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Mir-221 in the Detection of Head and Neck Squamous Cell Carcinoma

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Abstract: *Head and Neck Squamous cell carcinoma is the sixth most common cancer worldwide with increasing incidence in developing countries. Due to its late diagnosis and lack of availability of reliable biomarker for this disease, its incidence is still on rise.*

Aims & Objectives: *The aim of this study was to characterize the role of miR-221 in the tumor genesis of Head and neck squamous cell carcinoma (HNSCC). The objective of the study was to analyze the expression profile of miR-221 in HNSCC.*

Materials & Methods*: 43 Formalin-fixed paraffin embedded (FFPE) tissue samples (31 malignant HNSCC samples and 12 benign tumors from the same region) of both genders and aged 15-80 years were included in this study. 31 cases were malignant tumors were further consisted of 14 well-, 11 moderately- and 6 poorly-differentiated tumors. Total RNA was extracted using PureLink FFPE RNA Isolation Kit and Two- Step RT-PCR was performed. TaqMan primer/probe sets were used for the target miRNA- 221, while RNUB6 was the normalization control. Relative quantification was done to determine the level of expression of miRNA-221 by calculating* $\Delta\Delta Ct$ *and fold change difference according to Livak method.*

Results: *showed that the expression level of miR-221 is higher in malignant samples than benign control samples. Significantly higher expression was observed in moderately and poorly differentiated tumor categories of HNSCC. Gender-based expression comparison showed that females had higher level of expression, while it was found that its expression is high in late onset disease.*

Conclusion: *Our miRNA expression Profile provides a potential strategy for finding new head and neck squamous cell carcinoma (HNSCC) molecular targets. miR-221 could be regarded as potential diagnostic marker in HNSCC. Also, our results concluded that this miRNA does not show any differential expression between intra-oral and extra-oral sites of HNSCC.*

Keywords: *Head and neck squamous cell carcinoma, miR-221, Taqman assay, intra oral, extra-oral.*

I. Introduction

Head and neck squamous cell carcinoma is the sixth most common non-skin cancer worldwide (Matayoshi et al., 2013). It includes the carcinomas arising from the epithelium of the oral cavity, pharynx and larynx. The squamous cell carcinoma is the most common histological type, which occurs in the oral cavity, oropharynx, hypo pharynx, and larynx. Oral cavity and pharyngeal cancers are composed of the following tumor sites and associated International Classification of Disease codes: base of tongue (C01), unspecified or other areas of tongue (C02), gum (C03), floor of mouth (C04), palate (C05), unspecified and other areas of mouth (C06), tonsil (C09), oropharynx (C10), pyriform sinus (C12), hypopharynx (C13), and other and poorly-defined sites on lip, or in the oral cavity and pharynx (C14) (3).

Malignant neoplasms of the larynx are classified as follows: glottis (C32.0), supraglottis (C32.1), sub glottis (C32.2), laryngeal cartilages (C32.3), and unspecified and other sites of the larynx (C32.9, 33) (Pai and Westra, 2009). Hence, the term 'head and neck squamous cell carcinoma' (HNSCC) is frequently used to connote squamous cell carcinomas involving these anatomical sites (Ragin et al., 2007). After initiation, the disease seems to progress through a number of clinical and histopathological changes governed by specific genetic and epigenetic events including large chromosomal aberrations, gross genomic alterations, genome-wide cytosine hypomethylation and gene promoter specific hypermethylation causing inactivation of tumor suppressor genes or activation of proto-oncogene (Hasegawa et al., 2002). The major risk factor for the disease is tobacco and alcohol use, and the human papillomavirus infection (Avissar et al., 2009a). Each year, approximately 50,000 new patients are confirmed as having HNSCC, and patients are usually diagnosed at around 60 years of age. [1]

Incidence and Prevalence

In 2000 Head and Neck cancer was ranked as the eighth leading cause of cancer death worldwide. Due to this disease, approximately 481,100 new cases developed and 320,000 persons died, resulting in an average mortality rate of 7.3 and 3.2 per 100,000 males and females, respectively, and an average incidence rate of 8.8 and 5.1 per 100,000 males and females, individually.[2]

Pathogenesis of Head and Neck Sequamous cell Carcinoma

In oral carcinogenesis several factors are involved, such as age, gender, ethnicity, lifestyle, genetic background, status of health and exposure to one or more oncogenic factors (Llewellyn et al., 2004). For oral cancer, tobacco smoking and alcohol consumption have been well documented as major risk factor in several epidemiologic studies with dogmatic fraction of approximately 90% (Castellsague et al., 2004). However, 15-20% HNC have no known tobacco or alcohol exposure. [3]

Epigenetics

The field of epigenetics is concerned with heritable control of the genome that does not involve changes to the underlying DNA sequence (Turner, 2002).DNA methylation is one of the epigenetic mechanism that has been extensively researched. Via spontaneous deamination of cytosine to uracil over the course of evolution due to their hyper mutability, CpG dinucleotides are relatively exiguous in the genome overall but have a specific preponderance 5 in promoter regions. For the loss of tumor suppressor function promoter CpG island hypermethylation is now understood to be as important a mechanism as somatic mutation, as in the case of CDKN2A. [4]

Histone modification works in concert with DNA methylation to regulate transcription through various post-translational modifications on histones which alter their affinity for DNA, thereby forming either compact inactive chromatin or relaxed transcriptionally active chromatin. While these mechanisms act at the level of transcription, small regulatory RNA molecules act at the level of translation. Several of these small non-coding RNA species have been identified including microRNA (miRNA), small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA). [5]

Micro RNA

Micro RNAs (miRNAs) are the recently discovered class of small non coding RNAs, which regulate gene expression by binding to the expressed mRNAs at the 3'untranslated region (3'UTR) resulting in decreased protein expression either by translational repression or by mRNA degradation enhancement. Micro RNAs have variety of regulatory function and play a role in cancer initiation and progression controlling(Garzon et al., 2009). The mature miRNA, a 21-24 bp duplex, is processed step-wise from a primary transcript of 100s-1000s of nucleotides in length. [6]

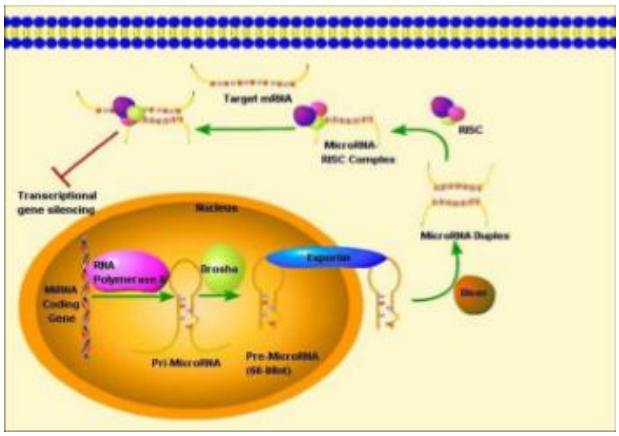

Figure 1.1: Biogenesis of MicroRNAs.

MicroRNAs in Cancer

The first corroboration of a connection between miRNA and human cancers came from findings in chronic lymphocytic leukemia. The miRNA genes that are located within regions of loss of heterozygosity, amplification, fragile sites, are more than 50%. Viral integration sites, and other cancer-related genomic regions (Mirnezami et al., 2009). Given that each miRNA has the potential to target a large number of mRNAs, any variations in miRNA expression level, disturbances in the processing of its precursors, or changes in the target sequence may deregulate normal cell processes (Calin et al., 2002). miRNAs act by repressing gene expression

through direct base-pairing interactions with their target mRNAs; there are several possible mechanisms through which miRNAs could affect tumorigenesis. Over expression, amplification, or loss of epigenetic silencing of a gene encoding a miRNA that targets one or more tumor suppressor genes could inhibit the activity of an anti-oncogenic pathway. By contrast, the physical deletion or epigenetic silencing of an miRNA that normally represses expression of one or more oncogenes might lead to increased protein expression and a gain of oncogenic potency (Ventura and Jacks, 2009). The first connection between miRNAs and cancer was established, after the discovery of mir-15 and mir-16, normally located in the chromosomal region 13q14, which were found to be deleted in more than half of the chronic lymphocytic leukemia cases. [7]

MicroRNAs in Head and Neck Squamous cell Carcinoma

There are a few numbers of studies investigating a role of miRNA in HNSCC. miRNA microarray performed on nine individual HNSCC cell lines was one of the first (Dillhoff et al., 2008). Relative to the expression of other miRNAs the expression of 33 miRNAs was determined to be high and 22 low. Markedly, one of the highly expressed miRNAs was miR- 21, a miRNA commonly up-regulated in cancer (Chang et al., 2008). In the detection of metastatic disease miRNAs seem to hold significant potential as a diagnostic tool. Abundantly expressed in HNSCC cells, miR-205 serves as a specific biomarker that could be used to detect cervical lymph node metastases from HNSCC, including OSCC sensitively and accurately (Fletcher et al., 2008). Analyzed miRNA expression profiles of HNSCC of the tonsil, base of tongue and post-nasal space, as well as their corresponding metastatic lymph nodes. The authors documented that each of the three primary sites maintained a distinct and specific miRNA expression profile, and that the miRNA expression profiles between primary cancer and its nodal metastatic disease were consistent (Barker et al., 2009), indicating that miRNA expression analysis has future utility as a diagnostic tool to establish whether the nodal metastasis is from the oral cavity, especially when the primary tumor cannot be identified on the basis of tumor biopsy samples. More excitingly, miRNA based classifier has been demonstrated to be more accurate than mRNA-based classifier to diagnose metastatic cancer of unknown primary origin. [8]

The aim of this study was to analyze the expression profile of miR-221 in HNSCC and compare it with benign lesions of the same region. Comparison of benign tumors and different categories of HNSCC tumors on the basis of histological differentiation. Comparison of benign tumors and malignant tumors on the basis of gender, age andsite of tumor.

II. Material and Methods

Study Design & Settings

A simple population based case-control study structure was selected. The study comprised of 43 subjects, 31 incident (2010-2013) cases of histopathologicaly confirmed head and neck squamous cell carcinoma (HNSCC), and 12 samples of benign lesions of head and neck region. The cases were identified from four medical facilities in Lahore viz: Services Institute of Medical Sciences, King Edward Medical University (Mayo Hospital), Ittefaq Hospital (Trust) and Post Graduate Medical Institute, Lahore, Pakistan.

After the consent of participating hospitals, the samples, in the form of formalin fixed paraffin embedded blocks (FFPE), were collected from the histopathology lab archives of each medical facility. The personal details of the patients were kept anonymous, and the cases were recruited against lab record number or archive number. Detailed procedural information, such as grossing examination detail, diagnosis, site of occurrence etc. was collected from respective hospital's database.

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 region, 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 23 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 comprised of both Independent variables and dependent variables. Independent Variables Age, Gender, Tumor Differentiation, Tumor Site were selected. Dependent variables were observed by the experimentation. Furthermore, secondary dependent variables were calculated from the observed values of primary dependent variables.

Cycle Threshold Value Ct

Ct values of target gene miR-221 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.

RNA Extraction

FFPE tissue blocks were further processed and total RNA was extracted from them. RNA extraction was done by using the PureLink 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 pieces of 10µm 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 gentle mixing every 2-3 minutes by tapping the tube. After this incubation a short spin was given to the tube to collect all the liquid at the bottom of tube.

20 µL Proteinase K was added to the tube containing the tissue, and mixed well by pipetting up and down to ensure that the tissue is well suspended in the liquid.

Tissue Digestion

After dewaxing and addition of Proteinase K, the mixture was incubated at 65ºC for overnight. This step was an extra addition to the manufacturer's protocol. The additional step has been reported to produce better amplification results in QRT-PCR. [9]

Binding and washing of RNA

All the following steps were performed at room temperature.

400 µL binding buffer and 800 µL of 100\% ethanol were added to the sample after overnight 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. A total of 3 washings 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. 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-221 while RNUB6 was taken as a normalization control taken as reference for the normalization of data.

Real-time PCR

Real time PCR was performed in Bio-Rad's CFX96 ™ Real-Time PCR Detection System using RT-PCR Kit. Primer/probe sets were used for target gene i.e. miR-221 while RNUB6 was taken as a normalization control.

Primer/Probe Sets

Primer/probe sets used were, TaqMan$^®$ MicroRNA Assays from Applied Biosystems®, while the Reporter/Quencher used were FAM/MGB-NFQ. Assay details of the primer sets used is illustrated in Table 1.1.

Table1.1: Assay Details of the Primer Sets Used

Gene Symbol	Type	Reporter/Quencher	Assay ID	Amplicon Size
miR-221	Target	FAM/MGB-NFQ	000524	76
RNUB6	Reference	FAM/MGB-NFQ	001093	64

Assay Protocol

Two-step reagents were used to amplify RNA samples with real-time amplification i.e. first reverse transcription was performed then the produced cDNA was preceded for real time amplification using TaqMan®

Universal PCR Master Mix along with the TaqMan® MicroRNA assays. 50 ng of total RNA input was used in every reaction of reverse transcription, incubating at 16ºC and 42ºC both for 30 minutes and then at 85ºC for 5 minutes. 10µl of produced cDNA was further used in amplification reaction. Cycling conditions used for QRT-PCR were as fallowing: 95ºC, 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. A schematic diagram of these cycling conditions is shown in Figure 1.2.

Step 1: First of all master mix was prepared, which included preparation of a cocktail including amplification reagents for both reverse transcription and chain polymerization separately in two reactions.

Step 2: Even distribution of master-mix to each reaction well and addition of cDNA samples.

Step 3: QRT-PCR Amplification.

In order to minimize errors of sample quantification and to correct for sample-to-sample variation in RT- PCR efficiency, housekeeping genes were used. Housekeeping genes are constitutive genes that are expressed in all cells of an organism under normal and pathophysiological conditions. In this study, RNUB6 was used as housekeeping gene as described in the Table 1.1.

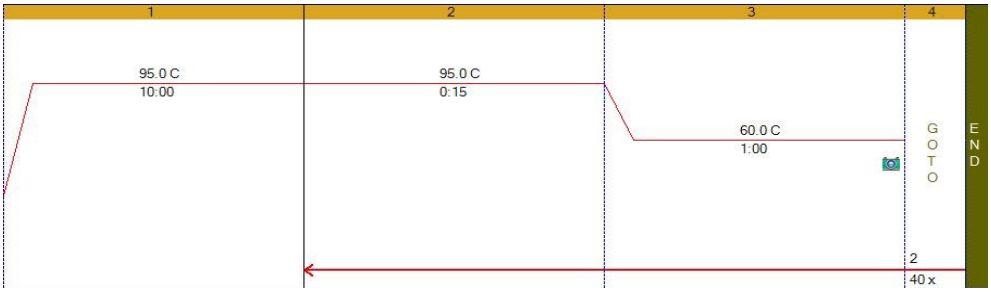

Figure 2.1: Schematic diagram of cycling conditions for QRT-PCR.

Analysis of qRT-PCR results

Relative quantification

Relative quantification or comparative quantification measures the relative change in mRNA expression levels. It determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of another RNA. The analysis result is a ratio: the relative amount (fold difference) of a target nucleic acid for equivalent amounts of control sample. The idea is to compare equal amounts of samples.

Mathematical model

To determine the level of expression, the difference (Δ) between Ct values was measured. The relative expression of a gene of interest (GOI) in relation to another gene, mostly to an adequate reference gene can be calculated on the basis of 'delta delta Ct' (ΔΔ Ct) values. [10]

The Livak Method (2− □ □*C t*)

The relative difference in expression level of target gene in different samples was determined using the steps below:

Firstly, the Ct of the target gene was normalized to that of the reference (ref) gene, for both the test sample (malignant) and the calibrator sample (benign).

Δ*Ct* (*test*) =*Ct* (*target, test*)-*Ct* (*ref, test*)

Δ*Ct* (*calibrator*) =*Ct* (*target, calibrator*)-*Ct* (*ref, calibrator*)

Secondly, the Δ Ct of the test sample (malignant) was normalized to the ΔCt of the calibrator (benign).

ΔΔ*Ct*=Δ*Ct* (*test*)-Δ*Ct* (*calibrator*)

Finally, the expression ratio was calculated:

Normalized expression ratio=2−ΔΔ*Ct*

The result obtained is the fold increase (or decrease) of the target gene in the test sample relative to the calibrator sample and is normalized to the expression of a reference gene. Normalizing the expression of the target gene to that of the reference gene compensates for any difference in the amount of sample tissue.

III. Results and Discussion

Descriptive Statistics

In this study, expression level of miR-221 was studied in 31 tumor samples from head and neck squamous cell carcinoma (HNSCC) patients and compared with12 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 23males (74.19%) and 8 females (25.80%). Mean age ±SD for HNSCC patients was 51.64 ±1.31.

Table 3.1: Descriptive statistics.

	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 3.2: Study Demographics

Group	Sr.#		Gender	Age	Site	Tumor Differentiation
Benign	1		Female	20	Left side mandible	Benign
	2		Female	15	Nasal polyp	Benign
	3		Female	30	Nasal polyp	Benign
	4		Female	40	Nasal polyp	Benign
	5		Female	18	Right Maxilla	Benign
	6		Female	40	Thyroid Adenoma	Benign
	7		Female	62	Thyroid Gland	Benign
	8		Male	22	Ameloblastoma	Benign
	9		Male	19	Nasal polyp	Benign
	10		Male	25	Nasal polyp	Benign
	11		Male	38	Right Mandible	Benign
	12		Male	20	Thyroid Tissue	Benign
	Total	N	12	12	12	12
Malignant	1		Female	45	Maxillary sinus	Well Differentiated
	2		Female	50	Tongue	Well Differentiated
	3		Female	50	Tongue	Well Differentiated
	4		Male	65	Laryngectomy	Well Differentiated
	5		Male	30	Larynx	Well Differentiated
	6		Male	42	Buccal Mucosa	Well Differentiated
	7		Male	65	Buccal Mucosa	Well Differentiated
	8		Male	35	Lower lip	Well Differentiated
	9		Male	48	Right Vocalcord	Well Differentiated
	10		Male	75	Scalp	Well Differentiated
	11		Male	45	Supra glotic mass	Well Differentiated
	12		Male	55	Supraglottic	Well Differentiated
	13		Male	35	Tongue	Well Differentiated
	14		Male	55	Tongue	Well Differentiated
	15		Female	50	Bucal Mucosa	Moderately Differentiated
	16		Female	80	Salivary Glands	Moderately Differentiated
	17		Female	28	Scalp	Moderately Differentiated
	18		Female	46	Toungue	Moderately Differentiated
	19		Male	68	Floor of Mouth	Moderately Differentiated
	20		Male	55	Larynx	Moderately Differentiated
	21		Male	55	Layrengectomy	Moderately Differentiated
	22		Male	45	Left Pyriform Fossa	Moderately Differentiated
	23		Male	40	Scalp biopsy	Moderately Differentiated
	24		Male	45	Tongue	Moderately Differentiated
	25		Male	40	Tongue	Moderately Differentiated
	26		Male	58	Larynx	Poorly Differentiated
	27		Male	74	Larynx	Poorly Differentiated
	28		Male	64	Maxilla	Poorly Differentiated
	29		Male	45	Tongue	Poorly Differentiated
	30		Male	67	Tongue	Poorly Differentiated
	31		Female	46	Buccal Mucosa	Poorly Differentiated
	Total	N	31	31	31	31
Grand Total		N	43	43	43	43

Gene Expression Profile of miR-221 in HNSCC Patients

The basic goal of this study was to find out the expression level of miR-221 in HNSCC samples and compare theses values with those of benign lesions. According to histopathological differentiation, tumor samples were divided into three categories, i.e., well differentiated, moderately differentiated and poor differentiated tumors. For the analysis of miRNA expression level of miR-221 in both cancerous and benign lesions, we have chosen the technique of real-time qRT-PCR, using TaqMan assays. Comparison of miRNA expression levels has also been done according to different parameters, such as, on the basis of gender, age, and site of the tumor (intra-oral or extra-oral). Fold change was calculated between malignant and benign tumors by Livak method (ref).

Comparison of miRNA Expression Profile of mir-221 between Patients having benign and Malignant HNSCC Tumors

As a first step, we sought to measure the difference in the expression profile of miR-221 in benign and malignant tumor samples. For this we calculated the ΔCt values by taking the differences in Ct values of target gene with reference gene in both benign and malignant samples, independently (table). Later, ΔΔCt was calculated by taking the difference in normalized ΔCt values of benign and malignant samples. Finally, the normalized expression values (NER) or fold change (FC) was calculated by using ΔΔCt values, as described in Materials and methods (Chapter 2). Table 3.3 shows the mean ± SD Ct values of benign and malignant tumor sample for miR-221. As shown in Figure3.1 the expression level of miR- 221 is higher in patients with malignant tumor with a fold change of 3.90, as compared to patients having benign tumors.

Table 3.3: Gene Expression Profile miRNA-221: Ct + SD values

		Benign	Malignant
miRNA-221	**N**	12	31
	C_T (Mean ± SD)	32.05 ± 3.84	30.77 ± 2.47

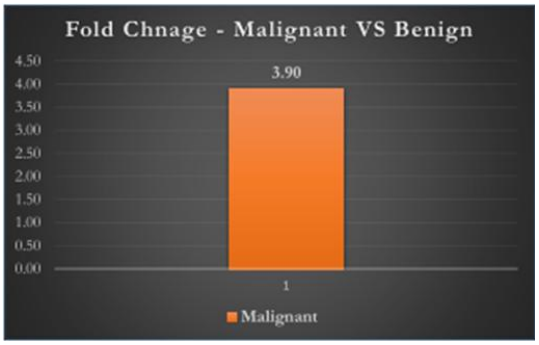

Figure 3.1. miRNA expression profile of miR-221.

Comparison of benign Tumors and different Categories of HNSCC Tumors On the basis of Histological Differentiation

On the basis of histological differentiation, malignant HNSCC tumors were categorized as well differentiated, moderately differentiated and poorly differentiated. According to our results, mir-221 showed gradually increasing expression levels in decreasing order of differentiation in malignant carcinomas of head and neck origin. In this regard, poorly differentiated neoplasms have been found to have high expression of miR-221, with a fold change of 7.35 as compared to patients having benign lesions of head and neck region, whereas, moderately differentiated and well differentiated had FC values 5.03 & 1.51, respectively (Table 3.4. & Figure 3.2).

Table 3.4: Comparison on basis of histological differentiation: Ct + SD values

			Differentiation		
		Benign	**Well**	**Moderately**	**Poorly**
miRNA-221	**N**	12	11	14	6
	C_T (Mean ± SD)	32.05 ± 3.84	31.78±1.17	30.34±3.07	29.80±2.74

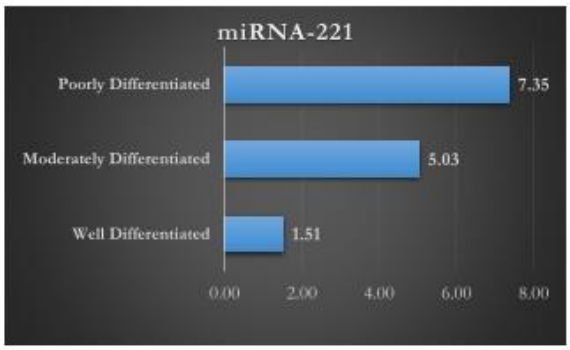

Figure 3.2: miRNA Expression Profile of miR-221 among Different Histopathological Categories of HNSCC.

Gender-based Comparison of Benign and Malignant HNSCC tumors.

In order to identify the miRNA expression profile on gender-based differences, we have also performed gender-based analysis in benign and malignant HNSCC tumors. In this regard we compared male specific miRNA expression in benign and malignant tumors and female miRNA expression in benign and malignant tumors. The expression level of miR-221 is higher in females with malignant lesions as compared to female having benign lesions of the head and neck region. Males having malignant lesions also have high expression of miR-221 as compared to benign lesions of head and neck region, however, the difference in both malesand females (malignant vs benign) is not significant (Table 3.5 & Figure 3.3).

Table 3.5: Gender-based: Ct +SD values

		Male		Female	
		Benign	Malignant	Benign	Malignant
miRNA-221	N	5	23	7	8
	C_T (Mean ± SD)	31.49 ± 4.96	30.77±2.40	32.44 ± 2.28	31.19 ± 1.99

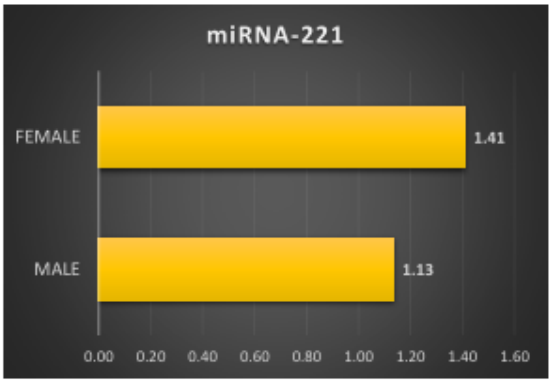

Figure 3.3: Gender-based gene Expression Profile of miRNA-221 among Benign and Malignant HNSCC.

Age-based Comparison of Benign and Malignant HNSCC Tumors.

Squamous cell carcinoma is considered a disease of old age; however, due to genetic predisposition it may occur at an early age as well. In our study we sought to determine the miRNA expression profile of miR-221 on the basis of age in HNSCC patients. We categorized our samples of malignant and benign tumors into two categories, i.e., patients having ages more than or equal to 40 years and those having age less than 40 years. We called them early onset and late onset tumors, respectively. Mir-221 fold difference in late onset malignant versus benign samples is very similar (4.84), to that of early onset tumors (below 40) (4.32). Table 3.6 shows the mean Ct ± SD values of miR-221 according to two age groups in benign and malignant tumor samples. Graphical representation is shown in Figure 3.4.

Table 3.6: Age-based expression profiles: Ct ± SD values

		< 40		40 or Above	
		Benign	Malignant	Benign	Malignant
miRNA-221	N	9	4	3	27
	C_T (Mean ± SD)	31.78 ± 3.58	31.84 ± 0.56	32.83 ± 3.69	30.74 ± 2.40

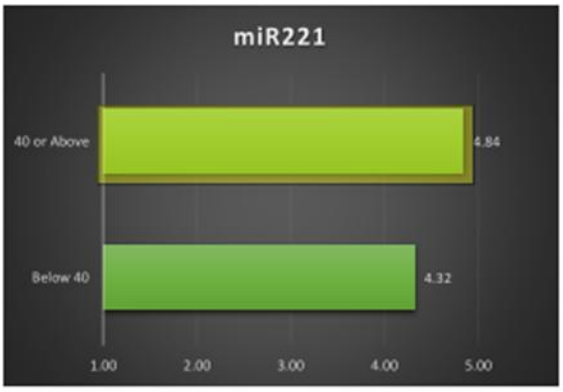

Figure 3.4: Age-based Gene Expression Profile of miR-221 among Benign and Malignant HNSCC.

Tumor Site-based Comparison of Benign and Malignant HNSCC Tumors

In order to find out 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 significant difference in the expression profile of miR-221 when compared between extra and intra-oral malignant and benign tumors. Table3.7 and Figure3.5 shows the visual comparison and means Ct ± SD values of miR-221 in benign and malignant tumors samples according to tumor sites.

Table 3.7: Tumor site based comparison: Ct ± SD values

		Intra Oral		Extra Oral	
		Benign	**Malignant**	**Benign**	**Malignant**
miRNA-221	**N**	4	19	8	13
	C_T (Mean ± SD)	33.24 ± 1.64	31.14 ± 1.68	31.44 ± 4.06	30.52 ± 2.95

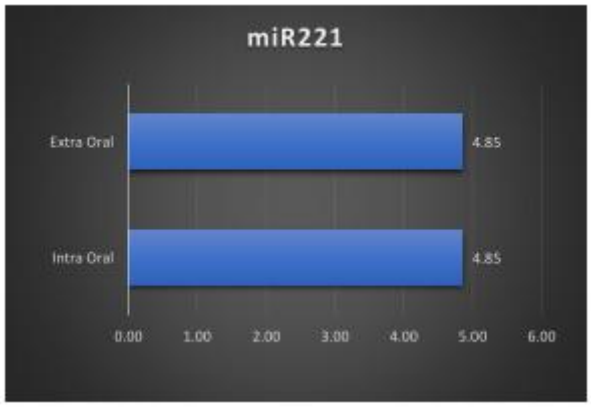

Figure 3.5: Tumor site-based Gene Expression Profile of mir-221 among Benign and M$alignant HNSCC.

IV. Conclusion

We concluded that, micro RNA expression profiling provides a potential strategy for finding new Head and neck squamous cell carcinoma (HNSCC) molecular targets. We have successfully shown that miR-221 are over expressed in head and neck squamous cell carcinoma (HNSCC) samples as compared to benign lesions of the same region.

We, therefore, conclude that this marker have the potential to be utilized as diagnostic biomarkers of HNSCC. A high degree of differential expression between malignant and benign tumors identified in our study indicates a definite role of this miRNA in HNSCC pathogenesis.

We also conclude, by looking at the expression profile in well, moderately and poorly differentiated categories of HNSCC, that this miRNA could possibly be associated with disease aggressiveness. Furthermore, miR-221 could be regarded as a specific marker of poorly differentiated HNSCC.

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

Future Lines of Research

The miRNA expression profiling using qRT-PCR provides an efficient way of confirming high throughput results obtained by expression microarrays. However, they only provide information regarding expression pattern of a particular gene at transcriptional level. Once, miRNA expression profiling is done using qRT-PCR, provided the results point in the right direction, the next logical step is to study what are the targets of these miRNAs. These targets will help us find out the pathways that these miRNAs are controlling at various steps of multi-step carcinogenesis.

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