   | 0.9 (0.76–1.05)    |
| CRC stage       |    | p = 0.52, p = 0.029| p = 0.02, p = 0.951| p = 0.53, p = 0.025|
| Local progression (T) |   | p = 0.16, p = 0.520| p = 0.0, p = 0.965| p = 0.0, p = 0.972|
| Lymph node status (N): |   |                    |                   |                    |
| N0              | 7  | p = 0.084          | p = 0.958         | 0.79 (0.65–0.92)   |
| N1/2            | 10 | 0.89 (0.7–1.07)    | 0.77 (0.51–1.17)  | 1.05 (0.8–1.3)     |
| Distant metastases (M): | |                    |                   |                    |
| M0              | 16 | p = 0.242          | p = 0.561         | 0.74 (0.58–0.94)   |
| M1              | 2  | 1.04 (0.85–1.22)   | 1.36              | 0.9 (0.75–1.05)    |
| IBD phenotype:  |    |                    |                   |                    |
| CD              | 14 | p = 0.347          | p = 0.339         | 0.755              |
| UC              | 11 | 1.22 (1.01–1.46)   | 1.22 (0.85–1.8)   | 1.28 (1.08–1.53)   |
| IBD activity⁴:  |    |                    |                   |                    |
| CD: CDAI        | 20 | p = 0.44, p = 0.052| p = 0.36, p = 0.118| p = 0.57, p = 0.009|
| UC: RI          | 12 | p = 0.34, p = 0.274| p = 0.0, p = 1    | p = 0.72, p = 0.009|

¹significantly different from CRC; ²significantly different from active IBD; ³significantly different from controls; ⁴correlation with clinical activity was calculated for all patients for whom it was available, also these with clinically inactive disease (CDAI < 150 or RI < 6). Data presented as means of normalized relative quantities (against geometric mean of SDHA, TBP and HPRT1) with 95% confidence intervals (95% CI) or as Spearman rank correlation coefficients (ρ).

CD – Crohn’s disease; UC – ulcerative colitis; CDAI – Crohn’s disease activity index; RI – Rachmilewitz index.
To further explore the association between popular reference genes and inflammation or tumor angiogenic potential, we investigated whether their expression was related to the levels of *IL-1β* and *CCL4* transcripts in whole blood and whether there was a correlation with circulating mediators of inflammation and angiogenesis.

Concerning whole blood, *ACTB* positively correlated with *CCL4*, exclusively in CRC patients (r = 0.57, p = 0.014), and with *IL-1β* in active IBD (r = 0.41, p = 0.42). It also tended to correlate with *IL-1β* in CRC (r = 0.45, p = 0.061). *B2M* correlated with *CCL4* and *IL-1β* neither in CRC nor in IBD patients. *GAPDH*, in turn, correlated exclusively with *CCL4* expression: positively in CRC (r = 0.58, p = 0.013) but negatively in IBD (r = −0.45, p = 0.023).

Table 4. Association between the whole blood expression of *ACTB*, *B2M* and *GAPDH*, and the systemic levels of inflammatory markers and growth factors.

| Marker | ACTB | B2M | GAPDH |
|--------|------|-----|-------|
| CRP    | r = 0.51, p = 0.0121 | –   | r = 0.63, p = 0.0011 |
| IL-1β  | r = 0.64, p = 0.0102 | –   | r = 0.74, p < 0.0001 |
| IL-4   | –    | –   | p = 0.62, p = 0.0531 |
| IL-6   | r = 0.54, p = 0.0322 | –   | p = 0.63, p = 0.0092 |
| IL-8   | r = 0.49, p = 0.0532 | –   | r = 0.64, p = 0.0461 |
| IL-12  | –    | –   | r = 0.71, p = 0.0231 |
| FGF2   | r = 0.65, p = 0.0072 | r = −0.49, p = 0.0552 | r = 0.53, p = 0.0352 |
| G-CSF  | r = 0.66, p = 0.0052 | r = −0.56, p = 0.0257 | r = 0.81, p < 0.0001 |
| GM-CSF | r = 0.65, p = 0.0062 | –    | r = 0.43, p = 0.0962 |
| MIP-1α | r = 0.63, p = 0.0092 | r = 0.65, p = 0.0441 |
| TNF-α  | r = 0.57, p = 0.0206 | r = −0.65, p = 0.0061 | r = 0.79, p < 0.001 |

1 correlation observed in IBD patients; 2 correlation observed in CRC patients. Data on circulating cytokines and growth factors was available for 26 patients (16 with CRC and 10 with IBD). Data presented as Pearson correlation (r) or Spearman rank correlation (p) coefficients.

**GAPDH, ACTB and B2M correlation with inflammatory and angiogenic indices**

A body of evidence has been gathered showing that there is no universal reference gene(s) that would be suitable for all tissues or pathophysiological conditions and that using invalidated normalizers, the expression of which is regulated under experimental conditions, might compromise the study leading to erroneous conclusions.1,6 There are no previous reports validating reference genes for whole blood analysis (encompassing a mixture of RNA from numerous blood cell types) in a mixed cohort including patients with inflammatory and cancer disease. Thus, we started our research from selection and validation of potential reference genes. As shown by the literature survey, *GAPDH, ACTB* and *B2M* are the most popular reference genes, frequently used single-handedly. Of these, *GAPDH* was rather poorly rated in blood-derived RNA studies, displaying high variability in neonatal whole blood with varying degrees of hypoxia and acidosis,19 whole blood of patients with multiple sclerosis,20 in neutrophils from healthy individuals,21 and in platelets22 or peripheral blood mononuclear cells23 from patients with a history of cardiac inflammatory conditions. It also fluctuated during the activation of T lymphocytes24 or LPS-stimulation of monocytes.25 Under some of these conditions, *ACTB*19,21,22,26 or *B2M*20,21,25 have been expressed stably, but similarly to *GAPDH, ACTB* expression has been altered upon the activation of lymphocytes24,27 or monocytes.25 In reticulocytes, in turn, *GAPDH* and not *B2M* have been found to be a better reference gene.18 Of other evaluated reference genes, *HPRT1*,18,21,26 *TBP*25 and *SDHA*18 have been repeatedly ranked well and, as such, they have been included in the current study.

There are several software programs available, which, using various algorithms, are dedicated to aiding in the evaluation of expression stability. We used the 4 most popular ones: NormFinder,16 geNorm utility in qBasePLUS,15 BestKeeper,17 and comparative ΔCt method,18 and employed...
RefFinder to summarize the results. Except for BestKeeper, all algorithms yielded concordant results with HPRT1, SDHA and TBP, outperforming ACTB, B2M and GAPDH. B2M was uniformly found the least stable of evaluated reference genes. All genes displayed the highest level of variability in IBD. Consequently, in studies involving patients with active disease normalization against 3 genes is optimal, while 2 – TBP and SDHA – are sufficient for CRC patients. To validate selected reference genes as normalizers, we analyzed expression of 2 target genes – IL-1β and CCL4 – using both absolute and relative quantification, the latter employing either a set of stable reference genes (HPRT1/SDHA/TBP) or ACTB, B2M and GAPDH individually. Only normalization against a set of reference genes yielded results of relative IL-1β and CCL4 expression analysis comparable to these obtained with absolute quantification of copy number. Normalization against GAPDH or B2M substantially overestimated between-group differences in CCL4 and underestimated differences in IL-1β expression, rendering these genes not suitable for whole blood expression analysis in IBD and CRC patients. However, the results obtained with ACTB as a reference did not differ much, although normalization against ACTB overestimated CCL4 downregulation in active IBD, falsely signifying the difference.

Random fluctuations in reference genes are likely to mask subtle alterations in expression of target genes but directional changes in genes used for normalization might result in invalid conclusions. It is evident now that, contrary to previous assumptions, GAPDH expression is tightly regulated at transcriptional and posttranslational levels. Consistently, several studies have demonstrated that GAPDH might be upregulated in response to hypoxia, a common feature of cancer and inflammation. Moreover, it has been implied that GAPDH overexpression may constitute a growth advantage for tumor cells. However, the effect of inflammation and cancer on whole blood expression of GAPDH as well as that of ACTB or B2M has not been evaluated yet. Hence, having validated set of stably expressed reference genes, we examined whether variability in GAPDH, B2M and ACTB in whole blood of CRC and IBD patients was random or specifically affected by the examined conditions.

Complementing the findings on GAPDH overexpression in colonic tumors at protein level, we found GAPDH expression to be upregulated in whole blood of CRC patients. The elevation was even more profound in IBD patients. Moreover, we showed that GAPDH upregulation was not random but corresponded with clinical activity of both CD and UC in IBD patients as well as with cancer advancement in CRC patients, in whom it tended to be associated with gaining metastatic potential rather than with local progression of the tumor. Furthermore, it positively and strongly correlated with a number of circulating inflammatory indices, either exclusively in IBD patients (CRP, IL-8, IL-12) or CRC patients (IL-6, FGF2, MIP-1α) or both (IL-1β, G-CSF, TNF-α), most of which display pro-angiogenic properties as well. As all these cytokines are interrelated, it is likely that some of GAPDH associations are mediated by others. Unfortunately, the number of observations is not sufficient to allow for a multivariate analysis, impeding identification of variables independently associated with whole blood GAPDH expression.

Our observations are consistent with the phenomena of tumor metabolism reprogramming, which occurs to satisfy increased energetic demands of proliferating cancer cells under diminished oxygen availability. One of its manifestations is the acceleration of glycolysis upon combined actions of mutations in c-myc proto-oncogene and the upregulation of hypoxia-inducible factor (HIF)-1α, the binding sites which can be found in GAPDH promoter. It also contains a binding site for c-jun/AP-1, transcription factor involved in inflammatory responses as well as tumor angiogenesis. Accordingly, new non-glycolytic functions have recently been attributed to GAPDH, e.g., in cell death regulation. Although the exact role it may play remains unclear, as GAPDH has been demonstrated to both induce cancer cell senescence and contribute to cancer progression in colon adenocarcinoma cell lines, GAPDH has been demonstrated to display pro-survival activities. B2M was uniformly selected as the least stable reference gene in the whole cohort, independently form statistical method of evaluation. It also displayed the highest degree of variability within each study group. Similarly to our previous observations in colorectal tissue (manuscript submitted), whole blood B2M was significantly downregulated in cancer but upregulated in active IBD. Accordingly, Bianchini et al. identified B2M as the most downregulated gene from among 19.2K genes screened and suggested that the phenomenon might be related to the escape mechanism from NK-mediated lysis developed by tumor cells. Shroult et al. showed that B2M downregulation in colorectal tumors was a strong prognostic indicator of lymph node metastases. In the investigated sample set, whole blood B2M did not correspond with cancer advancement nor was it correlated with clinical scores of IBD activity. Nevertheless, consistently with its observed underexpression in CRC and overexpression in IBD, B2M expression negatively correlated with FGF2, G-CSF and TNF-α in the former and positively with FGF2 and MIP-1α in the latter.

Expression of ACTB in whole blood was the least variable of the 3 most popular reference genes, and normalization against ACTB was even found superior using BestKeeper. However, although the differences between groups did not reach statistical significance when analyzed with ANOVA, ACTB clearly tended to be higher in active IBD than in controls (the difference was significant when t-test was used). Moreover, it positively correlated with CRP levels and clinical severity score in CD, rendering it unsuitable as a reference in IBD. In CRC, in turn, it tended to be higher in patients with lymph node metastases and...
positively correlated with inflammatory and angiogenic factors: IL-1β, IL-6, FGF2, G-CSF, GM-CSF, MIP-1α, and TNF-α.

Taken together, our results demonstrate that none of the most popular reference genes, namely, GAPDH, ACTB or B2M, are suitable for transcriptional studies on whole blood from CRC and/or IBD patients. Firstly, their expression differs between conditions, showing that bowel inflammation and cancer affect not only their tissue, but also whole blood expression. Even more importantly, however, the observed variability is not random, as the alterations in their expression levels correspond, to a varying degree, with CRC advancement and the clinical activity of IBD (GAPDH and ACTB) as well as correlate with the levels of circulating mediators of inflammation and angiogenesis. The clinical relevance of this observation needs to be elucidated; however, regardless of whether the ACTB, B2M, and GAPDH regulation is unintended or plays an active role in inflammatory responses and/or cancer, they definitely does not constitute good internal controls. The set of normalizers proposed here instead, that is, TBP, SDHA and HPRT1, have recently been successfully used by our group for the analysis of whole blood transcriptome of IBD patients.38,39

Conclusions

The expression of frequently used normalizers for whole blood transcriptomic analysis, that is, GAPDH, ACTB, and B2M, is directionally affected by bowel inflammation and cancer, rendering them unsuitable as references in CRC and IBD. The expression of HPRT1, SDHA and TBP was stable across CRC and IBD patients, allowing for their recommendation as normalizers in studies involving both groups of patients.

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