DOI: 10.7860/JCDR/2021/47575.15330

Original Article

Genetics Section

Gene Expression Profiling and Clinicopathological Importance of Fer1L4 and DANCR Long Non Coding RNAs in Patients with Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Introduction: Head and Neck Squamous Cell Carcinoma (HNSCC) entails a heterogeneous group of tumours that emerge from the interaction between molecular changes and environmental factors. Dysregulated long noncoding RNAs (LncRNAs) play a major part in tumourigenesis and could be used as cancer biomarkers and therapeutic aims.

Aim: To evaluate the expression of two lncRNAs named Fer-1 Like Family Member 4 (Fer1L4) and differentiation antagonising non protein-coding RNA (DANCR) in tumoural tissue of HNSCCs patients in comparison to Adjacent Non cancerous Tissues (ANCTs) to appraise their diagnostic power and the relationship with clinicopathological parameters.

Materials and Methods: The present case-control study was designed, in which fresh frozen cancerous tissues and ANCTs were taken from 50 sporadic HNSCC patients who were attended in Imam Khomeini and Amir Alam Hospitals (Tehran, Iran) from from January to December 2019. Real-time PCR was utilised for expression profiling of Fer1L4 and DANCR. By employing GraphPad Prism 8.0 GraphPad Software, Inc., San Diego, CA, the real-time quantitative PCR experiments(2-..Ct) method and the Mann-Whitney test were exerted to analyse the obtained data. The Receiver Operating Characteristic (ROC) curve analysis was employed for figuring out the discrimination potential of two selected lncRNAs between the subject tumour and ANCT.

Results: The expression of Fer1L4 was significantly down-regulated in tumoural tissues by analogy to ANCTs (p-value <0.0001) and statistically significant associations were found between the stage and grade status of the tumour with the relative expression of this lncRNA (p-value=0.008 and p-value=0.002 for stage and grade, respectively). The findings in this study indicated that the expression of DANCR was not statistically significant different in different tumoural tissues compared with ANCTs (p-value=0.46). ROC curve unraveled that the Fer1L4 had good diagnostic power Area Under Curve (AUC) 0.9252; p-value <0.0001. The expression of DANCR and Fer1L4 was significantly, respectively, higher and lower in samples with lymph node invasion and metastasis than that of the counterpart group. Concerning Human Papillomavirus (HPV) as an important exogenous factor for the development of HNSCC, DANCR and Fer1L4 were over-expressed and under-expressed, respectively in the HPV+group in comparison to HPV-.

Conclusion: This work represented that Fer1L4 could be used as a novel diagnostic biomarker for HNSCC. In addition, the statistically significant difference in the expression of Fer1L4 and DANCR in metastatic tumours demonstrated that these two lncRNAs are promising targets for therapeutic purposes.

Keywords: Biomarker, Differentiation antagonising non protein coding ribonucleic acid

INTRODUCTION

The HNSCC, the sixth most common cancer worldwide with over 880 000 new cases diagnosed annually, comprises 90% of malignancies of the ear-nose-and-throat anatomical region. These heterogeneous and highly aggressive groups of tumours involve different areas of the upper aero-digestive tract including sinonasal and oral cavities, nasopharynx, oropharynx, hypopharynx, and larynx [1]. The complex interplay between genetic susceptibility and environmental risk factors like long-term use of excessive alcohol and smoking or smokeless tobacco, which can have synergistic effects, as well as oncogenic HPV infection leads to the development of HNSCCs [2]. In parallel with falling in smoking-related HNSCCs, HPV-associated (HPV+) HNSCCs cases have been on the rise over the past decade, especially Oropharyngeal Squamous Cell Carcinomas (OPSCC), a sub population of HNSCCs. Determination of HPV status of patients is crucial because it is well documented that HPV+patients have a far more favorable prognosis and better response to chemoradiation than HPV- HNSCC counterparts; although, HPV-driven HNSCC is diagnosed at a younger age. The reason for this discrepancy in disease entity is not well understood; but, different molecular landscapes are the main proposed culprit [3].

Long-noncoding RNAs (lncRNAs), with more than 200 nucleotides in length, are a heterogeneous group of polyadenylated poorly-conserved regulatory RNAs which act in cis and/or in trans to modulate gene expression, either negatively or positively, at several levels including interaction with chromatin, direct binding to the promoter region, sponging the micro RNAs to act as competing endogenous RNAs (ceRNAs), affecting translation process by interfering in ribosome function, and determining cellular localisation of proteins [4]. Deregulated lncRNAs are crucially involved in the carcinogenesis process of different cancers consisting of HNSCCs. For instance, lncRNAs such as HOX Transcript Antisense RNA (HOTAIR), HOXA Transcript at the Distal Tip (HOTTIP), Urothelial Cancer Associated 1 (UCA1), Maternally Expressed 3 (MEG3), Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1), H19, CDKN2B antisense RNA 1 (CDKN2B-AS1), Growth Arrest Specific 5 (GAS5), Regulator of Reprogramming (linc-ROR), X-Inactive Specific Transcript (XIST), FOXC1 Upstream Transcript (FOXCUT), and Nuclear Paraspeckle Assembly Transcript 1(NEAT-1

are deregulated in HNSCCs) [5]. It is therefore not unexpected that these molecules could be ideal biomarkers for diagnosis, prognosis, and therapeutics response purposes [6]. Deregulated lncRNAs, based on their expression pattern in tumoural tissues versus ANCTs as well as their function, are categorised as oncogenic and tumour suppressor transcripts. Putatively, up-regulated and down-regulated lncRNAs were considered oncogenes and tumour suppressors, respectively, signifying them as novel therapeutics targets [7]. Only a few evidence propounded the distinct signature of lncRNAs in HPV+HNSCCs by analogy to HPV-; however, the underlying relation between lncRNAs and HPV is not fully understood [8,9].

It has been delineated that, except in glioma, the long noncoding RNA Fer1L4 acts as a tumour suppresser in a variety of cancers. Of note, in lung cancer, osteosarcoma, Esophageal Squamous Cell Carcinoma (ESCC), and hepatocellular carcinoma Fer1L4 plays a role as a competing endogenous RNA(ceRNA) to regulate the expression of Phosphatase and Tensin Homolog (PTEN). Subsequently, PTEN inhibits the PI3K/protein kinase B(AKT) signaling pathway which is involved in the proliferation and migration of cancer cells [10]. As of yet, the expression of Fer1L4 has not been investigated in HNSCCs. lncRNA Differentiation Antagonising Non-Protein-Coding RNA (DANCR), also named as Anti-Differentiation Noncoding RNA (ANCR), is up-regulated in diverse cancer types [11]. The DANCR has a critical oncogenic role in cancer progression by mediating the epithelial-mesenchymal transition and angiogenesis. From a mechanistic point of view, different action manners have been put forward for the regulation of cell proliferation, progression, and invasion by DANCR in Nasopharyngeal Carcinoma (NPC), a relatively rare head and neck neoplasm subsuming stabilisation of SRY-Box Transcription Factor 2(sox2) mRNA [12], binding to Enhancer of zeste homolog 2(EZH2) and down-regulation of PTEN [13], the regulation of AKT serine/threonine kinase phosphorylation and subsequently protein expression of PTEN [14], interaction with Signal transducer and activator of transcription 3(STAT3) to fortifying IL-6/JAK1/STAT3 signaling [15], and stabilisation of Hypoxia-inducible factors(HIF)-1α mRNA by interacting with Nuclear Factor(NF)90/NF45 to promote motility of Nasopharyngeal carcinoma(NPC) cells [16].

Therefore, in this study, the expression of Fer1L4 and DANCR, which both interact with PTEN, was evaluated in tumoural tissue of HNSCCs patients in comparison with ANCTs to assess their diagnostic power and the relationship with clinicopathological parameters. The aim of this work was to add more data in the current knowledge about the possible role of these two lncRNAs in the development of HNSCCs and consequently promote biomarker-based diagnosis and prognosis, improvement of therapeutic strategies, and finally lay the foundation for ongoing personalised medicine projects.

MATERIALS AND METHODS

This was a case-control study conducted in Imam Khomeini and Amir Alam Hospital (Tehran, Iran) from January 2019 to December 2019 after obtaining approval from Ethics Committee of the Tehran University of Medical Sciences (IEC registration number: IR.TUMS.MEDICINE.REC.1398.215).

Inclusion criteria: Fifty HNSCC patients were enrolled in the present study. Sporadic cases with a definite diagnosis of HNSCC, according to clinico-pathological data (like grading, staging and the anatomical regions which are involved), who were referred for the tumour removal and had not undergone chemo/radiotherapy before surgery were included in the present study. Definite pathological diagnosis and appropriateness of the excised tissue for expression analysis were the considerations of including patients in the study.

Exclusion criteria: Those patients who were having a family history of HNSCC and the presence of tumours in other sites were excluded from the study. Based on the site of the primary tumour, the HNSCC cases were categorised as the larynx, oral cavity, pharynx, and others. From all patients both fresh-frozen tumour and ANCTs tissues were obtained. ANCTs served as control.

Sample size calculation: was done using formulas previously described by Wang X and Ji X with the power of 80% and a 95% confidence interval [17].

The clinicopathological data was defined according to a previous study [18].

Expression Analysis of Fer1L4 and DANCR Genes

By using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was isolated from HNSCC tumour samples and ANCTs. The quantity and quality of extracted RNA were assessed utilising the NanoDrop instrument (Thermo Fisher Scientific, Inc.) and gel electrophoresis, respectively. For cDNA synthesis from about 2 μg of RNA, ExcelRT™ Reverse Transcription Kit (SMOBIO Technology, Inc, Beijing, China) was employed according to the manufacturer's protocol. The expression level of two selected lncRNAs in each sample was evaluated on a Rotor-Gene Q instrument (QIAGEN, Germany). Real-time PCR was done in triplicate using BioFACT™ 2X Real-Time PCR Master Mix (For SYBR Green I), low ROX (BIOFACT Co., South Korea). The SDHA was exploited as a housekeeping gene to calculate relative expression according to the real-time quantitative Polymerase Chain Reaction experiments(2ΔΔCt) method [19].

DNA Extraction and Determination of HPV Status

For the extraction of genomic DNA from fresh frozen tissue samples, TriPure™ Isolation Reagent was recruited according to the manufacturer's instruction. A nanodrop instrument (Thermo Fisher Scientific, Inc.) was utilised to measure the concentrations of extracted DNA. To amplify isolated DNA samples, the Ampliquality HPV-Type Express PCR kit was exploited giving to the manufacturer's recommendations. Briefly, the thermal PCR protocol was as follows: 10 minutes at 95°C for Deoxyribonucleic Acid(DNA)-polymerase activation, 50 cycles of a 30-seconds denaturation at 95°C, 30-seconds annealing at 50°C, and a 30-seconds elongation at 72°C, and eventually a final extension step of 72°C for 5-minutes. Size of amplification products were between 139-145 bp.

STATISTICAL ANALYSIS

At first, the normality of data was checked by the Kolmogorov–Smirnov test. Then, the Mann-Whitney test was applied to measure the expression level of lncRNAs Fer1L4 and DANCR. The ROC curve analysis was utilised for estimating the discrimination potential of two selected genes between the patient tumour and ANCTs. We used MedCalc Software version 10.3.2 (MedCalc, Mariakerke, Belgium) for calculation of cut-off according to Youden index. GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis and p-value ≤0.05 was considered statistically significant.

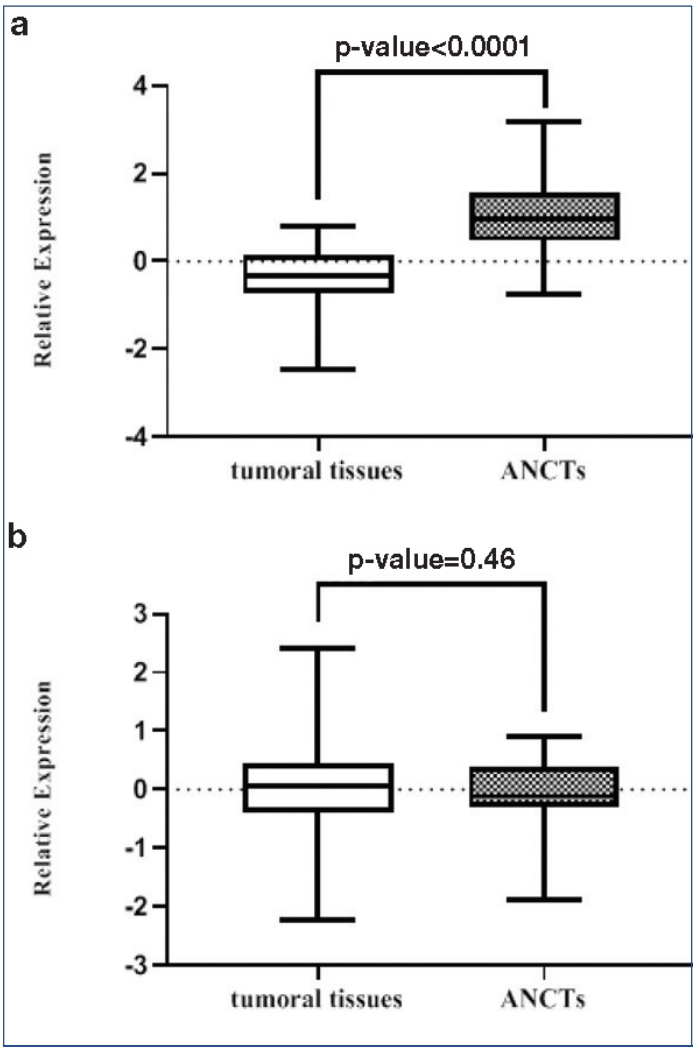
RESULTS

[Table/Fig-1] represents the demographical and clinicopathological data collected from HNSCCs patients. In order to examine the harnessing of Fer1L4 and DANCR as diagnostic biomarkers in HNSCC, real-time PCR was utilised for expression analysis of these two genes. As depicted in [Table/Fig-2], the expression of Fer1L4 is significantly down-regulated in tumoural tissues compared with ANCTs (p-value <0.0001). On the other hand, the results of present study showed that the expression of DANCR was not significantly significant different in tumoural tissues compared with ANCTs (p-value=0.46). The analysis of the area under curve (AUC) values of the ROC curve demonstrated that the Fer1L4 had good diagnostic power (AUC) 0.9252; p-value <0.0001 [Table/Fig-3].

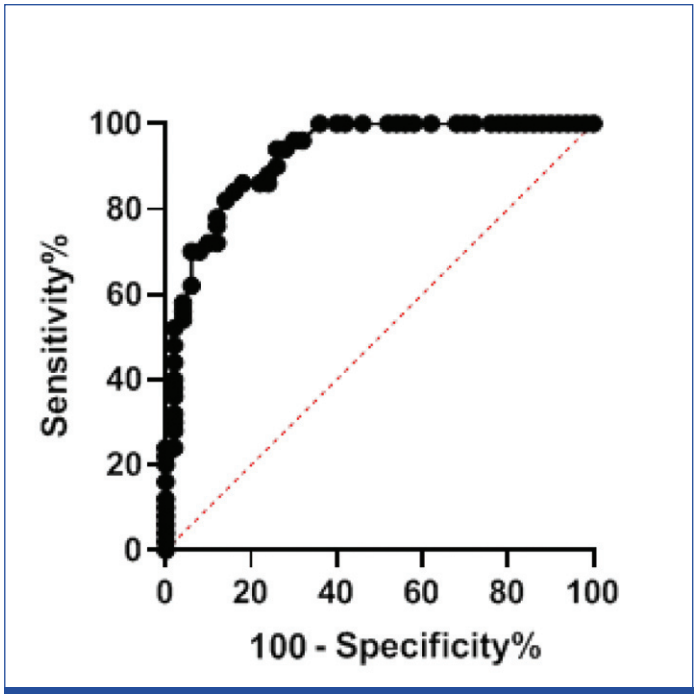
Uncovering the association between gene expression and clinicopathological features, presumably, throws light upon the patho-mechanism of genes. [Table/Fig-4] illustrates the results of the association analysis between relative expressions of two chose

Parameter	Data tabulaion	Tumour subjects (50)
Age	(≥60/<60)	30/20
Sex	(M/F)	33/17
Site of the primary tumour	Larynx	39
	Oral cavity	5
	Pharynx	4
	others	2
Tobacco smoking	M (Yes/No)	29/4
	F (Yes/No)	1/16
Alcohol consumption	M (Yes/No)	2/31
	F (Yes/No)	5/12
Human papilloma virus	(Positive/Negative)	16/34
Tumour size	(≥2.5 cm/<2.5 cm)	28/22
Lymph node invasion	(Yes/No)	25/25
Metastasis	(Yes/No)	25/25
Stage	I	5
	II	15
	III	24
	IV	6
Grade	Well differentiated	9
	Moderately differentiated	17
	Poorly differentiated	24

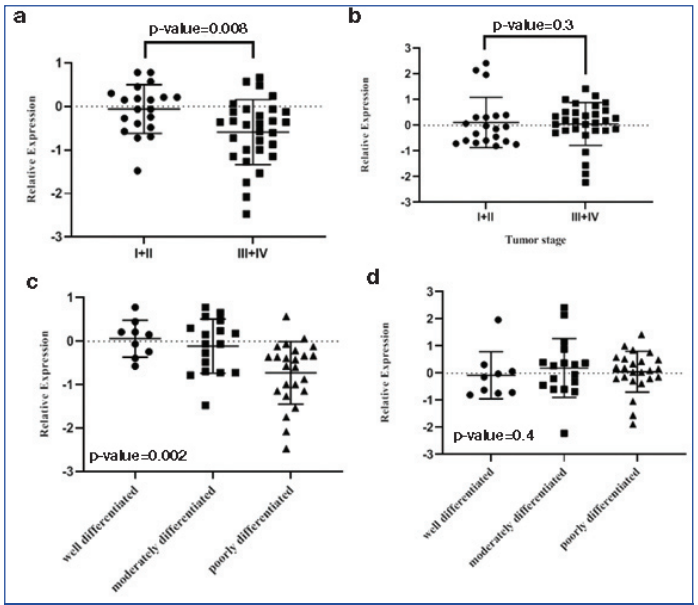
[Table/Fig-1]: The demographical and clinicopathological information of HNSCCs patients.
HNSCCs: Head and neck squamous cell carcinomas; M: Male; F: Female; HPV: Human papillomavirus



[Table/Fig-2]: Relative expression of (a) Fer1L4, and (b) DANCR in tumoural tissues compared to ANCTs. ANCTs, Adjacent Non cancerous Tissues (ANCT).



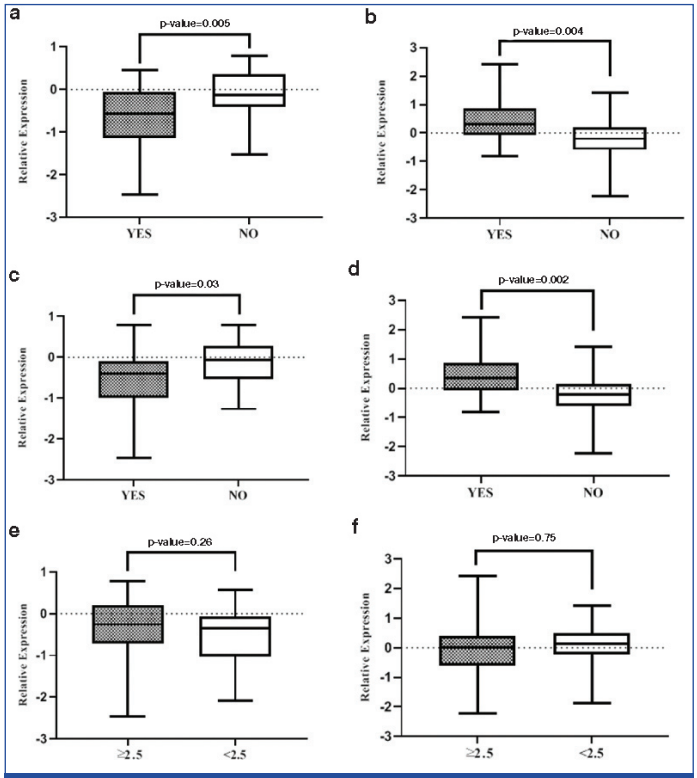
[Table/Fig-3]: The results of ROC curve analysis for evaluating the HNSCC diagnostic power of Fer1L4 expression levels, ROC.



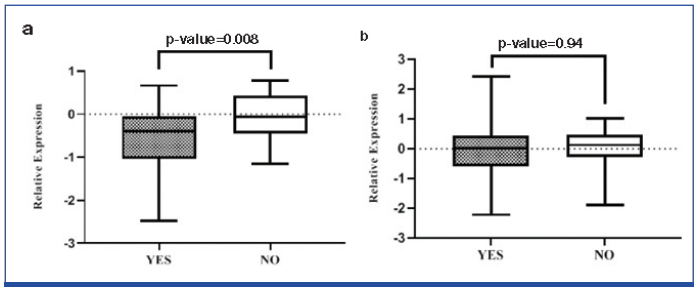
[Table/Fig-4]: The association between tumour stage and grade with expression level: (a) The expression of Fer1L4 is significantly lower in later than that the earlier stages; while (b) the expression of DANCR was not different between tumour stages; (c) The expression level of Fer1L4 is significantly reduced in tumour grades with poorly differentiated status; while (d) the expression of DANCR was not different between tumour stages.

lncRNAs and patients' stage and grade information. Statistically significant associations were found between the stage and grade status of the tumour and relative expression of Fer1L4 in all cancer samples (p-value=0.008 and p-value=0.002 for stage and grade, respectively). Contrarily, the association did not exist between DANCR expressions and patients' stage and grade (p-value=0.3 and p-value=0.4 for stage and grade, respectively). Furthermore, we evaluated the association between the characteristics of the Tumour, Node, and Metastasis (TNM) with the expression of two selected lncRNAs. Concerning metastasis, DANCR was over-expressed and Fer1L4 was down-regulated in metastatic tumours in comparison to non metastatic tumours (p-value for Fer1L4 and DANCR are, respectively, 0.005, 0.004,) [Table/Fig-5a,b]. The expression of DANCR was significantly higher in samples with lymph node invasion than of counterpart group; relevantly, Fer1L4 was significantly lower in samples with lymph node invasion (p-value for Fer1L4 and DANCR are, respectively, 0.03, 0.002) [Table/Fig-5c,d].

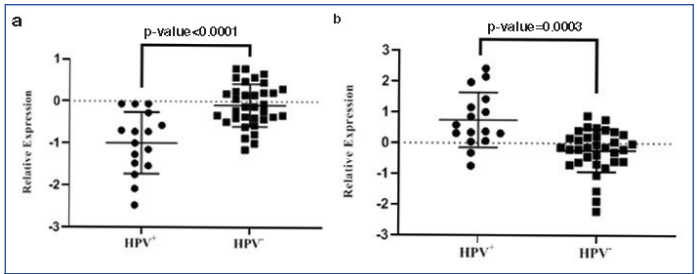
Of particular note, there was no association between tumour size (≥2.5 and <2.5 cm) and the expression of two selected lncRNAs [Table/Fig-5e,f]. Considering that the dynamics of gene expression is affected by tobacco smoking [20], the effect of this risk factor on the expression of the lncRNAs was assessed. Only, the expression of Fer1L4 was significantly lower in smoker than non-smoker patients (p-value=0.008) [Table/Fig-6]. We compared the expression level of Fer1L4 and DANCR genes between HPV+and HPV- groups to elucidate the putative effect of HPV status in patients on the expression of these lncRNAs. Results of the present study indicated that in the HPV+ group by analogy with HPV-, the DANCR and Fer1L4 were significantly over-expressed and significantly under-expressed, respectively (p-value for Fer1L4 and DANCR is <0.0001 and 0.0003, respectively) [Table/Fig-7].



[Table/Fig-5]: The association between the expression of (a) Fer1L4; and (b) DANCR with the occurrence of metastasis; (c) Fer1L4; and (d) DANCR with lymph node invasion; and (e) Fer1L4; and (f) DANCR with tumour size.

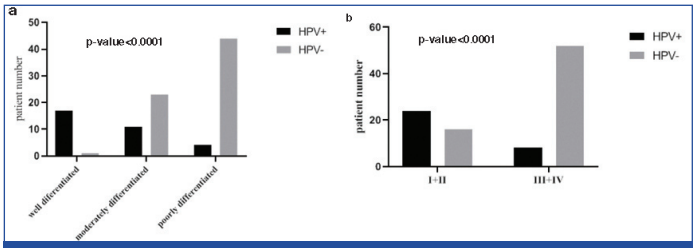


[Table/Fig-6]: The difference of expression of (a) Fer1L4; and (b) DANCR between smoker and non-smoker patients.



[Table/Fig-7]: The association between the gene expression and HPV status. (a) The expression of Fer1L4 was significantly lower in HPV+ group by analogy with HPV-. In contrast, (b) The expression of DANCR was significantly higher in HPV+ group by analogy with HPV-. HPV: Human papillomavirus.

As previously stated, HPV+HNSCC patients have improved clinical outcomes and better response to modality treatment; hence, we scrutinized the association between tumour grade and stage with HPV status. In agreement with previous studies, a positive association between HPV+ and low grade and low stage was found (p-value <0.0001 for both variables) [Table/Fig-8a,b].



[Table/Fig-8]: The association between HPV status and tumour grade and stage. (a) Most cases with low grade were HPV+ than that high grade group. (b) Most cases with low stage were HPV+ than that high stage group.
HPV: Human papillomavirus

DISCUSSION

Despite the major advances in diagnosis and therapy, alarmingly, up to 30% of HNSCC patients develop treatment failure and subsequent cancer relapse, and only 50% to 60% have a survival rate of 5 years after diagnosis, representing high morbidity and mortality of this malignancy [21]. The discovery that lncRNAs are linked to all six hallmarks of cancer has revolutionised the comprehension of molecular under-pinnings of tumourigenesis and stimulated the search for novel biomarkers and targeted therapy [22]. Thereby, in this work, the expression of Fer1L4, for the first time, and DANCR was evaluated in the HNSCCs.

The Fer1L4, in the present study, showed decreased expression in tumoural tissues compared with ANCTs [Table/Fig-2a] which is consistent with the well-established tumour suppressor activity of this lncRNA in most cancers. However, surprisingly, exploring the cancer genome atlas (TCGA) database indicated that Fer1L4 expression is higher in more advanced TNM stage of HNSCCs, suggesting that, most probably, Fer1L4 exerts different mechanisms in different stages of HNSCCs [23]. This result was in sharp contrast with the analysis of this study that indicated the lower expression of Fer1L4 in later than that the earlier stages [Table/Fig-4a]. We hypothesised that, plausibly, this lncRNA acts, as in many cancers, by regulation of PTEN/PI3K/AKT signaling pathway in earlier stages of tumourigenesis and in later stages via an unknown ceRNA mechanism. This is in conformity with the ceRNA system that unveiled a distinct mechanism of this lncRNA as an oncogene in glioma [24]. All in all, the existence of this controversy emanates from the paucity of data about Fer1L4 in HNSCCs and justifies more well-designed experiments. The result of constructed ROC manifested that Fer1L4 could be leveraged as a novel biomarker for diagnosis of HNSCC [Table/Fig-3] which is in keeping with a study on the ESCC [25]. The correlation between Fer1L4 expression and survival in cancers like kidney-renal clear cell carcinoma and bladder urothelial carcinoma is well established [26]. The patients of this study were not followed-up for overall survival and progression-free survival; but, the lower expression of Fer1L4 in metastatic and tumours with lymph node invasion informing a potential prognostic biomarker for HNSCC [Table/Fig-5a,c].

The existing evidence clearly suggests that evaluating the expression of DANCR in malignancies could be used as a biomarker for accurate diagnosis, prognostic assessments, and treatment response monitoring [27]. On the contrary, no difference was found in the expression of DANCR in tumoural tissues versus

ANCTs in the patients of this study [Table/Fig-2b]. This could be attributed to the low sample size of this study. Notwithstanding this discrepancy, the higher expression of DANCR, as a typical oncogenic lncRNA, in tumours with metastasis and lymph node invasion [Table/Fig-5b,d] mirrors that DANCR, at least partially, plays a pivotal role in the progression of HNSCC. Akin to other lncRNAs, DANCR is involved in lncRNA-MicroRNAs (miRNA)-mRNA regulatory networks, manifesting promising targets for early customised clinical interventions [28]. In the same vein, DANCR partakes in the progression and proliferation of specific HNSCC types like Oral Squamous Cell Carcinoma (OSCC) (through DANCR/miR-216a-5p/Bcl-2/KLF12 axis) [29] and Tongue Squamous Cell Carcinoma (TSCC) (through DANCR/miR-135a-5p/KLF8 axis) [30]. A 38,000 HNSCC cases per year are attributed to HPV [31]; relatedly, the expression of Fer1L4 and DANCR was different between HPV+and HPV- of patients [Table/Fig-7]. This reveals that these two lncRNAs would be an active participant in the mediation of HPV effect on HNSCC development.

Conventional cancer treatments such as surgical eradication, radiotherapy, and chemotherapy severely reduce the quality of life, and are, to some extent, largely ineffective [32]. The only FDA-approved targeted drug for HNSCCs is Cetuximab, a monoclonal antibody targeting epidermal growth factor receptor(EGFR) [33]. Limited efficacy of this drug has stimulated the researchers for identification of the novel targets; although, the heterogeneous nature of HNSCCs is the most important hurdle in this issue. The identification of lncRNAs as alternative therapeutic targets will be critical to improve the survival outcomes in individuals affected by this otherwise lethal disease. In lines, according to the results of this study, we propose that Fer1L4 and DANCR might be promising targets for the treatment of HNSCC.

Limitation(s)

It goes without saying that this work has some limitations. First, lncRNA expression studies are often strengthened by the support of functional assays; but, the function of Fer1L4 and DANCR at in-vitro or in animal models were not explored. Second, the sample size was too low to draw any firm conclusions about the diagnostic power of these two lncRNAs. Finally, the effect of alcohol consumption on the expression of Fer1L4 and DANCR was not evaluated. This is because in Iran, as its an Islamic country, many people deny alcohol drinking; thus, data of present study is not accurate in this regard.

CONCLUSION(S)

In this experiment, to the best of our knowledge, for the first time, the expression of Fer1L4 was evaluated in HNSCC. The findings pinpoint the recruitment of this lncRNA for the diagnosis of HNSCC. This is important on the account of improvement of current diagnostic workflow in terms of cost, feasibility, time. Besides, the association between the expression of Fer1L4 and DANCR with clinicopathological indices informs that dysregulation of these two lncRNAs is required for the development and progression of HNSCC tumours. As a correlate, restoration of the expression of Fer1L4 and DANCR should be envisaged as a novel therapeutic strategy in HNSCC. In aggregate, the present results pave the way for future studies, especially in the field of personalised medicine.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Nov 10, 2020
- Manual Googling: Feb 24, 2021
- iThenticate Software: Apr 07, 2021 (6%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Nov 09, 2020**
Date of Peer Review: **Dec 31, 2020**
Date of Acceptance: **Mar 17, 2021**
Date of Publishing: **Sep 01, 2021**