

Tumor Microenvironment in Head and Neck Squamous Cell Carcinomas

Review

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Abstract

Recent studies about solid tumors demonstrated that tumor microenvironment has an important role in tumor progression, aggressivity, and metastasis process, in addition to genetic aberrations and molecular alterations of cancer cells. Therefore, the crosstalks between cancerous and noncancerous cells and metabolic changes in tumor microenvironment cause significant detrimental

effects. The purpose of this review is to present the role and effect of noncancerous cells and their crosstalks with cancer cells, metabolic changes in tumor microenvironment, and to discuss the clinical significance of all these factors with respect to the current literature.

Keywords: Carcinoma, squamous cell of head and neck, head and neck neoplasms, tumor microenvironment

*“People should know the strategy: those who learn the strategy live and those who do not are doomed to perish!”
Sun Tzu, The Art of War.*

Introduction

Head and neck cancers define epithelial malignancies of the oral cavity, pharynx, larynx, paranasal sinuses, and nasal cavity. Head and neck cancers comprise 6% of all cancers and are in sixth place in order of frequency among all cancers. It is estimated that approximately 650,000 new cases develop worldwide each year and that cancer-related death occurs in approximately 350,000 cases (1). The most common histopathological type in head and neck cancers is squamous cell carcinoma, and besides genetic alterations, its known major risk factors among environmental factors are tobacco/cigarette consumption, alcohol consumption, and exposure to chemical and viral agents (2). In studies, particularly those conducted in recent years, the relationship between oral cavity and oropharynx squamous cell carcinomas and the human papilloma virus (human papilloma virus 16 and 18) has been clearly demonstrated (3).

It is known that the inactivation of tumor suppressor genes and/or the many genetic mutations and epigenetic changes with the activation of proto-oncogenes play a role in the development of head and neck squamous cell carcinomas. Telomerase reactivation has been revealed in approximately 90% of premalignant lesions developing in this region; this enzyme plays an important role in the continuity of telomeres and in the cell gaining immortality, specifically in deoxyribonucleic acid (DNA) (After a normal cell divides a maximum of 50–80 times, according to the Hayflick limit, it sustains apoptosis. However, cancer cells do not have this limit; therefore, they are defined as “im-

mortal.”) (4). The loss of 9p21 is among the most common (70–80%) genetic change in head and neck squamous cell carcinomas (5). Along with these, the inactivation of p16 and loss of 3p due to deletions, point mutations, or promoter hypermethylation play an important role in carcinogenesis (6). In 50% of head and neck cancers, a loss in 17p heterozygosity and TP53 point mutations can also be seen (7). In addition, the overexpression of Cyclin D1 was found to be closely associated with aggressive tumor characteristics (8).

In cancer, there are significant steps that cancer cells should surpass in the process, starting from carcinogenesis and continuing to distant metastases (Figure 1). They are as follows:

- (i) Local tumor proliferation and infiltration,
- (ii) Tumor cells' reaching the lymphatic/circulatory system (intravasation),
- (iii) Tumor cells' getting out of the circulation in the metastatic distant organ and reaching the tissue (extravasation), and
- (iv) Proliferation and progression in the organ with developing metastasis.

To successfully complete the above-mentioned challenging process, cancer cells need to gain some special abilities (9):

1. To show resistance to cell death (apoptosis),
2. To multiply many times while having immortality,
3. To hide from the devastating/destroying effects of the immune system,
4. To use cells in the immune system in their own favor,



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Received Date: 19.03.2015
Accepted Date: 23.04.2015

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 DOI: 10.5152/tao.2015.1065

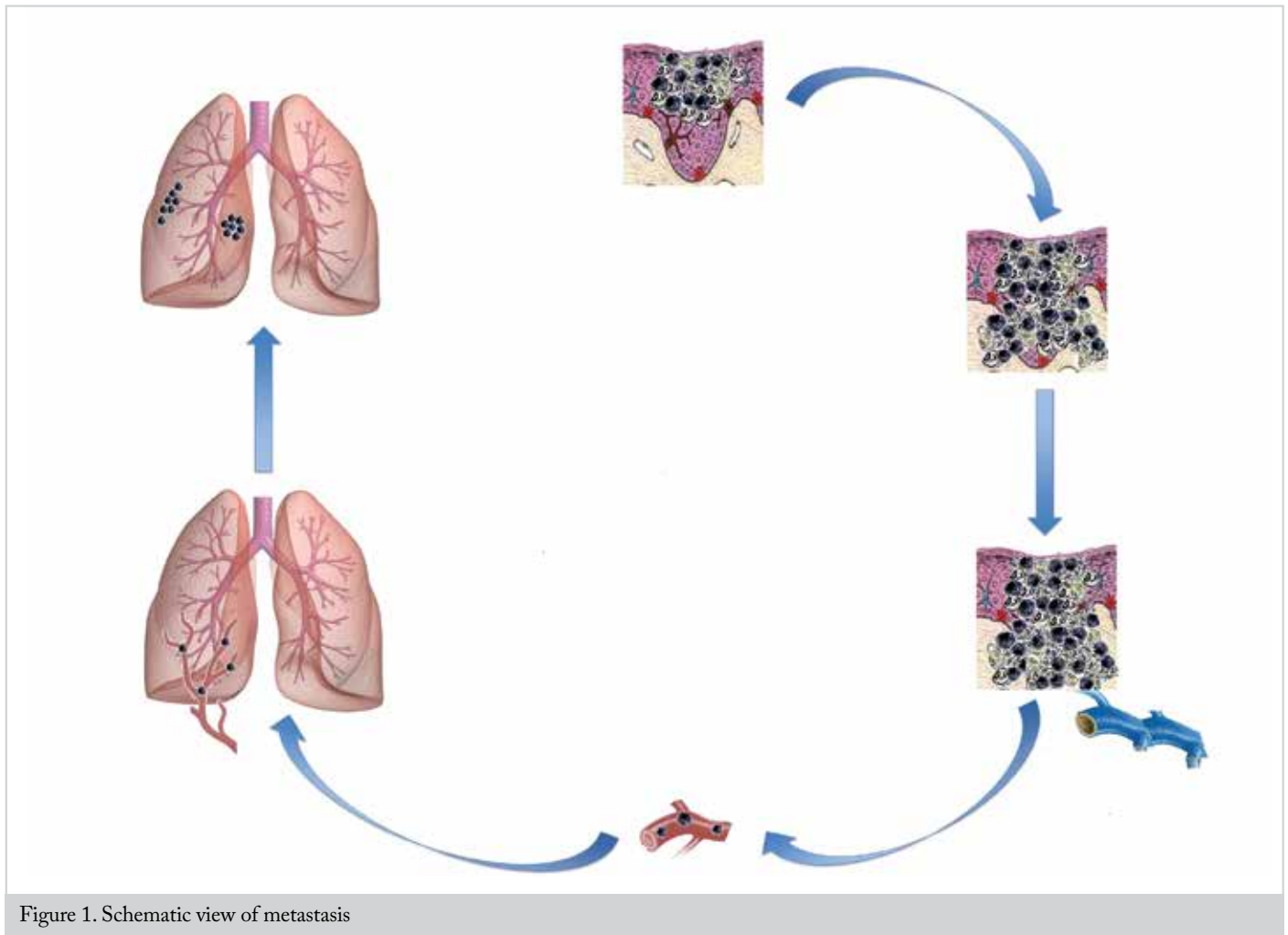


Figure 1. Schematic view of metastasis

5. To lose their sensitivity against growth suppressive cytokine/metabolites,
6. To be able to make changes in the intracellular energy system and metabolism,
7. To cause unstable genome mutation,
8. To gain the ability of epithelial–mesenchymal transition,
9. To gain the ability to invade tissues and to use surrounding cells (inflammatory cells, fibroblasts, etc.) in their favor in order to achieve this,
10. To increase the tumor–provoking inflammation,
11. To trigger/increase lymphangiogenesis and/or angiogenesis,
12. To make hematogenic and/or lymphatic metastasis,
13. To gain the ability of mesenchymal–epithelial transition, and
14. To be colonized by multiplying in remote organs.

The impact of the communication between cancer cells and cells located in the tumor microenvironment in cancer progression was emphasized in The 5th International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention, 2009 (10). In the article where basic formation mechanisms of cancer were presented, Hanahan and Weinberg (11) indicated the role

of the microenvironment in the development of abilities/attitudes or in the increase of their impact that cancer cells need for carcinogenesis, tumor growth, or metastasis.

The main objective of this review is to present the role and importance of the tumor microenvironment in carcinogenesis and cancer cell biology, tumor progression, metastasis process, treatment resistance, and prognosis in head and neck squamous cell carcinomas within the framework of current research and evidence.

An overview of the tumor microenvironment

The tumor microenvironment is an interactive, organized, and dynamic environment where cancer cells as well as many different cellular and biochemical structures exist together and are continuously in contact and interact with each another (12). The main structures in this area are the parenchyma, stroma, growth factors, lymphokines and cytokines, and inflammatory and matrix metalloproteinase enzymes (Figure 2). While cancer cells and cancer stem cells are located in the parenchyma, nonmalignant cells (inflammatory cells, cancer-associated fibroblasts, angiogenic vascular cells, and sometimes adipocytes) and extracellular matrix constitute the stroma. Inflammatory cells play an important role in providing mitogenic growth factors for

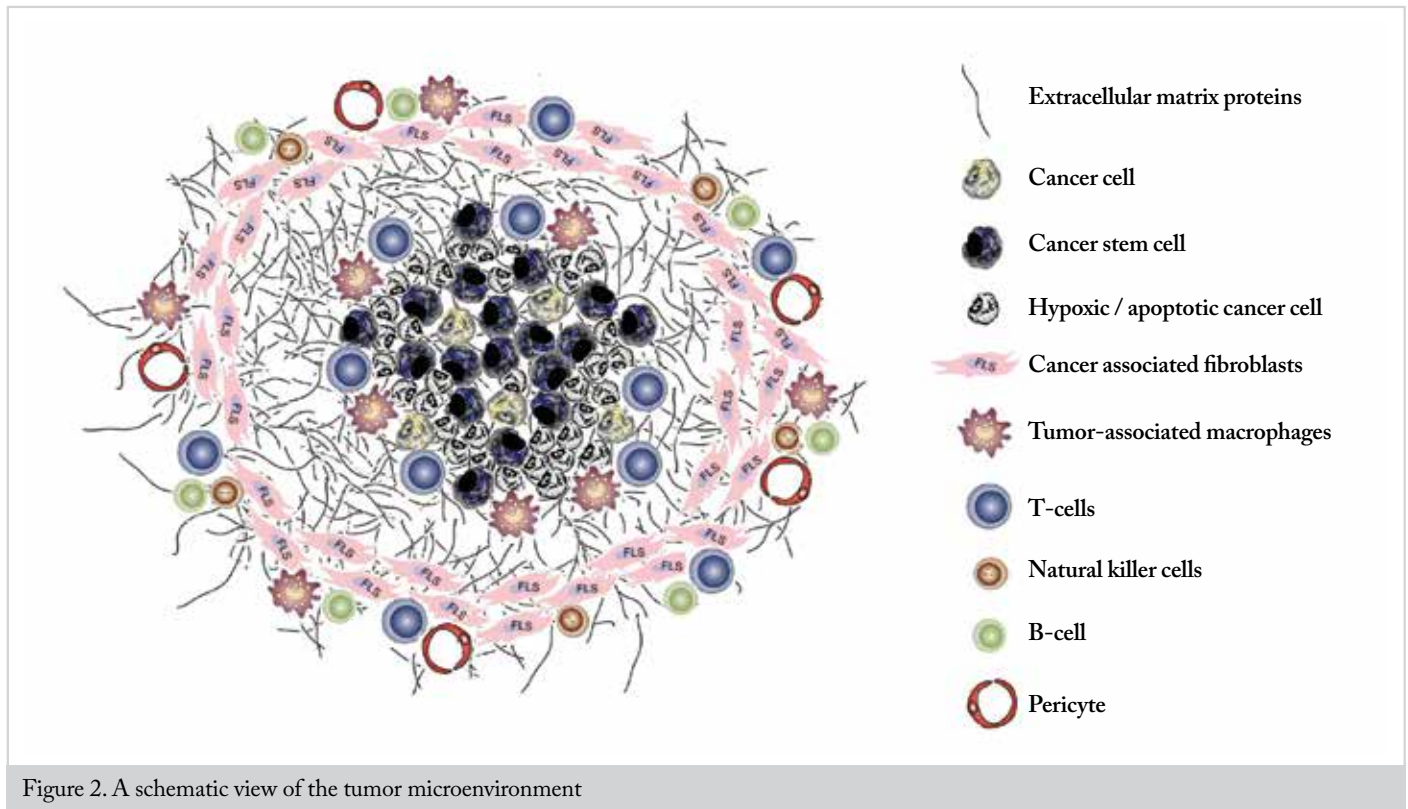


Figure 2. A schematic view of the tumor microenvironment

cancer cells, triggering angiogenesis and in cancer cells' showing resistance to cell death, escaping from the immune system, and gaining the ability of invasion/metastasis (13). Cancer-associated fibroblasts are also effective in reorganizing the cellular metabolism. The extracellular matrix is a dynamic structure with the role of a 3-dimensional pattern where all cells exist, and it contains many different proteins, glycoproteins, proteoglycans, and polysaccharides with different biochemical properties within the structure; mainly, these are collagen, fibrin, fibronectin, proteoglycans, and hyaluronan (14).

The effect of inflammatory cells around the microenvironment of head and neck squamous cell carcinoma tumors

The first hypothesis about the relationship between cancer development and inflammation was put forward by Virchow (15). Cancer cells safeguard their life and growth with chronic inflammation that they create in the microenvironment of the cancer cells. Though it is considered that inflammation creates a beneficial effect in the removal of pathological structures and in the destruction of cancer cells, inflammatory response that is unorganized and managed by the cancer cells creates an opportunity for the development of the tumor and for the cancer cells to evade the devastating effects of the immune system. Research conducted in head and neck cancers have clearly revealed that local, regional, and systemic inflammatory responses are dysfunctional (16, 17).

The major inflammatory cells that play a role in the tumor microenvironment are T lymphocytes, natural killer cells, and tumor-associated macrophages. The distribution of these cells in

the solid tumor microenvironment shows heterogeneity, while granulocytes, mast cells, and macrophages are located around tumors, and natural killer cells are located in the stroma and T lymphocytes are located in the microenvironment margins and lymph nodes (18). T lymphocytes are divided into two groups: cytotoxic CD8+ T lymphocytes and CD4+ T lymphocytes. CD4+ T lymphocytes are divided into two groups: T helper cells exhibiting different behaviors and characteristics and T regulatory cells. Cytotoxic CD8+ T lymphocytes play a major role in the antitumoral response and an active role in the destruction of tumor cells being recognized, and therefore their presence in the tumor microenvironment often indicates a favorable prognosis (19). In addition, T helper cells play a role in helping CD8+ T lymphocytes and in enhancing the antitumor effect. However, T regulatory cells are also effective in tumor progression because of their immunosuppressive effects and because they inhibit the functions of cytotoxic CD8+ T lymphocytes. As in many patients with solid tumors, functional disorders in circulating and tumor-infiltrating T lymphocytes have also been seen in patients with head and neck cancers (18, 20). Also, when blood samples taken from healthy individuals and patients with head and neck cancer were compared, the number of T regulatory cells was found to be higher by a significant ratio (21, 22). In a study in which they examined the number of T regulatory cells in tongue cancer, Hanakaw et al. (23) concluded that a high number of T regulatory cells are poor prognostic factors.

A high proportion of cytotoxic CD8+ T lymphocytes/T regulatory cells was reported to be a good prognostic factor in many solid tumor microenvironments, such as hepatocellular, breast,

and lung cancer (24). In the study they conducted in the tumor specimens of 87 oral cavity cancer patients, Watanabe et al. (25) found low stromal CD8+ T-lymphocyte counts and a high number of stromal T regulatory cells to be associated with low survival, but they determined that the total number of T regulatory cells did not have an impact. Besides, it was revealed that the worst prognosis was in the group where the ratio of "cytotoxic CD8+ T lymphocyte/Regulatory" cells was low, and it was emphasized that this proportion alone was an independent prognostic factor. However, studies conducted in oropharynx cancer revealed different results from the other head and neck cancers. Although it was determined that high value of cytotoxic CD8+ T lymphocytes is a positive prognostic factor in oropharyngeal cancer, the effect of the high value of T regulatory cells is controversial (26).

Tumor-associated macrophages (TAMs) play an important role in the extracellular matrix destruction and in restructuring of the tumor microenvironment, tumor cell motility, and triggering the angiogenesis. These cells are divided into two: proinflammatory (M1) and suppressive (M2) TAM. M1 TAMs demonstrate an antitumor effect thanks to the proinflammatory cytokines (IL-12, -23, interferon, etc.) they produce. However, M2 TAMs suppress the antitumoral effect of M1 TAMs, trigger angiogenesis, and lead to tumor progression with the suppressive cytokines (IL-10, tumor growth factor-beta, etc.) that they produce. Tumor-associated macrophages cluster particularly in solid tumors with a hypoxic and necrotic environment and, therefore, they are often detected in head and neck cancers. In a meta-analysis on this subject, considerable evidence was presented on the grounds that a high level of TAM in head and neck cancers causes the overall survival to be low (27).

Natural killer cells are involved in the detection and destruction of cancer cells. However, even if they are settled in the stromal tumor microenvironment, they are rarely in contact with cancer cells. They indicate good prognosis in many solid tumors, such as colorectal, lung, renal, and gastric cancer (12). However, it was demonstrated that a significant reduction in the number of natural killer cells occurred in the cases of head and neck cancer (28).

The effect of cancer-associated fibroblasts in the tumor microenvironment of head and neck squamous cell carcinoma

Normal fibroblasts in a healthy person play an important role in the formation of the architecture of the tissue that they are in by producing the important components (type IV collagen, laminin, cytokines, etc.) of the extracellular matrix. They are commonly located in the stroma along the respiratory system. Fibroblasts activated with the paracrine signaling that occurs with tissue damage change into the myofibroblasts and, thus, play a role in wound contraction and in regulating the fibrous and inflammatory response. They express α -smooth muscle actin and produce extracellular matrix components for the realization of these processes. In addition, fibroblasts proliferating for tissue repair in a cellular way sustain apoptosis with the completion of wound healing.

Cancer is characteristically defined as a "non-healing wound," and this process continues especially via cancer-associated fibroblasts (CAFs). CAFs located in tumor stromal microenvironment play a critical role; they are particularly effective in carcinogenesis, tumor progression, and metastasis (29, 30). In the CAF activity study, which they conducted with the samples taken from the head and neck cancer tumor stroma, Rosenthal et al. (31) revealed that CAFs play an important role in the production of many protease enzymes (membrane type 1-matrix metalloproteinase (MMP), MMP1, MMP2, MMP3, MMP9, urokinase, etc.) that help the tumor gain an invasive character and that are effective in remodeling of the extracellular matrix. Cancer-associated fibroblasts may be formed as a result of genetic changes that occur in normal fibroblasts, and in the exposure of epithelial cells to epithelial-mesenchymal transition and endothelial cells to endothelial-mesenchymal change, or may directly arise from mesenchymal stem cells (32). Undergoing myofibroblastic changes, these cells have a phenotype characterized by dense ultrastructural α -smooth muscle actin deposition. In addition, integrin α 6 overexpression, which is important in cell adhesion and surface signaling, is also determined (17).

Cancer-associated fibroblasts constitute 50–70% of the volume of many solid epithelial tumors, such as pancreas, stomach, and breast cancers (33). CAFs play an important role in the development of the desmoplastic response, which is a characteristic of head and neck cancers (dense collagen deposition and the stromal desmoplasia). Kawashir et al. (34) demonstrated that desmoplasia was apparent in aggressive tumors and the myofibroblasts proliferation in tumor stroma was helpful in tumor invasion and metastasis in a clinicopathological study on oral cavity cancers. In addition, they reported that the survival rate due to disease in the group of α -smooth muscle actin positive was statistically lower.

Hypoxia in the tumor microenvironment

Multiple metabolic changes develop depending on the interaction between cancer cells in the parenchyma and the stromal cells in the tumor microenvironment (Table 1). Hypoxia is the most important among these changes (35). Hypoxia ($pO_2 < 10$ – 15 mmHg) describes the deficiency in oxygen levels occurring due to the imbalance between the oxygen levels that are needed and that are provided for this tissue. This imbalance in oxygen levels may fundamentally occur from vascular or pulmonary diseases, of which there are three types (35–37):

- (i) Chronic hypoxia (diffusion-limited hypoxia): Low oxygen pressure (about 2–3%) due to long-term low oxygen diffusion. Although normal, healthy cells cannot show resistance to chronic hypoxia, cancer cells may continue to live in this environment with metabolic changes and the adaptation abilities they have.
- (ii) Acute hypoxia (perfusion-limited hypoxia): Reduction of oxygen pressure due to a sudden developing temporary/permanent reduction of the blood flow.
- (iii) Hypoxia with reperfusion: Development of reoxygenation along with the provision of the flow after acute

hypoxia. However, even if a physiological oxygen level is provided in this case, “reoxygenation injury” often develops due to the occurrence of free radical oxygen species. Free oxygen radicals occurring along with the reperfusion that develops after hypoxia in the tumor microenvironment may lead to chromosomal break, gene amplifications, and uncontrolled DNA replications.

Hypoxia in the head and neck squamous cell carcinoma tumor microenvironment: mechanisms, effects, diagnostic methods and clinical significance

When compared with the healthy tissues around many solid tumor microenvironments, such as breast, brain, cervix, prostate, rectum, and head and neck cancers, a significant level of hypoxia has been demonstrated (38). Heterogeneously distributed hypoxic areas at different levels were found in the microenvironment of 50–60% of locally advanced tumors (35). The basic mechanisms that play a role in hypoxia developing in the microenvironment of solid tumors are as follows:

- (i) Diffusion-limited hypoxia developing because of the distance (>70 microns) of cancer cells to micro-vessels.
- (ii) Perfusion-limited hypoxia developing as a result of functional and structural disorders (amorphous and chaotic vasculature system) in tumor micro-vascularization.
- (iii) The general condition of the patient, cancer-induced cachexia, and insufficiency in tissue oxygenation due to anemia occurring as a result of the treatment.

It was demonstrated that hypoxia in the tumor microenvironment causes genetic instability in cancer cells and a deterioration in gene expression, which play an important role in the tumor’s developing an invasion capability and gaining an aggressive character, radio-/chemotherapy treatment resistance, and metastasis development; all of which are negative prognostic factors (39–41). Therefore, detection of the presence and level of hypoxia in the tumor microenvironment is becoming increasingly important. Unfortunately, a “gold standard” technique is currently not available to determine the level of hypoxia in the tumor microenvironment. Currently, the main techniques used are those presented below (36, 42, 43):

- (i) Electrophysiological measurement techniques: This is performed directly and invasively in the tumor microenvironment with polarographic pO₂ electrodes. The ability to make direct measurements in the tumor microenvironment through this technique is its most important advantage. However, the heterogeneity of hypoxia in the tumor microenvironment and the difficulty of access to some areas are significant limitations.
- (ii) Exogenous hypoxia markers: This is an indirect and invasive technique. Hypoxic cells in the tumor microenvironment are marked by the intravenous administration of nitroaromatic markers (pimo-, miso-, eta-nidazol) that are selectively connected to hypoxic cells 24–48 hours prior to the biopsy.

- (iii) Radiological examinations: Direct and noninvasive dynamic contrast-enhanced magnetic resonance imaging is done with blood and tissue oxygen level specific magnetic resonance imaging techniques. Experimental and clinical studies on especially dynamic contrast-enhanced magnetic resonance imaging are widely available among these techniques. Dynamic contrast-enhanced magnetic resonance imaging can display the blood flow in the tumor microenvironment, vascular volume, and the permeability of blood vessels with high resolution.
- (iv) Nuclear medicine examinations: This is performed directly and noninvasively with positron emission tomography-based imaging methods, particularly together with radiotracers (18F-MISO, 18F-PHASE: 18F-HX4, 18F-EF5, etc.) specific to hypoxia. The most commonly used radiotracer for determining hypoxia is 18F-miso. Although promising results related to hypoxia measurement have been achieved in studies conducted using these radiotracers, the number of cases in the study is limited, and because they are not commonly found, the radiotracers could not enter routine clinical use.
- (v) Assessment of endogenous hypoxia associated markers: This is the indirect detection of hypoxia associated factors [hypoxia-inducible factor (HIF)-1, -2, -3, carbonic anhydrase-9 (CA-9), osteopontin (OPN), etc.] in tumor tissue. These biomarkers increase the correlations with the hypoxia in the tumor microenvironment. Thus, information may be obtained about the hypoxia level of the tumor by examining these biomarkers, and it is possible to then comment on the radio/chemotherapy resistance and prognosis. However, the most important limitation of these techniques is that different results can be obtained depending on the area of biopsy, due to heterogeneity of the tumor microenvironment. However, the potential increase in biomarkers due to causes other than hypoxia can also lead to detection failures of the hypoxia level.

Many signaling networks are activated with the development of hypoxia in the tumor microenvironment; mainly, these are HIFs, OPN, and CA-9. HIFs play the role of a “hypoxia sensor” in the tumor microenvironment. These proteins are transcriptional complexes and consist of two heterodimers: HIF- α and HIF- β (44, 45). Today, they are classified into three types of HIFs: HIF-1, -2, and -3. Among these, HIF-1 is the one on which most research and clinical trials have focused. Cells are continuously synthesizing HIF-1 α in normoxic environments, but they have a short duration of half-lives because they rapidly become hydroxylated depending on the reaction of oxygen with the aid of specific hydroxylase enzymes. The hydroxylated HIF-1 α protein becomes a substrate for the von Hippel-Lindau protein targeting this protein and is destroyed after the ubiquitination process. However, along with the reduction of the oxygen concentration in the environment (hypoxia/anoxia), HIF-1 α does not get hydroxylated and its concentration in the environ-

Table 1. Metabolic changes developing in the microenvironment of solid tumors

Increase in chaotic and amorphous vascular embodiment
Increase in perfusion failure
Increase in perfusion heterogeneity
Increase in the permeability of tumor microvascularization
Increase in interstitial fluid pressure
Increase in lactate production
Decrease in food provision
Decrease in partial oxygen pressure
Decrease in the high energy structure production
Decrease in extracellular matrix pH

ment increases because it is not destroyed. HIFs transcriptionally regulate the function of many genes and these genes play an important role in many stages of cancer development and progression, such as growth, development, apoptosis, epithelial-mesenchymal transition, invasion ability, angiogenic signaling, and the regulation of energy metabolism (Table 2) (44-46). In the study where they researched the levels of HIF-1 and -2 in head and neck cancer cell lines and tumor tissues, Beasley et al. (47) suggested that a significant increase occurred in both biomarker expressions.

OPN is a multifunctional, negatively charged, acidic protein. Although expressed at high levels, particularly in the bone, it can also be synthesized by many different cells (macrophages, endothelial cells, and smooth muscle and epithelial cells) (48). OPN is inversely correlated with the expression of the von Hippel-Lindau protein that plays an important role in the destruction of HIF. Thus, the increase in the level of OPN in the tumor microenvironment reduces the expression of the von Hippel-Lindau protein, and then, the destruction of HIF accordingly decreases. Le et al. (49) revealed the correlation between plasma OPN levels and tumor hypoxia in the study they conducted in the blood samples of patients with head and neck cancer.

CA-9 is a transmembrane glycoprotein that is a member of a large metalloenzyme family. The members of this family provide for the reversible transformation of CO₂ to HCO₃⁻; thus, they play a role in fulfilling many important functions such as pH regulation of the extracellular matrix and microenvironment, respiration, and calcification. The increasing HIF level along with hypoxia in the tumor microenvironment also triggers the expression of CA-9. It has been reported that the level of CA-9 was high and closely associated with adverse survival in many solid tumors such as cervix, kidney, stomach, lung, and head and neck cancers (50). In a study conducted in head and neck cancer cell lines and tumor specimens, it was found that CA-9 was expressed at an increased rate and was higher in advanced stage tumors (51).

The first study in which the clinical effects of hypoxia in head and neck cancers was examined was conducted in 1996 by Nordmark et al. (52), with measurements performed with Ep-

Table 2. Target genes where the hypoxia-inducible factor is effective in cancer development

Function	Target gene
Erythropoiesis/iron metabolism	Erythropoietin
	Transferrin
	Transferrin receptor
	Ceruloplasmin
Angiogenesis	Vascular endothelial growth factor
	Endocrine gland derived vascular endothelial growth factor
	Leptin
	Transforming growth factor β
Vascular tone	Nitric oxide synthase
	Heme oxygenase 1
	Endothelin 1
	Adrenomedullin
	α-adrenergic receptor
Matrix metabolism	Matrix metalloproteinases
	Plasminogen activator receptor and inhibitor
	Collagen prolyl hydroxylase
Energy metabolism	Adenylate kinase-3
	Aldolase-A, -C
	Carbonic anhydrase-9
	Enolase-1
	Glucose transporter-1,-3
	Glyceraldehyde phosphate dehydrogenase hexokinase 1.2
	Lactate dehydrogenase-A
	Pyruvate kinase M
	Phosphofructokinase L
	Phosphoglycerate kinase 1
	6-phospho fructo-2-kinase/fructose-2, 6-bisphosphate-3
	A cell proliferation/survival
Transforming growth factor-α	
Adrenomedullin	
Apoptosis	Bcl-2*
	Nip3-like protein X**

*B-cell lymphoma-2

**19 kD interacting protein-3

pendorf polarographic oxygen electrodes (KIMOC 6650; Sigma pO₂ Histogram; Eppendorf, Hamburg, Germany) in the lymph nodes of 34 advanced stage head and neck cancer patients in whom radiotherapy was planned. Experts determined the level of hypoxia as pO₂ of 2.5 mmHg in this study and divided the patients into two groups: those below and those above this level. In the results from this study, while locoregional

failure developed in 22% of patients with PO₂ of <2.5 mmHg, this rate was determined as 6% in patients with pO₂ of >2.5 mmHg. In addition, it was found that the pO₂ level was an independent factor for the radiotherapy response. Brizel et al. (53) determined pO₂ of 10 mmHg as the level of the hypoxia border in a study where they made polarographic measurements in both the primary tumor area and the lymph nodes of 63 head and neck cancer patients in whom radiotherapy was planned. In this study, it was concluded that 2 years of locoregional control (pO₂<10 mmHg: 30%, pO₂>10 mmHg: 73%) in disease-free patients (pO₂<10 mmHg: 26%, pO₂>10 mmHg: 73%) and overall survival (pO₂<10 mmHg, 35%, pO₂>10 mmHg: 83%) were negative in hypoxic patients. In conclusion, it was speculated that hypoxic examinations before treatment are a good method for patient choice. Although different hypoxia limits were determined in other studies, the common result is that hypoxia has a negative prognostic effect (54).

Moreover, in a randomized, double-blind, placebo-controlled study that the Danish Head and Neck Study Group published in 2005, 320 head and neck cancer patients were divided into three groups (high, medium, and low) according to their plasma OPN levels (55). At the end of this study, a high plasma OPN level was reported to be closely related to radiotherapy failure.

Conclusion

The inflammatory and non-inflammatory intercellular communication and environmental changes in head and neck squamous cell carcinomas in the tumor microenvironment play an important role in the progression and aggressiveness of cancer and the development of gaining the ability of making lymphatic and hematogenic metastasis. Inflammatory response dysfunction was demonstrated in head and neck cancers and functional disorders in circulating and tumor-infiltrating T lymphocytes. In addition, a low rate of "CD8+ cytotoxic T lymphocyte/T regulatory" was found to be a poor prognostic factor in head and neck cancers, except for the case of oropharynx cancers. Because head and neck cancers characteristically have a hypoxic environment, TAM clusters are often found in the tumor microenvironment. A high level of TAM leads to a low general survival. However, CAFs play an important role in the development of desmoplasia, which is a poor prognostic factor in head and neck cancers. Owing to these features, clarification of the changes of head and neck squamous cell carcinomas in the tumor microenvironment and development of targeted therapies are promising future treatment options.

Peer-review: Externally peer-reviewed.

Conflict of Interest: No conflict of interest was declared by the author.

Financial Disclosure: The author declared that this study has received no financial support.

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