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Human Papillomavirus Status of Head and Neck Squamous Cell Carcinoma Patients and its Association with Expression of PIK3CA and CRNDE

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Abstract

Background: Human papillomavirus (HPV) positive head and neck squamous cell carcinoma (HNSCC) have distinctive molecular features. We conducted the present work to evaluate the HPV status of HNSCC patients and its association with expression of PIK3CA and CRNDE genes.

Method: In the present case-control study, 50 fresh frozen tissue samples of HNSCC patients were collected from the patients referred to hospital for tumor removal. HPV typing was performed on DNA samples using a one-step polymerase chain reaction (PCR) followed by Reverse Line Blot method. Relative expression of PIK3CA and CRNDE genes was evaluated utilizing a real-time PCR method.

Results: Out of 50 patients, 14 (28%) were HPV positive and the most prevalent type was HPV 16. Both PIK3CA and CRNDE genes were upregulated in tumoral tissues compared with adjacent non-cancerous tissues (ANCTs) (*P*=0.0322 and 0.0005, respectively). Based on the area under curve (AUC) values, the diagnostic power of CRNDE (AUC= 0.676) was higher than that of PIK3CA (AUC= 0.604). Finally, the expression level of PIK3CA was significantly associated with HPV+ HNSCC (*P*=0.01).

Conclusion: We showed that the prevalence of HPV in HNSCC was within our local prevalence range. Moreover, the association of PIK3CA overexpression with HPV status implied distinctive molecular characteristics of HPV(+) HNSCC.

Keywords: HPV typing, Gene expression, Reverse Line Blot, ROC curve

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Introduction

Head and neck cancers comprise all the carcinomas located in the head and neck area, including the larynx, mouth cavity, nasal cavity, lips, nasal/paranasal sinuses, tongue, and

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salivary glands; over 90% of all cases are categorized as head and neck squamous cell-carcinomas (HNSCC).[1] Based on the newly-published GLOBOCAN 2018 report, HNSCC is the sixth more prevalent cancer with more than 800,000 new cases diagnosed annually.[2] As reported, the percentage of five-year survival for HNSCC patients was about 40%-50%.[1] The main risk factors predisposing to HNSCC comprise tobacco smoke exposure as a classic risk factor, excessive alcohol consumption (in 80% of cases), and HPV infections, which are considered as emerging risk factors.[3, 4]

Along with classification by anatomic sites, HNSCC is divided into two comprehensive categories, including HPV-associated (HPV+) and HPV-negative (HPV-) carcinomas. HPV+ carcinoma is usually detected in the oropharynx. Based on recent studies, there are convincing data demonstrating that HPV (+) and HPV(-) HNSCCs are separate subtypes with different molecular signatures, clinical presentation, and responses to therapy.[5] As we know, infections due to HPV are limited to the skin and mucous membranes. To date, more than 150 types of HPV have been detected, divided into high- and low-risk groups, out of which HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68, 69, and 73 have been categorized as high-risk HPV.[6] The most oncogenic subtype of HPV is HPV–16 that is detected in ~80% infected cases of the HPV + HNSCCs. Moreover, it has been shown that HPV+ HNSCC is typically identified in younger patients in their 6th decade of life.[7]

On the other hand, several genetic factors contribute to the pathogenesis of HNSCC. One of the more frequently altered (due to mutation or amplification of the 3q or overexpression) genes in about 55% of both HPV+ and HPV- HNSCC patients is PIK3CA.[8,9] The catalytic subunit alpha (p110α) of class IA PI3K (phosphoinositide 3-kinase) is the product of PIK3CA gene, which serves as an oncogene and its normal functions include the regulation of cell survival, growth, and metabolism. The activation of this gene is frequently seen in human tumors.[10] Activation of PIK3CA results in enhanced cellular growth, evasion of apoptosis, and invasion; therefore, it contributes to the formation and progression of tumors.[9]

In addition to conventional genetic factors, long non-coding RNAs (lncRNAs) as a new class of non-coding RNA, play an important role in the regulation of essential pathways in the development and progression of cancers, including HNSCC. Moreover, their tissue- and stage-specific expression pattern makes them useful biomarkers and therapeutic targets.[11-13] A lncRNA on chromosome 16, named "colorectal neoplasia differentially expressed" (CRNDE), has shown increased expression levels in several cancers, including CRC, GC, gliomas, and HCC. This overexpression suggests that CRNDE may be involved in various carcinogenesis features in tumoral cells, including proliferation, migration, invasion, metabolism, angiogenesis, and suppression of apoptosis. It has been indicated that CRNDE serves as a good diagnostic marker with excellent sensitivity and specificity.[12] Functional analysis showed that CRNDE exerts its oncogenic role in carcinogenesis by regulating PI3K/Akt/GSK3β-Wnt/β-catenin axis.[14,15]

In the present study, we evaluated the HPV status in the tissue samples of 50 HNSCC patients and the expression of PI3KCA and CRNDE as its upstream regulator in the cancerous tissues compared with the adjacent non-cancerous tissues (ANCT). We also investigated the correlation between the HPV status and their expression pattern.

Materials and Methods

Patients

The present work was a case-control study conducted on 50 HNSCC samples and 50 adjacent non-cancerous tissues (ANCTs) from the same patients who were referred to Cancer Institute, Imam Khomeini Medical Center, Tehran, Iran for tumor removal. The participants had no history of chemo/ radiotherapy prior to the excision of the samples. The collection of the samples was performed during surgery and the existence/absence of tumoral cells was confirmed by expert pathologists. The exclusion criteria

Table 1. Primer set sequences and the length of the products

Gene name	Sequence	Product length
PIK3CA	F: GATGCCACTGGAAATGTTGAAATGAAAA R: TAAGTCCCACACAGTCACCG	83 bp
CRNDE	F: GATGCCACTGGAAATGTTGAAATGAAAA R: ACATATTTAAACCACTCGAGCACTTTGA	125 bp
SDHA	F: ACGATTACTCCAAGCCCATCCA R: TTCCCAGTGCCAACGTCCA	97 bp

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *CRNDE*: Colorectal neoplasia differentially expressed; *SDHA*: Succinate dehydrogenase complex flavoprotein subunit A; F, Forward; R, Reverse; bp: Base pair

comprised having tumors in other sites and a family history of HNSCC. Appropriateness of the excised tissues for expression analysis and definite pathological diagnosis were considered as the inclusion criteria. The study protocol was approved by the Ethical Committee of Tehran University of Medical Sciences in compliance with the Helsinki Declaration (IR.TUMS.DENTISTRY.REC.1397.021). All the patients signed written informed consent forms.

DNA extraction and HPV typing

Genomic DNA was extracted from fresh frozen tissue samples using the TriPure™ Isolation Reagent according to the manufacturer's recommendations. The concentrations of the extracted DNA samples were measured with a nanodrop instrument (Thermo Fisher Scientific, Inc.). Amplification of the isolated DNA samples was done using the Ampliquality HPV-Type Express polymerase chain reaction (PCR) kit according to manufacturer's protocol. Briefly, the thermal protocol for the PCR experiments was as follows: 10 min at 95°C for DNA-polymerase activation, 50 cycles of a 30-sec denaturation at 95°C, 30-sec annealing at 50°C, and a 30-sec elongation at 72°C and eventually, a final extension step of 72°C for 5-min. HPV genotype was performed on the PCR products using the Single Step PCR and Reverse Line Blot method; 40 different genotypes of HPV were detected, including HPV 6, 11, 16, 18 , 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68a, 68b , 69, 70, 71, 72, 73, 81, 82, 83, 84, 87, 89, 90. Using this method, the HPV type was

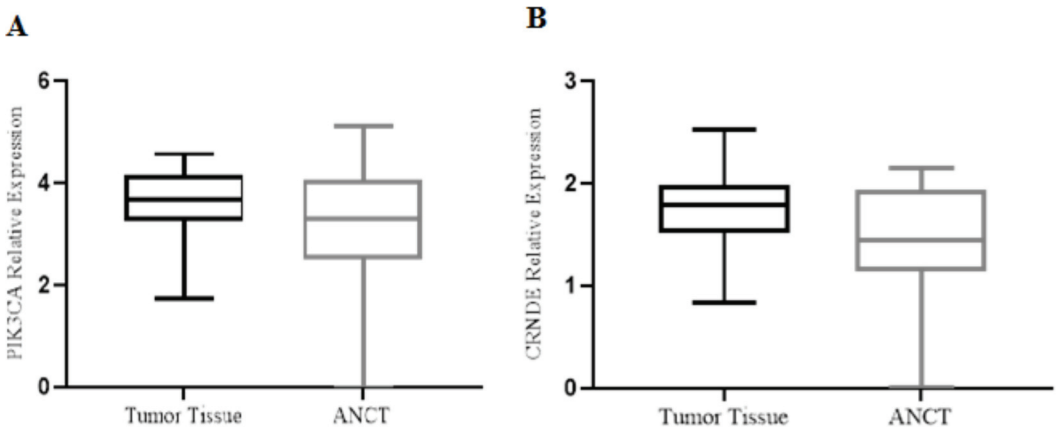

Figure 1. This figure shows the relative expression of lncRNAs in tumoral tissues (n = 30) and ANCTs (n = 30) as described by -Δ CT values (CT *SDHA*-CT target gene) in each group of the samples.

lncRNAs: Long non-coding RNAs; ANCTs: Adjacent non-cancerous tissues; *SDHA*: Succinate dehydrogenase complex flavoprotein subunit A; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; CRNDE: Colorectal neoplasia differentially expressed

reported as a band on a blot membrane corresponding to the genotype of HPV.

RNA extraction gene expression analyses

The same TriPure™ Isolation Reagent (Roche Molecular Diagnostics, Indianapolis, USA) was used to isolate total RNA from the fresh frozen tumoral tissues and ANCTs according to the manufacturer's instructions. Relative quantity and quality of the isolated RNA was measured with 1% agarose gel electrophoresis and then the isolated RNA samples were stored at ~80°C for subsequent evaluations. The cDNA synthesis was carried out using the PrimeScript™ RT reagent Kit (Takara) on 1µg of total RNA. The synthesized cDNA was stored at -20°C. Real-time PCR was run in duplicate employing SYBR® Premix Ex Taq™ II (Takara), according to the manufacturer's guideline. Rotor-Gene Q (Qiagen) instrument was used to perform real-time PCR amplification. SDHA expression level was used as an internal control for normalization in each run and the relative expression of the genes of interest (PIK3CA and CRNDE) was calculated utilizing the 2ΔΔCq method. Table 1represents the primer sequences and the length of the PCR products.

Table 2. Patients' demographic and clinical data

Variables	Values N(%)
Age	
≤40 year	8 (16%)
>40 year	42 (84%)
Gender	
Man	39 (78%)
Woman	11 (22%)
Site of the primary tumor	
Larynx	39 (78%)
Oral cavity	5 (10%)
Pharynx	4 (8%)
others	2 (4%)
Tumor grade	
Well-differentiated	18 (36%)
Moderately differentiated	20 (40%)
Poorly differentiated	12 (24%)
Tumor size	
≥2/5cm	29 (58%)
<2/5cm	21 (42%)
Lymphatic invasion	
Yes	12 (24%)
No	38 (76%)
Vascular invasion	
Yes	15 (30%)
No	35 (70%)
Lymph node involvement	
Yes	13 (26%)
No	37 (74%)

N: number; cm: centimeter

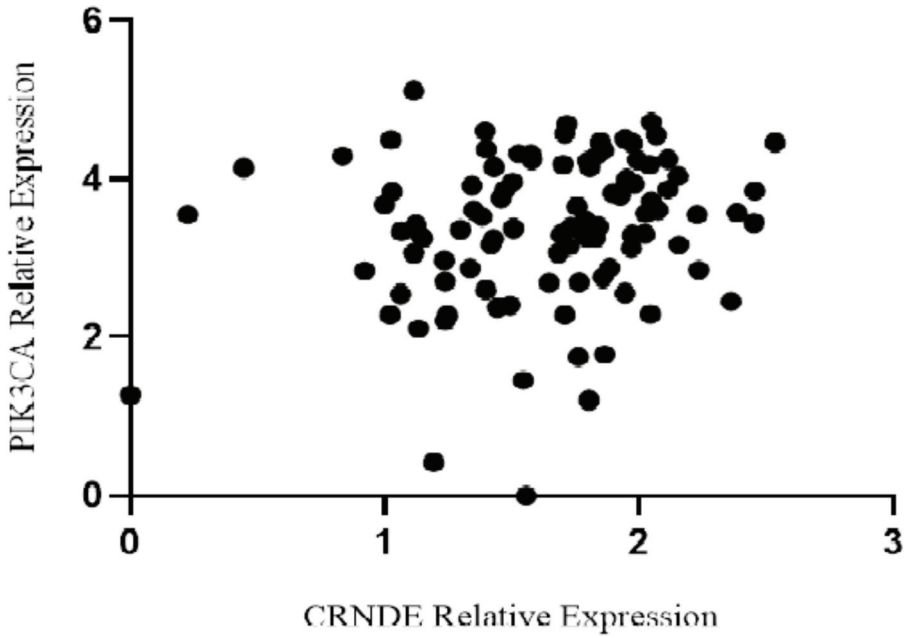

Figure 2. This figure shows the correlation between PIK3CA and CRNDE relative expressions.
PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; CRNDE: Colorectal neoplasia differentially expressed

Table 3. Relative expression of lncRNAs in the gastric cancer samples compared with ANCTs

Gene name	Expression ratio	*P*-value	CI (95%)
PIK3CA	2.489	0.0322	0.014-1855.514
CRNDE	2.038	0.0005	0.134-41.673

lncRNAs: Long non-coding RNAs; ANCTs: Adjacent non-cancerous tissues; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; CRNDE: Colorectal neoplasia differentially expressed; CI: Confidence interval
A *P*-value of less than 0.05 considered as significant.

Statistical analysis

Demographic and clinical data, as well as detected HPV types, were reported as the frequency and related percentage. Fold changes in lncRNAs expressions were assessed with REST 2009 software, considering the values of amplification efficiencies and cycle thresholds. The Shapiro-Wilk test was used to assess the normality of the distribution. To evaluate the significance of the difference in expression of genes, we employed the Student paired t-test. The correlation between the relative expression of CRNDE and PIK3A was assessed via the Pearson correlation test. The GraphPad Prism 8 software was used to perform statistical analyses. For estimation of the diagnostic power of each gene for the discrimination between the tumoral tissues and ANCT, the receiver operating characteristic (ROC) analysis was performed. For all the statistical tests, the level of significance was set at $P < 0.05$.

Results

Demographic and clinical data

Out of 50 HNSCC participants with squamous cell carcinoma of various locations of head and neck area, who were referred to Cancer Institute, Imam Khomeini Medical Center, 39 (78%) were men and 11 (22%) were women. Table 2 depicts the basic demographic and clinical data of the participants, including age, gender, site of the primary tumor, tumor grade, tumor size, lymphatic and vascular invasion, and lymph node involvement.

HPV typing

Generally, HPV DNA was detected in 14 out of 50 samples and therefore 28% of the cases were HPV positive. Checking the band on blot membranes against the interpretation transparent film revealed that HPV-16 was the most common tumor with 8 out of 14 (57.1%) of the HPV-positive tumors, including both single and multiple-genotype infection. Single-genotype infection by HPV 16, 18, 6, and 61 was observed in six, three, one, and one of the samples, respectively. Moreover, in three samples, we

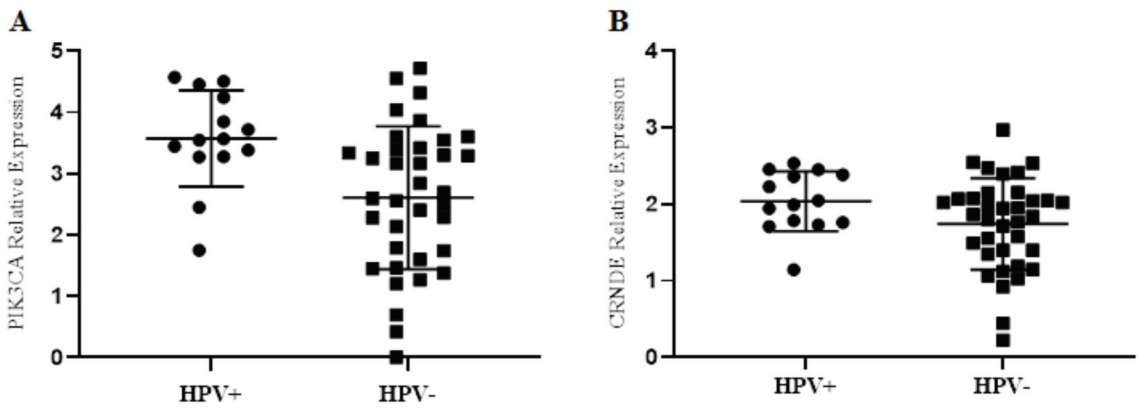

Figure 3. Association between HPV status and expression level of genes. A) The expression level of PIK3CA was associated with HPV+ HNSCC (*P*=0.0064). B) The expression level of CRNDE was not associated with HPV+ HNSCC (*P*=0.0931).
HPV: Human papillomavirus; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; CRNDE: Colorectal neoplasia differentially expressed

Table 4. The results of ROC curve analysis

Gene of interest	Associated criterion	AUC	J*	Sensitivity	Specificity	95% CI	*P*-value*
PIK3CA	>3.16	0.604	0.24	78	46	2.1 to >3.67	0.0737
CRNDE	>1.4	0.676	0.38	88	50	0.20 to 0.52	0.0013

AUC, the area under the curve; ROC, receiver operating characteristic; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; CRNDE: Colorectal neoplasia differentially expressed; CI: Confidence interval
*Youden index
**Significance Level *P* (Area = 0.5), Associated criterion: optimal cut-off point for gene expression

found multiple-genotype infections, including infection by HPV 6/16 in two samples, and HPV 6/11 in one case.

Relative expression of PIK3CA and CRNDE in HNSCC tumoral tissues compared with ANCTs

Our analysis revealed that compared with the ANCTs, the expression of PIK3CA and CRNDE was upregulated in tumoral tissues. In fact, statistical analyses showed that the PIK3CA expression level in the tumoral tissues was significantly higher than that of ANCTs (P = 0.0322). Additionally, the significant difference between the relative expression of CRNDE in the tumoral tissues compared with ANCTs was even more obvious (P = 0.0005). Table 3 illustrates the expression ratios and *P*-values for both genes. The relative expression of these genes is also shown in figure 1. Using the Pearson correlation test, we found no significant pairwise correlations between the expression level of PIK3CA and CRNDE in the tumoral tissues and ANCTs, with a correlation coefficient value of R= 0.1976 (P = 0.0487, and 95% CI = 0.001251 to 0.3793) (Figure 2).

ROC curve analysis

Using ROC curve analysis, compared with the PIK3CA gene, transcript levels of CRNDE lncRNA were found to have the highest sensitivity and specificity for discrimination of the tumoral tissues from ANCTs (88% and 50%, respectively). Besides, based on the area under curve (AUC) values, the diagnostic power of CRNDE (AUC= 0.676) was higher than that of PIK3CA (AUC= 0.604) (Table 4).

Association between the presence of HPV DNA and expression level of PIK3CA and CRNDE

After evaluating the association between HPV status and gene expression levels, the results revealed that the expression level of PIK3CA was significantly associated with HPV+ HNSCC (P = 0.01). However, the statistical analysis did not show any associations between the expression level of CRNDE and HPV status in HNSCC (P = 0.0931) (Figure 3).

Association between the clinicopathological features and the presence of HPV DNA and expression level of PIK3CA and CRNDE genes

As shown in table 5, there was a significant association between the presence of tumor grade (P = 0.0012), lymphatic invasion (P<0.0001), vascular invasion (P = 0.0002), and lymph node involvement (P = 0.0004). Our data showed that the upregulation of the PIK3CA gene was significantly associated with the site of the primary tumor (P = 0.0204), tumor grade (P = 0.0293), and tumor size (P = 0.0070). Statistical analysis revealed a significant association between the expression level of CRNDE gene and lymphatic invasion (P = 0.0012), vascular invasion (P = 0.0190), and lymph node involvement (P = 0.0022).

Discussion

Herein, we assessed the HPV status of 50 HNSCC patients using Single Step PCR and Reverse Line Blot method. We evaluated the expression level of PIK3CA gene and its upstream CRNDE lncRNA in the tumoral tissue compared to that of ANCTs and their diagnostic power for discrimination of tumoral from the normal tissues.

Results of the present study indicated that 28% of HNSCC patients were HPV positive, with HPV 16 as the most prevalent type. In 2014, Jalilvand and et al. performed a systematic review and found that HPV prevalence was 32.4% in head and neck cancers in the Iranian population. They also reported that the most frequent types of HPV

Table 5. The statistical association between the clinicopathological features and the presence of HPV DNA and expression level of PIK3CA and CRNDE genes in HNSCC patients

Clinicopathological feature	HPV +	PIK3CA expression	CRNDE expression
	P-value	*P*-value	*P*-value
Site of the primary tumor	0.8457	0.0204	0.1684
Tumor grade	0.0012	0.0293	0.0625
Tumor size	0.3408	0.0070	0.8570
Lymphatic invasion	<0.0001	0.8265	0.0012
Vascular invasion	0.0002	0.8945	0.0190
Lymph node involvement	0.0004	0.9452	0.0022

HPV: Human papillomavirus; HNSCC: Head and neck squamous cell carcinoma; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; CRNDE: Colorectal neoplasia differentially expressed; CI: confidence interval

in our population were HPV16 and 18.[16] In concordance with their data, we found a comparable frequency of HPV infection. In the study by Karbalaie et al. performed in 2017, three different methods were utilized, including HPV-16 specific conventional PCR, INNO-LiPA HPV genotyping assays, and PCR/. Their results showed that 3.2% of patients were HPV positive, which was validated by all the three methods. They detected 16, 2, 27, and 43 subtypes of HPV in five of their samples.[17] Different HPV typing methods that we used, which detected a wide spectrum of high-risk HPV types, may explain this huge difference between our HPV infection frequencies. Asvadi kermani et al. performed another investigation in 2011 on 40 FFPE blocks of HNSCC patients from northwest of Iran using Nested-PCR/sequencing and detected high-risk HPV in six(42.8%) patients, among whom HPV-18 was the most frequent type;[18] this indicates that the prevalence of HPV and the frequence of different types varies in different geographical regions of Iran. There are several studies in other countries on the HPV status in HNSCC patients.[19-21] In three recent studies in Bangladesh,[20] Romania,[21] and Sweden,[19] HPV prevalence was reported to be 21%, 12.2%, and 39 %, respectively. The prevalent HPV type in all the three countries was HPV-16.

HPV (+) HNSCC cancers have distinctive molecular features, including upregulation of p16IN4A, absence of p53 inactivating mutations, and alterations in PI3K/AKT and Wnt pathway.[22] Alterations in the PIK3CA gene, including overexpression, are the most frequent events in HNSCC following TP53 alterations.[9] As one of the important contributors to the PI3K/AKT pathway, we evaluated the expression level of PIK3CA in HNSCC patients. Our results showed that the PIK3CA was upregulated in HNSCC tumoral tissues compared with ANCTs. Two independent investigations on PIK3CA mRNAs in ESCC, as one type of HNSCC, determined that the expression level of PIK3CA in ESCC tissues was higher than that of adjacent normal esophageal epithelium, which implied that PIK3CA may contribute to the ESCC development as a key factor.[23,24] More recently, García-Escudero et al. analyzed whole-genome expression profiles of primary HNSCC tumors from The Cancer Genome Atlas and revealed an increased expression of PIK3CA gene and its association with poor prognosis. They also suggested that PI3KCA overexpression contributes to HNSCC tumors development through activation of the Hippo-YAP pathway which is known to be involved in organ size, stem cell maintenance, and tumorigenesis.[9] Functional analysis on a mouse model showed that while upregulation of PIK3CA alone could not cause HNSCC initiation, its overexpression triggered a significant increase in susceptibility to tumorigenesis in a mouse model of oral carcinogenesis. Furthermore, the investigation on human HNSCC clinical samples showed that PIK3CA protein levels are correlated with tumor progression through PI3K/PDK1 and TGFα signaling.[25] Yarbrough et al. using gene expression microarray data confirmed that HNSCC patients infected with HPV showed overexpression of PIK3CA compared with HPV(-)

HNSCC.[26] In concordance with their results, our study indicated that the upregulation of PIK3CA was associated with the HPV (+) status in HNSCC patients. It has been previously demonstrated that mutations of the PIK3CA genes, which activate the PI3K, are rare in HNSCC cell lines. Therefore, an alternative mechanism for activation of PI3K in HPV (+) HNSCC might be overexpression of PIK3CA.[26]

Aberrant overexpression of long non-coding RNA CRNDE (Colorectal Neoplasia Differentially Expressed) has been confirmed in various human cancers, which is correlated with advanced clinicopathological features and poor prognosis. CRNDE promotes cancer cell proliferation, migration, and invasion and suppresses apoptosis.[27] The first investigation which showed that lncRNA CRNDE might play an important role in cancer development was conducted by Liu et al. They confirmed the association of the increased level of CRNDE with CRC development and progression and stated that CRNDE lncRNA may serve as a potentially useful prognostic marker.[28] Recently, Ren et al. showed the increased expression level of CRNDE in tongue squamous cell carcinoma (TSCC) as a member of HNSCC and suggested that CRNDE plays an oncogenic role in the progression of TSCC through suppressing the expression of miR-384.[29] Other studies have exhibited that higher expression of CRNDE was in the tumoral tissues compared with normal tissues in several cancer types and indicates that CRNDE plays a pivotal role in promoting carcinogenesis.[30-32] In concordance with these studies, we showed that CRNDE was upregulated in tumoral tissues and suggested its putative role in HNSCC carcinogenesis. It may contribute to the carcinogenesis by affecting the activation of the Wnt/β-catenin signaling pathway. Dai et al. showed that the expression of CRNDE increased in OSCC tissues and cell lines compared with that in the normal controls. They suggested that silencing CRNDE may inhibit epithelial-mesenchymal transition and decrease the migration and invasion of human OSCC cells via repressing the Wnt/β-catenin pathway.[33]

To evaluate the diagnostic power of the evaluated PIK3A gene and CRNDE lncRNA in HNSCC, we performed ROC curve analyses. Based on the acquired *P* and AUC values, transcript levels of these two genes were poor diagnostic markers. On the other hand, a study suggested PIK3CA gene mutation and overexpression as biomarkers for targeted therapy for Chinese ESCC patients.[24] Moreover, other studies have indicated that CRNDE had diagnostic power for detecting cancerous tissues and serve as a novel diagnostic and prognostic biomarker.[28,31,32]

Conclusion

In conclusion, we showed that the prevalence of HPV in HNSCC was within our local prevalence range and as expected, HPV 16 was the most prevalent type. Additionally, the expressions of PIK3CA and CRNDE were upregulated and overexpression of PIK3CA was associated with the HPV status of the patients, which implies distinctive molecular characteristics of HPV(+) HNSCC. As a limitation of this study, the results of gene expression must be confirmed in the protein level. Further studies with more genes are needed in order to improve the sensitivity and specificity of this panel. A comprehensive study with a larger sample size and evaluation of a wider panel of genes may lead to more accurate results.

Acknowledgment

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Conflict of Interest

None declared.

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