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Analysis of biological and technical variability in gene expression assays from formalin-fixed paraffin-embedded classical Hodgkin lymphomas

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ABSTRACT

Formalin-fixed paraffin-embedded (FFPE) tissues are invaluable sources of biological material for research and diagnostic purposes. In this study, we aimed to identify biological and technical variability in RT-qPCR TaqMan® assays performed with FFPE-RNA from lymph nodes of classical Hodgkin lymphoma samples. An ANOVA-nested 6-level design was employed to evaluate *BCL2*, *CASP3*, *IRF4*, *LYZ* and *STAT1* gene expression. The most variable genes were *CASP3* (low expression) and *LYZ* (high expression). Total variability decreased after normalization for all genes, except by *LYZ*. Genes with moderate and low expression were identified and suffered more the effects of the technical manipulation than high-expression genes. Pre-amplification was shown to introduce significant technical variability, which was partially alleviated by lowering to a half the amount of input RNA. *Ct* and Cy_0 quantification methods, based on cycle-threshold and the kinetic of amplification curves, respectively, were compared. Cy_0 method resulted in higher quantification values, leading to the decrease of total variability in *CASP3* and *LYZ* genes. The mean individual noise was 0.45 (0.31 to 0.61 SD), indicating a variation of gene expression over ~1.5 folds from one case to another. We showed that total variability in RT-qPCR from FFPE-RNA is not higher than that reported for fresh complex tissues, and identified gene-, and expression level-sources of biological and technical variability, which can allow better strategies for designing RT-qPCR assays from highly degraded and inhibited samples.

1. Introduction

Formalin-fixed and paraffin-embedded (FFPE) tissues are invaluable sources of biological material for pathologic analysis and molecular diagnosis (Fairley et al., 2012). However, in this type of sample, recovery of RNA appropriate for molecular analysis is complicated by degradation and the cross-link between RNA and proteins after formaldehyde fixation (Masuda et al., 1999).

Reverse transcription quantitative real-time PCR (RT-qPCR) is the gold standard technique for gene expression analysis, however, in FFPE samples amplification is affected by both, degradation and the presence of co-extracted inhibitors, leading to amplification at high values of cycles of quantification (*Cq*) (Godfrey et al., 2000; Koch et al., 2006), hence associated with increased variability and loss of linearity.

Besides the technical restrictions imposed by the nature of the RNA-FFPE samples, there is an underscored aspect of gene expression studies, which is the complexity of the tissue investigated. This factor imposes the amount of biological variability to be surpassed in order to detect a meaningful biological difference (Kitchen et al., 2010). In this respect, classical Hodgkin lymphoma (cHL) is one of the most complex cancers known; where tumor cells account for only 0.5–2% of tumor mass and are surrounded by variable numbers and types of inflammatory cells (Steidl et al., 2011). Gene expression profiles (GEP) corresponding to both, tumor and microenvironment compartments have been identified in this disease and have shown to carry useful prognostic information (Chetaille et al., 2009; Sánchez-Aguilera et al., 2006). This has lead to an ongoing interest in the development of sets of qPCR assays based on FFPE-RNA (Sanchez-Espiridion et al., 2010; Scott et al., 2013;

Abbreviations: *BCL2*, B-cell CLL/lymphoma2; *CASP3*, caspase 3; cHL, classical Hodgkin lymphoma; *Cq*, cycles of quantification; *Ct*, cycle threshold; CV, coefficient of variation; FFPE, formalin-fixed and paraffin-embedded; GOI, genes of interest; *GUSB*, glucuronidase beta; *HMBS*, hydroxymethylbilane synthase; *IRF4*, interferon regulatory factor 4; LN, lymph node; *LYZ*, lysozyme (renal amyloidosis); MIQE, Minimum Information for Publication of Quantitative Real-time PCR Experiments; Pre-Amp, pre-amplification; qPCR, quantitative real time PCR; REFG, reference gene; RIN, RNA integrity number; RT, reverse transcription; RT-qPCR, reverse transcription quantitative real-time PCR; SD, standard deviation; *STAT1*, signal transducer and activator of transcription 1.

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Venkataraman et al., 2014) to be used for clinical prediction in cHL, but no study has yet evaluated the sources of variability in RT-qPCR assays from FFPE-RNA.

Different approaches have been proposed for evaluating the variability in RT-qPCR assays (Bengtsson et al., 2008; Tichopad et al., 2009; Weaver et al., 2010). Among them, the nested-ANOVA design is a hierarchical approach suited to quantify the biological differences among subjects and the technical noise introduced by sample processing; each subject receives one treatment condition, and errors are linearly accumulative in each level (Fisher, 1935; Quinn and Keough, 2002).

In this work, we applied experimental designs as well as quantification approaches to evaluate specific RT-qPCR assays from FFPE-samples from cHL lymph nodes, in order to obtain useful information for diagnostic and prognostic test development.

2. Material and methods

Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) is listed in Appendix A.

2.1. Ethics statement

This study has been approved by the Instituto Nacional de Câncer (INCA) Ethics Committee, and has been performed in accordance with the ethical standards of the Declaration of Helsinki. Samples from patients were used after signed informed consent.

2.2. Samples

FFPE lymph nodes (LN) from 25 cHL diagnosed at the INCA, Brazil were included in the initial phase of this study. For the ANOVA-nested PCR assay, three subjects with cHL of the nodular sclerosis histological subtype were selected from the initial group by an experienced pathologist (MHMB), based on extensive immunohistochemistry characterization (Barros et al., 2010; Barros et al., 2012), and availability of two different FFPE-blocks from the same LN with similar tumor/stroma proportions.

2.3. Extraction of total RNA from FFPE lymph nodes

Total RNA was obtained from five microtomized sections using a Master Pure™ RNA purification Kit, Epicentre, following the supplier instructions except by the use of 480 μl tissue-and-cell-lysis solution with 60 μl of 60 mg/ml proteinase K (Invitrogen) and incubation at 65 °C for 20 h (Chen et al., 2007), followed by a treatment with 5 U/μl *DNaseI* at 37 °C for 30 min. RNA was resuspended in 12 μl of RNase-free water (Appendix A).

2.4. Reverse transcription

Reverse transcription (RT) was performed with the High-capacity cDNA Archive kit (Applied Biosystems, Life Technologies) from 1 μg of total RNA in 20 μl final volume (10 μl of diluted RNA and 10 μl RT mix). The reaction was incubated at 25 °C for 10 min and at 37 °C for 120 min in a Veriti™ Thermal Cycler (Applied Biosystems).

2.5. Pre-amplification step

Pre-amplification (Pre-Amp) was performed with the TaqMan® PreAmp Master Mix (Applied Biosystems) in a 10 μl-multiplex reaction which included primers and probes for all assays. According to the supplier, multiplex assays were designed to include up to 100 primers/probe sets, without introducing significant variability (Applied, 2010). Pre-Amp products were diluted 1:20 in RNase/DNase-free water and used for qPCR analysis.

2.6. qPCR assays

PCR quantifications were performed in an ABI7000 (Applied Biosystems) using TaqMan® chemistry, in duplicate, using cycle threshold (*Ct*) with fixed thresholds. The mean qPCR accepted standard deviation (SD) was 0.15 cycles. Additionally, kinetic parameters of the curve were used to calculated the Cy_0 as described (Guescini et al., 2008), based on Richards' equation with five parameters. This method does not require the assumption of similar efficiency in amplification of the genes of interest (GOI) and reference genes.

GOI were selected based on previous descriptions of clinically relevant genes in cHL (Sánchez-Espiridión et al., 2009) (Appendix A), and *GUSB* and *HMBS* were used as reference genes (REFG). RT-qPCR assay efficiencies ranged from 94.9 to 100.8% (Appendix B, Fig. B.1). All values of relative expression were expressed as $2^{-\Delta Cq}$.

2.7. Performance of RNA extractions

RNA quantity and quality were evaluated by spectrophotometry (Nanodrop®, ND-1000 Spectrophotometer) at OD 260 and OD 260/280/230 ratios; and by microfluidics technology for RNA integrity number (RIN) algorithm (2100 Bioanalyzer, Agilent Technologies). *Ct*-values < 35 cycles of both REFG amplifications defined an "amplifiable" sample.

2.8. Nested assay

A nested 6-level design ($3 \times 2 \times 2 \times 2 \times 2 \times 2$) was used to investigate the source of variability (biological and technical), in which two different FFPE blocks from the same LN from 3 cHL cases were analyzed, with duplicated RNA extractions, followed by duplicated retrotranscriptions, Pre-Amp and qPCR steps (Fig. 1).

2.9. Statistical analyses

Mann–Whitney's test was used to analyze associations between dichotomous and continuous non-normal variables, Wilcoxon signed-rank test was used to test the relation between paired samples and Spearman's test was used for correlating continuous variables. The linear model of the biological and technical processing effects was calculated as described by Tichopad et al. (2009) as: $Cq_{ijklmn} = \mu + a_i + b_{j(i)} + c_{k(ji)} + d_{l(kji)} + e_{m(lkji)} + f_{n(mlkji)}$.

Analyses were performed by nested-ANOVA of 6 factors with random effects. Variance partition was calculated as: $100 \times \sigma^2_x/\sigma^2_{Cq'}$, being $x = i, j, k, l, m, n$; as described in Kitchen et al. (2010). Statistical analyses were carried out with GENEX enterprise (MultiD), SPC (BPI Consulting, LLC) and Statistical Package for the Social Sciences 20.0 (SPSS) software. Figures were constructed with the GraphPad Prism 6 and Photoshop CC software.

3. Results

3.1. RNA extraction and GOI expression

From the 25 selected cHL LN, RNA mean yield was 696.3 ng/μl ± 578.4 SD. DNA purity was acceptable, with means 260/280 OD ratio of 1.81 ± 0.16, and 260/230 OD ratio of 1.75 ± 0.36. RIN values ranged from 2.2 to 4.8 (mean 2.46).

Pre-Amp procedure resulted in a significant gain of sensitivity, the inclusion of this step leading to an average increase of 10.1 ± 1.5 *Ct* in *GUSB* amplifications.

Expression levels of GOI are shown in Table 1 and Fig. 2. Means varied from $2^{-\Delta Ct}$ 4.300 to −3.911, allowing genes to be classified in highly expressed (*LYZ*, *STAT1* and *IRF4*), moderately expressed (*BCL2*), and low expressed genes (*CASP3*).