In The Name of God

SHIRAZ UNIVERSITY OF MEDICAL SCIENCES

SCHOOL OF DENTISTRY

A Thesis Submitted in Partial Fulfillment for the Degree of

Doctor of Dental Medicine

Evaluation of the impact of sodium fluoride varnish on reduction of primary dental caries

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Thesis Number

Years/ Month 2022

ACKNOWLEDGEMENTS

First of all, I appreciate God for granting me power and patience to complete this thesis. I am dedicating this thesis to beloved people who have meant and continue to mean so much to me. Their presence continues to regulate my life. First and foremost, to my parents whose love for me knew no bounds and, who taught me the value of hard work. I wish to express my deepest thanks and gratitude to my supervisors for their efforts and guidance during this study. Finally, I thank all my beloved intimate friends who inspired me during my all life.

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Abstract

1 Introduction

Cancer remains a major cause of death worldwide and with an aging population, its annual toll of 8.2 million is only expected to increase. In this respect, carcinomas can be broadly divided into two groups: metastatic (the principal cause of cancer-related deaths) and no metastatic [1]. Traditionally, metastasis has been considered to occur in later stages of cancer progression; however, accumulating evidence has also described metastatic dissemination during early tumor formation [2]. During metastasis, disseminating cancer cells escape from primary tumors and acquire cellular traits that allow them to travel and colonize distant organs [3]. Cancer can be considered a very large and exceptionally heterogeneous family of malignant diseases, with squamous cell carcinomas comprising one of the largest subsets. All SCC lesions are thought to begin via the repeated, uncontrolled division of cancer stem cells of epithelial lineage or characteristics. SCCs arise from squamous cells, which are flat cells that line many areas of the body. Accumulation of these cancer cells causes a microscopic focus of abnormal cells that are, at least initially, locally confined within the specific tissue in which the progenitor cell resided. This condition is called squamous cell carcinoma in situ, and it is diagnosed when the tumor has not yet penetrated the basement membrane or other delimiting structure to invade adjacent tissues [4]. Once the lesion has grown and progressed to the point where it has breached, penetrated, and infiltrated adjacent structures, it is referred to as "invasive" squamous cell carcinoma. Once a carcinoma becomes invasive, it is able to spread to other organs and cause the formation of a metastasis [5, 6].

1.1 Squamous cell carcinoma

SCCs also known as epidermoid carcinomas, comprise a number of different types of cancer that result from squamous cells. These cells form the surface of the skin and lining of hollow organs in the body and line the respiratory and digestive tracts. Common types include:

- Squamous cell skin cancer: A type of skin cancer
- Squamous-cell carcinoma of the lung: A type of lung cancer
- Squamous cell thyroid carcinoma: A type of thyroid cancer
- Esophageal squamous cell carcinoma: A type of esophageal cancer
- Squamous-cell carcinoma of the vagina: A type of vaginal cancer

Despite sharing the name "squamous cell carcinoma", the SCCs of different body sites can show differences in their presented symptoms, natural history, prognosis, and response to treatment. Human papillomavirus infection has been associated with SCCs of the oropharynx, lung, fingers, and anogenital region. Head and neck squamous cell carcinoma (HNSCC) is a devastating malignancy that occurs in close proximity to vital structures. A projection for the year 2020 estimated that 53,260 new cases, and 10,750 annual deaths of oral and pharyngeal SCC will occur in the US [4-6].

1.2 Head and neck cancers

Head and neck squamous cell carcinoma (HNSCC) is diagnosed in 890,000 patients each year worldwide, ranking it as the sixth most common cancer in the world. HNSCC is a collection of cancers encompassing the oral cavity, nasopharynx, oropharynx, hypopharynx and larynx H&N cancers are the most common cancers in developing countries, especially in Southeast Asia. World-wide, the head and neck cancers form the sixth most common cancer. Head and neck cancer (HNC) is the most common cancer in developing countries. It is the most common cancer of males in India and the fifth most common in females [7]. Several studies have described HNSCC as an immune suppressive tumor The recent advent of immunotherapy showed unprecedented improvement in overall response of advanced stage malignancy. Further understanding of HNSCC tumor inflammation can provide the

basis for tumor stratification according to the underlying immune profile and hence may result in better treatment strategies and patient outcomes [8, 9].

1.2.1 Etiology

Cigarette-smoking and alcohol consumption are the main reasons for HNSCC in the Western population, whereas the use of smokeless tobacco and areca nut is the most common cause of HNSCC in Southeast Asia. The various forms in which smokeless tobacco is used in developing countries include khaini, mava, paan (betel quid), zarda, snuff, mashiri, etc [10, 11]. Nevertheless, around 5% of patients suffering from head and neck cancer are diagnosed before they reach the age of 45 years. The incidence in this subgroup appears to be consistently growing, a trend that is in contrast to what observed in the general population. This phenomenon seems to be mainly linked to an increase of oropharyngeal carcinomas (mainly human papilloma virus [HPV]-related) and of the oral cavity (apparently not related to any known virus), paralleled by a reduction of laryngeal and hypopharyngeal tumors [1].

Betel quid chewing is the most common form of tobacco chewing in the Asia-Pacific region. Betel quid consists of areca nut, betel leaf, catechu, and slaked lime. It has been reported from many countries like India, Pakistan, Bangladesh, Sri Lanka, Thailand, Cambodia, Malaysia, Indonesia, China, Philippines, Taiwan, Vietnam, and migrant populations in Europe, Africa, North America, and Australia About 10% of the world's population chew betel quid regularly. In one study conducted in Southeast Asia, the lower socio-economic groups had higher risk of developing HNC [12, 13]. Areca nut alone is a confirmed carcinogen and causally associated with a premalignant condition called oral submucous fibrosis (OSMF) and oral cancer. It is a chronic, debilitating disease of the aerodigestive tract owing to irreversible fibroelastic changes in the lamina propria which lead to stiffness of the oral mucosa resulting in progressive trismus. This is uncommon in the Western

world due to the rarity of areca nut use. In India alone, 5 million people (0.5% of the population of India) have OSMF. The high prevalence of tobacco usage has led to increases in disease burden and high health care costs in developing countries. There is a high incidence of smoking reported amongst youth from Bangladesh, India, and Indonesia. While the incidence of head and neck cancers is decreasing in E The buccal (cheek) mucosa is the most common site for oral cancer in South and Southeast Asia; in all other regions, the tongue is the most common site Europe and North America, it remains unabated in the developing world [13, 14]. The mean age of patients at presentation of head and neck cancers is the fifth and early sixth decades in Asian populations compared with the seventh and eighth decades in the North American population [14]. Regional variations in incidence and the site of occurrence relate to the major causes, which are alcohol and smoking in Western countries, and betel quid and tobacco chewing in South and Southeast Asia. Oral cancer mortality rates are influenced by oral cancer incidence, access to treatment, and variations in site distribution [15]. The observed trends in incidence and mortality among men and women are closely correlated with the patterns and trends in tobacco and alcohol use. An increasing trend in incidence has been reported in Karachi, Pakistan, and in Taiwan, China, caused by increases in tobacco and areca nut chewing and alcohol drinking (Table 1.1) [16].

Table 1-1. Risk factors associated with oral squamous cell cancer

Risk factors associated with oral squamous cell cancer
Smoked tobacco
Smokeless tobacco
Alcohol
Betel nut chewing
Human papillomavirus
Plummer-Vinson syndrome
Long-term immunosuppression
Ill-fitting dentures
Repeated trauma
Pipe smoking (carcinoma of the lip)
Chronic exposure to UV light (for carcinoma of the lip)

1.2.2 HUMAN PAPILLOMAVIRUS (HPV)

The overall prevalence of HPV in HNSCC is around 50%, with the highest prevalence in cancers of the tonsil and base of tongue. The rise in HPV-related cancers has been mainly attributed to the change in sexual practices in the Western world. These patients are younger, predominantly oropharynx involvement, equal gender distribution, and have better survival. HPV-16 is the most common type, being present in 30.9% of oropharyngeal carcinomas, 16% of oral cancers, and 16.6% of laryngeal cancers. Prevalence of HPV in oral cancers is similar in Europe (16%) and North America (16.1%), but greater in Asia (33%) [16, 17].

HPV-16 was most common, followed by HPV-18 and then cross-infection (16 and 18); 41% of patients had multiple HPV infections. Lesions of the tongue had the highest rate (9 of 11) of HPV infection. Another study showed a rate of HPV infections of 56.3% in cancers of the mandible, 37.5% in cheek, and 38.6% in maxilla. The study also reported that the advanced stages (III, IV) had higher infection rates as compared to earlier stage [11, 18].

Between developed and developing countries, there are not only differences in the age, subsite, and habit but also in the molecular biology. The prevalence of the p53 mutation is common in Europe and USA but rare in India. The recent data show the prevalence to be 81% in the Western world. Multiple genetic abnormalities are common in head and cancers in India and Southeast Asian [11, 19]. In oral cavity SCCs, gender distribution is different according to the age of onset taken into consideration: while in the elderly, males represent over 70% of cases, this percentage falls to 50-65% under 45 years of age. This difference has increased evidence when considering that the majority of non-smokers and non-drinkers young patients are females. These epidemiological features may thus represent the expression of aetiopathogenic differences distinguishing the young subgroup of patients from the elderly [17, 18]. It has been possible to observe a recent reduction in the number of smoke-related tumors. Nevertheless, oral cavity SCC, in particular of the mobile tongue, as well as those of the oropharynx, appear to have a progressively increasing incidence. While in oropharyngeal carcinomas this may be explained by an increasing exposure to the HPV, in the oral cavity, a specific pathogenic role for viral infections has yet to be demonstrated [11, 20, 21]. This involves the secretion of substances to degrade the basement membrane and extracellular matrix and also the expression/ suppression of proteins involved in the control of motility and migration. The tumor must also initialize angiogenesis, without which the tumor would fail to develop, as local diffusion for transport of nutrients to and removal of waste products from the tumor site would suffice for tumors up to 2 mm in diameter [22]. Once the tumor cell has arrived at a likely point of intravasation, it interacts with the endothelial cells by undergoing biochemical interactions (mediated by carbohydrate locking reactions, which occur weakly but quickly) develops adhesion to the endothelial cells to form stronger bonds, and thus penetrates the endothelium and the basement membrane; the process of

extravasation. The new tumor can then proliferate at this secondary focus [11, 23, 24].

1.2.3 Subtypes

One method of classifying squamous cell carcinomas is by their appearance under microscope. Subtypes may include:

- Adenoid squamous cell carcinoma (also known as pseudoglandular squamous cell carcinoma) is characterized by a tubular microscopic pattern and keratinocyte acantholysis.
- Basaloid squamous cell carcinoma is characterized by a predilection for the tongue base.
- Clear-cell squamous cell carcinoma (also known as clear-cell carcinoma of the skin) is characterized by keratinocytes that appear clear as a result of hydropic swelling.
- Signet ring-cell squamous cell carcinoma (occasionally rendered as signet ring-cell squamous cell carcinoma) is a histological variant characterized by concentric rings composed of keratin and large vacuoles corresponding to markedly dilated endoplasmic reticulum. These vacuoles grow to such an extent that they radically displace the cell nucleus toward the cell membrane, giving the cell a distinctive superficial resemblance to a "signet ring" when viewed under a microscope.
- Spindle-cell squamous cell carcinoma (also known as spindle-cell carcinoma) is a subtype characterized by spindle-shaped atypical cells [13, 22, 25]

1.2.4 Prevention

Studies have found evidences for an association between diet and skin cancers, including SCC. The consumption of high-fat dairy foods increases SCC tumor risk

in people with previous skin cancer. Green leafy vegetables may help prevent development of subsequent SCC and multiple studies found that raw vegetables, citrus fruits and non-citrus fruits are significantly protective against SSC risk. On the other hand, consumption of whole milk, yogurt, and cheese may increase SCC risk in susceptible people in addition, meat and fat dietary pattern can increase the risk of SCC in people without a history of SCC, but the association is again more prominent in people with a history of skin cancer. Tobacco smoking and a dietary pattern characterized by high beer and liquor intake also increase the risk of SCC significantly [22-25].

1.2.5 Presentation

Most head and neck cancers present with symptoms from the primary site for example, hoarseness, difficulty in swallowing, or pain in the ear. Enlargement of a cervical lymph node as the first presenting feature is not uncommon, particularly with certain “silent” sites the tongue base, supraglottis, and nasopharynx. Systemic metastases are uncommon at presentation (10%); however, synchronous or metachronous tumors of the upper aerodigestive tract occur in 10-15% of patients. Guidelines have been written for general medical and dental practitioners for referring patients with suspected malignancies of the head and neck, and most head and neck units have an open access clinic to see these patients urgently (Figure 1.1,1.2, and 1.3) [25, 26].

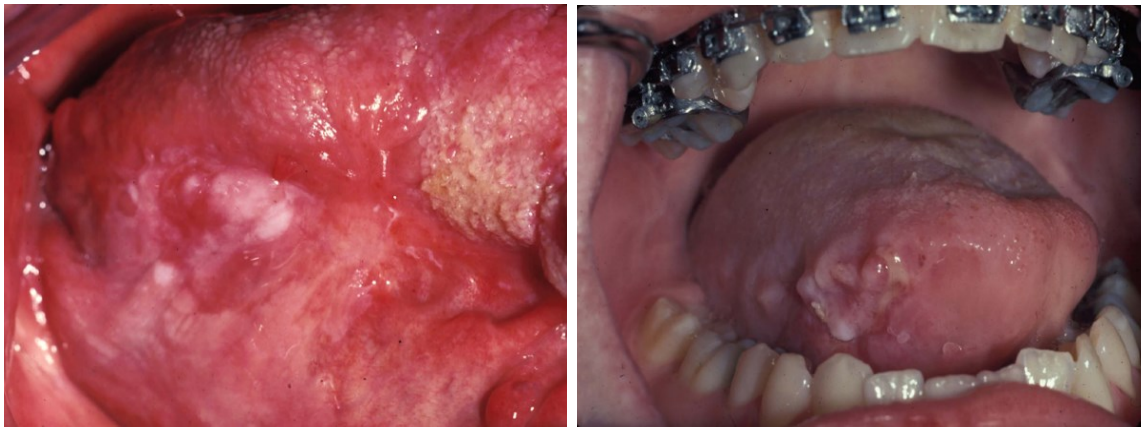


Figure 1-1. OSCC cases presented with indurated and ulcerated lesion on lateral border of tongue

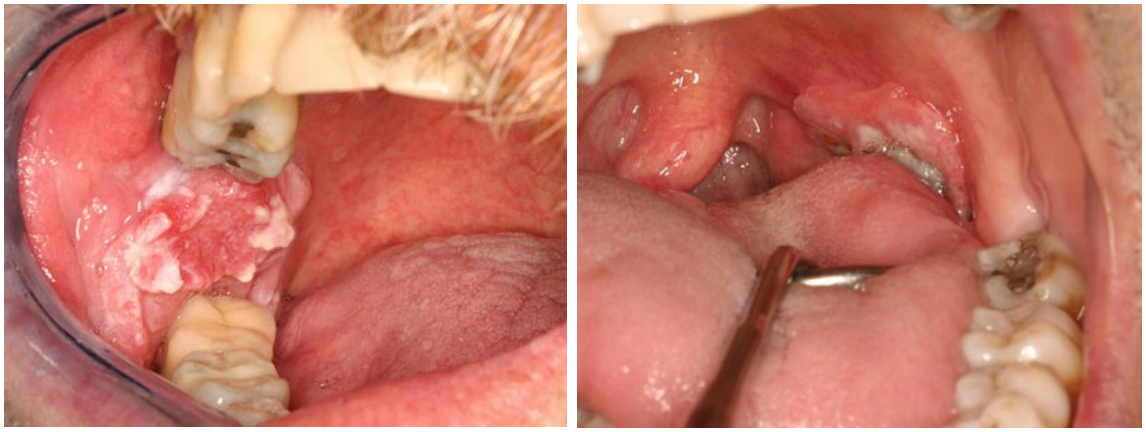


Figure 1-2. OSCC cases presented as tumoral mass in soft palate and retromolar

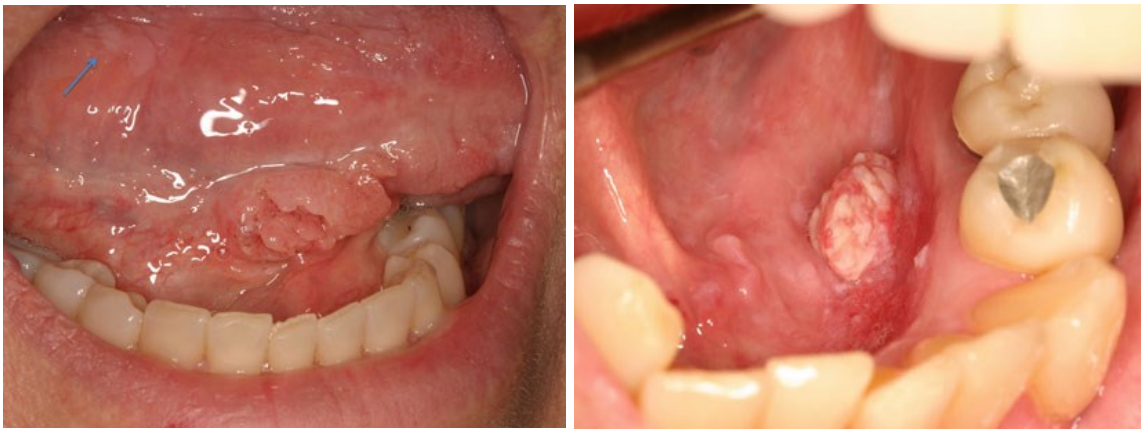


Figure 1-3. OSCC cases presented as verruciform pattern

1.3 Screening and early diagnosis

Early detection should be a priority, given the excellent prognosis of early-stage disease compared with the poor results in advanced stages. Screening is most cost effective when targeted at high-risk groups, for example, heavy drinkers and smokers [27]. A quick diagnosis is crucial to control a possible malignant transformation of oral premalignant diseases and for increasing the overall survival rate of the patients. Numerous techniques and methods like scraping the surface of the lesion analyzing the cytological characteristics of the oral premalignant lesions are essential for doing the right diagnosis. Nowadays, though the current standard of performing diagnosis in oral pathology is related to incisional biopsy with histology, this method is painful for patients and involves a delay in the diagnosis, although histology is fully done [27]. Primary and metastatic tumors are complex ecosystems composed of neoplastic cells, extracellular matrix (ECM), and "accessory" nonneoplastic cells, which include resident mesenchymal support cells, endothelial cells, and infiltrated inflammatory immune cells. Cross-talk between cancer cells and accessory cells fuels and shapes tumor development. During tumor formation, the tissue architecture evolves into a highly specialized microenvironment characterized by a corrupted ECM and chronic inflammation [28].

Cancer-associated inflammation, which is present at different stages of tumorigenesis, contributes to genomic instability, epigenetic modification, induction of cancer cell proliferation, enhancement of cancer anti-apoptotic pathways, stimulation of angiogenesis, and, eventually, cancer dissemination. Studies during the last two decades have demonstrated that inflammatory immune cells are essential players of cancer-related inflammation. Efforts have focused on understanding how immune cells impact tumor fate in different stages of disease: early neoplastic transformation, clinically detected tumors, metastatic dissemination, and therapeutic intervention [29].

1.4 Immune system challenges

An effective immune system must be able to interpret changes in the world around it and respond appropriately. To do this, it has to solve a number of specific problems.

1.4.1 Immune system Discrimination

Immune systems have an uneasy relationship with the environment. Most of the time an encounter with something new is harmless, but the small fraction of times that it is not can be very dangerous indeed. An effective immune system must be able to discriminate such differences, distinguishing self from non-self and distinguishing harmless non-self from dangerous non-self. For much of the 20th Century, research in immunology was focused on understanding how it achieved the former. It was spurred by an important early observation: that it was possible for animals to develop specific immune reactions against chemicals such as dyes, which had never existed in Nature. The ability to learn how to recognize these previously unknown structures implied that the immune system had solved the problem of how to classify and recall the shapes of individual molecules. Unravelling the biological machinery that achieves this was a signature achievement of 20th Century immunology. This remarkable flexibility underlines the fact that the immune system interrogates the fundamental building blocks of the environment. A process of recognition at a near molecular scale allows the immune system to exploit the fact that all organisms are defined by proteins encoded in their genes. As part of a defense against a potentially dangerous environment, each individual develops their own unique immune system, which acknowledges only itself. Everything finally that is not recognized might be a threat [30, 31]

1.4.2 Immune system Flexibility

The immune system's ability to adapt flexibly to strange environmental changes is critical in fighting infections and cancer. Because our bodies have a remarkable capacity for renewal, and almost every cell is a factory working day and night to turn over worn out molecules, breaking them down into building blocks that are reused to make replacements, infection or cancer can arise at any time. Every time a cell divides, there is a small chance that it may develop a random unpredictable mutation that will transform it into a cancer. Infections reproduce much more rapidly than their hosts and can change their appearance to allow them to evade recognition. An effective immune system must cope with this unpredictability [32]. It can picture this as an ongoing evolution of the environment and it presents a special challenge for an immune system. In contrast with most organs, such as the heart, which does the same job throughout life, the immune system needs to adapt to an environment that is always changing. This problem is solved by investing in strategies that exploit the power of random change itself. Using randomness in this way creates waste, but preserves responsiveness. Even identical twins, which share the same genes, have immune systems that become increasingly different from each other from birth to old age, as each twin independently makes hundreds of thousands of unique random responses to the environment. Our immune system is made up of both individual cells and proteins as well as entire organs and organ systems. The organs of the immune system include skin and mucous membranes, and the organs of the lymphatic system too. skin and mucous membranes are the first line of defense against germs entering from outside the body. They act as a physical barrier with support from the following:

- Antibacterial substances can kill germs right from the start. A certain enzyme found in saliva, the airways and tear fluid destroys the cell walls of bacteria.

- Mucus in the bronchi helps trap many of the germs we breathe in so they can be moved out of the airways by hair-like structures called cilia.
- Stomach acid stops most of the germs that enter the body in the food we eat.
- Harmless bacteria on our skin and many of the mucous membranes in our body also act as part of the immune system [32-34].

In addition, the reflexes that cause us to cough and sneeze help to free our airways of germs. The lymphatic system is composed of:

- Primary lymphoid organs: These organs include the bone marrow and the thymus. They create special immune system cells called lymphocytes.
- Secondary lymphoid organs: These organs include the lymph nodes, the spleen, the tonsils and certain tissue in various mucous membrane layers in the body (for instance in the bowel). It is in these organs where the cells of the immune system do their actual job of fighting off germs and foreign substances.

1.4.3 Memory cells

One of the most significant features of the immune response is its ability to retain a memory of previous infections. This both protects individuals from reinfection and limits the spread of infection in a community. Immune memory can be very long-lasting; when adults were studied, their memory for the measles infection was decaying so slowly, it would have taken over 3000 years to decrease by half. This goes well beyond life-long protection. These robust durable changes are the reason that, when we vaccinate, the protection this produces delivers long-term benefits. Within an individual, immune memory must be distributed throughout the body. Circulating antibodies travel in the blood, reaching everywhere the circulation does; memory also develops outside the bloodstream within tissues. Killer cells can remain on guard where the immune defenses broke down in the past, alert but not activated, ready to attack rapidly if reinfection occurs [35].

1.4.4 Immune system function

The immune system is a complex set of cellular, chemical, and soluble protein components designed to protect the body against foreign substances, including infectious agents and tumor cells, while not responding to self-molecules. Foreign or self-molecules (usually proteins or carbohydrates) that evoke specific immune responses are referred to as antigens. Immune cells are located throughout the body, either in discretely encapsulated organs such as the spleen and thymus or as diffuse accumulations of lymphoid and myeloid cells as found in association with the skin and gut where they are strategically placed to monitor the entry of foreign substances. Optimal function of the immune system requires that immune cells and cell products interact with each other in a sequential, regulated manner. The distinction between self and non self occurs through complex mechanisms that depend upon specific recognition molecules present on the surface of immunocompetent cells, in particular, T and B lymphocytes. Autoimmune disease occurs when the immune system attacks self-molecules as a result of a breakdown of immunologic tolerance to autoreactive immune cells. Nonspecific effector mechanisms that complement or amplify the specific T- and B-lymphocyte responses are also important in the immune response. These nonspecific entities serve as a first line of defense against potential pathogens and include other leukocytes such as macrophages, natural killer (NK) cells, and polymorph nuclear leukocytes, as well as soluble mediators that include complement and cytokines. A number of autoimmune diseases demonstrate characteristic aberrations in cytokine production, suggesting that these soluble mediators may play a role in both the initiation and pathogenesis of the disease [35-37].

All immune systems address and solve these challenges. How mammals achieve this complex task is the story of an integrated system of biological processes, often using strategies that surprised their discoverers, whose elegance and power continue to

provide new insights for students of immunology young and old [35, 36]. The immune system uses many different receptors to interrogate the environment. These are usually proteins and are found in the blood, in tissue fluids or bound to the cell surface. The antibody receptor, also called an immunoglobulin (Ig), was the first antigen-specific receptor to be characterized and is commonly drawn as a Y-shaped cartoon. Antibody research in the first half of the 20th Century focused on antibodies that could be purified from serum. Once the amino acids that made up the different chains were defined, attention turned to understanding how antibodies were produced by individual cells. It was discovered that, for most antibodies, their generation depended on co-operation between at least two types of cell: a cell that processed and presented targets (antigens) from the environment (antigen-presenting cell (APC)) and a lymphocyte that recognized the target antigen on the APC. This lymphocyte (now called a T-cell or T-lymphocyte) directed either the production of antibodies or killed the cell presenting the antigen. One early idea to explain how T cells determined what to respond to was that the immune system only presented antigens from infections, but this was wrong. To the surprise of many immunologists, studies in the 1980s which defined the receptor molecules on the surface of cells that controlled this targeting process revealed that essentially all cells presented antigens. A healthy immune system surveys these antigens constantly, but this does not provoke a response. This challenges the impression that the immune system spends most of its time doing nothing. Quite the opposite: it is constantly reviewing the environment, checking whether anything is amiss. The immune system has a carefully developed sense of self which it generates through a process of education [1, 3]. The T anti-tumor response mediated by the CD4 helper T cells and the CD8 cytotoxic cells are regulated by endogenous factors and other cells, such as Th17, Treg, MDSCs, etc., that will ultimately result in either a positive anti-tumor response or an immuno-suppressive response. Prior findings have implicated

the role of iNOS/.NO in the regulation and differentiation of those various immune cells. For instance, Niedbala et al., the lymphocyte's antigen receptors recognize a family of cell-surface molecules on APCs that are collectively known as major histocompatibility (MHC) determinants. In humans, these are also called human leucocyte antigens (HLAs). These molecules are a combination of material derived from the environment (the antigen), bound in the flexible jaws of the MHC molecule which hold it in place. The rest of the MHC molecule acts as a scaffold orientating the MHC–antigen complexes at a cell's surface where they can be scanned by a lymphocyte). In this way, cells display a constantly changing picture of the proteins that they are making as antigens loaded into their MHC molecules, and these antigens come from inside and around cells. This sampling strategy is effective because disguise is very difficult at the molecular level. A bacterial cell makes many proteins that do not resemble those made by an animal cell; a cell making a virus inside does not look like a normal cell. The immune system is a complex, balanced and organic entity. Under normal conditions, cells of the immune system recognize foreign antigens and destroy them, while playing a continuous role in immune regulation, it is vital to maintain a balanced immune response [32-35, 38].

1.4.5 Cancer-related inflammatory conditions

Since 1863, when Virchow first hypothesized that cancer develops as the product of unresolved inflammation, tumor-associated inflammation has been key to shaping our modern understanding of cancer progression. Today, it is accepted that chronic inflammation is a critical hallmark of cancer, with at least 25% of cancers associated with and possible underlying causes include microbial infections, autoimmunity, and immune deregulation. For example, human papilloma viruses (HPVs) induce inflammation and are responsible for 90%–100% of all cervical cancers Similarly, chronic infection with *Helicobacter pylori* elevates the risk for gastric cancer. In

addition, the immune deregulation seen in inflammatory bowel disease increases colorectal cancer incidence. The nonhuman form of sialic acid N-glycolylneuroaminic acid (Neu5Gc) in red meat can be incorporated into human tissue and recruit inflammatory cells. In this sense, diet may play a causal role in induction of cancer-associated inflammation. Importantly, tobacco and obesity, both of which induce low-grade inflammation, give rise to elevated risks of cancer; this evidence suggests that the majority of cancers is associated with unresolved inflammation [39-41]. While chronic inflammation has an important role in cancer, less is known about the impact of acute inflammation on tumor progression. For example, inducing acute inflammation locally in the bladder with a vaccine containing an attenuated *Mycobacterium bovis* strain successfully treats squamous cancer of the bladder [42]. Hence, with the infiltration of leukocytes and subsequent inflammation, the impact from inflammatory mediators can both initiate and, in certain cases, eliminate tumor cells and prevent tumor development. This dual role of inflammation also becomes evident in the clinic, where immunodeficient patients are more often diagnosed with cancer [43]. Whether or not inflammation is a cause or a consequence, the tumor microenvironment (TME) is compromised, triggering an immune inflammatory response, and histopathological analyses provide evidence for the presence of innate and adaptive immune cells in most human tumors, which are characterized as features of cancer progression [44].

1.5 Role of inflammatory cells during cancer progression

The presence of tumor-associated inflammatory cells (Figure 1.4) in tumors raises an important question, which is one of the most important challenges in oncology: How do cancer cells avoid destruction by the immune system? since inflammatory cells were first observed in human tumors, much effort has been invested in better understanding the complex role of inflammatory cells in carcinomas. It is currently accepted that an aberrant innate and adaptive immune response contributes to

tumorigenesis by selecting aggressive clones, inducing immunosuppression, and stimulating cancer cell proliferation and metastasis [45].

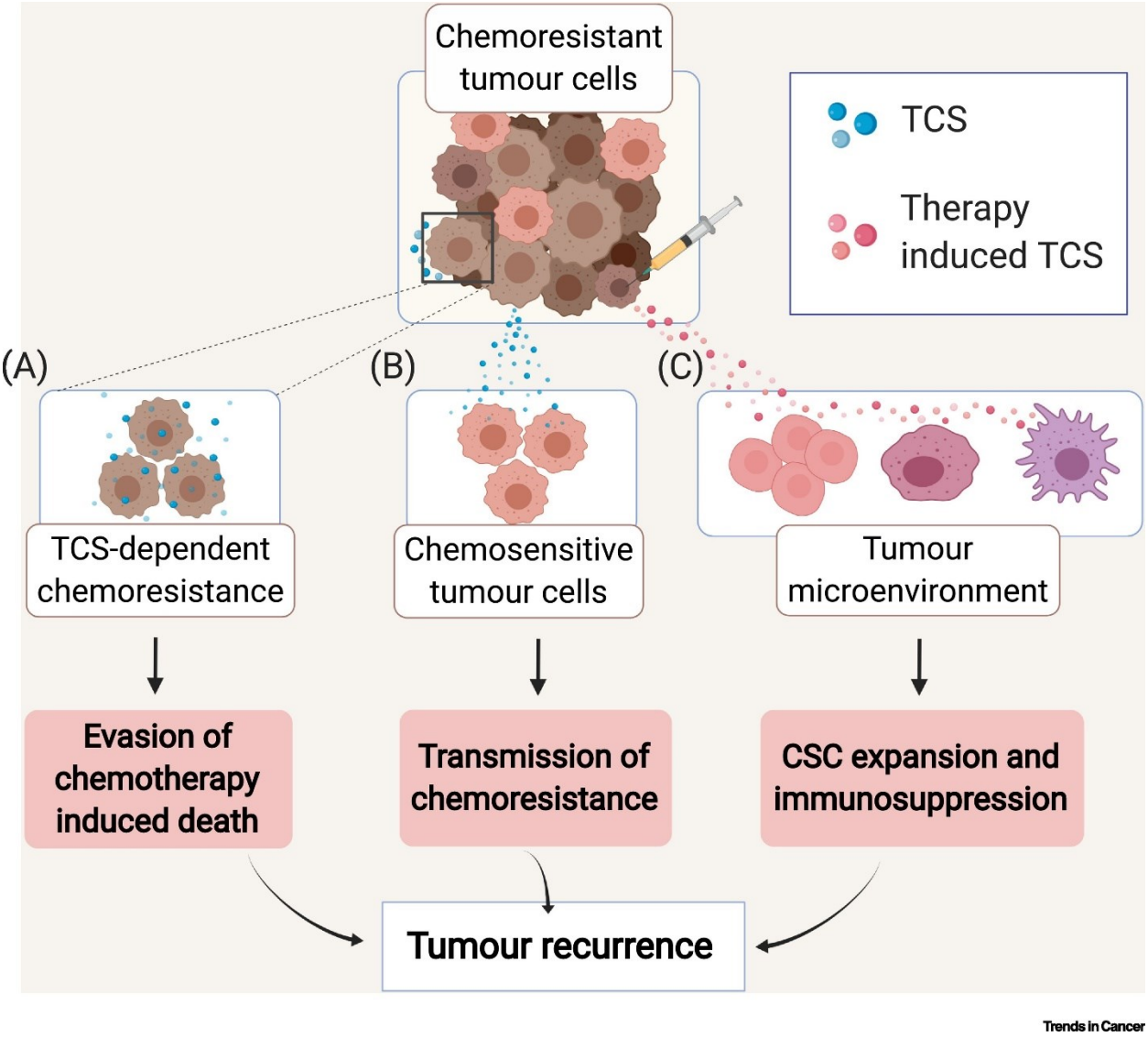
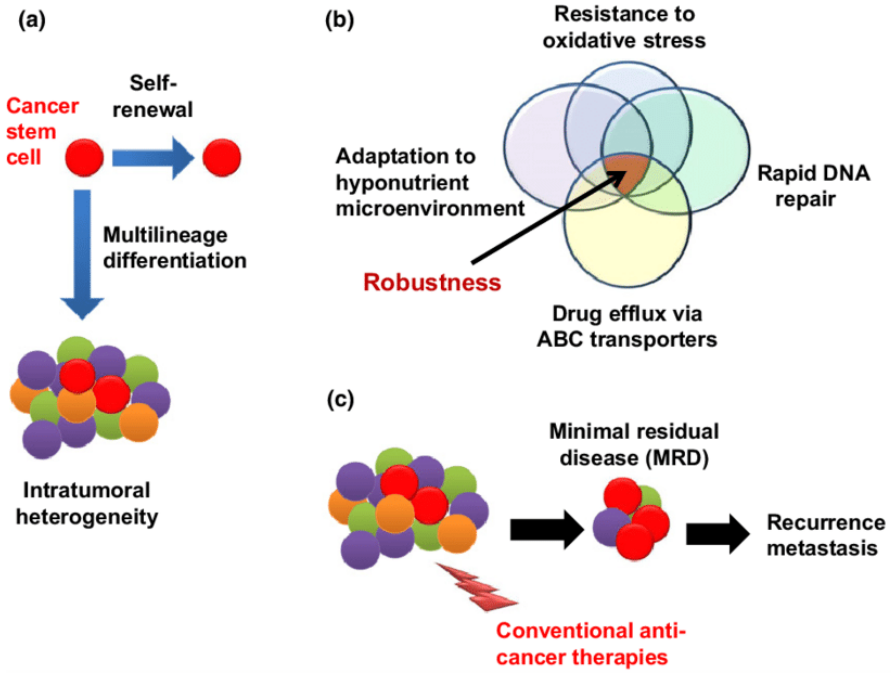


Figure 1-4. Tumor-associated inflammatory cells

During the early stages of tumor development, cytotoxic immune cells such as natural killer (NK) and $CD8^+$ T cells recognize and eliminate the more immunogenic cancer cells. This first phase of elimination selects the proliferation of cancer cell variants that are less immunogenic and therefore invisible to immune detection. As

the neoplastic tissue evolves to a clinically detectable tumor, different subsets of inflammatory cells impact tumor fate. For example, high levels of tumor-infiltrated T cells correlate with good prognosis in many solid cancers [46]. on the other hand, high levels of macrophage infiltration correlate with a worse prognosis [48]. Macrophages are innate immune cells that differentiate from circulating classical monocytes after extravasation into tissues. Upon differentiation, macrophages are equipped to sense and respond to infections and tissue injuries, playing a key role in tissue homeostasis and repair. As crucial drivers of chronic cancer-associated inflammation, their involvement has been described in every step of cancer progression, from early neoplastic transformation throughout metastatic progression to therapy resistance. In oncological patients and preclinical experimental models, high-grade tumor-associated macrophages (TAMs) correlate with poor prognosis and reduced overall survival [48, 49].



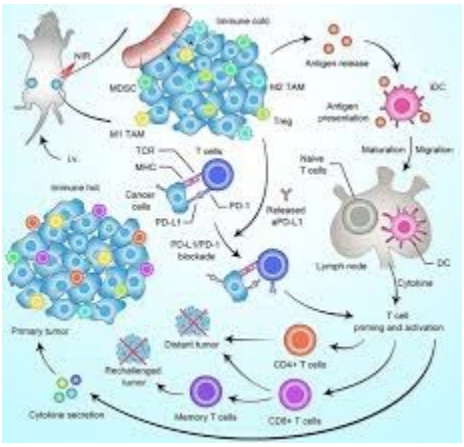


Figure 1-5. Oncological patients and preclinical experimental models

Similar to other tumor-associated immune cells, TAMs have been described mostly in primary tumors. However, understanding the roles of macrophages as promoters or inhibitors in the metastatic cascade and their role in metastasis is a growing field. In xenograft models of breast cancer, TAMs regulate invasion of stroma and intravasation of cancer cells via cell contact-mediated signaling [50]. Neutrophils are recognized as key players during inflammation. They are among the first immune cells to be recruited to damaged tissue, where they can eliminate pathogens and modulate inflammation by mechanisms such as phagocytosis, secretion of antibacterial proteins, deposit of neutrophil extracellular traps (NETs), and exocytosis of protease-containing granules. In cancer patients, high levels of tumor-associated neutrophils (TANs), high levels of neutrophilia, and/or high neutrophil/lymphocyte ratios have been associated with an adverse prognosis in different malignances. Similar to the M1/M2 phenotype of macrophages, it has been proposed that TANs exist in two polarization states, called “N1” and “N2,” to describe protumor and anti-tumor populations, respectively. This paradigm is still a matter of debate due to the lack of specific markers to identify these two populations. However, it is clear that TANs display functional heterogeneity. The recruitment of

TANs to the TME is thought be mediated mainly by CXCR2 ligands such as CXCL1, CXCL2 and CXCL5, secreted by cancer and stromal cells; TGF-β has also been associated with recruitment and reprogramming to protumor TANs [51, 52].

T cells are components of the adaptive immune system that act as orchestrators and effectors of immunity. Depending on the immunological context, T cells can acquire functional and effector phenotypes whose activity has direct inflammatory or anti-inflammatory consequences [53]. During the early stages of tumor initiation, if enough immunogenic antigens are produced, naïve T cells will be primed in the draining lymph nodes, followed by their concomitant activation and migration to the TME. From there, they mount a protective effector immune response, eliminating immunogenic cancer cells. Histopathological analyses of human tumors show that tumor-associated T cells extend beyond the invasive edge of the tumor and also predominate in its hypoxic core. A high level of T-cell infiltration in tumors is associated with a favorable prognosis in melanoma and breast, lung, ovarian, colorectal, renal, prostate, and cancer. Upon activation in the germinal centers in lymphoid organs, B cells expressing high-affinity antibodies differentiate into antibody-secreting plasma cells and memory B cells that mediate humoral immunity against pathogens [54]. Although, the presence of B cells in the TME has been described in different carcinomas (including melanoma and breast, ovarian, and prostate cancer, among others the role of B cells in cancer progression is much less understood than that of T cells. Accumulating evidence indicates that B cells promote and support tumor growth; for example, using a transgenic mouse model of epithelial carcinogenesis, Coussens and colleagues demonstrated that the lack of mature B cells decreases tumor progression. Notably, the adoptive transfer of B cells restores chronic inflammation, angiogenesis, and tumor growth. Different mechanisms have been described to explain the protumor role of B cells, from immunosuppression via secretion of IL-10 and TGFβ) to direct stimulation of tumor

cell proliferation by B-cell-derived IL-35 in human pancreatic neoplasia and *Kras*-driven pancreatic neoplasms in mice [56].

1.6 P53

The p53 gene like the Rb gene, is a tumor suppressor gene, i.e., its activity stops the formation of Tumor protein P53, also known as p53, cellular tumor antigen p53 (UniProt name), the Guardian of the Genome, phosphoprotein p53, tumor suppressor p53, antigen NY-CO-13, or transformation-related protein 53 (TRP53), is any isoform of a protein encoded by homologous genes in various organisms, such as *TP53* (humans) and *Trp53* (mice). This homolog (originally thought to be, and often spoken of as, a single protein) is crucial in vertebrates, where it prevents cancer formation As such, p53 has been described as "the guardian of the genome" because of its role in conserving stability by preventing genome mutation. Hence *TP53*is classified as a tumor suppressor gene. The name p53 was given in 1979 describing the apparent molecular mass; SDS-PAGE analysis indicates that it is a 53-kilodalton (kDa) protein. However, the actual mass of the full-length p53 protein (p53α) based on the sum of masses of the amino acid residues is only 43.7 kDa. This difference is due to the high number of proline residues in the protein, which slow its migration on SDS-PAGE, thus making it appear heavier than it actually is. In addition to the full-length protein, the human *TP53* gene encodes at least 15 protein isoforms, ranging in size from 3.5 to 43.7 kDa. All these p53 proteins are called the p53 isoforms. The TP53 gene is the most frequently mutated gene (>50%) in human cancer, indicating that the *TP53* gene plays a crucial role in preventing cancer formation. *TP53* gene encodes proteins that bind to DNA and regulate gene expression to prevent mutations of the genome tumors. If a person inherits only one functional copy of the p53 gene from their parents, they are predisposed to cancer and usually develop several independent tumors in a variety of tissues in early adulthood. This condition is rare, and is known as Li-Fraumeni

syndrome. However, mutations in p53 are found in most tumor types, and so contribute to the complex network of molecular events leading to tumor formation [57, 58]. The p53 gene has been mapped to chromosome 17. In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (cdk2). When p21 is complexed with cdk2 the cell cannot pass through to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the 'stop signal' for cell division. Thus cells divide uncontrollably, and form tumors. Help with unraveling the molecular mechanisms of cancerous growth has come from the use of mice as models for human cancer, in which powerful 'gene knockout' techniques can be used. The amount of information that exists on all aspects of p53 normal function and mutant expression in human cancers is now vast, reflecting its key role in the pathogenesis of human cancers. It is clear that p53 is just one component of a network of events that culminate in tumor formation [59, 60]. Cells are constantly exposed to a variety of cellular stresses such as DNA damage. These cellular stresses finally introduce the genomic aberrations including mutation, deletion and/or translocation into the cellular genome and thereby induce the genomic instability. Accumulation of the genomic aberrations often results in the development of cancers, Therefore, a proper stress response is required to maintain the genomic integrity and protect cells from malignant transformation.

In humans, a common polymorphism involves the substitution of an arginine for a proline at codon position 72 of exon 4. Many studies have investigated a genetic link between this variation and cancer susceptibility; however, the results have been controversial. For instance, a meta-analysis from 2009 failed to show a link for cervical cancer. A 2011 study found that the *TP53* proline mutation did have a profound effect on pancreatic cancer risk among males. A study of Arab women

found that proline homozygosity at *TP53* codon 72 is associated with a decreased risk for breast cancer. One study suggested that *TP53* codon 72 polymorphisms, MDM2 SNP309, and A2164G may collectively be associated with non-oropharyngeal cancer susceptibility and that MDM2 SNP309 in combination with *TP53* codon 72 may accelerate the development of non-oropharyngeal cancer in women. A 2011 study found that *TP53* codon 72 polymorphism was associated with an increased risk of lung cancer [59-61]. Meta-analyses from 2011 found no significant associations between *TP53* codon 72 polymorphisms and both colorectal cancer risk and endometrial cancer risk. A 2011 study of a Brazilian birth cohort found an association between the non-mutant arginine *TP53* and individuals without a family history of cancer. Another 2011 study found that the p53 homozygous (Pro/Pro) genotype was associated with a significantly increased risk for renal cell carcinoma [62, 63]. Due to the isoformic nature of p53 proteins, there have been several sources of evidence showing that mutations within the *TP53* gene giving rise to mutated isoforms are causative agents of various cancer phenotypes, from mild to severe, due to single mutation in the *TP53* . Tumors evolve through genetic and epigenetic changes that modify fundamental cellular programs of growth and proliferation, followed by selection of reprogrammed cells that best adapt to a variety of suboptimal or challenging conditions they encounter, either transiently or durably, during progression. The most frequently altered gene in human tumors is *TP53*encoding the p53 protein. *TP53* mutations are associated with adverse prognosis in many sporadic cancers, moreover germline *TP53* mutations are causative of the Li Fraumeni syndrome, a rare familial cancer predisposition [64, 65]. On this basis, specific missense p53 mutants have been reported to subvert crucial cellular pathways and to foster cancer cell proliferation and survival, promote invasion, migration, metastasis, and chemoresistance. Whereas several mutp53 neomorphic phenotypes contributing to tumor aggressiveness have been described,

our understanding of the mechanisms that determine cellular addiction to mutp53 expression for cancer maintenance and progression remains incomplete. Part of the tumor suppressive activities of wild-type p53 involves its capability to help the cell adapt to and survive mild stress conditions, including oxidative and metabolic stress .Remarkably, mutp53 becomes stabilized and activated in response to tumor-related stress conditions, similar to the wild-type counterpart .Alongside this notion, evidence is rising that mutp53 can provide cancer cells with the ability to cope with challenging conditions originated during tumorigenesis, including hyperproliferation-related DNA damage, oxidative and proteotoxic stress, nutrient fluctuations, physical constraints, stromal cues, and the anti-tumor immune response [66, 67]. Similar to wild-type p53, whose accumulation and activation are triggered by transformation-related stimuli, an array of inputs originated within the altered tumor context conspire to induce mutp53 protein stabilization and oncogenic functions. During oncogenic transformation the Hsp90 system is frequently hyper-induced, due to activation of the master transcription factor heat-shock factor-1 (HSF1) in response to multiple stress conditions .Significantly, high levels of oxidative stress—a major inducer of HSF1—are frequently associated with tumor growth and were shown to cause mutp53 protein stabilization in vivo .Moreover, mutp53 potentiates transcriptional induction of several heat-shock proteins by enhancing HSF1 stabilization and activation and participating into HSF1 transcriptional complexes at Hsp gene promoters ,thus generating a feed-forward circuit that sustains mutp53 accumulation [68, 69].

Many solid tumors undergo alterations of lipid metabolism, which contribute to cancer in multiple ways, e.g. by providing membrane lipids and supporting signaling pathways that promote proliferation and survival, EMT, cancer stem cells fate determination, and metastatic dissemination. HNSCC is a collection of cancers encompassing the oral cavity, nasopharynx, oropharynx, hypopharynx and larynx

[70]. The most common genetic aberrations are the overexpression of the epidermal growth factor receptor (EGFR) and inhibiting mutations of p53 in 95% and 75-85% of non-HPV-related cases respectively. Additionally, the PI3K/AKT/mTOR signalling pathway is the most commonly mutated signalling pathway with up to 62% of HNSCC patients showing activating mutations. The hyperactivation of this pathway contributes to increased cell growth, proliferation and cellsurvival as well as regulation of apoptosis and DNA damage repair [71, 72].

Due to the prevalence of p53 mutation, p53 is a frequent topic of many HNSCC studies. A large portion of those reports involves the reactivation of p53 which has become a promising treatment approach in combination with targeted therapies for HNSCC .In addition, p53 is responsible for the transcription activation of many genes involved in cell cycle arrest, apoptosis, senescence, DNA repair and metabolism .Indeed, activation of p53 occurs when either the cell accumulates too much DNA damage, an oncogene is activated, or in case of stressors such as nutrient deprivation or hypoxia .Depending on the severity of these activators, the cell will undergo different responses such as induction of cell cycle arrest, apoptosis, senescence, induction of protective antioxidant activities, DNA repair as well as the alteration of the mitochondrial respiration .In cancer cells without functional p53, the cell has a deletion in one of the *TP53* alleles and a mutated, non-functional form of *TP53* in the other allele. Consequently, the cell can accumulate additional mutations, without resulting in apoptosis or cell cycle arrest for repair, and further activate oncogenes that result in oncogenesis [73, 74]. The TP53 gene and its gene products control the cell cycle by inducing arrest in response to DNA damage and apoptosis if the damage is irreparable Inactivation of TP53 through mutations plays a critical role in early cancer development and progression. TP53 is a key tumor suppressor and TP53 mutation is the most common genetic change in malignancies,

involved in 50% of human cancers as well as up to 70% of head and neck squamous cell carcinomas (HNSCC) [75,76].

In HNSCC, there are other ways in which p53 is inactivated besides inactivating mutations of p53. Based on data acquired from a 243-patientcohort collected by the cancer genome atlas the highest percentage of mutations causing p53 inactivation in human papilloma virus (HPV) negative HNSCC occurs in *TP53* itself (84%) followed by *CDKN2A* (57%) which either contains a homozygous deletion or a mutation. A recent computational analysis of the TCGA database revealed that, although more or less severe depending on the types of mutations and localization of the mutation within the gene, patients with mutated *TP53* HNSCC were shown to have reduced overall survival. The 8 most common p53 mutations are all located in the DNA binding region which can be found between residues 103-292. Among these mutants are 2 groups, a group that has mutated the specific residues responsible for binding the DNA and a second group that changes the conformation of the DNA binding element thereby also preventing DNA binding [77, 78]. Because of the frequency and important biologic effects of TP53, it is clear that biomarkers such as p53 expression could provide useful clinical information if reliable and reproducible correlations with prognosis or treatment response could be demonstrated in large homogenous groups of patients. Most recently, we have shown that the immune response in the tumor microenvironment as reflected by tumor infiltrating lymphocytes (TILs) is a critical factor in predicting prognosis [79]. Because of the frequency and important biologic effects of TP53, it is clear that biomarkers such as p53 expression could provide useful clinical information if reliable and reproducible correlations with prognosis or treatment response could be demonstrated in large homogenous groups of patients. It has shown that the immune response in the tumor microenvironment as reflected by tumor infiltrating lymphocytes (TILs) is a critical factor in predicting prognosis. The role of p53 protein as a neoantigen in the tumor

microenvironment is currently unclear and it is unknown if over-expression of p53 protein can stimulate or suppress this critical cellular immune response. Evidence has suggested that p53-mediated responses may be responsible for the recruitment of TILs to the tumor microenvironment and can alter the immune response [80, 81].

2 Review Articles

Khadang et al (2007) studied 221 female patients with sporadic breast cancer and 205 healthy blood donors as control group were evaluated. DNA from peripheral blood mononuclear cells was extracted and amplified using allele-specific polymerase chain reaction. Frequency of homozygotic arginine at codon 72 was 37.6% in patients and 36.6% in controls, for homozygotic proline it was 13.1 and 19.5%, and for heterozygotic Arg/Pro it was 49.3 and 43.9%, respectively. No significant difference was found between patients and controls regarding allele frequencies. They concluded that mutation in codon 72 of TP53 gene was not associated with breast cancer in Iranian patients [82]. Poeta at al. (2007) evaluated 560 patients with squamous-cell carcinoma of the head and neck who were treated surgically with curative intent were enrolled in our prospective multicenter. TP53 mutations were found in tumors from 224 of 420 patients (53.3%). They also stated that disruptive TP53 mutations in tumor DNA are associated with reduced survival after surgical treatment of squamous-cell carcinoma of the head and neck [83]. Ghapanchi et al. (2009) investigated the association of p53 codon 72 polymorphism with OLP in Southern Iranian patients. Twenty-five patients with lichen planus and 93 healthy blood donors as control group were recruited. DNA from peripheral blood mononuclear cells was extracted and amplified using allele-specific polymerase chain reaction. The frequency of homozygotic arginine (Arg) at codon 72 was 44% in the patients and 37.6% in the controls; for homozygotic proline (Pro), it was 24% and 15.15%, respectively, and for heterozygotic arginine/proline, it was 32% and

47.3%, respectively. They concluded polymorphism in codon 72 of the TP53 gene was not associated with the OLP in Iranian patients [84]. Peltonen et al. (2011) reported that TP53 mutations in specific regions, including DNA-binding surface, to determine whether mutations at specific locations of TP53 could be used to help in setting up prognosis and response to therapy of head and neck squamous cell carcinoma patients. TP53 mutations in specific regions, including DNA-binding surface at specific locations of TP53 could be used to help in setting up prognosis and response to therapy of head and neck squamous cell carcinoma patients [85]. Etemad Moghadam and coworkers (2015) evaluated alterations in p53 and p27(KIP1) as important prognostic factor in Iranian oral squamous cell carcinoma. p53 and p27(KIP1) expression were found in 28.57% (8 positives versus 20 negative) and 67.85% (19 positive versus 9 negative) of OSCC cases, respectively. There was no significant association between these two proteins ($P = 0.371$), and neither of them showed a significant relationship with the studied clinicopathologic variables ($P > 0.05$). they reported that abnormalities in p53 and p27 (KIP1) may be involved in the development of OSCC [86].

Zhuo et al. (2016) study confirmed that mutations of the *TP53* tumor suppressor gene were the most frequent of all somatic genomic alterations in HNSCC, alluding to the importance of the *TP53* gene in suppressing the development and progression of this disease. Clinically, *TP53* mutations are significantly associated with short survival time and tumor resistance to radiotherapy and chemotherapy in HNSCC patients, which makes the *TP53* mutation status a potentially useful molecular factor for risk stratification and predictor of clinical response in these patients. In addition to loss of wild-type p53 function and the dominant-negative effect on the remaining wild-type p53, some p53 mutants often gain oncogenic functions to promote tumorigenesis and progression. Different p53 mutants may possess different gain-of-function properties. Therefore, mutant p53 is not just one protein but actually a

variety of proteins that contribute to an exceptionally vast network of tumor-promoting processes [87]. Kumari et al. (2018) studied the prostate cancer cases. Considering the important role of p53 inactivation in cancer development, restoration of wild-type p53 function by p53-reactivating compounds developed with different approaches, seems to be an attractive therapeutic strategy for prostate cancer therapy [88]. Melling et al. (2019) studied p53 inactivation in esophageal cancer and reported that heterozygous *TP53* deletions occurred in 40.9% in adeno carcinoma AC and in 19.4% in SCC. High-level p53 immunostaining was associated with shortened overall survival in AC and SCC while *TP53* deletions alone showed no correlation with survival. High-level p53 immunostaining in patients with AC was associated with advanced tumor (P=0.019) and, grading (P=0.027) and the resection margin status (P=0.006). Associations between p53 immunostaining and SCC were not found. *TP53* deletions were found to be associated with advanced tumor stages (P=0.028) and the presence of lymph node metastasis (P=0.009) in SCC. They concluded strong p53 immunostaining, but not *TP53* deletion alone, is associated with unfavorable outcomes and may therefore represent a clinically useful molecular marker in esophageal cancer [89]. Ziaran and coworkers (2020) analyzed the tumor tissues of 224 patients with urinary bladder cancer. They performed a histomorphologic analysis and immunohistochemistry for p53, Ki-67, and E-cadherin, which were selected as markers of the malignant process. They showed a relationship between bladder cancer and recurrence with these markers [90]. Farnoosh et al. (2021) evaluated several single nucleotide polymorphisms in breast cancer susceptibility polymorphisms within genes (*STK15, ERRs, ESR1, p53, SEP15, AURKA, SHBG, SRC, FAS, VEGF, XRCC1, GST, NFκB1, XPC, XRCC3*, sirtuin-3, *NKG2D*). They observed that the signaling pathways and antioxidant related genes are the main molecular processes associated with breast cancer progression. Further studies on types of polymorphisms in breast cancer could

validate the prognostic value of biomarkers [91]. Jamshidi et al. (2021) studied main genetic marker among Iranian patients with thyroid cancer. A significant relationship was found between SNP in codons 194, 280, and 399 (*XRCC*1), Allele 3434 Thr (*XRCC*7), GC or CC genotype 31, G/C and thyroid cancers [92].

3 Material and Method

3.1 AIM & objectives

There is a great interest in developing biomarkers and genes efficacy to enhance early detection and clinical management of squamous cell carcinoma. However, the developmental path towards a clinically valid biomarker remains extremely challenging. Ideally, the initial key step in moving discovered pathways towards clinical implementation, this study aimed to discover P53 in SCC and recurrent cancer. Emerging evidence indicates a potential role of P53in SCC immunopathology. We purposed to investigate the effect of this marker in both Scan recurrent SCC with the aim of characterizing their potential role in disease immunopathology. To the best of our knowledge, this is the first study of its kind.

3.2 Study Method

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran. Code #IR.SUMS.DENTAL.REC 1400.09 This study was supplied by Institue of Cancer researches of Medical Research of Shiraz University of Medical Sciences and desigend and excuded as a cross sectional study

3.3 Sampling

This cross sectional study was done in 2021-2022 and comprised patients, diagnosed with new-onset SCC and recurrent form by two independent pathologists, and healthy individuals without a history of cancer. The entire case group was selected, without any limitations in age and gender, from the Namazi, Khalili and Madar Va

Kodak Hospital affiliated with the Shiraz University of Medical Sciences. Healthy people were randomly selected from those referred to Shiraz dentistry school as controls. A total of 122 patients with SCC (78 primary including 28 females (35.9%) and 50 males, (6.1%)) aged 27-88 years (64.57 ±12.6) and 44 recurrent including 22 males (50%) and 22females (50%) constituted our study population. The healthy controls consist of 22 males (55%) and 18 females (45%). The examined subjects were categorized regarding age range to 2 subgroups, below 50 and over 50 years. The individuals voluntarily agreed to participate in this experiment as a part of a large prospective research project and completed the written informed consent form. Two pathologists confirmed the diagnosis of oral SCC. Clinical staging of cases was determined according to the tumor, node, and metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC). The histopathological grade of oral SCC was determined based on World Health Organization (WHO) criteria. Healthy or control group of this study consisted of volunteers whose medical history including no active oral ulcer, cancer, thyroid or metabolic disease. The donor health status was identified before venipuncture by case history. Every patient who had other kinds of oral cancer, thyroid disease, administration of anti-neoplastic chemotherapy, radiotherapy, or lichenoid lesions objectively related to exogamic agents, drugs or specific materials and allergens were excluded

3.4 Inclusion and exclusion criteria

Demographic data plus information regarding chief complaints of all subjects with biopsy-proven diagnoses of oral SCC were collected. Both primary tumor and recurrent samples with available Hematoxylin and Eosin (H & E) slides of the initial neoplasms were chosen and those with any of the following criteria were excluded from the study sample: incomplete medical and contact data, presence of a previously diagnosed cancer of any type and in any part of the body, history of

radio/chemotherapy prior to the first surgical treatment and specific systemic, or any genetic predisposing factors to development of cancer (e.g. Plummer-Vinson syndrome, xeroderma pigmentosum, or Li-Fraumeni syndrome). All H&E slides, obtained from the pathology archives of the respective clinics or hospital, were re-evaluated by two oral pathologists using a double-headed microscope (Olympus BX51) in order to confirm the initial diagnosis using the world health organization specifications [93]. Also, the tumors were graded according to the criteria proposed by Bryne et al. [94] and disagreements were resolved by consensus. The inclusion and exclusion criteria are listed in Table 3-1.

Table 3-1. Patient inclusion and exclusion criteria.

Criteria	Description
Inclusion	1-Histologically proven squamous cell carcinoma 2- Resect able tumor
Exclusion	(i) Pregnancy (ii) No disposing capacity or expected insufficient compliance (iii) Autoimmune disease and other cancers

The patients with any inflammatory or autoimmune disease were excluded from this evaluation. The healthy patients referring to dentistry school were matched by age and sex; they should be a non-smoker and fulfilled all exclusion criteria mentioned

above. Additional to any systemic disease, cancer and chemo or radiotherapy. All the research objects were the Iranian people who had no kinship with one another.

3.5 Sample collection and the procedure

Written consents were obtained from all participants after explaining the study protocol and objectives. Personal information of all patients remained confidential and in order to avoid loss of specimens, only paraffin blocks with adequate amount of neoplastic tissues were selected and sectioning was kept to a minimum.

3.6 Staining Procedure

Immunohistochemical staining procedures were performed according to the instructions provided by the manufacturers. In brief, 4-µm thick paraffin sections mounted on Poly-L-lysine-coated glass slides were dewaxed in xylene, dehydrated, placed in 0.1M citrate buffer (PH: 0.6) and microwaved twice for 15 minutes followed by blocking of endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for half an hour. Incubation with monoclonal antibodies against p27 (IB4, Novocastra) and p53 (DO-7, Dako SA, Glostrup, Denmark) was carried out using dilutions of 1:20 and 1:50, respectively. All sections were then treated with avidin-biotin-peroxidase complex followed by immersion in diaminobenzidine-H2O2 substrate (5 minutes, room temperature) for chromogen development and finally counterstained with hematoxylin. A known p53 positive oral SCC specimen were used. Substitution of primary antibody with nonimmune serum, served as negative control.

3.7 Immunohistochemical Analysis

- Labeling indices of all specimens were determined by two oral and maxillofacial pathologists using the abovementioned double-headed microscope without knowledge of their clinical features and outcomes. Any possible disagreements were resolved by consensus. A minimum of 300 cells in a total of 5 high power

fields (HPFs) were counted and cases with > 10% nuclear staining of tumor cells were considered positive for p27 [95].

- P53 labeling index was calculated by assessing nuclear immunopositivity in 1000 neoplastic cells in 10 HPFs which was considered positive where there was ≥ 10% immunostaining [96].

3.8 Statistical analysis

The results were analyzed by SPSS version 22 software. To compare the means of number and age between women and men who participated in the study and in the case and control groups, chi-square and ANOVA tests were used respectively and odd ratio OR, and to compare the means of P53 levels in the case and control groups Cox's proportional hazard regression and hazard ratio: HR was used. Also, Spearman correlation test was used to determine the correlation between means of P53 in each case and control. Fischer exact test was employed to assess significant correlations between the categorical variables. Survival data were evaluated using Cox regression analyses and $P < 0.05$ was considered statistically significant.

4 Result

4.1 Patients' Profile

Based on the inclusion and exclusion criteria, 178 cases were selected for this study. Unfortunately, because of unobtainable paraffin blocks, including those with insufficient residual tumor and changes in contact information, 16 cases were lost at the initial stage of the investigation; leaving 162 samples for evaluation. A total of 122 patients with SCC (78 primary including 28 females (35.9%) and 50 males, (6.1%)) aged 31-78 years (64.57 ±12.6) and 44 recurrent form including 22 males (50%) and 22 females (50%) constituted our study population. The healthy controls consist of 22 males (55%) and 18 females (45%). The examined subjects were

categorized regarding age range to 2 subgroups below 50 and over 50 years. (Table 4-1)

Table 4-1. The examined subject below 50 and over 50 years

	Tissue			
p	recurrence (n=۴۴)	primary (n=۷۸)		
۰/۱۲۸	۲۲ (۵۰)	۲۸ (۳۵/۹)	Female	Sex
	۲۲ (۵۰)	۵۰ (۶۴/۱)	Male	
۰/۲۷۶	۹ (۲۰/۵)	۲۳ (۲۹/۵)	۵۰ under	Age
	۳۵ (۷۹/۵)	۵۵ (۷۰/۵)	۵۰ over	

There was no significant difference regarding the mean age between male and female subjects in case subdivisions as well as cases and controls. (P >0.05). P53 expression were not seen in controls, on contrast 70 SCC cases (57.4%) showed P53 expression (P<0.001, OR =107.69) (Table 4-2).

Table 4-2. P53 expression

		Gene expression				
P	OR (95% CI)	+	-			
<۰/۰۰۱	۱۰۷/۶۹ (۶/۴۹-۱۷۹/۰)†	۰ (۰)	۴۰ (۱۰۰)	۴۰	control	Tissue
		۷۰ (۵۷/۴)	۵۲ (۴۲/۶)	۱۲۲	SCC	
۰/۳۹۰	۰/۷۲ (۰/۳۵-۱/۵۱)‡	۳۱ (۶۲)	۱۹ (۳۸)	۵۰	Female	Sex
		۳۹ (۵۴/۲)	۳۳ (۴۵/۸)	۷۲	Male	
۰/۰۰۱	۴/۵۲ (۱/۰۷-۱۲/۰۵)††	۲۶ (۸۱/۳)	۶ (۱۸/۸)	۳۲	۵۰ under	Age
		۴۴ (۴۸/۹)	۴۶ (۵۱/۱)	۹۰	۵۰ over	

In reviewing the clinical data of the study sample, we found the youngest patients (<50 years) had a higher rate of P53 expression (p=0.001. OR=4.52, 95% CI: [1.70-12.05],). Independent T test did not show a significant correlation regarding gender and P53 expression (p=0.39, OR=0.74, 95% CI: [0.35-1.51). (table4-2). Our cases were followed for a mean period of 13 months. Regression cox test did not demonstrate a significant difference in recurrence rate and P53 expression in both males and females, over expressed or under expressed gene cases (P=0.953, 95%

CI: 0.48 -2.00, HR=0.98) and age (p=0.223, HR=1.81, 95% CI: [0.70 -4.69]) (Figure 4-1 and Table4-3).

Table 4-3. P53 expression in both males and females, over expressed or under expressed gene

		Recurrence				
P	HR (95% CI)	+	-			
.7	.6	۲۲ (۳۶/۱) .5	۳۹ (۶۳/۹) .4	۶۱ .3	+ .2	
۰/۲۶۳	۰/۶۹ (۰/۳۶-۱/۳۲)‡	۲۱ (۵۱/۲)	۲۰ (۴۸/۸)	۴۱	Female	sex
		۱۷ (۲۷)	۴۶ (۷۳)	۶۳	Male	
۰/۲۲۳	۱/۸۱ (۰/۷۰-۴/۶۹)††	۶ (۲۰/۷)	۲۳ (۷۹/۳)	۲۹	۵۰ under	age
		۳۲ (۴۲/۷)	۴۳ (۵۷/۳)	۷۵	۵۰ over	

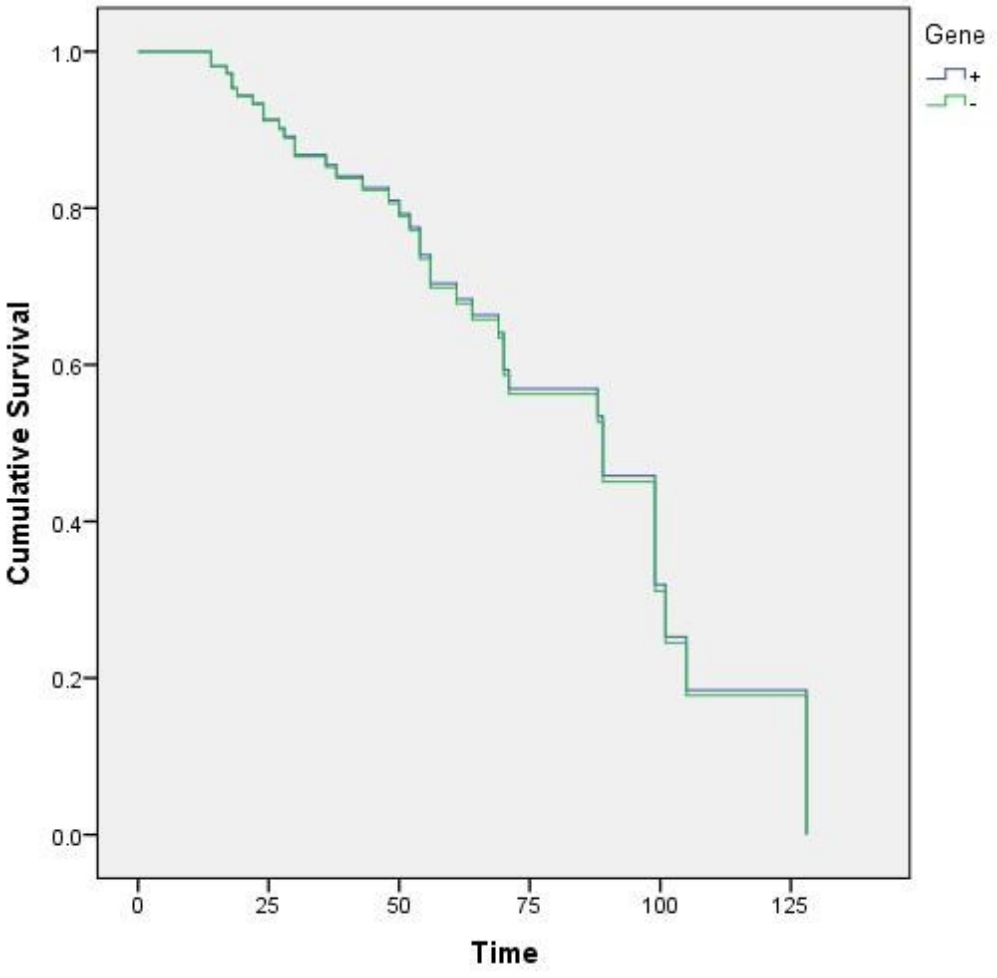


Figure 4-1. P53 expression in both males and females, over expressed or under expressed gene

4.2 Immunohistochemical Expression of p27 and p53

We observed p53 immunopositivity in 70 cases. A significant correlation between the expression of these proteins and variables of age, gender, local recurrence, or pathologic differentiation was not observed ($P > 0.05$).

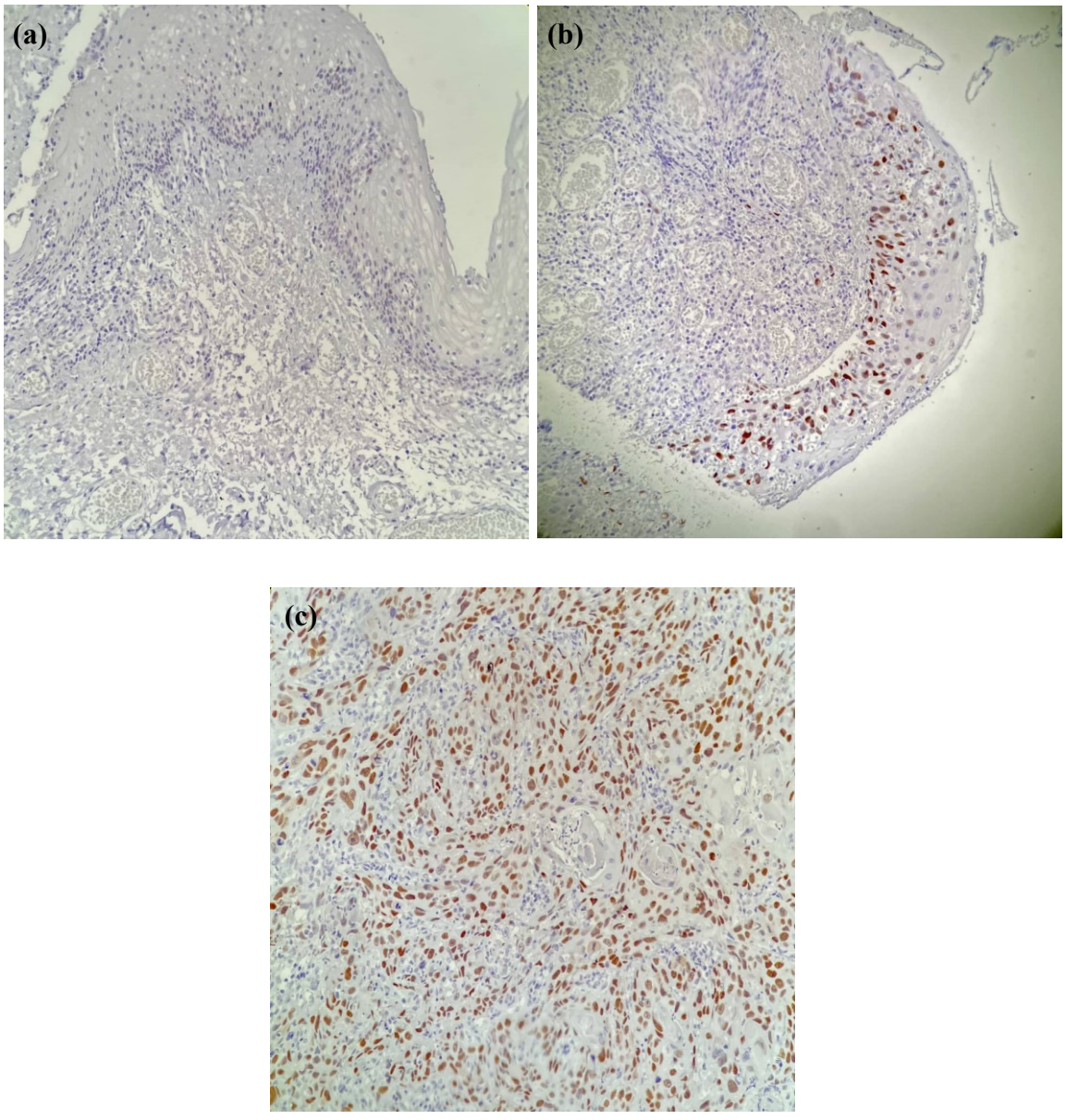


Figure 4-2. P53 immunopositivity in 70 cases

5 Discussion

SCC remains one of the leading causes of cancer-related deaths worldwide. In order to optimize treatment strategies, it is crucial that biomarkers are identified that associate with clinical outcome. Due to its critical role in combating tumor development and progression, the immune system has become an important focus in bioresearch. According to the results of this study, Statistical analysis showed a significant correlation between both groups regarding P53 expression in both cases and controls. ($p<0.001$); but this relation was not significant, regarding the gender in each group. To the best of our knowledge, there were no study about this marker and SCC in primary and recurrent form in Iran. Gene mutation and tumor microenvironment participate in several stages of cancer progression. There have been numerous surveys on the role of cell cycle-related factors in the head and neck and oral cavity of cancerous patients from various genetic backgrounds and geographic locations. However, their functional significance and clinical value remain unclear [96-98].

In the present study, we evaluated the status of p53 in a subset of Iranian patients and determined their correlation with different clinicopathologic features. Despite the proposition p53 may act in concert to induce malignant features like invasiveness and increased proliferation the number of studies simultaneously evaluating this protein is limited. Furthermore, we used previous studies on the expression of p53 in Iranian subjects with oral SCC. Despite the fact that other diagnostic methods like PCR may be more accurate than immunohistochemistry (IHC), it is noteworthy that IHC is more practical and cost efficient in addition to being less technique sensitive. This method is routinely performed in most laboratories and is easily completed on paraffin blocks, which are stored for all patients and retrievable at later dates [86]. According to our results, there was no significant association between p53 with

either patient tumor recurrence, which was in accordance with a considerable number of former investigations [97]. Conversely, there are some studies that have stated a negative effect of p53 expression on prognosis [99]. while Sauter et al. found that expression of this protein prolonged survival [100]. Immunohistochemistry detects inactivated and stabilized p53 protein, which can result from mutation, binding to products of viruses and cellular oncogenes or 'phosphorylation due to cdc2-like kinases [101]. Immunoreactivity to p53, either because of a functionally altered protein or accumulation of its wild-type form could be indicative of an assault on cellular deoxyribonucleic acid (DNA) or other events, some of which may be in favor of malignant transformation [102]. In cells with normally functioning p53, attack on DNA leads to G1 arrest followed by repair, senescence or apoptosis. Alteration of p53 protein could result in loss of its vital actions such as regulating proliferation, inhibiting migration and suppression of invasion. In addition, mutated p53 has been shown to promote metastasis [99]. Etemad moghadan et al in a research showed the trend towards a shorter survival of p53 positive cases that not evaluated in current study but it seems that their finding is in line with the known function of this protein [86]. There are a considerable number of reasons that might provide an explanation as to why a significant association was not found between p53 expression and survival in the previous investigations [103]. It seems that accumulation of wild-type p53, in which other molecules and pathways are responsible for carcinogenesis were seen in many studies [86, 97]. Immunoreactivity to p53, either because of a functionally altered protein or accumulation of its wild-type form could be indicative of an assault on cellular deoxyribonucleic acid (DNA) or other events, some of which may be in favor of malignant transformation. The complexity through which mutations affect proteins could help clarify the limited prognostic significance of p53 immunoreactivity. For example, mutations in

different codons exert different effects on the function of the protein. In addition, nonsense mutations or loss of both alleles can lead to lack of protein detection [104].

In the present study, tissue specimen in Iranian patients with SCC differ significantly to those in healthy controls that is not in line with Hedayatizadeh study in Mazandaran that did not showed a significant correlation between the genotype frequencies of a P53 polymorphism in Iranian patients with gastric cancer [105]. Eydian et al study revealed no association between P53 codon 72 polymorphism and increased risk of lung cancer in patients and controls but according to results of adenocarcinoma in never-smoker patients, it seems that environmental factors may have more important role than genetic susceptibility in Iranian population [106]. These discrepancies may be related to sample size, nature of the tumor and evaluated specimens that may be affect the results. Azarhoush et al. studied the relation between p53 and colorectal cancer in Northeast of Iran. They found that the P53 gene mutation could be considered as a prognostic factor affecting mortality in cancer and specifically colorectal cancer. Although the P53 protein expression shows no relationship with histopathological features, the mortality rate of the patients demonstrated a strong association [107]. This finding is in ordinance with our result in some manners. In cells with normally functioning p53, attack on DNA leads to G1 arrest followed by repair, senescence or apoptosis. Alteration of p53 protein could result in loss of its vital actions such as regulating proliferation, inhibiting migration and suppression of invasion. In addition, mutated p53 has been shown to promote metastasis [108]. Similar to previous reports we did not observe a significant relationship between p53 expression and recurrence [86]. However other studies, including a systematic review conducted by Gao et al. reported shorter survival rates. This discrepancy may be related to differences in the study population, tumor location, sample size and follow-up periods [109]. It is noteworthy

that similar to these investigations could be explained by the tumor suppressor and cyclin-dependant kinase (CDK) inhibiting activities of this protein [110]. Abdollahi et al. studied P53 in Iranian patients with breast cancer. they found that P53 and HER2 expressions were not t correlated with tumor grade, P53 expression was associated with poorer prognosis due to higher lymph node involvement and perineural invasion [111].

According to our results p53 could be considered as prognostic factors in oral SCC and its possible role in the development of this cancer. Considering the importance of p53 in oncogenesis and the discrepancy between findings of different studies, unification of laboratory techniques, detection methods and antibodies may be beneficial for future meta-analyses in this field. In current research we found a relationship between age and IHC marker that is in contrast with Abdollahi finding in Iranian women [111]. The different population and cancer may be related to this finding. Taken together, these data revealed that P53could act as a positive factor in primary and recurrent SCC. Further studies are being planned to prove the function of P53 on a larger and different communities in order to find the answer about diversity of researches. Current research did not find a relation p53 with staging and grading. It seems that a large amount of cases in different stages of dysplasia is necessary in order to find an accurate correlation between this p53 and oral malignancies. It is worth mentioning that owing to some limitation, we could not enroll more participants (financial, limited documented cases). It can be suggested that investigating patients in different stages of SCC should be included that can be helpful in determining the action. Finally, according to the results of this study, Statistical analysis showed an obvious expression of P53 in SCC cases but none of controls were expressed. This study had many limitation, imbalance between case and control participants count, Low sample size, financial restriction, and a different

ethnicity in Fars province. Further studies on larger sample size are necessary considering these problems.

6 Conclusion

The preliminary analysis of P53 proteins provides clues to elucidate mechanisms of development and progression of head and neck cancer Current research showed strong association between this protein and the disease, further studies on larger population including wide range of markers are necessary for more accurate results. According to our result P53 in partnership with other factors could be suggested as diagnostic marker in SCC. However, our investigation is just an initial step, and further studies are required to determine the potential roles of P53 as candidate protein in pathogenesis of SCC.

7 References

1-Siegel RL, Miller KD, Jamal A. 2016. Cancer statistics, 2016. *CA Cancer J Clan* 66: 7–30

2-Hosseini H, Obradovic MM, Hoffmann M, Harper KL, Sosa MS, Werner-Klein M, Nanduri LK, Werno C, Ehrl C, Maneck M, et al. 2016. Early dissemination seeds metastasis in breast cancer. *Nature* 540: 552–558.

3-Gonzalez H, Robles I, Werb Z. 2018. Innate and acquired immune surveillance in the postdissemination phase of metastasis. *FEBS J* 285: 654–664.

4--Martin TA, Jiang WG. Loss of tight junction barrier function and its role in cancer metastasis. Biochim Biophys Acta. 2009;1788:872–91. http://dx.doi.org/10.1016/j.bbamem.2008.11.005

5- Welch DR, Hurst DR. Defining the Hallmarks of Metastasis. Cancer Res. 2019;79(12):3011-3027. doi: 10.1158/0008-5472.CAN-19-0458

7- Laura QM, Chow MD. Head and Neck Cancer. *New Engl J Med* (2020) 382:60–72. doi: 10.3109/02841869609101661

8-Younis RH, Han KL, Webb TJ. Human Head and Neck Squamous Cell Carcinoma-Associated Semaphorin 4D Induces Expansion of Myeloid-Derived Suppressor Cells. J Immunol (2016) 196(3):1419–29. doi: 10.4049/jimmunol.1501293

9-Butterfield LH. The Society for Immunotherapy of Cancer Biomarkers Task Force recommendations review. Semin Cancer Biol (2017) 52(Pt 2):12–15. doi: 10.1016/j.semcancer.2017.09.006

10- Pai SI, Westra WH. Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. Annu Rev Pathol 2009;4:49-70

11- Joshi P, Dutta S, Chaturvedi P, Nair S. Head and neck cancers in developing countries. Rambam Maimonides Med J. 2014;5(2):e0009. Published 2014 Apr 28. doi:10.5041/RMMJ.10143

12- Angadi PV, Rao SS. Areca nut in pathogenesis of oral submucous fibrosis: revisited. Oral Maxillofac Surg. 2011;15:1–9. doi: 10.1007/s10006-010-0219-8

13-Agarwal AK, Sethi A, Sareen D, Dhingra S. Treatment delay in oral and oropharyngeal cancer in our population: the role of socio-economic factors and health-seeking behaviour. Indian J Otolaryngol Head Neck Surg. 2011;63:145–50. doi: 10.1007/s12070-011-0134-9.

14- Bray F, Ferlay J, Laversanne M, Brewster DH, Gombe Mbalawa C, Kohler B, Piñeros M, Steliarova-Foucher E, Swaminathan R, Antoni S, Soerjomataram I, Forman D. Cancer Incidence in Five Continents: Inclusion criteria, highlights from Volume X and the global status of cancer registration. Int J Cancer. 2015 Nov 1;137(9):2060-71. doi: 10.1002/ijc.29670. PMID: 26135522.

15- Jandoo T, Mehrotra R. Tobacco control in India: present scenario and challenges ahead. Asian Pac J Cancer Prev. 2008;9:805–10.

16- Tseng C H. 2013. “Oral Cancer in Taiwan: Is Diabetes a Risk Factor?” Clinical Oral Investigations 17 (5): 1357–64

17- Gillison ML. Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. Semin Oncol. 2004;31:744–54. doi: 10.1053/j.seminoncol.2004.09.011

18- Kaarthigeyan K. Cervical cancer in India and HPV vaccination. Indian J Med Paediatr Oncol. 2012;33(1):7-12. doi:10.4103/0971-5851.96961

19- National Cancer Control Programme - Home: National Portal of India. Available at www.archive.india.gov.in/sectors/health_family/index.php?id=11. (accessed April 13, 2014)

20- Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147(2):275-292. doi:10.1016/j.cell.2011.09.024

21- Poling JS, Ma X-J, Bui S, et al. Human papillomavirus (HPV) status of non-tobacco related squamous cell carcinomas of the lateral tongue. Oral Oncol 2014;50:306-10.

22-- Martin TA, Jiang WG. Loss of tight junction barrier function and its role in cancer metastasis. Biochim Biophys Acta. 2009 Apr;1788(4):872-91. doi: 10.1016/j.bbamem.2008.11.005. Epub 2008 Nov 14. PMID: 19059202.

23- Hormuth DA 2nd, Phillips CM, Wu C, et al. Biologically-Based Mathematical Modeling of Tumor Vasculature and Angiogenesis via Time-Resolved Imaging Data. Cancers (Basel). 2021;13(12):3008. Published 2021 Jun 16. doi:10.3390/cancers13123008

24- Jain R.K. Normalizing Tumor Microenvironment to Treat Cancer: Bench to Bedside to Biomarkers. J. Clin. Oncol. 2013;31:2205–2218. doi: 10.1200/JCO.2012.46.3653.

25- Ibiebele TI, van der Pols JC, Hughes MC, Marks GC, Williams GM, Green AC. Dietary pattern in association with squamous cell carcinoma of the skin: a

prospective study. Am J Clin Nutr. 2007 May;85(5):1401-8. doi: 10.1093/ajcn/85.5.1401. PMID: 17490979.

26- Grsic K, Opacic IL, Sitic S, Milkovic Perisa M, Suton P, Sarcevic B. The prognostic significance of estrogen receptor β in head and neck squamous cell carcinoma. Oncol Lett. 2016;12(5):3861-3865. doi:10.3892/ol.2016.5142

27- Bray F, Ren J S, Masuyer E, Ferlay J. 2013. "Estimates of Global Cancer Prevalence for 27 Sites in the Adult Population in 2008." International Journal of Cancer 132 (5): 1133–45

28- Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev*. 2018;32(19-20):1267-1284. doi:10.1101/gad.314617.118

29- Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. *Cell* 144: 646–674

30-Nicholson LB. The immune system. *Essays Biochem*. 2016;60(3):275-301. doi:10.1042/EBC20160017

31- Malech HL, DeLeo FR, Quinn MT. The Role of Neutrophils in the Immune System: An Overview. Methods Mol Biol. 2020;2087:3-10. doi: 10.1007/978-1-0716-0154-9_1. PMID: 31728979.

32- David D. Chaplin. Overview of the Immune Response. J Allergy Clin Immunol. Author manuscript; available in PMC 2010 Aug 18.

33- Jing Xu, Jiahui Wang, Xiaoli Wang, Ruoming Tan, Xiaoling Qi, Zhaojun Liu, Hongping Qu, Tingting Pan, Qingyuan Zhan, Yong Zuo, Wen Yang, Jialin Liu. Soluble PD-L1 improved direct ARDS by reducing monocyte-derived macrophages. Cell Death Dis. 2020 Oct; 11(10): 934. Published online 2020 Oct 30. doi: 10.1038/s41419-020-03139-9

34-Nicholson LB. The immune system. *Essays Biochem*. 2016;60(3):275-301. doi:10.1042/EBC20160017

35- Nicholson LB. The immune system. *Essays Biochem*. 2016;60(3):275-301. doi:10.1042/EBC20160017

36-Shao T, Verma HK, Pande B, et al. Physical Activity and Nutritional Influence on Immune Function: An Important Strategy to Improve Immunity and Health Status. *Front Physiol*. 2021;12:751374. Published 2021 Oct 8. doi:10.3389/fphys.2021.751374

37-Navasardyan I, Bonavida B. Regulation of T Cells in Cancer by Nitric Oxide. *Cells*. 2021;10(10):2655. Published 2021 Oct 5. doi:10.3390/cells10102655

38-Mahrosh HS, Salman M, et al. Identification of Peptides as Novel Inhibitors to Target IFN-*γ*, IL-3, and TNF-*α* in Systemic Lupus Erythematosus. *Biomed Res Int*. 2021;2021:1124055. Published 2021 Nov 13. doi:10.1155/2021/1124055

39- Beaugerie L, Svrcek M, Seksik P, Bouvier AM, Simon T, Allez M, Brixi H, Gornet JM, Altwegg R, Beau P, et al. 2013. Risk of colorectal high-grade dysplasia and cancer in a prospective observational cohort of patients with inflammatory bowel disease. *Gastroenterology* 145: 166–175 e168

40-Hussain SP, Amstad P, Raja K, Ambs S, Nagashima M, Bennett WP, Shields PG, Ham AJ, Swenberg JA, Marrogi AJ, et al. 2000. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. *Cancer Res* 60: 3333–3337

41-Samraj AN, Pearce OM, Laubli H, Crittenden AN, Bergfeld AK, Banda K, Gregg CJ, Bingman AE, Secrest P, Diaz SL, et al. 2015. A red meat-derived glycan promotes inflammation and cancer progression. *Proc Natl Acad Sci* 112: 542–547.

42- Askeland EJ, Newton MR, O'Donnell MA, Luo Y. 2012. Bladder cancer immunotherapy: BCG and beyond. *Adv Urol* 2012: 181987

43- Shalapour S, Karin M. 2015. Immunity, inflammation, and cancer: an eternal fight between good and evil. *J Clin Invest* 125: 3347–3355

44- Fridman WH, Pages F, Sautes-Fridman C, Galon J. 2012. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 12: 298–306

45- Palucka AK, Coussens LM. 2016. The basis of oncoimmunology. *Cell* 164: 1233–1247

46- Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V, Rabbe N, Laurans L, Tartour E, de Chaisemartin L, et al. 2008. Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. *J Clin Oncol* 26: 4410–441

47- Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. 2017. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* 14: 399–416

48- Noy R, Pollard JW. 2014. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* 41: 49–61.

49-Kitamura T, Qian BZ, Pollard JW. 2015. Immune cell promotion of metastasis. *Nat Rev Immunol* 15: 73–86.

50- Roh-Johnson M, Bravo-Cordero JJ, Patsialou A, Sharma VP, Guo P, Liu H, Hodgson L, Condeelis J. 2014. Macrophage contact induces RhoA GTPase signaling to trigger tumor cell intravasation. *Oncogene* 33: 4203–4212.

51- Kolaczkowska E, Kubes P. 2013. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 13: 159–175

52-Donskov F. 2013. Immunomonitoring and prognostic relevance of neutrophils in clinical trials. *Semin Cancer Biol* 23: 200–207

53- Speiser DE, Ho PC, Verdeil G. 2016. Regulatory circuits of T cell function in cancer. *Nat Rev Immunol* 16: 599–611.

54- De Silva NS, Klein U. 2015. Dynamics of B cells in germinal centres. *Nat Rev Immunol* 15: 137–148.

55- Pylayeva-Gupta Y, Das S, Handler JS, Hajdu CH, Coffre M, Koralov SB, Bar-Sagi D. 2016. IL35-producing B cells promote the development of pancreatic neoplasia. *Cancer Discov* 6: 247–255

56-Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, Malchinkhuu E, Wersto RP, Biragyn A. 2011. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting $CD4^+$ T cells to T-regulatory cells. *Cancer Res* 71: 3505–3515

57- Toufektchan E, Toledo F (May 2018). "The Guardian of the Genome Revisited: p53 Downregulates Genes Required for Telomere Maintenance, DNA Repair, and Centromere Structure". *Cancers*. 10 (5): 135. doi:10.3390/cancers10050135

58-Bourdon JC, Fernandes K, Murray-Zmijewski F, Liu G, Diot A, Xirodimas DP, Saville MK, Lane DP (September 2005). "p53 isoforms can regulate p53 transcriptional activity". *Genes & Development*. 19 (18): 2122–37

59- Levine AJ, Lane DP, eds. (2010). The p53 family. Cold Spring Harbor Perspectives in Biology. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. ISBN 978-0-87969-830-0.

60-Klug SJ, Ressing M, Koenig J, Abba MC, Agorastos T, Brenna SM, et al. (August 2009). "TP53 codon 72 polymorphism and cervical cancer: a pooled analysis of individual data from 49 studies". The Lancet. Oncology. 10 (8): 772–84

61- Sonoyama T, Sakai A, Mita Y, Yasuda Y, Kawamoto H, Yagi T, Yoshioka M, Mimura T, Nakachi K, Ouchida M, Yamamoto K, Shimizu K (2011). "TP53 codon 72 polymorphism is associated with pancreatic cancer risk in males, smokers and drinkers". Molecular Medicine Reports. 4 (3): 489–95.

62- Piao JM, Kim HN, Song HR, Kweon SS, Choi JS, Yun WJ, Kim YC, Oh IJ, Kim KS, Shin MH (September 2011). "p53 codon 72 polymorphism and the risk of lung cancer in a Korean population". Lung Cancer. 73 (3): 264–7.

63-Alawadi S, Ghabreau L, Alsaleh M, Abdulaziz Z, Rafeek M, Akil N, Alkhalaf M (September 2011). "P53 gene polymorphisms and breast cancer risk in Arab women". Medical Oncology. 28 (3): 709–15

64- Mantovani F, Walerych D, Sal GD. Targeting mutant p53 in cancer: a long road to precision therapy. *FEBS J.* 2017;284:837–50. doi: 10.1111/febs.13948

65- Kim MP, Lozano G. Mutant p53 partners in crime. *Cell Death Differ.* 2018;25:161–8. doi: 10.1038/cdd.2017.185

66- Mantovani F, Collavin L, Del Sal G. Mutant p53 as a guardian of the cancer cell. *Cell Death Differ*. 2019;26(2):199-212. doi:10.1038/s41418-018-0246-9

67-Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational landscape and significance across 12 major cancer types. *Nature.* 2013;502:333–9. doi: 10.1038/nature12634.

68- Suh YA, Post SM, Elizondo-Fraire AC, Maccio DR, Jackson JG, El-Naggar AK, et al. Multiple stress signals activate mutant p53 in vivo. *Cancer Res.* 2011;71:7168–75. doi: 10.1158/0008-5472.CAN-11-0459

69-Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. *EMBO Rep.* 2014;15:1243–53. doi: 10.15252/embr.201439246.

70- Marur S, Forastiere AA. Head and Neck Squamous Cell Carcinoma: Update on Epidemiology, Diagnosis, and Treatment. *Mayo Clinic Proc* (2016) 91:386–96. doi: 10.1016/j.mayocp.2015.12.017

71- Mullen PJ, Yu R, Longo J, Archer MC, Penn LZ. The interplay between cell signalling and the mevalonate pathway in cancer. *Nat Rev Cancer.* 2016;16:718–31. doi: 10.1038/nrc.2016.76

72- Zhou G, Liu Z, Myers JN. TP53 Mutations in Head and Neck Squamous Cell Carcinoma and Their Impact on Disease Progression and Treatment Response. *J Cell Biochem* (2016) 117:2682–92. doi: 10.1002/jcb.25592. TP53

73- de Bakker, Tycho et al. “Restoring p53 Function in Head and Neck Squamous Cell Carcinoma to Improve Treatments.” *Frontiers in oncology* vol. 11 799993. 6 Jan. 2022, doi:10.3389/fonc.2021.799993

74-Chin D, Boyle GM, Theile DR, Parsons PG, Coman WB. Molecular Introduction to Head and Neck Cancer (HNSCC) Carcinogenesis. *Br J Plast Surg* (2004) 57:595–602. doi: 10.1016/j.bjps.2004.06.010

75-Zhou G, Liu Z, Myers JN (2016) TP53 mutations in head and neck squamous cell carcinoma and their impact on disease progression and treatment response. J Cell Biochem 117: 2682-2692.

76-Kenzelmann Broz D, Attardi LD (2010) In vivo analysis of p53 tumor suppressor function using genetically engineered mouse models. Carcinogenesis 8: 1311-1318.

77- The Cancer Genome Atlas Network. Comprehensive Genomic Characterization of Head and Neck Squamous Cell Carcinomas. *Nature* (2015) 517:576–82. doi: 10.1038/nature14129

78-Caponio VCA, Troiano G, Adipietro I, Zhurakivska K, Arena C, Mangieri D, et al.. Computational Analysis of TP53 Mutational Landscape Unveils Key Prognostic Signatures and Distinct Pathobiological Pathways in Head and Neck Squamous Cell Cancer. *Br J Cancer* (2020) 123:1302–14. doi: 10.1038/s41416-020-0984-6

79-Nguyen N, Bellile E, Thomas D, McHugh J, Rozek L, et al. (2016) Tumor infiltrating lymphocytes and survival in patients with head and neck squamous carcinoma. Head and Neck 38: 1074-1084.

80-Wolf GT, Chepeha DB, Bellile E, Nguyen A, Thomas D, et al. (2015) The University of Michigan Head and Neck SPORE Program. Tumor infiltrating lymphocytes (TIL) and prognosis in oral cavity squamous carcinoma: A preliminary study. Oral Oncol 51: 90-95.

81-Couch ME, Ferris RL, Brennan JA, Koch WM, Jaffee EM, et al. (2007) Alteration of cellular and humoral immunity by mutant p53 protein and processed mutant peptide in head and neck cancer. Clin Cancer Res 13: 7199-7206.

82- Khadang B, Fattahi MJ, Talei A, Dehaghani AS, Ghaderi A. Polymorphism of TP53 codon 72 showed no association with breast cancer in Iranian women. Cancer Genet Cytogenet. 2007 Feb;173(1):38-42. doi: 10.1016/j.cancergencyto.2006.09.010. PMID: 17284368

83- Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, Westra W, Sidransky D, Koch WM. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007 Dec 20;357(25):2552-61. doi: 10.1056/NEJMoa073770. PMID: 18094376; PMCID: PMC2263014.

84- Ghabanchi J, Fattahi MJ, Mardani M, Tadbir AA, Paydar AA. Polymorphism of tumor protein p53 codon 72 showed no association with oral lichen planus in Shiraz, Iran. J Craniofac Surg. 2009 Nov;20(6):2168-70. doi: 10.1097/SCS.0b013e3181bf015e. PMID: 19884837.

85- Peltonen JK, Vähäkangas KH, Helppi HM, Bloigu R, Pääkkö P, Turpeenniemi-Hujanen T. Specific TP53 mutations predict aggressive phenotype in head and neck squamous cell carcinoma: a retrospective archival study. Head Neck Oncol. 2011 Apr 22;3:20. doi: 10.1186/1758-3284-3-20. PMID: 21513535; PMCID: PMC3094329

86- Etemad-Moghadam S, Keyhani A, Yazdani K, Alaeddini M. Status of p53 and p27(KIP1) in Iranian Patients With Oral Squamous Cell Carcinoma. Iran Red Crescent Med J. 2015 Oct 19;17(10):e19359. doi: 10.5812/ircmj.19359. PMID: 26568852; PMCID: PMC4640065

87- Zhou, Ge. “TP53 Mutations in Head and Neck Squamous Cell Carcinoma and Their Impact on Disease Progression and Treatment Response.” *Journal of cellular biochemistry* vol. 117,12 (2016): 2682-2692. doi:10.1002/jcb.25592

88- Kumari S, Sharma V, Tiwari R, Maurya JP, Subudhi BB, Senapati D. Therapeutic potential of p53 reactivation in prostate cancer: Strategies and opportunities. Eur J Pharmacol. 2022 Mar 15;919:174807. doi: 10.1016/j.ejphar.2022.174807. Epub 2022 Feb 10. PMID: 35151649.

89- Melling N, Norrenbrock S, Kluth M, Simon R, Hube-Magg C, Steurer S, Hinsch A, Burandt E, Jacobsen F, Wilczak W, Quaas A, Bockhorn M, Grupp K, Tachezy M, Izbicki J, Sauter G, Gebauer F. p53 overexpression is a prognosticator of poor outcome in esophageal cancer. Oncol Lett. 2019 Apr;17(4):3826-3834. doi: 10.3892/ol.2019.10020. Epub 2019 Feb 6. PMID: 30881503; PMCID: PMC6403495

90- Ziaran S, Harsanyi S, Bevizova K, Varchulova Novakova Z, Trebaticky B, Bujdak P, Galbavy S, Danisovic L. Expression of E-cadherin, Ki-67, and p53 in urinary bladder cancer in relation to progression, survival, and recurrence. Eur J Histochem. 2020 Mar 26;64(2):3098. doi: 10.4081/ejh.2020.3098. PMID: 32214283; PMCID: PMC7118433

91- Farnoosh G, Saeedi-Boroujeni A, Jalali A, Keikhaei B, Mahmoudian-Sani MR. Polymorphisms in genes involved in breast cancer among Iranian patients. Per Med. 2021 Mar;18(2):153-169. doi: 10.2217/pme-2020-0003. Epub 2021 Feb 10. PMID: 33565318.

92- Jamshidi M, Farnoosh G, Mohammadi Pour S, Rafiee F, Saeedi Boroujeni A, Mahmoudian-Sani MR. Genetic variants and risk of thyroid cancer among Iranian patients. Horm Mol Biol Clin Investig. 2021 Feb 8;42(2):223-234. doi: 10.1515/hmbci-2020-0051. PMID: 33544997.

93- Pinholt EM, Rindum J, Pindborg JJ. Oral cancer: a retrospective study of 100 Danish cases. *Br J Oral Maxillofac Surg.* 1997;35(2):77–80

94- Bryne M, Nielsen K, Koppang HS, Dabelsteen E. Reproducibility of two malignancy grading systems with reportedly prognostic value for oral cancer patients. *J Oral Pathol Med.* 1991;20(8):369–72

95- Kuo MY, Hsu HY, Kok SH, Kuo RC, Yang H, Hahn LJ, et al. Prognostic role of p27(Kip1) expression in oral squamous cell carcinoma in Taiwan. *Oral Oncol.* 2002;38(2):172–8.

96- Casalini P, Iorio MV, Berno V, Bergamaschi A, Borresen Dale AL, Gasparini P, et al. Relationship between p53 and p27 expression following HER2 signaling. *Breast.* 2007;16(6):597–605. doi: 10.1016/j.breast.2007.05.007

97- Perisanidis C, Perisanidis B, Wrba F, Brandstetter A, El Gazzar S, Papadogeorgakis N, et al. Evaluation of immunohistochemical expression of p53, p21, p27, cyclin D1, and Ki67 in oral and oropharyngeal squamous cell carcinoma. *J Oral Pathol Med.* 2012;41(1):40–6. doi: 10.1111/j.1600-0714.2011.01071.x. [PubMed] [CrossRef] [Google Scholar]

98- Geisler SA, Olshan AF, Weissler MC, Cai J, Funkhouser WK, Smith J, et al. p16 and p53 Protein expression as prognostic indicators of survival and disease recurrence from head and neck cancer. *Clin Cancer Res.* 2002;8(11):3445–53

99-Kato K, Kawashiri S, Yoshizawa K, Kitahara H, Okamune A, Sugiura S, et al. Expression form of p53 and PCNA at the invasive front in oral squamous cell carcinoma: correlation with clinicopathological features and prognosis. *J Oral Pathol Med.* 2011;40(9):693–8. doi: 10.1111/j.1600-0714.2011.01032.x

100- Sauter ER, Ridge JA, Gordon J, Eisenberg BL. p53 overexpression correlates with increased survival in patients with squamous carcinoma of the tongue base. *Am J Surg.* 1992;164(6):651–3

101- Mineta H, Borg A, Dictor M, Wahlberg P, Akervall J, Wennerberg J. p53 mutation, but not p53 overexpression, correlates with survival in head and neck squamous cell carcinoma. *Br J Cancer.* 1998;78(8):1084–90

102- Hasegawa M, Ohoka I, Yamazaki K, Hanami K, Sugano I, Nagao T, et al. Expression of p21/WAF-1, status of apoptosis and p53 mutation in esophageal squamous cell carcinoma with HPV infection. *Pathol Int.* 2002;52(7):442–50.

103- Skirnisdottir IA, Sorbe B, Lindborg K, Seidal T. Prognostic impact of p53, p27, and C-MYC on clinicopathological features and outcome in early-stage (FIGO I-II) epithelial ovarian cancer. *Int J Gynecol Cancer.* 2011;21(2):236–44. doi: 10.1097/IGC.0b013e31820986e5

104- Pim D, Banks L. p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. *Int J Cancer.* 2004;108(2):196–9. doi: 10.1002/ijc.11548

105- Hedayatizadeh-Omran, Akbar et al. "Association of P53 gene polymorphism with gastric cancer in Northern Iran as a high-risk region." *Biomedical reports* vol. 8,5 (2018): 433-438. doi:10.3892/br.2018.1070

106- Eydian Z, Asna'ashari AM, Behravan J, Sharifi-Rad J, Entezari Heravi R. Association of P53 codon 72 polymorphism and lung cancer in an ethnic Iranian population. Cell Mol Biol (Noisy-le-grand). 2016 Aug 29;62(9):34-8. PMID: 27585259

107-Azarhoush Ramin, Heidari Khatoun, Samadzadeh Soheila, Heidari Ahmad, Mehravar Fatemeh*Expression Of P53 Protein In Colorectal Cancer And Association With Prognostic Factors In Northeast Iran.

ACTA MEDICA IRANICA 2018 , Volume 56 , Number 3; Page(s) 161 To 165.

108- Aylon Y, Oren M. New plays in the p53 theater. *Curr Opin Genet Dev.* 2011;21(1):86–92. doi: 10.1016/j.gde.2010.10.002

109- Gao L, Gu W, Zheng J, Ren W, Chang S, Wang X, et al. Clinicopathological and prognostic significance of p27 expression in oral squamous cell carcinoma: a meta-analysis. *Int J Biol Markers.* 2013;28(4):e329–35. doi: 10.5301/jbm.5000035,
110- Zhang M, Li J, Wang L, Tian Z, Zhang P, Xu Q, et al. Prognostic significance of p21, p27 and survivin protein expression in patients with oral squamous cell carcinoma. *Oncol Lett.* 2013;6(2):381–6. doi: 10.3892/ol.2013.1381
111- Abdollahi, A., Sheikhbahaei, S., Safinejad, S., Jahanzad, I. Correlation of ER, PR, HER- 2 and P53 Immunoreactions with Clinico-Pathological Features in Breast Cancer. *Iranian Journal of Pathology*, 2013; 8(3): 147-152.