UNIVERSITY OF BERGEN

Frequent activation of mTOR pathway in oral squamous cell carcinoma cases from Nepal, but without association with prognosis

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Abstract

This master thesis is a research project and is a part of a larger study called “Molecular biomarkers in oral premalignant and oral cancer lesions”.

Background: Activation of mTOR pathway has been described as a common event in oral squamous cell carcinoma (OSCC) and expression of phosphorylated-S6 protein (pS6), one of the end-point indicators of the activation of mTOR pathway, has been shown to correlate with progression of OSCC in cohorts of patients from western world.

Aim: The aim of this study was to investigate pS6 as a putative predictor of OSCC progression in a cohort of patients from Nepal, with different demographic characteristics and etiological factors than the western cohorts previously investigated.

Materials and methods: Our cohort consists of tissue samples collected from the Nepalese population between the years 2011- 2014 (N = 71) after ethical approval from the regional authorities and informed consent from patients. Immunohistochemistry containing primary rabbit polyclonal antibodies and Dako En Vizion visualization system, were used to examine the expression of pS6. SPSS statistical package was used in this study to detect associations between pS6 expression with the 5-years survival rate (statistically significance: p-value <0.05) in cases of OSCC and clinico-pathological parameters.

Results: Age (p=0.006), lymph node metastasis (p=0.032) and stage (p=0.004) were found significantly associated with overall survival in Kaplan-Meier survival analysis. Only age and tumor stage, according to Cox-regression analysis, were independent predictors of overall survival. Gender, tobacco and alcohol misuse were not found associated with survival. The mean percentage of pS6 expressing tumor cells in the tumor center was 53.92% (SEM ± 3.86) with a higher value at the tumor invading front where the mean percentage was 56,43% (SEM ± 7.20). The percentage of positive pS6 cells in the tumor center were further analysed for correlations with

clinical parameters. A weak inverse correlation was found with lymph node metastasis ($p= 0.028$, Pearsons' $r=-0.287$). A trend for increased percentage of pS6 positive cells with tumor size and late stages, as well as in tissues from patients who died during the follow-up period, although this was not statistically significant ($p>0.05$).

Conclusions: Our results demonstrate that the activation status of mTOR pathway is present in many of the Napalese OSCC cases. Although the investigated samples show a high percentage of pS6 expression, pS6 as a cancer biomarker, does not seem to be a predictor of tumor progression or survival in this patient cohort.

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1. Background

1.1 Definition of oral squamous cell carcinoma (OSCC)

OSCC is one of the most common types of malignant oral cancer arising from the oral mucosal epithelium. This mucosal change makes up over 90% of the incidents of oral cancer (1). The 5-year overall survival of OSCC is considered to be 47%, while the disease-free survival rate is 74% (91).

1.2 Epidemiology

Head and neck cancers include areas in pharynx, larynx, nasal cavity, paranasal sinuses and oral cavity (2). Oral cancer is ranked as the 6th most common cancer type in the world. In India the prevalence of oral cancer is especially high, being the second country globally with the highest incidence of oral cancer cases (3). This can be explained by the presence of different etiological factors. Nepal is ranked as number 17 in the world (4) and within the country oral cancer is registered as the 8th most common type of cancer (5).

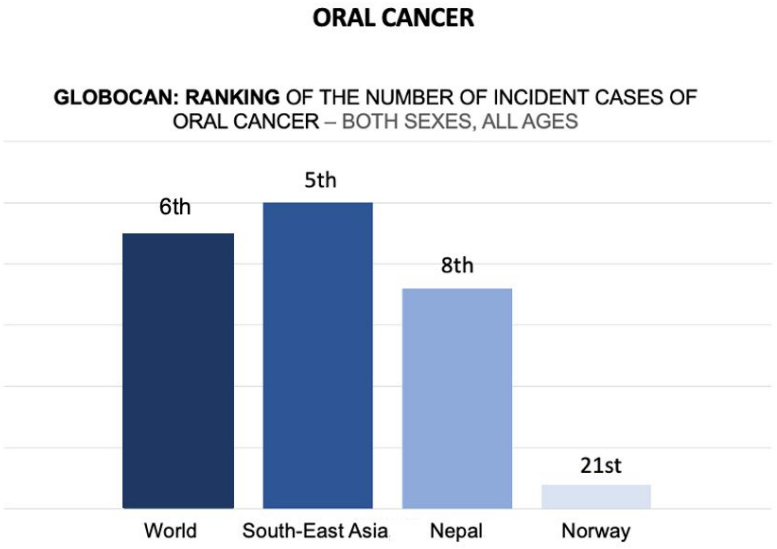


Figure 1:
Oral cancer ranked as the 6th most common type of cancer worldwide (3), the 5th in South-East Asia (6), the 8th in Nepal (5) and the 21st in Norway (7). The sources are respectively from 2020.

1.3 Geographic facts of Nepal

Nepal is located in the south of Asia, it borders China in the north and India in the west, east and south. Even though the country's total area is 147 180 km², which means its size is just above ⅓ of Norway´s total area, the country has a population of 30 327 877 people and agriculture makes up 75% of the population´s livelihood (8, 9).

1.4 Socioeconomic status in Nepal

According to the rating of the Human Development Index provided by The United Nations, Nepal ranks as number 145 out of 186 countries whereas 186 is the poorest developed country in the world regarding social and economic standards (10). A large portion of the population lacks health insurance to pay for medical treatments (11). As a result, it is reasonable to think that many people choose to give low priority to dental care, as it is not often categorized as life threatening.

1.5 Etiology/risk factors associated with OSCC

Oral cancer is a complex and multifactorial disease. Some generally known factors that increase the risk of oral cancer are: Smoking and smokeless tobacco, excessive alcohol consumption, bad oral hygiene and overexposure to sunlight (3).

1.5.1 The role of tobacco and alcohol consumption on OSCC

Studies show that there is a correlation between the use of tobacco and OSCC. In total, 90% of oral cancers have been estimated to be affected by tobacco and excessive alcohol consumption (12).

Betel quid is a type of smokeless tobacco that is popular in Asia (13). The reason why betel quid may have a higher risk for oral cancer than smoking tobacco, may be because it contains a different carcinogenic ingredient called 3-(methylnitrosamino)propionitrile (MNPN). It originates from arecoline, an alkaloid in the areca nut (14). Nevertheless, both smoking and smokeless tobacco use are considered harmful to health due to the chemical compounds in tobacco. According to the National Cancer Institute, there are 250 substances in tobacco that are categorized as harmful (15). The chemical compounds in tobacco that are

carcinogenic are known as the tobacco-specific N-nitrosamines (TSNAs) and are derived from nicotine, nor-nicotine, anatabine and anabasine (16).

1.5.2 Socioeconomic culture and tobacco consumption in Nepal

Studies show that there is a greater use of tobacco among the poor and lower educated as well as there are more males who consume tobacco, than females in Nepal. In 2016 there were 52,3% males who used some type of tobacco while females covered 8,4% (17). The cultural norm in Nepal may explain this difference. Traditional gender roles are still valued in many households and therefore it may be considered to be less acceptable for women to intake tobacco and alcohol (18). This is reflected in the number of men and women diagnosed with oral cancer where men make up 73% of the OSCC cases in Nepal (89). It has been reported that the majority of people in Nepal are diagnosed at an advanced stage of oral cancer (19). A contributing factor to this may be that the information related to risk factors of oral cancer is limited (20) compared to for instance human immunodeficiency virus (HIV) (21), acquired immune deficiency syndrome (AIDS) (21), tuberculosis (22) and cholera (23). The financial aspect of the medical treatments can be challenging for many people, especially in a low-income country such as Nepal. Many have therefore to go untreated.

Older age groups have similarly been associated with higher tobacco consumption. Over the last 10-year period smokeless tobacco has been an increasing trend compared to smoking tobacco in Nepal, especially among the age group 35 and older (17).

Figure 2: A) Betel quid (24). **B)** Smoking tobacco (25). **C)** Alcohol (26).

1.6 The Nepalese regulations on tobacco

Because of the well-known tobacco culture, the Napalese government, directed by the Ministry of Health and Population, propositioned a bill called “Tobacco Control and Regulatory Bill 2010”. The bill got approved in 2011 with the purpose of banning the consumption of tobacco in public spaces and private sales of cigarettes. Tobacco firms must cover 75% of their products with health warnings. All sales of tobacco are taxed, and tobacco advertisement is forbidden (27).

1.7 Normal oral mucosa

1.7.1 Clinical features

The clinical signs of an ideal healthy oral mucosa are tissues that appear symmetrical, with even, smooth surfaces that have a pink tone and are moisty (28). High levels of melatonin might be also common, and it will represent itself as areas of hyperpigmentation (29). However, irregularities that occur in the oral mucosa are not necessarily pathologic, such as Fordyce's granules or leukoedema.

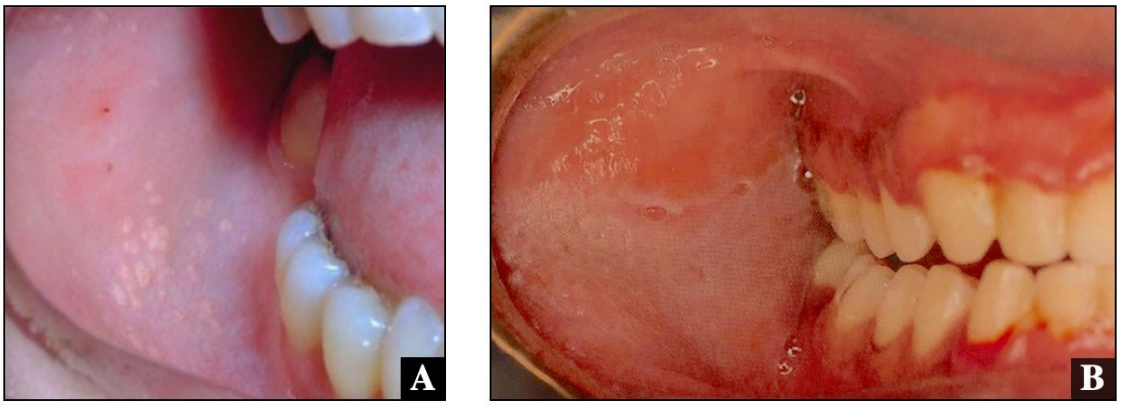


Figure 3: A) A clinical photo of Fordyce's granules. Published with the permission of Prof. Anne Christine Johannessen (30). **B)** Leukoedema observed clinically. Adapted from (31).

Topographical variations are found in oral mucosa. There can either be keratinized or non-keratinized epithelium. The areas that fall under the first are masticatory mucosa such as gingiva, hard palate and dorsum of the tongue. Non-keratinized epithelium can be found in lining mucosa such as lip mucosa, soft palate and buccal mucosa. Alveolar mucosa, the floor of the mouth and the ventral surface of tongue are also non-keratinized areas in the oral cavity (32, 33). Moreover, frictional

keratosis in areas of mucosa that is normally non-keratinized may arise due to continuous traumatic external stimuli (34). Non-keratinized epithelium tends to look softer and slightly darker in colour compared to keratinized mucosa, as the blood vessels underneath are more visible due to the lack of the superficial layer.

1.7.2 Histological features

The oral cavity has a protective barrier on the surface which is covered by stratified squamous epithelium, while the deeper layer consists of lamina propria. These two layers are what make up the oral mucosa. As mentioned above, the epithelium can either be keratinized or non-keratinized. It can further be divided into different layers (35). The main layers being:

- Stratum basale
- Stratum spinosum / prickle layer
- Stratum Granulosum (keratinized)
- Stratum Corneum (keratinized or non-keratinized)

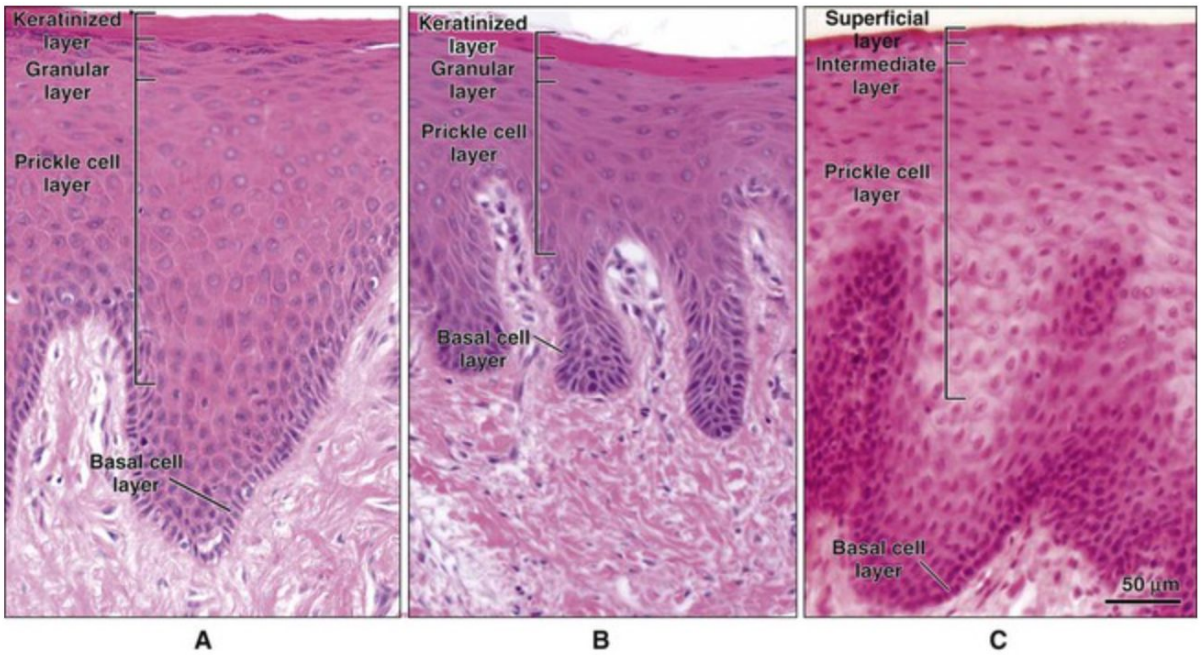


Figure 3: Histological image of the different layers in oral mucosa, keratinized and non-keratinized. **A)** Orthokeratinized epithelium: Characterized by the loss of nuclei in the keratinized layer. **B)** Parakeratinized epithelium: Characterized by the presence of nuclei in the layer of keratinization. **C)** Non-keratinized epithelium. Adapted from (36).

1.8 Pre-stage of OSCC

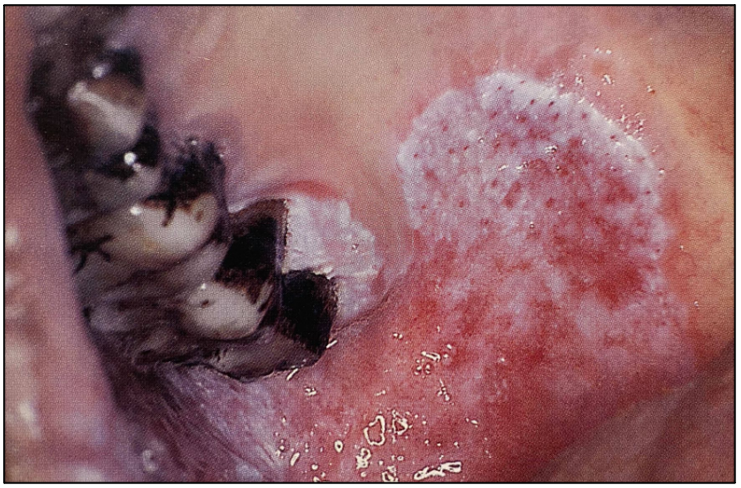


Figure 4: Oral lesion with premalignant potential (OPMD) in the form of speckled leukoplakia, observed in a patient who chews betel quid. Brown staining on the teeth is a result of the betel quid. Adapted from (37).

One of the most common oral lesions with premalignant potential (OPMD) is leukoplakia (34). About 10-15% of leukoplakias develop into oral cancer (38). Idiopathic leukoplakia has been defined by Regezi et al. as a “white lesion or plaque of the oral mucosa that cannot be rubbed off and cannot be characterized clinically as any other disease” (39).

1.8.1 Clinical features

Leukoplakia can appear as shallow lesions located on the oral mucosa, gingiva or/and the tongue. This mucosal change has a white appearance due to keratosis, with or without hyperplasia of the epithelium. Combined white and red lesions called erythroleukoplakia lesions and erythematous lesions called erythroplakia, may indicate the potential for cancerous development due to greater risk of being dysplastic (40). Although leukoplakia are mostly benign, some patients may therefore experience lesions that develop into malignancy. Leukoplakia is usually painless or slightly sore, and the texture of the area can be anything from smooth to uneven (41).

1.8.2 Histological features

Microscopically, leukoplakia may either have a normal dysplastic or a cancerous appearance. Dysplasia signifies disordered growth and abnormal epithelium which can further manifest in certain histological characteristics such as: (i) drop-shaped epithelial ridges, (ii) basal cell crowding, (iii) irregular stratification, (iv) increased and abnormal mitotic figures, (v) premature keratinization, (vi) nuclear pleomorphism and (vii) increased nuclear-cytoplasmic ratio. If there is dysplasia, the degree of epithelial dysplasia indicates how far the lesion has progressed, in terms of the extent to which the abnormal cells have replaced the normal epithelial cells. When the cells have penetrated the basal cell membrane, causing an infiltrative growth pattern, the patient will get the diagnosis of OSCC. Carcinoma in situ/severe dysplasia is the stage right before an invasion of abnormal epithelial cells into the underlying connective tissue (42).

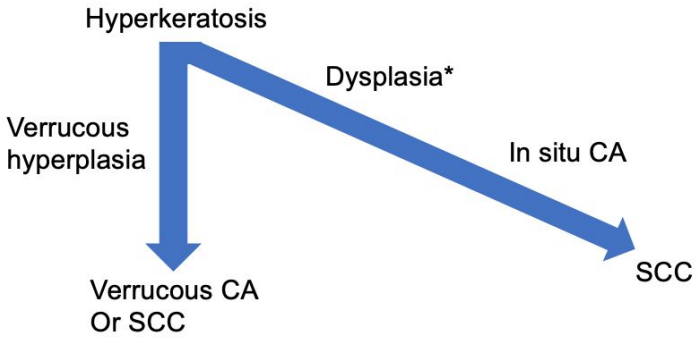


Figure 5: Illustrating possible development outcomes of hyperkeratosis lesions. Adapted from (38).

1.9 OSCC

1.9.1 Clinical features

OSCC can be located on the lips, gingiva, tongue, mobile part of the tongue, hard and soft palate, floor of the mouth and inside of the cheeks (43). The symptoms that may occur are mouth pain, ear pain or sore mouth, and lesions that do not heal. White/red patches inside of the mouth, loosening of teeth and a growth/lump inside the mouth are also symptoms that can occur (44). The lesion may become more prominent with rolled borders with necrosis at the center (Figure 6). These ulcers tend to be painful and look more inflamed (43).

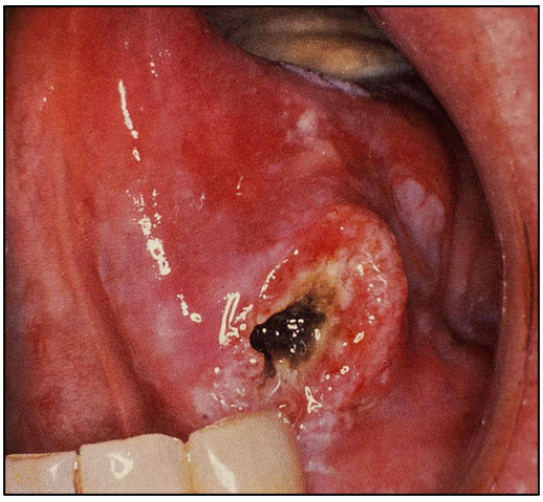


Figure 6: Advanced squamous carcinoma. Ulcer with rolled border and necrosis at the center. Surrounding area containing keratosis and erythema. Adapted from (43).

1.9.2 Histological features

OSCC is a pathological infiltrative growth of the oral mucous membrane, due to abnormal epithelial cells invading their surrounding tissue. After the abnormal cells penetrate the basal cell membrane they will form malignant tumor islands in the surrounding connective tissue and/or tissue of muscle (45). The tumor cells will induce development of their own blood vessels for nutrition (46). The patient's immune system is usually responding to the presence of cancer and this is usually observed as inflammatory cells infiltrate in the tumor stroma, as seen in the histological figure 8. The cancer cells are irregular and do not follow an organised structure, unlike normal epithelial cells (45).

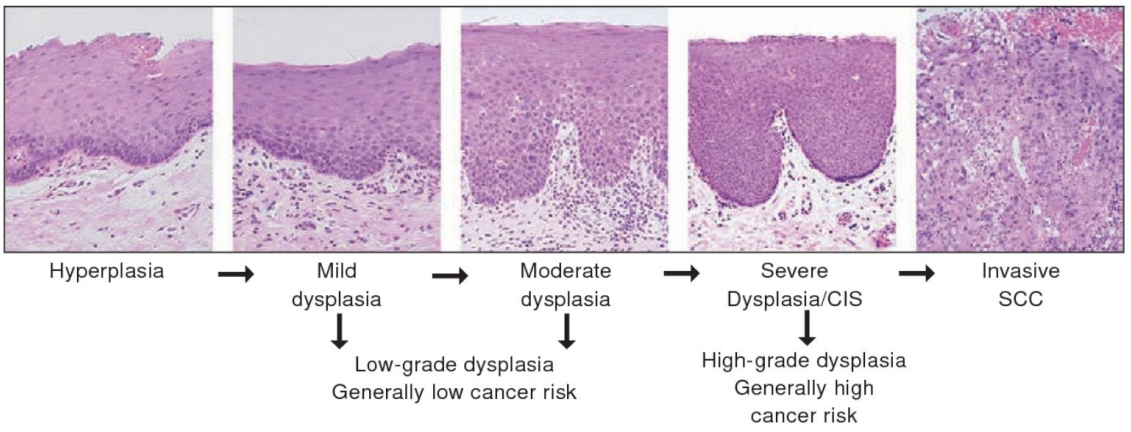


Figure 7: Histological image illustrating the stages of dysplasia resulting in OSCC and its appropriate cancer risk. Adapted from (47).

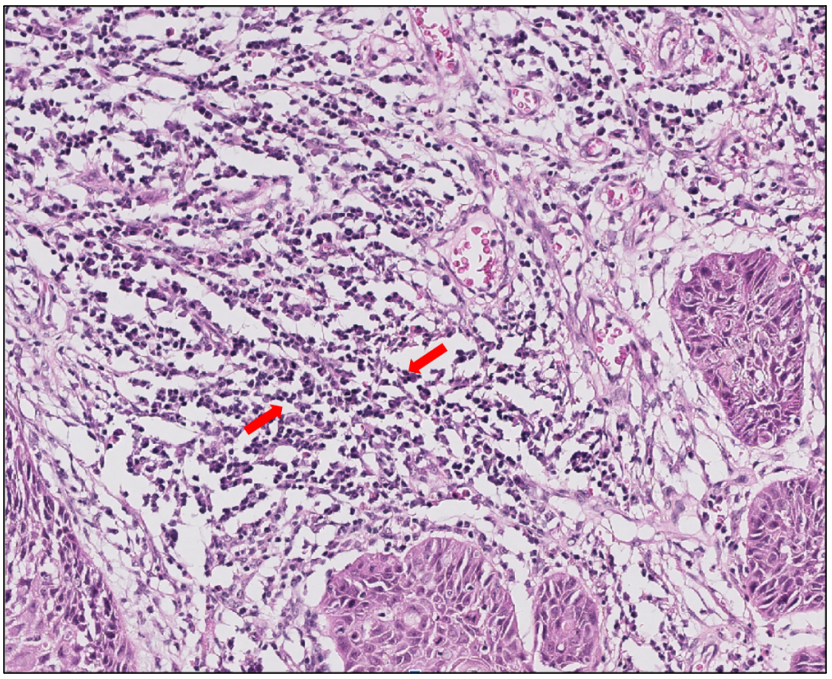


Figure 8: A representative picture from one of the patients in our cohort illustrating an increased amount of inflammatory cells in tumor tissue (red arrows).

Cell differentiation

OSCC can be divided into 3 grades. The grading system is based on the differentiation of the malignant cells, meaning their ability to differentiate and produce keratin (48).

Well-differentiated OSCC, low grade

Well differentiated OSCCs contain cells that can undergo a differentiation process similar to normal oral cells. These cells form keratin pearls. Compared to poorly differentiated cells, they usually spread and grow slower, which in turn gives the patient better prognosis (48).

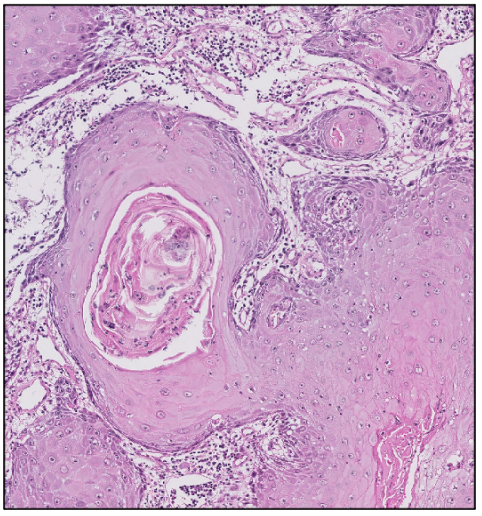


Figure 9: A hematoxylin-eosin stained slide of tumor tissue with a keratin pearl seen in the tumor center.

Moderately differentiated OSCC, intermediate grade

Being in the intermediate grade means that these cells have less ability to differentiate than the normal oral epithelial cells. Compared to well-differentiated OSCC, the moderately differentiated OSCC have less keratinization, more nuclear pleomorphism and higher mitotic activity. This comparison with these cells is opposite for the poorly differentiated cells. It has been reported that cases of moderately differentiated OSCC had a high recurrence rate (49).

Poorly differentiated OSCC, high grade

These OSCC contain more pleomorphic cells and have a more irregular form and they have little to no keratin formation. In most poorly differentiated carcinomas, the cytoplasm of the tumor cells is sparse and less visible. These cells have a tendency to metastase and infiltrate a larger portion of the surrounding tissue at an early stage, which results in poorer prognosis and therefore low survival rate as well (48).

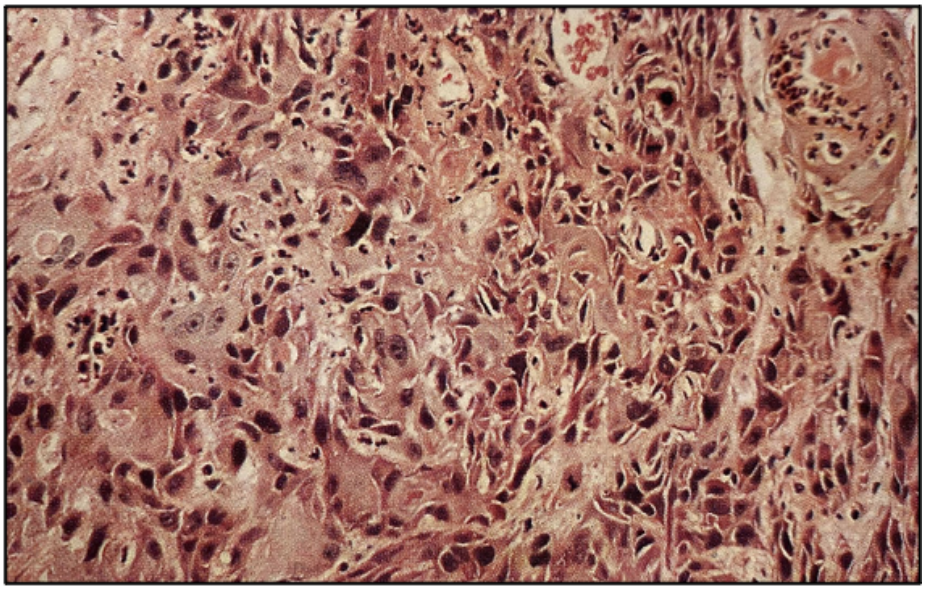


Figure 10: Squamous carcinoma containing poorly differentiated cells with hyperchromatic nuclei. Adapted from (48).

1.10 Tumor - Nodes - Metastasis (TNM)

1.10.1 TNM classification of malignant tumors

There is a standard system that is used to classify the extent of tumors, by examining the size and its spread into surrounding tissue. The higher the value is, the more severe the tumor is. This results in poorer prognosis and a more invasive treatment plan is required (50).

T: Tumor

T0: Carcinoma in situ
T1: Carcinoma infiltrative growth, perimeter < 2 cm
T2: Carcinoma infiltrative growth, perimeter 2–4cm
T3: Carcinoma infiltrative growth, perimeter > 4 cm
T4: Growth expanded into the bone, the muscle etc. This stage has the worst prognosis out of all the levels.

N: Noduli, lymph nodes (LN)

N0: No lymph nodes involved.
N1: Lymph nodes located on the same side as the tumor are involved.
N2: Lymph nodes located on both sides of the body are involved.
N3: The lymph node(s) is/are attached to the underlying tissue.

M: Metastases (happens when the tumor has grown past the lymph nodes).

M0: None
M1: Proven/Positive

What stage the tumor has progressed to depends on the combined TNM-values, as illustrated on the next page (51).

Tumor stage 1: T1, N0, M0

Tumor stage 2: T2, N0 or N1, M0

Tumor stage 3: T3-1, N0-1, M0

Tumor stage 4: T4, N0-1, M0 / T any, N2-3, M0 / T any, N any, M

1.11 Treatment of idiopathic white patches (leukoplakias) and OSCC

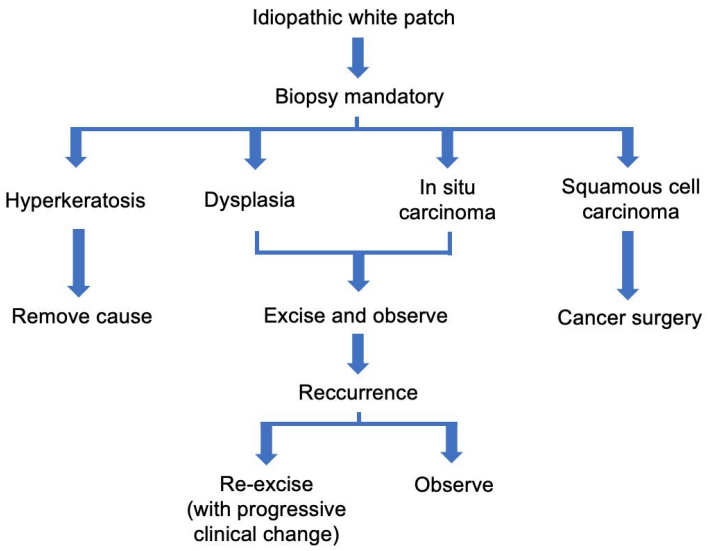


Figure 11: Showing a standard treatment pathway for idiopathic white patches after biopsy based on diagnosis. Adapted from (52).

The treatment of OSCC depends on the severity of the tumor. If the clinical practitioner suspects the tumor or the idiopathic white patches to be malignant, then the patient should be referred to a specialist - that would be to the regional head and neck oncological team. Where a biopsy will be taken to give the lesion a diagnosis.

If the practitioner has a low suspicion of the lesion being malignant the lesion can be observed (maximum 2 weeks). If the lesion progresses, a biopsy will be necessary for diagnostics. If it is indicative of malignancy, the patient will be referred to the regional head and neck oncological team for further evaluation.

Surgery is the preferred method for carcinomas that are small and easily accessible, verrucous or involve bone and have a high risk of later radionecrosis because of radiotherapy. However if the tumor responds poorly to surgery or metastasis to other areas that are less accessible, another option is to combine surgical treatment with radiotherapy.

Radiation therapy will give a more acceptable aesthetic and functional result compared to surgery, however it will also provide discomfort during the multiple treatment sessions. The downside of this treatment form is that radiation will damage the patient's normal cells as well as the neoplastic cells. Localizing and separating the healthy from the neoplastic tissue is therefore a crucial part of the treatment planning (53).

Chemotherapy is another treatment option for oral cancer. The treatment uses drugs containing cytotoxic agents. They can either be the primary therapy or it can be combined with radiation therapy and thereby be a radiation sensitizer. The latter gives a better survival rate for patients with extracapsular nodal extension and positive resection margin. For many cancer types that might especially have a high risk of recurrence and metastasis, adjuvant chemotherapy is given after the surgery to prevent this (54). However, for head and neck cancers this had not shown to give any benefits according to a meta-analysis of chemotherapy in head and neck cancer (55).

In Immunotherapy the patient's own immune system gets stimulated to be able to attack tumor cells. The immune system has checkpoints that regulate apoptosis, maintain homeostasis and prevent autoimmunity (56). Cancer cells survive by avoiding these checkpoints. Tumor cells have proteins on the surfaces that bind to immune checkpoint proteins located on the surfaces of the T-cells. This binding results in the T-cell getting inactivated, leading to the continued survival of the tumor cell (57). Prembrolizumat (keytruda) and Nivulomab (opdivo) are drugs used for the treatment of head and neck cancers. Their role is to inhibit this binding, and thereby allowing the T-cell to eliminate the cancer cell. In head and neck cancers, the use of checkpoint inhibitory drugs have had a positive impact (58).

1.12 The pathogenesis of OSCC

The development of OSCC has been reported by many studies to be caused by multiple genetic alterations in key genes controlling cell proliferations and survival (59, 60). These mutations are believed to cause dysregulation in cell proliferation and survival affecting cell invasion and metastasis. Further discovery has shown that most of these gene mutations take part in four major driver biologic processes. In 2013, Pickering and colleagues reported these four biologic processes (60):

(i) Mitogenic signaling was altered in 63% of the tumors.
(ii) Defective cell differentiation (9% NOTCH1 gene mutation and 66% predicted NOTCH signaling route alterations).
(iii) Nearly universal cell cycle-deregulation (94%).
(iv) Genomic instability, FAT1 (30%) and CASP8 (10%) due to the lack of candidate genes involved in detecting and repairing DNA damage, such as TP53.

Out of the four major driver biologic processes mentioned above, studies have shown that the PI3K pathway, a mitogenic signaling route, is harbouring the most genetic mutations in the head and neck cancers (60). This thesis about OSCC has therefore aimed to investigate the activation of the PI3K (mTOR) pathway and its putative role for prognosis.

1.12.1 mTOR pathway

mTOR is a serine/threonine protein kinase; a family member of the phosphoinositide 3-kinase (PI3K)-associated protein kinase, which has a key role in the cell physiology, as it is being involved in multiple cellular functions in the human body. In response to environmental cues from nutrient and hormonal stimuli, this protein kinase regulates the cell´s cytoskeleton, autophagy, metabolism, growth, proliferation and survival (61). Its activity has therefore further been reported to affect cancer development, obesity and aging (62).

1.12.2 mTORC1 and mTORC2

mTOR is a physical target of Rapamycin, which is a compound with the ability to prevent cell growth and proliferation by inhibiting signal transduction pathways (62).The mTOR component is made out of two protein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). They have different sensitivities to Rapamycin; mTORC1 being sensitive to the compound whereas mTORC2 mostly being insensitive to it (61).

First, mTORC1 integrates growth factors, ATP production, oxygen levels and amino acids, which are all important signals in biologic processes. The downstream targets of this specific protein complex have in turn the ability to regulate cellular growth and proliferation due to it being involved in protein synthesis, biosynthesis of lipids, autophagy and energy metabolism. The protein synthesis is promoted by mTORC1´s direct phosphorylation of S6 kinase 1 and the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). This enables 4E-BP1 to bind to the cap-binding protein eIF4E. The eIF4E complex will further initiate the cap-dependent mRNA translation. Cell division and proliferation is dependent on the protein synthesis. Second, mTORC2 is mainly affected by growth factors, and it controls cellular survival, metabolism and cytoskeleton organization by activating the Akt protein kinase and the SGK1 kinase (61).

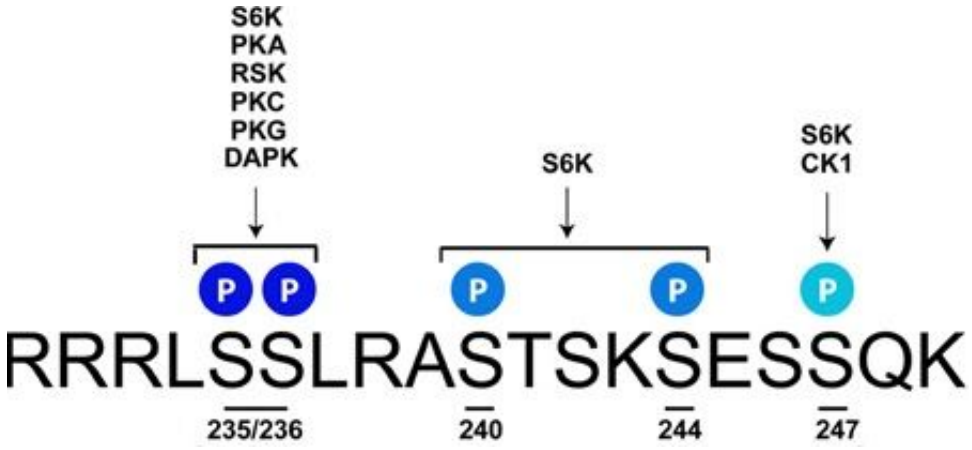


Figure 12: Shows the S6 phosphorylation serine residual sites. Adapted from (63).

Phosphorylation is a mechanism in cellular processes that regulates protein function and signal transduction pathways (64). Both protein complexes have the ability to catalyze the phosphorylation of different substrates. Protein S6, which is a compound of the 40S ribosomal subunit, plays an important part in translation and protein biosynthesis as it is one of the downstream effectors of mTORC1 (65).

S6 can, during the mitogenic stimulation, get phosphorylated at their serine residual sites, such as Ser-235, Ser-236, Ser-240 and Ser-244 - those being reported as the key downstream effectors of mTORC1 activity (66, 69). The phosphorylation step is done by the upstream components, comparatively the p70S6 kinases and the p90 ribosomal S6 kinases (67).

1.12.3 Dysregulated mTOR pathway

In certain human diseases, such as OSCC, the mTOR pathway is dysregulated, respectively in 90 -100% of all head and neck cancers (68, 84). When it is an aberrant activation of the phosphorylation of S6 in the (PI3K)/mTOR pathway, it can cause genetic alterations in head and neck cancers. The protein has also the ability to get phosphorylated by mTOR-independent pathways such as oncogenic Ras/extracellular signal-regulated kinase (ERK), tumor promoting phorbol esters, serums and growth factors (69). However, the PI3K/mTOR pathway is considered to be the most frequently activated signaling route in head and neck cancer (60, 87). According to a research published in 2013, it was reported that activation of the PI3K pathway caused by genetic mutations had the highest percentage (30,5%) compared to other mitogenic signaling pathways, such as MAPK and JAK-STAT (>10%) in the head and neck. PIK3CA was registered as the most mutated gene in the network, covering 12,6% of the alterations. Overall, this may indicate that the maintenance of the activation of the malignant conditions causing different genetic changes, regarding the PI3K pathway, is happening in correlation to PIK3CA mutations (60).

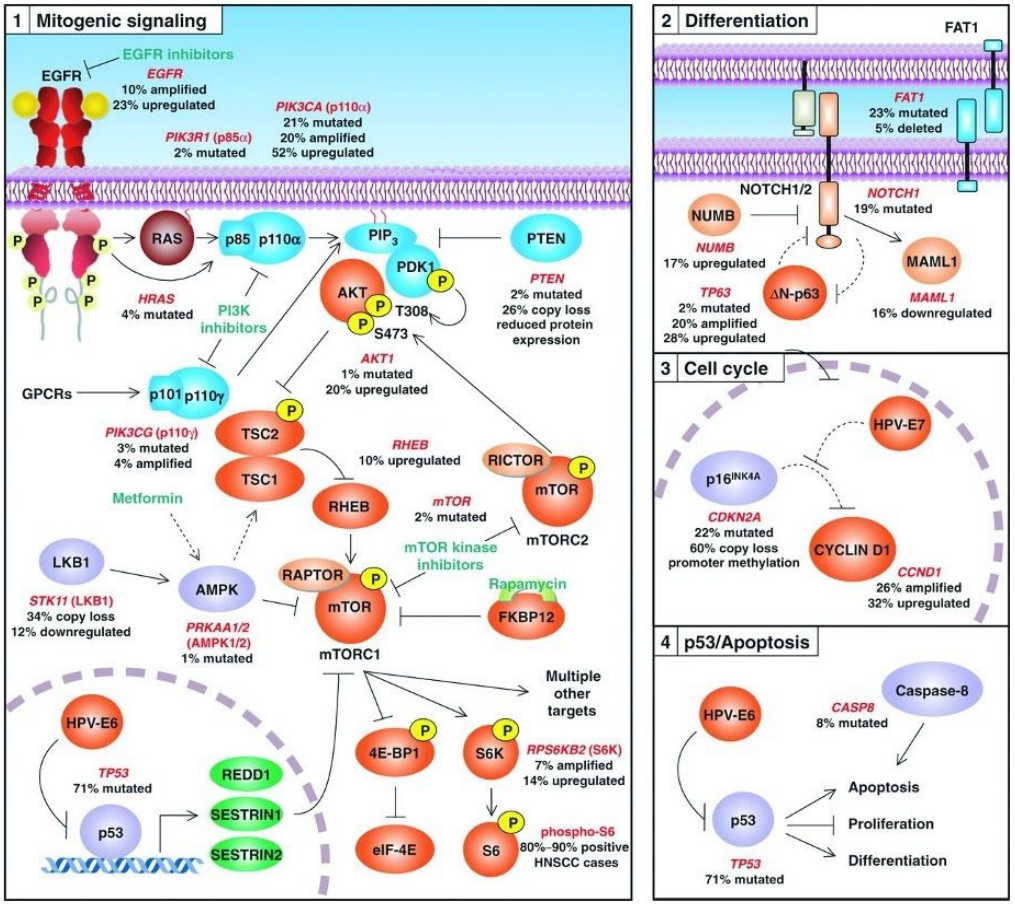


Figure 13: Demonstrating the oncogenome of head and neck cancer with alterations in the key genes. Adapted from (70).

1.12.4 pS6 as a cancer biomarker

The amount of pS6 in a cell marks its mTORC1 activity (71). pS6 has therefore been used as a cancer biomarker for detecting the status of activity in the mTOR pathway in cases of OSCC. Studies have found overexpression of pS6 being present in different human cancers including cases of OSCC and further the possibility of pS6 being significant for the evaluation of prognosis (72-75). For instance in one of the studies, expression of pS6 was detected in 100% of the cases of dysplasia and within these there were 88,67% of OSCC cases. Only 50% of cases of normal oral mucosa were reported to display pS6 expression (73). Low expression of pS6 has further been reported to cause reduction in biologic processes such as cell proliferation, cell growth and protein synthesis (76). Nevertheless, all this data has been generated on cohorts of patients from the western world. None investigated pS6 expression in OSCC cohorts from other parts of the world.

2. Hypothesis and specific aims of the study

2.1 Hypothesis

Deregulation of the mTOR pathway is a common molecular event and the expression of pS6 can be used to evaluate the prognosis of patients with oral squamous cell carcinoma in South Asia as well.

2. 2 Specific aims

1. Examine the expression of p6S in formalin fixed paraffin embedded (FFPE) material of OSCC from a cohort of patients from Nepal.
2. Examine the correlation between the expression pattern of pS6 and the clinicopathological parameters and survival of OSCC patients from a cohort of patients from Nepal.

3. Methods and statistics

3.1 “Molecular biomarkers in oral premalignant and oral cancer lesions”: An ongoing project

The tissue material that was used for this study was retrieved between the years 2011- 2014 and it came from Nepalese patients. The Ethics Committee for Medical and Health research in West Norway (2011/1244 REK vest) and Nepal Health Research Council (ref.526/2012) had approved the use of the material. This study is a part of a bigger and ongoing project called, “Molecular biomarkers in oral premalignant and oral cancer lesions". Tissues were collected after the patient's consent.

3.2 Inclusion and exclusion criteria

Several criteria had to be fulfilled for the specimens to be included in the study: The patients had to have a confirmed diagnosis of OSCC and be above 18 years old. They should not have received any prior treatment with chemotherapy or radiation before the surgery, and there should be enough tissue material accessible for analysis.

The patients that fell outside these criteria were not included in the study. This included patients who would not give their consent, cases where relevant clinical data were missing, such as use of smoking and alcohol or cases that were positive for HPV infection. Some tissue samples that were originally part of the cohort, had to be excluded during quantification (10 specimens). Because the specimens were torn by the staining procedure or due to the presence of too many inflammatory cells (leukocytes) in the specimen and thereby making the areas for analysis not representable.

3.3 Description of cohort

The clinicopathological parameters in this study are age, gender, alcohol and smoking habits, tumor differentiation, tumor stage and lymph node metastasis.

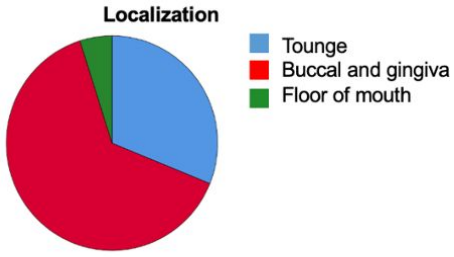


Figure 14: Pie chart representing localization distribution.

Tissue specimens from 71 patients were used in the study. The samples were taken from various regions of the oral cavity, such as buccal mucosal or gingiva (63.9%), tongue (31.1%) and floor of the mouth (4.9%).

Of the 71 specimens, 10 were excluded due to the reasons mentioned earlier. This resulted in 61 approved tissue samples that could be used to examine the expression of pS6. The cohort consisted of 13 females and 48 males, between the ages of 35-75 (mean age 56) with varying use of alcohol (65,6% misused alcohol) and smoking (36,1%). Of the 61 patients, 29.5% were still alive at the end of the study (5 missed values). Figure 15 A illustrates the distribution of the substance use between the genders.

A

Clinical Data	Male	Female	Total
Tobacco	15	6	21
Alcohol	3	0	3
Tobacco + Alcohol	14	5	19
Non-Alcohol + No Tobacco	16	2	18
Total	48	13	61

B

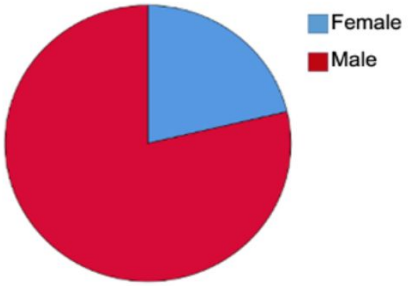


Figure 15: A) Table representing the patients tobacco and alcohol habits. **B)** Pie chart demonstrates the gender distribution within the cohort.

Most of the patients presented with big tumours (54.1% with T4, 26.2% with T3, 16.4% with T2 and 3.3% only with T1); 85.2% were at a late stage, but only 42.6% of the cases had lymph node metastasis at presentation.

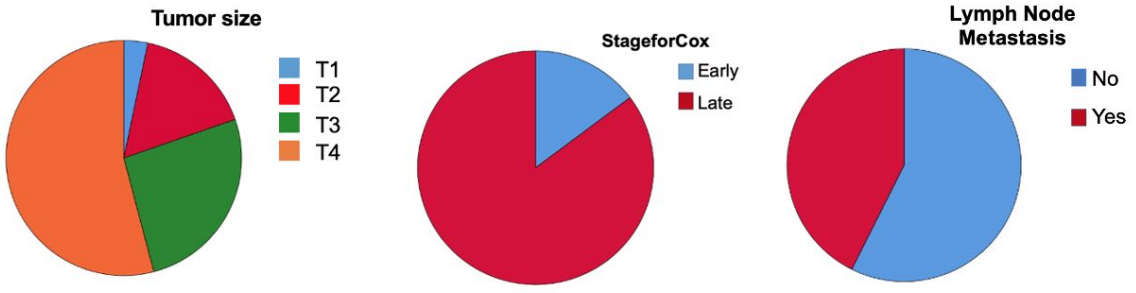


Figure 16: Distribution of tumor sizes, cases of late stage and early stage, cases with and without lymph node metastasis.

Most of the cases were well-differentiated OSCC, as shown in Table 2. All of the cases presented here are valid, meaning only cases with all of the variables available were included. The variables being age, gender, smoking/non-smoking, alcohol/non-alcohol, differentiation and metastasis status.

Differentiation					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	High	38	62.3	62.3	62.3
	Medium	20	32.8	32.8	95.1
	Poor	3	4.9	4.9	100.0
	Total	61	100.0	100.0	

Figure 17: The table shows the different differentiation levels. *Frequency:* The number of cases within its respective category. *Percent*: Frequency represented as percentage. *Valid percent*: The percentage of cases left when cases with missing clinical data were excluded. *Cumulative percent*: Summarizes the percentage of each category from the top to the bottom of the table, resulting in 100%

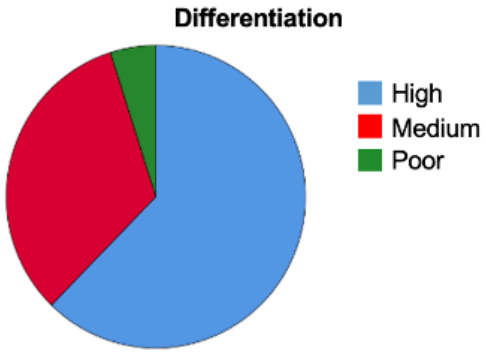


Figure 18: Pie chart representing distribution of differentiation within the cohort.

3.4 Method

The preferred method to examine the expression of pS6 in carcinogenic cells is immunohistochemistry (IHC). The technique builds upon the antibody's ability to bind to its complementary antigen which in turn will give us the necessary information about the distribution and localization of PS6 protein. This technique is a well-established method, with its own advantages and disadvantages. The advantages being that it is possible to use both fresh and formalin fixed samples. Since our tissues come from Nepal, formalin fixed paraffin embedded (FFPE) samples were used. The procedure is inexpensive, but the equipment needed is costly, although IHC could also be done manually, making it an affordable method that can be used in low-income countries. Infectious agents are dead, and there is no risk to human health. The disadvantage of IHC is that the method is sensitive, exposed to human errors and the quantification of the results can be difficult.

3.5 Protocol

The protocol for pS6 was adopted from an existing master's thesis on International Health from University of Oslo (UIO). About 4-5 microns thick sections of the FFPE were used for IHC. The sections were afterwards placed on glass-slides and incubated at 56°C for 1-2 hours. This will make the tissue adhere better to the glass-slides.

3.5.1 Deparaffinization and antigen retrieval procedure

The first step after incubation is to remove the paraffin which the tissue sample is embedded in. For this, the glass-slides were placed in a Xylene solution and later in Ethanol. The next step is to expose the tissue's antigens by boiling the slides in a Citrate-buffer with pH 6. The high temperature will break the bridges made by formalin between the proteins in the fixed tissue, and thereby expose the desired protein, in this case pS6.

3.5.2 Choice of dilution

Before having all the tissue samples exposed to IHC, four went through the staining protocol to determine which dilution would be sensitive enough to stain the relevant

protein in the cancer cells. For this we used two different antibody concentrations, 1:100 and 1:200. The result showed that 1:100 was specific and sensitive enough. Negative and positive controls from breast cancer tissue were also included.

3.5.3 Primary and secondary antibody

To examine the expression of pS6, specific primary rabbit polyclonal antibodies were used. Primary antibody is the first antibody that binds to the antigen in the tissue. The secondary antibody, which is a part of a complex consisting of a polymer backbone (dextran) and enzymes, binds to the primary antibody (77). The enzymes in the complex are the structures that will convert 3,3' diaminobenzidine (DAB) chromogen to a compound with a brown colour and thereby resulting in the visual exposure of pS6. The purpose of using "peroxidase block" is to inactivate the peroxidase enzyme in the tissue to prevent it from being stained by 3,3′-(DAB) which in turn will make the antibody more visually clear (78).

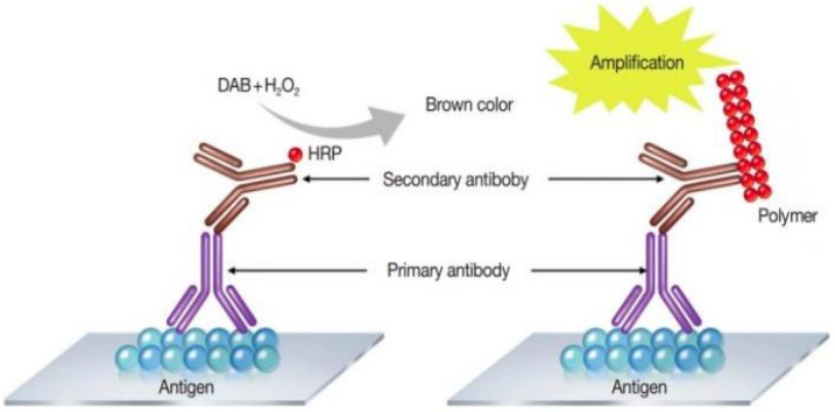


Illustration of polymeric amplification system. DAB, diaminobenzidine; HRP, horseradish peroxidase.

Figure 19: Adapted from (79).

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^{0}C, 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		

Figure 20: Detailed immunohistochemistry staining protocol used for pS6 (Ser235/236).

3.6 Scanning of slides and digital quantification of IHC stained slides

3.6.1 QuPath cell detection

To quantify the cells with cytoplasm/membranous staining, we used QuPath open-source software created by the Center for Cancer Research and Cell Biology, Queen's university Belfast (80). The purpose of the quantification is to find the percentage of the number of positive cells, meaning the amount of the expression of pS6. Before quantification certain parameters, such as brightfield (H-DAB), magnification and brush tool had to be standardized. Two layers from the basal cell membrane and a minimum of 1000 cells were required per slide. The areas that should be evaluated for expression of pS6 were tumor front and tumor center.

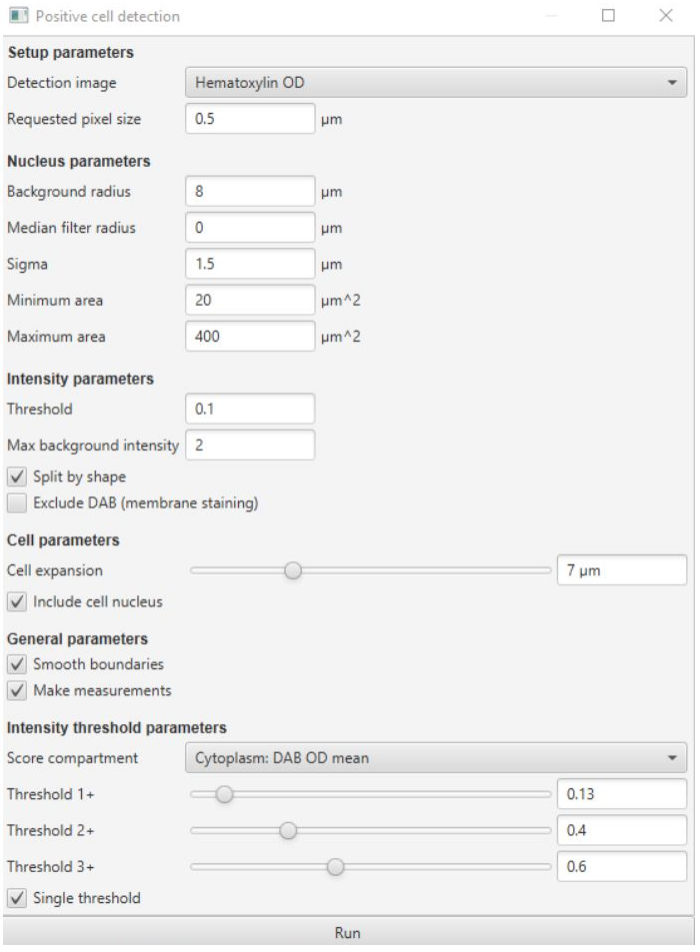


Figure 21: The "positive cell detection" function in QuPath with the parameters used for quantification.

3.6.2 Tumor invading front

Tumor front is topographically defined as the area of invasive tumor islands and the underlying stromal tissue situated at the outmost 200 µm of the tumor from the most invasive cells. This was firstly demarcated with a guideline.

Tumor front is characterized by aggressive tumor cells that are poorly differentiated. These cells infiltrate surrounding tissue and show a high grade of cell dissociation. It is therefore believed that the tumor front is the most relevant part of a tumor to be analysed when investigating biomarkers for prognosis (81).

3.6.3 Tumor center

The area of the tumor center is generally composed of cells that are more differentiated cancer cells, which means that they are often organized in networks containing tumor islands and keratin pearls. Keratin pearls usually do not have the same amount of nutrients as the cells in the tumor invading front. Cases where there is too little nutrients can result in necrosis which can often be detected in especially large cancers. The tumor center reflects therefore more the history of a tumor while the tumor front is the most active and new part of a tumor and thus might better represent the current status of a tumor (82).

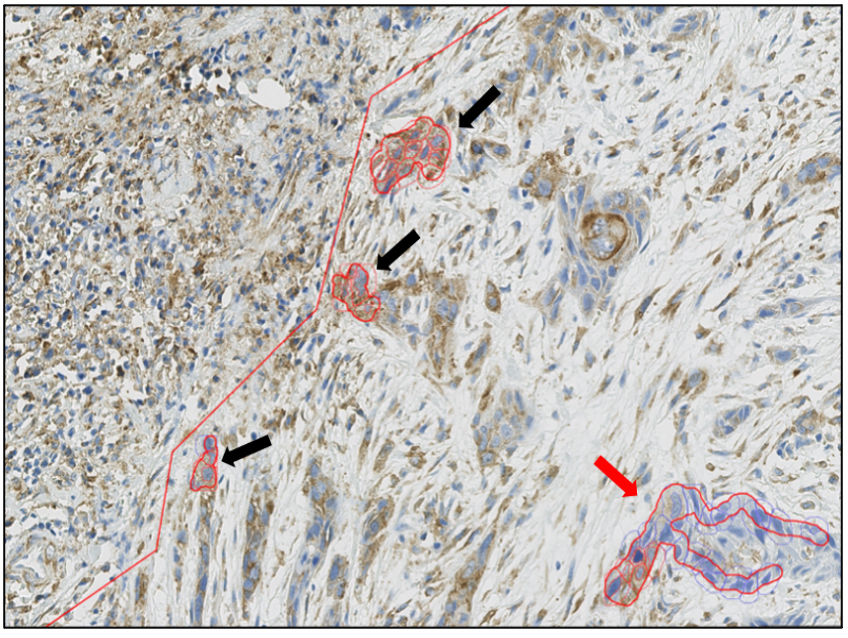


Figure 22: Immunohistochemical analysis of pS6 protein in a OSCC tissue sample. Red guideline to locate tumor invading front. Red circles dividing the tumor front and stroma, and thereby being a guide for the annotation of tumor front. Black arrows illustrating tumor invading front, whereas red arrow showing an island in tumor center.

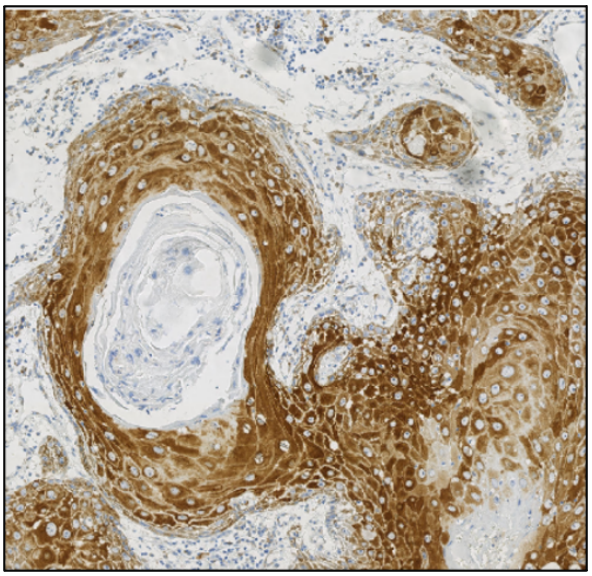


Figure 23: Showing a keratin pearl in high pS6 expressed tumor center.

4. Results

4.1 Clinical-pathological correlations and survival analysis

Age (p=0.006), lymph node metastasis (p=0.032) and stage (p=0.004) were found significantly associated with overall survival in Kaplan-Meier survival analysis. Cox regression showed that only age and stage were independent predictors of overall survival. People who were 56 years or younger had a longer survival rate than those who were older (Figure 24). The same pattern was found with lymph node metastasis (Figure 25) and stage (Figure 26), where the presence of lymph node metastasis and late stage were associated with reduced survival time.

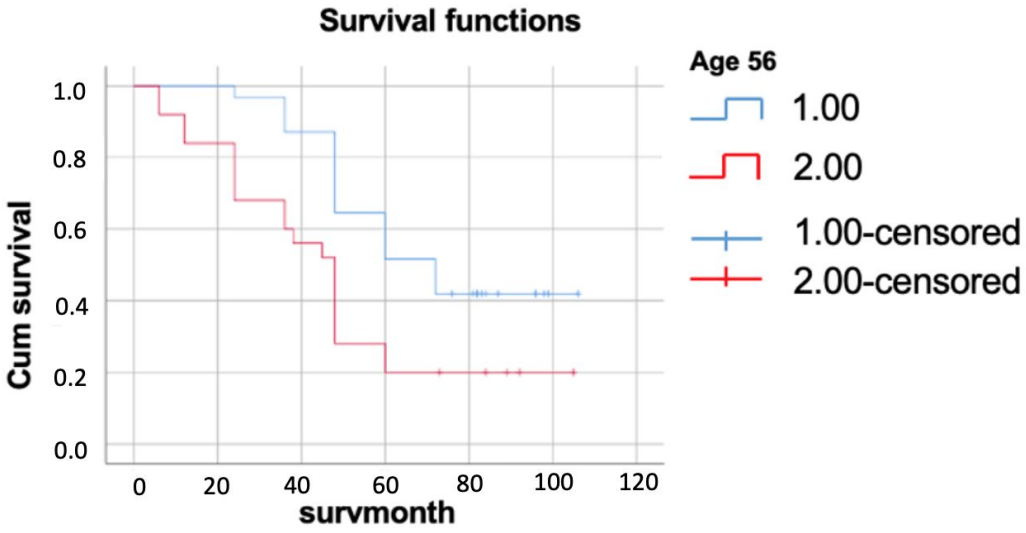


Figure 24: Demonstrates the survival rate according to age. Value 1.00 indicates patients aged 56 and under, while 2.00 represents patients older than age 56. 1.00-censored represents people aged 56 and under, who did not survive, while 2.00-censored represents people over 56 years, who did not survive.

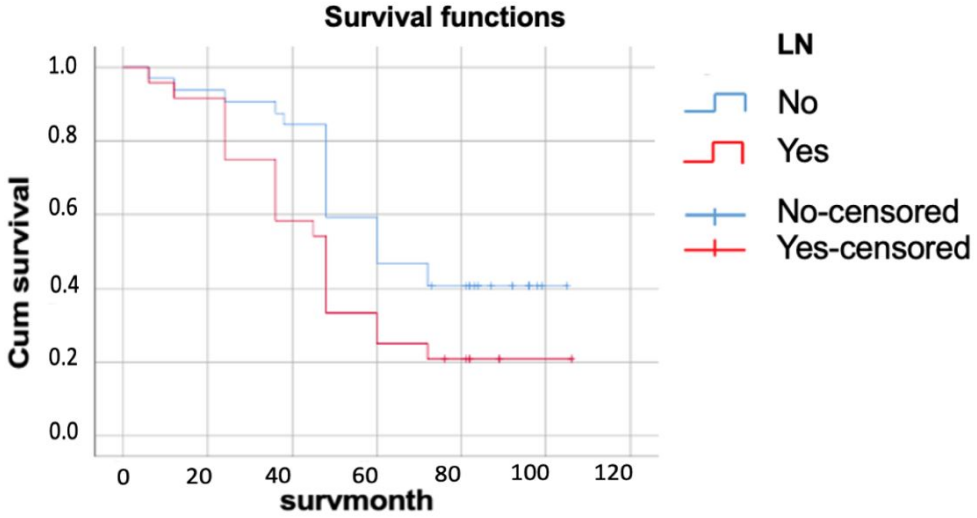


Figure 25: Demonstrates the survival rate of people with and without lymph node metastasis. No-censored represents people without lymph node metastasis, who did not survive, while yes-censored represents people with lymph node metastasis, who did not survive.

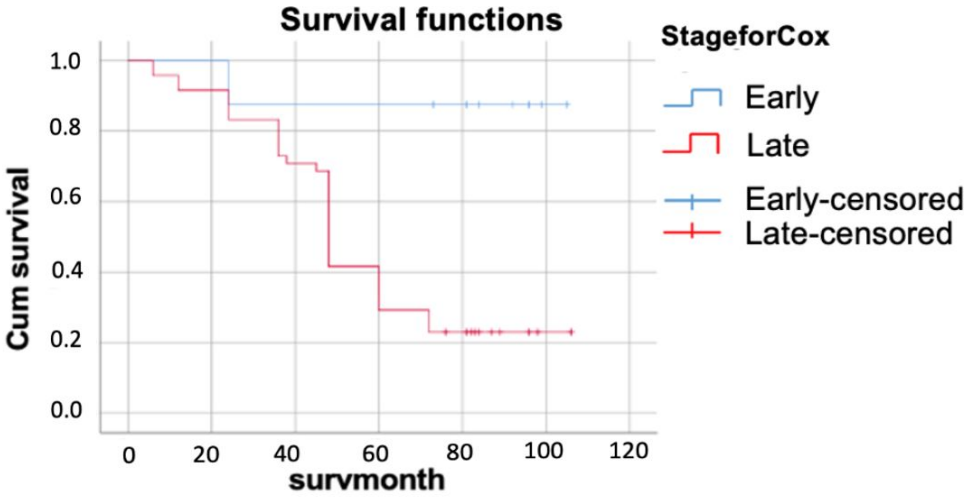
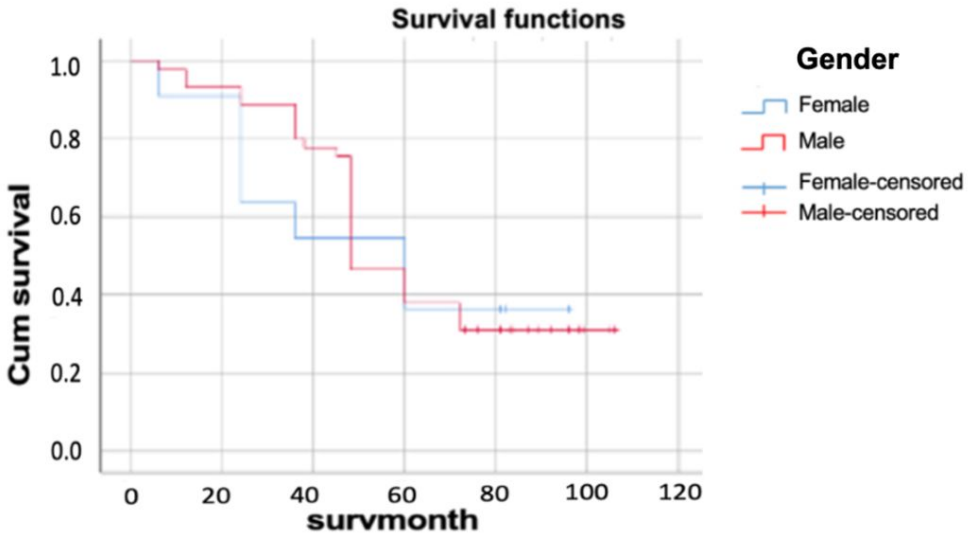


Figure 26: Illustrates that the prognosis is significantly better by diagnosis at an early stage of OSCC compared to diagnosis at a late stage. Early-censored and late-censored symbolizes the people who died.

Gender, smoking or alcohol misuse were not found associated with survival, as demonstrated by Figure 27.

A



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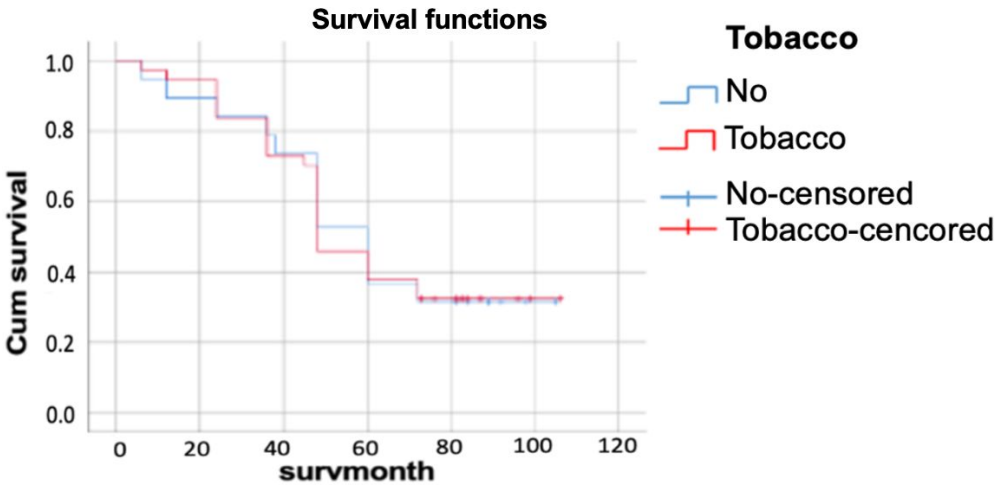

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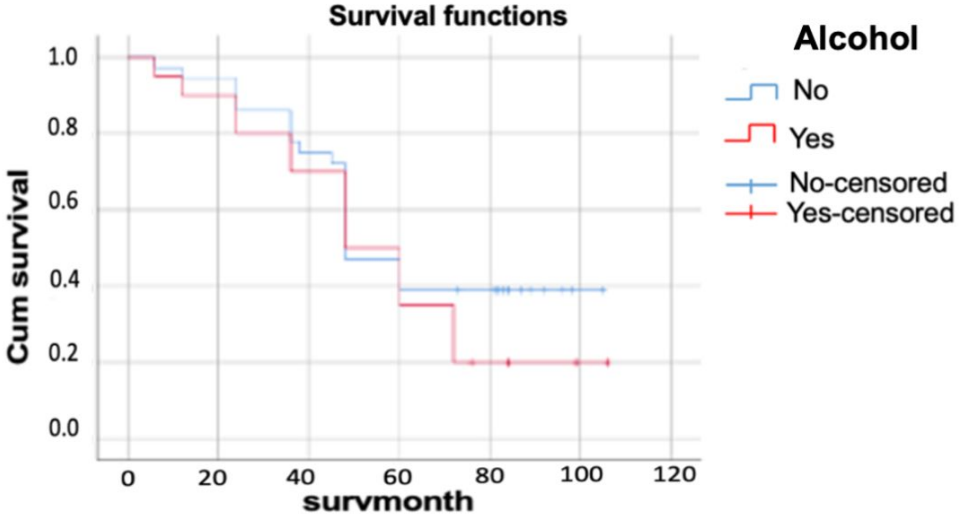
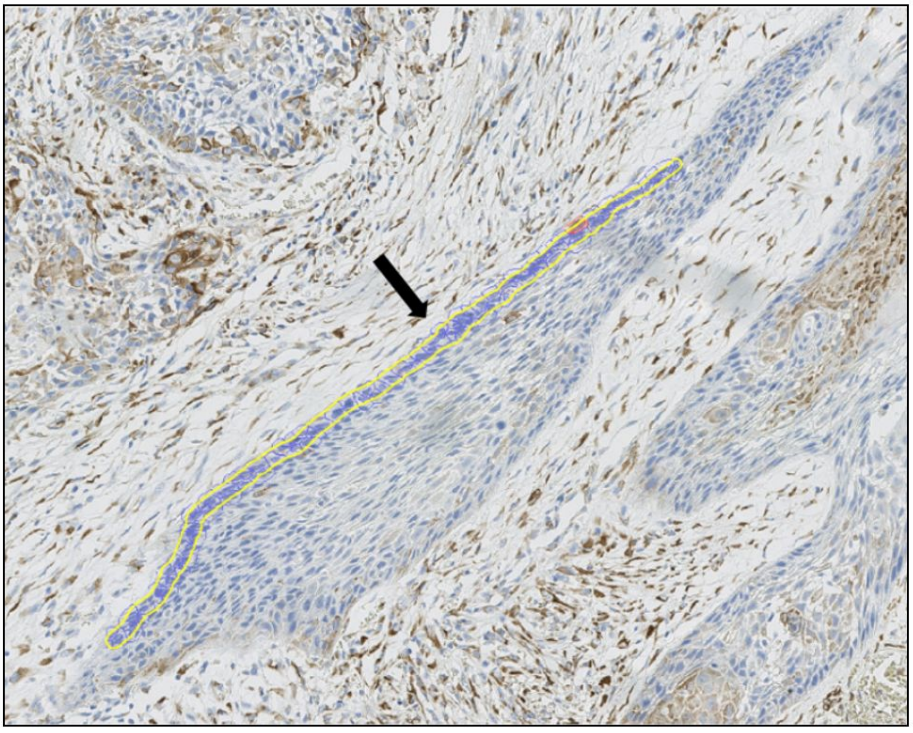


Figure 27: A) Graph illustrates survival rate of OSCC among male and females. **B)** Graph demonstrates the survival rate of OSCC among non-tobacco and tobacco users. **C)** Graph illustrates survival rate of OSCC among alcohol users and non-alcoholics.

4.2 Description of pS6 staining

Immunohistochemistry allowed the tumor cells that contained pS6 to be visible by staining them brown. Cells that were negative for the expression of pS6 remained blue. Various amounts of pS6 expression were found in all 61 cases both for tumor center and tumor invading front. In most of the cases the expression was located in the basal epithelial layer, however structures surrounding them such as salivary gland and stroma also displayed positivity for pS6.

A



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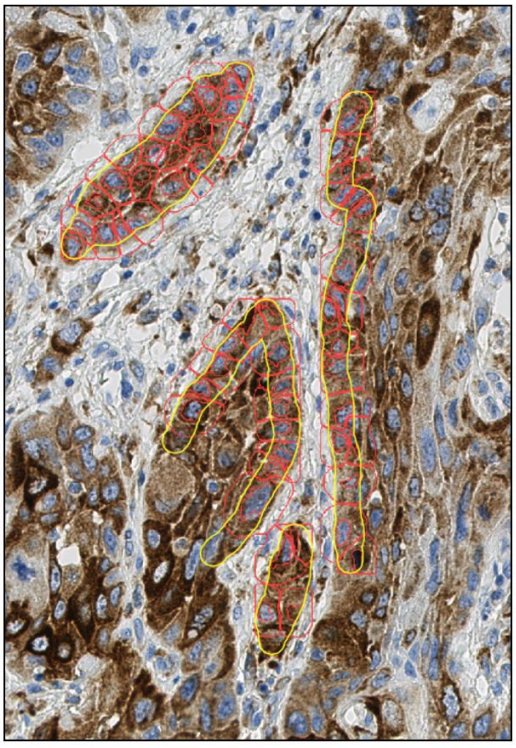

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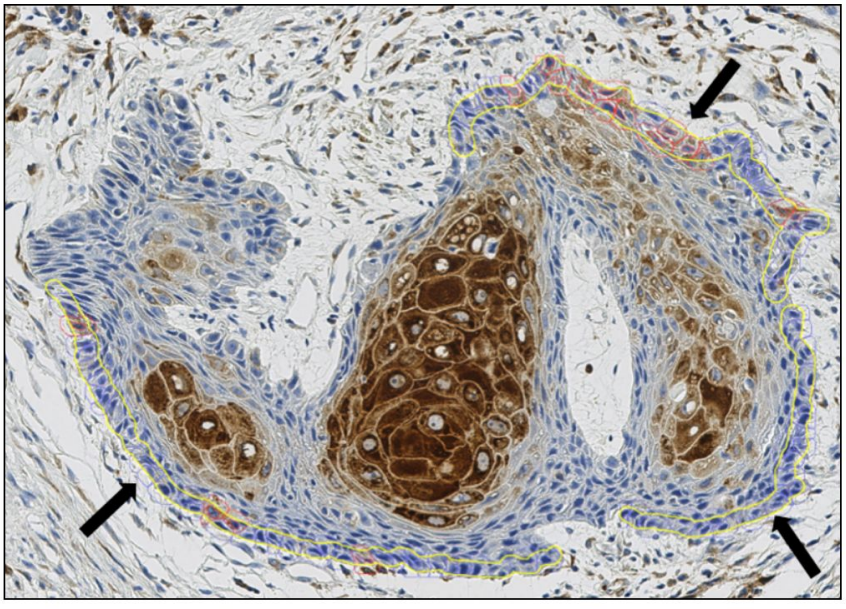


Figure 28: A) Illustrating a tumor island with mostly negative pS6 expression in the quantified area (black arrow). Positive pS6 expressed stroma is however quite visible. **B)** Demonstrating quantified areas of tumor center with a high amount of pS6 positive cells. **C)** Showing a tumor island with both positive and negative pS6 expressions.

4.3 Expression of pS6

The 59 cases were quantified to identify the amount of positive pS6 cells in the tumor center. Only 22 of these 59 cases presented a tumor front, and pS6 was also quantified at tumor invading front in these cases. Results showed that the mean percentage of pS6 expression in the tumor center was 53.92% (SEM ± 3.86) with a higher value at the tumor invading front where the mean percentage was 56,43% (SEM ± 7.20). The standard deviation of the mean within a dataset is an indication of the spread of data, the smaller the value, the more accurate the dataset. The data here show that there was more variation at the tumor front.

A

Statistics			
		pS6 centre	pS6 invading
N	Valid	59	22
	Missing	2	39
Mean		53.9224	56.4386
Std. Error of Mean		3.86645	7.20729
Median		51.4500	57.9900
Std. Deviation		29.69876	33.80519
Variance		882.016	1142.791
Percentiles	25	26.5000	28.5700
	33	41.2720	34.2732
	50	51.4500	57.9900
	75	76.5600	92.9825

The black curves in graph B and C, represent the normal distribution of positive cells. According to graph B, the percentage of positive cells were relatively normally distributed for tumor center. This pattern was not seen at tumor invading front, as demonstrated in figure 29 graph C. Here the percentage of positive cells curved towards the high value spectrum. The median is used as a measure of centrality and it is more stable to extreme observations in terms of measure errors when finding the mean value, compared to the average. In this case, the median was found to be 51.45 for pS6 in the tumor centre and 57.99 for pS6 in the tumor invading front, which might be a more appropriate way of expressing the data at the tumor front.

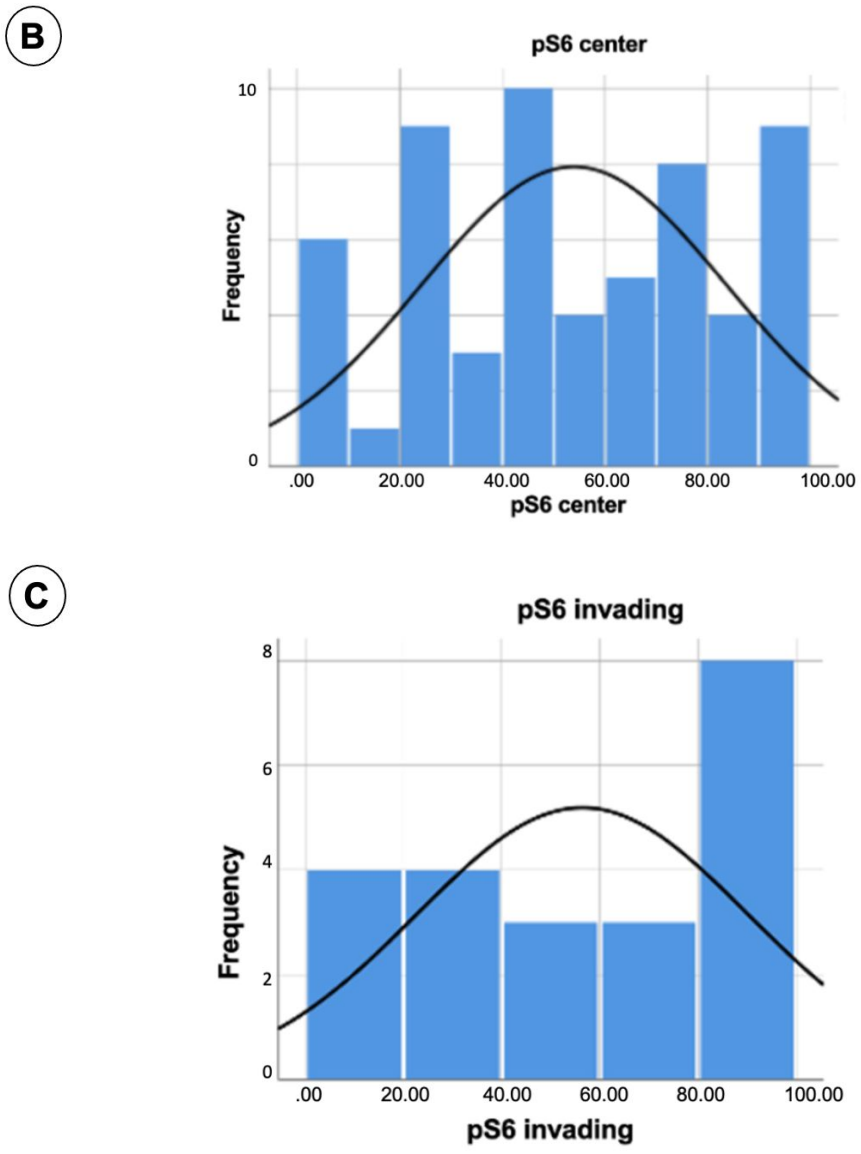


Figure 29: Table A) Cohort statistics regarding the expression of pS6. **B)** The distribution of ps6 expression in tumor center. **C)** The distribution of positive ps6 cells in the tumor invading front.

To discover any possible existence of correlation between two values, those being pS6 centre and pS6 invading in this case, data in SPSS for Pearson´s r, Sig (2-tailed) and N value were used. The results showed generally higher percentages at the tumor front than in the center of the same tumor. However a strong correlation between the percentage of positive cells in both tumor invading front and tumor centre was observed ($p<0.001$, $r=0.806$).

Correlations

		pS6 centre	pS6 invading
pS6 centre	Pearson Correlation	1	.806**
	Sig. (2-tailed)		.000
	N	59	20
pS6 invading	Pearson Correlation	.806**	1
	Sig. (2-tailed)	.000	
	N	20	22

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 30: Table illustrating the values for interpreting the correlation between tumor center and tumor invading front regarding pS6 expression.

Because there were very few (n=22) cases with quantifiable tumour invading front, further analysis of this region would not provide a representable outcome. Statistics illustrated below demonstrate therefore values retrieved from analysis performed only on tumor center.

4.4 Correlations of % pS6 positive cells with clinical parameters and overall survival

The analysis showed that the clinical parameters, such as age, gender, smoking and alcohol use and tumor characteristics, had no correlation with the percentage of positive pS6 cells. It did however present a weak inverse correlation with lymph node metastasis (p= 0.028, Pearsons' r=-0.287). Figure 29 table A shows that the percentage of positive cells in the tumor center, within the 25 % of the patients who had the lowest occurrence of percentage of positive cells, was 26.5%. In the bar chart below, the patient cohort was divided into two groups (red=1.00 and blue=.00). The red signifying patients with higher than 26.5% pS6 positive cells, whereas blue represents patients with the percentage of positive cells equal to 26.5% or less. There was higher pS6 expression among patients who had N2 compared to N1. A similar comparison in amount of pS6 expression between the groups was observed for patients without lymph node metastasis as well. There were also registered more cases of pS6 expression for patients who had no lymph node metastasis in the red group compared to patients in the red group with lymph node metastasis.

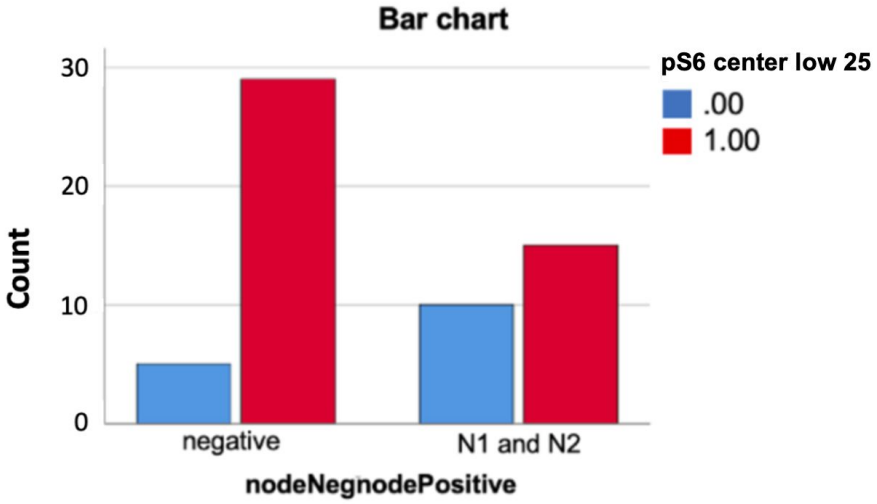
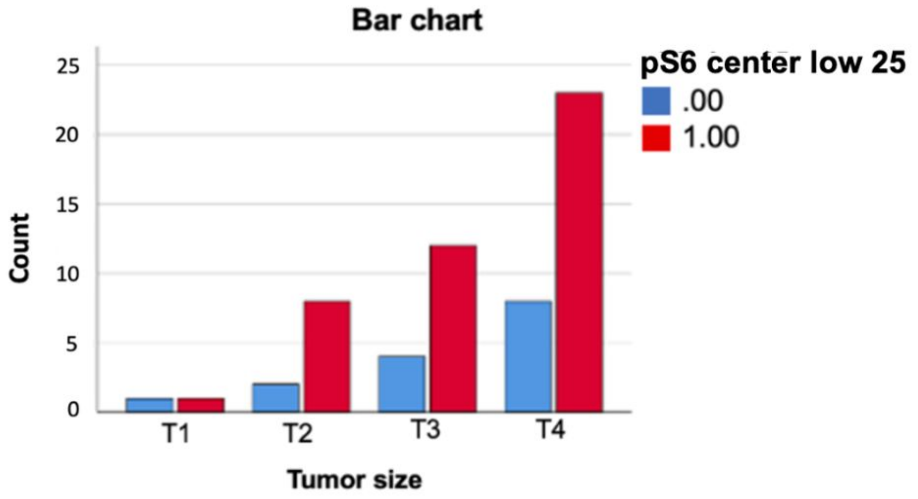


Figure 31: Bar chart representing the expression of pS6 in patients with positive and negative lymph node metastasis. Results showing patients with no metastasis having a higher percentage of pS6.

An increase in tumor size, showed an increase in the expression of pS6. The same pattern was found in cases where the tumors were in late stages as well with

patients who died during the study. Although this was not statistically significant (p>0.05).

A



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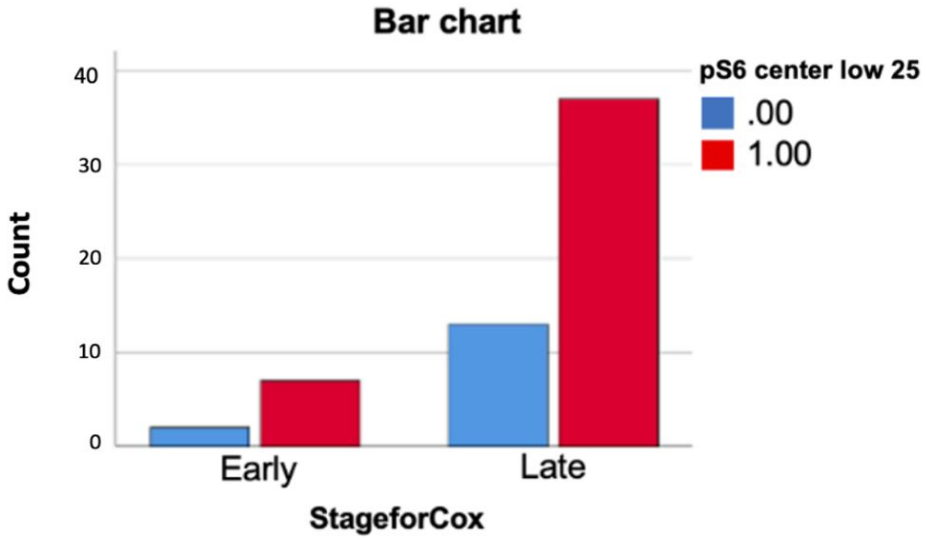

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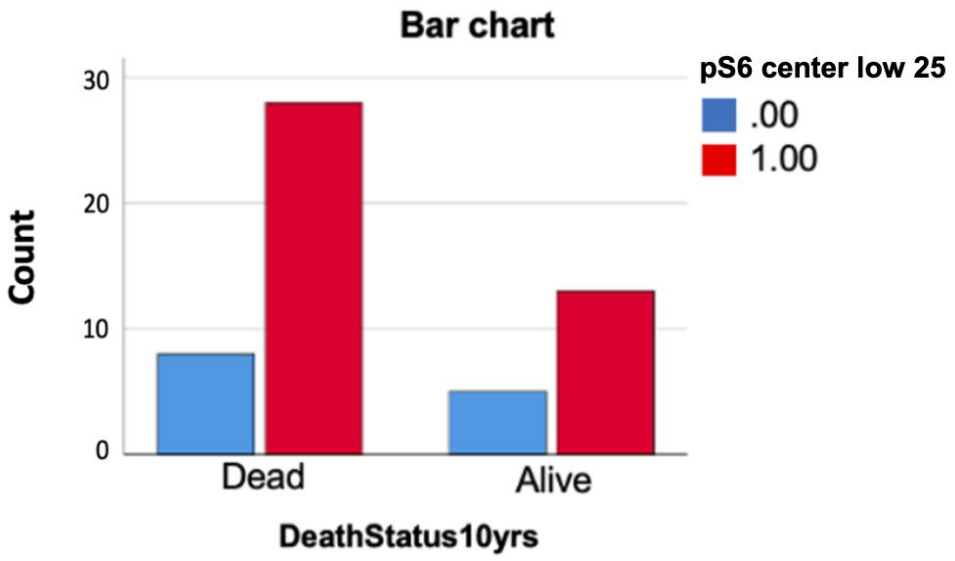
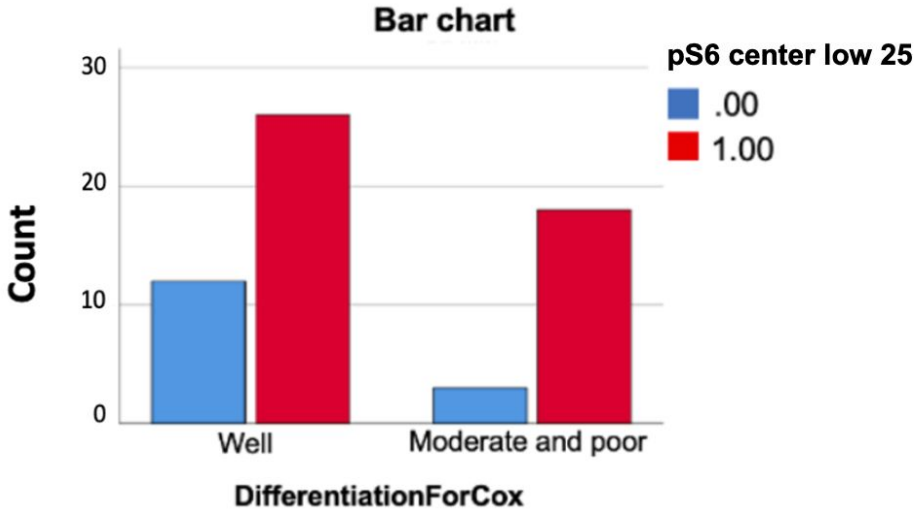


Figure 32: A) Illustrating tumor size being proportional to expression of pS6. **B)** Bar chart demonstrating that cases of later stages grow in proportion to positive ps6 expression. **C)** Indicating an increased level of ps6 expression for candidates who died before completing the study at 10 years.

Buccal and gingiva located cancers had greater expression of pS6 meanwhile cancers located in the floor of the mouth had the lowest. Both localization and differentiation status were found to have no correlation regarding pS6 expression ($p>0.05$).

A



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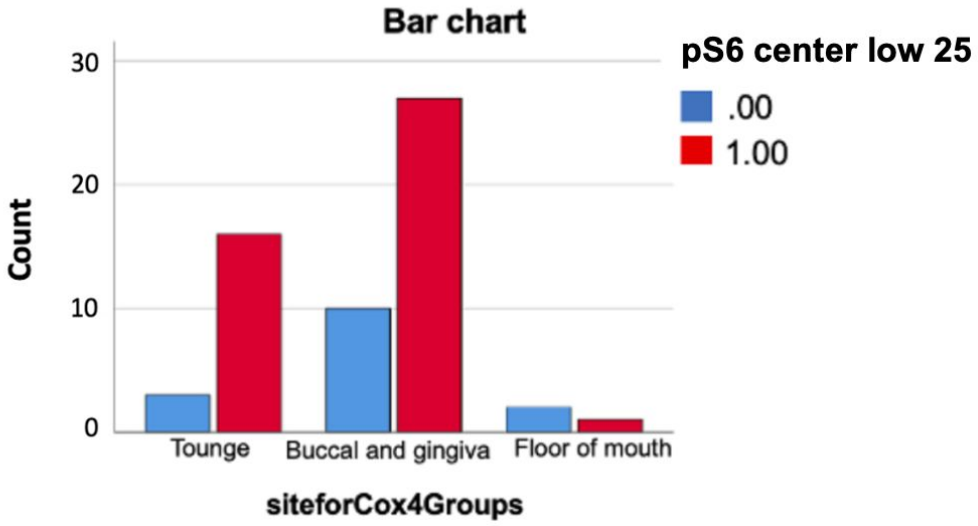

Figure 33: A) Bar chart demonstrating the level of pS6 expression among the different cancer sites. **B)** Bar chart demonstrating the level of pS6 expression among the differentiation groups.

As the graph below demonstrates, patients with a low percentage of pS6 positive cells in tumor center were provided with a greater survival chance, but this was not statistically significant.

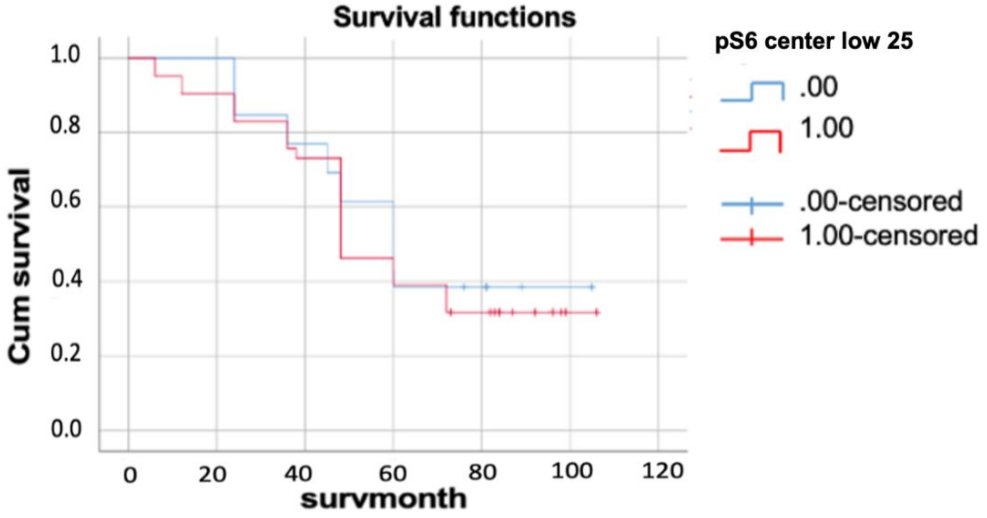

Figure 34: Graph illustrating survival benefits between patients with low and high ps6 expression to be of insignificant value.

5. Discussion

Oral squamous cell carcinoma is one of the most common types of oral cancer which can result in high morbidity and mortality. As for many cancer types, the cells in OSCC undergo genetic alterations which lead them to have unpredictable growth patterns and thereby are difficult to treat. Studies have shown that many factors take part in influencing the prognosis of cancer. Factors such as alcohol intake and tobacco use are carcinogenic and will worsen the prognosis (83).

Treatment of cancer has evolved throughout the years and continues to evolve. One major problem related to the treatment plans is that they are not cancer-cell-specific (53). This results in not only the removal of damaged cells, but also the patient's healthy cells. Prognostic biomarkers can help solve this problem. By studying the expression of certain proteins in cancer cells, one would learn more about how aggressively cancer cells grow and infiltrate surrounding tissue, and thus how aggressive the treatment should be. This can in turn help to predict the prognosis and decide the extent of the treatment.

An association between pS6 and the pathogenesis of OSCC has been suggested by numerous studies through the years. Activation of mTOR pathway followed by the phosphorylation of the S6 protein were indicated to be an early and common event in the disease. In five investigations by Chakraborty et al. (84), Chaisuparat et al. (73), Martins et al. (85), de Vincente et al. (75) and Tamatam (86) who did respective analysis of pS6 expression, it was reported higher pS6 expression in cases of OSCCs than in normal mucosa. Similar to the previous mentioned studies the current project observed high pS6 expression in most of the tissues of OSCC, although we did not compare the levels in OSCC lesions with the levels in the adjacent normal mucosa, due to lack of normal mucosa tissues in our tissue samples. This indicates that the mTOR classical pathway is also activated in the OSCC lesions from patients from South Asia, with similar demographic characteristics and etiological factors.

Contrasting the study by Tamatam (86), the mean percentage of pS6 expression was found to be higher at tumor invading front (56.44%) compared to tumor center (53.9%) (Figure 29 A). Although the difference between the two locations was small, the observation of greater pS6 expression at the tumor invading front was expected, as it is known to be the most active and proliferative of the two. In our study, a correlation between high pS6 expression and less lymph node metastasis was found. Whether this finding has any biological or prognostic meaning has to be further investigated in larger cohorts of patients from similar demographic populations. However, a study on a Caucasian cohort of head and neck cancers from Spain found also expression of pS6 to correlate inversely with presence of lymph node metastasis (87). This controversy needs further investigation.

The distribution of pS6 positive cells in the tumor invading front was not investigated for clinical correlations in this study since there were very few cases with tumor front and the values were as it skewed toward the high value spectrum (Figure 29 C). A greater cohort of patients may give a more expected normal dispersal, as it was observed for the values at the tumor center (Figure 29 B).

As statistics earlier demonstrated, age (Figure 24), tumor stage (Figure 25) and lymph-node metastasis (Figure 26) were found to predict statistically significant overall survival. The explanation for why younger age groups have a better survival rate, could be that they have a more effective immune system that is able to combat the cancer. Additionally, in those cases, it may be common to observe a highly increased amount of inflammatory cells which is often recognized by oral pathologists as a positive trait regarding fighting cancer, as the body detects the tumor cells as pathological (Figure 8). In addition, the bodies of younger persons are also more able to withstand the aggressive treatments available for OSCC (88). Patients who presented carcinoma at stage I and II, had also a better survival rate than those in stage III and IV. Late stage cancers have progressed in size, lymph node involvement and metastasis to surrounding organs. Managing the tumor would therefore be more difficult in such late cases.

In agreement with studies on a number of registered cases of OSCC between male and female, this cohort provided a similar distribution where the men made up the

majority (48 men, respectively 13 female) (Figure 15). This observation supports previous reports that there is a trend of more men being diagnosed with OSCC in Nepal compared to women (89).

Neither gender (Figure 27 A), alcohol intake (Figure 27 B) or tobacco consumption (Figure 27 C) were able to predict overall survival in our study. Based on literature, chewing tobacco is moderate to low carcinogenic, whereas betel quid with tobacco is highly carcinogenic (13). Frequent use increases the cancer risk (15). Since the tobacco type and the consumption quantity was not registered, a possible explanation for tobacco being statistically insignificant, can be due to low tobacco consumption and/or the use of low carcinogenic tobacco among the patients of the study. Although it can not be ruled out that the size of the cohort, may be another important explanation why the mentioned clinical risk factors above were found not to be significant for the overall survival as in other studies. In the study there were only 22 of 61 patients who consumed alcohol, whereas 40 of 61 of them used tobacco (Figure 15 A).

According to our analysis, there was no statistically significant association between the expression of pS6 in tumor center and overall survival ($p<0.05$), although a trend was shown towards higher percentage of positive pS6 cells in bigger tumors and patients that died during the follow up period. Similarly, an earlier study has shown that there is no significance between positive pS6 in tumor invading front and overall survival (86). This might support our findings since we found a strong correlation in our study was observed between positive pS6 cells at tumor invading front and tumor center. Interestingly, other studies have also detected better survival to be correlated with pS6 expression in OSCCs and laryngeal carcinomas (75, 87). The different outcome between different studies and ours, might come from the use of different antibodies for detection of pS6 and different ways of quantification. However, whether pS6 is a prognosticator of better or worse survival is a controversial issue and needs further investigation.

Nevertheless, mTOR pathway mutations have been suggested by studies to be a predictive biomarker, due to the PIK3CA mutated gene’s different sensitivity for

activation of pS6 expression in the mTOR pathway. mTOR-based targeted therapy in head and neck cancers has further reported to be effective (60, 90).

6. Conclusion

Our results demonstrate that the mTOR pathway is activated in many of the OSCC cases from Nepal, as shown by the high percentage of pS6 positive cells in the tissues investigated. However, pS6 does not seem to be a predictor of tumor progression or survival in this patient cohort. The value of pS6 as a prognostic biomarker in OSCC is still controversial and needs further studies on bigger cohorts of patients.

7. Limitations

Certain limitations regarding this study were observed. The acquisition of information about the overall activation status to mTOR in the current study was limited, as pS6 only provided information about activation of mTORC1. To get a better understanding of oral cancer, the knowledge around the activation status of mTORC2 will be required as well.

This project will only provide limited conclusions as it is a retrospective study and it was performed on a limited number of cases. In addition, because type and amount of tobacco use were not documented among the patients, this brings a limitation when interpreting the results from the study.

Extensive research on mTOR investigation on larger cohorts of patients is therefore needed in the future.

8. References

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