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 on rise.  Aims & Objectives: This study was aimed to study the expression of miR-21 in the tumor genesis of HNSCC, to study the miRNA expression profile of miR-21 between control and tumor samples, to study the expression profile of miR-21 benign tumors and different categories of HNSCC Tumors on the basis of Histological Differentiation, gender-based Comparison of Benign and Malignant HNSCC Tumors, age-based Comparison of Benign and Malignant HNSCC Tumors, tumor Site-based Comparison of Benign and Malignant HNSCC Tumors.  Study Design: Case-control study.  Study Setting: The University of Lahore.  Period: June -2014 June -2105.  Materials & Methods: In this research, 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. By calculating __Ct and fold change difference according to Livak method. late onset disease the Relative quantification was done to determine the level of expression of miRNA-221. Tumor site did not show any effect on miR-21 expression levels.  Results: Our results showed that the malignant samples have higher expression level of miR-21 then benign control samples. Significantly higher expression was observed in moderately and poorly categories of HNSCC. Gender-based expression showed that females had higher level of expression, while it was found that its expression is high in late onset disease. Tumor site did not show any effect on miR-21 expression levels.  Conclusion: Our miRNA expression profile provides a potential strategy for finding new head and neck squamous cell carcinoma (HNSCC) molecular targets. miR-21 could be regarded as potential diagnostic marker in HNSCC.  Keywords: Head and Neck Squamous Cell Carcinoma (HNSCC), miR-221, Taqman Assay, Intraoral, Extra-oral.

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. 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%.

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. HPV is a circular DNA virus that can exist as an episome or integrate into the host genome. 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. Remarkably HPV-positive HNSCC patients respond more favorably to treatment with cisplatin and radiation and display overall improved survival compared to their HPV-11 negative counterparts.

MiRNAs include a category of endogenous small non-coding RNAs that have only been discovered within the last couple of decades. Since their discovery, a great deal has been learned about their biogenesis, mode of action in the genome, and their involvement in both common and pathological processes. The mature miRNA, a 21-24 bp duplex, is processed step-wise from a primary transcript of 100s-1000s of nucleotides in length.

The first research that indicated the relation between miRNA and human cancers 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. 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.

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. A key methodology is to profile the mature miRNAs in specific tissue types at various disease stages. 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. One of the first was a miRNA microarray performed on nine individual HNSCC cell lines (Dillhoff et al., 2008). The expression of 33 miRNAs was determined to be high and 22 low relative to the other miRNAs expression. Notably, one of the highly expressed miRNAs was miR-21, a miRNA commonly unregulated in cancer. Another study involving microarray of four normal mucosa, four primary HNSCC tissues and four HNSCC cell lines revealed differential expression of nine miRNAs, including overexpression of miR-21, in tumor tissue. Additionally, this study found that miR-21 overexpression in HNSCC cells induced increased proliferation and that inhibition of miR-21 increased apoptosis and cytochrome c release. A third study also required to determine miRNA profiles of HNSCC, concluding that combined expression 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.

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 histopathologically confirmed head and neck squamous cell carcinoma (HNSCC), and 12 samples of benign lesions of head and neck
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. Histopathologically, these were negative for any type of malignancy.

Study Variables
Study variables comprised of both Independent variables and dependent variables.

Independent Variables
Fallowing variables were selected as Independent variables:

Age
Gender
Tumor Differentiation
Tumor Site

Dependent Variables
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-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:

Deparaffinization
• 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 deparaffinized by addition of 300 µL melting buffer to specimens and incubating at 72°C for 10 minutes, with intermittent mild mixing every 2-3 minutes by tapping the tube. After this incubation a quick spin was given to the tube to collect all the liquid at the bottom of tube.
• The tube containing the tissue, was added by 20 µL Proteinase K 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 65°C 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 real-time RT-PCR (Abramovitz et al., 2008).

Binding and washing of RNA
All the following steps were performed at room temperature.
• 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.
This can be done by breaking the adsorption, 50 µL RNase-free water was applied to the column which was pre-heated at 65°C, 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 performed in Bio-Rad’s CFX96™ Real-Time PCR Detection System using AgPath-ID™ One-Step RT-PCR Kit. Primer/probe sets were used for target gene i.e. miR-21 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-I.

RESULTS
3.1 Descriptive Statistics
In the present study, expression levels of miR-21 were studied in 31 tumor samples 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 ∆∆Ct.

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 (FC) between male benign and male malignant and female benign and female malignant categories.

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. In our studies we check the miRNA expression profile of miR-21 on age based differences 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 are those having less than 40 years. We called them early onset and late onset tumors, respectively.

Age-based gene expression profile of miR-21 among benign and malignant HNSCC. The bars represent 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.
SQUAMOUS CELL CARCINOMA

<table>
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<tr>
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<th>Benign</th>
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<tr>
<td>N</td>
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<td>31</td>
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<tr>
<td>Mean Age±SD</td>
<td>29.08 ± 1.36</td>
<td>51.64 ± 1.31</td>
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<tr>
<td>Gender (%)</td>
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<td>5(41.66%)</td>
<td>7(58.33%)</td>
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Table-I. Descriptive statistics

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<tr>
<td>N</td>
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<tr>
<td>Ct (Mean±SD)</td>
<td>33.15 ± 2.50</td>
<td>32.68 ± 1.17</td>
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<td>Ct (Mean±SD)</td>
<td>31.02 ± 3.10</td>
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Table-II. Comparison on basis of histological differentiation: ΔCt SD values

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<td>Ct (Mean±SD)</td>
<td>33.02 ± 2.27</td>
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Table-III. Gender based comparison of benign and malignant HNSCC tumors.

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<td>Ct (Mean±SD)</td>
<td>33.33 ± 3.68</td>
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Table-IV

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<td>Ct (Mean±SD)</td>
<td>32.77 ± 2.56</td>
<td>31.28 ± 3.09</td>
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Table-V. Tumor site based comparison: Ct ± SD values

**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 malignant lesions were classified into the categories of well differentiated, moderately differentiated and poorly differentiated following the WHO classification. The result shows similarity with previous studies in HNSCC where miR-21 show high expression in tumors as compared to normal tissue. MiR-21 was significantly upregulated in HNSCC is shown by quantitative real time PCR analysis. MicroRNA profiling of breast, cervical, and ovarian tumors; glioblastomas; and head and neck primary tumors and cell lines, amongst others, has shown that miR-21 is commonly up-regulated in cancer. There are several targets of miR-21 which has been experimentally validated; many of them are tumor suppressor genes.

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 the development of targeted therapeutic modalities that profoundly benefit cancer patients.

The result shows similarity with previous studies in HNSCC where miR-21 show high expression in tumors as compared to normal tissue. MiR-21 was significantly upregulated in HNSCC is shown by quantitative real time PCR analysis. MicroRNA profiling of breast, cervical, and ovarian tumors; glioblastomas; and head and neck primary tumors and cell lines, amongst others, has shown that miR-21 is commonly up-regulated in cancer. There are several targets of miR-21 which has been experimentally validated; many of them are tumor suppressor genes.

CONCLUSIONS

We concluded that this marker have the potential to be applied as diagnostic biomarkers of HNSCC. 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
that this miRNA could not differentially expressed between extra-oral HNSCC and intra-oral carcinomas.

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REFERENCES


Each betrayal begins with trust.

– Martin Luther –

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