RESEARCH ARTICLE

Expression of Radioresistant Gene PEG10 in OSCC Patients and Its Prognostic Significance

Shankar Sharan Singh[1], Rajendra Kumar[1], Vandana Singh Kushwaha[1], Madan Lal Brahma Bhatt[1]*, Anshuman Singh[2], Anupam Mishra[3], Hari Ram[4], Devendra Parmar[2], Rajiv Gupta[1]

Abstract

Background: Oral squamous cell carcinoma (OSCC) is one of the most common forms of cancer occurring worldwide. PEG10 is well known as a paternally expressed gene from a newly recognized imprinted region at human chromosome 7q21. Previous study had demonstrated that the significant expression of PEG10 was found in radioresistant OSCC cell line and its expression was significantly associated with poor survival in several cancers. Therefore it has been evaluated as a potential marker in OSCC patients undergoing radiotherapy. **Objective:** This study was conducted to analyze the mRNA expression of PEG10 in OSCC and its expression in relation to clinicpathological features, radiotherapy treatment response and survival. **Methods:** This study included tissue specimens obtained via biopsy of 118 patients with OSCC who were recommended for radiotherapy treatment and 80 healthy control tissues analysis of mRNA expression of PEG10 was done by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Patients were treated with 70 Gy of radiation dose by shrinking field technique using Cobalt-60 teletherapy machine. **Results:** Significantly higher mRNA expression of PEG10 was found in OSCC patients when compared with matched controls. High level of PEG10 mRNA expression showed a significant correlation with lymph node metastasis ($p = 0.0047$) and tumor stage ($p = 0.0499$). Multivariate Cox regression analysis revealed that high level of mRNA expression of PEG10 was significantly associated with poor survival ($p < 0.05$). Our research demonstrated that the expression of PEG10 was higher in radioresistant tumor. **Conclusion:** We observed significantly increased expression of PEG10 in context of lymph node status, advanced stage and poor survival in our study. Thus PEG10 gene can be used as potential predictive and prognostic biomarker in OSCC patients undergoing radiotherapy.

Keywords: Oral sqamous cell carcinoma (OSCC)- Paternally Expressed Gene 10 (PEG10)- Radiotherapy response

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Introduction

Oral squamous cell carcinoma (OSCC) is one the most common neoplasm of the head and neck (Zhou et al., 2016). An estimated 263,900 new cases and 128,000 deaths from oral cavity cancer (including lip cancer) occurred in 2008 worldwide (Ahmedin et al., 2011).

Several risk factors like use of tobacco, including smokeless tobacco and excessive consumption of alcohol are the major risk factors in oral cancer (Sankaranarayanan et al., 1999; Ko et al., 1995). Late diagnosis and resistance to radiotherapy, chemotherapy are the major cause of poor prognosis in oral cancer patients (Julianaet al., 2012). Recently, several studies have analyzed the biomarkers for predicting the prognosis of OSCC but no single study has found the appropriate result for clinical application.

Previously a microarray based study revealed that the expression of six genes including Paternally Expressed Gene 10 (PEG10) had significant up-regulation in OSCC cell line. This study had demonstrated that the significant expression of PEG10 was found in radioresistant cells when compared with radiosensitive cells in a dose and time dependent manner (Ishigami et al., 2007).

PEG10 was first identified by Ono et al., (2001). PEG10 is well-known as a paternally expressed gene from a newly recognized imprinted region at human chromosome 7q21. PEG10 shows parent of origin specific expression in monochromosomal hybrids (Ono et al., 2001; Sun et al., 2006). PEG 10 mRNA is expressed in normal tissues of brain, kidney, ovary, spleen, thymus, testis, lymphoblasts, endothelial cells and placenta (Okabeet al., 2003; Kainzet al., 2007). Okabeet al., (2003) found that overexpression of PEG10 may block TGF-β signaling pathway via binding to TGF-β receptors II and may cause decrease in cell death through interaction with SIAH1 (antisense S-oligonucleotides) in human

*[1]Department of Radiotherapy, [3]Department of Otorhinolaryngology, [4]Department of Oral and Maxillofacial Surgery, King George's Medical University, [2]Developmental Toxicology Division, CSIR-Indian Institute of Toxicology Research, M.G. Marg, Lucknow, India. *For Correspondence: drmlbhatt@yahoo.com*

hepatocellular carcinoma.

PEG10 is the essential protein for cell proliferation, migration, invasion, and its expression may help to predict the radiotherapy treatment response for the OSCC patients and their prognosis. In this study we examined the expression of PEG10 in OSCC and analyzed its expression in relation to clinicopathological features, radiotherapy treatment response and survival. We evaluated the prognostic significance of PEG10 in OSCC patients.

Materials and Methods

Study Population

We recruited 118 histopathologically confirmed tissue samples from OSCC patients (who were recommended for the Radiotherapy treatment) as well as eighty matched controls for the evaluation of PEG10 mRNA expression. Healthy matched individuals (n=80) attending dental procedures for dental implant or benign cyst were included as control group (for tissue samples). The tissue specimens were collected from the department of Radiotherapy and Oral and Maxillofacial Surgery, King George's Medical University, Lucknow, India. The OSCC tissue specimens were derived from the buccal mucosa, tongue (excluding base of tongue), hard palate, floor of mouth, alveolus and lip. All the collected tissues were instantly stored in trizol solution at -80oC deep freezer. Ethical approval was obtained from the Institutional Ethics Committee (IEC number XLIV ECM/B-P11) before the start of the study. Informed written consent was taken from all the participants before their inclusion into the study. The characteristics of the OSCC patients including age, clinical stage (TNM classification defined by the American Joint Committee on Cancer AJCC 2010, VIIth edition) (Edge SB and Compton CC, 2010) and adverse oral habits (tobacco and alcohol) were assessed by radiation oncologist. Patients with more than 18 years of age, normal hematological, renal and liver function test were included. Patients with history of prior chemotherapy, radiotherapy and surgery and distant metastasis were excluded from the study.

Treatment and Response Assessment

Patients of OSCC were subjected to radiotherapy using tele-cobalt radiotherapy machine (Theratron 780 E, AECL, Ottawa) in the Department of Radiotherapy. A dose of 70 Gy of radiation was delivered to all the patients in 7 weeks with 2 Gy fraction size, 5 days a week (46 Gy to primary and whole neck followed by 24 Gy after sparing the cord). The response to treatment was assessed by World Health Organization (WHO) criteria after one month of completion of treatment (Hoogstraten et al., 1979).

Patients were followed up after treatment and evaluated for survival. The primary endpoints were treatment response and overall survival (OS) from the date of diagnosis to the date of death from any cause and patients were censored who were alive on the date of last follow up examination.

Preparation of cDNA

The tissue specimens of OSCC patients and controls were collected in TRIzol Reagent (Ambion, Life Technology) and immediately homogenized with T25 basic homogenizer (IKA Labortechnik, Stanfen, Germany). Total RNA was isolated according to TRIzol Reagent protocol and stored at -80°C for further use. The total RNA of each sample was reverse transcribed to cDNA using cDNA synthesis kit (Thermo Scientific, EU, Lithuania) according to the manufacturer's protocol.

Analysis of mRNA expression by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

qRT-PCR analysis was performed according to the manufacturer's protocol (Takara SYBR Fast qPCR kit, Takara Biosystem). The qRT-PCR reactions were performed using Applied Biosystem 7900 HT real time PCR system and reactions were carried out in triplicates. Beta-actin was taken as an internal control and used to normalize ratio between samples. The primer sets specific for PEG10 (Forward: 5'-GACTCCGGCTTTGACACAACA-3', Reverse: 5'-AACGCTGGAGCCACCAGTAA-3') and beta actin (Forword: 5'-GCACAGAGCCTCGCCTT-3', Reverse: 5'-GTTGTCGACGACGAGCG-3') were used. An initial incubation of enzyme denaturation at 95°C for 10 minutes followed by 45 rounds of amplification at 95°C (10 sec) for denaturation, 58-65°C (10 sec) for annealing and 72°C (30 sec) for extension for performing qRT-PCR. Relative change in mRNA level between tumor and matched normal control tissue were calculated by using $2^{-}\Delta\Delta ct$ method.

Statistical analysis

One way ANNOVA using Turkey's Multiple Comparison test was used to compare the mRNA expression levels between different groups in both cases and controls. Comparisons were made between categorical groups by chi-square ($\chi 2$) test. Survival curves were plotted compared by Log rank (Mantel-Cox: $\chi 2$) test using Kaplan-Meier method. Cox-regression analysis was conducted to find out independent prognostic factors of overall survival. A two-tailed $p<0.05$ was considered statistically significant.

Results

The demographic and clinicopathological characteristics of OSCC patients are summarized in (table 1). The age range was 22-72 years (mean ± SD, 44.9 ± 7.2 years) for OSCC patients and 21-70 years (mean ± SD, 42.1 ± 13.9 years) for controls. The number of male patients was 72.9% which was higher than the female patients. Majority of the patients had carcinoma of buccal mucosa (52.5 %) followed by tongue (24.6 %) as primary disease sites. It was observed that most of the patients (63.6%) had habit of tobacco chewing. The distributions of OSCC according to histopathological grading were 58 patients (49.2%) with well differentiated histology, 42 patients (39.8%) were moderately differentiated and 13 patients (11.0%) were poorly differentiated. Out of

Table 1. Demographic and Clinicopathological Characteristic of OSCC Patients (n=118)

Characteristic /category	No. of patient (%)
Age (Years)	
20-30	14 (11.8)
31-40	29 (24.6)
41-50	37 (31.4)
51-60	27 (22.9)
61-70	11 (9.3)
Sex	
Female	32 (27.1)
Male	86 (72.9)
Tobacco Chewing	
No	43 (36.4)
Yes	75 (63.6)
Smoking	
No	57 (48.3)
Yes	61 (51.7)
Alcohol Consumption	
No	62 (52.5)
Yes	56 (47.5)
Primary Site	
Buccal Mucosa	62 (52.5)
Oral tongue	29 (24.6)
Alveolus	18 (15.3)
Retromolartrigone	4 (3.4)
Lips	3 (2.5)
Hard Palate	2 (1.7)
SCC Differentiation	
Well	58 (49.2)
Moderate	47 (49.8)
Poor	13 (11.1)
Tumor Size	
T1	5 (4.2)
T2	25 (21.2)
T3	37 (31.4)
T4	51 (43.2)
Lymph node status	
N0	39 (33.1)
N1	42(35.6)
N2	30 (25.4)
N3	7 (5.9)
Stage	
Early	12 (10.2)
Advanced	106 (89.8)
Radiotherapy Treatment response	
Responder's	67 (56.8)
Non-Responder's	51 (43.2)

total 118 patients, T4 tumor size were the commonest (43.2%), Lymph node positive (66.1%), and advanced stage, (stage III and IV) (89.3%). qRT-PCR study revealed highly significant ($p<0.001$) mRNA expression of PEG10 in OSCC patients cases as compared to matched controls. Moreover the mRNA expression of PEG10 was higher in 70.3% (83/118) patients and 29.7% (35/118) patients had low PEG10 mRNA expression (Table 2).

The association between PEG10 mRNA expressions (Low and high) and clinicopathological characteristics was done using $\chi 2$ test and shown in (Table 2). Overexpression of PEG10 significantly correlated with lymph node metastasis (N1 and N2) vs lymph node negative (N0) ($p=0.0047$) and advanced Stage (III and IV) vs early stage (I and II) ($p=0.049$). Out of 118 patients, 67 (56.8%) patients were responders (complete response and partial response) and 51 patients (43.2%) were non responders (no response or progressive disease) (Table 3). The increase in expression of PEG10 mRNA was found to be highly significant ($p<0.001$) in non responders when compared to responder OSCC patients.

The follow up time for patients that were used for analysis of PEG10 mRNA expression ranged from 10 month to 73 months (mean: 42.7 months, median: 45 months). During this period, 70 (59.3%) patients were alive and 48 (40.7%) were died. Survival rate for low and high expression of PEG10 were 77.1% and 51.8% respectively after a median follow up of 45 months. The PEG10 mRNA expression significantly correlated with survival and patient with high expression had shortened survival (log-rank, $P = 0.003$) as observed by Kaplan-Meier analysis (Figure 1).

To find out independent prognostic factors of overall survival, univariate and multivariate Cox regression analysis were done which is summarized in (Table 4). The univariate Cox regression analysis revealed significant ($p=0.008$, $p=0.097$ and $p=0.003$) association of overall survival with lymph node metastasis, stage and PEG10 mRNA expressions. In multivariate Cox regression analysis, the expression of PEG10 mRNA remained as a significant and independent predictor of survival after adjustment for age, sex, adverse habits and clinicopathological parameters, (HRR=2.982, 95% CI, 1.278-6.957, P=0.011).

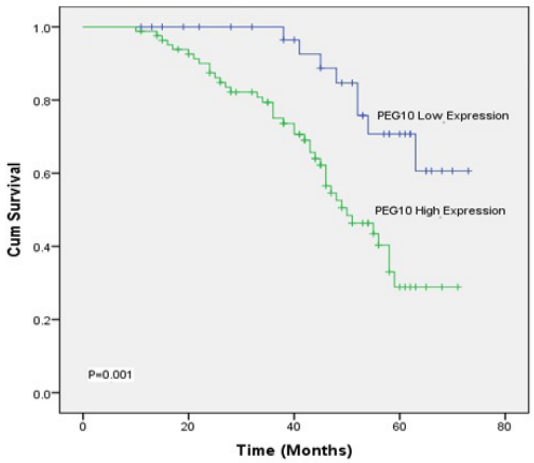

Figure 1. Overall Survival Curve of OSCC Patients According of PEG10 Mrna Expression Using Kaplan-Meier Estimate with Log

Table 2. Correlation of PEG10 Gene Expression with the Clinicopathological Characteristics

Variables	Category	Number of patients (%)	PEG10 mRNA expression (n, %) Low level of expression	high level of expression	P value
Total		118	35 (29.7)	83 (70.3)	
SCC Differentiation	Well	58 (49.2)	19 (32.7)	39 (67.2)	p= 0.6012
	Poor and Moderate	60 (50.8)	16 (26.7)	44 (73.3)	
Tumor size	T1-T2	30 (25.4)	12 (40)	18 (60)	p= 0.2285
	T3-T4	88 (74.6)	23 (26.1)	65 (73.9)	
Lymph node metastasis	Negative	40 (33.9)	19 (47.5)	21 (52.5)	**p=0.0047**
	Positive	78 (66.1)	16 (20.5)	62 (79.5)	
Stage	Early stage	12 (10.2)	7 (58.3)	5 (41.7)	**p= 0.0499**
	Advanced stage	106 (89.8)	28 (26.4)	78 (73.6)	

Significant p values were highlighted in bold.

Discussion

Our research demonstrated significant increase in the mRNA expression of PEG10 in tissue samples from patients diagnosed with OSCC. The significant expression of PEG10 was also found previously in different types of human cancers such as hepatocellular carcinoma, gallbladder cancer, leukaemia, lung cancer, breast cancer, prostate cancer and pancreatic cancer (Liet al., 2006; Liu et al., 2011; Tsou et al., 2003; Li et al., 2016; Denget al., 2014).

Radiation therapy has an important contribution in the treatment of oral cancer but sometimes cancer cells do not respond to radiotherapy (Ishigami et al., 2007; Terakado et al., 2004). Current study demonstrated that the expression of PEG10 was higher in radioresistant tumor.

Table 3. Correlation of Clinicopathological Characteristics and PEG10 Expression with Overall Survival Using Cox's Regression Analysis (n=118)

Predictors	Number of patients(%)	Univariate (unadjusted)		Multivariate** (adjusted)	
		OR (95% CI)	p value	OR (95% CI)	p value
Total	118				
Age (yrs):					
<=45	67 (56.8)	1.0 (Ref)		1.0 (Ref)	
>45	51 (43.2)	1.411 (0.796-2.500)	0.238	1.679 (0.905-3.118)	0.101
Sex:					
Female	32 (27.1)	1.0 (Ref)		1.0 (Ref)	
Male	86 (72.9)	0.898 (0.481-1.677)	0.736	0.625 (0.315-1.242)	0.18
Primary site:					
Buccal mucosa	62 (52.5)	1.0 (Ref)		1.0 (Ref)	
Other*	56 (47.5)	1.166 (0.660-2.058)	0.596	1.011 (0.561-1.825)	0.97
SCC Grade:					
WD	58 (49.2)	1.0 (Ref)		1.0 (Ref)	
MD/PD	60 (50.8)	1.243 (0.700-2.207)	0.458	1.317 (0.720-2.407)	0.371
Tumor size:					
T1/T2	30 (25.4)	1.0 (Ref)		1.0 (Ref)	
T3/T4	88 (74.6)	1.725 (0.835-3.566)	0.141	1.838 (0.813-4.153)	0.143
Lymph node metastasis:					
Negative	40 (33.9)	1.0 (Ref)		1.0 (Ref)	
Positive	78 (66.1)	2.515 (1.274-4.967)	0.008	1.880 (0.835-4.231)	0.127
Stage:					
I/II	12 (10.2)	1.0 (Ref)		1.0 (Ref)	
III/IV	106 (89.8)	2.702 (.836-8.731)	0.097	1.032 (0.237-4.500)	0.966
PEG 10:					
Low	35 (29.7)	1.0 (Ref)		1.0 (Ref)	
High	83 (70.3)	3.240 (1.505-6.979)	0.003	2.982 (1.278-6.957)	0.011

CI, Confidence interval; Bold, Significant association (P<0.05); Reference category, 1.0; * Others, Tongue, Alveolus, Floor of mouth, Lip, soft palate; **Adjusted for age, sex and Clinicopathological parameters.

Several studies have demonstrated that PEG10 expression can be regulated by the proto-oncogene, c-MYC, via the binding of the c-MYC oncoprotein to the E-box-containing region of the first intron of PEG10 (Li et al., 2006; Ip et al., 2007; Jie et al., 2007). Previous reports indicated that the expression of PEG10 was significantly associated with poor survival in gall bladder carcinoma and hepatocellular carcinoma patients (Liu et al., 2014; Bang et al., 2014). It was also found that overexpression of PEG10 enhanced cell proliferation, migration and invasion in breast cancer (Li et al., 2016) and it plays a crucial role in human lung cancer proliferation, progression, prognosis and metastasis as well (Deng et al., 2014).

Liu et al., (2014) demonstrated that overexpression of PEG10 showed significant association with large tumor size, lymph node metastasis and advanced tumor stage and worse overall survival in patients with adenocarcinoma of gall bladder. In current study, we found the significant over expression of mRNA PEG10 in advanced stage and lymph node metastasis. This observation reflects the mRNA activity of PEG10 and its association with the tumor progression and poor prognosis in OSCC.

Currently, there are no specific biomarkers available that can reliably predict the outcome of radiotherapy treatment response in OSCC. Ishigami et al., (2006) revealed that inhibition of

PEG10 gene enhances radiosensitivity in human oral squamous carcinoma cell line. No single study on OSCC patients has revealed significant association of PEG10 mRNA expression with radiotherapy treatment response. In this study, significantly higher mRNA expression of PEG10 was found in non responder when compared to responders undergoing radiotherapy indicating that increase in the expression of PEG10 is associated with poor response in OSCC patients.

No previous study demonstrated any association between overexpression of PEG10 and clinical outcome as well as survival in oral cancer patients. In the current study, we observed the association between overexpression of mRNA PEG10 and clinical response and as well as poor survival. This implies that significance of using PEG10 as a predictive marker for response. These observations may further provide support that PEG10 is involved in the invasion and nodal metastasis of OSCC and may play a decisive role in the overall therapeutic outcome in OSCC.

We conducted the study in 118 oral cancer patients from India and found that PEG10 is overexpressed in OSCC patients. We also observed poor survival in patients where PEG10 were overexpressed as compared with low expression, suggesting the possibility of resistance to radiation in these patients and documenting the prognostic role of over expressed PEG10 in OSCC. However, the limitation of this study is the expression of PEG10 may or may not translate to patients of other ethnicities due to variability in genetic constitution and disease presentation differences. It requires more studies with larger patient populations to understand genetic variability of PEG10 expression among individuals in order to develop useful therapeutic approach and its correlation with radioresistance.

PEG10 may help in finding the pattern of radio-resistance in OSCC cases on the basis of clinicopathological parameters and could be found suitable for the use as a molecular marker to evaluate the response of OSCC undergoing radiotherapy. In conclusion, we observed significant increase in the mRNA expression of PEG10 in OSCC patients when compared to matched controls. We also found that overexpression of PEG10 gene was found to be significantly higher in lymph node positive when compared to lymph node negative OSCC patients. The PEG10 mRNA expression was significantly higher in non responders as compared to responders patient after radiotherapy treatment. Since the overexpression of PEG10 mRNA levels alter many signaling pathways found to be involved in the development and prognosis of OSCC, which may lead to the consideration that altered level of this gene may be helpful in predicting radiotherapy treatment response in OSCC patients. These results support that PEG10 could be used as potential biomarker for the OSCC patients undergoing radiotherapy treatment.

Statement conflict of Interest

The authors declare no conflict of interests for this research.

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