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Quantitative analysis of lncRNA in formalin-fixed paraffin-embedded tissues of oral squamous cell carcinoma

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ABSTRACT

The study evaluated expression profiles of few regulatory lncRNAs in oral squamous cell carcinoma and normal mucosa adjacent to oral cancer using paired fresh frozen and formalin-fixed paraffin-embedded (FFPE) tissues stored at a different duration of time (1–5 years) using real-time quantitative PCR. The quantity and quality of total RNA isolated from FFPE tissues was less compared with that of fresh frozen tissues, which resulted in a noncorrelation of quantification cycle values. Following normalization, the expression of lncRNAs in the paired tissues did not differ significantly. The differential expression of the lncRNAs in the study was consistent with The Cancer Genome Atlas head and neck squamous cell carcinoma database. The study findings demonstrate the possibility of performing accurate quantitative analysis of lncRNAs using short amplicons and standardized real-time quantitative PCR assays in oral squamous cell carcinoma FFPE samples.

METHOD SUMMARY

An optimized TRI reagent modified protocol for RNA isolation and real-time quantitative PCR was carried out to identify the lncRNAs in formalin-fixed paraffin-embedded tissues of oral squamous cell carcinoma and the tissue of normal mucosa adjacent to oral cancer. An endogenous control gene (*GAPDH*) was used to normalize the expression level. The expression levels of formalin-fixed, paraffin-embedded tissues were compared with paired fresh frozen tissues. The relative expression of lncRNAs levels in the study samples were compared with The Cancer Genome Atlas dataset of head and neck squamous cell carcinomas. Gene stability analysis was carried out for all lncRNAs and reference gene by a comprehensive ranking method using a web-based bioinformatics tool, RefFinder.

KEYWORDS:

formalin-fixed paraffin-embedded tissues • fresh frozen tissues • lncRNAs • oral squamous cell carcinoma • real-time quantitative PCR

Gene transcriptional quantification methods have become important tools in both understanding the molecular events that occur in oral squamous cell carcinoma (OSCC) and identifying diagnostic and therapeutic targets [1].

RNA has traditionally been thought to be more prone to degradation than DNA and proteins. But major advances in RNA isolation and quantitation methods, particularly reverse transcription real-time quantitative PCR (RT-qPCR), have revealed RNA's versatility and stability [2]. In the post-genome-sequencing era, it is identified that only a small subset of RNA encodes proteins, while the majority of RNA (98%) is transcribed from the genome but not translated into proteins; this RNA is known as ncRNA [1]. lncRNAs is a recently identified novel class of ncRNAs having more than 200 nucleotides known to be implicated in the initiation and progress of OSCC [3].

Since RNA profiling studies are traditionally conducted on fresh frozen (FF) tissues, their application in clinical settings has been constrained by the scarcity of clinical samples. Additionally, FF samples are difficult to obtain for large-scale studies and expensive to store. Hence, the development of molecular tests for clinical use based on archival materials such as archived formalin-fixed paraffin-embedded (FFPE) tissues would be extremely beneficial [1]. Furthermore, FFPE samples can be very useful in retrospective studies as they are associated with follow-up details. But unfortunately, RNA extracted from archival FFPE tissues frequently suffers chemical modification, crosslinking and degradation over time as a result of fixation and archiving methods [4].

Although partial RNA degradation is inevitable, optimized normalization strategies in RT-qPCR will effectively compensate for experimental errors, such as the inherent variability of RNA, variability of extraction protocols that may copurify inhibitors, and different reverse transcription and PCR efficiencies [5,6].

In recent years, many articles have been published about attempts to understand RNA expression patterns using paired FF and FFPE tissues identifying mRNAs or stable noncoding small RNAs like miRNAs [7–10]. However, it remains unclear whether newly identified ncRNAs, such as lncRNA and circRNA, remain stable for downstream molecular analysis in FFPE tissues [2]. Few studies have analyzed the expression of selected lncRNAs using paired FF and FFPE tissues of brain [2] and glioblastoma [11] using small nonoverlapping amplicons and have demonstrated the possibility of lncRNA analysis in FFPE tissues. But whether FFPE specimens are suitable for expression studies of lncRNA in OSCC has not been systematically investigated yet.

Although there have been few studies on gene expression profiling and miRNA [12] and gene expression profiling in FFPE tissues of tongue carcinomas [13] using matched FF tissues, there have been no studies in head and neck tumors or oral cancer that have investigated whether FFPE specimens are acceptable for lncRNA expression analyses using matched (or paired) FF tissues. Since OSCCs have a higher degree of tumor heterogeneity than other types of cancer [14], and also because long-chain RNA molecules, such as lncRNA, are prone to degradation, it must be thoroughly investigated whether FFPE tissues are appropriate for lncRNA expression investigations in OSCC.

Hence, in the present study, we have assessed the feasibility of using FFPE tissues to perform expression profiles of frequently used regulatory lncRNAs (HOTAIR, MEG3, H19, MALAT1) in OSCC and normal mucosa adjacent to oral cancer (NM) by performing optimized RT-qPCR with the help of paired FF and FFPE tissue samples.

Materials & methods

Tissue specimens

Thirty matched pairs of FF and FFPE tissues of OSCC (60 samples) and NM (60 samples), a total of 120 samples, were retrieved from the Department of Oral Pathology and Microbiology. These tissues were collected and stored from patients who underwent surgery at the Shri Dharmasthala Manjunatheshwara Craniofacial Surgery Unit during 2017–2021. NM samples were histologically confirmed tissues adjacent to OSCC cases and hence age and gender matched to OSCC samples. The histopathological evaluation of all the samples was carried out by an experienced oral pathologist to confirm the OSCC diagnosis and tumor content ($\geq$80%). Fresh OSCC tissues were stored in RNAlater™ solution (Takara Bio, Shiga, Japan) at -80°C and FFPE blocks were stored in the dark at room temperature (24 $\pm$ 2°C).

Institutional Ethical Committee approval was obtained to conduct the study (ref. no. IRB no. 2020/S/OP/71). Written informed consent from OSCC patients was taken except for deceased patients who could not be traced or contacted.

RNA isolation

All materials and working surfaces were treated with RNase kill (Himedia Pvt. Ltd, Mumbai, India) prior to sample handling to minimize the risk of RNA degradation.

The FF tissues stored in RNAlater™ solution (Takara Bio) were washed with 1× phosphate-buffered saline thrice, and total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) following the protocol by Iker *et al.* [15].

For extraction of RNA from FPPE tissue, 6–8 μm size sections were cut from each archival block and five to eight sections were transferred into a new tube containing 1 ml of xylene and then washed by ethanol. RNA was isolated following the optimized TRI reagent modified protocol published previously by us [16]. The quantity and quality of the total RNA extracted was analyzed by Bio-Spectrophotometer (Eppendorf, model no. 6136, Hamburg, Germany) in nanograms and the integrity of RNA was determined by using 1% denaturing agarose gel electrophoresis. Bands were visualized by staining in ethidium bromide stain.

lncRNA primer design

Primer sequences for lncRNAs and an endogenous control gene (*GAPDH*) used to normalize the expression level were custom-designed (Supplementary Table 1). Primers were designed using a software tool (https://bioinfo.ut.ee/primer3-0.4.0/) and were checked for specificity using a basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

cDNA synthesis & RT-qPCR

All RNA extracts were prepared at 1 μg/μl per sample and transcribed into cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara) as per the manufacturer's instructions. cDNA sample was diluted with nuclease-free water (1:10) and stored at -20°C until further use.

RT-qPCR (Rotor-Gene Q6 MDx-Qiagen, Hildon, Germany) was performed with TB Green Mix (Takara) in a total volume of 20 μl. Each experiment was carried out in triplicates. A light cycler melting curve analysis was performed which resulted in single product-specific melting temperatures as follows: initial denaturation -95°C for 5 min, denaturation -95°C for 5 s, annealing -60°C 10 s. Amplicons were analyzed by high resolution melting at 1°C resolution from 60 to 95°C to determine the absolute melt curve of each amplicon. No primer dimers were generated during the applied 40 real-time PCR amplification cycles, and the specificity of RT-qPCR products were documented with high-resolution gel electrophoresis and resulted in a single product with the desired length.

In the present study, to determine the reference gene for normalization, the preliminary study was carried out on ten paired FF and FFPE tissues of OSCC, and expression analysis was carried out for commonly used reference genes *GAPDH*, *HPRT1* and *ACTB*. *GAPDH* showed fairly constant quantification cycle (Cq) values and was the most stable expression when analyzed using RefFinder and was

selected for further analysis (data not presented). The relative lncRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method by Schmittgen and Livak [17] and compared among the study groups.

The flow chart of the method carried out is represented in Supplementary Figure 1.

Statistical analysis

The numerical data were expressed in mean ± SD. The statistical significance of differences in the resulting data was analyzed with GraphPad and SPSS software, with $\alpha = 0.05$. The total RNA concentration and expression levels (Cq values) of lncRNAs were compared between the paired FF and FFPE tissues in both OSCC and NM groups by paired *t*-test. Interclass correlation coefficient analysis was used to correlate the Cq values of lncRNA between the FF and FFPE groups. Wilcoxon's signed-rank sum test was used to analyze the difference in lncRNA expression among archived FFPE tissue samples of different years.

The relative expression (fold change) of lncRNAs in OSCC cases was compared between FF and FFPE tissues by unpaired *t*-test. Gene stability analysis was carried out for all lncRNAs and reference genes by a comprehensive ranking method using web-based RefFinder which compares geNorm, NormFinder, Best Keeper and comparative ΔCq.

Relative expression of lncRNAs levels in the study samples were compared with The Cancer Genome Atlas (TCGA) dataset obtained using the University of California Santa Cruz Xena platform for head and neck squamous cell carcinomas (HNSCCs). TCGA box plots were generated using the ggplot2 package (v 3.3.5) in the R programming language. The dplyr package (v 1.0.9) was also used for data-wrangling tasks, which included filtering, sorting and summarizing the data to prepare them for analysis. The obtained data were log2 transformed and relative expressions were compared using unpaired *t*-test.

Results & discussion

Identifying and validating molecular markers in FFPE tissues is a hotly debated and exciting area of research right now. Extensive collections of FFPE clinical samples are readily available worldwide providing an excellent resource for retrospective and follow-up studies. Although FF tissue samples are known to harbor good-quality RNA, there are only a few institutions worldwide that have large frozen-tissue banks associated with long-term clinical follow-up [18].

In OSCC, despite the continuous advancement in multimodality treatment, satisfactory survival rates have not been achieved, owing to diagnosis at advanced stages of the disease, resulting in poor outcomes [19]. The retrospective studies play a very important role to identify the molecular factors which influence the survival of patients with OSCC. Previous research has shown that the ncRNAs, such as lncRNAs, play vital regulatory roles in the cellular physiological process and could also act as an miRNA sponge [20]. In this setting, FFPE tissues can provide an opportunity to investigate lncRNA expression correlating to clinical outcomes.

Few recent studies have demonstrated the possibility of accurate expression analysis of selected lncRNAs using paired FF and FFPE tissues. Lv *et al.* have demonstrated the possibility of performing accurate quantitative analysis of MALAT1 using short amplicons and standardized RT-qPCR assays in autopsy-derived FFPE brain tissue samples compared with paired FF tissues [2]. Esteve-Codina *et al.* have suggested that RNA-Sequencing of FFPE glioblastoma specimens provides reliable gene expression of lncRNAs like lincRNA compared with paired FF tissues [11]. Rentoft *et al.* have reported the possibility of gene expression profiling and miRNA expression analysis in FFPE tissues of tongue carcinomas by microarray using a limited number of paired FF tissues [12,13]. The authors have suggested the appropriate quality assurance steps to be taken to ensure sample consistency for quality measurement and confirmation by quantitative PCR. However, there are no studies reported on investigating lncRNAs in OSCC tissues using paired FF and FFPE tissues. Although few researchers have used FFPE tissues of OSCC for lncRNAs analysis in the past studies [21,22], it is not clear whether genomic data obtained from FFPE tissue is as reliable as that obtained from FF tissue. Hence, the present study was undertaken to analyze lncRNA from paired FF and FFPE OSCC tissues systematically.

RNA quantity, quality & integrity analysis

Isolation of good quantity and quality for RNA from FFPE tissues has always been a challenge. The present study employed our previously published optimized TRI reagent protocol for RNA isolation to identify lncRNA in OSCC tissues [16].

Total RNA was successfully extracted from FFPE tissues and its paired FF tissues of NM and OSCC samples. The mean concentrations of total RNA of FFPE tissues of NM and OSCC tissues were 132.2 ± 130.1 ng/μl and 308.7 ± 227.1 ng/μl, which was significantly lower ($p < 0.05$) than that of FF tissues of NM and OSCC tissues with 336.2 ± 304.8 ng/μl and 447.1 ± 314.8 ng/μl, respectively. RNA purity was assessed using the 'nanodrop' method. The mean A260/A280 ratio of the FF and FFPE tissues of NM was 1.79 and 1.60, and of OSCC was 1.83 and 1.7, respectively. The mean A260/A230 ratio of the FF and FFPE tissues of NM was 0.76, 0.37, and of OSCC was 1.2, 0.9, respectively (Figure 1). The integrity of RNA was verified by resolving in 1% formaldehyde agarose gel electrophoresis (Supplementary Figure 2).

The both quantity and quality of RNA extracted from FFPE tissues in the present study were found to be low but comparable to FF tissues. Lower RNA yields in FFPE tissues in the present study are consistent with previous reports. Abrahamsen *et al.* have suggested the amount of RNA that can be extracted from FFPE tissue represents only a fraction and crosslinks prevent this RNA from being extracted [23]. Previous studies have shown low RNA yield in the FFPE samples with pronged storage time [24,25]. The reason for the

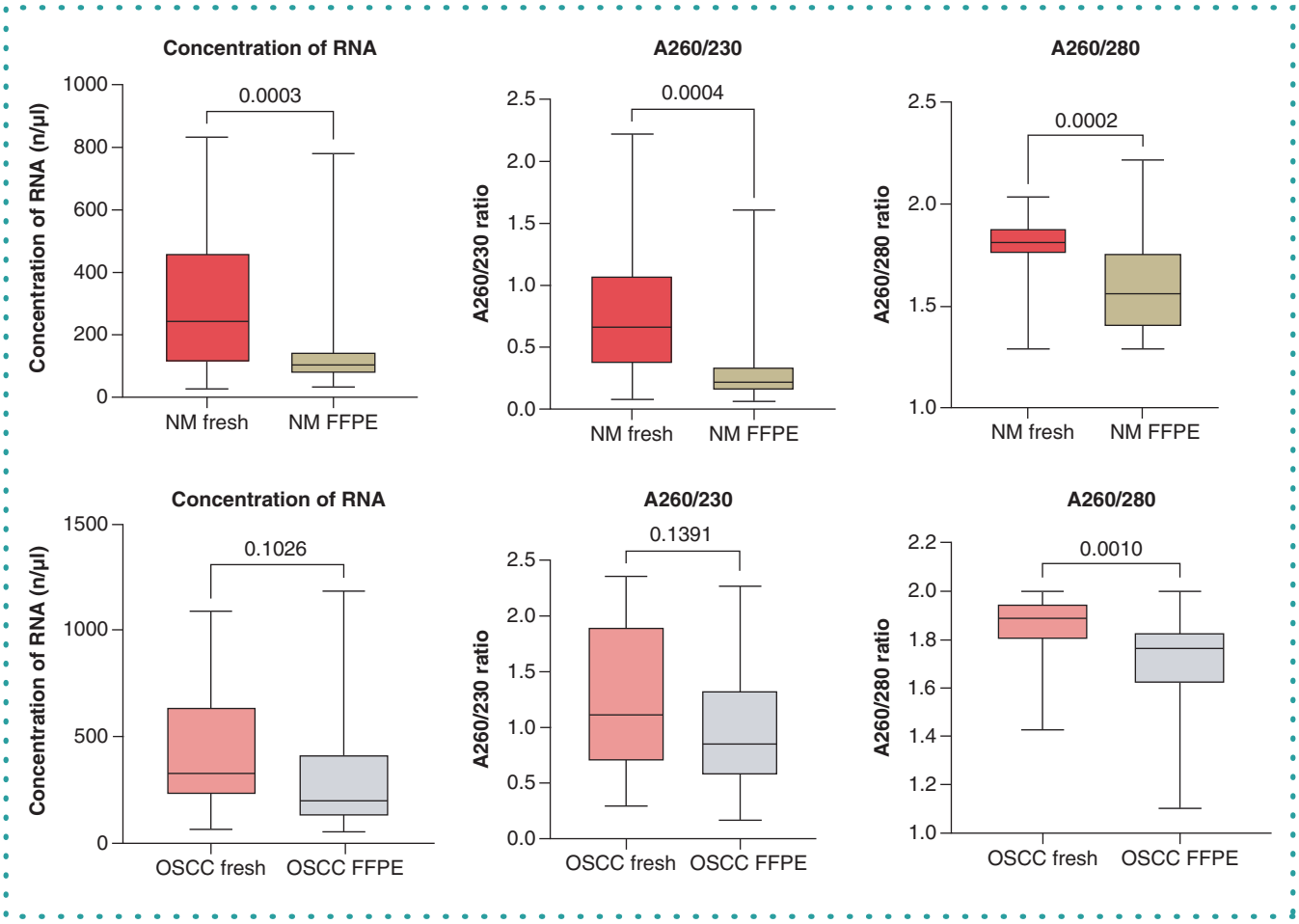

Figure 1. Comparison of quantity and quality of total RNA in paired fresh frozen and formalin-fixed paraffin-embedded tissues of normal mucosa and oral squamous cell carcinoma.
FF: Fresh frozen; FFPE: Formalin-fixed paraffin-embedded; NM: Normal mucosa; OSCC: Oral squamous cell carcinoma.

low RNA yield may be due to preanalytical factors such as postmortem interval, fixation time and storage conditions that can affect RNA integrity, amplification success and quantitative analytical results of RNA [26,27].

The RNA data of FFPE tissues of NM and OSCC cases were compared among tissues with different durations of storage time (1–5 years). Although there were variations in the total quantity of RNA in all age categories of samples, there was no significant change in quantity, quality and integrity of RNA content with the duration of storage (Supplementary Tables 2 & 3). This finding may be due to the fact that all the samples were from the same center where all samples were fixed, processed and stored in tightly controlled conditions.

RT-qPCR & expression stability

RT-qPCR is a powerful tool that can show gene expression in a highly efficient manner even using short RNA fragments for amplification. This property is highly suited to FFPE tissue since FFPE-derived long-chain RNAs like lncRNAs are generally partially degraded [2]. However, systematic evaluation using paired FF and FFPE tissues is required.

Amplification was successful for all lncRNAs and reference gene in all tissues. The dissociation curves of the RT-qPCR experiment showed single peaks representing the unique amplicons suggesting the designed primers were specific and efficient for quantitative analysis.

Comparison & correlation of lncRNA & reference gene expression in paired FFPE & FF tissues

The present study analyzed the expression of four lncRNAs in 30 each FF and FFPE pairs of OSCC and NM tissues by RT-qPCR. The *GAPDH* was used as an endogenous control gene (reference gene). Many different algorithms and software are available to evaluate the stability of reference genes; however, the results are not always consistent, and a lot of discrepancies are found in conclusions among researchers [28]. *GAPDH* showed the most stable expression when analyzed using RefFinder in the pilot study and hence was selected for normalization.

Table 1. Comparison of quantification cycle values of lncRNAs in paired formalin-fixed paraffin-embedded and fresh frozen tissues of normal mucosa cases.

lncRNAs	Normal mucosa		p-value (paired *t*-test)†
	Paired paraffin tissues	Fresh frozen tissues	
GAPDH	25.69 ± 1.51	25.19 ± 1.69	0.153
HOTAIR	27.70 ± 1.05	27.26 ± 1.31	0.077
MEG3	26.65 ± 1.54	26.90 ± 1.89	0.227
H19	20.75 ± 1.40	21.14 ± 1.76	0.195
MALAT1	25.76 ± 1.47	23.75 ± 2.68†	0.001

Values are expressed as mean ± SD; n = 30.
†$p < 0.001$ is significant.

Table 2. Comparison of quantification cycle values of lncRNAs in paired formalin-fixed paraffin-embedded and fresh frozen tissues of oral squamous cell carcinoma cases.

lncRNAs	Oral squamous cell carcinoma cases		p-value (paired *t*-test)†
	Formalin-fixed paraffin-embedded tissues	Fresh frozen tissues	
GAPDH	27.05 ± 1.13	26.06 ± 2.21†	0.044
HOTAIR	28.01 ± 1.23	27.53 ± 1.14†	0.005
MEG3	27.36 ± 1.19	27.07 ± 2.43	0.547
H19	21.15 ± 1.52	21.25 ± 1.43	0.790
MALAT1	25.05 ± 1.14	22.90 ± 1.64†	0.000

Values are expressed as mean ± SD; n = 30.
†$p < 0.05$ is significant.

Table 3. Interclass correlation coefficient analysis of lncRNA expression (quantification cycle values) in paired formalin-fixed paraffin-embedded and fresh frozen tissues of normal mucosa.

	Intraclass correlation coefficient	95% CI		F-test with true value 0			
		Lower bound	Upper bound	Value	df1	df2	p-value
GAPDH	0.339	-0.019	0.619	2.024	29	29	0.031
HOTAIR	0.377	0.025	0.646	2.211	29	29	0.018
MEG3	0.783	0.592	0.890	8.199	29	29	0.000
H19	0.487	0.159	0.718	2.897	29	29	0.003
MALAT1	0.128	-0.238	0.462	1.292	29	29	0.047

$p < 0.05$ is significant.

Cq values of lncRNAs in paired FFPE and FF tissues of NM and OSCC are represented in Tables 1 & 2. The findings depicted that the Cq values of only MALAT1 were significantly reduced ($p = 0.001$) in FF tissues of NM as compared with its paired FFPE tissues, whereas the Cq values of GAPDH, HOTAIR and MALAT1 were significantly reduced ($p < 0.05$) in FF tissues of OSCC compared with its paired FFPE tissues.

The results suggested that mean raw Cq was slightly higher in FFPE samples compared with FFPE samples except for MEG3 and H19 in NM tissues and H19 in OSCC tissues. The previously published studies have reported the median Cq values approximately 5 units higher in FFPE material than in FF samples [29–31]. Since the RNA extracted from archival FFPE specimens is partially degraded, this loss of intact amplicon template explains the raw Cq shift observed in FFPE specimens compared with the matched FF samples. The present study finding also showed samples with a greater degree of RNA degradation had higher Cq values, confirming the inverse relationship between average Cq and the quality of RNA.

Interclass correlation coefficient analysis of expression of paired FF and FFPE tissues showed a significant fair-to-good correlation in NM tissues. However, except for HOTAIR, there was no significant correlation in the expression of lncRNAs in paired OSCC tissues (Tables 3 & 4).

In the paired tissues of NM and OSCC, the interclass correlation coefficient analysis revealed a varied correlation of Cq values. These outcomes might be a result of the difference in RNA degradation rates between FF and FFPE tissue. However, the levels of expression for each lncRNA were conserved (Tables 5 & 6), indicating that normalization techniques can compensate for changes in Cq values, as has been also documented by other investigators [29–32].

Table 4. Interclass correlation coefficient analysis of lncRNA expression (quantification cycle values) in paired formalin-fixed paraffin-embedded and fresh frozen tissues of oral squamous cell carcinomas.

	Intraclass correlation	95% CI		F-test with true value 0			
		Lower bound	Upper bound	Value	df1	df2	p-value
GAPDH	-0.064	-0.410	0.298	0.880	29	29	0.633
HOTAIR	0.722	0.493	0.857	6.190	29	29	0.000
MEG3	-0.001	-0.356	0.354	0.998	29	29	0.502
H19	0.044	-0.316	0.393	1.093	29	29	0.406
MALAT1	0.072	-0.290	0.417	1.156	29	29	0.349

$p < 0.05$ is significant.

Table 5. Comparison of relative expression (fold change) of lncRNAs in paired formalin-fixed paraffin-embedded and fresh frozen tissues of oral squamous cell carcinomas.

lncRNAs	Oral squamous cell carcinoma cases		p-value (independent sample t-test)†
	Expression in formalin-fixed paraffin-embedded tissues	Expression in fresh frozen tissues	
HOTAIR	3.10 ± 2.44	2.52 ± 2.06	0.306
MEG3	1.49 ± 1.32	1.36 ± 1.38	0.621
H19	1.70 ± 1.46	1.54 ± 1.27	0.637
MALAT1	6.47 ± 5.34	6.11 ± 6.20	0.754

Values are expressed as mean ± SD; n = 30.
†$p < 0.05$ is significant.

Table 6. Differential expression of lncRNA in paired formalin-fixed paraffin-embedded and fresh frozen tissues of oral squamous cell carcinomas.

lncRNA	Fresh frozen tissues	p-value†	Formalin-fixed paraffin-embedded tissues	p-value†
HOTAIR	Overexpressed	0.0045	Overexpressed	0.0002
MEG3	Underexpressed	0.0915	Underexpressed	0.2869
H19	Underexpressed	0.1704	Underexpressed	0.5984
MALAT1	Overexpressed	0.0015	Overexpressed	0.0002

†Unpaired t-test.
$p < 0.05$ is significant.

Comparison of relative expression of lncRNAs in FF & FFPE tissues of OSCC

After the normalization, though there was a numerical increase in the fold change of lncRNAs expression in FFPE tissues compared with FF tissues of OSCC, there was no statistically significant difference (Table 5). HOTAIR and MALAT1 were significantly overexpressed and H19 and MEG3 were underexpressed but not significantly in both FFPE and FF tissues (Table 6).

The above results indicate that normalization and steady expression are crucial in establishing accurate expression in FFPE tissues of OSCC using short amplicons. This result is consistent with other research that has shown that short amplicons can accurately replicate linear transcript expression levels in both FF and FFPE tumor specimens [33,34]. Some previous study observations have shown inhibition of quantitative PCR increased with greater amplicon length [33,35]. Rentoft *et al.* have shown by applying normalization methods, they could demonstrate miRNAs in archived FFPE tissues of OSCC, and NM for up to 11 years [12]. Furthermore, Kong *et al.* have innovatively demonstrated 100% accurate quantification of long-chain RNAs with three nonoverlapping short amplicons in standardized-preserved FFPE tissues of colorectal carcinomas and adjacent normal tissues [33].

Gene stability analysis

Gene stability analysis was carried out by a comprehensive ranking method using web-based bioinformatics RefFinder software tool for lncRNAs HOTAIR, MEG3, H19, MALAT1 and reference gene *GAPDH* in both paired FF and FFPE tissues of NM and OSCC. Results depicted that HOTAIR was found to be the most stable lncRNA studied in FFPE tissues of NM followed by *GAPDH*, MALAT1, H19 and MEG3 with geomean ranking values 1.00, 2.06, 2.45, 4.23 and 4.73, respectively. HOTAIR was found to be the most stable lncRNA studied in FF tissues of NM followed by *GAPDH*, H19, MEG3 and MALAT1 with geomean ranking values 1.00, 1.86, 2.71, 4.00 and 5.00, respectively (Supplementary Figure 3).

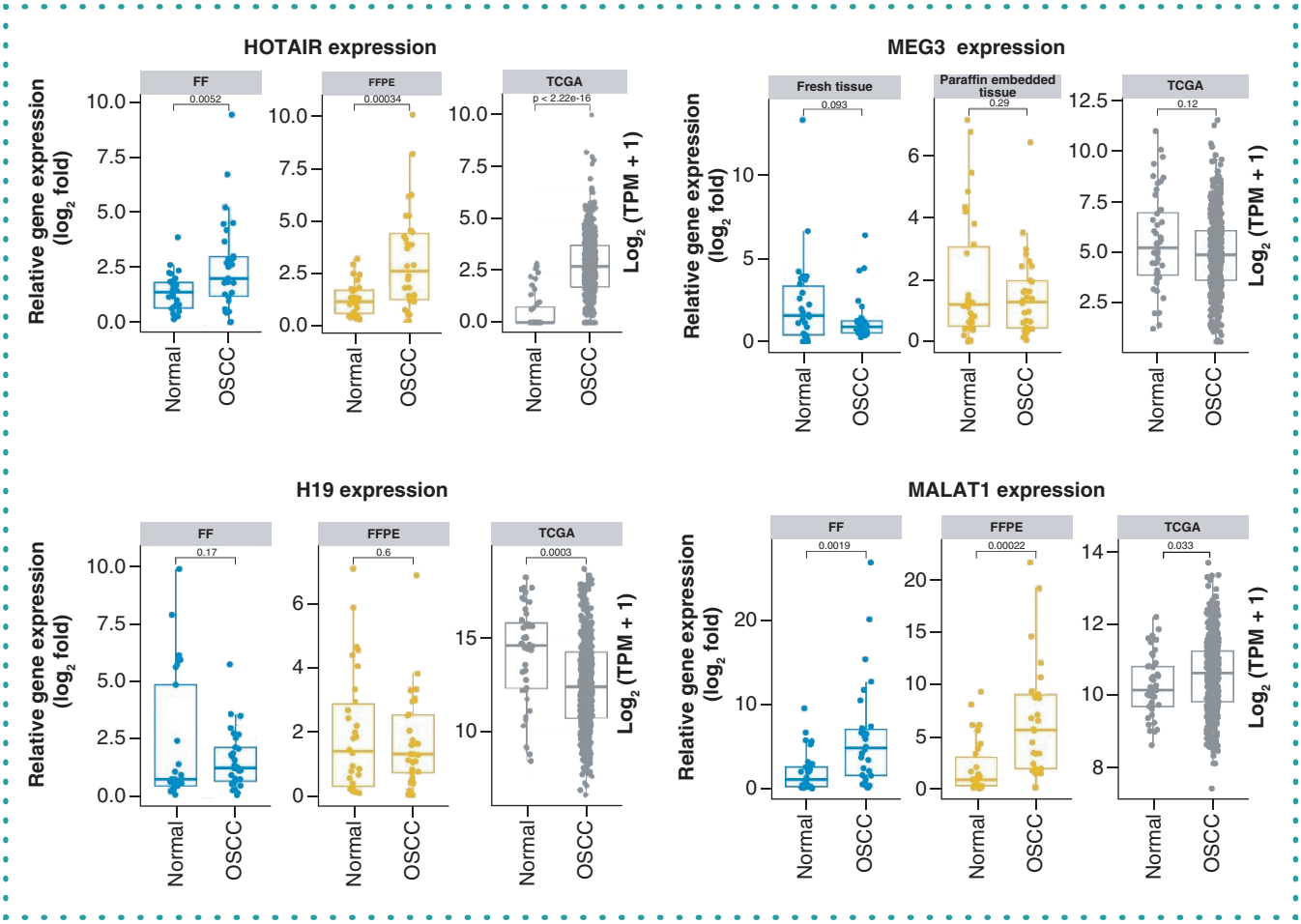

Figure 2. Comparison of lncRNA expression in head and neck squamous cell carcinoma datasets from The Cancer Genome Atlas database (517 oral squamous cell carcinoma and 44 normal [healthy patient data]) and study samples.
***p $<$ 0.05 is statistically significant.**
HNSCC: Head and neck squamous cell carcinoma; OSCC: Oral squamous cell carcinoma; TCGA: The Cancer Genome Atlas.

Similarly, MEG3 was found to be the most stable lncRNA studied in FFPE tissues of OSCC cases followed by HOTAIR, *GAPDH*, H19 and MALAT1 with geomean ranking values 1.73, 2.00, 2.91, 2.99 and 3.34, respectively. MALAT1 was found to be the most stable lncRNA studied in FF tissues of OSCC followed by *GAPDH*, HOTAIR, H19 and MEG3 with geomean ranking values 1.41, 1.86, 3.34, 3.36 and 3.41, respectively (Supplementary Figure 4).

The comprehensive geomean rankings using web-based RefFinder were below 5, and suggested excellent expression stability and accurate RT-qPCR protocol.

Comparison of relative expression of lncRNA of study samples with HNSCC-TCGA data sets & other studies

Relative expression of lncRNAs levels in the study samples were compared with TCGA dataset of HNSCCs and showed similar expression levels. However, MEG3 and H19 expression levels in our study samples were not significant (Figure 2).

HOTAIR was significantly overexpressed in the present study, which was in corroboration with TCGA database and other studies [12,36–42]. MALAT1 was significantly overexpressed in our study, the same as HNSCC-TCGA datasets and other studies [40,43,44]. However, three studies [28,36,45], including two Indian studies [36,37], with similar sample size have reported contrasting results. MEG3 was underexpressed in our study but was not significant. Significant underexpression of MEG3 was found in HNSCC-TCGA datasets and similar consistent underexpression is reported by other researchers [40,42,46,47]. However, Arunkumar *et al.* have found nonsignificant dysregulation of MEG3 like our study [37]. H19 in the present study was underexpressed but not significantly. TCGA data sets and other studies have also shown significant underexpression [36,37].

These variations in lncRNAs dysregulation is may be due the fact that HNSCC is a heterogeneous group of tumors with different epigenetic alterations and tissue-specific expression of lncRNA. Moreover, the habits in OSCC patients like tobacco and alcohol may also influence the expression of lncRNAs [48].

Conclusion

Our study findings demonstrate the possibility of performing accurate quantitative analysis of lncRNAs using short amplicons and standardized RT-qPCR assays in OSCC FFPE samples.

Future perspective

The future study will analyze more lncRNAs using FFPE samples preserved for a longer duration (up to 10–12 years) and using longer amplicons. In addition, the study will also analyze the clinicopathological features and treatment outcome associated with the differentially expressed lncRNAs using FFPE tissues. In addition, we will also try to elucidate the impact of differentially expressed lncRNAs on clinic-pathological features and response to treatment in patients with OSCC.

Executive summary

Background

- Identifying and validating molecular markers in formalin-fixed paraffin-embedded (FFPE) tissues is a hotly debated and exciting area of research right now.
- lncRNAs expression has not yet been systematically studied in FFPE tissues of oral squamous cell carcinoma (OSCC).

Materials & methods

- The present study assessed the feasibility of using FFPE tissues to perform expression profiles of frequently used regulatory lncRNAs in OSCC and normal mucosa adjacent to oral cancer using paired fresh frozen (FF) and FFPE tissue.
- Thirty matched pairs of FF and FFPE tissues from OSCC (60 samples) and normal mucosa adjacent to oral cancer (60 samples) stored at a different duration of time (1–5 years) were analyzed by real-time quantitative PCR (RT-qPCR) for expression of lncRNAs HOTAIR, MEG3, H19 and MALAT1.
- The differential expression of the lncRNAs in the study were compared with with The Cancer Genome Atlas head and neck squamous cell carcinoma database.

Results & discussion

- The quantity and quality of total RNA isolated from FFPE tissues was less compared with that of FF tissues, which resulted in a noncorrelation of quantification cycle values.
- Following normalization, using *GAPDH* as reference gene, the expression of lncRNAs in the paired tissues did not differ significantly.
- Gene stability analysis using web-based RefFinder showed geomean values less than 5, suggesting excellent expression stability and accurate RT-qPCR protocol.
- HOTAIR and MALAT1 were significantly overexpressed and MEG3 and H19 were underexpressed but not significantly in paired tissues.
- The differential expression of the lncRNAs in the study was consistent with The Cancer Genome Atlas head and neck squamous cell carcinoma database.

Conclusion

- The study findings demonstrated the possibility of performing accurate quantitative analysis of lncRNAs using short amplicons and standardized RT-qPCR assays in OSCC FFPE samples.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2023-0033

Author contributions

Concept and design: K Kumar, K Hallikeri and A Oli. Acquisition of data: K Kumar, M Goni and A Jain. Analysis and interpretation of data: M Goni, A Oli, J Poyya and K Kumar. Drafting the manuscript: AS Shilpasree and PK Javaregowda. Critical revision of manuscript: PK Javaregowda, M Goni, A Jain and J Poyya. Funding, administration, technical or material support: AS Shilpasree and PK Javaregowda. Statistical analysis: J Poyya, K Hallikeri and A Oli.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Institutional Ethical Committee approval was obtained to conduct the study (ref. no. IRB no. 2020/S/OP/71). Written informed consent from OSCC patients was taken except for deceased patients who could not be traced or contacted.

Open access

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Papers of special note have been highlighted as: •• of considerable interest

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