Investigation of pS6 protein as a potential prognostic biomarker in oral squamous cell carcinoma

Dhanalakshmi Tamatam

Under the guidance of:
Principal supervisor: Associate Prof. Dipak Sapkota (UiO)
Co-supervisor: Prof. Daniela Elena Costea (UiB)

Master's thesis submitted as a part of the Master of Philosophy Degree in International Community Health
120 credits

Department of Community Medicine
Institute of Health and Society
Faculty of Medicine
University of Oslo, Norway

May 2020

This page is intentionally left blank

Acknowledgements

I would like to express gratitude to God for blessing me all through my career and giving me strength to achieve my aims and dreams.

It is with a humble sense of gratitude that I acknowledge my esteemed main supervisor Associate Prof. Dipak Sapkota, for his valuable guidance, unlimited support, constructive suggestions and professional insight which helped to bring this thesis to a successful completion. I am also indebted for his kindheartedness, tireless pursuit for academic excellence throughout my master's thesis. I also would like to acknowledge and express gratitude to my co-supervisor Prof. Daniela Elena Costea for her continuous support, giving me the benefits of her knowledge and experience and friendly supervision. I sincerely thank them for their guidance, endless support, constant encouragement, involvement with their originality has triggered and nourished my intellectual maturity that I will benefit from, for a long time to come.

I am indeed thankful to Associate Prof. Burcu Senguven Tokozlu, who always had a kind word of encouragement and advice. Her critical advice and teachings helped me tremendously in my endeavor. I express my earnest gratitude to Siren Fromreide, chief engineer at Gade's pathology laboratory, University of Bergen, for her invaluable support and teachings involved at the start of the thesis project. I would like to thank my group members and especially Sushma Pandey Dhakal for always being supportive and for teaching to use QuPath software. I would personally thank my course co-coordinator Terese Eriksen for being very patient and providing timely information and encouragement throughout my course. Not to forget the program leader Associate Prof. Elia Mmbaga for his valuable suggestions, encouragement and cheering nature.

I thank Prof. Ganesh Acharya for getting me connected with my main supervisor at the right time.

I would like to thank my parents and my brother who have been pillars of strength throughout my life. Without their love, blessings, encouragement and sacrifice I would not have reached this stage, cannot thank enough. I would take this opportunity to thank Abhisheak and his parents who always stood by.

Finally, I dedicate this piece of my work to all the oral squamous cell carcinoma patients out there.

Dhanalakshmi Tamatam
Oslo, Norway
May 2020.

Abbreviations

AJCC	American Joint Committee on Cancer
DOI	Depth of invasion
ENE	Extranodal extension
FFPE	Formalin fixed paraffin embedded
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HSV	Herpes simplex virus
IHC	Immunohistochemistry
IARC	International Agency for Research on Cancer
mTOR	mammalian (mechanistic) Target of Rapamycin
mTORC1	mammalian (mechanistic) Target of Rapamycin Complex 1
mTORC2	mammalian (mechanistic) Target of Rapamycin Complex 2
OSCC	Oral squamous cell carcinoma
pS6	Phospho-S6-ribosomal protein
PI3K	Phosphoinositide-3-kinase
REMARK	Recommendations for Tumor Marker Prognostic Studies
ROI	Region of interest
SSE	Stratified squamous epithelium
ST	Smokeless tobacco
TSNA	Tobacco-specific-nitrosamines
TNM	Tumor-node-metastasis
TIF	Tumor invading front
TC	Tumor center
UICC	Union for International Cancer Control

This page is intentionally left blank

Abstract

Head and neck cancers are malignancies that arise in pharynx, larynx, paranasal sinuses, nasal cavity and oral cavity (1). Oral cancers account for 40% of head and neck cancers and consist of malignancies arising from buccal mucosa, gingiva, floor of the mouth, tongue, palate, and lip (1, 2). The most common histological variant of oral cancer is oral squamous cell carcinoma (OSCC). The global incidence rate of OSCC is high particularly in the developing countries (3). It is growing at an alarming rate, indicating the need for more efficient methods for prevention, early detection and management. Despite the easier access and clear visibility of the oral cavity, OSCCs are usually diagnosed at advanced stage. In addition, the metastatic spread would also affect the 5-year survival and most of the times recurrence and metastasis is seen in the first two years after initial diagnosis (4). Patients who survive more than 5 years have high risk for recurrence, loco-regional metastasis to lymph nodes and often have a compromised quality of life.

Though there is advancement in diagnostic and treatment strategies, no reliable and established methods are currently available to stratify the OSCC patients and to predict prognosis. From few decades, tumor-node-metastasis (TNM) staging and histopathological grading systems were the standard tools used to predict prognosis and to guide the selection of appropriate treatment methods. More recently, depth of invasion (DOI) and extranodal extension (ENE) parameters were included in the latest edition of TNM staging. Despite their inclusion, they still do not provide a robust stratification of OSCC patients. There were also proposal to use a few other grading systems (various pathological parameters), such as tumor budding, pattern of invasion and perineural invasion as a prognostic parameters for OSCC, but they were shown to be indecisive and disputable (5-7).

Ribosomal protein S6 is a key downstream molecule of mechanistic/mammalian target of rapamycin (mTOR) pathway in OSCCs. Despite the fact that phosphorylation of S6 (pS6) is one of the end-point indicators of the activation of mTOR pathway, the major oncogenic (mitogenic) pathway in OSCC, there is a paucity of studies done on its prognostic significance in OSCC. Therefore, using immunohistochemistry (IHC), the

current study aimed to examine the expression profile and prognostic significance of pS6 (at 235 and 236 serine sites) protein in OSCC. Cytoplasmic expression of pS6 at Ser235/236 was detected in 80.2% of the OSCC cases at tumor center (TC) and in 66.3% of samples at the tumor invading front (TIF) region. The higher expression of expression of pS6 at TIF correlated with the worst pattern of invasion (p=0.012). Additionally, higher expression of pS6 at TIF was marginally associated with reduced overall- and recurrence free-survival probabilities for OSCC patients. In conclusion, our study corroborates previous findings indicating that activation of the mTOR signaling is a common event in OSCC. Correlation between high pS6 expression at TIF with the worst pattern of invasion and reduced probabilities for overall and recurrence free survival indicate that activation of mTORC1 arm of mTOR pathway might contribute to an aggressive tumor phenotype. In future, validation of these findings using a large cohort of patients might be useful in prognostication and guiding therapy for OSCC patients.

Table of Contents

1. Background 9
   1.1. Histology of normal oral mucosa 9
   1.2. Epidemiology of OSCC 11
   1.3. Etiology/risk factors associated with OSCC 12
   1.4. Pathogenesis of OSCC 14
   1.5. Management and prognosis of OSCC 18
   1.6. Histopathological prognostic indicators of OSCC 20
   1.7. Cancer biomarkers 21

2. Justification and aims of the study 23
   2.1 Hypothesis 23
   2.2 Aim of the study 23
   2.3 Specific aims 23

3. Materials and Methods 24
   3.1 Ethical considerations 24
   3.2 Patient Cohort 24
      3.2.1 Inclusion criteria 24
      3.2.2 Exclusion criteria 25
   3.3 Study design 25
   3.4 Statistical power calculation 25
   3.5 Choice of method 25
   3.6 The use of FFPE archival tissues 26
   3.7 Selection and validation of primary antibody 26
   3.8 Specimen preparation 27
   3.9 IHC protocol 27
      3.9.1 Scanning of slides and digital quantification of IHC stained slides 29
      3.9.2 QuPath cell detection 29
   3.10 Statistical Analysis 31

4. Results 32
   4.1 Clinicopathological characteristics of the patient cohort 32
   4.2 Expression of pS6 in paratumor epithelium and normal structures 36
   4.3 Expression of pS6 in OSCC 37
   4.4 Association between the expression of pS6 and clinicopathological variables of OSCC 40
   4.5 Association between pS6 expression and OSCC patient survival 40

5. Discussion 43

6. Conclusion 46

7. Limitations 47

8. Future perspectives 47

9. References 48

1. Background

1.1. Histology of normal oral mucosa

The normal oral mucosa is made up of an outer stratified squamous epithelium (SSE) separated from its underlying connective tissue by a basement membrane (Fig. 1). The mucosa of oral cavity can be divided into three main types: masticatory, specialized and lining. The first two types are keratinized while the lining mucosa is non-keratinized (8). The non-keratinized mucosa lacks the process of keratinization and the lining membrane is more elastic in nature (9).

The stratified squamous oral epithelium consists of different cell layers (Fig. 2):

- Stratum Basale
- Stratum Spinosum
- Stratum Granulosum (only in the keratinized mucosa)
- Stratum Corneum (in the keratinized mucosa)/Superficial layer (in the non-keratinized mucosa)

In both the types of mucosa, the most superficial layers are regularly shed by losing its cellular content, contributing to the protective function of oral mucosa. The cycle of superficial shedding is balanced by the regeneration at the basal and para-basal cells, and the balance of the two is crucial to maintain the integrity of SSE (9).

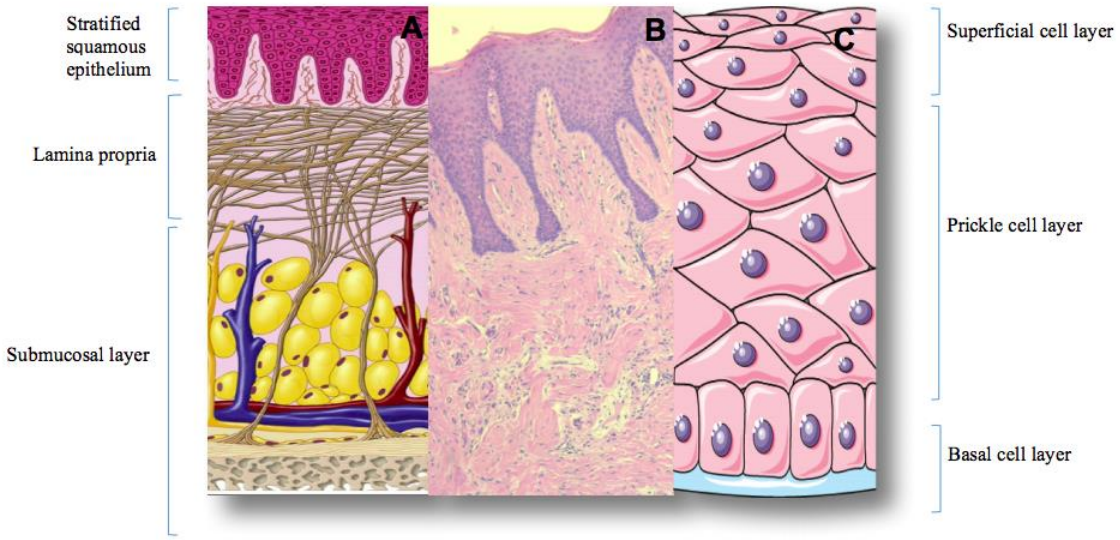


Figure 1: (A) Pictorial representation of different layers of oral mucosa; (B) Hematoxylin and Eosin stained normal human oral mucosa; and (C) Layers in stratified squamous epithelium. Source: Adapted from (10, 11).

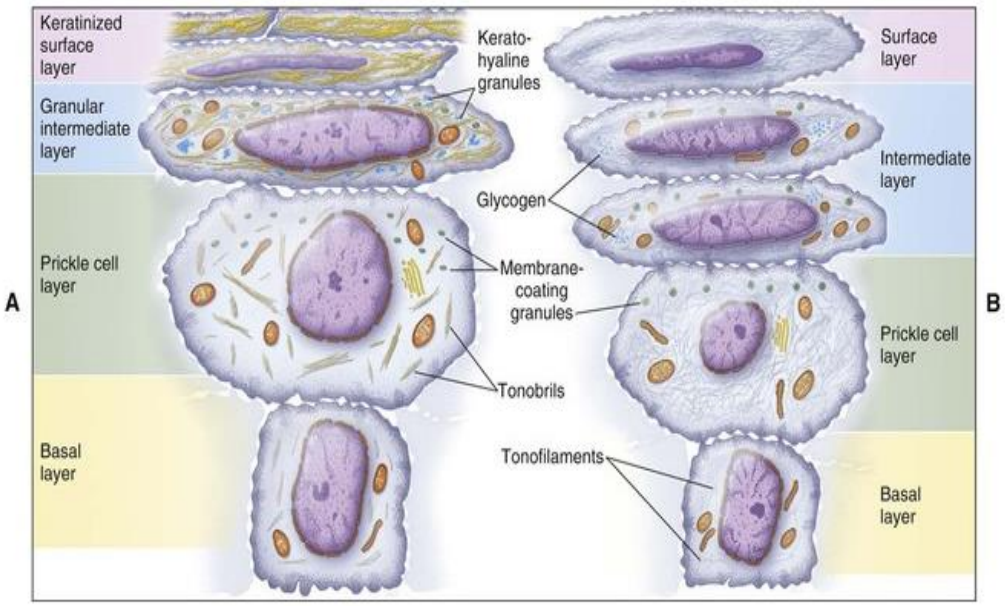


Figure 2: Structural features of oral epithelial cells in consecutive layers. (A) Ortho-keratinized oral epithelium; and (B) Non-keratinized oral epithelium. Source: Adapted from (11).

Oral epithelium has finger like projections called rete ridges that protrude into the lamina propria and form inter-lockings that provide more surface area so that the epithelium can

withstand the masticatory forces without being displaced (8). The cells in the basal layer are attached to the basement membrane by their membrane structures called hemi-desmosomes and are responsible in maintaining the homeostasis of cell proliferation. The entire mitotic activity takes place in the basal and para-basal cell layers, and the cell division is tightly regulated. Few basal cells remain as stem cells that can further respond to stimulus, while few others start dividing into daughter cells. The ones which are committed to differentiate are called transient amplifying cells and through further differentiation and maturation they become adult squamous cells and start moving in upward direction to the more superficial cell layers becoming flatter and finally shed on the surface (5, 8, 12-14).

The underlying connective tissue consists of lamina propria and submucosal layer (Fig. 1). Fibroblasts are the predominant cell type found in the connective tissue. In addition, there are some inflammatory cells, blood and lymph vessels, nerves, structural fibers and minor salivary glands in the connective tissue. In the submucosal layer, the connective tissue is loosely arranged and it consists of muscles, adipose tissue and bone depending on the location within the oral cavity (15).

1.2. **Epidemiology of OSCC**

Head and neck cancers encompass malignancies that arise in the oral cavity, nasal cavity, para nasal sinuses, pharynx and larynx. Oral cancers represent 40% of all head and neck cancers and consist of the malignancies arising in the lip, tongue, floor of the mouth, gingiva, palate and buccal mucosa (1, 2). The most frequent neoplasms arising from the oral epithelium are OSCCs, representing more than 90% of all oral cancers. When oral cancers are combined with the oropharynx cancers, these two together constitute the 8th most common cancer type with estimation of 447,571 new cases and approximately 228,389 deaths every year (16-18). Nevertheless, OSCC is the third most common cancer type in the developing countries (for example, South and Southeast Asian countries: Pakistan, India, Sri Lanka, Taiwan; African countries: Sudan, *etc*) (19). Incidence rate is rapidly increasing in low income countries and about 66% of the OSCC cases reported annually occur in the developing countries. More than half of those cases are from India (20, 21). In Europe, 98% of diagnosed patients are over the age of 40 years (2, 19, 22, 23) (Fig. 3).

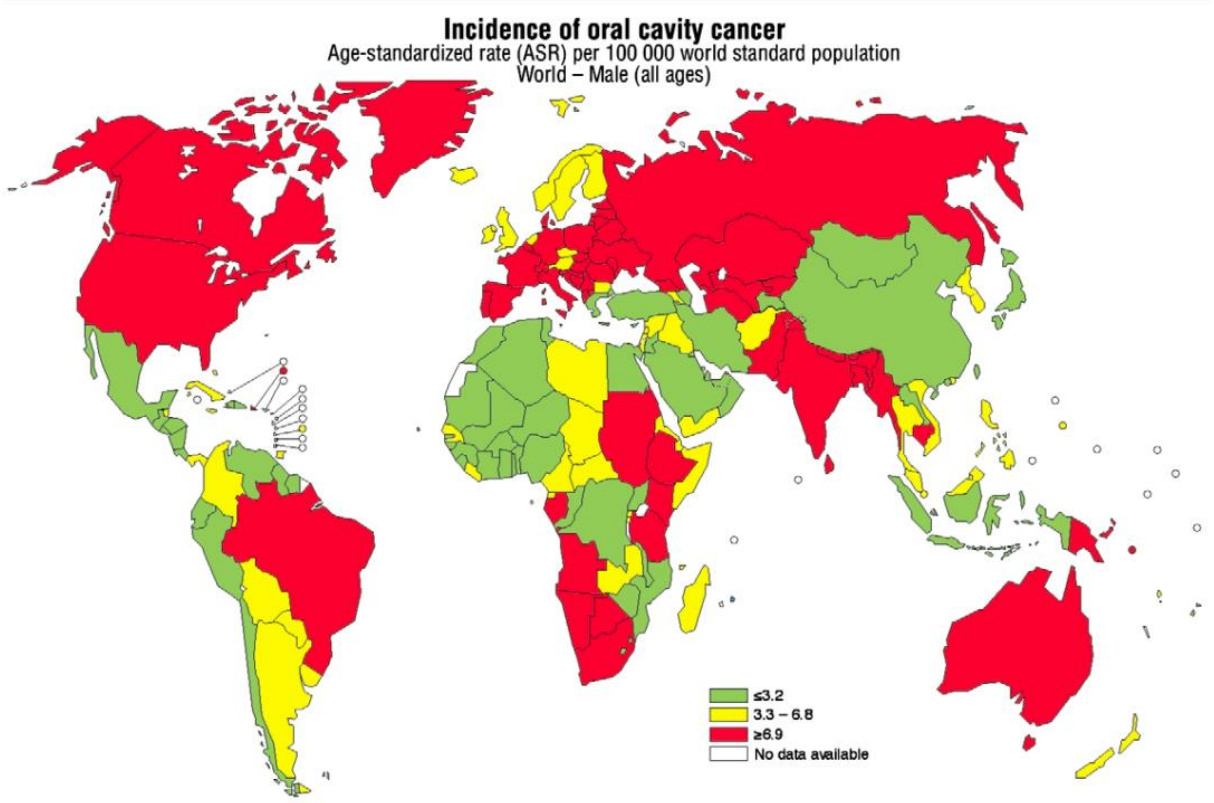


Figure 3: Schematic representation showing the incidence of oral cavity cancer expressed by age-standardized rate among men in different countries in the world. Source: Adapted from (24).

1.3. **Etiology/risk factors associated with OSCC**

The etiology of OSCC is considered to be multifactorial. Several etiological factors namely the use of tobacco (smoked and smokeless) and alcohol, dietary deficiencies or imbalances (micronutrient deficiency), persistent chronic inflammation, poor oral hygiene, bacterial infection, ultraviolent sun rays (lip cancer), genetic predisposition, *etc* have been linked with the development of OSCCs (25-27). The wide geographical variations in the incidence of OSCCs have been linked with the country specific risk factors, for instance: betel quid chewing and use of smokeless tobacco (ST) are common in South and Southeast Asian countries (28) and toombak in the Sudan (29).

Along with commercially produced cigarettes, the use of home-made cigarettes (such as Bidi: a low grade tobacco rolled into the leaf of Tendu - Fig. 4A and 4B) are popular in countries like India. In addition, ST products such as Gutkha/Paan-masala (products consisting of areca-nuts,

tobacco, slaked lime, catechu and spices) are common in Indian subcontinent (Fig. 4C) (30, 31). In South East Asian countries, the use of ST especially the betel quid chewing (ingredients: betel leaf, areca-nut, slaked lime, may or may not contain tobacco, and other flavoring agents like sweeteners depending on local preferences) (32) (Fig. 4D) along with the habit of alcohol drinking is associated with 75% of OSCC cases reported (33).

Figure 4: Illustration of transcultural tobacco products (A) Flakes of tobacco rolled into the leaf of tendu at cottage industries/home produced; (B) Commercially available bidi, thin cigarettes made of low grade tobacco; (C) Commercially available Gutkha/Paan-masala is a popular form of ST used in Indian sub-continent; and (D) Betel quid with or without tobacco products, most commonly used by rural women as a household practice and it is also popularly used in urban settings during celebrations. Source: Adapted from (34, 35).

Interestingly, the use of the smokeless form of tobacco is also common in developed countries, such as USA, Sweden and Norway. However, the carcinogenic substances (tobacco-

specific-nitrosamines (TSNA) in the Swedish snus and the other smokeless forms of tobacco used by Americans have been reported to be much lower (36). Although the use of Swedish snus is related to the development of white lesions in oral mucosa, it's carcinogenic role in OSCC is debated (37).

1.4. **Pathogenesis of OSCC**

Cancer is suggested to develop due to mutations of key genes involved in cell proliferation and survival. Accumulation of such mutations leads to uncontrolled cellular growth, proliferation, invasion and metastasis (38, 39). Hundreds of genetic alterations have been identified by integrative genomic characterization of OSCC (40-42). However, most of these alterations fell within four major driver biologic processes (Fig. 5):

(i) Mitogenic signaling (63%), with particular emphasis on aberrant activation of the phosphoinositide-3-kinase (PI3K)/mTOR pathway (including 11% with mutations of *PIK3CA*, encoding the catalytic subunit of PI3Kα).

(ii) Defective cell differentiation (including 9% with *NOTCH1* gene mutations and 66% with predicted NOTCH signaling pathway alterations).

(iii) Nearly universal (94%) cell-cycle deregulation due to inactivation of the *CDKN2A* (p16INK4A) tumor suppressor gene by copy number loss or promoter methylation, together with *CCND1* (CYCLIN D1) amplification.

(iv) Genomic instability caused by loss of *TP53* and other candidate genes, such as those involved in DNA damage recognition and repair. This study also identified two additional key genes likely affecting cell-cell communication and cell death: *FAT1* (30%) and *CASP8* (10%), respectively. In a pathway-specific effort, Lui and colleagues (43) studied targetable mitogenic signaling routes genomically altered in head and neck cancers including the MAPK, JAK/STAT, and PI3K pathways. Among these, the PI3K pathway harbored the highest percentage of mutations (30.5%), whereas the MAPK and JAK/STAT pathways were mutated in less than 10% of the cases, further emphasizing that PI3K is the most altered mitogenic signaling pathway in head and neck cancer. *PIK3CA* was the most commonly mutated gene in the pathway (12.6%), and mutations in *PI3K* genes were the only identifiable oncogenes in 20% of the Human Papilloma Virus (HPV) positive

tumors, suggesting that *PI3K* fuels the growth of these HPV associated head and neck cancers. However, the emerging picture is that *PIK3CA* mutations are not the only genetic alterations resulting in the persistent activation of *PI3K* and its downstream targets, including AKT and mTOR, in head and neck squamous cell carcinomas (HNSCC). Indeed, the AKT/mTOR pathway may represent the most frequently activated signaling route in both HPV-ve and HPV+ve HNSCCs [>80% of HNSCC cases(44)] (45, 46), suggesting that multiple genetic and epigenetic changes may act in concert with *PIK3CA* mutations in order to activate the pathways causing malignancies (Fig. 5).

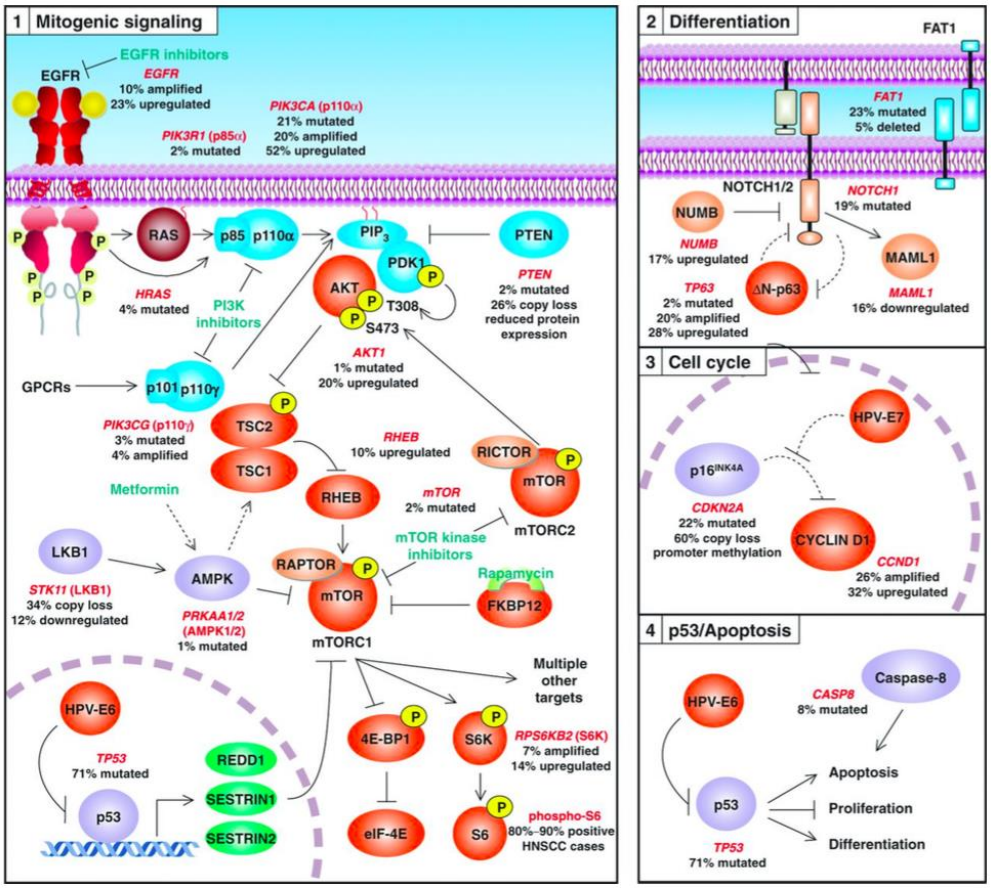
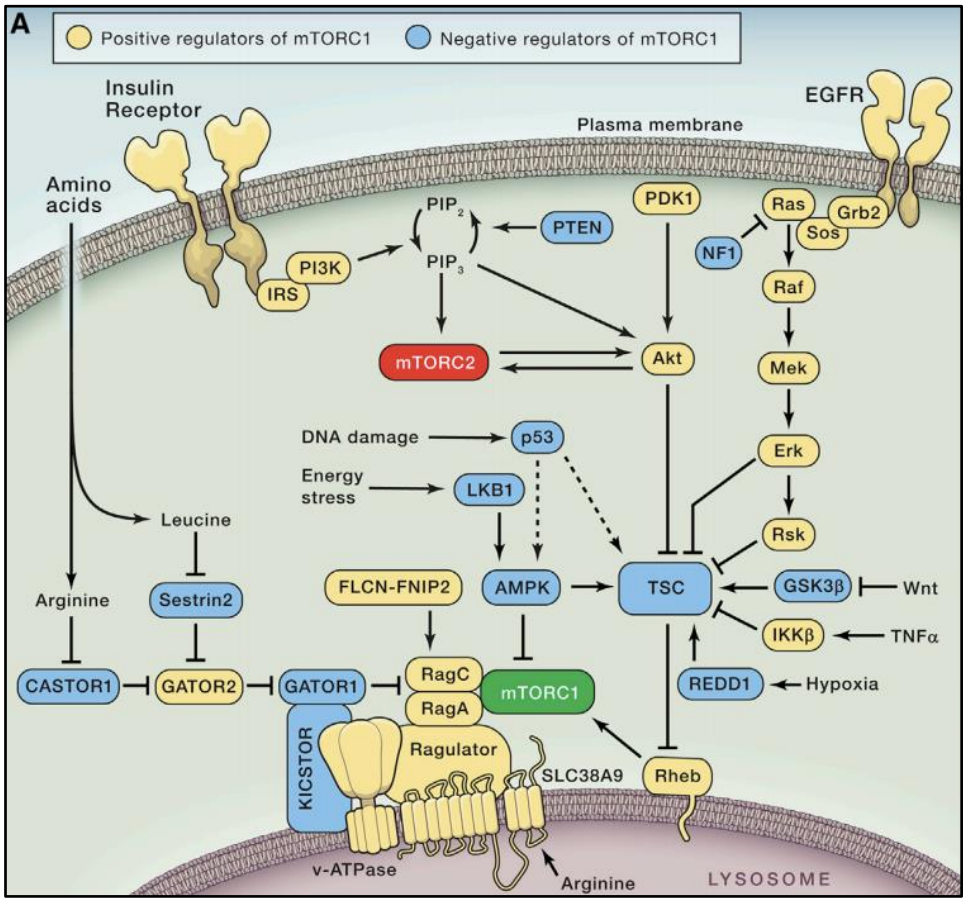


Figure 5: The head and neck cancer oncogenome. Alterations found in each key gene are shown. Copy loss refers to homozygous and heterozygous gene deletion. Data were extracted from the publicly available Cancer Genome Atlas consortium (http://cancergenome.nih.gov/) HNSCC provisional dataset containing CNA, mutational, and gene expression data from 295 HNSCC samples. Source: Adapted from (47).

mTOR, a key molecule in the PI3K/AKT/mTOR pathway, is a protein kinase involved in multiple cellular functions related to normal development and carcinogenesis. mTOR is a key component of two protein complexes, mTOR complex 1 and 2 (mTORC1 and mTORC2). The mechanism of action of this pathway is still evolving. There are many upstream regulators stimulating both mTORC1 and mTORC2 directly and indirectly (Fig. 6A). Important downstream signaling molecules of mTORC1 are involved in protein synthesis, lipid synthesis, autophagy and energy metabolism (48). Whereas downstream targets of mTORC2 are responsible for cell survival/metabolism and cytoskeletal organization. mTORC1 directly phosphorylates 4E(eIF4E) binding protein and further initiates cap-dependent protein translation (49). The S6 protein chosen for this study is one of the downstream targets of mTORC1 that is mainly involved in translation and protein synthesis.



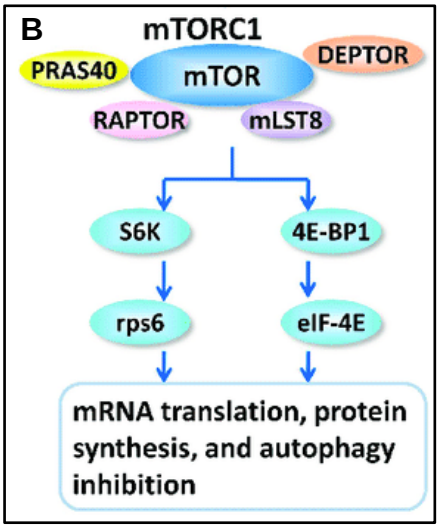


Figure 6: mTOR signaling network (A) Upstream of mTORC1 and mTORC2, positive regulators of mTORC1 signaling are shown in yellow, while negative regulators are shown in blue whereas mTORC1 and mTORC2 are shown in green and red, respectively; and (B) Illustration showing the downstream components of mTORC1. Source Adapted from (50, 51).

During the mitogenic stimulus, the C-terminal of serine residues of S6 gets phosphorylated at the sites Ser-235, Ser-236, Ser-240, Ser-244, and Ser-247 (Fig. 7) by the p70 S6 kinases and p90 ribosomal S6 kinases. The modification in these sites initiates the cap-binding protein (mRNA translation) activity (52, 53). Although S6K is the predominant kinase responsible for the phosphorylation of Ser235 and Ser236 sites in S6, mTOR independent molecules/pathways such as oncogenic Ras, phorbol esters and serum growth factors can also phosphorylate S6 (48, 54). S6K mediated phosphorylation of S6 leads to increased mRNA biogenesis, translation and protein synthesis (Fig. 6B). Protein synthesis is crucial process during the cell division and cell proliferation. Aberrant activation of S6 can fuel the uncontrolled cell proliferation, thereby promoting tumor growth. In parallel with this suggestion, high expression of S6 has been reported in various other human cancers including HNSCC (45, 55, 56). In addition, experiments in mice have indicated that lack of S6 protein could lead to the reduced cell proliferation, cell growth and protein turnover (57).

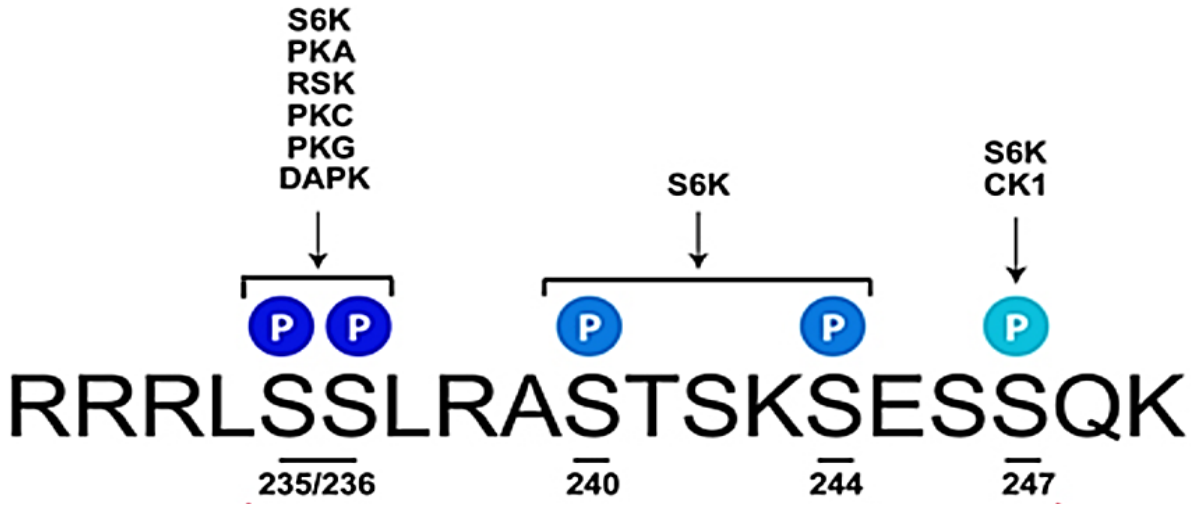


Figure 7: The picture depicts the serine residual sites in S6 protein that can be phosphorylated by upstream signaling. Source: Adapted from (53).

1.5. Management and prognosis of OSCC

On clinical examination, oral cavity is directly visible. Despite of its direct visibility, most of the OSCC cases are diagnosed at advanced stages in most countries, including Norway (58). During the early stages, OSCC mainly presents as white/red lesions, ulcers or painless exophytic mass. However, during late stages, OSCC can lead to problems in swallowing and speech (59, 60). OSCC is a highly invasive malignant tumor and often metastasizes to cervical lymph nodes leading to reduced patient survival. In spite of the recent improvements in diagnostic aids and treatment methods, the survival of OSCC patients has not improved significantly and approximately 62% (61) of OSCC patients survive the span of 5 years from the time of diagnosis (62).

The conventional method of managing and evaluating the prognosis of OSCC are based on clinical examination and TNM classification system (Fig. 8).

T: Primary tumor size			
T1	Size ≤ 20 mm and DOI ≤ 5 mm		
T2	Size ≤ 20 mm and DOI 5-10 mm or size 2-4 cm and DOI ≤ 10 mm		
T3	Size > 40 mm or DOI >10 mm		
T4a	Tumor invades through cortical bone, into deep/extrinsic muscles of the tongue, maxillary sinus, or skin of face		
T4b	Tumor invades masticator space, pterygoid plates, or skull base, or encases internal carotid artery		
N: Regional lymph nodes			
N1	Single ipsilateral LN, ≤ 30 mm, no ENE		
N2a	Single ipsilateral LN, 30-60 mm, no ENE or ≤ 3mm, with clinical ENE		
N2b	Multiple ipsilateral LNs, ≤ 60 mm, no ENE		
N2c	Bilateral or contralateral LNs, ≤ 60 mm, no ENE		
N3a	Any LN > 60 mm, no ENE		
N3b	Any LN with clinical or pathological ENE		
M: Distant metastasis			
M0	No distant metastasis		
M1	Distant metastasis		
Stage-I	T1	N0	M0
Stage-II	T2	N0	M0
Stage-III	T1/T2	N1	M0
	T3	N0/N1	M0
Stage-IV	T4	Any N	M0
	Any T	N2/N3	M0
	Any T	Any N	M1

DOI - Depth of invasion; LN – Lymph node; ENE – Extranodal extension

Figure 8: TNM classification and clinical staging guidelines for carcinomas of oral cavity (Eighth edition). Source: Modified from (63).

The TNM classification was introduced in 1987 by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) (64). This classification gives an information about the size of the primary tumor, involvement of local or regional lymph nodes and if it has been metastasized to distant sites. TNM classification has been widely used in deciding the treatment option and evaluating the prognosis of OSCC. According to this system of classification, OSCC can be considered as either early stage without involvement of regional lymph nodes and metastasis or late stage with the involvement of lymph nodes and with or without metastasis to distant site. Ideally, patients diagnosed at very early stages should have better prognosis. However, unfortunately, 35% of the OSCC patients diagnosed and treated at early stages have been shown to have a poor prognosis (65).

Biopsy is considered to be the gold standard in diagnosis. Histopathological features such as differentiation, tumor depth and tumor budding, together with TNM staging, are valuable in predicting prognosis. However, evaluation of histopathological features alone has been reported not to be sufficient to provide information on the biological behavior and complexity of the OSCC (65-68).

The main treatment option for OSCC is surgical resection. Radical surgical resection of the oro-facial structures (jaws, tongue) affects esthetics and function (mastication, speech, etc), thereby severely compromising the overall quality of life. However, if the tumor is in advanced stage, surgery can be combined with either radiotherapy, chemotherapy or both. More targeted therapies combined with chemotherapy or radiotherapy were expected to yield superior results, but, most likely because of the heterogeneity of the OSCC, such treatments are not successful due to development of drug resistance (69-72). Hence, there is a great need for development of more precise and targeted therapy for treating the patients. Also investigating the OSCC cases at the very molecular, genetic and epigenetic level and understanding the mechanism will help to understand the tumor biology better and that would eventually aid in planning a more effective treatment design (41, 42, 73). Such information would also be valuable to identify molecular biomarkers which can be used not only to predict disease prognosis, but also to stratify patients into subgroups so that appropriate treatment and follow up can be implemented.

1.6. **Histopathological prognostic indicators of OSCC**

From decades, histopathological evaluation of OSCC has been used for diagnosis and prognosis of disease. Although a number of histopathological features of the tumor has been suggested, their routine use for OSCC prognosis is disputable.

Some of the most commonly suggested and used histopathological features with prognostic values are:

- **Depth of invasion:**

  It is the distance from the surface of the tumor to its most invading point. There is an association between the depth of invasion and OSCC prognosis. Patients with deeply

invaded tumors along with lymph node metastasis have been shown to have a poor prognosis (74). In the recent TNM staging (8th edition) this parameter has been included.

- **Extranodal extension:**

  The spread of cancer cells out of the capsule of involved lymph node is called extranodal extension. Extranodal extension is associated with poor prognosis in OSCC (75). It has been included in the recent edition of TNM staging as one of the potential parameters.

- **Pattern of invasion:**

  OSCC cells at the invasive front region have been suggested to undergo epithelial mesenchymal like transition, thereby promoting cellular motility and invasion (76). Based on the pattern of invasion at the invading front area, OSCC can be categorized into cases where cancer cells form small group of cells or islands, or cases with cancer cells in bigger cluster/groups or with pushing borders (77). The presence of a degree of tumor budding, defined as "a single cancer cell or a cluster of <5 tumor cells present in the stroma at the invasive tumor front" were also shown to be associated with poor prognosis in OSCC (78).

1.7. Cancer biomarkers

Off late, there is a shift in the trend of health care from 'one size fits all' approach to a more personalized care. Nevertheless, the success of these efforts will hugely depend on availability of biomarkers able to stratify the patients. In general, biomarkers are biological molecules/parameters (such as DNA, mRNA or proteins, etc) that can be measured objectively and can be used as indicators to differentiate between normal and abnormal biological processes (diagnostic biomarkers) and to predict the course of a disease (prognostic biomarker) or the response to a treatment (predictive biomarker). Biomarkers can aid in detection and determine the disease onset, progression, interventions, treatment outcomes, prognosis, and stratification (79). To be applicable in the clinical setting, it is important for the biomarkers to possess high predictive accuracy, should be easily measurable and

reproducible, minimally invasive and easily accepted by both patients and physicians (80). Among these categories, protein biomarkers are very well known for their analytical instrumentation which in-turn helps in identification of the content and amount of protein distributed in the complex biological samples (80).

2. Justification and aims of the study

OSCC is growing rapidly at the frightening rate. The routine diagnostic methods are inadequate to reflect and predict the precise molecular mechanism underlying OSCC. This emphasizes the need for better understating of OSCC biology and altered signaling pathways. Key protein molecules in the altered signaling pathways have been increasingly recognized to provide earlier and more precise diagnosis, and to assign patients to the best-targeted treatment modalities available for avoiding ineffective overtreatment.

2.1 Hypothesis

The expression pattern of pS6 (ser235/236) can be used to predict prognosis of OSCC patients.

2.2 Aim of the study

To examine the expression pattern of pS6 (ser235/236) using IHC and to examine the correlation between its expression and clinicopathological parameters and survival of OSCC patients.

2.3 Specific aims

1. To examine the expression of pS6 in formalin fixed paraffin embedded (FFPE) specimens of OSCC using IHC and image analysis tool QuPath.
2. To examine correlation(s) between the expression pattern of pS6 and clinicopathological parameters and survival of OSCC patients.

3. Materials and Methods

3.1 Ethical considerations

For this study, all OSCC specimens were retrospectively collected from the diagnostic archive at the Department of Pathology, Haukeland University Hospital, Bergen, Norway. The current project is a part of an established and ongoing project, and was approved by the Regional Ethical Committee in West Norway (REK Vest 13.01.2016.685695 2010/481). Consent was obtained from all of the patients used in the study.

3.2 Patient Cohort

The biopsy specimens from a total of 147 OSCC patients were used. Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria (81) was followed. All the samples used were histopathologically confirmed cases of primary OSCC without prior treatment. Samples included were from different regions of oral cavity (tongue, gingiva, palate, floor of the mouth, buccal mucosa and lip). All samples were collected during the period between 1998 and 2012. The clinicopathological data for this cohort were retrieved from the Haukeland University Hospital Record System (DIPS) and from the Pathology Report System (DOCULIVE). The corresponding FFPE blocks were obtained from the hospital archive. Individually, the FFPE blocks were screened for HPV infection by using p16 *(p16INK4a)* as a surrogate marker by performing IHC. Positive (samples with >70% p16 positive cells, both cytoplasmic and nuclear) tested samples were excluded. In addition, all samples were closely examined by an oral pathologist using their respective hematoxylin and eosin sections in order to ensure they contained sufficient tumor cells/areas and stromal structure.

3.2.1 Inclusion criteria

- Patients with confirmed diagnosis of OSCC.
- Patients with >18 years.
- Patients who hadn't received any chemotherapy or radiation therapy before surgery.
- Patients who consented to use their samples for research in case of alive patients.
- Patients for whom sufficient tissue material was available for analysis.

3.2.2 Exclusion criteria

- Any missing clinical data to its relevant sample blocks or vice versa.
- Patients who refused to give the consent.
- Any p16 positive cases.

3.3 Study design

This was a retrospective cohort study of patients diagnosed with OSCC during the period 1998 to 2012. Expression of pS6 in FFPE specimens was analyzed in laboratory and the expression pattern was examined for association with patient's clinicopathological data and survival.

3.4 Statistical power calculation

As the availability of samples are predetermined, hence statistical power analysis is not applicable to this particular study. However, based on our experience on similar studies and literature, the current sample size should provide enough statistical power.

3.5 Choice of method

As the aim of the study was to examine the expression of pS6 in the cancerous cells, IHC technique was chosen. IHC works on the principle of antigen antibody reaction and gives an insight into the protein content and distribution (Fig. 9). Though it is one of the commonly used histopathological methods, similar to other techniques, it possesses its own advantages and disadvantages. To mention some of its merits, it preserves the tissue morphology and provides sufficient information on the amount of protein content and distribution in the tissue samples. This method is more comprehensive and affordable to be implemented in the developing countries where OSCC is a significant issue. Following are some of the limitations of IHC: it is a sensitive method, duplicability and precision of the outcomes varies on discrete aspects from formalin fixation, handling, processing, antigen retrieval method, antibody selection, duration of exposure during each step, visualization and quantification of the final stained expression (82-85). Hence, optimization of the staining protocol with use of

appropriate positive and negative controls is extremely important to establish a robust and reproducible IHC protocol. Strictly adhering to the established IHC protocol and use of fresh reagents are also important to minimize the staining variations across runs.

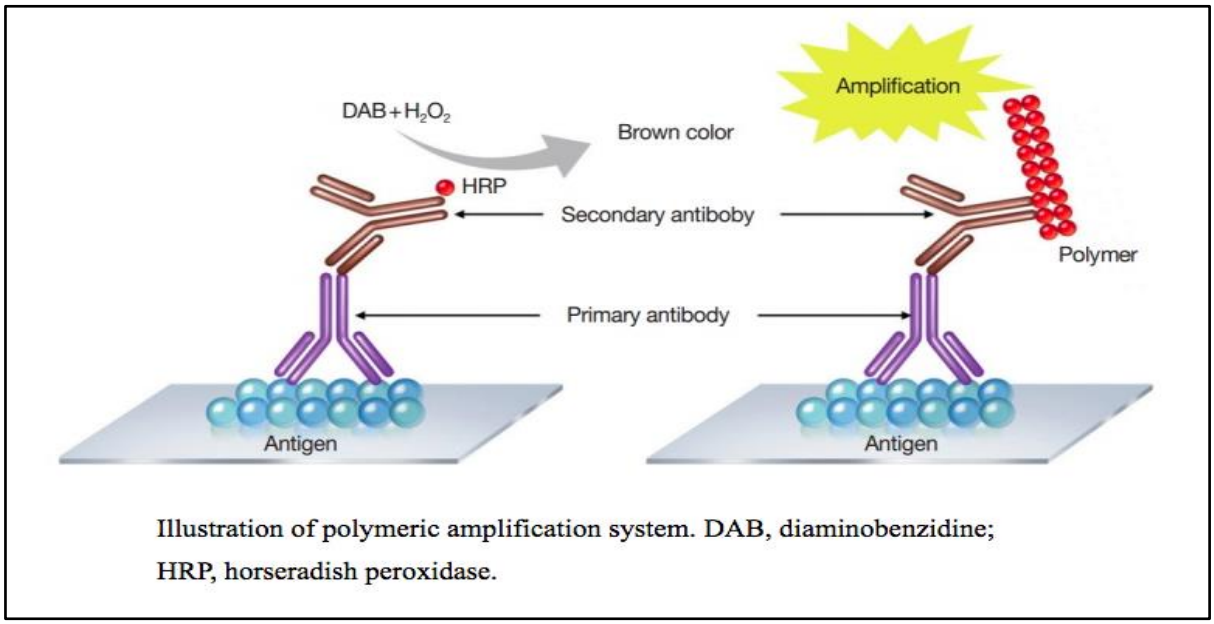


Figure 9: Illustration showing the principle of IHC. Source: adapted from (82).

3.6 The use of FFPE archival tissues

We have used 3-4 microns thick FFPE sections of OSCC samples for IHC. Type of the fixative used and the duration of fixation of specimens can affect the outcome of IHC (86). A few reports have stated that either under or over fixation will alter the results as it interferes in the protein crosslinking (86-88). In addition to fixation, appropriate paraffin embedding step is crucial to preserve the morphology of tissue. The samples used in the current study were fixed in 10% neutral buffered formalin and embedded in paraffin following the standard protocols at the Department of Pathology, Haukeland University Hospital.

3.7 Selection and validation of primary antibody

Based on literature search on the functional biomarkers related to the key pathways altered in OSCC, pS6 is one of the key downstream protein and an end-point indicator of activated status of mTORC1. The anti-pS6 (Ser235/236) antibody (Manufacturer: Cell Signaling

Technology, Massachusetts, USA. Catalogue number: 4858S) detects the endogenous levels of ribosomal protein S6 only when it is phosphorylated at Serine 235 and 236 sites. To establish a reproducible IHC protocol for anti-pS6 (Ser235/236) antibody, IHC optimization was done testing various antigen retrieval reagents, different antibody concentrations and incubation times and temperatures; along with the use of appropriate positive and negative controls. As the positive assay control, we used FFPE sections from human breast carcinoma, tonsil, and oral cancer tissues with known positive expression. For negative assay control, we used the same samples as for positive but incubated without primary antibody.

3.8 Specimen preparation

After shortlisting the FFPE tissue blocks, with the aid of manual microtome (Leica Microsystems, UK) a series of thin sections ranged between 3-4µm were prepared and mounted on to the superfrost coated glass slides from the Thermo Scientific Superfrost Plus, USA. All the slides were incubated at 56^0 C for 1-2 hours to melt the residual paraffin and later it was stored in the slide box at 4^0 C until it was used for immunohistochemistry staining. All this work was performed at the Gade Laboratory for Pathology, Department of Clinical Medicine at University of Bergen.

3.9 IHC protocol

The sectioned samples were brought to Sapkota's laboratory, Institute of Oral Biology, University of Oslo. Reagents required for the IHC procedure were pre-ordered from Agilent-Dako. Reagents from antigen retrieval to the secondary antibody all were from the manufacturer Agilent Technologies, USA. The IHC staining was performed to detect the antigen ribosomal protein S6 that are specifically phosphorylated at the site of 235 and 236. As a positive control, oral cancer samples were used, which showed good expression.

Detailed immunohistochemistry staining protocol for pS6 (Ser235/236)

After cutting sections, put them in incubator at 56 ºC for 1-2 hours		
Xylene	In ventilation	2 x 5 min
Ethanol	Absolut 100%	2 x 3 min
Ethanol	96%	3 min
Ethanol	70%	3 min
Distilled water		Rinse
Retrieval of the antigen	Ag. Ret. **pH 6** citrate (S2369) from Agilent-Dako (Pressure cooker)	25 min total
Cooling	Let it be on the bench for cooling	15-20 min
Wash	Slightly pouring tap water	Until room temperature
Put sections in wash buffer, wipe around the tissue and draw around the tissue sections with Agilent-Dako Pen		
Inactivation of peroxidase	Use peroxidase block from the EnVision$^+$ kit from Agilent-Dako	5 min
Wash	TBST	10 min (shaking)
Block with goat serum	Normal goat serum by Agilent-Dako, X0907 10 % in 3 % BSA	30 min
Primary antibody:	Diluted in antibody diluent (S0809) from Agilent-Dako **1:100** (Room temperature for 1 hour followed by overnight incubation at 4^0C, again followed by room temperature for additional 1 hour)	60 min – overnight – 60 min
Wash	TBST	10 min (shaking)
Secondary antibody	EnVision HRP Rabbit (K4003) from Agilent-Dako	30 min
Wash	TBST	10 min (shaking)
Visualisation	Agilent-Dako DAB (K3468) 1 drop DAB+ 1 ml buffer	10 min (look at the slides)
Wash	Distilled Water	5 min (shaking)
Counterstain	Hematoxylin (S3301) from Agilent-Dako	5 sec
Wash	Running tap water	10 min
Mounting of sections	Ethanol 70%	30 sec
	Ethanol 96%	1 min
	Ethanol 100%	1 min
	Ethanol 100 %	1 min
	Xylene	2 min
	Xylene (new)	2 min
Mount sections with pertex		

3.9.1 Scanning of slides and digital quantification of IHC stained slides

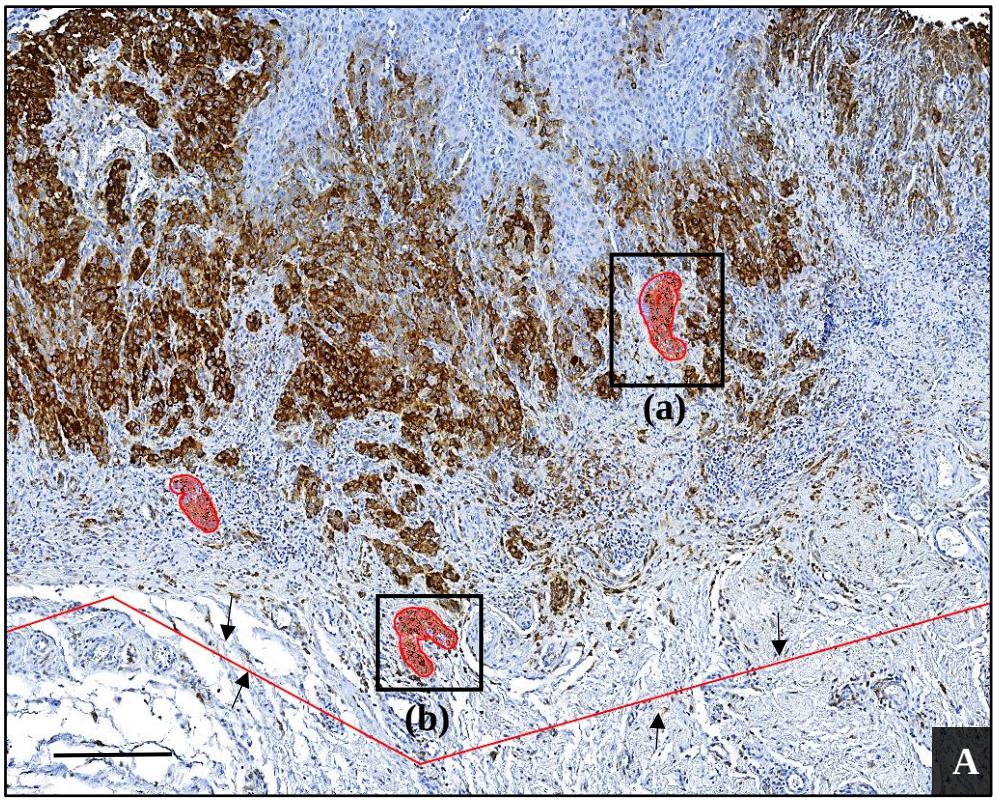
All the slides were digitally scanned at 40x magnification using Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc., Vista, CA, USA). There are different methods to quantify IHC staining: digitalized, semi-quantitative and quantitative. We opted for semi-quantitative method as it is objective thereby reducing intra- and inter- observer bias, is less laborious, and also suited best to work with our timeframes. We used QuPath opened-source software (version 2.00.m5 for Windows) developed by the Center for Cancer Research and Cell Biology at Queen's University Belfast (89).

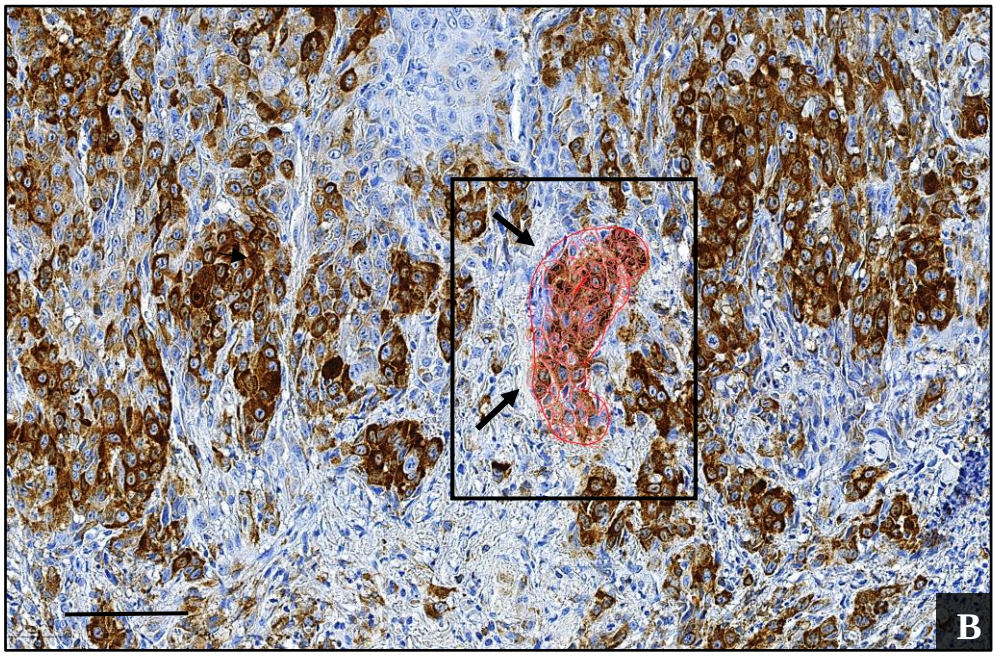
3.9.2 QuPath cell detection

QuPath's cell detection command was applied to identify the cells with cytoplasm/membranous staining. This command estimates the extent of each cell based upon a constrained expansion of the nucleus region, and calculates morphology, including nucleus area, circularity, staining intensity for hematoxylin and DAB, and nucleus/cell area ratio. Before the image analysis, a detailed evaluation of protocol was prepared and calibration between observers (DEC, DS and DT) was done. Thereafter, blinded for the clinicopathological information, the IHC evaluation was done by DT.

Cytoplasmic expression of pS6 was evaluated both at the TIF and the corresponding TC. TIF represents the interface between the most invasive epithelial tumor islands and the underlying stromal tissue (10). Compared to the TC, the TIF is believed to contain the most aggressive tumor cells and better reflects the biological behavior of tumors (90-94). Firstly, a line of demarcation between the tumor and normal stromal structure was drawn as a guide to select the regions of interest (ROI) at the TIF (Fig. 10). ROI consisting of 3-4 most outermost layers of cancer cells at the invading island was marked using a brush tool with 150 pixels. At least three random ROIs were marked at TIF areas at 18x magnification. In a similar way, four random ROIs, best representing in the central region, were selected. A minimum of 500 and up to 1000 cells were selected from each slide for analysis. A total of 147 OSCC cases from Bergen were initially included in the study. Among them, 15 cases with no visible tumor tissue on Hematoxylin and Eosin slides were excluded. Of the remaining 132 samples, all were used

for IHC bio-image analysis. Sections which were unquantifiable or completely destroyed after the staining were excluded.





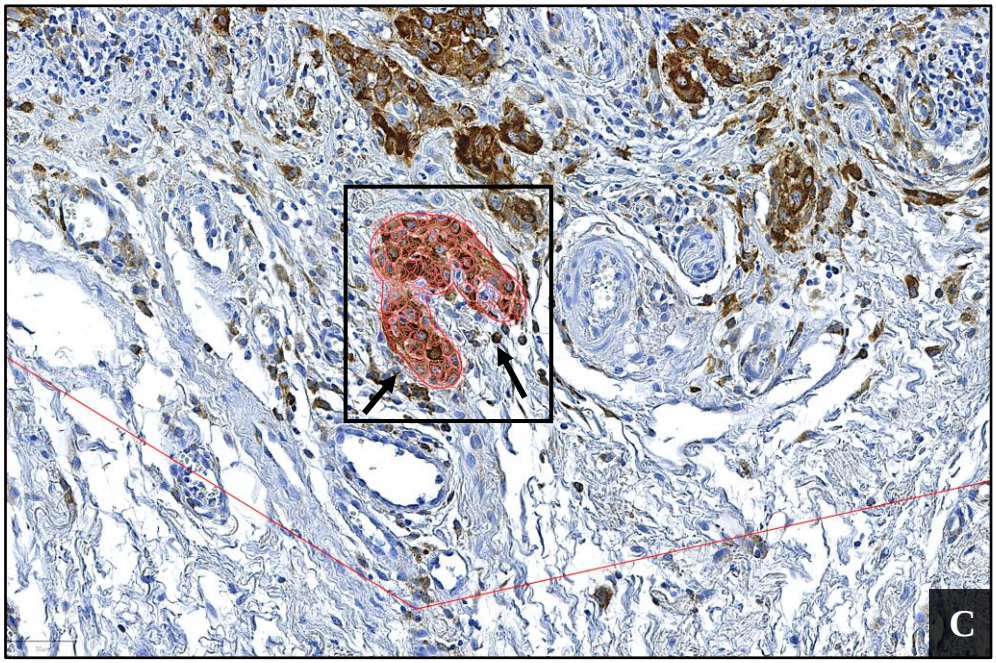


Figure 10: Immunohistochemical analysis of pS6 (Ser235/236) protein in the OSCC tissue specimens. (A) Red line (pointed by black arrows) indicates an area of demarcation between the TIF and stroma drawn and serves as a guide for the annotation of TIF ROIs. (B) and (C) show the magnified images of area (a) and (b) respectively. (Scale bar used for (A) is 200µm, whereas for (B) and (C) is 50µm).

3.10 Statistical Analysis

The statistical analysis was performed using Statistical Package for Social Sciences using version 26 (SPSS Ver. 26, IBM, NY, USA). The distribution of the S6 expression data was found to be non-normal (postively skewed with histogram). Therefore, Wilcoxon matched-pair (non-parametric) test was used to examine the difference in the expression of pS6 in TC and corresponding TIF. Further, OSCC specimens were divided into high and low expression groups using the 66.6th percentile of pS6 staining as a cut off value. OSCC cases were also categorized based on other varialbes such as age, gender, history of habbits like smoking and alcohol, histological degree of differentiation, worst pattern of invasion, tumor budding, depth of invasion, recurrance and lymph node metastasis. Chi Square test was used to examine the association between dichotomized clinicopathological varibales and the expression of pS6. Association beween expression status of pS6 in OSCC patients and 5-years-and the recurrance free- survival was examined using Kaplan-Meier analysis (log-rank test). <0.05 p-value was considered to be statistically significant.

4. Results

4.1 Clinicopathological characteristics of the patient cohort

A total of 147 OSCC cases from Bergen were initially included in the study. Among them, 15 cases with no visible tumor tissue on hematoxylin and eosin slides were excluded. Of the remaining 132 samples used for IHC analysis, (92, 69.7%) samples consisting of clear TIF were used for cross-tabulation and survival analysis. Of them, pS6 expression data both at the TIF and corresponding superficial/central areas/TC were available in 89 cases. The clinicopathological characteristics of the patient cohort are summarized in Table 2. Briefly, the mean age of the patients was 67.14 (range 27 - 93) years. (50, 54.9%) of the cases were males (Fig. 11A). Number of cases (52, 56.5%) with late stage disease was higher as compared to the cases (40, 43.5%) with early stage. Tongue was most commonly affected site (45, 49.5%). Tumor spread to lymph nodes was seen in 34 (37%) of patients (Fig. 11B). Advanced worst pattern of invasion was seen in 71 (89.9%) of the patients (Fig. 11C). Tumor recurrence was seen in 40 (43.5%) of the cases. Majority (77, 83.7%) of the cases were histologically well differentiated. At the time of diagnosis, tumor with >4mm depth of invasion was found in 34, (37%) of the cases. In 39 (42.4%) of the cases, tumor budding with more than or equal to 5 tumor buds were noticed.

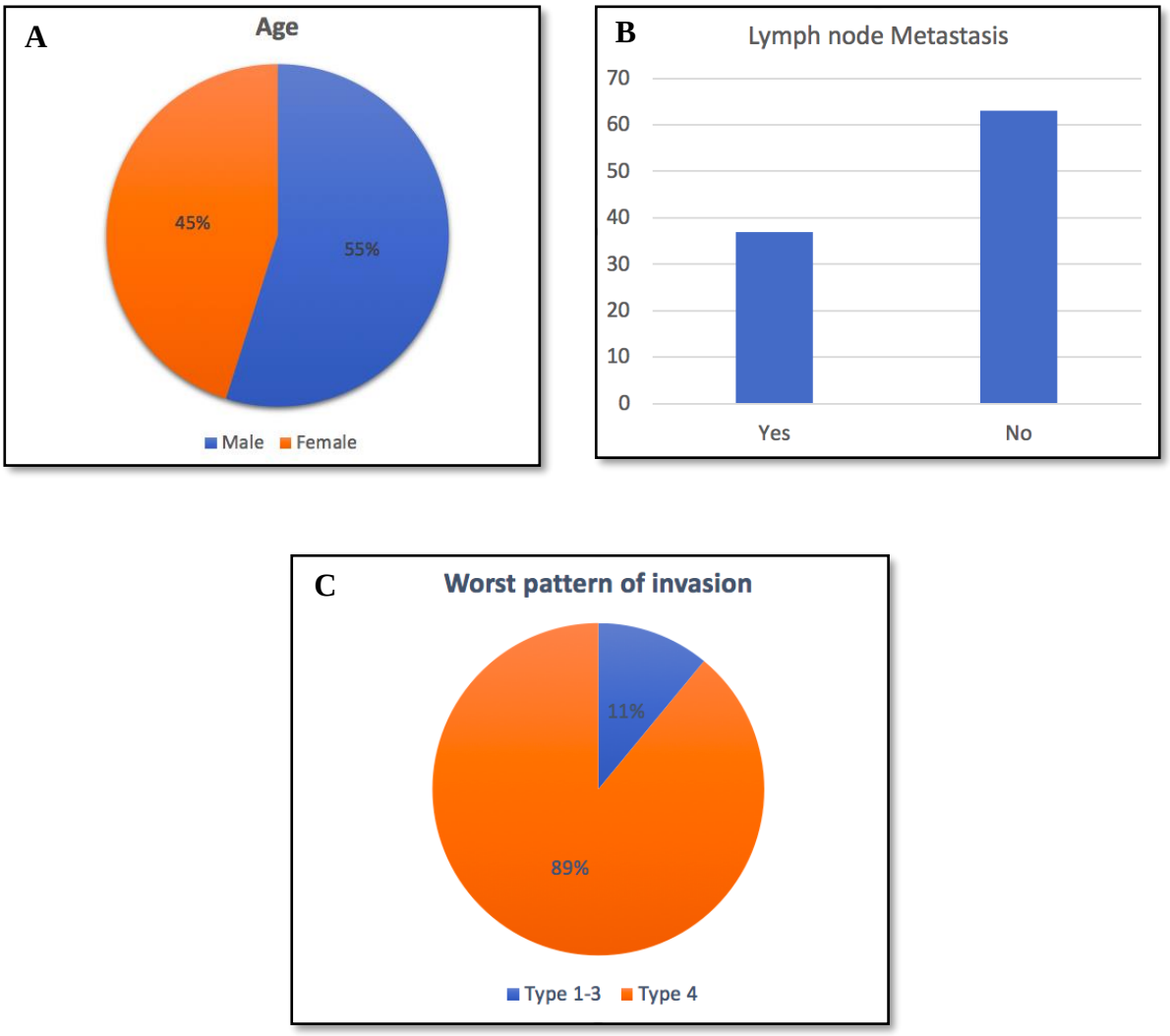


Figure 11: (A) Pie chart representing gender distribution among the cohort; (B) Bar graph depicting the lymph node metastasis among OSCC patients; and (C) Pie chart illustrating the worst pattern of invasion among the OSCCs.

Table 2: Cytoplasmic expression of pS6 and clinicopathological variables of the OSCC patients from Bergen, Norway.

	Scoring at TIF		
Variables	N	%	*P*
[a]Age (years)			
≤67	50	54.9	0.186
>67	41	45.1	
[b]Smoking			
No	24	33.8	0.678
Yes	47	66.2	
[b]Alcohol use			
No	32	60.4	0.874
Yes	21	39.6	
Tumor site			
Tongue	45	49.5	0.208
Gingiva and buccal mucosa	35	38.5	
Floor of the mouth	7	7.7	
Palate, lip and oro-pharynx	4	4.4	
Tumor stage coded			
Early (stage 1&2)	40	43.5	0.117
Late (stage 3&4)	52	56.5	
Lymph node metastasis			
No	58	63.0	0.114
Yes	34	37.0	
Tumor budding scored			
Low budding (< 5 buds)	40	43.5	0.265
High budding (≥ 5 buds)	39	42.4	
Unquantifiable	13	14.1	
[b]Worst pattern of invasion			
Type 1-3	8	10.1	***0.012***

Type 4	71	89.9	
Depth of invasion coded			
Superficial (< 4mm)	26	28.3	0.978
Deep (≥ 4mm)	34	37.0	
Unquantifiable	32	34.8	
Death end of 5 years			
Dead	58	63.0	0.114
Alive	34	37.0	
Recurrence			
No	52	56.5	0.498
Yes	40	43.5	
Histological degree of differentiation			
Well	77	83.7	0.974
Moderate to poor	15	16.3	

[a] OSCC patients are categorized based on their mean age.

[b] Some data on smoking, alcohol and worst pattern of invasion were not available for all the OSCC cases in this cohort.

4.2 Expression of pS6 in paratumor epithelium and normal structures

Predominantly cytoplasmic expression of pS6 was noticed in the supra basal cells in the para tumor epithelium (Fig. 12 A), In addition, S6 expression was found in some of the inflammatory cells, skeletal muscle (Fig. 12B) and nerve bundles.

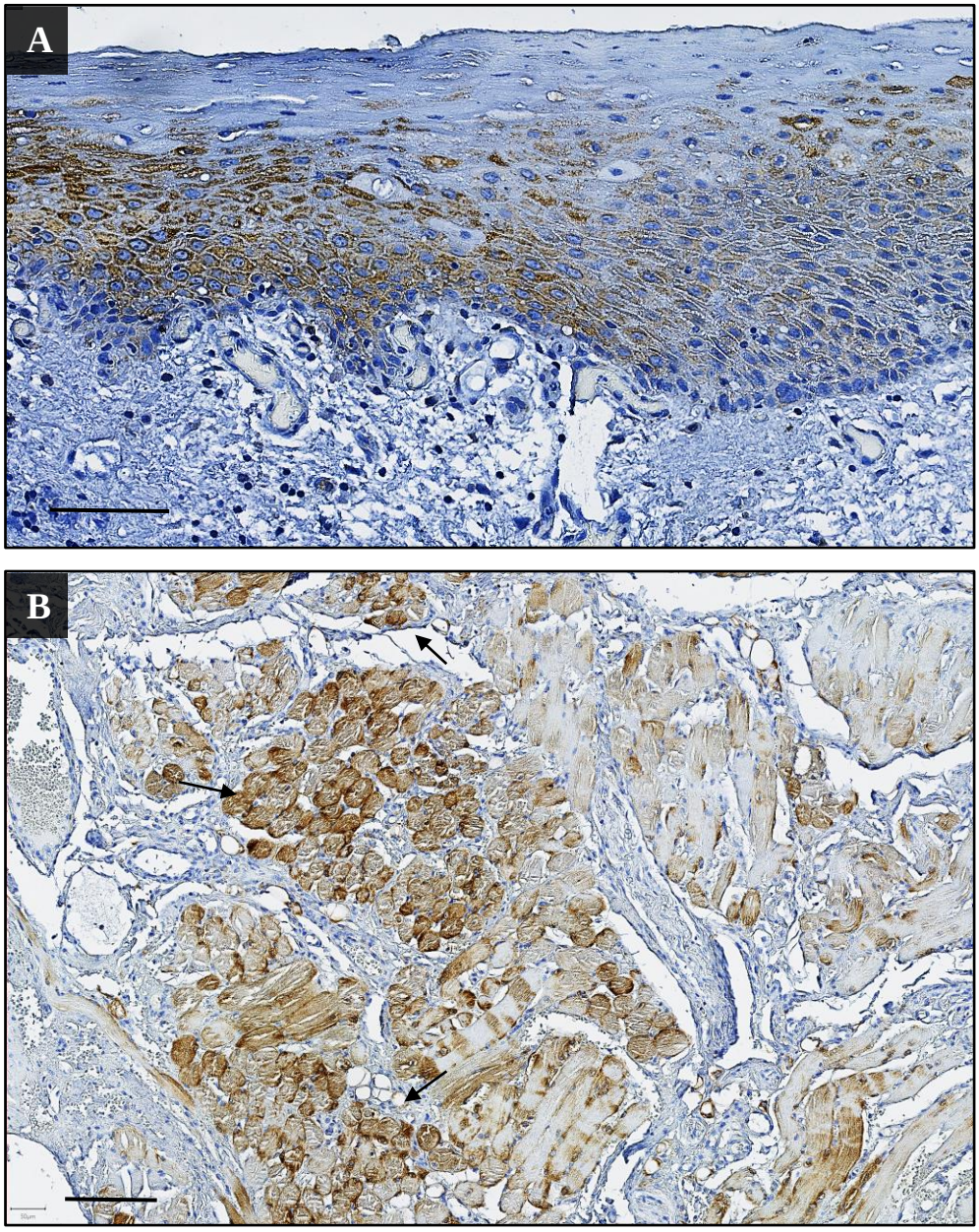
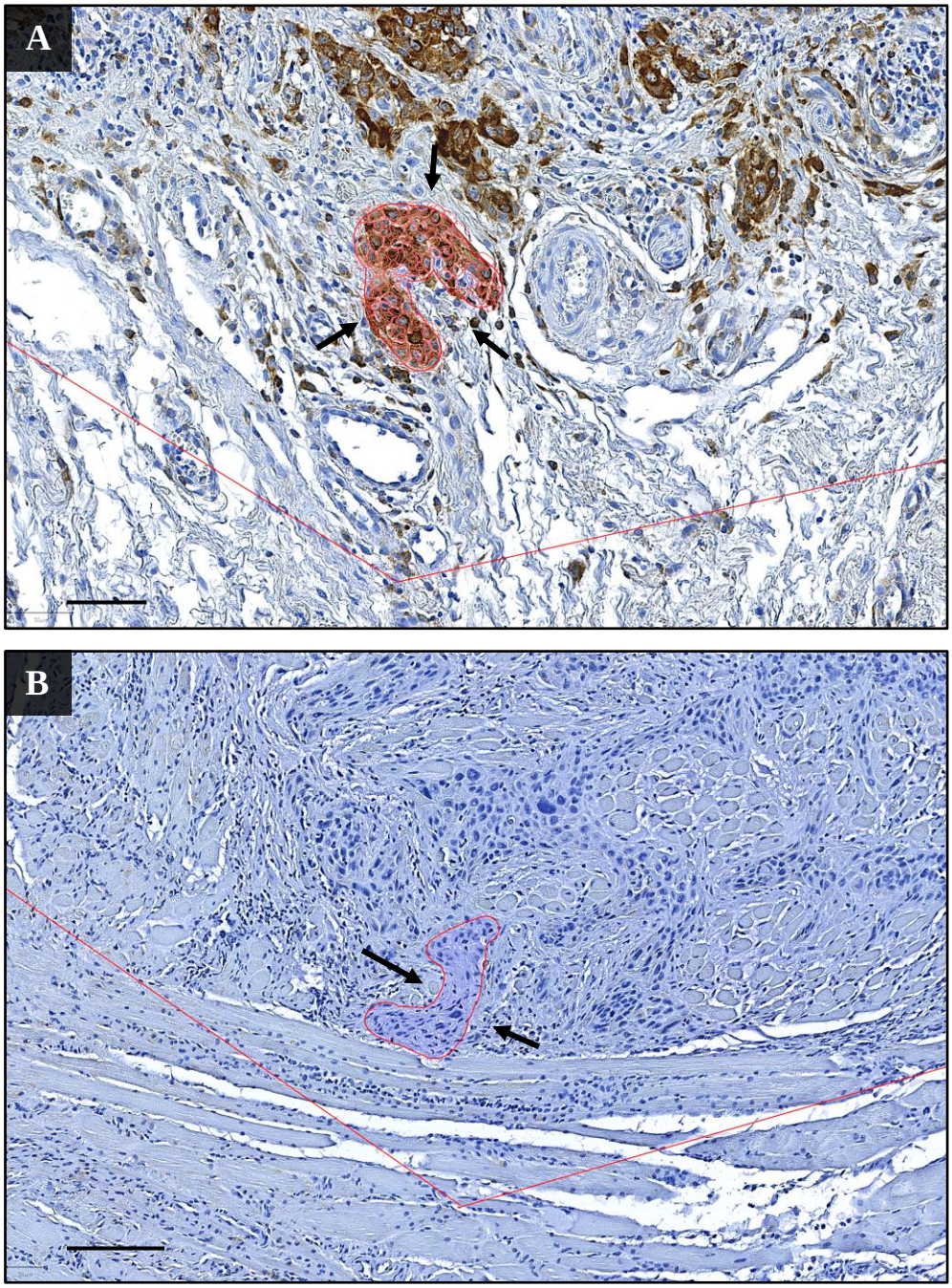


Figure 12: (A) Cytoplasmic expression of pS6 protein in the parabasal layers in paratumor epithelium; and (B) pS6 expression was also noticed in skeletal muscles. (Scale bar used: 50µm).

4.3 Expression of pS6 in OSCC

The expression of pS6 was highly variable among the OSCCs (Fig. 13). Out of 132 cases included for IHC, 8 samples were excluded while analyzing as either they were unquantifiable or destroyed. In the 124 cases analyzed, pS6 was quantifiable at TC in 121 OSCCs, whereas at TIF in 92 of cases. pS6 both at the TC and corresponding TIF was quantifiable in 89 cases. Out of 121 cases, pS6 expression was found positive in 97 (80.2%) cases at TC. Similarly, out of 92 cases, in 61 (66.3%) cases showed positive expression of pS6 at TIF.

Pair-wised comparison showed that the mean of the number of positive cells was significantly higher ($p=0.012$) in tumor center (19.6%) as compared to the corresponding TIF (13.4%) (Fig. 14). It was interesting to note that in a subgroup of OSCC cases, the % of pS6 positive cells at TIF was clearly higher as compared to that at corresponding TC (Fig. 14, marked by a black box). As the tumor cells at the TIF are believed to be the most aggressive and biologically more relevant cell types (90-94), pS6 expression only at the TIF will be considered in the subsequent analysis.


A
B

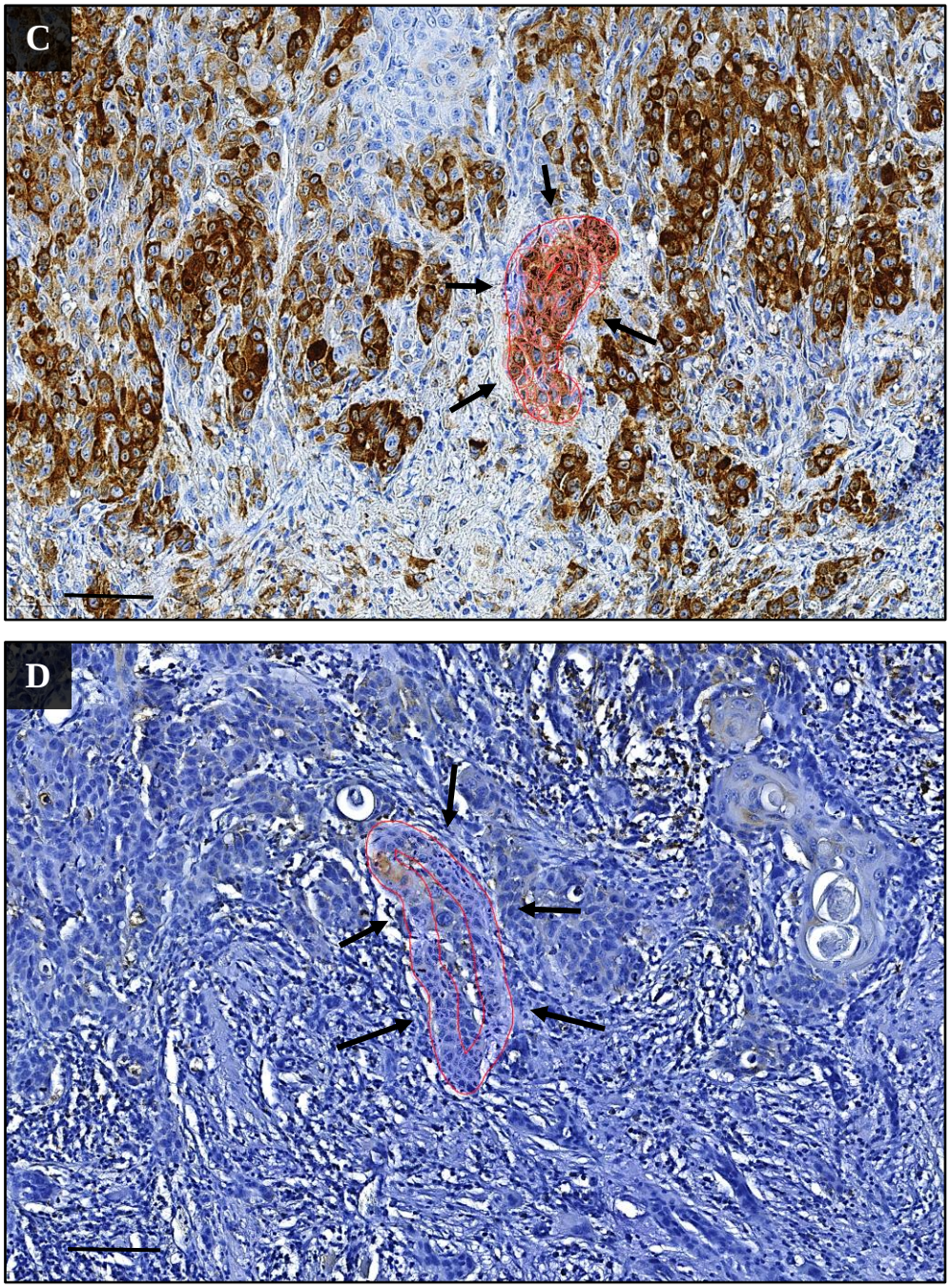


Figure 13: Immunohistochemical staining showing expression of pS6 protein in OSCC specimens. Images showing high and no/weak expression of the pS6 at TIF region (A and B); and at TC (C and D). (Scale bar used: 50µm).

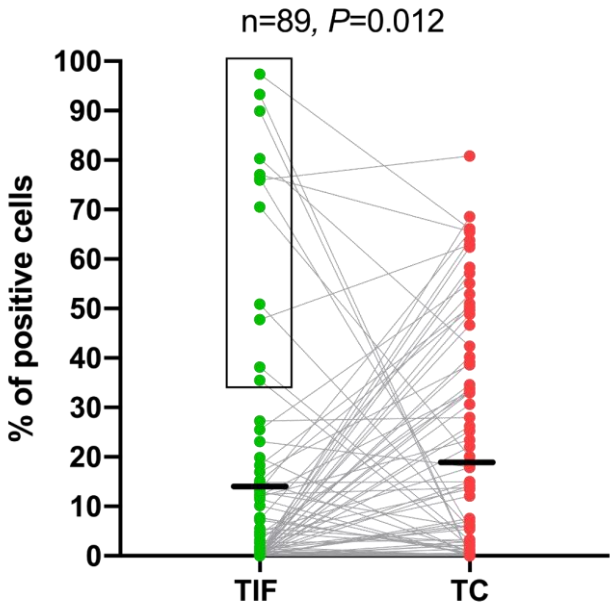


Figure 14: Figure showing distribution of the % of pS6 positive cells at TIF and corresponding TC. Wilcoxon matched-pair test was used for statistical analysis. The horizontal bars represent means.

4.4 Association between the expression of pS6 and clinicopathological variables of OSCC

Expression of pS6 at TIF was examined for association with clinicopathological variables. High expression of pS6 at TIF was positively associated with the worst pattern of invasion (p=0.012). Similarly, a positive association was also found with lymph node metastasis (p=0.114) and tumor stage (p=0.117), but the results were not significant.

4.5 Association between pS6 expression and OSCC patient survival

Out of 92 cases, 58 (63%) were dead and 34 (37%) were censored within the 60 months of follow up. Kaplan-Meyer analysis showed that the patients with higher expression of pS6 (median survival months of 6.1) at the TIF had a lower 5-years survival (Fig. 15) probability as compared to the patients with lower pS6 expression (median survival months 16.2). However, the results were not significant (Log Rank test, p=0.206). Similarly, patients with higher

expression of pS6 at TIF showed a trend for a lower recurrence-free survival (Fig. 16) probability as compared to the patients with lower pS6 expression (Log Rank, p=0.189).

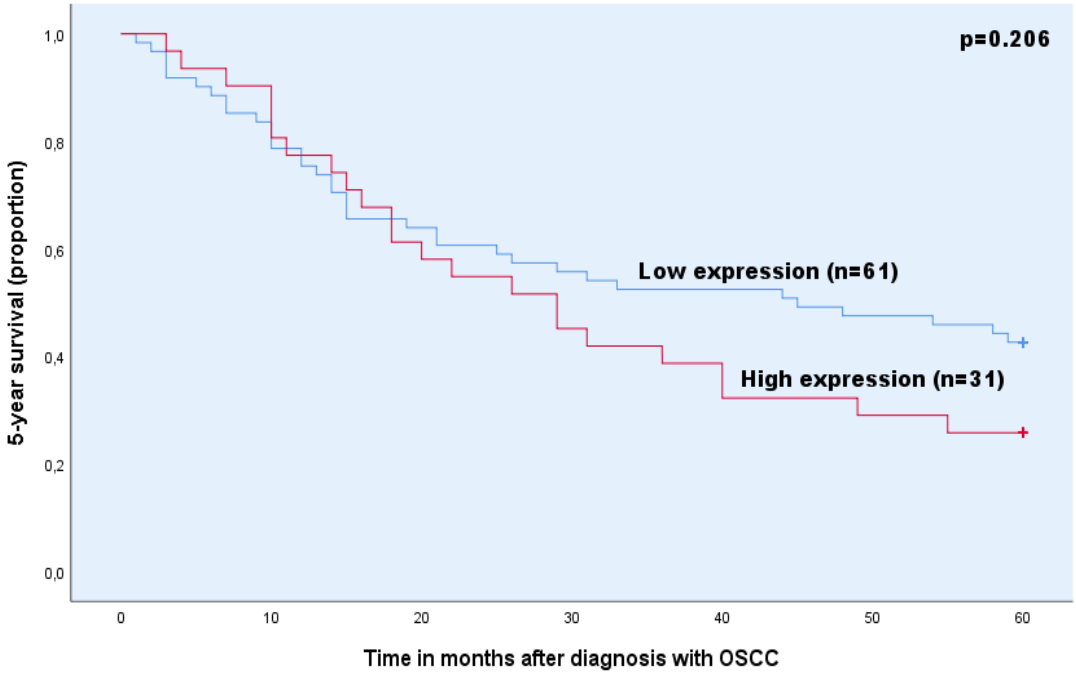


Figure 15: Kaplan Meier plot showing better 5-years overall survival probabilities for OSCCs with lower pS6 expression at TIF as compared to that with cases with high pS6 expression. 66.6th percentile of pS6 expression was used as a cut off.

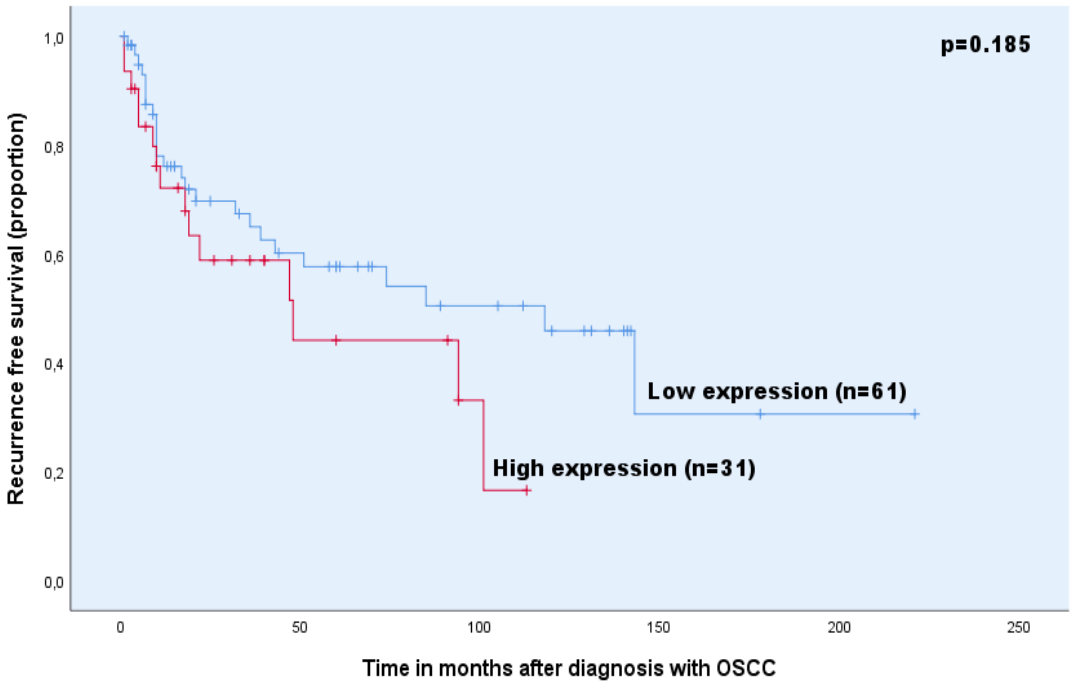


Figure 16: Kaplan Meier plot showing better recurrence free survival probabilities for OSCCs with lower pS6 expression at TIF as compared to that with cases with high pS6 expression. 66.6th percentile of pS6 exression was used as a cut off.

5. Discussion

From last few decades, there has been significant progress made in understanding and identifying prognostic biomarkers for predicting the aggressiveness of OSCC. TNM staging, which recently got upgraded with two more histopathological parameters: depth of invasion and extranodal extension, is still widely used as the only prognostic tool. Nevertheless, it fails to adequately explain the biological behavior of tumors and hence it is not possible to categorize patients into molecular subtypes and plan the treatment accordingly. Therefore, having a robust prognostic biomarker will aid in stratification of the OSCC patients based on the biological behavior of the tumor, so that suitable treatment and /or follow up strategies can be implemented. This will also allow the possibility for molecular targeted therapy along with adjuvant radiotherapy and/or chemotherapy.

The mTOR signaling pathway is very crucial to several biological processes such as mRNA biogenesis, initiation of translation process, protein turnover, cell proliferation and motility; and it is related to a number of disease processes. Accordingly, activation of mTOR pathway has been shown in several cancer types, including HNSCC (45, 55, 56). Being a complex signaling network with a number of interconnected upstream and downstream molecules, it is challenging to identify and precisely target the component(s) of mTOR pathway for cancer management. In this context, protein molecules such as S6, representing one of the end-point markers of mTOR activation can be a relevant option. The phosphorylation sites of S6 at Ser235/236 and Ser240/244 have been shown to be important downstream effectors of mTOR activity. Phosphorylation of S6 at Ser235/236 and Ser240/244 has been reported as markers of response to treatment with the PI3K inhibitor BYL719 in various human cancers (95-97) and also as predictive markers for targeted mTOR therapies in numerous other cancers (98-100).

A number of previous studies have suggested a link between pS6 and OSCC pathogenesis. In a study by Chakraborty et al, high levels of pS6 were observed in cell lines from patients with HNSCC (101). In another study by Chaisuparat et al, the IHC expression of pS6 (Ser240/244) was reported in 50% of cases in normal oral mucosa, in 100% of cases of oral epithelial dysplasia and in 88.67% of OSCC cases (102). Similarly, Martins et al reported expression of

pS6 (Ser240/244) and several other components of AKT/mTOR pathway in 52.9% in non-dysplastic oral tissue, 70.8% of cases in oral epithelial dysplasia and 77.4% of cases in OSCC (103). de Vicente et al showed that expression of pS6 (Ser235/236) and pS6 (Ser240/244) was found in 83% and 88% of OSCCs, respectively (104). These findings indicate that activation of pS6 is an early and common event in HNSCC/OSCC pathogenesis. In agreement with the above studies, pS6 expression was found in 66.3% of OSCC cases at TIF and 80.2 % of cases at TC. The mean percentage of pS6 positive tumor cells at TIF was found to be lower than that of the corresponding TC of OSCCs (Fig. 14). Given the more aggressive phenotype of tumor cells at TIF as compared to TC, the above observation was unexpected. However, interestingly, in a subset of OSCC cases, the number of pS6 positive cells was higher than the corresponding TC (Fig. 14). Such OSCC subsets were not analyzed against cases with opposite pS6 expression pattern in the current study and warrants future studies.

We found a significant positive association between the expression of pS6 (Ser235/236) and the worst pattern of invasion in OSCC. Similarly, trends for positive association were found between the expression of pS6 and lymph node metastasis and tumor stage. These observations indicate that pS6 positive cells at the TIF might have a more invasive/aggressive phenotype. Indeed, previous studies have functionally linked pS6 with aggressive tumor phenotype such as cell proliferation, cell motility and invasion in esophageal squamous cell carcinoma (105). In line with these suggestions, higher pS6 expression at TIF was associated with lower overall and recurrence free survival probabilities in the current study. Nevertheless, those results were not statistically significant and analysis of a larger number of OSCC cases is necessary to substantiate them.

Similar to our findings, a previous studies has reported that expression of pS6 was correlated with poor prognosis in nasopharyngeal carcinoma (106) and in renal cell carcinoma (107). In contrast, positive expression of pS6 was associated with better survival in OSCCs (104) and laryngeal carcinomas (108) although the results were not statistically significant. de Vicente et al showed that a higher expression of pS6 correlated with smaller tumors and absence of node involvement. Moreover, they reported an inverse association between the expression of pS6 and disease specific survival of OSCC patients (104) . One of the explanations for the differences in the results between theirs and our studies could be related to the area of tumor

tissue analyzed. In the current study, whole FFPE sections were used, and pS6 at TIF was used for analysis, whereas the region of evaluation is difficult to ascertain in their study as the authors used tissue microarrays for IHC.

The current study focused on the activation of one of the arms (mTORC1) of mTOR pathway. Although activation status of S6 can be considered as an indicator of mTORC1 activation, it does not provide the information on the activation status of the other arm of mTOR, the mTORC2. Hence, future studies including IHC for phosphor-AKT desirable so that OSCC patients can be stratified based on the activation status of mTOR pathway. Such information will be valuable not only to predict the prognosis but also for guiding the treatment.

6. Conclusion

In conclusion, our study corroborates previous findings indicating that activation of the mTOR signaling is a common event in OSCC. Correlation between the high pS6 expression at TIF with the worst pattern of invasion and reduced probabilities for overall and recurrence free survival indicate that activation of mTORC1 arm of mTOR pathway might contribute to aggressive tumor phenotype. In future, validation of these results using a large cohort of patients might be useful in prognostication and guiding therapy for OSCC patients.

7. Limitations

Some limitations of this study need to be acknowledged. The nature of this study was retrospective and therefore only limited conclusions can be drawn. The current study has examined the activation status of only mTORC1 arm and therefore it will not be possible to identify OSCC cases with activation of mTORC2 arm of mTOR pathway. The confounding effects of other clinicopathological variables (such as stage, age, etc) for survival has not been examined in the current study as none of the examined variables with Kaplan-Meyer/Log-rank result in statistically significant results thereby precluding the establishment of Multivariate cox model.

8. Future perspectives

1. Longitudinal studies with more samples.
2. Functional studies using in vitro and in vivo models

9. References

1. Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. The Lancet. 2008;371(9625):1695-709.
2. Dobrossy L. Epidemiology of head and neck cancer: magnitude of the problem. Cancer Metastasis Rev. 2005;24(1):9-17.
3. Scully C, Bagan J. Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. Oral Dis. 2009;15(6):388-99.
4. Sakamoto Y, Matsushita Y, Yamada S, Yanamoto S, Shiraishi T, Asahina I, et al. Risk factors of distant metastasis in patients with squamous cell carcinoma of the oral cavity. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016;121(5):474-80.
5. Weise JB, Rudolph P, Heiser A, Kruse M-L, Hedderich J, Cordes C, et al. LOXL4 is a selectively expressed candidate diagnostic antigen in head and neck cancer. European journal of cancer. 2008;44(9):1323-31.
6. Almeida ADS, Oliveira DT, Pereira MC, Faustino SES, Nonogaki S, Carvalho AL, et al. Podoplanin and VEGF-C immunoexpression in oral squamous cell carcinomas: prognostic significance. Anticancer research. 2013;33(9):3969-76.
7. Lin NN, Wang P, Zhao D, Zhang FJ, Yang K, Chen R. Significance of oral cancer-associated fibroblasts in angiogenesis, lymphangiogenesis, and tumor invasion in oral squamous cell carcinoma. Journal of Oral Pathology & Medicine. 2017;46(1):21-30.
8. Jones KB, Klein OD. Oral epithelial stem cells in tissue maintenance and disease: the first steps in a long journey. Int J Oral Sci. 2013;5(3):121-9.
9. Nanci A. Ten Cate's Oral Histology. 9th Edition ed: Elsevier; 2017. 15th April 2020.
10. Ahmed IA. Identification of prognostic biomarkers for oral squamous cell carcinoma [Doctoral Thesis]. Bergen: University of Bergen; 2018.
11. Dentistry P. Cellular Events in Maturation. Fastest Clinical Dentistry Insight Engine2015.
12. Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsagué X, Laporte L, et al. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. Lancet Oncol. 2014;15(12):1319-31.
13. Alves AM, Diel LF, Lamers ML. Macrophages and prognosis of oral squamous cell carcinoma: A systematic review. J Oral Pathol Med. 2018;47(5):460-7.
14. Fujita Y, Okamoto M, Goda H, Tano T, Nakashiro K-i, Sugita A, et al. Prognostic significance of interleukin-8 and CD163-positive cell-infiltration in tumor tissues in patients with oral squamous cell carcinoma. PloS one. 2014;9(12).
15. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell, 5th edn. Garland Science. New York. 2008.
16. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018;68(6):394-424.
17. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, et al. Global cancer observatory: cancer today. Lyon, France: International Agency for Research on Cancer. 2018.
18. Warnakulasuriya S, Greenspan JS. Epidemiology of Oral and Oropharyngeal Cancers. Textbook of Oral Cancer: Springer; 2020. p. 5-21.
19. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer. 2015;136(5):E359-E86.
20. Gupta B, Johnson NW, Kumar N. Global epidemiology of head and neck cancers: a continuing challenge. Oncology. 2016;91(1):13-23.

21. Ghantous Y, Abu IE. Global incidence and risk factors of oral cancer. Harefuah. 2017;156(10):645-9.
22. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. CA: a cancer journal for clinicians. 2017;67(1):51-64.
23. Mehanna H, Paleri V, West C, Nutting C. Head and neck cancer—Part 1: Epidemiology, presentation, and preservation. Clinical Otolaryngology. 2011;36(1):65-8.
24. Petersen PE. Oral cancer prevention and control–the approach of the World Health Organization. Oral oncology. 2009;45(4-5):454-60.
25. Warnakulasuriya S. Risk factors for oral cancer. British journal of healthcare management. 2009;15(11):557-62.
26. Chen F, He B-C, Yan L-J, Qiu Y, Lin L-S, Cai L. Influence of oral hygiene and its interaction with standard of education on the risk of oral cancer in women who neither smoked nor drank alcohol: a hospital-based, case-control study. British Journal of Oral and Maxillofacial Surgery. 2017;55(3):260-5.
27. Perera M, Al-hebshi NN, Speicher DJ, Perera I, Johnson NW. Emerging role of bacteria in oral carcinogenesis: a review with special reference to perio-pathogenic bacteria. Journal of oral microbiology. 2016;8(1):32762.
28. Nair U, Bartsch H, Nair J. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. Mutagenesis. 2004;19(4):251-62.
29. Idris A, Prokopczyk B, Hoffmann D. Toombak: a major risk factor for cancer of the oral cavity in Sudan. Preventive medicine. 1994;23(6):832-9.
30. Petti S. Lifestyle risk factors for oral cancer. Oral Oncol. 2009;45(4-5):340-50.
31. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma—an update. CA: a cancer journal for clinicians. 2015;65(5):401-21.
32. Guha P. Betel Leaf: The Neglected Green Gold of India. Journal of Human Ecology. 2006;19(2):87-93.
33. Petti S, Masood M, Scully C. The magnitude of tobacco smoking-betel quid chewing-alcohol drinking interaction effect on oral cancer in South-East Asia. A meta-analysis of observational studies. PloS one. 2013;8(11).
34. Scully C, Bedi R. Ethnicity and oral cancer. The lancet oncology. 2000;1(1):37-42.
35. Gorde S. Travel and Documentary Photography. Beedi - Indian Cigarrete: Wordpress; 2015. p. 2.
36. Zatterstrom UK, Svensson M, Sand L, Nordgren H, Hirsch JM. Oral cancer after using Swedish snus (smokeless tobacco) for 70 years - a case report. Oral Dis. 2004;10(1):50-3.
37. Hirsch JM, Wallström M, Carlsson AP, Sand L. Oral cancer in Swedish snuff dippers. Anticancer Res. 2012;32(8):3327-30.
38. Califano J, Westra WH, Meininger G, Corio R, Koch WM, Sidransky D. Genetic progression and clonal relationship of recurrent premalignant head and neck lesions. Clinical Cancer Research. 2000;6(2):347-52.
39. JM Braakhuis B, René Leemans C, H. Brakenhoff R. A genetic progression model of oral cancer: current evidence and clinical implications. Journal of oral pathology & medicine. 2004;33(6):317-22.
40. Pickering CR, Zhang J, Yoo SY, Bengtsson L, Moorthy S, Neskey DM, et al. Integrative genomic characterization of oral squamous cell carcinoma identifies frequent somatic drivers. Cancer discovery. 2013;3(7):770-81.
41. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154-7.

42. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157-60.
43. Lui VW, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. Cancer discovery. 2013;3(7):761-9.
44. Amornphimoltham P, Patel V, Sodhi A, Nikitakis NG, Sauk JJ, Sausville EA, et al. Mammalian target of rapamycin, a molecular target in squamous cell carcinomas of the head and neck. Cancer research. 2005;65(21):9953-61.
45. Molinolo AA, Hewitt SM, Amornphimoltham P, Keelawat S, Rangdaeng S, García AM, et al. Dissecting the Akt/mammalian target of rapamycin signaling network: emerging results from the head and neck cancer tissue array initiative. Clinical Cancer Research. 2007;13(17):4964-73.
46. Molinolo AA, Marsh C, El Dinali M, Gangane N, Jennison K, Hewitt S, et al. mTOR as a molecular target in HPV-associated oral and cervical squamous carcinomas. Clinical cancer research. 2012;18(9):2558-68.
47. Iglesias-Bartolome R, Martin D, Gutkind JS. Exploiting the head and neck cancer oncogenome: widespread PI3K-mTOR pathway alterations and novel molecular targets. Cancer discovery. 2013;3(7):722-5.
48. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149(2):274-93.
49. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nature reviews Molecular cell biology. 2009;10(5):307-18.
50. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;168(6):960-76.
51. Fan B, Sun Y-J, Liu S-Y, Che L, Li G-Y. Neuroprotective Strategy in Retinal Degeneration: Suppressing ER Stress-Induced Cell Death via Inhibition of the mTOR Signal. International Journal of Molecular Sciences. 2017;18:201.
52. Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, et al. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. J Biol Chem. 2007;282(19):14056-64.
53. Nakashima A, Tamanoi F. 8 - Conservation of the Tsc/Rheb/TORC1/S6K/S6 Signaling in Fission Yeast. In: Tamanoi F, Hall MN, editors. The Enzymes. 28: Academic Press; 2010. p. 167-87.
54. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nature reviews Molecular cell biology. 2011;12(1):21-35.
55. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase–AKT pathway in human cancer. Nature Reviews Cancer. 2002;2(7):489-501.
56. Clark C, Shah S, Herman-Ferdinandez L, Ekshyyan O, Abreo F, Rong X, et al. Teasing out the best molecular marker in the AKT/mTOR pathway in head and neck squamous cell cancer patients. The Laryngoscope. 2010;120(6):1159-65.
57. Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, et al. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes & development. 2005;19(18):2199-211.
58. Larsen IK, Myklebust TÅ, Johannesen TB, Møller B, Hofvind S. Stage-specific incidence and survival of breast cancer in Norway: The implications of changes in coding and classification practice. The Breast. 2018;38:107-13.
59. Güneri P, Epstein JB. Late stage diagnosis of oral cancer: components and possible solutions. Oral oncology. 2014;50(12):1131-6.

60. De Paz D, Kao H-K, Huang Y, Chang K-P. Prognostic stratification of patients with advanced oral cavity squamous cell carcinoma. Current oncology reports. 2017;19(10):65.
61. Jordan JARJSRCK. Oral Pathology. 7th ed: Elsevier Health Sciences; 2015.
62. Silverman Jr S. Demographics and occurrence of oral and pharyngeal cancers: the outcomes, the trends, the challenge. The Journal of the American Dental Association. 2001;132:7S-11S.
63. Dirven R, Ebrahimi A, Moeckelmann N, Palme CE, Gupta R, Clark J. Tumor thickness versus depth of invasion – Analysis of the 8th edition American Joint Committee on Cancer Staging for oral cancer. Oral Oncology. 2017;74:30-3.
64. Huang SH, O'Sullivan B. Overview of the 8th edition TNM classification for head and neck cancer. Current treatment options in oncology. 2017;18(7):40.
65. Bavle RM, Venugopal R, Konda P, Muniswamappa S, Makarla S. Molecular classification of oral squamous cell carcinoma. Journal of clinical and diagnostic research: JCDR. 2016;10(9):ZE18.
66. Thompson LD. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. Ear, Nose and Throat Journal. 2006;85(2):74-5.
67. De Cecco L, Nicolau M, Giannoccaro M, Daidone MG, Bossi P, Locati L, et al. Head and neck cancer subtypes with biological and clinical relevance: Meta-analysis of gene-expression data. Oncotarget. 2015;6(11):9627.
68. Massano J, Regateiro FS, Januário G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontology. 2006;102(1):67-76.
69. Denaro N, Russi EG, Adamo V, Merlano MC. State-of-the-art and emerging treatment options in the management of head and neck cancer: news from 2013. Oncology. 2014;86(4):212-29.
70. Dzioba A, Aalto D, Papadopoulos-Nydam G, Seikaly H, Rieger J, Wolfaardt J, et al. Functional and quality of life outcomes after partial glossectomy: a multi-institutional longitudinal study of the head and neck research network. Journal of Otolaryngology-Head & Neck Surgery. 2017;46(1):56.
71. Rampias T, Giagini A, Siolos S, Matsuzaki H, Sasaki C, Scorilas A, et al. RAS/PI3K crosstalk and cetuximab resistance in head and neck squamous cell carcinoma. Clinical Cancer Research. 2014;20(11):2933-46.
72. Boeckx C, Baay M, Wouters A, Specenier P, Vermorken JB, Peeters M, et al. Anti-epidermal growth factor receptor therapy in head and neck squamous cell carcinoma: focus on potential molecular mechanisms of drug resistance. The oncologist. 2013;18(7):850.
73. Network CGA. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517(7536):576-82.
74. Mücke T, Mitchell DA, Ritschl LM, Tannapfel A, Wolff K-D, Kesting MR, et al. Influence of tumor volume on survival in patients with oral squamous cell carcinoma. Journal of cancer research and clinical oncology. 2015;141(6):1007-11.
75. Mermod M, Tolstonog G, Simon C, Monnier Y. Extracapsular spread in head and neck squamous cell carcinoma: a systematic review and meta-analysis. Oral oncology. 2016;62:60-71.
76. Wang C, Huang H, Huang Z, Wang A, Chen X, Huang L, et al. Tumor budding correlates with poor prognosis and epithelial-mesenchymal transition in tongue squamous cell carcinoma. Journal of oral pathology & medicine. 2011;40(7):545-51.
77. Chang YC, Nieh S, Chen SF, Jao SW, Lin YL, Fu E. Invasive pattern grading score designed as an independent prognostic indicator in oral squamous cell carcinoma. Histopathology. 2010;57(2):295-303.

78. Chatterjee D, Bansal V, Malik V, Bhagat R, Punia RS, Handa U, et al. Tumor Budding and Worse Pattern of Invasion Can Predict Nodal Metastasis in Oral Cancers and Associated With Poor Survival in Early-Stage Tumors. Ear, Nose & Throat Journal. 2019;98(7):E112-E9.
79. Henry NL, Hayes DF. Cancer biomarkers. Molecular Oncology. 2012;6(2):140-6.
80. Chen XH, Huang S, Kerr D. Biomarkers in clinical medicine. IARC Sci Publ. 2011(163):303-22.
81. Altman DG, McShane LM, Sauerbrei W, Taube SE. Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK): explanation and elaboration. PLoS Med. 2012;9(5):e1001216-e.
82. Kim SW, Roh J, Park CS. Immunohistochemistry for Pathologists: Protocols, Pitfalls, and Tips. J Pathol Transl Med. 2016;50(6):411-8.
83. Yaziji H, Barry T. Diagnostic Immunohistochemistry: what can go wrong? Adv Anat Pathol. 2006;13(5):238-46.
84. Wester K, Wahlund E, Sundstrom C, Ranefall P, Bengtsson E, Russell PJ, et al. Paraffin section storage and immunohistochemistry. Effects of time, temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen. Appl Immunohistochem Mol Morphol. 2000;8(1):61-70.
85. Grillo F, Bruzzone M, Pigozzi S, Prosapio S, Migliora P, Fiocca R, et al. Immunohistochemistry on old archival paraffin blocks: is there an expiry date? Journal of Clinical Pathology. 2017;70(11):988-93.
86. Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med. 2011;135(5):537-43.
87. Xie R, Chung JY, Ylaya K, Williams RL, Guerrero N, Nakatsuka N, et al. Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. J Histochem Cytochem. 2011;59(4):356-65.
88. Oyama T, Ishikawa Y, Hayashi M, Arihiro K, Horiguchi J. The effects of fixation, processing and evaluation criteria on immunohistochemical detection of hormone receptors in breast cancer. Breast Cancer. 2007;14(2):182-8.
89. Bankhead P, Loughrey MB, Fernandez JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017;7(1):16878.
90. Bryne M, Boysen M, Alfsen CG, Abeler VM, Sudbo J, Nesland JM, et al. The invasive front of carcinomas. The most important area for tumour prognosis? Anticancer Res. 1998;18(6b):4757-64.
91. Wagner VP, Webber LP, Curra M, Klein IP, Meurer L, Carrad VC, et al. Bryne's grading system predicts poor disease-specific survival of oral squamous cell carcinoma: a comparative study among different histologic grading systems. Oral Surg Oral Med Oral Pathol Oral Radiol. 2017;123(6):688-96.
92. Sawair FA, Irwin CR, Gordon DJ, Leonard AG, Stephenson M, Napier SS. Invasive front grading: reliability and usefulness in the management of oral squamous cell carcinoma. J Oral Pathol Med. 2003;32(1):1-9.
93. Sapkota D, Bruland O, Parajuli H, Osman TA, Teh M-T, Johannessen AC, et al. S100A16 promotes differentiation and contributes to a less aggressive tumor phenotype in oral squamous cell carcinoma. BMC Cancer. 2015;15(1):631.
94. Bryne M, Koppang HS, Lilleng R, Kjaerheim A. Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. J Pathol. 1992;166(4):375-81.

95. Elkabets M, Pazarentzos E, Juric D, Sheng Q, Pelossof RA, Brook S, et al. AXL mediates resistance to PI3Kα inhibition by activating the EGFR/PKC/mTOR axis in head and neck and esophageal squamous cell carcinomas. Cancer cell. 2015;27(4):533-46.
96. Elkabets M, Vora S, Juric D, Morse N, Mino-Kenudson M, Muranen T, et al. mTORC1 inhibition is required for sensitivity to PI3K p110α inhibitors in PIK3CA-mutant breast cancer. Science translational medicine. 2013;5(196):196ra99-ra99.
97. Bosch A, Li Z, Bergamaschi A, Ellis H, Toska E, Prat A, et al. PI3K inhibition results in enhanced estrogen receptor function and dependence in hormone receptor–positive breast cancer. Science translational medicine. 2015;7(283):283ra51-ra51.
98. Iwenofu OH, Lackman RD, Staddon AP, Goodwin DG, Haupt HM, Brooks JS. Phospho-S6 ribosomal protein: a potential new predictive sarcoma marker for targeted mTOR therapy. Modern pathology. 2008;21(3):231-7.
99. Zhang M, Fu W, Prabhu S, Moore JC, Ko J, Kim JW, et al. Inhibition of polysome assembly enhances imatinib activity against chronic myelogenous leukemia and overcomes imatinib resistance. Molecular and cellular biology. 2008;28(20):6496-509.
100. Papageorgiou A, Avruch J. A genome-wide RNAi screen for polypeptides that alter rpS6 phosphorylation. mTOR: Springer; 2012. p. 187-214.
101. Chakraborty S, Mohiyuddin SA, Gopinath K, Kumar A. Involvement of TSC genes and differential expression of other members of the mTOR signaling pathway in oral squamous cell carcinoma. BMC cancer. 2008;8(1):163.
102. Chaisuparat R, Rojanawatsirivej S, Yodsanga S. Ribosomal protein S6 phosphorylation is associated with epithelial dysplasia and squamous cell carcinoma of the oral cavity. Pathol Oncol Res. 2013;19(2):189-93.
103. Martins F, de Sousa SC, dos Santos E, Woo SB, Gallottini M. PI 3K–AKT–mTOR pathway proteins are differently expressed in oral carcinogenesis. Journal of Oral Pathology & Medicine. 2016;45(10):746-52.
104. de Vicente JC, Peña I, Rodrigo JP, Rodríguez-Santamarta T, Lequerica-Fernández P, Suárez-Fernández L, et al. Phosphorylated ribosomal protein S6 correlation with p21 expression and inverse association with tumor size in oral squamous cell carcinoma. Head Neck. 2017;39(9):1876-87.
105. Kim S-H, Jang YH, Chau GC, Pyo S, Um SH. Prognostic significance and function of phosphorylated ribosomal protein S6 in esophageal squamous cell carcinoma. Modern Pathology. 2013;26(3):327-35.
106. Chen J, Hu C-F, Hou J-H, Shao Q, Yan L-X, Zhu X-F, et al. Epstein-Barr virus encoded latent membrane protein 1 regulates mTOR signaling pathway genes which predict poor prognosis of nasopharyngeal carcinoma. Journal of translational medicine. 2010;8(1):30.
107. Pantuck AJ, Seligson DB, Klatte T, Yu H, Leppert JT, Moore L, et al. Prognostic relevance of the mTOR pathway in renal cell carcinoma: implications for molecular patient selection for targeted therapy. Cancer. 2007;109(11):2257-67.
108. García-Carracedo D, Villaronga MÁ, Álvarez-Teijeiro S, Hermida-Prado F, Santamaría I, Allonca E, et al. Impact of PI3K/AKT/mTOR pathway activation on the prognosis of patients with head and neck squamous cell carcinomas. Oncotarget. 2016;7(20):29780-93.