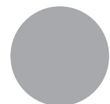
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SQUAMOUS CELL CARCINOMA;
miR-21 IN THE DETECTION OF HEAD AND NECK SQUAMOUS CELL CARCINOMA

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ABSTRACT… Background: Head and Neck Squamous cell carcinoma is the sixth most common cancer globally with increasing frequency in developing countries. Despite huge advancement in surgery, radiotherapy and chemotherapy there is a little changed in the overall survival rate for patient with HNSCC over the past few decades. Due to its late diagnosis and lack of availability of reliable biomarker for this disease, its incidence is still [illegible] rise. **Aims & Objectives:** This study was aimed to study the expression of miR[illegible] genesis of HNSCC. The objective of the study is to analyze the expr[illegible] of miR[illegible] in HNSCC, to study the miRNA expression profile of miR-21 b[illegible] control and tumor sam[illegible], to study the expression profile of miR-21 benign [illegible] different categori[illegible] HNSC[illegible] tumors on the basis of Histological Differ[illegible] gender-based Comp[illegible] ison [illegible] gn a[illegible] Malignant HNSCC Tumors, age-[illegible] comparison of Benign [illegible] M[illegible] ant [illegible] C[illegible] mors, [illegible] or Site-based Com[illegible] Benign and Malignant [illegible] Tu[illegible] s. **St[illegible] D[illegible] n:** C[illegible] control st[illegible] **[illegible] Setting:** The Universit[illegible] e. **P[illegible] d:** J[illegible] 14 [illegible] 05. **M[illegible] ials & [illegible] thods:** In this researc[illegible] Form[illegible] -fi[illegible] para[illegible] emb[illegible] ed (F[illegible]) [illegible] e sa[illegible] s (31 malignant HN[illegible] mple[illegible] 2 b[illegible] n tumors [illegible] the [illegible] both gen[illegible] and ag[illegible] y[illegible] w[illegible] in[illegible] t[illegible] 31 c[illegible] we[illegible] alignant tumors w[illegible] ther [illegible] of 1[illegible] mo[illegible] a[illegible] differ[illegible] ated tumors. Total R[illegible] xtracted using [illegible] Link [illegible] A [illegible] it [illegible] -Step RT-PCR wa[illegible]. TaqMan primer/ [illegible] be [illegible] were [illegible] th[illegible] rg[illegible] miRNA- 221, while [illegible] the normalization control. [illegible] y calc[illegible] ng ___ [illegible] fold change difference[illegible] to Livak method. late onset disease [illegible] e Rela[illegible] quan[illegible] cation was done [illegible] the level of expression of [illegible] 221. Tumor [illegible] id not show any effect [illegible] expression levels. **Results** [illegible] ur r[illegible] ults [illegible] owed that the malignant sam[illegible] higher expression level of [illegible] IR- [illegible] than [illegible] nign [illegible] samples. Significan[illegible] expression was observed [illegible] mo[illegible] ra[illegible] y a[illegible] poorly categories [illegible] f HNSCC. [illegible] expression showe[illegible] that females ha[illegible] higher le[illegible] l of expression, while it was [illegible] and that its expressio[illegible] s hig[illegible] late onset dis[illegible] se. Tu[illegible] or [illegible] e did not show any effect on miR-21 [illegible] pression le[illegible]. **Conclu[illegible] on:** [illegible] m[illegible] xpression profile provides a potential [illegible] rategy [illegible] finding new head and n[illegible] k squamous cell carcinoma (HNSCC) molecular targets. [illegible] R-21 co[illegible] d be [illegible] rded [illegible] potential diagnostic marker in HNSCC.

K[illegible] words: Head and Neck Squamous Cell Carcinoma (HNSCC), miR-221, Taqman Assay, Intraoral, Extra-oral.

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INTRODUCTION

Head and Neck cancer is increasingly becoming a critical public health issue in the world, correlated with high incidence and mortality rates mainly in developing countries.[1] Human papillomavirus (HPV) infection, alcohol and tobacco use are the major risk elements for this disease.[2] A full understanding of how these exposures effects cellular functions and the molecular basis for their risk remains evasive. For improving early diagnosis, predicting prognosis, and establishing effective therapeutics, knowledge of the molecular nature of HNSCC carcinogenesis is critical. Defining genetic biomarkers for HNSCC, several attempts have been made for at.[3]

Each year worldwide approximately 650,000 people are diagnosed with HNSCC, making it the eighth most common cancer. Remarkably, the incidence in men is more than twice that in

women.[4] In the United States, HNSCCs represent 3.1% of all incident malignancies with 35,720 new cases and 7,600 deaths expected in 2009. The overall 5-year survival rate for HNSCC is 60%.[5]

The majority of cervical cancers is caused by Human papillomavirus (HPV), particularly type 16, previously recognized as the oncogenic virus, has emerged as the cause of a distinct form of HNSCC, generally stirring in the oral cavity and oropharynx.[6] HPV is a circular DNA virus that can exist as an episome or integrate into the host genome.[7] The clinical and molecular characteristics of HPV-associated HNSCCs are disparate from those with alcohol and tobacco-related etiologies as they do not show the identical genetic and epigenetic alterations characteristic of HPV-negative tumors.[8] Remarkably HPV-positive HNSCC patients respond more favora[illegible] to treatment with cisplatin and [illegible] and display overall improved [illegible] compared to their HPV-11 neg[illegible]unterparts.[9]

M[illegible]s include a cate[illegible]og[illegible] sma[illegible] no[illegible]coding R[illegible] that [illegible]ve onl[illegible] been [illegible]co[illegible]re[illegible] wit[illegible] the l[illegible] c[illegible]ple [illegible]eca[illegible]s. S[illegible]e [illegible]ei[illegible] disc[illegible]very, a [illegible]ea[illegible] has bee[illegible] earr[illegible] about their [illegible]ogene[illegible] n[illegible] e [illegible] on in the genome and [illegible]eir inv[illegible]en[illegible]t in both comm[illegible] pathol[illegible]ical pr[illegible]cesses. The [illegible]NA, a 21-24 b[illegible] duplex, is [illegible] step-wise from a primary [illegible]00s-1000s of nucleotid[illegible]n length.[10]

The first research that [illegible]dicate[illegible] the relation between miRNA and human [illegible]ancers was in 2000. This study found miR-15 and miR-16-1 in the most commonly deleted region, 13q14, in CLL; subsequently, their frequent deletion or down-regulation has been detected in the majority of CLL cases.[11] miR-15 and miR-16-1 function as tumor suppressors, and their expression inversely correlates with anti-apoptotic BCL2 expression where inhibition of BCL2 by miR-15 and miR-16-1 enhance apoptosis in leukemic cells. Thus, somatic deletion of miR-15 and miR-16-1 facilitates leukaemogenesis as bypass apoptosis.[12]

MiRNA can change cellular behavior to a specific drug or class of drugs not only through survival or apoptotic signaling but also by DNA repair and interfering with drug targets.[13] A key methodology is to profile the mature miRNAs in specific tissue types at various disease stages.[14] For several reasons, however, miRNAs detection is technically challenging. Due to the short length of mature miRNAs, very little sequence is available to design complementary microarray and perform reliable amplification or labeling of each miRNA without leading signal bias.

There are a lesser number of studies investigating a role of miRNA in HNSCC. [illegible] first was a miRNA microarray [illegible] on ni[illegible]ndividual HNSCC [illegible] (Dillhoff et al., [illegible]8). The [illegible] of 33 miRNAs [illegible] term[illegible]d to be high and 22 lo[illegible] [illegible]ive [illegible] t[illegible] othe[illegible] iRNAs expressio[illegible] [illegible]ably [illegible] o[illegible] [illegible]ly e[illegible]essed m[illegible] wa[illegible] miR[illegible] a [illegible] co[illegible]monly u[illegible]gulated i[illegible] anc[illegible] An[illegible] udy in[illegible]ving mi[illegible] arr[illegible] of [illegible] no[illegible] mucosa, four [illegible] ary [illegible] C [illegible]sue[illegible] and four HNS[illegible] lines [illegible]e[illegible] differential ex[illegible] nine miRNAs, including ove[illegible] of miR-21, in tumor tissu[illegible]ally, this study fo[illegible] that miR-[illegible] overexpression in HN[illegible]CC cell[illegible] induced increased prolifer[illegible] and th[illegible] inh[illegible] of miR-21 increas[illegible]d a[illegible]op[illegible]sis [illegible]nd cy[illegible]chr[illegible] release. [illegible] third study [illegible]so [illegible]equ[illegible]ed to [illegible]etermine miRNA [illegible]ofile[illegible] of [illegible]NSCC, [illegible]oncluding that combined e[illegible]pression of miRNAs let-7d and miR-205 was a predictor of prognosis. Interestingly, though this study also found miR-21 to be expressed at consistently higher levels in HNSCC tumors compared to normal tissues, one of the findings on which its conclusion is based, namely that miR-205 is down-regulated in tumor, contradicts with the previous miRNA study which found miR-205 to be significantly overexpressed 16.

MATERIALS AND METHODS

A simple population based case-control study structure was selected. The study comprised of 43 subjects, 31 incident (2014-2015) cases of histopathologicaly confirmed head and neck squamous cell carcinoma (HNSCC), and 12 samples of benign lesions of head and neck

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region.

As per the study design sampling was done for two groups, malignant cases and benign controls. A total of 43 samples of HNSCC were collected in the form of FFPE tissue blocks collected and fixed after resection, 31 among these cases represented HNSCC of various sites in head and neck section, these 31 malignant samples further consisted of 14 well differentiated 11 moderately differentiated and 6 poorly differentiated tumors. As control specimens a total of 11 non-malignant tissue representatives were included in the study, these tissues were from the same regions as cases to minimize the bias in the study. Histopathologicaly, these were negative for any type of malignancy.

Study Variables

Study variables comprised of both Indepen[illegible] variables and dependent variables[illegible]

Independent Var[illegible]

Follo[illegible] [illegible]ables were select[illegible] [illegible]ent var[illegible]es:

Age
Gend[illegible]
Tumor [illegible]fferent[illegible]
Tumor [illegible]e

Depende[illegible] Variables

Dependen[illegible] were observed by [illegible] experimen[illegible]tion. Furthe[illegible]mor[illegible] secondary dependent variables we[illegible] calcula[illegible]d f[illegible] the observed values of primary [illegible]pende[illegible]t variables.

Cycle Threshold Value Ct

Ct values of target gene miR-21 were obtained along with the Ct values of reference gene (RNUB6), after obtaining these values for all experimental groups they were used in a mathematical model to calculate the Normalized Expression Ratio (NER) for the target genes.

METHODS

RNA Extraction

Further processing of FFPE tissue blocks was done and total RNA was extracted from them. RNA extraction was done by using the Pure Link FFPE RNA Isolation Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions with some minor changes. Detailed procedure is described below:

Deparafinization

- 10-15 parts of 10 μm sections of FFPE tissue samples were taken into a sterile, RNAse-free 1.5 ml micro-centrifuge tube. Tissue was deparafinized by addition of 300 μL melting buffer to specimens and incubating at 72ºC for 10 minutes, with intermittent mild mixing every 2-3 minutes by tapping the tube. After this incubation a quick spin was [illegible]ven to the tube to collect all the li[illegible] [illegible] the [illegible]ottom of tube.
- The t[illegible]taining the tissue was [illegible]ded by [illegible] μL Proteinase K an[illegible] mi[illegible]ll by [illegible]etting up and do[illegible] [illegible]ure [illegible] t[illegible] issu[illegible] well s[illegible] the [illegible].

Tis[illegible] Digesti[illegible]

A[illegible]te[illegible] w[illegible]ng a[illegible] ad[illegible] of Proteinase [illegible]e [illegible]tu[illegible] w[illegible] incubated at 65ºC fo[illegible]ight. This s[illegible] was an extra addi[illegible] manufacturer's protocol. The [illegible] step has been reported to pr[illegible]ter amplification resu[illegible]eal-time [illegible]CR (Abramovitz et al., 20[illegible]).

Binding an[illegible] wa[illegible]hi[illegible]g o[illegible] RNA

[illegible] the followi[illegible] ste[illegible] w[illegible]re pe[illegible]ormed at room te[illegible]per[illegible]ure.

- 400 μL binding buffer and 800 μL of 100% ethanol was added to the sample after over night incubation and was mixed well by vortexing.
- The sample from above step was added to the spin cartridge inserted to a collection tube provided with the kit, to bind total RNA with the column.
- 3 washings in total were given with 500 μL of wash buffer provided with the kit. This step washed away the high and low molecular weight proteins.

Elution of RNA

The washed column was processed to elute the total RNA adsorbed to the column membrane.

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This can be done by breaking the adsorption, 50 μL RNAse-free water was applied to the column which was pre-heated at 65ºC, and centrifuged at maximum speed to collect the total RNA in a 1.5 mL elution tube.

Analyzing RNA Yield

RNA yield was analyzed with the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA), using The Qubit® RNA Assay Kit.

Reverse transcription

The recovered miRNA out of the total RNA isolated was reverse transcribed using specified primers provided by the Applied Biosystems® using TaqMan® MicroRNA Reverse Transcription Kit for miR-21 while RNUB6 was taken as a normalization control taken as reference for the normalization of data.

Real-time PCR

Real time PCR was perform[illegible]n Bio-Rad's CFX96 ™ Real-Time P[illegible]ection System usi[illegible] AgPath-ID™ [illegible] RT-PCR Kit. P[illegible]rob[illegible] set[illegible]used for target g[illegible] [illegible]2 whi[illegible] R[illegible]B6 was taken as [illegible]rmal[illegible]ion [illegible]

Pri[illegible]r/Prob[illegible]e[illegible]

Prim[illegible]probe[illegible] were, [illegible]qMan$^®$ Micr[illegible]NA As[illegible]s [illegible]plied Biosystem[illegible] while [illegible]e Rep[illegible]r/Quencher used [illegible]AM/ MGB-N[illegible]Q. Assay details of [illegible]er sets used is illustr[illegible]ed in Tab[illegible]

RESULTS

3.1 Descriptive Statistics

In the present study, expre[illegible]ion le[illegible]ls of miR-21 were studied in 31 tumor sa[illegible]ples from head and neck squamous cell carcinoma (HNSCC) patients and compared with 12 samples constituting benign lesions of head and neck origin. The age range of study participants was 15-80 years. The benign group includes 5 males (41.66%) and 7 females (58.33%) with the mean age ±SD 29.08 ±1.36. Among 31 HNSCC patients there were 23 males (74.19%) and 8 females (25.80%). Mean age ±SD for HNSCC patients was 51.64 ±1.31.

Comparison of Benign Tumors and Different Categories of HNSCC Tumors On the Basis of Histological Differentiation

miRNA expression profile of miR-21 among different histopathological categories of HNSCC. The bars represent fold change (FC) between Well, Moderately, and Poorly Differentiated HNSCC and benign tumors. FC values were calculated using normalized expression ratios by first calculating ΔΔCt.

Gender-based Comparison of Benign and Malignant HNSCC Tumors

Gender-based gene expression profile of miR-21 among benign and malignant HNSCC. The bars represent fold change (F[illegible]) [illegible]een male benign and male malig[illegible] [illegible] fem[illegible]e benign and female mal[illegible]t categories.

[illegible] based com[illegible] [illegible] ben[illegible] and malignant [illegible]mors

Sc[illegible]s c[illegible]arc[illegible] is [illegible]red a[illegible]ease o[illegible] ho[illegible]er, [illegible] to g[illegible]edis[illegible]ition it [illegible] occur [illegible] e[illegible]. [illegible] our stu[illegible] we [illegible] t[illegible] miR[illegible] expression profile [illegible]21 [illegible]sed differences in [illegible] patients, [illegible] categorized ou[illegible] of malignant and benign tu[illegible] two categories i.e. patients [illegible] more than or equal [illegible] 40 years are those having less [illegible] 40 [illegible]rs. [illegible]ed them early onse[illegible] la[illegible] o[illegible]et tum[illegible]rs, resp[illegible]tively.

[illegible]ge-based [illegible]ene [illegible]ssion profile of miR-21 among ben[illegible]d malignant HNSCC. The bars r[illegible]resent fold change (FC) between patients having less than 40 years and greater than 40 years of age.

Tumor Site-based Comparison of Benign and Malignant HNSCC Tumors

In order to inquire the differences in expression profile on the basis of anatomical localization of the tumor, we have divided our samples into two categories i.e. extra oral neoplasm and intra oral neoplasm. Our result did not show a differential expression profile of miR-21 in extra and intra oral malignant is compared to benign tumors.

	Benign		Malignant	
N	12		31	
Mean Age±SD	29.08 ± 1.36		51.64 ±1.31	
Gender (%)	Male	Female	Male	Female
	5(41.66%)	7(58.33%)	23(74.19%)	8(25.80%)

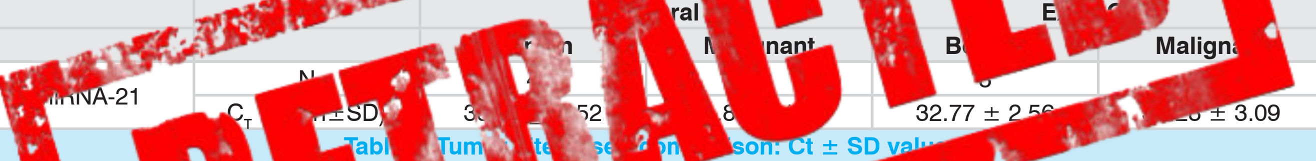
Table-I. Descriptive statistics

		Benign	Malignant		
			Well	Moderately	Poorly
miRNA21	N	12	11	14	6
	C_T (Mean±SD)	33.15 ± 2.50	32.68±1.17	31.02±3.10	30.30±2.73

Table-II. Comparison on basis of histological differentiation: ΔCt SD values

		Male		Female	
		Benign	Malignant	Benign	Malignant
miRNA-21	N	5	23	7	**8**
	C_T (Mean±SD)	33.31 ± 2.50	31.50 ± 2.46	33.02 ± 2.27	31.98±2.12

Table-III. Gender based comparison of benign and malignant HNSCC tumors



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		< 40		[illegible] Above	
		Benign	Malignant	Benign	Malignant
miRNA-21	N	9	[illegible]	3	27
	C_T (Mean±SD)	33.08 ± [illegible]	32.89 ± 0.69	[illegible]	[illegible]44 ± [illegible]

Table-IV

		[illegible]ral		E[illegible]	
		[illegible]	Malignant	B[illegible]	Maligna[illegible]
miRNA-21	N	[illegible]	[illegible]	[illegible]	[illegible]
	C_T (Mean±SD)	3[illegible]52	[illegible]8[illegible]	32.77 ± 2.56	[illegible] ± 3.09

Tabl[illegible] Tum[illegible] [illegible]son: Ct ± SD valu[illegible]

Figure-A

Figure-B

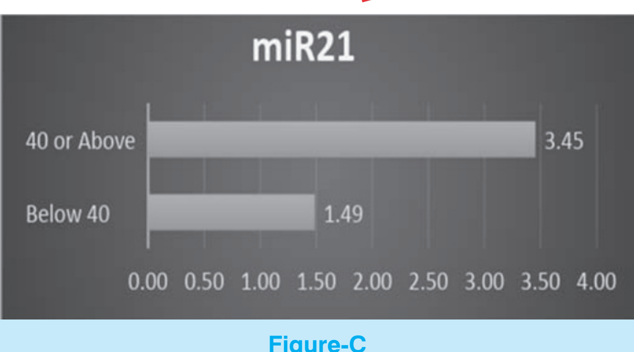

Figure-C

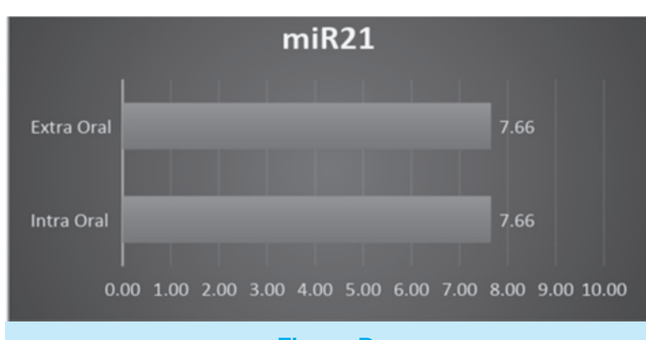

Figure-D

DISCUSSION

Head and Neck cancer is increasingly becoming a very severe public health issue globally, correlated with high incidence and mortality rates mainly in developing countries (Jefferies and Foulkes, 2001). Human papillomavirus (HPV) infection, alcohol and tobacco use are the major risk factors for this disease (Pai and Westra, 2009). In the discovery of new biomarker for HNSCC, we studied the expression profile of miRNA-21, as a candidate biomarker of this type of cancer. miRNA expression profile as a well known method for the identification of a potential biomarker for various cancers. To studied miRNA expression, required extracted RNA from different sources, including direct tumors samples from patients in the form of fresh frozen tissue or formalin fixed paraffin embedded tissues (FFPE). Representative tissue sections were obtained from Paraffin-embedded blocks and the histologic diagnosis were confirmed by an experienced pathologist. The malig[illegible] lesions were classified into the [illegible] of well differentiated, mod[illegible] differentiated and poorly diff[illegible] following the W[illegible] classifi[illegible]. The result sho[illegible] w[illegible] p[illegible]ous studies in HN[illegible]re [illegible] sho[illegible] h[illegible] expressi[illegible] tu[illegible]s as c[illegible]pare[illegible] m[illegible] tis[illegible]e.[16] Mi[illegible]s [illegible]can[illegible] upre[illegible]la[illegible] HN[illegible]C is s[illegible]y [illegible]ntitati[illegible]eal[illegible]e PCR ana[illegible]is.[17] M[illegible]R[illegible] p[illegible] of [illegible]east, cervical and [illegible]arian t[illegible]o[illegible] glioblastomas; a[illegible] and n[illegible]k prim[illegible] tumors and [illegible], amongst others, [illegible]as shown th[illegible] is commonly up-regulate[illegible] in [illegible]There are several targ[illegible] of miR-21 [illegible] has been e[illegible]perim[illegible]tally validat[illegible]; many of them are tumo[illegible]ppres[illegible]r ge[illegible].[19]

Our results showed that poorly differentiated HNSCC had the highest expression of MiR-21 when compared with benign tumors of same region. Furthermore there was trend of increasing expression with decreasing order of differentiation. In such a way that among the three categories of malignant neoplasms, well differentiated tumors had the lowest expression, then moderately and then poorly differentiated categories. This result might reflect toward the fact that expression levels of miR-21 may be correlated with the loss of differentiation and hence could be considered a marker of aggressive tumor behavior. According to our results, miR-21 showed gradually increasing expression level in decreasing order of differentiation in malignant carcinoma of head and neck region. In our results poorly differentiated HNSCC has the highest miR-21 expression as compared to the benign tumors of the same region.

One of the study reported that tumors regressed completely in few days when miR-21 was inactivated, there result demonstrate that tumor can be addicted to oncomiRs, and this study emphasizes the absolute dependence of at least some cancers on miR-21 for maintenance of the malignant phenotype. Oncogene addiction of some tumors has allowed [illegible]de[illegible]opment of targeted therape[illegible]dalities tha[illegible]rofoundly benefit [illegible]atients.[20]

The result sho[illegible]arit[illegible]it[illegible]revio[illegible] studies in HNSC[illegible]ere [illegible]-21 [illegible]w[illegible]gh e[illegible]ession i[illegible]s as[illegible]mp[illegible] to [illegible]rn[illegible] tissu[illegible] MiR-2[illegible]was sign[illegible]ntly[illegible]reg[illegible]n HN[illegible]C is s[illegible]n [illegible]qu[illegible]tativ[illegible] time PCR an[illegible]is.[21] Mi[illegible]R[illegible] pr[illegible]ng of breast, [illegible], and [illegible]va[illegible]umors; gliobl[illegible], and head and neck primary [illegible] and cell lines, amongst othe[illegible]own that miR-21 is [illegible]monly up-[illegible]ated in cancer.[18] There [illegible]re s[illegible]vera[illegible] targets of miR-21 which has [illegible]e[illegible] e[illegible]pe[illegible]men[illegible] validated; many of [illegible]em [illegible]re [illegible]um[illegible] suppresso[illegible]es.[19]

CON[illegible]US[illegible]NS

[illegible]e concluded that this marker have the potential to be applied as diagnostic biomarkers of HNSCC. We We additionally conclude, by looking at the expression profile in well, moderately and poorly differentiated categories of HNSCC, that both of this miRNA could possibly be associated with disease aggressiveness. Gender-based comparison showed that miRNA expression program in female HNSCC patients is completely different than in male HNSCC patients. The differential regulation of our studied genes in late and early onset disease showed that they are much more implicated in the pathogenesis of late-onset disease. Also, our results concluded

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that this miRNA could not differentially expressed between extra-oral HNSCC and intra-oral carcinomas.

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