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Deregulation of Beclin 1 in patients with tobacco-related oral squamous cell carcinoma

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ABSTRACT

Autophagy is a physiologically regulated and evolutionary conserved process that plays a critical role in degradation of cytoplasmic proteins and other macromolecules within the lysosomes. Beclin-1, the mammalian orthologue of yeast Atg6, is an important mediator of autophagy that has been studied in many human cancers. However, the expression of Beclin-1 has not yet been investigated in oral cancer. We for the first time investigated the expression of Beclin-1 in serum and tissues and correlated it with the clinic-pathological features of oral cancer patients. m-RNA expression of Beclin-1 was evaluated in tumor and normal areas of surgical specimens from 10 oral cancer patients by real-time PCR. Approximately, 8-fold lower expression ($p < 0.001$) of Beclin-1 mRNA was observed in tumor tissue as compared to the normal tissue. Serum levels of Beclin-1 were evaluated by SPR and ELISA. No significant difference was observed in serum Beclin-1 levels in patients as compared to healthy subjects, similarly no correlation was found between serum levels and clinic-pathological parameters such as stage, lymph node involvement and tumor size. Our results demonstrate that down-regulation of Beclin-1 may play an important role in the development and progression of oral cancer possibly by dysregulation of autophagy in tumor cells.

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1. Introduction

Oral squamous cell carcinoma (OSCC), which falls in the head and neck cancer category, represents one of the six most common cancers in the world [1]. Word wide, there are 400,000 new cases each year and most of these cases have been reported from Asian countries [1]. In India, it is the leading cancer among males and the third most common malignancy in females [2]. In spite of many advances in treatment modalities, the five-year survival rate is considerably lower than that for other cancers, such as those of colorectal, cervix and breast origin [3]. There is a lack of early detection markers and many patients show drug-resistance to the available chemotherapy [4]. Therefore, determining the expression profiles of key molecules involved in the survival pathways holds a great deal of promise in the diagnosis and prognosis of these patients.

Autophagy is a highly regulated process of degradation and recycling of cellular constituents, participating in organelle turnover, and contributing in the bioenergetic management of starvation [5]. Besides its specific role in adaptation to nutrient deprivation and growth factor depletion [6], it is also observed in numerous pathological processes, including myopathy [7], neurodegenerative disorders [8], tuberculosis [9], cancer [10], and other clinical conditions. Accumulating evidences point to the importance of autophagy in the regulation of cancer development and progression and in determining the response to anticancer therapy.

Beclin 1, the mammalian orthologue of the yeast Atg6/Vps30 gene, is an essential mediator of autophagy [11–13] and also has an important role in tumor suppression [12,14]. It is part of a type III phosphatidylinositol 3-kinase complex required for autophagic vesicle formation [12,15–17]. The human Beclin 1 gene has been mapped to a tumor-susceptibility locus on chromosome 17q21, and its heterozygous disruption resulted in reduced autophagy, increased cellular proliferation and spontaneous tumor development in mice [14,18]. It has been reported to be monoallelically deleted in approximately 40% of prostate, 50% of breast, and 75% of ovarian cancers [11,19]; moreover, decreased expression of Beclin 1 is also observed in many types of cancers including human colon cancer [20], brain tumors [21], and hepatocellular carcinoma [22]. Thus it appears reasonable to presume an important role of Beclin 1 gene in promoting oncogenesis. In contrast, increased expression of Beclin 1 has been detected in malignant colorectal and gastric epithelial cells compared to normal colon and stomach mucosa

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[23]. Thus it appears that the tumor suppressor function of Beclin 1 may be tissue-specific [24].

To the best of our knowledge, the expression of Beclin-1 has not been fully studied in oral cancer. We therefore investigated the expression of Beclin-1 in serum and tumor tissues and correlated the results with clinico-pathological characteristics of oral cancer patients.

2. Materials and methods

2.1. Patients and controls

This study was performed on 70 patients of biopsy proven oral squamous cell carcinoma of various sites (Table 1) attending the Out Patient Department of Otorhinolaryngology of the All India Institute of Medical Sciences (AIIMS, New Delhi, India). Tumor, node and metastasis (TNM) classification and clinical staging of the tumor were done as per American Joint Committee on Cancer criteria. None of the patients had received any anti-inflammatory or anti cancer treatment prior to the study. The control group included 60 age, sex and ethnicity matched healthy subjects. Ten millilitre of blood was aseptically collected from each individual by venipuncture; RNA and serum was separated and stored at −20 °C until use.

The study protocol was approved by 'Ethics Committee' of AIIMS and informed consent was obtained from all participants.

2.2. Cloning, expression and purification of Beclin-1

True Clone™ (Origene, USA) was used as template for PCR amplification of full-length (1353 bp) open reading frame (ORF) of *beclin-1* cDNA. The sense primer (5′-GGATCCATGGGAGGGTCTAA-GACGTCC-3′) containing a *BamH1* site (underlined) and the anti-sense primer (5′-TCATTTGTTATAAAAATTGTGAGGAC-3′) were used in a PCR reaction mixture (25 μl) containing 1× PCR buffer, 1 mM $MgSO_4$, dATP, dGTP, dCTP, dTTP (0.3 mM each), 50 ng of the template DNA, 12.5 μmol of each primer, and 1 U of *Pfx* DNA polymerase (Invitrogen Life Technologies, USA). The resulting blunt-ended PCR products were resolved on 0.8% agarose gel. Gel purified 1353 bp product was modified using the standard A-tailing procedure and directly ligated into the pGEM-T Easy vector using the commercially available kit (Promega, USA). The plasmids were digested with *BamH1* and *Sal1* (NEB, USA) and gel-purified fragments were then inserted into bacterial expression vector, pET-28a after *BamH1* and *Sal1* digestion.

In order to obtain full-length protein BL21 RPC + cells were made competent and transformed with expression vector containing the insert. Cells were grown until they reached 0.6 OD and then were induced for 4 h with 1.0 mM isopropyl-*P*-thiogalactoside (IPTG). The bacterial pellet thus obtained, was dissolved in lysis buffer containing 50 mM Tris–Cl (pH 7.0) and protease inhibitor cocktail (Sigma Aldrich, USA) and sonicated on ice until it was no longer viscous. The preparation was centrifuged for 20 min at 8000 rpm (4 °C) and the pellet obtained was dissolved in urea containing buffer (50 mM Tris–Cl (pH7.0) and 8 M urea) and kept in shaking conditions at 37 °C for 1 h to dissolve the inclusion bodies and to facilitate release of protein. It was then centrifuged at 8000 rpm for 20 min and the supernatant containing the protein was loaded onto a Ni^{2+}-NTA column (Quiagen), pre-equilibrated with urea buffer (50 mM Tris–Cl (pH 7.0) and 8 M urea). Flow through was collected and the resin was washed with 10 bed volume of lysis buffer (8 M Urea, 150 mM NaCl and 20 mM Tris–Cl (pH 8.0), 1 mM PMSF, 20 mM Imidazole), 10 bed volume of washing buffer (8 M Urea, 150 mM NaCl and 20 mM Tris–Cl (pH 8.0), 1 mM PMSF, 40 mM Imidazole). Elution was performed with 8 M Urea, 150 mM NaCl and 20 mM Tris–Cl (pH 8.0) and 300 mM Imidazole. Urea and imidazole were removed gradually over 48 h of dialysis at 4 °C. Finally Beclin-1 was present in 50 mM Tris and 150 mM NaCl. It was then aliquoted into 50 μl fractions and stored at −70 °C until further use.

2.3. Separation of serum from blood

Ten milliliters of venous blood was collected from each individual in vaccutainers under aseptic conditions. It was allowed to settle down for 1 h at room temperature. The blood was then centrifuged at 3000 rpm for 20 min to settle down the pellet. Serum was collected from the supernatant and stored at −70 °C in multiple aliquots until further use.

2.4. RNA isolation from blood and tumour tissues and cDNA synthesis

Total cellular RNA was extracted from peripheral blood mononuclear cells (PBMC) of 10 patients and 10 healthy subjects and tissues from tumour and normal region of patients using Trizol reagent (Sigma, USA) as per the manufacturer's instructions. The amount of RNA was quantified spectrophotometrically at 260 nm absorbance and the purity of RNA was evaluated by the ratio of the absorbance at $A_{260/280}$. About 1–5 μg of total RNA was reverse-transcribed using the Revertaid H minus cDNA synthesis kit (Fermentas, USA) as per the manufacturer's instructions.

2.5. Quantitative real-time PCR for Beclin-1

To quantitatively determine the level of mRNA expression of *Beclin-1*, the primers were tested empirically for amplification from 100 ng of cDNA. Optimal annealing temperature was determined by testing primers for generation of single band on gels. The relative amplification efficiencies of the primers were tested and found to be similar.

Quantitative real-time PCR reactions were performed on the I cycler (Bio-rad, USA), iQ5 Multicolor Real-time PCR Detection system. For SYBR Green based PCR reaction (Biorad, USA), the reaction contained, 5 pmol forward and reverse primers, 100 ng cDNA 1× SYBR Green mix and water added upto 25 μl. The primers for Beclin-1, F-5′-CAA GAT CCT GGA CCG TGT CA-3′, R-5′-TGG CAC

Table 1
Clinicopathological features of patients with oral squamous cell carcinoma.

Characteristics	Patients
Age(yrs)	
Range	22-70
Mean ± SD	50.4 ± 13.11
	No. (%)
Gender	
Males	68(97.2%)
Females	2(2.8%)
Tobacco usage	
Users	63 (90%)
Non-users	07 (10%)
Tumour status	
T1 + T2	24(34.2%)
T3 + T4	46(65.8%)
Node status	
N0	29(41.4%)
N+	41(58.6%)
Clinical stage	
Stage I + II	19(27.1%)
Stage III + IV	51(72.8%)
Site of tumor	
Buccal mucosa	28 (40.3%)
Tongue	25 (35.6%)
Lip and alveolus	17 (24.1%)

TTT CTG TGG ACA TCA-3′ and β-actin, F-5′-AGA AAA TCT GGC ACC ACA CC-3′, R-5′-TAG CAC AGC CTG GAT AGC AA-3′. The PCR cycling parameters included initial denaturation at 95 °C for 10 min, annealing at 56 °C for 25 s and extension at 72 °C for 30 s. After amplification, a melting curve analysis was performed by collecting fluorescence data using β-actin as an internal control. The comparative threshold cycle method ($2^{-\Delta\Delta CT}$), which eliminates the need for standard curves, was used to enable quantification of the mRNA of these genes. All tests were performed in duplicate.

2.6. Estimation of serum Beclin-1 by surface plasmon resonance (SPR) technique

The serum levels of Beclin-1 were measured by the biosensor-based SPR technique using an automatic apparatus SensiQ Pioneer (ICx Technologies, USA) as described earlier [4]. Briefly, in the first step, preparation of carboxylated (COOH1) sensor chip (ICx Nomadics, USA) surface and immobilization of the anti-Beclin-1 antibody (BD Biosciences, USA) was performed by the method of amine coupling. Experiments were performed at 25 °C in HBS-EP buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA and 0.005% P20 (surfactant). 0.4 M EDC and 0.1 M NHS solutions were mixed and diluted to 1:100 in water and injected (2 minutes at 50 μL/minute) over the surface to activate it. Next, anti-Beclin-1 antibody (typically 100 μg/ml in 10 mM acetate buffer, pH 4.5) was injected (15 minutes at 10 μL/minute). One mloar ethanolamine, pH 8.5, was then injected for 4 minutes at a flow rate of 25 μL/minute in order to cap any residual NHS-esters. Under these conditions 9743 RU of anti-Beclin-1 antibody was immobilized on the sensor chip.

Next, six different concentration of rBeclin-1 in HBS-EP buffer (pH 7.4) were passed over the immobilized Beclin-1 antibody and the corresponding RU were obtained. A standard curve was plotted with RU against the concentrations of rBeclin-1.

Serum samples diluted in HBS-EP buffer (1:99) was passed (40 μl) over the immobilized Beclin-1 antibody on the activated sensor chip at the flow rate of 10 μl/min and RU for each sample was recorded. After each binding event the sensor surface was regenerated with 1 mM NaOH and 10 mM glycine-HCl. HBS-EP buffer and recombinant Beclin-1 were run along with the serum as negative and positive controls respectively. The concentration of the serum Beclin-1 was derived from the standard curve.

2.7. Circulating levels of Beclin-1 by ELISA

ELISA was performed with the collected serum samples from patients and the healthy volunteers according to the following protocol. Hundred μl of 1:10 dilution of serum in coating buffer was coated for 16 h at 4 °C. Blocking of plate was done with 3.5% BSA at 37 °C for 2 h after washing with 0.05% PBS-T (3 times). 1:6000 dilution of monoclonal anti-Beclin-1 antibody (from BD Biosciences) was made in blocking buffer and 100 μl was added to each well and incubated at 37 °C for 2 h. After three washes with 0.05% PBS-T, and 100 μl of 1:10,000 dilution of HRP conjugated anti-mouse antibody was added to each well and kept at 37 °C for 2 h. After washing 100 μl of HRP-substrate was added to each well and incubated at 37 °C for 10 min. The reaction was stopped and the plate was analyzed at 490 nm.

2.8. Immunohistochemistry

Immunohistochemical detection of Beclin-1 was carried out on 5 micron paraffin sections using Streptavidin – biotin Universal Detection Kit (Immunotech, France). Briefly, after sequential rehydration through acetone, ethanol and distilled water, the endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol at room temperature for 5 minutes. The sections were washed with water and antigen was retrieved by heating sections in microwave (700 W) in 10 mM citrate buffer (pH 6.0) for 20 min. Before incubation in antibody solution, the sections were covered with protein blocking agent (PBA) for 5 min at room temperature, excess PBA was taped off and sections were covered with mouse monoclonal anti-Beclin-1 antibody (1:50 dilution; BD Biosciences, USA) for 2 h in a wet chamber. The sections were washed and treated with biotinylated secondary antibody (anti-mouse 1gG) for 30 min at room temperature followed by treatment with streptavidin-peroxidase for 30 min at room temperature. The colour was developed using freshly prepared chromogen (DAB). The sections were counter stained with Mayer's haematoxylin and mounted with D.P.X. mountant. Negative control was prepared similarly by omitting primary antibody. Slides were examined under light microscopy and scored positive in case with positive staining of more than 5% cells in three randomly selected areas of dense staining.

2.9. Statistical analysis

All values are expressed as mean ± S.D. Statistical difference between two variables was evaluated by unpaired t-test. The values were considered significant if the probability was <0.05 ($P < 0.05$).

3. Results

3.1. Clinical profile of oral cancer patients

The clinico-pathological features of oral cancer patients have been shown in Table 1. The age of the patients ranged from 22 to 70 years with a mean age of 50.4 ± 13.11 years. Amongst 70 patients 68 (97%) were males and 63 (90%) were tobacco users for at least 6 months. As per TNM staging, majority (72.8%) of patients presented with late stage (stages III and IV) tumours. Buccal mucosa was the most commonly affected sites (40.3%) followed by tongue (35.6%), besides lip and alveolus being the less commonly affected sites (24.1%).

3.2. Lower m-RNA expression of Beclin-1 in human oral cancer tissues

Beclin-1 mRNA expression was evaluated in surgical specimens from 10 oral cancer patients. Results showed a remarkable decrease in Beclin-1 expression in tumor area as compared to those of the normal area (Fig. 1). The expression of Beclin-1 in cancer tissues was approximately 8 ($P < 0.0001$) times lower as compared the normal tissue. However, no significant difference was found in mRNA levels of Beclin-1 from PBMCs of oral cancer patients and healthy subjects (data not shown).

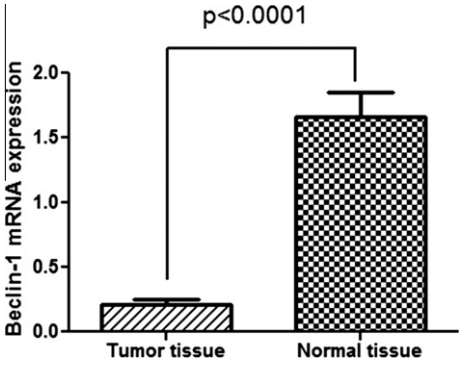

Fig. 1. Beclin-1 m-RNA expression in tumor and normal area of oral tissues.

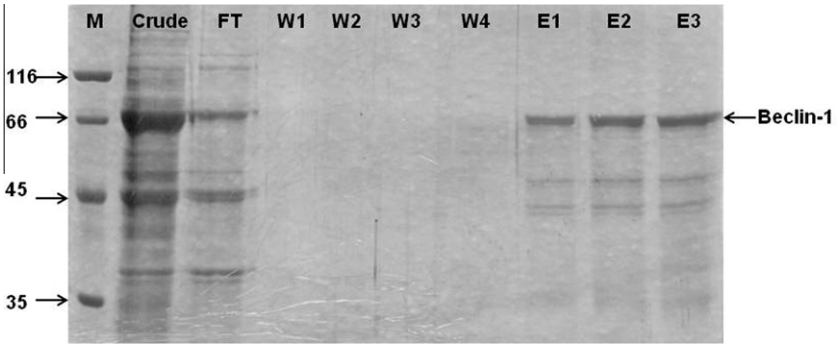

Fig. 2. SDS–PAGE showing purification of Beclin-1 protein through Ni-NTA column. M: protein molecular weight marker, Crude: IPTG-induced *E. coli* cell lysate which was passed through Ni-NTA column, FT: Flow Through obtained after passing crude lysate from Ni-NTA column, W1–W4: washes 1-4, E1–E3: Eluents from the Ni-NTA column containing the Beclin-1 protein.

3.3. Cloning, expression and partial purification of Beclin-1

The beclin-1 gene was successfully cloned into pET28a expression vector and expressed in *E. coli* BL21 RPC + strain. The his-tagged protein was purified from the lysate of *E. coli* using Ni-NTA column chromatography. Fig. 2 shows the different eluted fractions of the protein. The purified protein was then used for further experiments as standard to draw standard curve.

3.4. Circulating levels of Beclin-1 estimated by SPR

The serum levels of Beclin 1was quantitated by SPR technique. Fig. 3A shows the immobilization of Beclin-1 antibody on the surface of the sensor chip. The SPR signal for immobilization was 9743 RU. A standard curve of concentration of Beclin-1 vs RU was plotted by passing six different concentration of pure recombinant Beclin-1 as shown in Fig. 3B. The concentration of serum Beclin-1 in patients was derived from the standard curve using RU obtained from binding of serum over the Beclin-1 antibody (Fig. 3C). Oral cancer patients showed a decreasing trend in circulating Beclin-1 levels as compared to the healthy subjects (17.3 ± 4.7 vs. 19.1 ± 3.4 μg/ml; $p < 0.16$), although the difference was not statistically significant (Fig. 4A). However, circulating Beclin-1 levels did not show significant variation in relation to tumor size, cervical lymph node involvement and stage of disease as an independent variable.

Immunohistochemistry of biopsy samples from tumor and normal areas of oral cancer patients revealed weak expression of Beclin-1 in normal areas but none in the tumor areas (data not shown).

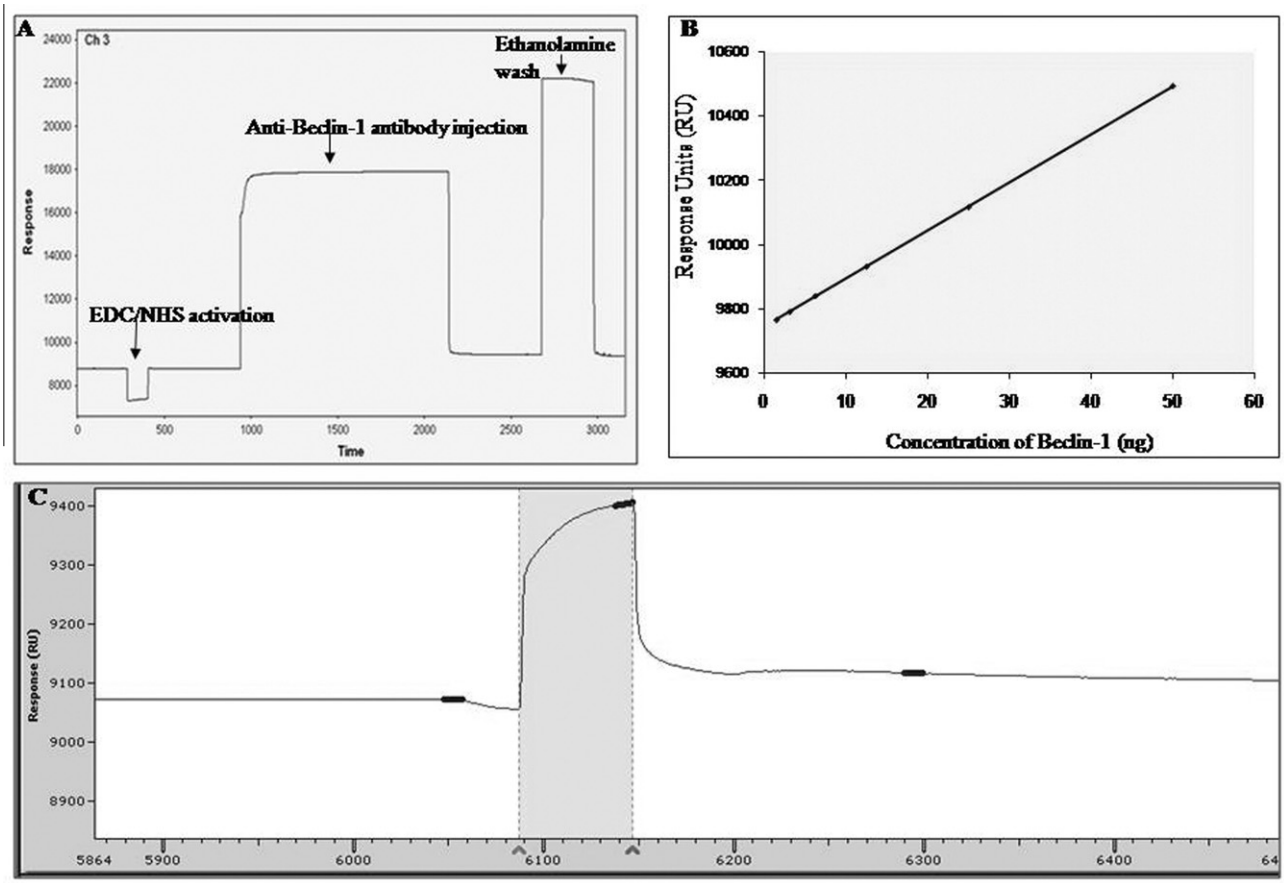

Fig. 3. Evaluation of serum levels of Beclin-1 by SPR. (A) Sensogram showing immobilization of Beclin-1 antibody on the sensor chip. The Beclin-1 antibody was immobilized on the sensor chip by amine coupling method as described in the materials and method section. (C) Standard curve of RU vs concentration of Beclin-1 (μg/ml). (C) Response curve for binding of serum Beclin-1 to the anti-Beclin-1 antibody on the sensor chip.

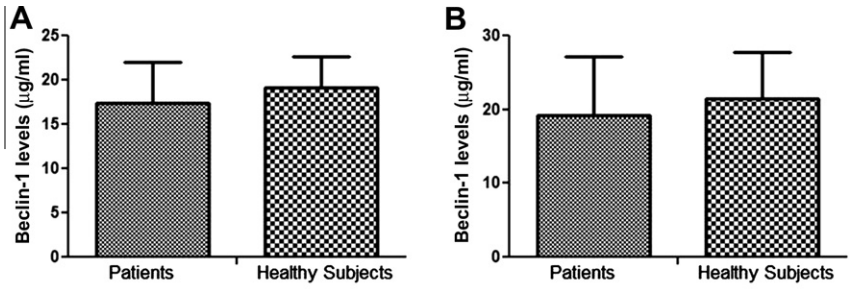

Fig. 4. Bar diagrams showing mean ± SD of serum levels of Beclin-1in oral cancer patients and healthy subjects by (A) SPR and (B) ELISA.

Table 2
Comparitive serum levels of Beclin-1 by SPR and ELISA.

Group	*n*	Assay	Mean ± SD
Healthy subjects	20	SPR	19.10 ± 3.4
	60	ELISA	21.50 ± 6.3
Patients	30	SPR	17.37 ± 4.77
	70	ELISA	19.25 ± 7.9
Tumor size			
T1 + T2	13	SPR	17.53 ± 4.7
	24	ELISA	22.23 ± 6.7
T3 + T4	17	SPR	17.07 ± 5.02
	46	ELISA	22.0 ± 6.3
Lymph node			
N0	13	SPR	17.38 ± 4.74
	29	ELISA	22.91 ± 7.6
N+	17	SPR	16.91 ± 5.05
	41	ELISA	21.60 ± 5.5
Clinical stage			
Early	7	SPR	17.58 ± 3.6
	19	ELISA	23.07 ± 6.4
Late	23	SPR	17.07 ± 5.12
	51	ELISA	21.7 ± 6.5

3.5. Serum levels of Beclin-1 by ELISA

Fig. 4B shows the serum levels of Beclin-1 by ELISA. As with SPR, a decreasing trend of Beclin-1 levels was seen in patients as compared to healthy subjects. However, no correlation was found between Beclin-1 serum levels and tumor size, cervical lymph node involvement and stage of the disease in oral cancer patients by either technique (Table 2). SPR data was almost same as that obtained by ELISA.

4. Discussion

The role of autophagy in cancer development and progression is still unknown. Beclin-1 levels appear to be one of the critical factors that affect the induction of autophagy. It serves as a scaffold, which, by binding to other proteins, forms pre-autophagosomal structures. In this study we have demonstrated approximately eight times lower Beclin-1 m-RNA expression in tumor areas of oral tissues as compared to the normal area. However, no significant difference was found in Beclin-1 mRNA levels from PBMCs of patients and healthy subjects. Majority of reports have demonstrated decreased beclin-1 expression in various human malignancies as compared to adjacent normal tissues [11,21]. These studies also point to the role of Beclin-1 in the regulation of cancer development and progression. Higher Beclin-1 levels also have been linked to better prognosis [21,25–30]. Indeed, allelic deletions of Beclin-1 are common in breast cancer cell lines and promote tumourigenesis in experimental models [14]. Moreover, micro RNAs, such as miT-30a, also seem to be involved in the negative regulation of Beclin-1 [31].

In one study, Ahn et al. [23] reported increased Beclin-1 expression in colorectal and gastric cancer cells as compared to normal epithelial cells. Therefore, it appears that both under- and over expression of Beclin-1 may exist in human carcinomas. There are several proposed mechanisms to describe how autophagy induced by beclin-1 prevents tumorigenesis. While autophagic cell death could be one of the reasons, it could also eliminate damaged organelles to remove sources of genotoxic materials such as damaged DNA products. In contrast, cancer cells use autophagy for its own survival by targeting oxidative sources like damaged mitochondria and other organelles [32].

In this study we for the first time, estimated serum Beclin-1 levels by SPR and ELISA. A decreasing trend of serum levels of Beclin-1 were observed in patients as compared to healthy subjects by both techniques although the difference was statistically not significant. However, no correlation was observed between Beclin-1 levels and clinical parameters such as tumor size, cervical lymph node involvement and stage of oral cancer as independent variables. Similar to our results, Ahn et al. [23] reported no significant correlation between beclin-1 expression in gastrointestinal neoplasms and clinicopathological characteristics, such as invasion, metastasis, and stage. Beclin-1 is reported to reside in the trans-Golgi network, endoplasmic reticulum and the mitochondria [15,33]. It is therefore expected that Beclin-1 may not enter into circulating body fluids. In this study we used SPR technique for quantitative measurements of Beclin-1 in the serum and compared the data with that observed by ELISA. SPR is a technique mostly used for studying protein–protein interactions. In our earlier study we have shown that it can be used for quantitative assessment of serum levels of COX-2 in oral patients and healthy subjects [4]. The main advantages of SPR over ELISA are real-time detection of proteins, requirement of small sample quantities and re-usability of the sensor surface once the antibody is immobilized. Results of the present and our earlier studies [4] suggest that SPR can be developed as an additional technique for quantitative assessment of different proteins in different body fluids.

In conclusion, our limited data demonstrates that expression of Beclin-1 is downregulated in oral squamous cell carcinoma. m-RNA and/or protein levels of Beclin-1 from tissue as compared to serum levels could play a possible role in the development and progression of oral cancer possibly by inhibition of autophagic pathway.

Acknowledgments

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