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REVIEW

Consensus reference gene(s) for gene expression studies in human cancers: end of the tunnel visible?

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Abstract
Background Gene expression studies are increasingly used to provide valuable information on the diagnosis and prognosis of human cancers. Also, for in vitro and in vivo experimental cancer models gene expression studies are widely used. The complex algorithms of differential gene expression analyses require normalization of data against a reference or normalizer gene, or a set of such genes. For this purpose, mostly invariant housekeeping genes are used. Unfortunately, however, there are no consensus (housekeeping) genes that serve as reference or normalizer for different human cancers. In fact, scientists have employed a wide range of reference genes across different types of cancer for normalization of gene expression data. As a consequence, comparisons of these data and/or data harmonizations are difficult to perform and challenging. In addition, an inadequate choice for a reference gene may obscure genuine changes and/or result in erroneous gene expression data comparisons.
Methods In our effort to highlight the importance of selecting the most appropriate reference gene(s), we have screened the literature for gene expression studies published since the turn of the century on thirteen of the most prevalent human cancers worldwide.
Conclusions Based on the analysis of the data at hand, we firstly recommend that in each study the suitability of candidate reference gene(s) should carefully be evaluated in order to yield reliable differential gene expression data. Secondly, we recommend that a combination of *PPIA* and either *GAPDH, ACTB, HPRT* and *TBP*, or appropriate combinations of two or three of these genes, should be employed in future studies, to ensure that results from different studies on different human cancers can be harmonized. This approach will ultimately increase the depth of our understanding of gene expression signatures across human cancers.

Keywords Reference genes · Normalizer genes · Endogenous controls · qPCR · Gene expression · Human cancers

1 Introduction

Gene expression analyses require normalizations across different samples, which involves standardization of data against a set of reference points in any differential expression strategy. This is usually done using 'invariant' housekeeping genes (also referred to as endogenous controls). An inherent property of housekeeping genes is that they maintain the basic metabolic functions of a cell at a similar level under different conditions and perturbations. This is presumably achieved by keeping their gene expression level invariant [1]. Hence, housekeeping gene expression levels are chosen as endogenous references for gene expression data normalization. However, recent technological advances have raised the question of choice and reliability of these endogenous genes as references. An increasing number of reports show that housekeeping genes may also be subject to variation in expression in different disease states and experimental conditions, as well as between subjects, tissues, model systems etc. [2–13]. Housekeeping genes may also exhibit expression variation in the same type of cancer when located in different tissues or organs. Exposure of human peripheral blood lymphocytes

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(HPBL) to environmental stresses, including ionizing radiation, is also known to activate signal transduction pathways, which may result in complex patterns of gene expression changes [14–17]. In a recent study, using quantitative real-time PCR (qPCR), we have compared the expression of 6 different housekeeping genes in human blood cells exposed to ^{60}Co γ-rays for their suitability as reference or normalizer gene. We found that *GAPDH*, either alone or in combination with the *18S rRNA* gene, suited best [18]. Others found that in colon cancer several housekeeping genes, mainly those coding for metabolic enzymes, show considerable expression changes under different conditions [19]. It is apparent that there is currently no single housekeeping gene that meets the criteria of being stably, abundantly and consistently expressed under various conditions, i.e., criteria that are required for serving as a consensus reference or normalizer gene [20].

In any gene expression study, the selection of an appropriate reference or normalizer gene(s) is critical for a reliable and accurate interpretation of the data. In the past, several bioinformatics tools have been developed for the delineation of the best reference or normalizer gene(s) for expression studies, including *BestKeeper, geNorm* and *NormFinder*. Each of these tools applies different and highly complex mathematical algorithms to finally achieve the goal set. For example, the *BestKeeper* tool estimates the geometric mean of the most suitable pair of genes by correlating the average variations of candidate genes [21]. To minimize variations across samples, the *geNorm* tool employs multiple reference genes to derive a geometric mean [12]. The *NormFinder* tool applies a mathematical model to analyse sample subgroups and their intra- and inter-group expression variation, thereby preventing the selection of co-regulated genes [22]. Since the choice of a reference gene, or a set of reference genes, is inherent to these complex algorithms, the same gene expression data set may yield different results by using different reference gene(s) [18].

Many attempts have been made over the last few years to settle the issue by finding the most appropriate reference gene(s) for gene expression analysis purposes. A consensus will facilitate the harmonization of data emerging from different studies across the globe, and consolidate our grip on the fundamental understanding of (alterations in) gene expression, especially in cancer. Recently, the use of 3 reference genes selected by at least three stability algorithms for reliable interpretation of gene expression data has been recommended [23]. A survey of published results shows, however, that a wide range of different reference or normalizer genes are in use for normalization of gene expression data in different cancer studies. At least 50 housekeeping genes have so far been tested and/or used in studies dealing with the most prevalent human cancers (listed in Table I). This review collates all available data published on this issue since the turn of the century to (i) highlight the wide range of reference genes employed in the normalization of qPCR data in the most prevalent human cancers and (ii) to extract consensus reference genes for normalization.

Based on worldwide data available on the incidence and prevalence of human cancers (compilation up to 2012) from the World Health Organization (WHO; http://www.who.int/mediacentre/factsheets/fs297/en/) and the World Cancer Research Fund International (WCRF; http://www.wcrf.org/int/cancer-facts-figures/worldwide-data/), we chose the 13 most common human cancers, accounting for nearly 70 % of the total global cancer burden, for this study. To retrieve and collate the relevant literature for the selected cancers, a search strategy was devised wherein the PUBMED database of the National Library of Medicine, NIH Bethesda, Maryland, USA, was the primary source. The search was restricted to full text papers published from 2000 onwards. In addition, a Google web-based search was employed to retrieve published data that were not covered by the PUBMED database. Some highly relevant cross references from these papers are also included in this review. The keywords utilized for the searches were Validation, Reference Genes, Housekeeping Genes, Human Cancer, Real Time PCR, Endogenous Controls, Normalizers, and Evaluation, both alone and in combination with specific cancer types.

2 Breast cancer

Breast cancer is the most common invasive cancer of high prevalence in women [24]. Breast cancer-related gene expression studies by qPCR have utilized a wide range of endogenous control genes. In an evaluation study by Lyng et al. [25], the best reference gene appeared to be *PUM1*, or the average of 3 genes, i.e., *TBP, RPLPO* and *PUM1* (Table 2). In another evaluation study on breast cancer the *MRPL19* and *PPIA* genes (Table 2) were identified as the most stable and reliable candidate reference genes [26]. Gur-Dedeoglu et al. [27] reported *ACTB* and *SDHA* to be the most suitable reference genes, among 18 endogenous reference genes tested, for the normalization of qPCR data in breast cancer tissues using both the *geNorm* and *NormFinder* software tools (Table 2). In another study 5 reference genes were identified, i.e., *ACTB, RPS23, HUWE1, EEF1A1* and *SF3A1* (Table 2), as potential normalizer genes for the experimental and clinical analyses of breast cancer samples [28]. On the other hand, the *18S rRNA* gene was found to be the most suitable reference gene in the MCF-7 breast cancer-derived cell line, while the *GAPDH* gene was recommended for the MDA-MB-231 breast cancer-derived cell line [29]. In an earlier study, however, *GAPDH* was not recommended as a reference gene in breast cancer [30]. More recent studies on breast cancer have employed a wide range of reference genes, such as *ACTB* [31, 32], *GAPDH* [33, 34], *APP* [35], *RPLPO* [36] and *β-*

globin [37], as well as the averaged expression of the *GAPDH, HPRT1* and *B2M* genes [38] for normalization purposes.

3 Cervical cancer

Cervical cancer is another leading causes of cancer-related death in women, worldwide. In a validation study by Daud et al. aimed at identifying the best reference genes in cervical cancer *GAPDH*, followed by *RPLPO*, were found to be the best candidate reference genes (Table 2) [39]. In clinical cervical tissue samples, *EEF1A1* was recommended as a reference gene by Shen et al. [40], while the combined use of *EEF1A1* and *GAPDH* may serve as a reliable normalization strategy (Table 2). In several other studies on cervical cancer reference genes such as *GAPDH* [41] or *ACTB* [42, 43] were employed.

4 Colon cancer

Colon cancer, or colorectal cancer (CRC), is also one of the most common causes of cancer-related death in developed countries [44]. In different studies on CRC, the use of different housekeeping genes as reference genes has been suggested. In an evaluation study of several of these housekeeping genes 3 of them, i.e., *PMM1, ACTB* and *PSMB6*, showed least variation and were, therefore, considered as the most reliable reference genes for the analysis of CRC samples [45]. Three other reference genes (i.e., *UBC, GAPD* and *TPTI*) were recommended for CRC by another research group [22]. In yet another study by Jacob et al. aimed at selecting reference genes [23], *HSPCB, YWHAZ* and *RPS13* were found to be the most stably expressed genes in at least a subset of CRC-derived cell lines (Table 2). One recent study has advocated the use of *GUSB* and *ACTB*, but not *B2M*, as internal reference genes for CRC gene expression studies [46]. On the contrary, *B2M* was reported to be the best reference gene for gene expression studies in primary human CRCs by Dydensborg et al. [47]. After evaluating 13 potential candidate genes, a combined use of the *PPIA* and *B2M* genes (Table 2) was recommended as reference for human CRC samples by Kheirelseid et al. [48]. Of the 16 genes in metastatic and non-metastatic CRC specimens studied, the use of 2 pairs of genes, i.e., *HPRT1-PPIA* and *IPO8-PPIA*, was recommended by Sorby et al. [49]. They were found to serve as the most suitable combinations (Table 2). In other CRC studies different endogenous reference genes such as *GAPDH* [50], *ACTB* and *GUSB* [51] were employed as normalizer genes.

5 Esophageal cancer

Esophageal cancer is the eighth most frequently diagnosed cancer worldwide [52] and, due to its poor prognosis, it is the sixth most common cause of cancer-related death [53, 54]. Of the 21 genes evaluated as best endogenous reference genes in primary esophageal cancer tissue specimens, the highest stability was observed for *GAPDH*, followed by *CETN2* [43]. In several other studies on esophageal squamous cell carcinoma, *GAPDH* [55–59] or *ACTB* [60–62] were used as reference genes. As shown in Table 2, a triple normalization with 3 reference genes, i.e., *PPIA*, *ALAS1* and *ACTB*, was recommended for human esophageal adenocarcinomas specimens by Slotta-Huspenina et al. [63]. In some other studies, *GAPDH* [64–66] and *18S rRNA* [67] were employed as reference genes for esophageal adenocarcinomas.

6 Kidney cancer

A study by Jung et al. [5] aimed at identifying suitable reference genes for gene expression analyses in renal cell carcinoma, which is the most common type of kidney cancer, proposed 2 housekeeping genes, i.e., *PPIA* and *TBP*. Both genes were recommended as reference genes for data normalization, either as single genes or in combination, with a preference for the latter (Table 2). Previously, one report indicated that the *18S rRNA* and *cyclophilin A* (*CyPA*) genes were the most suitable reference genes for micro-dissected kidney biopsies [68]. In other studies on renal cell carcinomas, *GAPDH* was used as a reference gene [69, 70], whereas another recent study revealed that *PPIA* and *RPS13* served as the most suitable combination for the normalization of gene expression data in clear cell renal cell carcinoma (ccRCC) tissues [71].

7 Liver cancer

Liver cancer, or hepatic cancer, is one of the leading causes of cancer-related death globally [72]. In a selection study aimed at identifying optimal reference genes for expression profiling of liver diseases, 10 housekeeping genes were evaluated across 67 liver tissue samples using the *geNorm* software tool [73]. By doing so, it was found that the *HMBS* and *UBC* gene pair served as the most accurate normalization factor in qPCR analyses (Table 2). For the normalization of microarray-based gene expression data, Lee et al. identified 3 housekeeping genes, i.e., *CGI-119, CTBP1* and *GOLGA*, that showed stable expression across the liver cancer tissues tested [74]. One evaluation study recommended a combination of the *TBP* and *HPRT1* genes (Table 2) for the normalization in hepatocellular carcinoma (HCC) data [75]. This recommendation was supported by another study that showed that the *TBP* and *HPRT1* genes were stably expressed and, hence, served as reliable reference genes for qPCR-based gene expression normalization in hepatitis B virus (HBV)-related HCC specimens compared to the *18S rRNA* and *ACTB* genes [76].

Another recent study recommended *CTBP1* as the best candidate reference gene in human male HBV infection-related HCC with cirrhosis [77]. Also, a combination of 2 other genes, *SFRS4* and *RPL41*, has been recommended for HCC by Waxman et al. [78]. On the other hand, Cicinnati et al. [79] concluded from an evaluation study that *HMBS* was the single best reference gene for gene expression studies in HCC (Table 2). The latter authors also suggested a combination of the *HMBS, GAPDH* and *UBC* genes for primary liver cancer samples, and a combination of the *HMBS, B2M, SDHA* and *GAPDH* genes for liver cancer-derived cell lines.

8 Lung cancer

Lung cancer, or pulmonary carcinoma, is one of the most common cancers and is a leading cause of mortality among men worldwide [80]. A wide range of reference genes has been used for the normalization of gene expression data in lung cancer studies. In a study on non-small cell lung cancer (NSCLC), the best reference gene was found to be *HPRT1* followed by *GAPDH* [81], even though another gene, i.e., *B2M*, was previously preferred by Heighway et al. [82]. In a panel of 6 investigated reference genes by another group, *HPRT1* was found to be the most stably expressed gene in NSCLC (Table 2), followed by *RPLP0* and *ESD* [83]. As also shown in Table 2, 6 housekeeping genes, i.e., *RPLPO*, *UBC*, *GAPDH*, *MT-ATP6*, *CASC3* and *PES1*, were identified as reliable reference genes for analysis of the NSCLC-derived cell line A549 by Sharungbam et al. [84]. Besides, *18S rRNA*, *POLR2A, ESD* and *YAP1* were found to be the most stably expressed genes in primary lung cancer specimens [85]. A sequencing-based approach suggested 4 genes, i.e., *NDUFA1, RPL19, RAB5C* and *RPS18*, as suitable reference genes for normalization of data in primary lung cancer tissues [86]. In a DNA microarray-based expression profiling study of lung cells, the *SPCS1* and *HADHB* genes were found to show a high level of expression stability [87], and in a recent qPCR-based expression study of lung squamous-cell carcinomas, *ACTB*, *EEF1A1*, *FAU*, *RPS9*, *RPS11* and *RPS14* were identified as ideal reference genes [88].

9 Lymphoma

In studies on lymphoma, a wide range of endogenous genes, such as *B2M* [89–91], *RPS9* [92], *GAPDH* [93–96] and *ACTB* [97], have been used as references. In an evaluation study on several lymphoma-derived cell lines, the *RPL13A* gene was found to suit best as reference gene out of 4 genes tested, i.e., *ACTB, HPRT1, HMBS* and *RPL13A* (Table 2) [98]. A study on non-Hodgkin's lymphomas, on the other hand, indicated that the expression of target genes should be normalized against the *PRKG1* and/or the *TBP* genes [99].

10 Ovarian cancer

Ovarian cancer is another leading cause of cancer-related death in women, worldwide [100]. A recent analysis of different housekeeping genes [101] identified *IPO8* as the most suitable reference gene for this malignancy, followed by *RPL4* (Table 2). In different evaluation studies of endogenous references (shown in Table 2), the *GUSB, PPIA* and *TBP* genes were reported to be most suitable for expression normalization in serous ovarian cancer [102], whereas the *PPIA, RPS13* and *SDHA* genes were recommended for use in ovarian cancer-derived cell lines by Jacob et al. [23]. In some of the more recent ovarian cancer studies, the reference genes employed were *PRLPO* [103], *ACTB* [104–106], *GAPDH* [107–109], *18S rRNA* [110], *GUSB* and *PPIA* [111]. *GAPDH* was also employed in a study on ovarian cancer-derived cell lines, such as the SKOV3 and HO8910 cell lines and the highly invasive HO8910-PM cell line [112]. In yet another evaluation study on ovarian tissues, the *RPLPO* and *RPL4* genes were recommended as the best combination of reference genes [113].

11 Pancreatic cancer

Pancreatic cancer has one of the highest mortality rates among all cancers for both men and women, worldwide. The results of an evaluation study of endogenous reference genes indicated that *EIF2B1, ELF1, MRPL19* and *POP4* were the most stably expressed genes in pancreatic cancer and, as such, should be used for the normalization of qPCR-based expression data (Table 2) [114]. In another study, *RPL37A, RPLPO* and *CASC3* were recommended as reference genes for the analysis of pancreatic cancer-derived cell lines [84]. The *18S rRNA* and *QRRS* genes were also found to exhibit expression variation of less than 10 % and, therefore, they were recommended as reference genes for the analysis of pancreatic carcinoma tissues [45]. In other primary pancreatic carcinoma studies, the *GAPDH* gene was used as a single reference gene [115], alone or together with *PSMB6* [116]. Only recently it was reported that the expression of the *PPM1* gene is more stable than that of the *GAPDH* gene in pancreatic cancer [117].

12 Prostate cancer

Prostate cancer, or carcinoma of the prostate, is one of the leading causes of cancer death in males, worldwide [118]. In

an evaluation study of 16 candidate housekeeping genes in prostate cancer, the best reference gene was found to be *HRPT1*, alone or in combination with *ALAS1* and *K-ALPHA-1* (Table 2) [119]. Another study recommended a combination of the *GAPDH* and *SDHA* genes for normalization of mRNA levels of target genes in primary cultures of prostate cancer cells transfected with siRNAs (Table 2) [120]. Yet another report showed that the *ACTB* gene was abundantly and stably expressed in prostate-derived cells and, thus, was considered suitable for use as a reference gene [121]. However, the same gene was not recommended for primary prostate cancer tissue samples. qPCR-based gene expression analyses of prostate cancer cell lines have been conducted using the *18S rRNA* gene as the reference [122], and a comparison of *B2M* gene expression levels between healthy volunteers and patients with prostate cancer revealed a lack of significant variation, including the absence of an effect of hormonal treatment [123]. Another study on prostate cancer showed variation in expression levels of some commonly used endogenous control genes between aerobic and hypoxic samples [13]. In several additional qPCR-based prostate cancer expression studies, the housekeeping genes used as references included *GAPDH* [124–128], *ACTB* [129, 130], *RPS14* [131] and *S19* [132].

13 Stomach cancer

Stomach cancer, or gastric cancer, also belongs to one of the most common cancers across the globe [133]. For the identification of valid reference genes in stomach cancer-derived cell lines, a combination of the *GAPDH* and *B2M* genes has been recommended for normalization (Table 2) [134]. Besides, a combination of the *RPL29* and *B2M* genes for comparisons between normal and stomach cancer tissues has been proposed (Table 2). In another report, combinations of pairs of reference genes, i.e., *GAPDH-B2M* or *ACTB-B2M*, were recommended for gene expression analyses in gastric tissues and cell lines (Table 2) [135]. Another evaluation by Zhao et al. [136] revealed *18S rRNA* as the most stably expressed gene when compared to *GAPDH, ACTB* and *RPII* and, thus, to be most suited for the normalization of qPCR-based expression data in gastric cancer samples (Table 2). An earlier study, however, identified 3 other genes (i.e., *PMM1, ADA* and *SDHA*) as candidate reference genes for the normalization of expression data in stomach cancer [45].

14 Thyroid cancer

In a recent evaluation study aimed at selecting the best candidate reference gene for thyroid cancer [137], *ACTB* was found to be suited best among several other candidates tested [138]. In a comparative study on normal thyroid tissues using the *NormFinder* software tool, it was found that the *ACTB* gene was most stably expressed when compared to 5 other candidate genes (Table 2). A similar study also suggested that the *ACTB* gene was more stably expressed than the *TBP, GAPDH* and *B2M* genes in primary cultures of thyroid cells [139]. Moreover, *ACTB* has been employed as a reference gene in thyroid cancer gene expression studies by different groups [140–142]. Several additional studies on primary thyroid cancers [143–147] and thyroid cancer-derived cell lines [148, 149] have employed *GAPDH* as a reference gene. Additionally, also other housekeeping genes such as *18S rRNA* [150] and *G6PDH* [151] have been employed as references in thyroid cancer-related studies.

15 Discussion

It is obvious from the above overview that there is no single gene, or set of genes, that has in the past been universally applied as endogenous reference or normalizer in gene expression studies of the most common human cancers. Over 50 different reference genes have so far been used (listed in Table 1). Different software tools have been employed by researchers to evaluate suitable reference genes for data normalization, yielding a number of possible candidates for particular human cancers and/or its corresponding normal tissues in different studies (Table 2). Consequently, different reference genes have been used by different researchers for gene expression studies in the same human cancers. This makes harmonization or inter-comparison of valuable gene expression data difficult and challenging.

Differences in the metabolism of cancer cells may contribute to variation in the expression levels of housekeeping genes, even within the same organ, suggesting that cancer-related studies are mostly case-specific [152, 153]. This variation may even be more prominent when comparing one cancer type with another. In colon cancer, for example, it has been suggested that such variation may also, at least partly, be due to chromosomal aberrations resulting in gains or losses of segments containing the relevant gene(s) [154]. In such situations, it is recommended to consider the genotype of the tissue samples so that extra or missing chromosomal parts (i.e., copy number variation) can be taken into account. Obviously, the outcome of reference gene evaluations may also vary depending on the validation methodology used.

Based on our present evaluation, we find that all three software packages used for validation in cancer-related gene expression studies, i.e., *geNorm, NormFinder* and *BestKeeper*, using three different and complex algorithms and efficiency-corrected values [155], ranked the genes in similar and/or comparable patterns. This clearly suggests that the choice of the software package is not the primary

Table 1 Reference genes used for gene expression studies

Gene names	Full gene names	References
ACTB	β-Actin	[27]
ADA	Adenosine deaminase	[45]
ALAS1	Aminolevulinate, delta-, synthase 1	[63]
B2M	β2-Microglobulin	[135]
CASC3	Cancer susceptibility candidate 3	[84]
CETN2	Centrin-2	[45]
CGI-119	Transmembrane BAX inhibitor motif containing 4	[74]
CTBP1	C-terminal binding protein 1	[74]
EEF1A1	Eukaryotic translation elongation factor 1 α 1	[40]
EIF2B1	Eukaryotic translation initiation factor 2B, subunit 1 α	[114]
ELF1	E74-like factor 1	[114]
ESD	Esterase D	[85]
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	[18, 134]
GOLGA1	Golgi autoantigen, golgin subfamily a, 1	[74]
G6PD	Glucose-6-phosphate dehydrogenase	[151]
GUSB	Glucuronidase, beta	[102]
HMBS	Hydroxymethylbilane synthase	[79]
HPRT1	Hypoxanthine phosphoribosyltransferase 1	[76, 83]
HSPCB	Heat shock protein 90 kDa alpha, Class B member 1	[23]
IPO8	Importin 8	[49]
K-ALPHA-1	Tubulin, alpha 1b	[119]
MRPL19	Mitochondrial ribosomal protein L19	[26]
MT-ATP6	Mitochondrial ATP synthase F0 subunit 6	[18]
NDUFA1	NADH dehydrogenase (ubiquinone) 1 α subcomplex	[86]
PES1	Pescadillo homolog 1	[84]
PMM1	Phosphomannomutase 1	[45]
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	[85]
POP4	Processing of precursor 4, ribonuclease P/MRP subunit	[114]
PPIA	Peptidylprolyl Isomerase A	[48]
PRLPO	Prolactin-like protein O	[103]
PRKG1	Protein kinase, cGMP-dependent, type I	[99]
PSMB6	Proteasome subunit beta type-6	[116]
PUM1	Pumilio homolog 1	[25]
QRRS	Glutaminyl-tRNA synthetase	[45]
RAB5C	Ras-related protein	[86]
RPL4	Ribosomal protein L4	[101]
RPL19	Ribosomal protein L19	[86]
RPL13A	Ribosomal protein L13a	[98]
RPL29	Ribosomal protein L29	[134]
RPL37A	Ribosomal protein L37a	[84]
RPL41	Ribosomal protein L41	[78]
RPLPO	Large ribosomal protein	[39]
RPS13	Ribosomal protein S13	[23]
RPS14	Ribosomal protein S14	[124]
RPS18	Ribosomal protein S18	[86]
rRNA18S	18S ribosomal RNA	[85, 136]
SDHA	Succinate dehydrogenase complex, subunit A	[120]
SFRS4	Serine/arginine-rich splicing factor 4	[78]
TBP	TATA-binding protein	[5, 75]
TPT1	Tumor protein, translationally-controlled 1	[22]
UBC	Ubiquitin C	[73]
YAP1	Yes-associated protein 1	[85]

The table presents a consolidated alphabetical list of genes used as endogenous references or normalizers in gene expression studies related to thirteen main human cancers

factor causing variability in the outcome of gene expression data analyses. Therefore, it appears that taking only the highest ranking reference or normalizer gene, as revealed by either one of the three software packages, could be the primary source of variability. To counter this adverse effect, it would be best to consider a set, or subset, of candidate reference genes for normalization, as suggested by Chervoneva et al. [156]. They reported a robust and comprehensive technique to evaluate normalizing factors, which was based on all possible subsets of reference genes, rather than addressing the stability of individual reference genes. Consequently, reference or normalizer gene(s) with a low degree of variability got automatically included in the algorithm. We suggest that the use of at least a pair of reference or normalizer genes with distant functions will help to level the influence of a possible co-regulation between the reference gene(s) and the gene(s) under investigation [152]. For even more stringent and accurate results, it is recommended to employ at least three reference or normalizer genes [21, 157] and three different evaluation algorithms in a typical gene expression study [23].

16 Conclusions and future perspectives

Upon careful scrutinizing the currently published data, we find that the *PPIA* gene (Table 1) is the only reference or normalizer gene that has been found suitable in several gene expression studies of at least five of the most prevalent human cancers, i.e., breast, colon, esophagus, kidney and ovary cancers (Table 3). The *PPIA* gene is a widely conserved eukaryotic gene that encodes a member of the peptidyl-prolyl cis-trans isomerase family of proteins. It catalyzes cis-trans isomerization of proline imidic peptide bonds in oligo-peptides to accelerate protein folding (http://www.ncbi.nlm.nih.gov/gene/5478). Further analysis indicates that the other most common reference genes for the normalization of gene expression data in several human cancers are *GAPDH* (cervix, lung, prostate and stomach cancers), *ACTB* (breast, esophagus,

Table 2 Human cancers and reference genes selected for gene expression studies

Cancer type	Endogenous controls, reference or normalizer genes	Software employed	References
Breast	*PUM1*	NormFinder	[25]
	TBP, RPLO & PUM1	geNorm	[25]
	MRPL19 &PPIA	geNorm, NormFinder	[26]
	ACTB & SDHA	geNorm, NormFinder	[27]
	ACTB, RPS23, HUWE1, EEF1A1 & SF3A1	NormFinder	[28]
Cervix	*GAPDH &RPLPO*	geNorm, NormFinder	[39]
	EEF1A1 & GAPDH	geNorm, NormFinder	[40]
Colon	*B2M &PPIA*	geNorm, NormFinder	[48]
	HPRT1 & PPIA	geNorm	[49]
	IPO8 &PPIA	NormFinder	[49]
	HSPCB, YWHAZ & RPS13	geNorm,NormFinder, BestKeeper	[23]
Esophagus	*PPIA , ALAS1, ACTB*	geNorm	[63]
Kidney	*PPIA & TBP*	geNorm, NormFinder	[5]
	RPS13 & PPIA	geNorm	[71]
Liver	*HMBS & UBC*	geNorm	[73]
	TBP&HPRT	geNorm, NormFinder	[75, 76]
	HMBS	geNorm, NormFinder	[79]
	CTBP1	geNorm, NormFinder	[77]
Lung	*HPRT1 & ESD*	geNorm, NormFinder	[83]
	RPLPO, UBC, GAPDH, CASC3, MT-ATP6, PES1	geNorm	[84]
	18S rRNA, POLR2A,YAP1, ESD	geNorm	[85]
	ACTB, EEF1A1, FAU, RPS9, RPS11, RPS14	geNorm, NormFinder	[88]
Lymphoma	*RPL13A*	geNorm	[98]
Ovary	*IPO8, RPL4*	geNorm,NormFinder, BestKeeper	[101]
	PPIA, RPS13, SDHA	geNorm,NormFinder, BestKeeper	[23]
	GUSB, PPIA, TBP	geNorm,NormFinder	[102]
	GUSB & PPIA	NormFinder	[102]
	RPLP0 & RPL4	geNorm,NormFinder	[113]
Pancreas	*ELF1,EIF2B1, POP4, MRPL19*	geNorm, NormFinder	[114]
Prostate	*HPRT1*	geNorm, NormFinder	[119]
	GAPDH & SDHA	NormFinder	[120]
Stomach	*GAPDH & B2M; RPL29 & B2M*	geNorm, NormFinder	[134]
	ACTB & B2M	geNorm, NormFinder, BestKeeper	[135]
	18S rRNA	geNorm	[136]
Thyroid	*ACTB*	NormFinder	[138]

The table presents a list of 13 main types of human cancer along with the most suitable reference gene(s) that were evaluated by different research groups and found to be suitable for gene expression studies using the software tools indicated

stomach and thyroid cancers), *HPRT1* (colon, liver, lung and prostate cancers) and *TBP* (breast, kidney, liver and ovary cancers) (Table 3). Interestingly, the ribosomal RNA gene family, including the *18S rRNA* gene (Tables 1 & 2) that is abundantly expressed and, hence, widely used as a normalizer gene in numerous studies, seems to have very limited preference in studies related to human cancers (Table 4). The same holds for over two dozen other genes, including *MT-ATP6, IPO8* and *UBC*, which were found suitable for gene expression studies in only 1, or at most 2, different human cancers (Table 4).

From the analysis presented in this review, it is evident that there is not one single consensus endogenous reference gene to normalize gene expression data in different human cancers.

Table 3 Most commonly selected reference genes for gene expression studies

Endogenous control, reference or normalizer gene	Human cancer types	References
PPIA	Breast	[26]
	Colon	[48, 49]
	Esophagus	[63]
	Kidney	[5]
	Ovary	[23, 102]
GAPDH	Cervix	[39, 40]
	Lung	[84]
	Prostate	[120]
	Stomach	[134]
ACTB	Breast	[27, 28]
	Esophagus	[63]
	Stomach	[135]
	Thyroid	[138]
HPRT	Colon	[49]
	Liver	[75, 76]
	Lung	[83]
	Prostate	[119]
TBP	Breast	[25]
	Kidney	[5]
	Liver	[75, 76]
	Ovary	[102]

The table presents the five most suitable reference genes, each one of which has been selected by different research groups for normalization of gene expression data in studies of four or more types of human cancers

Table 4 Least commonly used reference genes for gene expression studies

Endogenous control, reference or normalizer gene	Cancer types	References
ALAS1	Esophagus	[63]
B2M	Colon	[48]
	Stomach	[134, 135]
CASC3	Lung	[84]
CTBP1	Liver	[77]
EEF1A1	Cervix	[40]
	Breast	[28]
EIF2B1	Pancreas	[114]
ELF1	Pancreas	[114]
ESD	Lung	[83, 85]
GUSB	Ovary	[102]
HMBS	Liver	[73, 79]
HSPCB	Colon	[23]
HUWE1	Breast	[28]
IPO8	Colon	[49]
	Ovary	[101]
MRPL19	Breast	[26]
	Pancreas	[114]
MT-ATP6	Lung	[84]
PES1	Lung	[84]
POLR2A	Lung	[85]
POP4	Pancreas	[114]
PUM1	Breast	[25]
RPL4	Ovary	[101]
RPL13A	Lymphoma	[98]
RPL29	Stomach	[134]
RPS13	Colon	[23]
RPS23	Breast	[28]
SF3A1	Breast	[28]
UBC	Liver	[73]
	Lung	[84]
YAP1	Lung	[85]
YWHAZ	Colon	[23]
18S rRNA	Lung	[85]
	Stomach	[136]

The table presents a list of reference genes that have been selected for normalization of gene expression data by different research groups in studies on limited types (1 or at most 2) of human cancers

However, a combination of *PPIA* with either the *GAPDH*, *ACTB*, *HPRT1* or *TBP* genes, or their suitable combinations, should be able to cover 11 of the 13 most common human cancers included in this review (i.e., breast, cervix, colon, esophagus, kidney, liver, lung, ovary, prostate, stomach and thyroid cancers) (Table 3). Furthermore, we found that the choice of the software package (i.e., *geNorm, NormFinder* or *BestKeeper*) for the validation of cancer-related gene expression data does not seem to have any influence on the final outcome. Therefore, we recommend that future gene expression studies in human cancer should seriously consider using the five reference or normalizer genes listed in Table 3 (in combinations of at least 2 or preferably 3) for normalization of the data. Once adopted by different researchers the final output is anticipated to be harmonized, which will lead to an increase in the depth of our understanding of the mechanisms underlying cancer, as well as its use in diagnostics and prognostics. We strongly recommend to first evaluate the suitability of the above five reference genes (preferably in appropriate combinations) using any of the available software packages for selecting 2 or 3 reference genes for normalization. Even if another reference gene, besides the above mentioned genes, is proposed, we recommend that it should first be evaluated in order to avoid obscuring real gene expression changes and/or yielding erroneous gene expression data. Such an approach would also meet the MIQE guidelines for qPCR data analysis [157].

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Author's contributions RNS and STV conceived the project, designed the study, analyzed data and interpreted its significance. STV, SN, JK and MK searched and collated the literature, and performed preliminary data analyses. RNS and STV wrote the manuscript.

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