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Long noncoding RNA hot air an independent predictive marker of overall survival and loco-regional recurrence in oral squamous cell Carcinoma

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ABSTRACT

Introduction: Identifying suitable predictive biomarkers is critical for preventing recurrence and improving the survival rate in individuals with Oral Squamous Cell Carcinomas (OSCC). HOTAIR is a novel type of long non-coding RNA (lncRNA) that has been linked to initiation and progression in OSCC. Most previous studies on HOTAIR have included a small number of OSCC cohorts, only a few studies have performed multivariate analyses adjusting for confounding factors, and there has been little emphasis on HOTAIR's potential to predict loco-regional recurrence.

Methodology: The study included 96 OSCC Formalin Fixed Paraffin Embedded (FFPE) tissue samples and gender and age-matched 30 normal mucosa who had received surgical therapy. HOTAIR expression in OSCC was analysed using qRT-PCR and correlated with clinic-pathological variables, risk practices, loco-regional recurrence, and Overall Survival (OS).

Results: HOTAIR was overexpressed in OSCC compared to NM. High HOTAIR expression was related to regional lymph node metastases, perineural invasion, and locoregional recurrence. The Kaplan-Meier curve followed by Log-rank test survival estimates revealed a significantly associated with high HOTAIR expression and poor OS ($p<0.05$). In multivariate analysis, lymph node metastasis (Hazard ratio 4.39, $p \leq 0.001$, 95% confidence interval, 1.775-10.886) and high HOTAIR expression (Hazard ratio 3.337, $p \leq 0.001$, 95% confidence interval, 1.396-7.973) were the independent predictors of poor OS. Age, TNM staging, and high HOTAIR expression were all independent predictors of loco regional recurrence.

Conclusion: The current study establishes HOTAIR as a novel prognostic marker for OSCC and suggests a therapeutic strategy for targeted therapy of OSCC.

Keywords: Oral Squamous Cell Carcinomas (OSCC), long non-coding RNA, reverse transcriptase PCR, HOTAIR expression, loco-regional recurrence, oval all survivals

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INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is one of the most prevalent malignancies in the world accounting for approximately 90% of all cancers originating in the oral cavity [1, 2]. According to the Global Cancer Observatory (GCO) 2020, OSCC is the second greatest cause of cancer-associated morbidity and mortality in India, accounting for one-third of all OSCC cases globally [3]. Despite significant advances in the cancer treatments, the overall survival remains below 50% mainly due to lymph node metastasis and loco-regional recurrence/relapse [1]. Notably, OSCC prevalence is considerably greater in Indian rural populations and disadvantaged strata due to overexposure to risk factors such as tobacco and betel quid, lack of knowledge about diseases, and inadequate good medical health care [4]. Hence, discovering suitable predictive biomarkers is a key to prevent recurrence and improve the survival of patients with OSCC.

Till recently, genes and their related proteins studies were the centre point of oncologic studies. However, these studies cannot fully explain initiation and progression of cancers [5]. According to large-scale gene annotation projects, 80% of the human genome is transcribed into functional RNAs, but only <2% of genes are translated into proteins. The remaining RNAs are known as non-coding RNAs (ncRNAs) [6]. The novel class of non-coding RNAs is called long non-coding RNA (lncRNA) made up of more than 200 nucleotides that have recently been linked to cancer initiation and progression in a variety of cancers [7].

HOTAIR (HOX Transcript Antisense Intergenic RNA) was the very earliest lncRNA discovered to have a retro trans positional function recognized to have critical involvement in controlling cell proliferation, differentiation, and metastasis of Head and Neck Squamous Cell Carcinomas (HNSCC) [8]. The over expression of HOTAIR is known to be associated with clinico-pathological characteristics like size of the tumour, clinical stage, and survival in HNSCC [9, 10].

HOTAIR is the most commonly researched lncRNA and the only transcript with prognostic value evaluated in many independent cohorts of OSCC and other cancer patients [7-10]. However, most studies that have analysed survival included a small number of OSCC cohorts. Only a few researches have done multivariate analysis adjusting the confounding factors and not given emphasis on ability of HOTAIR to predict loco-regional recurrence [11-

15]. More research is required to confirm the role of HOTAIR as an independent predictive biomarker. Hence, the present study included Indian cohort of 96 OSCC patients with long term follow-up analysing the expression of HOTAIR with Overall Survival (OS) and loco-regional recurrence. To further enhance the clinical significance of our study results and the importance of HOTAIR, we investigated the TCGA (The Cancer Genome Atlas) data sets for HOTAIR expression and overall survival.

MATERIAL AND METHODS

OSCC tissues and control tissues

The study included 96 OSCC Formalin Fixed Paraffin Embedded (FFPE) tissue samples and age and gender matched 30 Normal Mucosae (NM) adjacent to oral cancer tissues obtained from Department of Oral and Maxillofacial Pathology and Oral Microbiology. SDM College of Dental Sciences and Hospital. The clinico-pathological details, treatment and follow up details were retrieved from SDM Craniofacial Surgery and Research Centre (A Constituent Unit of Shri Dharmasthala Manjunatheshwara University), Dharwad, India.

The study included OSCC patients treated with Radical Neck Dissection (RND) or Modified Radical Neck Dissection (MRND), with or without adjunct radiation or radio-chemotherapy, and who had at least 3 years of follow-up. The exclusion criteria were neoadjuvant radio and chemotherapy, patients suffering from immunodeficiency conditions or other malignancies, patients with distant metastasis and incomplete data or follow-up.

The study was approved by Institutional Ethical Committee (ref. no. IRB no. 2020/S/OP/71). Except for deceased patients and those who could not be located or contacted, all study participants provided written informed permission.

RNA isolation and quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

FFPE tissues of OSCC and NM were sectioned to 5 μm-10 μm thicknesses for RNA isolation. Our previously reported optimized modified TRI reagent protocol was employed for total RNA isolation [16]. The quantity and quality of isolated RNA was determined using a bio-spectrophotometer (Eppendorf, Germany) and 1% formaldehyde Gel Electrophoresis. Isolated RNA was used for cDNA synthesis using a Prime-script cDNA synthesis kit (TaKaRa, China).

qRT-PCR was carried out following our previously published protocol 16 on Rotrar Gene 6 Pleix- Qiagen thermocycler (Manchester, UK) using custom designed primers (HOTAIR

- Forward: 5'-GCAGTGGAATGGAACGGATT-3', HOTAIR
- Reverse: 3'-ATCAGACTCTTTGGGGCCTT-5'). Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as control gene GAPDH
- Forward: 5'- GGGGAAGGTGAAGGTCGGAG-3', GAPDH
- Reverse: 3'ACGGTGCCATGGAATTTGCC-5')

Agarose gel electrophoresis done to confirm the molecular weight of the qRT-PCR end product. The relative HOTAIR expression in OSCC tissue samples was calculated by 2-ΔΔct method. The differential expression of HOTAIR in OSCC compared to NM was calculated and was compared with clinico-pathological factors, risk habits, loco-regional recurrence and oval all survival [17-19].

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (version 9.0.0, San Diego, CA, USA) and the SPSS software program. The experimental data were reported as mean ± SD. A p-value of <0.05 was statistically significant.

Descriptive analysis was performed on demographics, clinic pathological features, behaviours, loco-regional recurrence status, follow-up details, and HOTAIR expression using contingency tables. OS was computed from the surgery date to the last follow-up or death date. Patients who died due to causes other than OSCC were censored. The cutoff value for HOTAIR expression was established by creating the Receiver Operating Characteristic (ROC) curve and categorized as low and high expression. chi-square or χ^2 test, Fisher's exact test and student's two-tailed unpaired t-test were used to compare the clinico-pathological parameters and its correlation with HOTAIR expression. The predictive relevance of HOTAIR expression level in relation to the OS of patients was assessed using the Kaplan-Meier analysis and the log-rank test. Univariate and Multivariate Cox regression analysis was carried out to analyse the ability of HOTAIR to predict the overall survival and loco-regional recurrence after adjusting the all-confounding factors.

Bioinformatics analysis

The study used the UCSC Xena platform to access the TCGA (The Cancer Genome Atlas) (https://tcga-data.nci.nih.gov) dataset for OSCC. The dataset was then filtered to include only samples from primary solid tumour and normal patients. The HOTAIR raw mRNA expression data were downloaded and normalized by ($\log^2$ (data+1)) for further statistical analysis. Box plots were generated to visualize the HOTAIR expression levels using ggplot2 (v 3.3.5) of R programming language. Additionally, the dplyr (v 1.0.9) package was used for data wrangling tasks. To assess the Overall Survival (OS), patients were categorized into low and high expression levels groups. Kaplan Meier survival curves were estimated using the survival package of R programming, and the implementation of the log-rank test. Specifically, the survfit function was used to generate the survival curves, and the survfit function was employed to perform the log-rank test and survival distributions were compared between the two groups. The survival package provides tools for calculating and visualizing survival data, while the ggplot2 package was utilized for enhanced graphical representation of the survival curves.

RESULTS

Expression of HOTAIR in OSCC and its correlation with clinico-pathological parameters

Expression of HOTAIR was detected in 96 OSCC FFPE tissue samples and NM by RT-qPCR and was normalized using endogenous control gene GAPDH. HOTAIR was overexpressed in OSCC compared with NM ($p<0.005$). Cut off value of HOTAIR expression 2.10 was obtained by constructing the

ROC curve. The HOTAIR was highly expressed in OSCC cases with positive regional lymph node metastasis, Perineural Invasion (PNI) and patients with loco-regional recurrence ($p<0.05$) (Table1).

Tab. 1. Correlation of HOTAIR expression and clinico-pathological factors in OSCC

Parameter		HOTAIR		n (%)	p-Value (χ^2)
		Low (<2.10)	High (≥ 2.10)		
Age	<50	20	26	46	0.884
	≥ 50	21	29	50	
Gender	Male	36	50	86	0.622
	Female	5	5	10	
Habit	Tobacco chewing	15	27	-	0.437
	Smoking	9	7	-	
	Chewing + smoking	9	13	-	
	Chewing + smoking + alcohol	3	1	-	
	No habit	5	7	-	
Duration of Habit	Up to 15 years	24	17	41	0.458
	More than 15 years	28	27	55	
Site	BM with or without GBS	28	42	70	0.102
	Tongue	12	7	19	
	Hard Palate	0	3	3	
	Alveolus and lip	1	3	4	
Lymph node Metastasis	No	26	25	51	0.041
	Yes	15	30	45	
TNM staging	Stage 1	6	5	11	0.616
	Stage 2	6	5	11	
	Stage 3	20	33	53	
	Stage 4	9	12	21	
Histopathology grade	Well differentiated	26	40	66	0.354
	Moderately differentiated	12	10	22	
	Poorly differentiated	3	5	8	
PNI	No	33	34	67	0.049
	Yes	8	21	29	
PVI	No	38	46	84	0.185
	Yes	3	9	12	
Recurrence status	No	33	8	41	0.013
	Yes	31	24	55	

HOTAIR expression and survival analysis

The average overall survival period of OSCC cases was 88.5 months ± 29.65 months. Kaplan Meier curve followed by log rank test analysis showed high HOTAIR expression was significantly associated with poor OS (38.9 months) compared with patients with low HOTAIR expression to (94.6 months). These findings imply that HOTAIR is overexpressed in OSCC and may be related with disease severity, which leads to poor survival in OSCC patients. Univariable Cox regressions analysis for risk factors showed that age >50 years, lymph node metastasis, PNI and high HOTAIR expression (≥ 2.10) were associated with poor OS. However, Multivariate analysis showed lymph node metastasis (Hazard ratio 4.39, $p \leq 0.001$, 95% confidence interval, 1.775-10.886) and high HOTAIR expression (Hazard ratio 3.337, $p \leq 0.001$, 95% confidence interval, 1.396-7.973) were independent predictor with poor OS (Table 2).

HOTAIR expression as an independent predictor of loco-regional recurrence of OSCC

The average recurrence period of OSCC cases was 16.6 months. The multivariate Cox regression analysis demonstrated age of the OSCC patients >50years, Higher TNM staging and high HOTAIR expressions were independent predictor of loco-regional recurrence of OSCC (Table 3).

Tab. 2. Univariate and multivariate Cox regression analysis of prognostic factors for overall survival in Oral squamous carcinomas

		Univariate				Multivariate			
		HR	95.0% CI for HR		P -value	HR	95.0% CI for HR		P-value
			Lower	Upper			Lower	Upper	
Age	AGE (<50 years ≥ 50 years)	0.501	0.276	0.909	0.023	0.64	0.28	1.459	0.288
Gender	(Female *vs.* Male)	0.792	0.282	2.222	0.657	0.281	0.045	1.742	0.173
Habits	No habits	Reference category				Reference category			
	Tobacco chewing	0.807	0.354	1.843	0.611	0.849	0.236	3.05	0.802
	Smoking	0.327	0.104	1.026	0.055	0.499	0.096	2.586	0.408
	Tobacco chewing and smoking	0.867	0.348	2.159	0.759	0.808	0.199	3.272	0.765
	Chewing + smoking + alcohol	0	0	-	0.979	0	0	-	0.974
Duration of Habit	Habit (<15 years *vs.* ≥15 years)	1.158	0.649	2.066	0.62	2.068	0.878	4.867	0.096
Site	BM with or without GBS	Reference category				Reference category			
	Tongue	0.202	0.062	0.656	0.008	0.139	0.036	0.535	0.064
	Hard Palate	0.632	0.086	4.644	0.652	0.564	0.047	6.774	0.652
	Alveolus and lip	0.511	0.07	3.734	0.509	0.906	0.1	8.173	0.93
TNM staging (AJCC, 7th edition)	STAGE I	Reference category				Reference category			
	STAGE II	1.188	0.361	3.909	0.777	0.646	0.134	3.103	0.585
	STAGE III	1.461	0.555	3.847	0.443	0.639	0.168	2.428	0.511
	STAGE IV	1.706	0.569	5.112	0.34	1.22	0.288	5.177	0.787
Histopathology grading	Well differentiated	Reference category				Reference category			
	Moderately differentiated	0.861	0.445	1.668	0.658	0.702	0.283	1.738	0.444
	Poorly differentiated	0.721	0.551	2.135	0.567	0.837	0.621	1.82	0.267
PNI	PNI (yes *vs.* No)	2.627	1.462	4.721	0.001	1.379	0.569	3.347	0.477
PVI	PVI (yes *vs.* No)	2.051	0.95	4.429	0.067	0.43	0.106	1.741	0.237
Lymph node metastasis	(Yes *vs.* No)	2.777	1.515	5.09	0.001	4.396	1.775	10.886	0.001
Treatment	Surgery	Reference category				Reference category			
	Surgery + Radiotherapy	0.776	0.37	1.629	0.502	0.848	0.337	2.134	0.726
	Surgery + Radiotherapy+ Chemotherapy	1.57	0.747	3.301	0.234	0.923	0.313	2.726	0.885
Locoregional Recurrence	Locoregional recurrence status (Yes *vs.* No)	1.36	0.755	2.448	0.306	0.721	0.331	3.125	0.932
HOTAIR	<2.10 *vs.* ≥ 2.10	1.335	1.16	1.538	0	3.337	1.396	7.973	0.007

Tab. 3. Multivariate Cox regression analysis of loco-regional recurrence of OSCC

	HR	95.0% CI for Exp(B)		Sig.
		Lower Bound	Upper Bound	
Age recoded (<50 years ≥ 50 years)	0.954	0.918	0.991	0.016
Gender (Female *vs.* Male)	3.982	0.426	0.226	0.226

Habit	4.504	0.473	0.191	0.191
(Habit 1 *vs.* Habit 2 *vs.* Habit 3 *vs.* Habit 4)				
Duration of habit	4.58	0.327	0.376	0.376
(<15 years *vs.* ≥ 15 years)				
Site (Site 1 *vs.* Site 2 *vs.* Site 3 *vs.* Site 4)	3.558	0.742	0.112	0.112
TNM staging	1.707	1.039	2.806	0.002
(Stage 1 *vs.* Stage2 *vs.* Stage 3 *vs.* Stage 3 *vs.* Stage 4)				
Histopathology	0.357	0.038	0.368	0.368
(Well *vs.* Moderate *vs.* Poor)				
PNI	1.058	0.145	0.956	0.956
(Yes *vs.* No)				
PVI	3.762	0.569	0.246	0.246
(Yes *vs.* No)				
Lymph node metastasis	0.261	0.031	0.215	0.215
(Yes *vs.* No)				
Treatment	4.877	0.403	0.213	0.213
(Treatment1 *vs.* Treatment 2 *vs.* Treatment3 *vs.* treatment4)				
HOTAIR (<2.10 *vs.* ≥ 2.10)	2.084	1.442	3.013	0

Correlation of HOTAIR expression in patients with the history of risk habits

HOTAIR expression was matched to oral risk habits in patients with OSCCs. HOTAIR expression has no significant relationship with risk practices (data not shown).

Bioinformatics analysis

HOTAIR was overexpressed in OSCC cases compared to adjacent Normal Mucosa (NM) and was significantly correlated to higher histopathological grade in TCGA data sets (p-value=0.0069, ANOVA, Supplementary file 3) It was found that HOTAIR over-expression was significantly associated with poor OS in OSCC (p-value=0.0027, Supplementary file: 4) but there was no association with loco-regional recurrence/DFS (Disease Free Survival) (Figure 1).

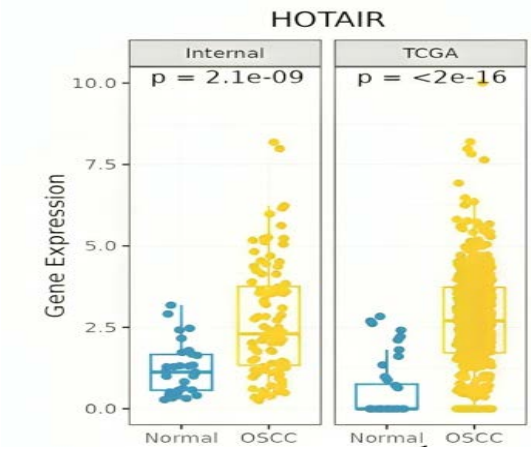


Fig. 1. Box plot showing the comparison of expression of HOTAIR in OSCC compared to normal (NM) in study cases (Internal) (OSCC, n=96 and Normal n=30) and TCGA data base (OSCC, n= 517 and Normal, n= 44)

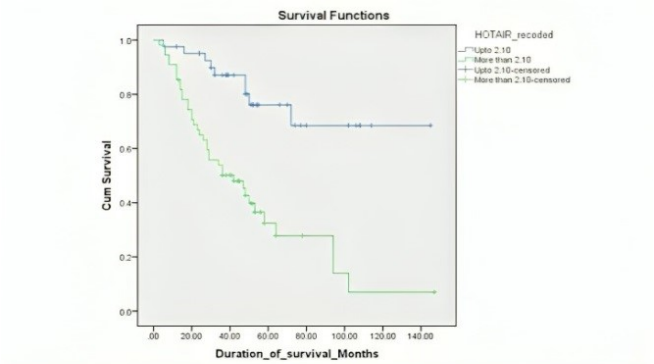
DISCUSSION

HOTAIR is 2.2 kb long non-coding RNA having 6 exons and transcribed by RNA polymerase II from the HOXC gene antisense strand located on chromosome 12q13.13.18,19 Recent evidences have shown HOTAIR dysregulation is involved in various signalling pathways and correlated with OSCC initiation and prognosis making it a potential predictive prognostic biomarker [20, 21].

The HOTAIR is known to influence the pathogenesis of OSCC by cooperation with different chromatin modifying complexes, mainly Polycomb Repressive Complex 2 (PRC2) and Lysine-Specific Demethylase 1(LSD1). HOTAIR causes a silencing effect on PRC2 through tri-methylation of histone H3K27 and LSD1 complex by H3K4 demethylation. Furthermore, HOTAIR can serve as a competitive endogenous RNA (Sponging) in OSCCs, regulating microRNAs such as miR-106a-5p, 22 miR-10123, and miR-723. In HNSCC, it plays a crucial role in the Epithelial Mesenchymal Transition (EMT) by inhibiting E-cadherin expression through interaction with EZH2 [22-24]. Under expression

of HOTAIR can increase apoptosis, resulting in mitochondrial membrane potential alterations and Mitochondrial Calcium Uptake 1 (MICU1)-dependent cell death. It also contributes to the growth and metastasis of HNSCC via a regulatory loop involving HuR, moreover, it can sponge mir-7, inhibiting its inhibitory action on HuR expression [25].

Most studies have documented overexpression of HOTAIR in OSCC8-13, 21, 22, l25 and this is also reported in our study in the study cases (Internal) and in TCGA data sets (Figure 2). HOTAIR has been found to be strongly linked with clinicopathological characteristics such as larger tumour size 11, 26, 27 positive lymph node metastasis 12, 26, 27, 28 higher clinical stage 11, 12, 26, 27 and histopathological grading 12, 26, 27 in OSCC patients. In the current investigation, HOTAIR was substantially linked with positive lymph node Metastases and Perineural Invasion (PNI). Previous studies did not include the histopathological metric PNI, which is proved to be an independent predictor of prognosis for patients with OSCC. Bioinformatics analysis from the TCGA data base showed significant correlation of high HOATAIR expression with only tumour histopathological grade.



Means and Medians for Survival Time

HOTAIR_recoded	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Upto 2.10	113.017	9.357	94.677	131.357				
More than 2.10	51.856	6.580	38.959	64.752	42.000	9.943	22.512	61.488
Overall	77.231	6.866	63.773	90.689	64.000	10.865	42.704	85.296

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	19.740	1	.000
Breslow (Generalized Wilcoxon)	16.078	1	.000
Tarone-Ware	17.647	1	.000

Test of equality of survival distributions for the different levels of HOTAIR_recoded.

Fig. 2. Kaplan-Meier curve and Log–rank (Mantel-Cox) test survival estimates of OSCC cases correlated to HOTAIR Expression

lncRNAs generally have a mild-to-moderate expression level depended on type of cell differentiation and is tissue-type specific, and can alter rapidly in response to exogenous environmental factors such as risk habits [10]. However, there is limited research on the link between lncRNAs and oral risk habits few studies have made an attempt to correlate risk habits like tobacco and alcohol with HOTAIR expression in OSCC [11, 12, 15]. The two Indian studies, Arun Kumar et al., have found significant higher expression of HOTAIR with tobacco chewing and Vishwakarma et al., have found reduced expression in smokers [15, 26-28]. However, in our study (Both Internal and TCGA data base) have found no significant correlation of HOTAIR expression with any of the risk habits. The variations in results may be attributable to interactions between risk habits and oral anatomical sub sites [10].

Although, HOTAIR is one of the most investigated lncRNAs, there have been few studies on the possible function of HOTAIR expression as a predictor of poor prognosis, with the majority focusing on OS and Relapse-Free Survival (RFS) [11-15]. Whenever HOTAIR expression and OS was found, it was consistently related to the presence of 2 or 3 parameters like tumour size, lymph node involvement and higher TNM stage. Hence, these potential confounding factors need to be adjusted. However, only a few studies have used multivariate analysis to account for these potential confounding factors [10]. In the present study, High expression of HOTAIR and lymph node metastasis were found to be the independent predictive marker for OS in multivariate analysis (Table 2). Similarly, high expression of HOTAIR along with higher age, clinical staging were the independent predictors of loco-regional recurrence (Table 3).

Many studies have acquired data from public transcriptome data sets and have done gene expression profiling in OSCC [29, 30]. Present study analysed the TCGA datasets and found HOTAIR overexpression was significantly correlated with poor OS and same is also demonstrated by Lu et al., [31].

CONCLUSION

The findings in the present study indicate HOTAIR is a novel prognostic marker. The studies like ours provide support to therapeutic strategy for the personalized targeted treatment for OSCC. Further research needed to examine the underlying process of HOTAIR role in the regulation of progression of OSCC and to develop novel therapeutic strategies to reduce the recurrence and improve the prognosis of OSCC. We suggest study of large OSCC sample size from standardized multicentre belonging to different race and ethnicity in order to confirm study findings.

Limitations and future perspectives

The current study did not examine HOTAIR expression levels in vitro (cell lines) or regulatory signalling pathways that could be involved in the development and progression of OSCC. In recent years, we have made significant a dvances i n o ur u nderstanding of HOTAIR's role in tumour cell biology. Identifying the regulatory pathways that lead tumour to overexpress HOTAIR and by reducing its function, could lead to novel therapeutic targets.

Furthermore, it is recently recognized that HOTAIR expression is may led to the development of chemo resistance and regulates the efficacy of chemotherapeutic drugs like cisplatin [32]. Hence, it can be used with other drugs to make malignant tumours more sensitive to chemotherapy. Currently, advanced genetic engineer-ing methods like shRNA and siRNA can be utilized to block HO-TAIR, potentially leading to advances in personalized, precision medicine and enhancing the prognosis of HNSCC and OSCC.

DISCLOSURE STATEMENT

Conflict of interest

The authors declare that they have no conflict of interest.

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