Analytical Cellular Pathology

Inflammation, Immunity, and Intercellular Communication Within the Tumor Microenvironment

Lead Guest Editor: Andreea Cristiana Didilescu
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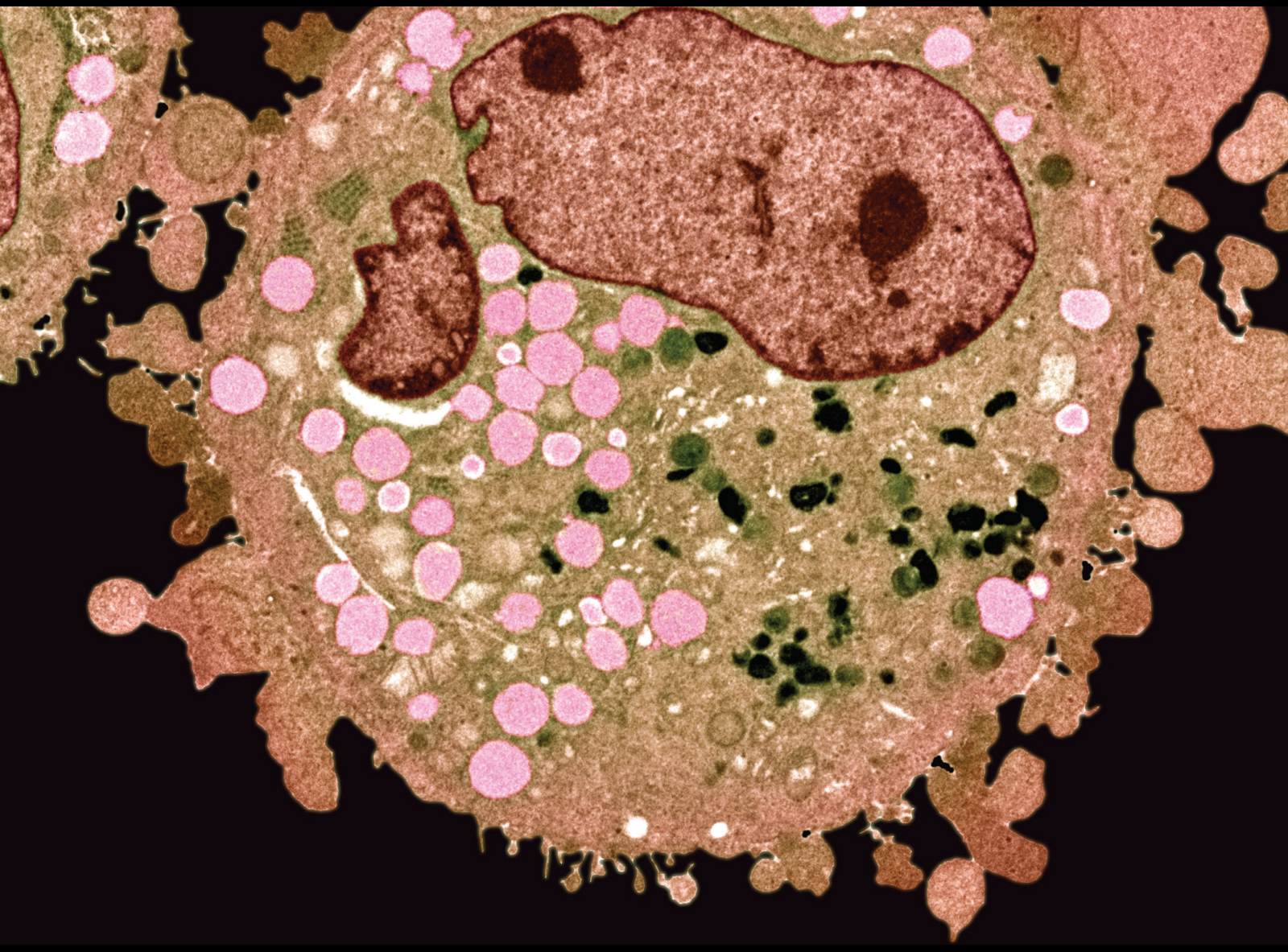

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Review Article

IL-6 Signaling Link between Inflammatory Tumor Microenvironment and Prostatic Tumorigenesis

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Benign prostatic hyperplasia and prostate cancer are tumoral pathologies characterized by the overexpression of inflammatory processes. The exploration of tumor microenvironment and understanding the sequential events that take place in the stromal area of the prostate could help for an early management of these pathologies. This way, it is feasible the hypothesis that normalizing the stromal environment would help to suppress or even to reverse tumor fenotype. A number of immunological and genetic factors, endocrine dysfunctions, metabolic disorders, infectious foci, nutritional deficiencies, and chemical irritants could be involved in prostate tumor development by maintaining inflammation, affecting local microcirculation, and promoting oxidative stress. Inflammatory processes activate hyperproliferative programs that ensure fibromuscular growth of the prostate and a number of extracellular changes. Acute and chronic inflammations cause accumulation of immunocompetent cells in affected prostate tissue (T cells, macrophages, mastocytes, dendritic cells, neutrophils, eosinophils, monocytes). Prostate epithelial and stromal cells, peri-prostatic fat cells, prostatic microvascular endothelial cells, and inflammatory cells produce cytokines, generating a local inflammatory environment. Interleukin-6 (IL-6) proved to be involved in the prostate tumor pathogenesis. IL-6 ability to induce pro- and anti-inflammatory responses by three mechanisms of signal transduction (classical signaling, transsignaling, cluster signaling), to interact with a diversity of target cells, to induce endocrine effects in an autocrine/paracrine manner, and the identification of an IL-6 endogenous antagonist that blocks the transmission of IL-6 mediated intracellular signals could justify current theories on the protective effects of this cytokine or by alleviating inflammatory reactions or by exacerbating tissue damage. This analysis presents recent data about the role of the inflammatory process as a determining factor in the development of benign and malign prostate tumors. The presented findings could bring improvements in the field of physiopathology, diagnosis, and treatment in patients with prostate tumors. Modulation of the expression and activity of interleukin-6 could be a mean of preventing or improving these pathologies.

1. Prostatic Microenvironment

The tumor microenvironment forms a biological barrier around the tumor, which protects the tumor from the action of the host's defense mechanisms or the action of antitumor drugs. The prostate tumor microenvironment should be considered as a dynamic structure, consisting of extracellular matrix (three-dimensional network of collagen, fibronectin, sialoproteins, laminin, osteocalcins, cadherins, osteonectins, and vitronectins), constituent cells (endothelial cells, fibroblasts, myofibroblasts, and immune and mesenchymal cells), and soluble factors (cytokines, chemokines, growth factors, metalloproteinases) [1, 2]. A characteristic of the prostate tumor microenvironment is that it contains the same

elements as the normal microenvironment, but with specific properties that promote inflammation (benign enlargement, hyperplasia, prostatic intraepithelial neoplasia) [3, 4].

2. Chronic Inflammation and Prostatic Tumorigenesis

Inflammation is considered the key in BPH development in latest clinical and experimental studies. BPH is a chronic, slow-progressing disease present in most men over 50 years, characterized by growth of prostate epithelial and stromal cells, which can cause urinary tract blockage and reduced urinary flow intensity. The urinary function impairment predisposes men with benign prostate hyperplasia to an increased risk of urinary tract infection and acute urine retention [5]. Meanwhile, the prostate cancer is less symptomatic because of its peripheral development, having a 97% of cases of adenocarcinoma and 3% of other tumor types. However, the inflammation plays a key role in the evolution of the disease, being correlated with the tumor aggressivity [6].

Although prostatic tumor pathogenesis is not fully known, most recent studies suggest that a better understanding of the factors that contribute to the inflammatory response regulation will provide more information to the pathogenesis of prostatic tumors thus, contributing to the development of new therapeutic strategies. Inflammatory processes activate hyperproliferative programs that ensure the creation of a suitable microenvironment for the initiation and progression of tumors. There are a lot of speculations regarding the signaling of inflammation and the activation of specific and nonspecific immune defence mechanisms in the prostate tissue (Figure 1).

Infectious agents, urinary reflux, nutritional deficiencies, metabolic syndrome, ageing process, alkaloids in tobacco, cigarette smoke, and activation and assembly of inflammasomes were considered triggers for alteration of prostatic immune system via different molecular pathways, involving the development of inflammatory infiltrates [5–13]. Researching the relationship between smoking and sex steroid hormone in prostatic pathology showed impaired metabolism of sex steroid hormones and high levels of inflammation in prostatic tissue [13].

In the inflamed prostatic tissue, B and T activated lymphocytes, macrophages, mastocytes, neutrophils, dendritic cells, and monocytes were detected. Those activated cells produce cytokines (IL-1 alpha, IL-1 beta, IL-6, IL-8, IL-18), chemokines, growth factors, IFN gamma, TGF beta, and MCP-1 which provide fibromuscular growth of the prostate via STAT-1/NF-κB signaling [5–8, 10, 11, 13–16]. Prolonged persistence of cytokines in the affected prostate tissue determines chronic inflammation, mediated by JAKs/STATs, MAPKs, and PI3/Akt [5, 17, 18]. Inflammatory factors induce the expression of COX-2 and iNOS that leading to growing the prostate cell proliferation rate [5, 15, 16, 19]. Local hypoxia could play a role as an inflammation mediator, developing neovascularization and differentiation from fibroblasts to myofibroblasts and some extracellular changes [19–21]. In affected prostate tissue, the activity of some proteolytic enzymes is overregulated, while some active substances are released in the intercellular spaces [22]. In this process, free radicals of oxygen and nitrogen are produced in excess and maintain chronic inflammation and induce oxidative degradation of biomolecules, disorganization of the extracellular matrix (EMC), and degradation of cellular architecture [1, 2, 12, 17–19]. All these phenomena lead to the proliferation of prostate cells, increased prostate volume, alteration of the epithelial barrier, urinary obstruction, vascularization disorder, apoptosis, cellular survival, and autophagy process (Figure 1).

3. Interleukin-6 Biologic Activities

A number of recent findings support the central role of IL-6 in prostate tumor pathology. The functional characteristics of IL-6 and its specific receptor (IL-6R) determine various biological responses in prostate tumors, probably explained by IL-6's ability to promote pro- and anti-inflammatory responses [7, 8, 23, 24], to interact with a diversity of target cells [10, 25–27], to induce autocrine and paracrine effects in prostate tissue [12, 24, 28], and to activate intracellular signaling pathways (Jak/S) [10];

IL-6 promotes three mechanisms for transmitting intracellular signals [7, 10, 25–27], being known an IL-6-induced signal buffer system [7, 8, 25, 26]; all the reactions modulated by IL-6 being produced in waterfall [7, 29].

IL-6 is a pleiotropic mediator, with multiple effects in the host's immune including inflammatory responses to different stimuli [7, 8, 10]; it plays the role of an endogenous pyrogen factor, triggering the fever process [7]. IL-6 is active in very low concentrations, participating in synergistic and antagonist processes, in the elaboration of the body's response to stress caused by inflammation [7, 8]. IL-6 secretion is fast and short-lived in an inflammatory, autoimmune, or oncogenetic process.

3.1. IL-6 and Its Receptors. IL-6 cytokine is synthesized as a precursor of 212 amino acids, with a molecular weight of 21.5-28.0 kDa depending on O- and N-glycosylation grade. IL-6 is mostly secreted by macrophages and also by T and B lymphocytes, monocytes, fibroblasts, endothelial cells, keratinocytes, synoviocytes, condrocytes, epithelial cells, mesangial cells, podocytes, astrocytes, stromal cells, adipocytes, and malignant cells. Thus, IL-6 is produced in response to infections, inflammation, trauma, autoimmune processes, and tumorigenesis [25, 30, 31]. IL-6 secretion is stimulated by endotoxins, cytokines, and microorganisms. Some proteins and microRNA control IL-6 synthesis at transcriptional and posttranscriptional levels. IL-6 mRNA formation is stimulated by NF-IL6 (nuclear factor of IL-60), Tax (transactivator protein), TAT (transactivator of the transcription), HBVX (hepatitis B virus X protein), Ahr (aryl hydrocarbon receptor), GR (glucocorticoid receptor), ER (estrogen receptor), Rb (retinoblastoma), and PPARα (peroxisome proliferator–activated receptor α) and inhibited by microRNA (miR-155, miR-146a/b, miR-223). A group of factors provide IL-6 mRNA stability: ORF (open reading frame), p38, and arid5a; IL-6 mRNA complex disintegration:

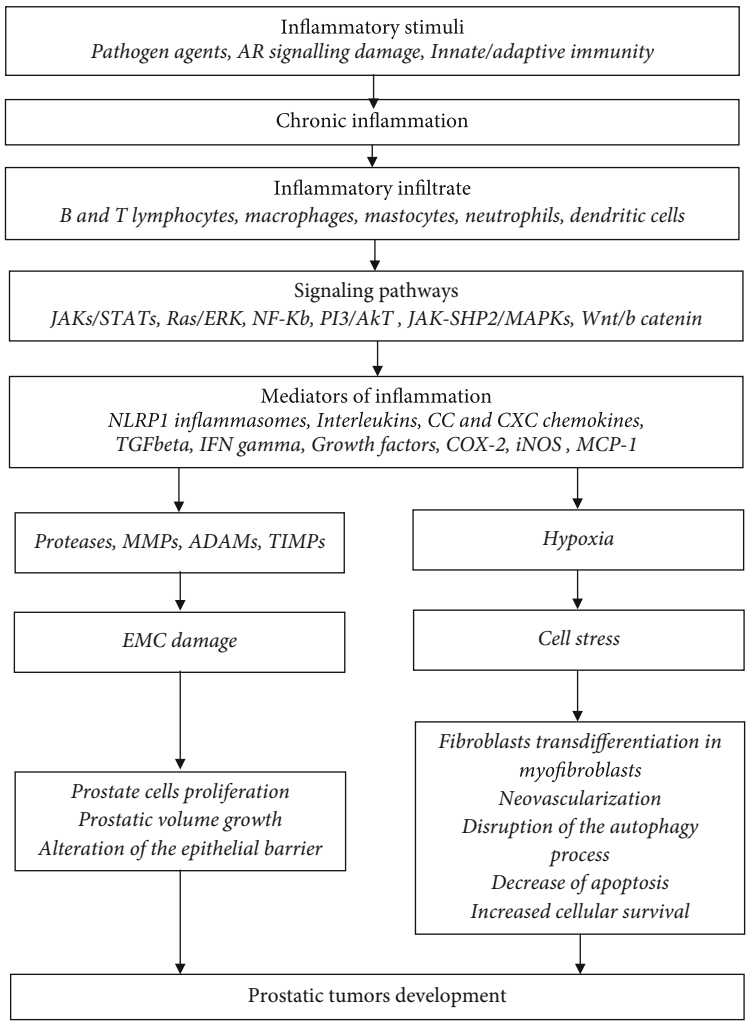

Figure 1: The inflammation role in prostate tumor development and evolution. AR-, JAK-, STAT-, ERK-, NF-κB-, PI3-, Akt, SHP, MAPK, NLRP, CC, CXC, TGF, IFN, COX, iNOS, MCP, MMP, ADAM, TIMP, EMC.

TTP (tristetraprolin), BRF (butyrate response factor), miR-365, and miR-608 [30, 32].

The specific receptor for IL-6 (IL-6R) is a membrane protein complex composed of two structural and functional subunits: a specific ligand subunit (IL-6Rα) and a signaling subunit (gp130). The IL-6Rα subunit is 80 kDa α chain, noted mMIL-6R or CD126. The gp130 signal subunit is a 130kYes b chain, noted CD130, the common component of several receptors, such as those linked to IL-11, IL-21, and IL-31. The active IL6-R receptor is on the surface of a limited number of cells (some lymphocytes, hepatocytes, neutrophils, monocytes/macrophages, and podocytes), while gp130 is ubiquitous on the surface of cell membranes. Both IL-6Rα and gp130 can be cleaved, with the rapid appearance in circulation of soluble protein components, noted sIL-6R and sgp130 [25, 30, 31].

IL-6R soluble receptors are considered regulators of cytokine signaling and inflammatory events. Soluble IL-6 receptors (sIL-6R) have been identified in blood and urine. In humans, two distinct mechanisms induce sIL-6R secretion: the mIL-6R proteolytic splitting and mRNA IL-6R alternative splicing. The proteolytic cleavage of sIL-6R is activated of metalloproteinases (ADAM-10, ADAM-17) and serine proteases derived from neutrophils (cathepsin G, proteinase 3, neutrophilel elastase, neutrophil serine protease 4) [25, 29, 31]. Soluble glycoprotein sgp130 has been described as a specific inhibitor of IL-6-mediated signaling. It is found naturally in plasma. In humans, sgp130 is generated mainly by alternative splicing and not by proteolytic splitting [25, 26].

3.2. IL-6 Signaling Pathways

3.2.1. IL-6 Classical Signaling. The classical signaling pathway (cis-signaling) mediated by IL-6 is functional in cells expressing CD126 and CD130. In cells that respond directly to IL-6 (macrophages, neutrophils, T helper cells, podocytes, hepatocytes), it binds to mIL-6Rα. This complex interacts with the homodimer consisting of two subunits gp130 and initiates the transduction of the signal. Intracellular signaling is carried out by the Janus Kinase-Signal transducer and transcription activator (JAK-STAT), mitogen-activated

kinase protein (MAPK), and phosphatidylinositol kinase 3/Akt kinase (PI3-AKT) [14, 27]. Though, IL-6 induces intracellular overregulation of cytokine signaling suppressor (SOCS-3) transcription genes by STAT3. This mechanism mediated by IL-6 is the classical signaling pathway.

The attachment of IL-6 to mIL-6R is critical for inducing the activation of transcription factors—nuclear factor-kappa B (NF-κB), C/enhancer binding protein beta (C/EBP beta), CCAAT/enhancer binding protein delta (C/EBPdelta), cAMP responsive element binding protein 1 (CREB1), Jun D proto-oncogene (junD), v-Fos FBJ murine osteosarcoma viral oncogene homolog (c-Fos), and Jun oncogene (c-Jun)—depending on cell type and ligand specificity (Figure 1). Classical signaling induces in hepatocyte synthesis of acute phase proteins, proteins with anti-inflammatory and washable properties for immune defense. IL-6 classical signaling involved cell survival and proliferation regulation, epithelial cell regeneration, antiapoptotic signaling, and mitosis development [7, 10, 25, 27, 31, 33].

3.2.2. IL-6 Transsignaling. The alternative signaling path (transsignaling) mediated by IL-6 requires the formation of a hexamer in which IL-6, sIL-6R, and gp130 interacts in a 2 : 2 : 2 [14] stochiometry. In the extracellular environment, sIL-6R binds to IL-6. This activated IL-6/sIL-6R complex interacts whether with the ubiquitous gp130 on the cell membrane or with circulant sgp130. IL-6/sIL-6R complex membrane-gp130 link is the alternative signaling pathway that allows IL-6 to modulate a wide spectrum of target cells and is called transsignaling and also for inhibiting IL-6 activity. Transsignaling is regulated by proinflammatory cytokines (IL-1 beta, TNF alpha), bacterial toxins, cell cholesterol depletion, PKC agonists, protease inhibitor, and degradated nucleic acids.

Transsignaling was described in inflammatory and autoimmune processes, lately being studied in cancer. The alternative signaling path mediated by IL-6 results in the regulation of neutrophil-monocyte transition, blocking the apoptosis of T cells and reducing the differentiation of Treg cells. In some situations, transsignaling produces protective effects by alleviating inflammatory reactions, and in other conditions, it exacerbates tissue destruction, by blocking inflammatory reactions [8, 10, 14, 31]. IL-6 transpresentation (cluster signaling) involves firstly IL-6 and IL-6R interaction, a complex located on cell membrane and secondly the link with sgp130 subunit, expressed on others cell membrane [7, 34]. The IL-6 signaling modulation was well documented in the latest studies [7].

The role of endogenous inhibitors in IL-6 signaling has been investigated in various biological systems. Endogenous negative feedback adjusters of the IL-6 signal can be grouped into as follows:

(i) *STAT Protein Inhibitors*. Prevent STAT-DNA interaction by blocking STAT dimerization

(ii) *SH2-Protein Tyrosine Phosphatases*. Modulate tyrosine residue dephosphorylation, critical elements for kinases activation

(iii) *Intracellular Suppressors of IL-6 Signaling (SOCS-1, SOCS-3)*. Inhibit JAK activation and disrupt cell cycle and apoptosis [35]

Special attention was paid to the sgp130 subunit, extracellular suppressor of IL-6-induced signal. The circulating protein sgp130 is in competition with membrane gp130 for linking with IL-6/sIL-6R complex. IL-6 classical-mediated signaling via membrane IL-6R receptors is not affected by circulating levels of sgp130. An excess of sgp130 leads to competitive in vivo inhibition of IL-6/sIL-6R complex. Thus, sgp130 is considered a natural antagonist of IL-6/sIL-6R complex that can prevent systemic transsignaling and cluster signaling within inflammatory diseases [7, 8, 14, 25–27] (Figure 2).

Recently, it has been reported that sIL-6R to sgp130 serum ratio could be associated with overexpression of a particular IL-6 signaling pathway [8]. When the amount of sgp130 is greater than sIL-6R, the simultaneous activation of classical signaling and IL-6-mediated transsignaling takes place. When the amount of sIL6R is greater, IL-6-mediated transsignal is overexpressed. Consequently, pharmacological modulation of the sIL-6R/sgp130 ratio could prevent excessive organ damage during active inflammatory events [8].

3.3. IL-6 and IL-6R in the Prostate

3.3.1. IL-6 and IL-6R Expression in Prostate Tumours. IL-6 is secreted by prostate cells, periprostatic fat cells, prostate microvascular endothelial cells, and inflammatory and immune cells recruited into the assaulted tissue microenvironment [26, 27, 31]. IL-6 and its receptor expression was investigated in normal prostate tissue, benign and malignant prostate lesions, slow-growing androgen-sensitive prostate cell lines (LNCaP), and rapid-growth androgen-insensitive prostate cell lines (DU145, PC3) [28, 35, 36].

In normal prostate tissue, IL-6 was immunolocalized mostly in basal epithelial cells and poorly expressed in stromal cells, while gp130 was detected only in stromal cells [27]. IL-6R has been identified in epithelial and stromal cells [35]. The most important source of IL-6 in prostate tissue is macrophages/monocytes, being involved in the inflammatory response, though, secreting cytokines and MMPs [14, 26]. Monocytes, macrophages, and neutrophils express IL-6R, which ensures the functioning of the classical IL-6-mediated signaling pathway. In benign prostate hypertrophy, IL-6 was preferentially immunolocated in basal epithelial cells, and gp130 was limited to epithelium and stroma [27].

In malignant prostate tissue, IL-6 is intensely expressed in adenocarcinoma, being secreted preferentially by glandular cells, and its level is elevated in patients with a low prognosis. IL-6 was detected in all cell types, and its immunostability increased with the degree of Gleason. The gp130 subunit was detected in stroma and epithelium, and its expression increased with Gleason grade [27]. IL-6R receptors were intensely expressed in malignant prostate cells [27]. The IL-6/IL-6R signaling pathway could be considered a molecular marker associated with the progression

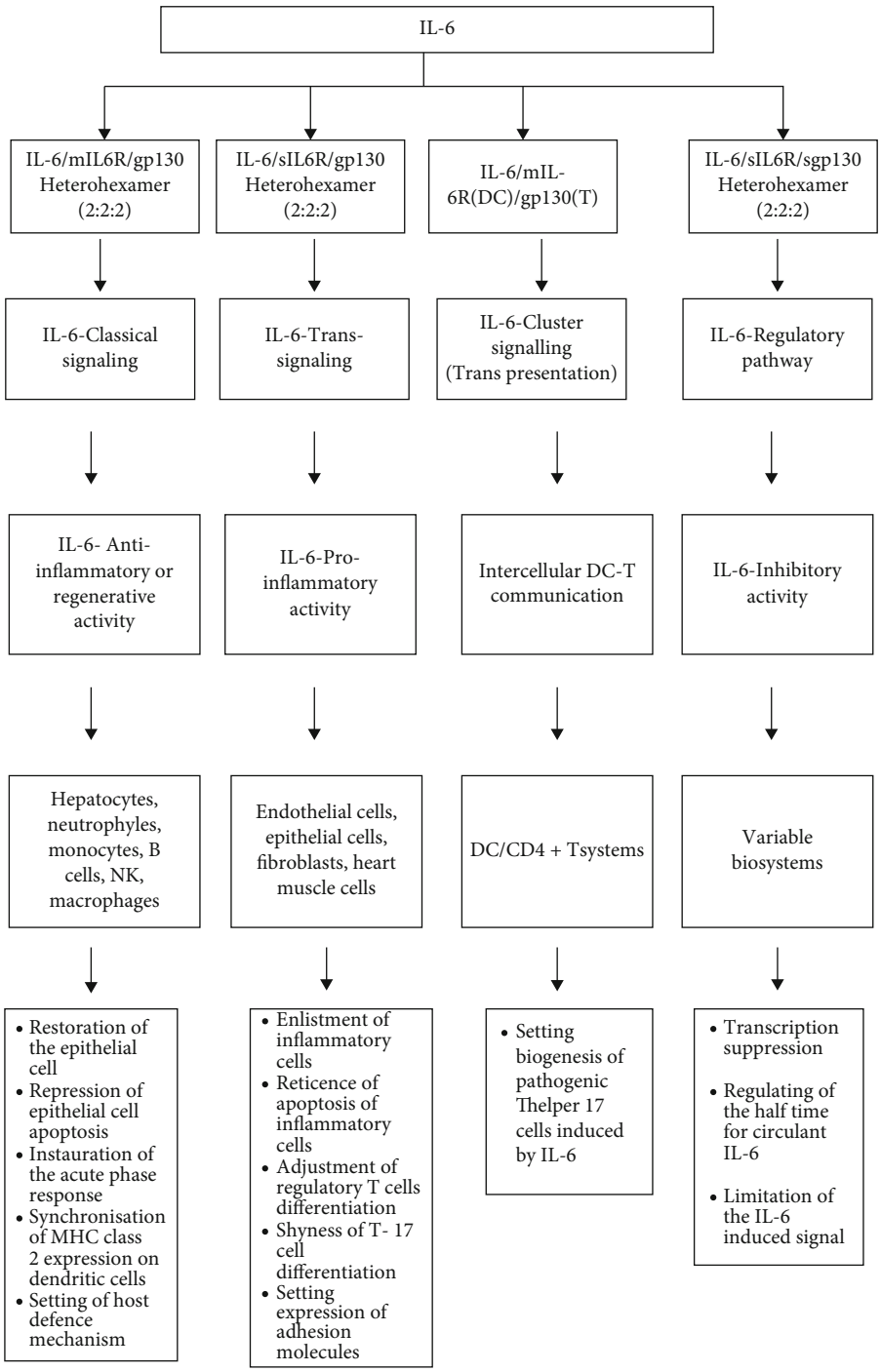

Figure 2: Schematic illustration of IL-6 signaling. IL-interleukin; IL-6R-IL6 receptor; mIL-6R-membranar-IL-6R; gp130-glycoprotein; sIL-6R-soluble IL-6R; sgp130-soluble gp130; mDC-dendritic cell membrane (transmitting cells); T-T cells (received cells).

of prostate cancer. Serum concentrations of IL-6 and sIL-6R were correlated with advanced stages of the disease and a poor prognosis in prostate cancer patients [27].

3.3.2. IL-6 and IL-6R Synthesis Regulation in Prostate Cancer. Regulation of IL-6 synthesis and secretion in prostate cancer is the result of several cellular processes, some of which being interconnected. One interesting connection was TGF beta—IL-6. Serum TGF beta was overexpressed in prostate cancer patients, and it increased IL-6 expression. TGF beta acted as an inhibitor of prostate cancer growth in vitro. In vivo, TGF beta activated the angiogenic cascade, suppressed the immune responses, and induced the expression of matrix metalloproteinases, though causing tumor growth. Though, IL-6 production might stimulate angiogenesis [27].

IL-6 study in LNCaP-IL-6+ cells culture showed that IL-6 production might be a consequence of reduced

retinoblastoma protein expression. It is also important to note that the presence of andrographolide inhibited IL-6 expression. These findings are consistent with the inhibition of NF-κB activity by dihydrotestosterone. PC-3 and DU-145 AR-negative cells secreted high levels of IL-6. IL-6 production was stimulated by JunD and protein kinases [35]. In LNCaP, DU145, and PC3 cell lines, this cytokine and its specific receptors were widely expressed, but they were absent in normal prostate epithelial PZ-HPV-7 culture cells [27]. DU145 and PC3 cell lines proliferated in response to IL-6 stimulation. As a result, IL-6 acted as an autocrine and/or paracrine proliferative factor in prostate cell lines [28].

There is evidence that IL-6 acts as a paracrine growth inhibitor under certain conditions and as an autocrine growth stimulant in other situations. IL-6 might have different functions in the proliferation of prostate cell lines according to the phenotypic characteristics of the cells and the microenvironment of the culture system [27, 28]. Prostate microvascular endothelial cells synthesize and secrete IL-6 in a paracrine manner, under the induction of IL-1, LPS, TNF alpha, and IL-4. IL-6 modulates processes such as andrographolide subregulation, leukocyte recruitment at the site of inflammation, chemokine secretion, expression of endothelial surface adhesion molecules, neovascularization and angiogenesis, and metastasis. Endothelial cells do not express IL-6R, so IL-6 regulates endothelial function only through transsignaling, being involved in multiple biologic processes in prostate tumors [24, 31].

3.4. Biologic Processes Mediated by IL-6 in Prostate Tumors. The inflammatory response in prostate is ensured by the active complex IL-6/IL-6R. The involvement of IL-6 in benign prostatic hyperplasia is complex. IL-6 has multiple, distinct, or even contradictory pathophysiological effects in benign prostatic hyperplasia. At this level, IL-6 is secreted by prostate cells, periprostatic fat cells, prostate microvascular endothelial cells, and inflammatory and immune cells recruited into the aggressed tissue microenvironment [26, 27, 31].

In chronic inflammatory diseases, IL-6 appears to be a reliable indicator of future inflammatory reactions [14, 37, 38]. IL-6 mainly modulates the body's immune and inflammatory responses. IL-6 exerts proinflammatory effects, being synthesized in response to infections, inflammation, or trauma. Cytokine IL-6 is considered the most important inducer of hepatic production of acute phase reactants: fibrinogen, serum amyloid A, haptoglobin, C-reactive protein, complement, and hepcidin [32].

IL-6/IL-6R modulated innate and adaptive immunity in the prostate. IL-6/ILs-6R was involved in regulation of acute inflammatory responses (chemokine production, T-helper 2 cell cytokine production, leucocyte chemotaxis) and immune processes (neutrophil-mediated immunity, immunoglobulin secretion, hepatic immune response) in cell activation and proliferation (B cell activation, T-cell proliferation and differentiation, platelet activation, osteoblast differentiation), in apoptotic processes, in DNA replication, in signaling pathways (ERK1 and ERK2 cascade, JAK/STAT cascade, MAPK cascade, cytokine and chemokine mediated signaling pathway, activation of NF-kappaB transcription factor, peptidyl-serine and peptidyl-tyrosine phosphorylation), in gene expression, and in angiogenesis (VEGF production) [5, 23, 25, 30]. The cytokine IL-6 stimulated humoral and cellular immune responses by acting on both B and T lymphocytes, promoting their growth and differentiation. IL-6 was involved in regulating the differentiation of CD4 + lymphocytes into regulatory (Treg) and helper17 (Th17) T cells. IL-6 and TGF beta triggered Th17 differentiation by increasing ROR expression and attenuating Treg generation by STAT3. Th17 cells secreted proinflammatory cytokines and initiated inflammatory responses. IL-6 modulated apoptosis by producing IL-2 and activating STAT3 and activated the generation of Th2 cytokines via the C/EBP transcription factor. IL-6 was involved in B cell-induced inflammation through follicular Th cells. As a result, IL-6 plays a key role in the prostate-mediated immune response at the prostate level [27, 31].

3.4.1. Biologic Processes Mediated by IL-6 in Prostate Hypertrophy. An important role in BPH development and progression is played by inflammation. Histopathological examination of BPH showed infiltration of lymphocytes and macrophages around the glandular area, cells that secreted high levels of IL-6 and CXCL8, secondarily, with epithelial and stromal cell growth [5]. Thus, IL-6 was produced in the presence of systemic insults (bacterial and viral infections, inflammation, trauma, altered AR signaling, hypoxemia, toxins, oxidized lipids, advanced glycosylation end products), quickly reaching detectable serum levels. IL-6 regulated macrophage/monocyte/dendritic cell differentiation, by triggering and stimulating the expression of M-CSF receptors (on monocytes), complement receptors, Fc, and F4/80 receptors (on macrophages), via JAK/STAT signaling. Moreover, IL-6 stimulated the in vivo and in vitro expression of the genes Jun-C, Jun-B, Jun-D, Jak, Egr, lysozyme, ferritin (on macrophages), and MCP-1 (on monocytes) [26, 27, 31, 39].

Clinical and laboratory data showed a statistically significant positive correlation between IL-6 and prostate volume and a weak positive correlation with IPSS (International Prostatic Symptom Score). During treatment with dutasteride, IL-6 showed a progressive decrease in patients with BPH [37]. The positive, statistically significant, correlations between the acute phase reactants and clinical elements such as IPSS, PVR (postvoiding residue), and prostate volume sustain the role of inflammation in BPH development. The acute phase positive proteins (CRP, ferritin, and ceruloplasmin) were characterized by elevated serum values and a positive correlation with the IPSS score and prostate volume, while the negative acute phase proteins (transferrin and albumin) showed decreased levels in BPH patients. In patients with BPH, IL-6 regulated the status of iron and zinc. In condition of high inflammation and high levels of IL-6, the liver decreased the synthesis of albumin, fibronectin, ferroportin, ZIP14, and transferrin, in turn initiating liver regeneration processes [32].

IL-6/IL-6R regulated the cellular response to infectious agents (bacteria, fungi, protozoa, viruses) detected in BPH patients. IL-6 is involved in defense response to Gram negative and positive bacterium, to protozoa, and to virus. IL-6

also modulated the cellular response to antibiotics, glucocorticoids, insulin, estradiol stimulus, HGF, IL-1, TNF alpha, LPS, nutrient levels, and cytokines. Moreover, it has been suggested that chronic retention of T. vaginalis in the prostate is associated with BPH development [5, 33]. T. vaginalis-infected BPH-1 cells could induce inflammatory responses. In addition, T. vaginalis-stimulated BPH-1 cells produced proinflammatory cytokines and induce monocyte and mast cell migration. Though, increased IL-6 promoted the development of benign prostatic hyperplasia and prostate cancer [5, 11, 19, 33]. When BPH-1 cells and prostate epithelia were treated with IL-6, cell proliferation was increased. IL-6 activated intracellular signaling via JAK/STAT, Ras/ERK, or PI3K/Akt [32]. In T. vaginalis-infected BPH-1 cells, increased levels of IL-6 and high expression of JAK2 and phosphorus STAT3 were detected. Moreover, the treatment with a JAK2 inhibitor reduced IL-6 production. These results suggested that JAK2/STAT3 signaling was involved in the production of IL-6 [5, 33] in BPH patients. BPH-1 cells infected with T. vaginalis produced cytokines, such as CXCL8, CCL2, IL-1 β, and IL-6, through cellular signaling pathways involving ROS, MAPK, and NF-κB. T. vaginalis infection in patients with BPH by the effects mentioned above could be responsible for the development of lower urinary tract symptoms [5, 33]. IL-6/IL-6R stimulated androgen hormone synthesis and AR expression. IL-6 functioned as a paracrine growth factor for LNCaP and as an autocrine growth factor for DU145 and PC3 but had no stimulatory effect on BPH-derived epithelial cells [28]. Some interesting studies sustained also IL-6 role in BPH development, by reducing dihydrotestosterone production after dutasteride treatment, increase of antioxidant capacity, reduction of prooxidant levels, and progressive reduction of serum IL-6 [37, 39].

3.4.2. Biologic Processes Mediated by IL-6 in Prostate Cancer. The expression of IL-6 and IL-6R in prostate cancer, as well as the role of IL-6 as a growth factor in prostate cancer, was well documented. IL-6 protein levels were correlated with disease stages and high levels of IL-6 with poor prognosis. Some studies analyzed the autocrine and paracrine effects of IL-6 in IL-6 positive LNCaP cells. IL-6 induced the growth of neuroendocrine cells, overexpressed in castration-resistant prostate cancer (CRPC). IL-6 stimulated androgen and androgen receptor synthesis in prostate cancer cells. IL-6 was a potent inducer of the protein encoded by the S100P gene (S100P) that was upregulated in CRPC and metastatic prostate cancer and therefore was involved in regulating androgen sensitivity. Constitutive expression of IL-6 in the prostate, similar to chronic inflammation, activated STAT3, reprogrammed the transcription of a set of genes, activated IGF, and amplified inflammation in the prostate and periprostatic adipose tissue [26, 40–43].

Because IL-6 is a cytokine produced by many cell types in inflamed prostate tissue, numerous studies suggest a direct link between chronic inflammation and prostatic tumor development. In vivo and in vitro studies sustain the above findings by the following results results: activation of STAT3 in prostate stromal cells, infiltration of many types of inflammatory cells in the prostate, and the presence of inflammatory cells in peri-prostate adipose tissue. IL-6 is considered the best-known activator of STAT3. In normal prostate cells, STAT3 was inactive, and SOCS3 was overexpressed; SOCS3 interacted with gp130 and prevented IL-6 signaling and STAT3 activation. In chronic inflammation, STAT3 was activated by blocking the SOCS3 gene via hypermethylationand IL-6-mediated signaling was constitutively expressed [7, 10, 26, 40–43].

IL-6 also mediated a series of anti-inflammatory effects, like completing the inflammatory cascade by suppressing the IL-1 and TNF synthesis, simultaneously with the stimulation of IL-1R alpha synthesis in different types of cancer. The anti-inflammatory activity of IL-6 included the activation of STAT3, which involved the regeneration of epithelial cells and the induction of the acute phase hepatic response [7, 10, 25]. IL-6 had an anti-inflammatory role in myeloid cells. In these cell cultures, the exposure to IL-6 before stimulation with microbial products (e.g., lipopolysaccharide (LPS)) inhibited class II major histocompatibility complex (MHC) expression and proinflammatory mediators in bone marrow-derived dendritic cells. In fact, IL-6-deficient mice showed a higher-class II MHC expression on dendritic cells than that in wild-type mice, whereas mice with enhanced IL-6 signaling caused by the loss of the SOCS3-binding site in gp130 (F759 mice) showed a lower expression. IL-6 reduced the level of cystatin C, an endogenous inhibitor of cathepsins, thereby increasing cathepsin S activity and subsequent degradation of MHC class II components in IL-6-treated dendritic cells. Similarly, prolonged action of IL-6 had been shown to mimic the anti-inflammatory effects of IL-10, which also activated STAT3, in macrophages. Moreover, the anti-inflammatory effects of IL-6 were manifested in a murine model of allergic asthma. In this model, IL-6-deficient mice showed exaggerated lung inflammation whereas lung-specific overexpression of IL-6 reduced the disease symptoms. Importantly, IL-6 stimulation was also known to suppress T-cell-receptor-mediated signaling via SOCS3. Thus, direct IL-6 stimulation in certain immune cell populations could induce an anti-inflammatory signal in prostate cancer and hypertrophy [44–46].

Because IL-6 is a cytokine produced by many cell types in the inflamed hyperpasic prostatic tissue, a lot of studies suggested a direct link between inflammation and prostatic tumors. The regulation of the inflammasome activity at posttranslational levels (phosphorylation, ubiquitination, de-ubiquitination, proteolytic processing, *S*-nitrosylation, and ADP-ribosylation) [45]; regulation of NLRP1, caspase-1, and cytokines (IL-18 and IL-1β) [46]; and modulation of anti- and proinflammatory activities of IL-6 [5, 7, 14, 46] might offer innovative therapeutic targets for prostatic tumor-related inflammation.

4. Conclusions and Future Directions

The study of inflammation in prostate tumors is very actual and a continuously research area. Blocking inflammation could allow the identification of control factors that react quickly, and though, inflammation takes place within certain

limits, allowing the body to regenerate its affected tissues. The ability of interleukin-6 to induce pro- and anti-inflammatory activities by using three mechanisms of information transduction (classical signaling, transsignaling, cluster signaling), the ability of interleukin-6 to interact with a variety of target cells to induce endocrine effects in autocrine/paracrine manner, and the identification of an endogenous antagonist that blocks the transmission of interleukin-6-mediated intracellular signals could justify current theories on the protective effects of IL-6 cytokine in prostate tumors, by attenuating inflammatory reactions in certain conditions or exacerbating tissue damage in other conditions. Antitumor therapeutic methods targeting the tumor microenvironment tend the inversion of the tumor microenvironment into normal microenvironment. Immunotherapy focused on IL-6 involvement in prostate tumor could help the urologists and oncologists for a better management of the patients with prostatic tumors.

Abbreviations

BPH: Benign prostate hyperplasia
IL-6: Interleukin-6
IFN gamma: Interferon gamma
TGF beta: Transforming growth factor beta
MCP-1: Monocyte chemoattractant protein-1
STAT-1: Signal transducer and activator of transcription 1
NF-κB: Nuclear factor kappa B
JAKs: The Janus kinases
MAPK: Mitogen-activated protein kinase
PI3: Phosphoinositide 3-kinases
Akt: Protein kinase B
EMC: Extracellular matrix
AR: Androgen receptor
ERK: Extracellular signal-regulated kinases
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
SHP: Tyrosine phosphatase
NLRP: Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing
CC: β-Chemokine
CXC: α-Chemokines
COX: Cyclooxygenase
iNOP: Inducible nitric oxide synthase
MMP: Matrix metalloproteinase
ADAM: A disintegrin and metalloproteinase
TIMP: Tissue inhibitors of metalloproteinase
NF-IL6: Nuclear factor of IL-60
Tax: Transactivator protein
TAT: Transactivator of the transcription
HBVX: Hepatitis B virus X protein
Ahr: Aryl hydrocarbon receptor
GR: Glucocorticoid receptor
ER: Estrogen receptor
Rb: Retinoblastoma
PPARα: Peroxisome proliferator–activated receptor α
ORF: Open reading frame
p38: Protein kinase
arid5a: AT-rich interaction domain 5A
IL-6 mRNA: Complex disintegration
TTP: Tristetraprolin
BRF: Butyrate response factor
PI3-AKT: Phosphatidilinositol kinase 3/Akt kinase
SOCS-3: Cytokine signaling suppressors
C/EBP beta: C/enhancer binding protein beta
CCAAT: Enhancer-binding proteins
CREB1: cAMP responsive element binding protein 1
junD: Jun D proto-oncogene
c-Jun: Jun oncogene
mDC: Dendritic cell membrane (transmitting cells)
T-T cells: Received cells
LNCaP: Androgen-sensitive prostate cell lines
DU145, PC3: Rapid growth cell lines insensitive to androgen
PZ-HPV-7: Cellosaurus cell line
ZIP14: Zrt- and Irt-like–Zn transporter
CXCL8: C-X-C motif chemokine ligand 8
PVR: Postvoiding residue
EMP: Erythroblast macrophage protein
MHC: Major histocompatibility complex.

Data Availability

The studies used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Antitumoral and Anti-inflammatory Roles of Somatostatin and Its Analogs in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the most common primary liver cancer and affects about 8% of cirrhotic patients, with a recurrence rate of over 50%. There are numerous therapies available for the treatment of HCC, depending on cancer staging and condition of the patient. The complexity of the treatment is also justified by the unique pathogenesis of HCC that involves intricate processes such as chronic inflammation, fibrosis, and multiple molecular carcinogenesis events. During the last three decades, multiple in vivo and in vitro experiments have used somatostatin and its analogs (SSAs) to reduce the proliferative and metastatic potential of hepatoma cells by inducing their apoptosis and reducing angiogenesis and the inflammatory component of HCC. Most experiments have proven successful, revealing several different pathways and mechanisms corresponding to the aforementioned functions. Moreover, a correlation between specific effects and expression of somatostatin receptors (SSTRs) was observed in the studied cells. Clinical trials have tested either somatostatin or an analog, alone or in combination with other drugs, to explore the potential effects on HCC patients, in various stages of the disease. While the majority of these clinical trials exhibited minor to moderate success, some other studies were inconclusive or even reported negative outcomes. A complete evaluation of the efficacy of somatostatin and SSAs is still the matter of intense debate, and, if deemed useful, these substances may play a beneficial role in the management of HCC patients.

1. Introduction

Liver cancer is the fourth most frequent type of cancer and has been a rising cause of concern for the global medical community [1]. Despite significant diagnostic and therapeutical advances, hepatocellular carcinoma (HCC) still has recurrence rates of over 50%, even after aggressive treatments, such as curative resection [2–4]. Recent studies show that the majority of patients are at risk of recurrence, and this is attributable to intrahepatic metastasis or multicentric hepatocarcinogenesis [5].

HCC, the most frequent primary liver cancer, is more common in males and certain ethnic groups [6]. Its incidence increases markedly among patients with chronic viral hepatic infections and cirrhosis. Up to 8% of cirrhotic patients might develop HCC at some point in their lives

[1]. Despite these viral causes, chronic alcohol consumption remains the leading cause of hepatocellular carcinoma [7], and this is in part explained by the fact that alcoholism is by far more prevalent than hepatitis B and C infections [8]. The risk of HCC is also increased by metabolic conditions such as diabetes mellitus and obesity. This etiological variety implies that patients with HCC will also carry the burden of the underlying disease, and their management will require a more complex approach, often involving an interdisciplinary team to address cardiovascular, neurological, metabolic, or renal complications as well as any other associated pathologies [9–11]. Also, the presence of rare subtypes of HCC or atypical presentations can further delay the treatment and worsen patient prognosis [12, 13].

There are several therapies employed against HCC, ranging from potentially curative to palliative and symptomatic [14]. The most promising treatments are those involving liver resection, transplantation, and local ablation [15], but for a host of causes, only about 20% of the HCC patients can benefit from such radical treatments [16, 17]. Consequently, most HCC patients will be subjected to palliative or symptomatic treatment, with survivability of about 40%, at most [18]. Treatment options are evaluated based on the patient's condition and the severity of the carcinoma itself, among other factors [19]. While there are various evaluation systems employed, the BCLC staging system is the most widely adopted in Europe and the USA and is of interest to all medical specialties involved in the management of HCC patients [14, 20].

Due to the limited therapeutic options, especially in advanced HCC, and the relatively low efficiency of the available drugs in terms of improving overall survival, there is a high need for the development of new treatments [21]. A large number of new molecular targeted drugs are tested in clinical trials [22]. Recently, several alternative treatments for HCC have come into focus. The use of oncolytic viruses, the application of stem cell research and peptide vaccines, and even the modulation of the intestinal microbiota have been considered [23]. The use of natural compounds is increasingly investigated and also seems promising, especially when used in association with conventional treatment [24–27].

Another important therapeutic solution for HCC may be the use of somatostatin and its analogs (SSAs), which have been employed mostly in patients towards the later stages of HCC. In this review, we summarize the published experimental and clinical results and present the current research on the correlation between the action of somatostatin and SSAs in HCC and the expression of somatostatin receptors (SSTRs).

2. Somatostatin and Hepatocellular Carcinoma

2.1. General Actions of Somatostatin. Somatostatin can be thought of as a wide-ranging inhibitory peptide, with diverse functions depending on the target tissue [28]. More specifically, it may function as a neurotransmitter, a neuromodulator, an endocrine hormone, or a paracrine factor, while its role as a trophic factor has also been proposed [29, 30]. Physiologically, its levels are very low due to its prompt degradation by ubiquitous peptidases [31]. It exists in two isoforms known as SRIF14 and SRIF28—SRIF standing for somatotropin release inhibitory peptide. It is noteworthy that the inhibitory effects of somatostatin are directed not only towards the release of peptides per se but also towards the target tissues; it is possible for somatostatin to simultaneously and independently block the release of gastrin and of gastric secretion [32]. Frequently, the inhibitory effects of somatostatin are so potent as to be able to inhibit the associated peptides regardless of the type and intensity of the stimuli [28].

Somatostatin was isolated from the gastrointestinal tract, genitourinary system, heart, eyes, thyroid, thymus, and skin [33–35]. The presence of somatostatin was also identified in the central nervous system, where the concentration of somatostatin is sufficient to inhibit growth hormone release from the pituitary gland via the hypothalamic-pituitary axis [36].

2.2. Somatostatin in the Treatment of Hepatocellular Carcinoma. The importance of neuroendocrine factors in various cancers has been emphasized in recent research [30, 37, 38]. Studies on somatostatin, ever since its discovery, have demonstrated its inhibitory effects on the secretion of numerous hormones, as mentioned above. So, it is reasonable to assume that its administration by reducing glucagon, gastrin, and insulin levels will have an adverse effect on the cells targeted by these polypeptides acting as trophic factors [39, 40]. This might exert at least an antiproliferative if not an apoptotic effect.

It is known that one of the major antiproliferative actions of somatostatin is exerted via the mitogen-activated protein kinase (MAPK) pathway, which leads to cell cycle arrest in the G1 phase [41]. This has been shown to occur due to the upregulation of the p21cip1/waf1 and p27kip1 kinase inhibitors [42]. Nevertheless, there are other signaling mechanisms associated with this function, as we will further elaborate in this paper. The specific mechanism depends upon the type of receptor expressed in the studied cells [43]. Somatostatin can induce apoptosis, either by following a pathway involving the p53 protein [44] or by other p53-independent mechanisms [45, 46]. It can also exert the same effects by increasing the expression of the Fas-Fas ligand system, which promotes apoptosis [47].

The antineoplastic effect of somatostatin may be attributed to its potential to modulate immune pathways, although further research is required on the topic [48]. It is also possible that somatostatin reduces oxidative stress [49] and NO production [50], which further contribute to the antineoplastic effect.

Somatostatin also reduces the levels of proinflammatory cytokines in rat liver stellate cells [51], and this anti-inflammatory action may be beneficial in HCC. Other effects on stellate cells have also been reported, but this remains subject to further research [52, 53]. Furthermore, somatostatin may reduce the activity of MMPs which are associated with Kupffer cells [43].

The mentioned antitumoral effects of somatostatin on HCC are presented in Table 1.

Table 1: Classification of therapeutic actions of somatostatin on HCC.

Level	Effect type	Mechanism	Reference
Cellular	Antiproliferative	MAP kinase pathway—G1 phase arrest	[41, 42]
		Other mechanisms (specific receptors)	[43]
		Trophic factor secretion inhibition	[39, 40]
	Apoptotic	p53-dependent	[44]
		p53-independent	[45, 46]
		Trophic factor secretion inhibition	[39, 40]
		Fas-Fas ligand expression increase	[47]
Systemic	Antineoplastic	Immune pathway modulation	[48]
		Reduction of oxidative stress	[49]
		Reduction of NO production	[50]
	Anti-inflammatory	Decrease of proinflammatory cytokine levels	[51]
		Potential direct effect on stellate cells	[52, 53]
		Reduction of Kupffer cell-related MMP activity	[43]

The current research consensus for the HCC metastasis seems to indicate that the PEBP1 (RKIP) gene is the main culprit behind the invasive behavior. In general, the expression of PEBP1 is found to be much lower in cancer cells [54], and therefore, its upregulation might limit the metastatic potential. PEBP1 can act by inhibiting metastasis, acting as a tumor suppressor gene [55]. Huang et al. showed that increased concentration of somatostatin in SK-Hep-1 and HepG2 cancer cell lines can be correlated with increased expression of the PEBP1 gene and therefore with decreased invasive and metastatic potential [56].

Apart from influencing the endogenous expression of specific proteins which regulate the invasive and metastatic potential of the HCC cells, somatostatin may also be used for the direct and indirect downregulation of proteins related to the destruction of the extracellular matrix, the prerequisite for cancer invasion and metastasis. Highly invasive cancers such as HCC are characterized by abnormal activity levels of both intracellular and extracellular molecules. A paradigm of intracellular proteins was that of the RKIP as mentioned before, while extracellular related molecule research focuses on matrix metalloproteinases (MMPs) [56].

Recent data has shown MMPs as promising targets to avert or minimize the invasiveness of cancer cells, while the overexpression of these enzymes presents a direct correlation with cancer metastasis prognosis [57, 58]. In the pathogenesis of HCC, MMPs are involved in many processes related directly or indirectly to pathogenesis, such as fibrosis, weakening of the matrix, and tissue destruction [59–61]. While antibodies against these specific enzymes are currently experimented with, their use in integrated clinical practice remains elusive [62]. Somatostatin may regulate the activity of MMPs directly or indirectly, via IGF [63].

Therefore, somatostatin exhibits two types of antimetastatic potential effects: the direct control on antimetastatic genes and the influence on the activity of specific enzymes that promote metastasis. These functions of somatostatin are also exerted by its analogs, and there were several reports of metastasis halt or even reversal [64, 65]. However, no definitive evaluations for this effect of either somatostatin or SSAs are available at the moment, and further research is required on the subject.

2.3. Somatostatin Analogs in the Treatment of Hepatocellular Carcinoma. In modern clinical practice, somatostatin has been replaced, in many instances, by analogs due to the easier administration and the potentially severe side effects of using pure somatostatin [66]. More specifically, somatostatin administration must be performed intravenously, due to its half-life of approximately 3 min; therefore, its window of action is rather limited. Moreover, postinfusion hypersecretion rebound is also present, and growth hormone and insulin are secreted in pathological values, shortly after the activity of the administered somatostatin has ended [67].

There are several somatostatin-derived synthetic analogs available for the treatment of HCC. Early research has shown that somatostatin analogs inhibit tumor growth in animals [68, 69]. However, a consensus on the efficacy of such a treatment has not been reached, mainly due to the different research parameters which make comparison and evaluation of results difficult [1].

While there is little difference in the affinity of somatostatin receptors for SRIF-14 and SRIF-28, there is a marked affinity difference in the binding of the somatostatin analogs. All somatostatin analogs have a very high affinity for SST_1 and generally higher affinity than somatostatin for the other SSTRs. Whereas octreotide and lanreotide have a great affinity for SST_2 and SST_5 and to a lesser extent SST_3 [70], pasireotide has a high affinity for receptors SST_1, SST_3, and SST_5 [71]. Since different types of SSTs have been identified in different types of HCC, it may be of paramount importance to identify the type of SST or SSTs which are more prevalent and adjust the SSA treatment accordingly [52, 72–75].

The numerous experiments on the action of somatostatin and its analogs (e.g., [44, 52, 76–85]) did not always exhibit a unified corpus of conclusions. While many researchers replicated the originally determined antiproliferative and/or apoptotic effects, others did not manage that, and some even had conflicting results, depending on the dose of somatostatin or SSA employed.

However, it is the general research consensus that both somatostatin and its analogs exert a direct antiproliferative and apoptotic effect, which is modulated by the different types of somatostatin receptors [43]. The different mechanisms associated with these actions seem to be SSTR-dependent. While some SSTRs may be implicated in the same type of responses, they are associated with different signaling pathways, as seen in Table 2.

Similar to somatostatin, SSAs can induce cell cycle arrest by stopping the hepatoma cells in the G_1 phase, through specific receptors and the corresponding signaling pathways [86, 87, 93, 95]. The activation of $SSTR_2$ and/or its heterodimerization with $SSTR_3$ can induce apoptosis [42, 94]. The activation of these receptors yields the same results, whether it is performed by somatostatin or by SSAs. Inhibitory effects of octreotide and other analogs on liver tumors have been reported [96–98]. The inhibition of trophic factor secretion by somatostatin and its analogs might act as an antiproliferative and an apoptotic factor on HCC [39, 40].

Somatostatin analogs also activate the Fas-Fas ligand system [47], which induces the formation of the death-inducing signaling complex and forms an integral part of the anticancer immune function [99]. The ligation of Fas with FasL results in the activation of caspase-mediated apoptosis [100–102]. However, such mechanisms depend upon the serum levels of the analog. For the specific case of the octreotide, in vitro studies have revealed a concentration threshold, which if exceeded, the octreotide exerts antiproliferative effects, but if not, it actually promotes proliferation [43, 96].

Recent data showed that opioids bind to the somatostatin receptors, inducing the PTP signaling cascade [103]. The opioid growth factor and its receptor are also capable of halting cancer cell proliferation by inhibiting DNA replication [104]. This is especially important since functional opioid receptors have not been identified in HCC cell lines [43].

Octreotide has also shown direct and indirect inhibitory effects on angiogenesis [105–107]. Direct inhibition involves the SSTRs while indirect effects occur through inhibition of the vascular endothelial growth factor or of the adenylyl cyclase [39, 108, 109]. An octreotide and celecoxib combination has been successfully employed as an antiangiogenic agent [110].

It is possible that the SSA-induced immune pathway modulation may exert an antineoplastic effect, but this needs to be corroborated by further research [48, 111, 112].

Octreotide reduces the inflammatory component of HCC through a dual effect of decreasing the concentration of proinflammatory cytokines while increasing the anti-inflammatory cytokines [113]. It was also suggested that liver macrophages are downregulated by octreotide [114]. In addition, the TGFb1 secretion by the Kupffer cells is inhibited when these cells are exposed to octreotide, and this may contribute to the anti-inflammatory effects of SSAs [114]. SSAs also demonstrate antineoplastic effects by reducing oxidative stress and NO production [49, 50, 115].

2.4. In Vitro and Animal Experiments Involving Somatostatin Analogs. The initial success of somatostatin analogs in in vitro experiments in the treatment of other types of carcinomas [93] led to the investigation of the potential of this compound on patients suffering from HCC [77]. Initial reports showed that octreotide exerts dose-dependent apoptotic effects on Bel-7402 hepatoma cells, therefore introducing it as a potential antineoplastic drug [80]. Further research used the longer-lasting lanreotide, in a series of in vitro and in vivo experiments; the in vitro data showed lanreotide to exert a dose-dependent apoptotic potential on human HepG2 cells in the S-phase [78]. Wang et al. [79] determined that octreotide induces apoptosis and dose-dependent inhibition of cell proliferation on SMMC-7721 HCC cells. They also recorded tumor growth inhibition when xenografting the cell line to mice which was considered a consequence of an octreotide-induced decrease in DNA synthesis.

The study of Liu et al. [44], using octreotide on both normal liver cells and HCC cells, verified the apoptotic effect on the pathological cell lines and correlated it with the expression of $SSTR_3$, which is uniquely expressed in those cells. Again, the effect was dose-dependent. In most of these studies, a decrease in the synthesis of α-fetoprotein, a marker for proliferative activity, was observed. However, Reynaert et al. [52] did not manage to replicate the antiproliferative effect of somatostatin analogs, when using specific SSTR agonists, but noted that the metastatic potential of the cells was significantly reduced and correlated with the expression of $SSTR_1$.

Hua et al. [83] did not succeed in reproducing the results of previous studies [44, 77, 78] on the Bel-7402 cell line and noted no quantifiable apoptotic effect but noticed that after exposure to octreotide, the $SSTR_2$ expression levels were decreased. On the other hand, when these cells were xenografted to rats, it prevented the growth of the xenograft and HCC development, similar to previous reports [52].

An interesting experiment was performed by Xie et al. [82] who used two cell lines, HepG2 and HepG2x, the latter having a transfected HBV X gene. This gene codes for a small peptide, of 154 amino acids, which stimulates several cellular transduction pathways, in many cell types, including hepatocytes [116]. They noted that the apoptosis of the first cell line was significantly increased, but the second cell line was unresponsive, even when octreotide was used in combination with lamivudine, an antiretroviral medication, frequently used to treat AIDS/HIV and chronic hepatitis B. This was positively correlated with the decreased expression of $SSTR_2$ and $SSTR_5$ in the cells transfected with the HBV X gene.

Grant et al. [88] used a HEK 293 cell clone which expressed both a hemagglutinin- (HA-) tagged $SSTR_2$ and a c-Myc-tagged $SSTR_5$. This cell clone was specifically chosen for its relatively low SSTR expression levels, compared to physiological conditions [117]. They determined that the p21 and p27Kip1 cyclin-dependent inhibitors, associated with $SSTR_2$, were involved in cell cycle arrest. Ma et al. used the SMMC-7221 cell line and found that apoptosis positively correlates with the dosage and exposure time to octreotide and is achieved through the activation of the Fas-FasL ligand system [47]. Tsagarakis et al. reported that high doses of octreotide inhibited proliferation, while low doses of octreotide promoted proliferation in the HepG2 line [96].

Table 2: Specific effects of SSTR stimulation and associated signaling pathways [66, 86–93].

Receptor	Strongest agonist	Signaling pathway	Effect	Reference
$SSTR_1$	All SSAs	Tyrosine phosphatase SHP-2 stimulation Induction of MAPK-ERK pathway and p21:Waf1:Cip1 Adenylyl cyclase modulation	Cell cycle arrest Reduced metastatic potential	[41, 52]
$SSTR_2$	Vapreotide	Modulation of the $ERK_{1/2}$ pathway and activation of SHP-1, SHP-2, and PTPη Modulation of the p21 and p27kip1 pathways Adenylyl cyclase modulation	Apoptosis Cell cycle arrest; antineoplastic	[82, 83, 87, 88]
$SSTR_3$	Lanreotide	Adenylyl cyclase modulation	Apoptosis	[42, 94]
$SSTR_4$	Octreotide	MAP kinase pathway, Ca^{2+}-channels, K^+-channels, and Na^+-H+ antiporter Adenylyl cyclase modulation	Cell cycle arrest	[41]
$SSTR_5$	Octreotide	Guanylate cyclase inhibition and MAP kinase-ERK pathway Adenylyl cyclase modulation	Cell cycle arrest Antineoplastic Apoptosis	[41, 82, 84]

Klironomos et al. used hepatic stellate cells isolated from rats, to study the effects of somatostatin in their proliferation in correlation with the expression of SSTRs [85]. They determined that the effects of octreotide were subject to the cytokine microenvironment of those cells, but they noted that collagen production was reduced, mirroring the results of the research of Reynaert et al. who used somatostatin on the same cell line [118].

By using octreotide, lanreotide, and SOM230, another somatostatin analog, Lü et al. replicated the apoptotic effect of earlier researches using the Bel-7402 cell line [84]. Their study had also an in vivo component where the tumor cell lines were xenografted on mice, and it was observed that survival and quality of life were improved. These effects were attributed to the variations of SSTR expression in the cancer cells.

Octreotide also inhibited tumor progress in rats after partial hepatectomy [119], and further experiments confirmed these findings [120, 121]. The combination of a COX 2 inhibitor with SSAs demonstrated antiproliferative effects [98], a combination also proving successful in rabbits [97]. Following transcatheter arterial embolization, a combination treatment of octreotide with celecoxib also inhibited metastasis and angiogenesis [122]. A recent experiment on Sprague-Dawley rats has also raised the possibility of using octreotide preventatively in nonalcoholic steatosis, to prevent HCC development [123].

Lanreotide was also proven to have antiproliferative and apoptotic actions [124] and also to decrease fibrosis and angiogenesis in a series of animal experiments [125, 126]. In addition, lanreotide administration in rats was proven to prevent malignant transformation, an effect associated, most probably, with the reduction of oxidative stress [115]. The results of the mentioned in vitro experiments with SSAs are summarized in Table 3.

2.5. Clinical Evidence Involving Somatostatin Analogs as Single Treatment. An early clinical trial on patients with HCC using subcutaneous octreotide reported an improved median survival rate, compared to the control group; also, there were reports of tumor size decrease, and in some patients, the tumor disappeared [77]. The study concluded that octreotide administration can improve life expectancy and the quality of inoperable patients.

Positive results of octreotide were also reported in a retrospective study although the small size of the studied sample does not allow for a statistical evaluation [127].

Several case reports recorded excellent results when using octreotide for the treatment of advanced HCC [64, 128]; lanreotide was also found effective in metastatic HCC, and the positive response was correlated with $SSTR_2$ expression [65].

In the clinical trial of Raderer et al., intramuscular injection of lanreotide was used in patients with inoperable HCC and resulted in partial response to treatment and improvement in the quality of life for some patients [78]. The consensus of the researchers was that, most likely, the doses of lanreotide administered were suboptimal and that higher doses might achieve more significant results. However, further clinical trials using octreotide demonstrated an overall increase in survivability [129–136].

Conversely, there were clinical trials that reported no significant difference between the control and the treated group [137–140]. The use of pasireotide as a second-line treatment was mostly unsuccessful in another trial [141]. However, possible explanations for these results include an improper choice of the control and the treatment groups and the trial parameters. Furthermore, there were retrospective observational studies that found octreotide administration to be ineffective in altering the survival rate of the patients, albeit 40% of them were alcoholics, which is a mitigating factor in the potential success of such therapies [103].

A study on less than 30 patients tested octreotide and found limited beneficial clinical results [142]. Although the results may seem disheartening, as pointed out by Samonakis et al., the choice of patients and the statistical processing of results may leave a lot to be desired [143]. The study of Cebon et al. [144], which used octreotide, mentioned that patients reported improvement in some symptoms, but it

Table 3: In vitro experiments with SSAs and their results.

Somatostatin analog	Cell line	Mechanism	Result	References
SSA RC-160+CCK	Chinese Hamster Ovary (CHO) cells	Inhibition of CCK-induced intracellular cGMP formation and activation of p42-MAP kinase phosphorylation and activity	Inhibition of cell proliferation in response to the administration of cholecystokinin	[93]
Lanreotide	Human HepG2 cells	Potential action through the SST3 and/or insulin and IGF	Antiproliferative effect proportional to the SSA dose established	[78]
Octreotide	Human BEL-7402 cells	Potentially correlated to the antineoplastic effect of SSA	Antiproliferative effect proportional to the SSA dose established	[80]
Octreotide	SMMC-7721 HCC cells	Antineoplastic action potentially attributable to decreased DNA synthesis	Antiproliferative and apoptotic effect and also decreased tumor growth in xenografted mice	[79]
SSA RC-160	Chinese Hamster Ovary (CHO) DG-44 cells	A Gi/o protein-coupled receptor inhibits cell proliferation via ERK signaling	Inhibition of cell proliferation	[87]
Octreotide	Human HepG2, SMMC-7721, and L-02 cells	Some mechanism most probably associated with SST3	Antiproliferative effect proportional to the SSA dose established	[44]
Receptor agonists	Human HepG2, HuH7, and hepatic stellate cells (HSCs)	Signaling pathways linked to SSTs	Reduced migration of cancer cells but no antiproliferative effect observed	[52]
Octreotide± lamivudine	Human HepG2 and HepG2x	Signaling pathways linked to SSTR2 and SSTR5	Increased apoptotic effect on the HepG2 cell line	[82]
L-779,976	HEK 293 cell clone	Inhibiting adenylate cyclase, activating ERK1/2, and inducing the cyclin-dependent kinase inhibitor p27(Kip1)	Inducement of cell cycle arrest	[88]
Octreotide	SMMC-7221 cells	Activation of the Fas-FasL ligand system	Inducement of apoptosis	[47]
Octreotide	Human Bel-7402	Some mechanisms linked to SSTR2	No antiproliferative effect but no xenografted HCC development	[83]
Octreotide	Human HepG2 cells	Caspase-mediated signaling pathways	Inhibition of proliferation at high octreotide doses and proliferation of promotion at low octreotide doses	[96]
Octreotide	Rat hepatic stellate cells (HSCs)	A mechanism related to the cytokine environment of HSCs	Varied effect. General reduction in collagen synthesis related to PDFG and TGFb1	[85]
Octreotide, lanreotide, SOM230	Human Bel-7402 cells	Some mechanisms linked to SSTR expression variation	Apoptotic effect observed and improved survivability and life quality after the xenograft on mice	[84]

did not record any overall amelioration in the quality of life of the patients examined, while some also minor anticancer activity of the octreotide was registered. Overall, it was believed, however, that this trial had not been a success. Another factor that evaluates the results of this study was the variable length of the treatment, which was subject to disease progression and/or toxicity of the compound or withdrawal at the patient's or the doctor's discretion. In addition, 22% of the patients were alcoholics, a factor that must be considered in the objective assessment of the trials' results. The study of Dimitroulopoulos et al. [145] reported the doubling of the survival rate of patients with hepatitis-induced cirrhosis, who expressed SSTRs. Patients with a lack of SSTR expression did not respond to the treatment.

A small observational study using octreotide by Shah et al. [146] had some mixed results, with 6 patients, out of the original 22, surviving past 10 months, with advanced HCC developed on hepatitis B infection, and being of Asian descent, potentially indicating a racial aspect of the response to therapy.

2.6. Clinical Evidence Involving Somatostatin Analogs in Combined Treatment. Following the encouraging results of using SSAs in the treatment of HCC, further clinical trials were developed combining SSAs with other forms of treatment.

In one trial, octreotide was combined with tamoxifen but failed to provide any clear benefit, when compared to the results of the control group, which was treated solely with tamoxifen [147]. Pan et al. used tamoxifen, combining it with octreotide and chemotherapy, and demonstrated positive results in about 40% of the patients whose treatment included octreotide [81]. About 52% of the participants of this trial were alcoholics. Octreotide was also combined with sorafenib, and moderately positive results were achieved, but

Table 4: Clinical trials evaluating the role of SSAs in the treatment of HCC.

Somatostatin analog	Trial type	Trial length	Patients/ controls	Result	References
Octreotide	R	≤4 years	28/30	[P] Median survival levels of treated patients increased significantly	[77]
Lanreotide	NR	Variable	21/0	[N] Insignificant improvement in most patients; minor life quality improvement of some patients	[78]
Octreotide	R	Variable	12/13	[P] Overall increase in the survivability of treated patients	[129]
Octreotide	R	7 mo.	35/35	[N] No tumor regression, and no improvement in life quality of the patients	[137]
Octreotide/lanreotide	NR	n/a	32/27	[P] Overall improved survival rate of the SSA-treated patients and superior life quality	[130]
Octreotide	NR	6 mo.	63/0	[N] No significant prolongation of survival observed	[156]
Octreotide+tamoxifen	R	3 mo.	24/15	[P] Response of 43% of the patients treated with octreotide and doubling of their survival	[157]
Octreotide	R	Variable	32/33	[P] Improvement of the survival rate of the treated group	[131]
Octreotide	NR	Variable	30/0	[P] Increase of survivability and life quality of patients	[136]
Octreotide	R	Variable	20/25	[P] Improvement of the survival rate of the treated group	[132]
Octreotide	NR	32 mo.	41/33	[N] Similar survivability between the treated patients and the control group treated with TACE	[140]
Octreotide	NR	2 years	26/0	[N] Very limited beneficial response to treatment	[142]
Octreotide	NR	≤12 mo.	63/0	[N] No improvement of patient life quality and minor anticancer activity of octreotide	[144]
Octreotide±rofecoxib	R	min. 6 mo.	71/0	[P] Increased survivability in patients with high IGF and VEGF levels	[149]
Octreotide	R	3 years	31/30	[P] Response of those patients expressing SSTRs and doubling of the survival rate	[145]
Octreotide+tamoxifen	R	Variable	56/53	[N] No clear benefits in patient survival	[147]
Octreotide	R	Variable	60/59	[N] No significant improvement and no objective tumor regression	[138]
Octreotide	R	Variable	16/14	[P] Moderate increase of the survival rate of the treated group	[134]
Octreotide	NR	54 mo.	35/0	[P] Significant tumor regression (14%) and clear clinical benefits (80%) in association with VEGF levels	[154]
Octreotide	NR	72 mo.	95/0	[P] Positive results for the group receiving the octreotide treatment	[127]
Octreotide	NR	30 mo.	22/0	[P] Positive results for 6 patients of Asian descent who had hepatitis B-induced cirrhosis	[146]
Octreotide	R	2 years	135/137	[N] No improvement in patient survival rate and negative consequence on patient life quality	[139]
Octreotide+sorafenib	NR	Variable	50/0	[P] Slightly positive results on the survivability of some patients	[148]
Octreotide	R	Variable	21/24	[P] Increase of survival rate of the treated group and significant 1-year survival increase	[133]
Octreotide+sorafenib	NR	Variable	50/0	[P] Reduction of oxidative stress in the treated group, potentially signifying an antineoplastic effect	[153]
Octreotide+heparin	NR	1 year	84/63	[P] Significant reduction in tumor metastasis of the treated group	[150]
Octreotide	NR	5 years	99/0	[P] Higher survivability in patients with higher SSTR expression	[155]
Pasireotide+everolimus	NR	Variable	24/0	[N] No clear benefit from the combination of pasireotide and everolimus was discerned	[151]
Octreotide+celecoxib	R	3 years	35/36	[P] Prolonged overall survival, enhanced tumor response, and reduced postembolization syndrome of the treated patients	[152]
Pasireotide	NR	≤54 mo.	20/0	[N] Limited clinical benefit of pasireotide as a second- or third-line treatment	[141]

Abbreviations: [P]: positive results; [N]: negative results; R: randomized study; NR: nonrandomized study; mo.: months; n/a: not available.

further evaluation of the potential of this pharmacological combination is required [1, 148].

Octreotide was also tested in combination with rofecoxib in the randomized trial of Treiber et al. [149]. Positive results were associated with the IGF and VEGF levels of the patients, indicating the potential antiangiogenetic effect of the applied combination. When octreotide was combined with heparin, in posttranscatheter arterial chemoembolization (TACE)

patients, during the yearly follow-up, the metastasis incidence decreased in the treated patients; the control group had been treated with heparin only [150].

Everolimus and pasireotide were also tested in combination, but no clear benefit could be discerned and a quarter of the treated patients developed hyperglycemia [151]. About 60% of the patients were alcoholics, and treatment discontinuation was brought about by disease progression. Finally, lanreotide and celecoxib were combined with quite positive results; serum VEGF levels were correlated with positive response [152]. The combination of octreotide with sorafenib seemed to reduce serum NO levels, and this is possible evidence of a reduction of oxidative stress, thus signifying a potential antineoplastic effect [153].

Moreover, SSAs were also considered supplementary therapeutic means to surgical interventions in several clinical trials. Montella et al. used octreotide following radiofrequency ablation, with positive results, in inoperable patients [154]. Additionally, Liu et al. tested the effects of octreotide administration, following curative surgery on HCC hepatitis B-positive patients, and determined that survivability was higher in those patients whose HCC cells had high SSTR expression [155]. However, neither of these two studies were randomized nor had a control group; therefore, supplementary data is required for a more accurate assessment of their results. The trials mentioned above are summarized in Table 4.

It should be noted that a number of studies reporting negative results for the use of SSAs were conducted on patients belonging to the most advanced stage of the disease (i.e., BCLC stage D), where survival is very limited and the treatment is usually symptomatic, not contributing to the improvement of life expectancy. Therefore, it appears that SSAs are more suitable for HCC patients belonging to BCLC stages where kinase inhibitors, monoclonal antibodies, immune checkpoint inhibitors, or other new agents are employed, an opinion also shared by other authors [43].

3. Conclusions

Both somatostatin and its analogs have proved useful, in a clinical setting, for the treatment of various malignancies, and their use in oncologic therapy is still a subject of research. From both in vivo and in vitro experiments, it has been determined that both somatostatin and SSAs share apoptotic, antiproliferative, antineoplastic, and anti-inflammatory properties, although some of these effects can be exerted via different mechanisms. A distinct difference is that the antiangiogenic effect has so far been associated only with SSAs.

The results of the clinical trials involving SSAs are mixed, mostly due to the heterogeneity of the trials in regard to design, lot selection, and inclusion criteria. However, the positive reports are encouraging. There seems to be potential use for SSAs, especially considering that many patients may be ineligible for chemotherapy. SSAs may also provide a viable alternative to other emerging therapies for HCC with minor or major side effects where the gravity depends mostly on the specifics of the patient.

Overall, somatostatin and its analogs appear to be promising candidates in the treatment of HCC, and further studies regarding their effectiveness and safety may reveal their definitive role in the multimodal approach of HCC patients.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Argyrios Periferakis, Georgios Tsigas, and Aristodemos-Theodoros Periferakis contributed equally to the work and share first authorship.

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Review Article

The Pivotal Immunomodulatory and Anti-Inflammatory Effect of Histone-Lysine N-Methyltransferase in the Glioma Microenvironment: Its Biomarker and Therapy Potentials

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Enhancer of zeste homolog 2 (EZH2) is a histone-lysine N-methyltransferase that encrypts a member of the Polycomb group (PcG) family. EZH2 forms a repressive chromatin structure which eventually participates in regulating the development as well as lineage propagation of stem cells and glioma progression. Posttranslational modifications are distinct approaches for the adjusted modification of EZH2 in the development of cancer. The amino acid succession of EZH2 protein makes it appropriate for covalent modifications, like phosphorylation, acetylation, O-GlcNAcylation, methylation, ubiquitination, and sumoylation. The glioma microenvironment is a dynamic component that comprises, besides glioma cells and glioma stem cells, a complex network that comprises diverse cell types like endothelial cells, astrocytes, and microglia as well as stromal components, soluble factors, and the extracellular membrane. EZH2 is well recognized as an essential modulator of cell invasion as well as metastasis in glioma. EZH2 oversecretion was implicated in the malfunction of several fundamental signaling pathways like Wnt/β-catenin signaling, Ras and NF-κB signaling, PI3K/AKT signaling, β-adrenergic receptor signaling, and bone morphogenetic protein as well as NOTCH signaling pathways. EZH2 was more secreted in glioblastoma multiforme than in low-grade gliomas as well as extremely secreted in U251 and U87 human glioma cells. Thus, the blockade of EZH2 expression in glioma could be of therapeutic value for patients with glioma. The suppression of EZH2 gene secretion was capable of reversing temozolomide resistance in patients with glioma. EZH2 is a promising therapeutic as well as prognostic biomarker for the treatment of glioma.

1. Introduction

Gliomas are primary brain malignant tumors which are often triggered by malignant modification of neural stem cells, progenitor cells, and differentiated glial cells such as astrocyte, oligodendrocyte, and ependymal cells [1–4]. These lesions are histologically grouped into Grades I-IV according to the World Health Organization (WHO) criteria [4, 5]. Most frequently, Grade I gliomas are detected in children and they mostly have good outcomes [1, 4]. However, Grade II gliomas are often associated with hypercellularity and have a 5-8-year average survival rate [4, 6]. Furthermore, Grade III comprises astrocytoma or anaplastic astrocytoma based on histological classification [4]. They are depicted with hypercellularity, nuclear atypia, and mitotic characters [4]. The anaplastic astrocytoma has a 3-year average survival rate [1, 7, 8]. Glioblastoma multiforme (GBM) comprises Grade IV gliomas [1, 4].

Enhancer of zeste homolog 2 (EZH2) is a histone-lysine N-methyltransferase that encrypts a member of the Polycomb group (PcG) family [9–11]. EZH2 is an enzyme that is encrypted by the EZH2 gene in humans. It is found on chromosome 7q35, and it contains 20 exons as well as 19 introns [9–11]. EZH2 is made up of multimeric protein

complexes as well as associated with the preservation of the transcriptional suppressive state of genes over consecutive cell productions [9–11]. EZH2 is the catalytic subunit of the Polycomb repressive complex 2 (PRC2) which mediates the suppression of target genes that are associated with essential cellular processes via trimethylation of histone H3 on Lys 27 (H3K27me3) [12, 13].

EZH2 forms a repressive chromatin structure which eventually participates in regulating the development as well as lineage propagation of stem cells and glioma progression [12, 14]. Furthermore, EZH2 is involved in glioma initiation and progression as well as in the formation, maintenance, and plasticity of GSCs [15]. Thus, EZH2 may be a possible biomarker and therapeutic target in gliomas as well as in the development of novel treatment schemes that target both the genetic and epigenetic mechanisms of gliomagenesis. Further, the up- and downregulation of the EZH2 in *in vitro* as well as *in vivo* studies may be of a diagnostic as well as therapeutic biomarker in glioma treatment.

This review explores the fundamental immune and inflammatory players regulated by EZH2. The "Boolean logic" was used to search for articles on the subject matter. Most of the articles were indexed in PubMed and PMC with strict inclusion criteria being the immunomodulatory and anti-inflammatory effect of EZH2 in the glioma microenvironment which may be of biomarker and therapeutic importance. Search parameters were EZH2 and/or the posttranslational modifications, microenvironment, signaling pathways, biomarker, and therapy in gliomas.

2. Polycomb Group

PcG proteins are fundamental epigenetic modulators which constitute transcriptional repressors as well as crucial modulators of cell fate in cancer development [16, 17]. PcG proteins initiate their repressive actions via the formation of two distinctive protein multimeric complexes such as PRC1 and PRC2 in mammals [16, 18]. The PRC1 configuration is usually inconstant, and the mammalian core PRC1 is made up of B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), ring finger protein (RING) 1 proteins such as RING1A and RING1B, chromobox (CBX), polyhomeotic (PH) proteins like PH1 and PH2, nervous system Polycomb 1 (NSPC1), or Polycomb group ring finger (Pcgf) 1 and Pcgf2 (MEL18) proteins [16]. The core subunits of mammalian PRC2 are often made up of EZH2 or EZH1, embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12), retinoblastoma protein-associated protein 46/48 (RbAp46/48), AE binding protein 2 (AEBP2), Polycomblikes (PCLs), and Jumonji and AT-rich interaction domain containing 2 (JARID2) [16, 19].

3. EZH2

EZH2 is the catalytically active domain of the PRC2 complex that partakes in transcriptional repression of precise genes via trimethylation of lysine 27 and, to a slighter extent, lysine 9 of histone H3 [20, 21]. EZH2 is a highly maintained histone methyltransferase (HMTase) which is capable of stimulating H3K27me3 as well as inhibiting transcription and secretion of target genes mediating several fundamental biological processes such as cell cycle modulation, cell fate assessment, senescence, cell proliferation, differentiation, apoptosis, and glioma progression [16, 17, 22].

Anomalous EZH2 secretion was extensively associated with a comprehensive array of aggressive as well as metastatic malignancies with poor outcomes because it was a core epigenetic modulator [16, 23, 24]. Studies have demonstrated that EZH2-mediated H3K27me3 acts as a docking location for PRC1 chromodomain-containing protein CBX as well as accelerates the preliminary recruitment of PRC1 that catalyzes H2AK119ub to conserve a repressed state of target genes [16, 25–28]. This therefore indicates a common as well as classic modulatory model that PRC1 functions downstream of PRC2 [16].

The EZH2-mediated methyltransferase complex in the cytoplasm was capable of stimulating actin polymerization, cellular adhesion, and migration resulting in glioma dissemination [29, 30]. Studies have established that 3-deazaneplanocin A (DZNep), S-adenosylhomocysteine-(SAM-) competitive inhibitors like GSK343, GSK126, and EPZ-6438, and the stabilized α-helix of EZH2 peptide (SAH-EZH2) are the three types of potent EZH2 inhibitors [31–33]. The SAH hydrolase inhibitor DZNep triggers the buildup of SAH resulting in a by-product blockade of the SAM-dependent methyltransferase action like EZH2 [31–33].

SAM is the general methyl donor for HMTase reaction; SAM-competitive inhibitors are the key routes for EZH2 inhibition due to their high selectivity for EZH2 [31, 32]. Yu et al. established that the introduction of GSK343 in glioma cells expressively reduced H3K27 methylation as well as coprecipitation with EZH2-H3 in a time-dependent manner and decreased the quantities of core units of PRC2 [31]. Furthermore, GSK343 treatment in normal glioma cells not only reduced the protein quantities of EZH2 but also downregulated the secretion of c-MYC [31].

4. Posttranslational Modifications

Posttranslational modifications (PTMs) are covalent processing actions that transform the structure and function of a protein via the proteolytic cleavage as well as addition of a modifying group, like acetyl, glycosyl, methyl, and phosphoryl, to one or more amino acids [16, 34, 35]. PTMs are often revisable or irreversible, and they participate in several critical biological processes by expressively affecting the structure as well as dynamics of proteins [16, 35]. PTMs often influence several protein behaviors as well as characteristics such as enzyme function and assembly, protein-protein interactions, protein lifespan, protein solubility, protein folding, protein localization, cell-cell as well as cell-matrix interactions, molecular trafficking, and receptor activation [16, 35].

PTMs are distinct approaches for the adjusted modification of EZH2 in the development of cancer [16]. E2 factors (E2Fs) are capable of binding to the promoter of EZH2 resulting in the transactivation of its secretion at the

transcriptional level [16, 36]. The amino acid succession of EZH2 protein makes it appropriate for covalent modifications, like phosphorylation, acetylation, O-GlcNAcylation, methylation, ubiquitination, and sumoylation [37]. Thus, the most studied EZH2 PTMs include acetylation, phosphorylation, ubiquitination, sumoylation, and O-GlcNAcylation [16, 38–42].

Acetylation is a reversible and key type of PTM which involves the modulation of gene secretion primarily via the regulation of core histone tails by histone acetyltransferases (HATs) or histone deacetylases (HDACs) (Figure 1) [16, 43, 44]. Acetylation influences a series of cellular processes such as proliferation, apoptosis, differentiation, metabolism, and transcriptional modulation [45, 46]. Wan et al. demonstrated that EZH2 was acetylated by acetyltransferase P300/CBP-associated factor (PCAF) and was deacetylated by deacetylase SIRT1 (Figure 1) [39]. It was further established that PCAF was capable of interrelating with EZH2 resulting in the acetylation of EZH2 mainly at lysine 348 (K348) which triggers a reduction in EZH2 phosphorylation at T345 as well as T487 and augments EZH2 stability without either altering its interaction with other PRC2 complex members such as SUZ12 and EED or influencing its site and HMTase activity (Figure 1) [16].

O-GlcNAcylation refers to protein glycosylation with β-N-acetyl-D-glucosamine which is a reversible as well as a dynamic PTM activity universally observed in both the cytosol and the nucleus [47, 48]. It was established that EZH2 was capable of interrelating substantially with OGT as well as OGT-dependent O-GlcNAcylation of EZH2 at serine 75 (S75) which was necessary for the conservation of EZH2 protein stability and successive formation of H3K27me3 resulting in tumorigenesis (Figure 1) [42]. It was further demonstrated that O-GlcNAcylation of EZH2 at S75 inhibits phosphorylation at the same location obligatory for EZH2 degradation or shields EZH2 from other modifications at other locations that are advantageous for EZH2 degradation [16].

Phosphorylation normally transpires when protein kinases insert phosphate groups in an ATP-dependent approach to serine (Ser), threonine (Thr), tyrosine (Tyr), and histidine (His) residues of substrates, which triggers a conformational modification in the structure of several proteins resulting in their activation or deactivation and thus creating differences in the biological properties of their targets as well as binding affinities [49, 50]. Studies have demonstrated that phosphorylation of EZH2 at serine 21 (pS21 EZH2) was possible under different conditions. Signal transducer and activator of transcription (STAT) 3 S27 phosphorylation was capable of triggering arsenic- (As^{3+}-) mediated growth stimulation via JNK pathways which resulted in AKT activation via upregulation of the negative AKT modulator miR-21 leading to pS21 EZH2 as well as oncogenesis (Figure 1) [16, 51, 52].

As^{3+}-stimulated pS21 EZH2 was principally localized in the cytoplasm contrary to the notion that EZH2 was mainly a nuclear protein (Figure 1) [16, 51, 52]. Studies are needed on the role of As^{3+} initiation of AKT-dependent pS21 EZH2 via the stimulation of the JNK-STAT3-AKT signaling axis in glioma. Chen et al. demonstrated that EZH2 contains one perfectly matched (Thr350) and two imperfectly matched (Thr421 as well as Thr492) CDK phosphorylation motifs (K(R)S(T)PXK(R)) which are extremely evolutionally preserved from fruit flies to humans [16, 53]. Furthermore, mutation of Thr350 to alanine (T350A) led to about 60% decrease in CDK1-mediated EZH2 phosphorylation, while only about 30% or no decrease in phosphorylation was detected in T421A as well as T492A mutants which signify that Thr350 was a major CDK-mediated phosphorylation site [53].

It was established that JAK2-stimulated phosphorylation at EZH2 Y641 (pY461 EZH2) triggered EZH2-β-TrCP intercommunication resulting in β-TrCP-mediated EZH2 ubiquitination as well as proteasomal degradation which triggered downregulation of EZH2 protein stability as well as H3K27me3 hypoactivity, signifying phosphorylation-dependent EZH2 ubiquitination [16, 54, 55]. In ubiquitination, Ub covalently attaches to the modified proteins and modulates their stability and functions as well as localizes their involvement in several cell functions as well as diseases, expressly in cancer development [54, 55].

Ubiquitination transpires via the stimulation of a cascade of enzymatic reactions dependent on three obligatory enzymes like ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (Figure 1) [16, 54, 55]. Microarray studies of U87MG glioma cells after EZH2 silencing revealed a robust transcriptional reduction of the AXL receptor kinase [56]. Histone modification was associated with the positive modulation of AXL by EZH2 [56, 57]. The knockdown of AXL imitated the anti-invasive properties of EZH2 silencing, and AXL secretion was detected in human gliomas with elevated EZH2 secretion [56, 57].

Studies have demonstrated that SMAD ubiquitination regulatory factor-2 (SMURF2), β-TrCP (FBXW1), Casitas B-lineage lymphoma (c-Cbl) protein, and PRAJA1 function as dynamic EZH2 ubiquitin E3 ligases (Figure 1) [40, 58–60]. Ub E3 ligase PRAJA1 triggered ubiquitination-proteasome pathway-mediated EZH2 protein degradation [59]. Also, SMURF2 interacts with EZH2 resulting in the stimulation, ubiquitination, and proteasome-mediated degradation of EZH2 at lysine 421 leading to upregulation of its target gene PPARγ (Figure 1) [40]. A recent study established that EZH2 acts as a substrate for Skp/cullin/F-box protein (SCF) and ubiquitin E3 ligase β-TrCP (Figure 1). Furthermore, EZH2 was expressly interrelated with β-TrCP resulting in β-TrCP-mediated EZH2 ubiquitination [58].

Sumoylation is an extremely preserved enzymatic cascade in which a tiny ubiquitin-like modifier (SUMO) protein is enzymatically conjugated to the ε-amino group of certain lysine residues [61]. Sumoylation was primarily authenticated to be linked to the modulation of EZH2 activity (Figure 1) [41]. It was established that EZH2 had several SUMO-modified locations or diverse configurations of sumoylation on the same location because EZH2 displayed several bands of modifications in both western blot analysis and *in vitro* sumoylation assay [16].

DNA methylation is an enzyme-mediated chemical modification of DNA by the insertion of a methyl group

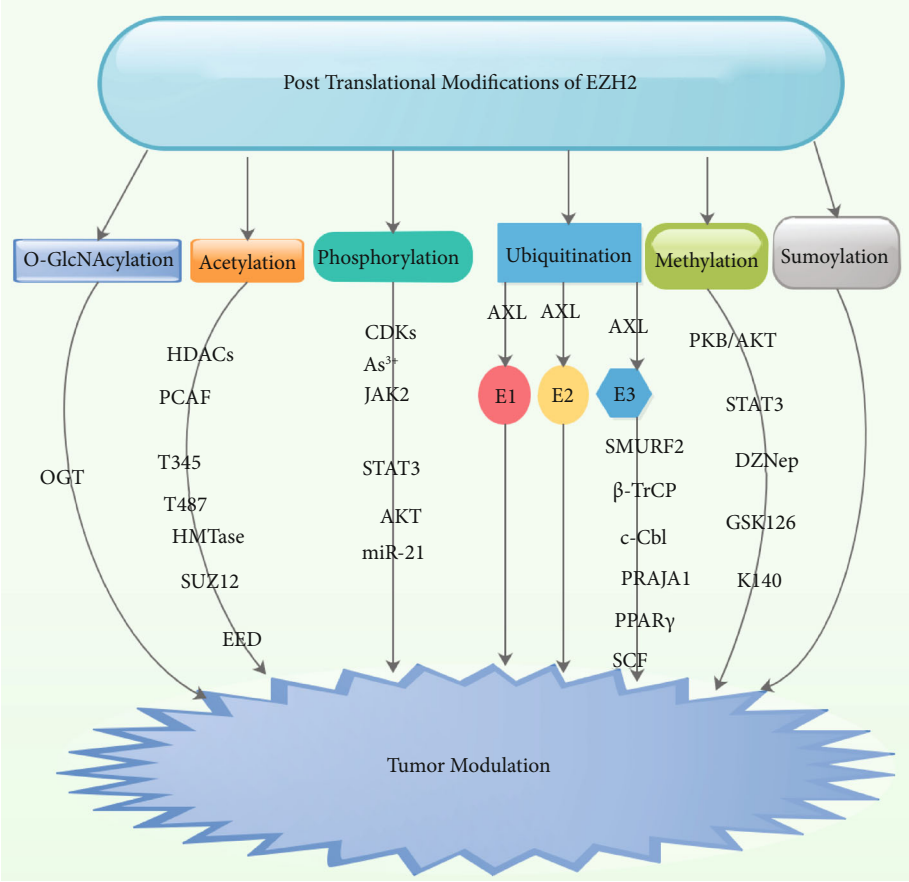

Figure 1: The posttranslational modifications of EZH2 and cascades.

from S-adenosyl-L-methionine substrates to the 5-position of cytosine (5-methylcytosine (5mC)) [56, 62]. It was established that DNA methylation can directly modulate gene secretion by repressing the binding of fundamental transcription factors as well as by indirectly recruiting methyl-CpG-binding domain (MBD) proteins to the promoter gene (Figure 1) [56]. Studies have demonstrated that EZH2 was capable of modulating oncogenic gene secretion by mediating the DNA methylation level [9, 63]. Furthermore, EZH2 was capable of methyltransferase activity and the oversecretion of EZH2 was capable of modulating transcription of downstream genes via DNA methylation [20].

Protein kinase B (PKB)/AKT-induced pS21 EZH2 was capable of accelerating EZH2-STAT3 intercommunication (Figure 1), stimulated EZH2-mediated STAT3 methylation, and augmented STAT3 activity in glioblastoma multiforme (GBM) stem-like cells (GSCs) [64]. This indicates that the AKT-pS21 EZH2-STAT3 signaling axis is a prospective modulator of GSC tumor malignancy and an auspicious therapeutic target for GBM [64]. Furthermore, EZH2 binds to and methylates STAT3, leading to augmented STAT3 activity via upregulation of tyrosine phosphorylation of STAT3 [64].

Kim et al. demonstrated that the EZH2 blockade significantly reduced universal levels of H3K27 trimethylation and p-STAT3 in GSCs [64]. Also, p-STAT3 in GSCs was precipitously reduced when either DZNep or GSK126 was introduced (Figure 1) [64]. They detected oversecretion of EZH2 S21 stimulated STAT3 methylation as well as augmented STAT3 activity [64]. They concluded that EZH2 S21 phosphorylation was a molecular switch that accelerates STAT3 methylation [64]. Yang et al. demonstrated that K140 methylation of STAT3 destabilizes STAT3 tyrosine phosphorylation resulting in a negative influence on STAT3-dependent transcription [65].

It was further established that K140 methylation functions as a negative influence in the STAT3 signaling cascade which is directly opposite to K180 STAT3 methylation by EZH2 [64]. Ott et al. indicate that EZH2 stimulates transcription of AXL mRNA in a methylation-independent manner [57]. It was established that H3K27M blocked the enzymatic action of the PRC2 via communication with the EZH2 subunit [56]. Transgenes comprising lysine-to-methionine substitutions at other known methylated lysines like H3K9 and H3K36 are adequate to trigger a specific decrease in methylation via the blockade of SET-domain enzymes [56].

5. EZH2 and Glioma Microenvironment

The glioma microenvironment is a dynamic component that comprises, besides glioma cells and GSCs, a complex network that comprises diverse cell types like endothelial cells, astrocytes, and microglia as well as stromal components,

soluble factors, and the extracellular membrane (ECM) [2, 66–68]. Furthermore, glycolytic metabolism was almost 3 times higher in normal brain tissue compared to GBM and was modulated by oncogenes like phosphoinositide 3-kinase (PI3K), AKT, and hypoxia-inducible factor 1 (HIF1) [69, 70]. Studies have shown that BGB324 and BMS-777607 are targets for AXL that suppressed multiple malignant activities like growth, migration, and invasion in GBM [71–73].

Ott et al. demonstrated that the blockade of EZH2 decreased glioma cell proliferation as well as invasiveness [57]. They indicated that EZH2 triggers glioma invasiveness via transcriptional regulation of AXL (Table 1) [53]. Yen et al. revealed that n-butylidenephthalide (BP) targeting AXL (Table 1) decreased brain tumor migration and invasion as well as prolonged animal survival in orthotic GBM animal models [71]. They further disclosed that BP was capable of downregulating EZH2 secretion and inhibiting the secretion of AXL in a dose-dependent manner of GSCs [71]. Jin et al. demonstrated that EZH2 influenced hypoxia, acidic stress, and nutrient restriction which promoted GSC maintenance (Table 1) [74]. Moreover, the RNF144A-BMI1 regulatory mechanism was capable of empowering GSCs to reside in stressful microenvironments [74].

Studies have shown that BMI1 was capable of modulating tumor induction and growth in a genetically engineered murine model of GBM as well as human stem-like glioma lines [74–77]. Also, BMI1 binds and modulates the promoters of several genes, including TGF-β, which was intensely associated with the mesenchymal phenotype [74, 78]. GBM cells are capable of experiencing molecular subtype transitions under the influence of a diverse tumor milieu resulting in diverse consequences between the *in vitro* and *in vivo* experiments by BMI1 and EZH2 inhibitor administration (Table 1) [74]. Studies have shown that STAT3 signaling was capable of modulating mesenchymal transformation of gliomas [64]. Furthermore, STAT3 downstream genes were extremely secreted in the mesenchymal GBM subtype [64, 79].

Studies further revealed that INK4B-ARF-INK4A, which encodes three distinct proteins, $p15^{INK4b}$, $p14^{ARF}$, and $p16^{INK4a}$, p57, bone morphogenetic protein receptor 1B (BMPR1B), MyoD, and RUNX3 are all negatively modulated by EZH2 (Table 1) [69, 80, 81]. Liu et al. also established that the E-cadherin gene (CHD1) (Table 1) is associated with epithelial-mesenchymal transition (EMT), invasion, and migration in an essential inhibitory target of EZH2 [82]. Furthermore, molecules like BIM, TNF-related apoptosis-inducing ligand (TRAIL), and FBO32 which are associated with apoptosis are suppressed by EZH2 (Table 1) [83, 84]. Lu et al. demonstrated that Vasohibin1, a molecule meticulously linked to tumor angiogenesis, was also suppressed by EZH2 [85]. Zhou et al. demonstrated that EZH2 and MICU1 were obligatory in conserving mitochondrial membrane potential stability (Table 1). Also, they were capable of modulating tumor growth via regulation of a mitochondrial-dependent cell-death pathway [86].

Pang et al. discovered that oxygen consumption rates were decreased in knockdown EZH2 GBM cells (Table 1), which indicates a deficiency in the TCA cycle [69]. Furthermore, oversecretion of EZH2 exerted a negligible influence on mitochondrial oxidative capacity [69]. However, the oversecretion of EZH2 triggered glycolytic metabolism which resulted in a significant increase in cellular deoxyglucose uptake as well as the activities of key enzymes associated with glycolysis and lactate production (Table 1) [69]. Thus, EZH2 was capable of modulating the Warburg effect in GBM [69].

Table 1: Immune/inflammatory factors and their influential effects on EZH2.

Immune/inflammatory factor	Effect of factors on EZH2 at a tumor milieu	Citations
Nutrients	Inhibitory	74
Hypoxia	Inhibitory	74
Acidic stress	Inhibitory	74
AXL	Inhibitory	53
n-Butylidenephthalide	Inhibitory	71
STAT3	Inhibitory	64, 79
INK4B-ARF-INK4A	Inhibitory	69, 80, 81
p57	Inhibitory	69, 80, 81
BMPR1B	Inhibitory	69, 80, 81
MyoD	Inhibitory	69, 80, 81
RUNX3	Inhibitory	69, 80, 81
CHD1	Inhibitory	82
TRAIL	Inhibitory	83, 84
FBO32	Inhibitory	83, 84
Vasohibin1	Inhibitory	85
MICU1	Inhibitory	86
Oxygen (O_2)	Facilitatory	69
Deoxyglucose	Facilitatory	69
HIF1α	Facilitatory	69
PHD1-3	Facilitatory	69
EAF2	Inhibitory	69
siRNA	Inhibitory	87
DZNep	Inhibitory	87
GSK343	Inhibitory	31
CDKN2A	Inhibitory	12, 93
BRAF V600E	Facilitatory	93
iNOS	Inhibitory	94
TNF-α	Inhibitory	94
HCMV	Facilitatory	95
BMI1	Facilitatory	74

Pang et al. further observed that exogenous oversecretion of EZH2 augmented HIF1α secretion under normoxia (Table 1) [69]. HIF1α modulation typically depends on oxygen-dependent protein stability [69]. It was established that HIF1α was hydroxylated by a family of oxygen-dependent prolyl hydroxylases (PHD1-3) resulting in the binding of pVHL to HIF1α for ubiquitination as well as proteasomal degradation under normoxic conditions (Table 1) [69]. Pang et al. also detected that the secretion of tumor suppressor protein EAF2 was repressed by EZH2 because

the depletion of EZH2 correlated with the stimulation of EAF2 (Table 1) [69].

Wang et al. observed that EZH2 was crucial for glioma cell aerobic glycolysis [87]. They detected that the blockade of EZH2 activity by siRNA as well as DZNep reduced the magnitude of glycolysis under basal conditions (Table 1), the glycolytic capacity, and the glycolytic reserve [87]. Yu et al. established that GSK343, a blocker of EZH2 (Table 1), inhibits the proliferation, invasion, and cancer stem-like phenotypes and reverses mesenchymal transition of glioma cells *in vitro* as well as *in vivo* [31]. CDKN2A is a tumor suppressor gene that encodes for p16 protein and functions as a cellular senescence as well as a negative modulator of cell cycle progression. Several studies have detected CDKN2A deletion/loss of p16 protein secretion in high-grade gliomas [12, 88–90].

Purkait et al. detected that about 80% of samples with p16 loss with deficiency of CDKN2A homozygous deletion exhibited robust EZH2 secretion [12]. They suggest that EZH2 mediated downregulation of p16 secretion in the samples [12]. The mechanism of the EZH2-mediated blockade of p16 secretion was a result of the repressive chromatin mark H3K27me3 triggering the recruitment of the PRC1 complex. Studies have demonstrated that p16 acts as a negative modulator of cell cycle progression from the G1 phase to the S phase [91, 92]. Thus, high-grade glioma cells may elude the p16 cell cycle checkpoint either via the homozygous deletion of CDKN2A or via the EZH2-mediated transcriptional knockout of p16 protein secretion which means that loss of p16 protein secretion augments proliferative activity (Table 1) [12].

Wang et al. demonstrated that EZH2 staining was expressively dissimilar in epithelioid cells as well as low-grade sections of five biphasic epithelioid glioblastoma (EGBM) samples [93]. They further observed robust EZH2 secretion in epithelioid cells with a high Ki67 index but not in low-grade lesions which suggested that EZH2-positive cells are associated with intratumoral heterogeneity as well as the malignant progression of the tumor [93]. They also observed a coexistence of robust EZH2 secretion, BRAF V600E, and CDKN2A/B deletions in EGBM samples, but no negative correlations between robust EZH2 secretion and CDKN2A/B deletions were detected (Table 1) [93].

Yin et al. established that the EZH2 blockade in GBM stimulation promoted the elevation of M1 markers like iNOS and TNF-α as well as the decrease in a pool of M2 markers in murine microglia and human PBMC-derived macrophages (Table 1) [94]. They further observed that the EZH2 blockade in GBM cells augmented the phagocytic capabilities of cocultured microglia via the stimulation of iNOS [94]. Mechanic studies revealed that the knockdown of EZH2 blocked the secretion of anti-inflammatory factors while promoting the secretion of proinflammatory factors in GBM cells [94]. The EZH2 blockade in GBM facilitated the polarization shift of microglia as well as PMMC-derived macrophages resulting in an upsurge of M1 markers and a decrease of M2 markers [94].

Ahani et al. demonstrated that human cytomegalovirus (HCMV) gene products are capable of facilitating the PI3K/AKT pathway which was associated with apoptosis, angiogenesis, invasion, and immune evasion resulting in tumor growth [95]. They further established that HCMV was capable of facilitating the progression of GBM via upregulation in the secretion of the EZH2 gene because they observed oversecretion of EZH2 in HCMV-positive GBM models compared to HCMV-negative GBM models (Table 1) [95]. Bioinformatics analysis revealed that miR-133b was capable of influencing the EZH2 gene in glioma. EZH2 was aberrantly secreted in glioma as well as contributed to the invasive and metastatic capabilities of GBM [71].

6. EZH2 Signaling Pathways in Glioma

EZH2 oversecretion was implicated in the malfunction of several fundamental signaling pathways like the wingless-related integration site (Wnt)/β-catenin signaling, rat sarcoma (Ras) and NF-κB signaling pathways, PI3K/AKT pathway, β-adrenergic receptor signaling, and bone morphogenetic protein (BMP) as well as NOTCH signaling pathways in cancers [16, 96–99]. The Wnt/β-catenin signaling pathway partakes in the development of the central nervous system and is linked to oncogenesis in many cancers [100, 101]. It was established that downstream of EZH2 had inhibitory effects on glioma growth via the blockade of the β-catenin signaling pathway [87, 100]. Also, the Wnt/β-catenin signaling pathway was associated with GBM progression [100, 102].

Domenis et al. demonstrated that exosomes derived from mesenchymal stem cells (MSCs) were capable of facilitating glioma development [103]. Xu et al. demonstrated that MSC-derived exosomes oversecreting miR-133b were capable of blocking the progression of glioma via the EZH2-Wnt/β-catenin signaling pathway [100]. They indicated that MSC-derived exosomal miR-133b was capable of blocking glioma cell proliferation, invasion, and migration as well as tumor growth via the suppression of EZH2 through the blockade of the Wnt/β-catenin signaling pathway in *in vitro* as well as *in vivo* experiments (Figure 2) [100]. The EZH2-miR-328/β-catenin signaling cascade could act as an innovative therapeutic biomarker for glioma. Also, inhibition of EZH2 was linked to the suppression of glioma growth via the inhibition of the β-catenin signaling pathway (Figure 2) [87, 100].

Chen et al. demonstrated that the EGFR/NEAT1/EZH2/β-catenin axis in GBM bestowed an oncogenic activity in GBM that is of novel therapeutic potential (Figure 2) [104]. Wang et al. detected that miRNAs like miR-1224-3p, miR-328, and miR-214 are repressed by EZH2 which was also modulated by β-catenin secretion via its 3′UTR in gliomas (Figure 2) [87]. They indicated that miR-328 served as a tumor inhibitor by abolishing EZH2 activities on glucose metabolism in glioma cells [87]. They further identified an EZH2/miRNA/β-catenin feedforward loop associated with the oversecretion of EZH2, β-catenin, and miRNA repression in glioma glucose metabolism (Figure 2) [87]. Juan et al. demonstrated that miR-214 negatively modulated EZH2 secretion by targeting the EZH2 3′UTR [105]. Thus, EZH2 and miR-214 form a modulatory loop controlling PcG-dependent gene secretion [87].

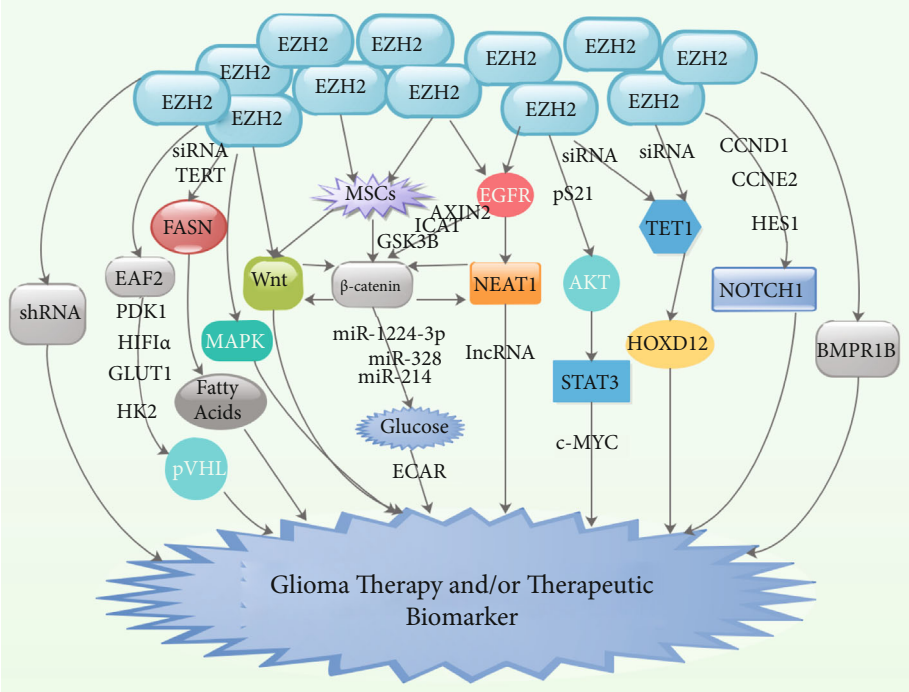

Figure 2: Signaling pathways via which EZH2 modulates the glioma microenvironment.

AKT-stimulated pS21 EZH2 was capable of accelerating EZH2-STAT3 intercommunication and augmenting EZH2-mediated methylation as well as activities of STAT3 resulting in the facilitation of GSC self-renewal as well as GBM tumor progression (Figure 2) [38]. It was demonstrated that EZH2 was capable of interacting with DNA methyltransferases (DNMTs) resulting in an influence on DNMT activity [56]. Furthermore, the binding of DNMTs to respective EZH2-repressed genes depended on the existence of EZH2 [56]. Cartron et al. demonstrated that EZH2 downregulation influenced the ten-eleven translocation 1 (TET1)/EZH2 intercommunications in U251 cells [106]. They further demonstrated that downregulation of EZH2 reduced TET1 recruitment on the HOXD12 genes [106]. They indicated that reduction of TET1/EZH2 recruitment on the HOXD12 gene in cells treated with siRNA-EZH2 generated an upsurge in methylation of the HOX12D gene (Figure 2) [106]. They concluded that EZH2 functions as an anchor for TET1 recruitment on the HOXD12 genes [106].

Chen et al. exhibited that administration of melatonin expressively influenced sphere morphology, EZH2-STAT3 intercommunications, and STAT3 activity in AKT1-oversecreted GSCs (Figure 2) [107]. They indicated that melatonin was capable of influencing the AKT-EZH2-STAT3 signaling axis resulting in robust impairment of GSC self-renewal as well as cancer-initiating capacity. Also, administration of melatonin blocked EZH2 S21 phosphorylation as well as EZH2-STAT3 intercommunication [107]. However, AKT1 oversecretion annulled this effect which means that AKT was a fundamental downstream effector of melatonin in GSCs.

A study established that PI3K/AKT signaling was augmented in about 90% of GBM samples [108]. Chen et al. observed that AKT oversecretion distinctly augmented EZH2 S21 phosphorylation concentrations, EZH2-STAT3 intercommunications, and STAT3 activity but downregulated H3K27me3 concentrations [107]. Thus, AKT-STAT3-EZH2 signaling and EZH2 phosphorylation participated in cancer stem cell (CSC) growth as well as carcinogenesis, and administration of melatonin blocked EZH2 S21 phosphorylation as well as EZH2-STAT3 intercommunications and modulated histone modifications resulting in the blockade of tumor initiation as well as propagation [107]. Yu et al. discovered that EZH2 participated in glioma tumor progression via EZH2-STAT3-c-MYC dependent pathways (Figure 2) [31].

Kim et al. established that the blockade of AKT signaling reduced STAT3 activity via EZH2 phosphorylation, indicating that PI3K/AKT signaling was an upstream modulator of the EZH2-STAT3 intercommunication in GSCs (Figure 2) [64]. They revealed that EZH2 S21 phosphorylation was obligatory for the EZH2-STAT3 intercommunication as well as augmentation of STAT3 activity, and the AKT blockade *in vivo* essentially stopped pS21 EZH2 (Figure 2) [64]. Thus, pS21 EZH2 secretion is a potential therapeutic biomarker via the PI3K/AKT axis [64]. Studies have shown that stimulation of the PI3K/AKT pathway in glioma was linked to an unfavorable clinical prognosis when various PI3K/AKT inhibitors were subjected to clinical trials [64, 108].

Zheng et al. established that melatonin extremely decreased NOTCH1 and other NOTCH1 signaling pathway components like CCND1, CCNE2, and HES1, which are modulated by NOTCH1 in GSCs (Figure 2) [109]. They observed that the active NOTCH1 protein portion and NOTCH intracellular domain 1 (NICD1) were deregulated, signifying that NOTCH1 was capable of mediating the

activities of EZH2 upon administration of melatonin [109]. They further revealed that EZH2 modulated NOTCH1 secretion by directly interrelating with the NOTCH1 promoter [109]. They emphasized that a substantial correlation in the secretion of EZH2 and NICD1 was detected in tumor samples from GBM patients, signifying the existence of the EZH2-NOTCH1 signaling pathway in malignant gliomas [109].

Natsume et al. showed that biological interconversion between GSCs and differentiated non-GSCs correlated with the gain or loss of EZH2/PRC2-mediated H3K27me3 on pluripotent or development-related genes like NANOG, Wnt1, and BMP5 [110]. Pang et al. detected a substantial inverse correlation in the secretion of EZH2 and EAF2 which signifies that EAF2 influenced EZH2 activities [69]. Furthermore, EAF2 binds to and stabilizes pVHL resulting in a disruption of the HIF1α-mediated hypoxia signaling pathway (Figure 2) [111]. H3K27ac coinhibition augmented the efficacy of EZH2 inhibitors and also triggered the MAPK pathway in some cancers, signifying that the blockade of EZH2 resulted in a feedback stimulation of certain signaling pathways in a context-dependent manner (Figure 2) [112, 113].

NF-κB is a major modulator of fundamental cell processes like inflammation, proliferation, and apoptosis [112, 114]. Several stimuli, comprising inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β, which induce the classical pathway, are capable of triggering the NF-κB signaling [112, 115]. It was established that SAH was able to trigger the stimulation of the NF-κB pathway resulting in endothelial dysfunction as well as stimulation by partially blocking the enzymatic activity of EZH2 [112, 116]. Jiang et al. implicated SOX9 as a fundamental downstream target of EZH2 in rat cells [117]. Also, the blockade of EZH2 reduced the concentration of H3K27me3 at the SOX9 promoter region as well as augmented SOX9 secretion in rat endplate chondrocytes (EPCs) [112, 117].

Min et al. demonstrated that EZH2 triggered Ras and NF-κB by epigenetically inhibiting DAB2IP, which stimulates the molecular mechanism through which an epigenetic modulator triggers these two major signaling pathways [26]. Studies on EZH2-Ras-NF-κB signaling pathways in glioma are warranted. ADRB2 is a G protein-coupled receptor (GPCR) of the β-adrenergic signaling pathway. Yu et al. demonstrated that ADRB2 is a target for EZH2-mediated transcriptional repression [96]. Nevertheless, no studies on EZH2-ADRB2 signaling in glioma exist [96]. Thus, studies on EZH2-ADRB2 signaling in glioma are warranted. The hallmark of cAMP/β-adrenergic signaling is its capability of blocking cell proliferation in certain types of cells while activating cell growth in others [118]. Studies on EZH2-β-adrenergic receptor signaling in glioma are needed.

7. EZH2 as a Biomarker in Glioma

EZH2 is well recognized as an essential modulator of cell invasion as well as metastasis in glioma [71, 100]. It was established that EZH2 was more secreted in GBM than in low-grade gliomas as well as extremely secreted in U87 human glioma cells [42]. Orzan et al. demonstrated that EZH2 was upregulated in malignant gliomas [119]. They established that EZH2 secretion was 26.62 ± 19.90-fold elevated in 57 GBM specimens compared to normal brains [119]. They also evaluated EZH2 levels in nine low-grade gliomas and detected that the secretion of EZH2 was 4.26 ± 2.90-fold elevated compared to normal brains, which was significantly lower than that in GBM [119]. This signified that EZH2 secretion was linked to glioma malignancy [119].

Ott et al. observed a robust EZH2 secretion in GBMs while the secretion was low in Grade III astrocytoma as well as absent in Grade II astrocytoma [57]. They indicated that the knockdown of EZH2 suppressed glioma cell proliferation as well as invasiveness, and it also suppressed AXL receptor kinase secretion [57]. Wu et al. also detected elevated secretion of EZH2 in gliomas and suggested that EZH2 participated in the modulation of glioma development [120]. It was established that BMI1 and EZH2 secretion in glioma tissues were expressively elevated compared to those in nonneoplastic brain tissues [56]. Furthermore, upregulations of BMI1 and EZH2 proteins were both expressively associated with advanced WHO grades as well as low Karnofsky [56].

Wu et al. observed that the overall survival of patients with elevated BMI1 protein secretion or elevated EZH2 protein secretion was apparently lower than those with low secretions [120]. It was established that EZH2 was capable of stimulating aerobic glycolysis in tumors [121, 122]. Also, EZH2 was capable of switching mitochondrial respiration to glycolysis *in vitro* by augmenting the level of H3K27me3 at EAF2 promoter areas in GBM cells [121, 122]. This inhibited the transcription of EAF2 as well as triggered the HIF1α signaling pathway resulting in the transcription of downstream genes like HK2, glucose transporter 1 (GLUT1), and PDK1 which are associated with metabolism (Figure 2) [121, 122]. Thus, EZH2 was capable of accelerating tumorigenesis as well as the malignant progression of tumor cells via the stimulation of the Warburg effect [122].

Specifically, the blockade of EZH2 activity inhibited aerobic glycolysis in glioma cells [121, 123]. It was discovered that the glycolytic capability and reserve were both reduced when the concentrations of EZH2 are reduced in U87 as well as U251 glioma cells [121, 123]. EZH2 was capable of binding to the miR-328 promoter and downregulating miR-328 via a recognized H3K27me3 modification fashion [123]. Furthermore, miR-328 was also capable of blocking the secretion of β-catenin [121, 123]. Also, the EZH2/miRNA/β-catenin pathway triggered an upsurge in the extracellular acidification rate (ECAR) resulting in an augmentation in the glycolytic capability (Figure 2) [121, 123]. It was revealed that telomerase reverse transcriptase (TERT) and EZH2 jointly stimulated PCG-1α resulting in the secretion of fatty acid synthase (FASN) in glioma having (TERT) promoter mutations [121, 124].

Higher EZH2 concentrations in TERT mutants participated in gliomagenesis via epigenetic reprogramming of H3K27me3 modification marks because EZH2 silencing influenced not only TERT secretion but also lipid metabolism [121, 124]. Also, the pharmacological blockade of human TERT

repressed the secretion of EZH2 as well as FASN and reduced the buildup of fatty acids [121, 124]. Nevertheless, reduced secretory levels of TERT as well as FASN and decreased levels of intracellular fatty acids were detected upon siRNA-mediated EZH2 silencing (Figure 2) [121, 124]. Thus, EZH2 endorses the synthesis of fatty acid as well as lipid buildup through the TERT-EZH2 pathway (Figure 2) [121, 124].

Fan et al. demonstrated that in experiments involving EZH2 siRNA and controls, the percentage of cells in the G1 phase exhibited a steady rising trend, while the percentage of cells in the S, G2, and M phases reduced concordantly, implying G1 arrest [9]. It was established that EZH2 siRNA was capable of blocking the progression of the cell cycle via the inhibition of transition from the G1 phase to the S and G2 phases [9, 56]. Furthermore, the silencing of EZH2 secretion by using RNA interference in U87 human glioma cells triggered apoptosis and cell cycle arrest in the G0/G1phase [9, 56]. Also, the knockdown of EZH2 modified the mitochondrial membrane potential as well as endorsed the expression of cytochrome c from the mitochondria [9, 56].

Zhang et al. established that decreased secretion of EZH2 modified Bax as well as Bcl-2 protein levels and triggered the stimulation of caspase 9 and caspase 3 [125]. Smits et al. demonstrated that the blockade of EZH2 *in vivo* by systemic DZNep treatment in a U87-Fluc-mCherry GBM xenograft mouse imaging model led to inhibition of tumor growth [126]. Wang et al. demonstrated that lncRNA transcribed from the 5-prime end of the HOXA transcript HOXA11-AS participated in the malignant progression of GBM [127]. Chen et al. established that nuclear enriched abundant transcript 1 (NEAT1) was a preserved lncRNA in diverse species and EZH2 was a hypothetical NEAT1-binding protein (Figure 2) [104].

Chen et al. specified that the GBM-linked lncRNA NEAT1 was an oncogenic factor that was modulated via the EGFR pathway and triggered tumorigenesis by acting as a scaffold as well as recruiting the chromosome modification enzyme EZH2 to knock down target-specific genes like AXIN2, ICAT, and GSK3B which facilitated β-catenin nuclear transport (Figure 2) [104]. Zheng et al. exhibited that EZH2 secretion correlated with GSC proliferation, self-renewal, and GSC marker secretion, signifying that EZH2 regulated the "stemness" of the GSCs [109]. They observed that melatonin reduced GSC viability or self-renewal to an analogous level in both the control and EZH2-oversecreted cells [109].

Purkait et al. demonstrated that EZH2 was not secreted by the normal brain, reactive glial tissue, and circumstantial nonneoplastic glia [12]. EZH2 was variably secreted in the nuclei of tumor cells in Grades II to IV gliomas [12]. They indicated that the secretion of EZH2 was slightly irregular, with a low labeling index (LI) in Grade II gliomas, while its secretion became more regular as well as widespread with high LI in higher-grade gliomas [12]. Their finding suggests that aberrant secretion of EZH2 was associated with malignant progression [12]. Moreover, EZH2 protein secretion correlated with mRNA secretion in their study [12]. Furthermore, they indicated that EZH2 immunohistochemistry was capable of differentiating nonneoplastic reactive glial proliferation from gliomas, thus showing its diagnostic application in routine neuropathology practice [12]. Also, high LI of EZH2 was a potential indicator supporting the diagnosis of higher-grade gliomas like Grades III and IV gliomas [12].

8. EZH2 and Glioma Therapy

EZH2 is both a promising therapeutic target and a prognostic factor in brain tumors [56, 128]. It was established that *in vitro* administration of the EZH2 inhibitor DZNep was capable of suppressing the proliferation potency of GSCs in an analysis of brain glioma [20, 63]. Furthermore, the suppression of EZH2 gene secretion was capable of reversing temozolomide (TMZ) resistance in patients with brain glioma [9, 63]. CSCs have been implicated in tumor recurrence after treatment, and their extreme chemoresistance and radiation resistance require alternative therapeutic schemes that are capable of effectively eradicating them (functional or physical) [20, 129].

Studies have demonstrated that c-MYC downregulation was capable of abolishing tumorigenicity exhibited by EZH2-depleted glioblastoma CSCs. It was further observed that complete loss of tumor-initiating capacity was capable of causing disruption in c-MYC in GBM CSCs (Figure 2) [20, 130, 131]. Furthermore, the knockdown of EZH2-mediated BMPR1B stimulated maintenance of CSCs in a subset of GBM, signifying that BMPR1B was responsible for the reduction in tumorigenic potential in EZH2-knockdown BT-CSC (Figure 2) [81]. Suvà et al. identified elevated secretion of EZH2 in tumor cells but no detectable secretion in the adjacent brain parenchyma in paraffin-embedded immunohistochemistry of GBM samples [20]. They indicated that pharmacologic as well as shRNA-mediated depletion of EZH2 in GBM CSCs decreases their capability to form new spheres *in vitro* and new tumors *in vivo* (Figure 2) [20].

TMZ is an oral chemotherapy agent which works by sensitizing the tumor cells to radiation with reduced side effects. TMZ has become the standard therapeutic option for GBM treatment [9, 132, 133]. Tumor recurrence and resistance remain key challenges with TMZ therapy although it has made an impact on the survival of several patients. Fan et al. established that silencing of EZH2 secretory levels was linked to a TMZ-resistant phenotype in GBM cells during gene secretory analysis of both the TMZ-sensitive and TMZ-resistant GBM cell lines [9]. Furthermore, EZH2 was extremely secreted in multidrug-resistant human glioblastoma cells U251/TMZ as well as U87/TMZ [9].

Fan et al. specified that administration of EZH2 siRNA into glioma cells effectively as well as quickly silenced EZH2 resulting in a reduction in mRNA and protein levels by about 70%, signifying the effective inhibitory effects of EZH2 siRNA [9]. They further indicated that EZH2 was capable of modulating cellular proliferation because downregulation of EZH2 was capable of decreasing the cell growth viability of U251/TMZ as well as U87/TMZ cells by about 30-40% [9]. Also, the knockdown of EZH2 resulted in reduction of MDR, MRP, and BCRP mRNA and protein

levels leading to a decrease in efflux pump activity as well as augmented sensitivity to chemotherapy in GBM cells [9]. Thus, the anti-MDR influence of the EZH2 deletion was mediated by MDR, MRP, and BCRP [9].

Cheng and Xu demonstrated that the blockade of EZH2 secretion expressively repressed proliferation as well as tumorigenic efficiency of glioma cells [63]. They indicated that the blockade of EZH2 secretion was capable of downregulating the levels of numerous oncogenes including c-MYC and AKT [63]. Wu et al. further established that glioma patients with combined oversecretion of BMI1 as well as EZH2 proteins had the shortest overall survival. Moreover, secretion of BMI1 as well as EZH2 was observed as an independent prognostic factor for overall survival in glioma patients [120].

Natsume et al. demonstrated that the biological transformation between GSCs and differentiated non-GSCs is plastic in nature and escorted by gain or loss of PRC2-mediated H3K27me3 on pluripotency [110]. They further exhibited that EZH2 was extremely secreted in murine as well as human GSCs [110]. Also, administration of suberoylanilide hydroxamic acid triggered upregulation of PRC2 anticipated target genes, GSC disruption, and reduced secretion of EZH2 and stem cell marker CD133 [110]. Studies further demonstrated that the blockade of EZH2 secretion by shRNA was associated with a substantial reduction in the proliferation of glioma cells (Figure 2) [76, 119]. Furthermore, the blockade of EZH2 suppressed GBM tumor growth [76, 119].

Ahmad et al. established that treatment schemes targeting the disruption of EZH2-TERT-lipid metabolism interaction are capable of exhibiting intrinsic specificity for TERT mutant tumors as compared to TERT wild-type GBM tumors (Figure 2) [124]. Kim et al. demonstrated that the EZH2 and STAT3 signaling pathways are essential treatment targets for GBMs [64]. Kim et al. further established that targeting EZH2 may efficiently block oncogenic activities of both the EZH2 and STAT3 pathways (Figure 2) [64]. Yu et al. established that GSK343 was a feasible therapeutic approach as well as an imperative tool to understand the oncogenic function of EZH2 in glioma (Figure 2) [31]. Karlowee et al. discovered that notwithstanding varying age as well as tumor grades, EZH2 secretion was robust in high-grade glioma as well as in patients with a worse outcome [134]. Thus, EZH2 is a promising therapeutic as well as prognostic biomarker for the treatment of glioma.

9. Conclusions

EZH2 is well recognized as an essential modulator of cell invasion as well as metastasis in glioma. EZH2 oversecretion was implicated in the malfunction of several fundamental signaling pathways like Wnt/β-catenin signaling, Ras and NF-κB signaling, PI3K/AKT signaling, β-adrenergic receptor signaling, and BMP as well as NOTCH signaling pathways. EZH2 was more secreted in GBM than in low-grade gliomas as well as extremely secreted in U251 and U87 human glioma cells. Thus, the blockade of EZH2 expression in glioma could be of therapeutic value for patients with glioma. The suppression of EZH2 gene secretion was capable of reversing TMZ resistance in patients with brain glioma. EZH2 is a promising therapeutic as well as prognostic biomarker for the treatment of glioma.

Abbreviations

DZNep:	3-Deazaneplanocin A
AEBP2:	AE binding protein 2
As^{3+}:	Arsenic
BMI1:	B cell-specific Moloney murine leukemia virus integration site 1
BMP:	Bone morphogenetic protein
BMPR1B:	Bone morphogenetic protein receptor 1B
CSCs:	Cancer stem cells
CBX:	Chromobox
FBXW1:	β-TrCP
DNMTs:	DNA methyltransferases
c-Cbl:	Casitas B-lineage lymphoma
EZH2:	Enhancer of zeste homolog 2
EED:	Embryonic ectoderm development
E2Fs:	E2 factors
ECM:	Extracellular membrane
CHD1:	E-cadherin gene
EMT:	Epithelial-mesenchymal transition
EGBM:	Epithelioid glioblastoma
EPCs:	Endplate chondrocytes
ECAR:	Extracellular acidification rate
FASN:	Fatty acid synthase
GBM:	Glioblastoma multiforme
GSCs:	GBM stem-like cells
GPCR:	G protein-coupled receptor
GLUT1:	Glucose transporter 1
HMTase:	Histone methyltransferase
HATs:	Histone acetyltransferases
HDACs:	Histone deacetylases
HIF1:	Hypoxia-inducible factor 1
HCMV:	Human cytomegalovirus
JARID2:	Jumonji and AT-rich interaction domain containing 2
LI:	Labeling index
MBD:	Methyl-CpG-binding domain
MSCs:	Mesenchymal stem cells
NSPC1:	Nervous system Polycomb 1
TRAIL:	TNF-related apoptosis-inducing ligand
BP:	n-Butylidenephthalide
NICD1:	NOTCH intracellular domain 1
NEAT1:	Nuclear enriched abundant transcript 1
PH:	Polyhomeotic
PcG:	Polycomb group
PRC2:	Polycomb repressive complex 2
Pcgf:	Polycomb group ring finger
PCLs:	Polycomblikes
PTMs:	Posttranslational modifications
PCAF:	P300/CBP-associated factor
pS21 EZH2:	Phosphorylation of EZH2 at serine 21
pY461 EZH2:	Phosphorylation at EZH2 Y641
PI3K:	Phosphoinositide 3-kinase
PHD:	Prolyl hydroxylases
RING:	Ring finger protein

RbAp46/48: Retinoblastoma protein-associated protein 46/48
STAT: Signal transducer and activator of transcription
SMURF2: SMAD ubiquitination regulatory factor-2
SCF: Skp/cullin/F-box protein
SAH: S-Adenosylhomocysteine
SUZ12: Suppressor of zeste 12
Ser: Serine
Thr: Threonine
Tyr: Tyrosine
H3K27me3: Trimethylation of histone H3 on Lys 27
TET1: Ten-eleven translocation 1
TNF-α: Tumor necrosis factor-α
TERT: Telomerase reverse transcriptase
TMZ: Temozolomide
Wnt: Wingless-related integration site.

Data Availability

No data was used in this paper.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors contributed toward literature search, drafting, and critical revision of the paper and agreed to be accountable for all aspects of the work.

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Research Article

Assessment of Immune Cell Populations in Tumor Tissue and Peripheral Blood Samples from Head and Neck Squamous Cell Carcinoma Patients

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Head and neck squamous cell carcinoma (HNSCC) is a common type of cancer worldwide. Strong connections have been revealed between immune cells and the pathogenesis of HNSCC. Important differences regarding the levels of immune cell subpopulations in both peripheral circulation and tumor microenvironment were emphasized, with some of them having prognostic significance. In our study, we performed an analysis of immune changes in the tumor tissue and the peripheral blood of untreated HNSCC patients, investigating the proportions of different immune cell populations in these two compartments. The local infiltrating lymphocytes were mainly cytotoxic T cells ($CD8^+$). We have also revealed an increased level of B lymphocytes ($CD19^+$) in the tumor microenvironment. In peripheral blood, the most important lymphocyte subtype was represented by the helper T lymphocytes ($CD4^+$). We also found an increased proportion of circulating NK cells ($CD56^+$). Our results showed significant differences between all investigated lymphocyte subtypes in the peripheral blood and the tumor tissue of untreated HNSCC patients, suggesting that the local and systemic expressions of antitumor immune responses are different and that investigation of immune cell proportions in peripheral circulation has different cues that do not reflect the immune infiltrate pattern within the tumor microenvironment. Further studies are necessary to unveil the complex interplay involving local and systemic events in the immune system's fight against cancer.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is an epithelial type of cancer, with a high prevalence and an increasing incidence worldwide. The immune inflammatory factors are among the most important actors in the onset and progression of cancer [1–6], and numerous studies support important connections between immune cells, especially lymphocytes, and the pathogenic mechanisms of HNSCC [7–11].

Progression from early stages to advanced locoregional disease is associated with a significant alteration in the number and function of immune cell populations in peripheral blood, correlated with the inability of the immune system to limit the evolution of the tumor, facilitating tumor growth [7]. Moreover, tumor-infiltrating immune cells have attracted a special attention in scientific research, due to their impact on tumor development and progression [12, 13]. Multiple research findings suggest that there is a close relationship between local tumor inflammatory infiltrate, local disease control, and patient survival [7, 10]. However, the complexity of the immune carcinogenic interplay in HNSCC is not fully unveiled yet. Various populations and subpopulations of lymphocytes, such as cytotoxic T lymphocytes ($CD8^+$), helper T lymphocytes ($CD4^+$), and B lymphocytes, along with other types of immune cells, such as NK cells, acting in the tumor microenvironment may exert coordinated or sometimes even contrary responses [7, 10].

Peritumoral infiltration rich in total T lymphocytes ($CD3^+$), as well as particularly in cytotoxic T lymphocytes ($CD8^+$), main actors in tumor surveillance, was correlated with a favorable prognosis in HNSCC [14]. Helper T lymphocytes ($CD4^+$) mediate antitumor immunity [15]; however, in HNSCC, the prognostic significance of their presence in the tumor microenvironment is not yet settled [16].

The role of infiltrating B lymphocytes in HNSCC is still uncertain. However, there are results showing a better prognosis associated with an increased density of intratumoral B cells together with a high infiltrate of cytotoxic T lymphocytes ($CD8^+$) [17] supporting further studies in this direction.

Natural killer (NK) $CD56^+$ cells are leading actors of the innate immune system, having an effective role in tumor immunosurveillance, alongside their equivalents in the adaptive immune system—the cytotoxic T cells ($CD8^+$) [7, 18, 19]. Several studies have emphasized an improved disease control and a better outcome associated with an increased intratumor density of NK cells in HNSCC patients [20, 21]. However, other research has revealed tumor resistance strategies, suggesting a supporting role of NK cells in tumor progression [22, 23].

In HNSCC, a high variability of immune cell subpopulations was observed, partially correlating with the prognosis of patients. The information presented above demonstrates that a real representation of the antitumor response capacity is a topic of major interest. Moreover, an important issue is whether in HNSCC the proportion of circulating immune cells provides a relevant picture of the immune infiltrate in the tumor microenvironment or each of these two immune-related investigations portrays different points of view of a complex process with distinct local and systemic expressions.

In our study, we have investigated the differences between the distribution of immune cell populations in tumor tissue and peripheral blood samples from treatment-naïve HNSCC patients.

2. Materials and Methods

2.1. Study Protocol. In this study, we have included patients with operable forms of HNSCC treated in the Department of Oral and Maxillofacial Surgery, "Carol Davila" Central Military Emergency Hospital, Bucharest. The study was conducted in accordance with the Declaration of Helsinki (1964), with the approval of the Local Ethics Committee (No. 25/November 27, 2017). All patients included in the study were informed of the study protocol and signed the informed consent form.

All patients met the following inclusion criteria: histopathological confirmed diagnosis of HNSCC, in operable stages, that did not receive any previous treatment. Patients with unresectable or metastatic tumors, with other types of malignancy, immunological conditions, and other severe, decompensated conditions or with incomplete medical records were excluded.

All patients underwent a thorough preoperative evaluation, which included, in addition to the usual investigations, the collection of peripheral blood samples to determine circulating lymphocyte subtypes using the flow cytometry technique.

After radical resection of the tumor, histopathological examination of the excision specimen was performed, with a subsequent immunohistochemical study.

Surgical treatment was followed by oncological therapy and/or active follow-up program, according to national therapeutic guidelines.

2.2. Flow Cytometry Analysis. Based on the expression of surface markers, immunophenotyping allows quantification by flow cytometry of main lymphocyte subsets from whole hemolyzed blood: T lymphocytes (CD45+CD3+), B lymphocytes (CD45+CD3-CD19+), helper T lymphocytes (CD45+CD3+CD4+), suppressor/cytotoxic T lymphocytes (CD45+CD3+CD8+), and NK cells (CD45+CD3-CD16+CD56+).

In order to determine the percentages of these subsets, a BD Multitest IMK Kit (IVD) (Becton Dickinson) was used. EDTA-anticoagulated whole peripheral blood was incubated with a mixture of monoclonal antibodies (CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC; CD3-FITC/CD16+CD56-PE/CD45-PerCP/CD19-APC) for 15 min at room temperature and in the dark, followed by red blood cell lysis and flow cytometry analysis (BD FACSCanto II, Becton Dickinson). BD FACSCanto clinical software was used for sample acquisition and data analysis; daily check-up of cytometer performances was performed using 7-Color Setup Beads (BD Biosciences).

2.3. Histopathologic Examination. The surgical specimens were immediately immersed in 10% buffered formalin and

sent for histopathologic diagnosis. The macroscopic examination and selection of the fragments were performed according to the national and international protocols; further, the tissue fragments were manually processed and paraffin embedded. The paraffin blocks were cut with a semiautomated Rotary Microtome Leica RM2245; 3 μ sections were obtained, taken on regular slides for routine and special stains and on precoated slides for immunohistochemical tests.

Several immunohistochemical (IHC) stains were performed for CD4, CD8, CD19, and CD56 (see Table 1). Novolink Polymer (Leica/Novocastra) and DAB chromogen were used as the detection system.

Immunohistochemical analysis was performed using an Olympus BX41 microscope; CD4, CD8, CD19, and CD56 were evaluated in lymphoid cells. All the markers were evaluated as the number of positive cells per high-power field (HPF) (0.55 mm in diameter) both in the intratumor location and in the invasion front, with the number of the positive cells being appreciated in hot spot by counting 10 adjacent HPF.

2.4. Statistical Analysis. For statistical analysis, we used GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). We evaluated the normality of the data distribution using the Kolmogorov-Smirnov test. The differences between the lymphocyte subtypes in peripheral blood and tumor tissue were assessed by the paired t-test (normal distribution) or the Wilcoxon test (nonnormal distribution). The results are presented as mean ± standard deviation (SD); p values < 0.05 were considered statistically significant.

3. Results

3.1. Patient Characteristics. A total of 10 patients with operable HNSCC were included in the study, 7 males and 3 females, with a mean age of 67.6 ± 14.25 years, ranging from 45 to 86 years old. The primary tumor was staged from T1 to T4a and involved different head and neck subsites: lower lip, buccal mucosa, tongue, gingiva, and retromolar mucosa. In three patients, cervical lymph node involvement was confirmed. Histology analysis of the specimen revealed that 50% of the lesions were moderately differentiated, 40% were well differentiated, and 10% were poorly differentiated. Descriptive data of the patient cohort is presented in Table 2.

3.2. Immunohistochemical Analysis of Resected HNSCC Tumors. Immune cell subtypes evaluated within the inflammatory infiltrate in the tumor microenvironment focused on T cell subpopulations ($CD4^+$, $CD8^+$), total B cells ($CD19^+$), and NK cells ($CD56^+$) (Figure 1 and Table 3). The number of positive cells in the tumor microenvironment was evaluated for all the tumor samples. We traced different distribution patterns of lymphocytes within the tumor tissue. Thus, T lymphocytes ($CD4^+$ and $CD8^+$) represented the main immune cell type in the peritumoral infiltrate, with cytotoxic T lymphocytes ($CD8^+$) being the vast majority of these cells, with a rigorously spread pattern within the tumor tissue. The B cell population ($CD19^+$) represented almost 40% of the tumor immune infiltrate analyzed in our study, following a distinct pattern of organization, in small peritumoral aggregates. NK cells ($CD56^+$) were present as isolated cells, rarely found in the tumor microenvironment.

Table 1: Specific details of immunohistochemical markers.

Marker	Clone	Host	Dilution	Pretreatment*	Producer
CD3	LN10	Mouse	1 : 500	HIER, citrate, pH 6	Leica
CD19	BT51E	Mouse	1 : 100	HIER, citrate, pH 6	Leica
CD4	4B12	Mouse	1 : 200	HIER, EDTA, pH 8	Leica
CD8	4B11	Mouse	1 : 100	HIER, EDTA, pH 8	Leica
CD56	CD564	Mouse	1 : 200	HIER, citrate, pH 6	Leica

*HIER: heat-induced epitope retrieval.

3.3. Comparative Investigation of Immune Cell Populations in Tumor Tissue and Peripheral Blood Samples of HNSCC Patients. Analysis of lymphocyte subtypes showed significant differences between peripheral blood and tumor tissue in HNSCC patients (see Table 3 and Figure 2).

In peripheral blood, the most important lymphocyte subtype was represented by helper T lymphocytes ($CD4^+$), with a proportion significantly higher than the tumor tissue ($p < 0.0001$).

In contrast, cytotoxic T lymphocytes ($CD8^+$) represented over half of the lymphocyte populations in the tumor infiltrate and were markedly increased compared to peripheral circulation ($p = 0.0002$).

Also, the level of B lymphocytes ($CD19^+$) was significantly higher in tumor tissue than in peripheral blood ($p = 0.0020$).

As for NK cells ($CD56^+$), their proportion was much lower in the tumor infiltrate compared to peripheral circulation ($p = 0.0003$).

4. Discussion

Alteration of cellular and humoral immune responses has been indicated as important players in the development and progression of HNSCC [7, 24]. Systemic immunity may affect the clinical evolution of head and neck cancer [25], and the local immunity is essential in the control of tumor growth and invasion capacity [7, 24].

In our study, we performed an analysis of immune changes in the peripheral blood and the tumor tissue of untreated HNSCC patients, investigating the distribution of different immune cell populations in these two compartments.

The local infiltrating lymphocytes were mainly cytotoxic T cells ($CD8^+$). We have also revealed an increased level of B lymphocytes ($CD19^+$) in the tumor microenvironment.

$CD8^+$ lymphocytes are main effectors in antitumor protection [26], and other studies have also revealed their higher levels in the inflammatory infiltrate of HNSCC [27, 28]. An

Table 2: Clinical and histopathological details of HNSCC patients.

Patient #	Gender	Age	Tumor location	Clinical staging	Histological differentiation
1	M	80	Buccal mucosa	T2N0M0	WD
2	M	60	Retromolar mucosa	T3N2M0	PD
3	M	80	Lower lip	T1N0M0	WD
4	M	51	Tongue	T2N2M0	WD
5	M	65	Gingiva	T2N0M0	MD
6	F	85	Lower lip	T2N0M0	MD
7	F	86	Lower lip	T1N0M0	WD
8	F	54	Tongue	T2N0M0	MD
9	M	45	Buccal mucosa	T3N1M0	MD
10	M	71	Gingiva	T4aN1M0	MD

M: male; F: female; WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated.

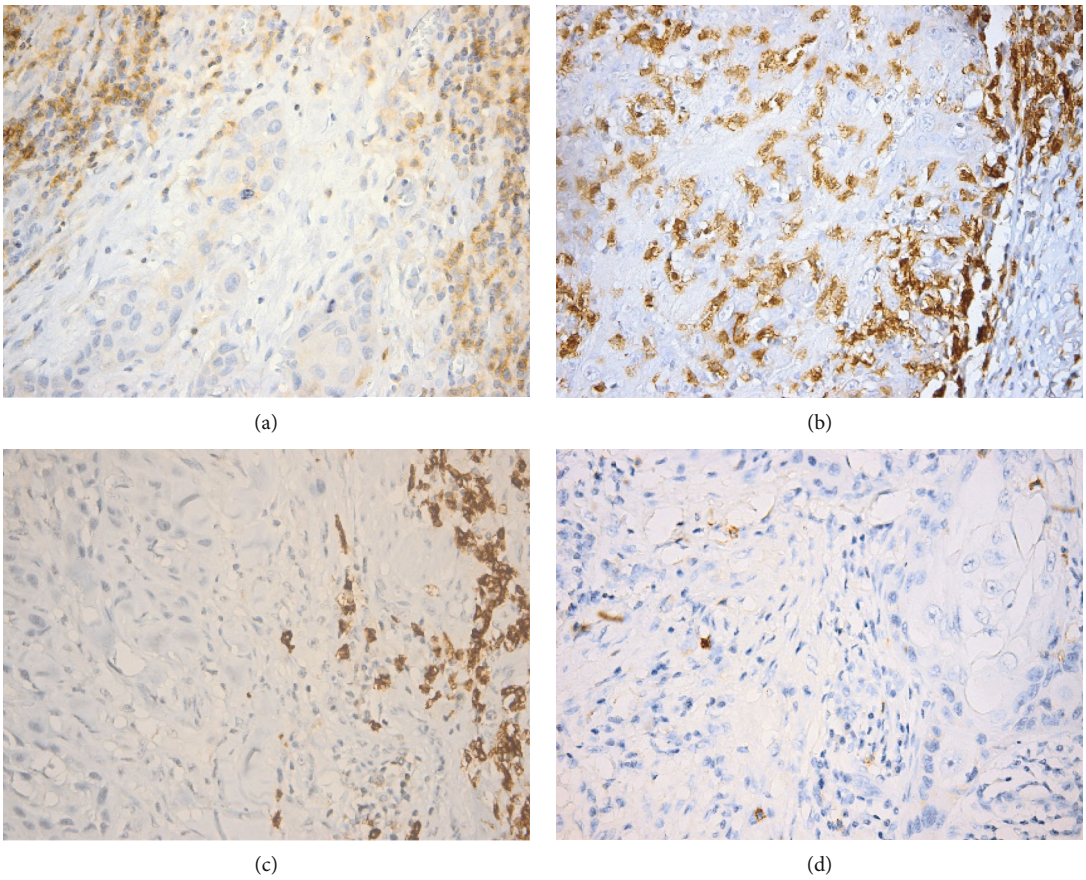

Figure 1: Immune cell subtypes evaluated within the inflammatory infiltrate in the tumor microenvironment of head and neck squamous cell carcinoma (HNSCC). (a) ($CD4^+$) T lymphocytes within the peritumoral inflammatory infiltrate. (b) Numerous ($CD8^+$) T cells within the intratumor inflammatory infiltrate (E). (c) ($CD19^+$) B lymphocytes, mostly forming small aggregates present within the peritumoral inflammatory infiltrate. (d) Few ($CD56^+$) NK cells within the peritumoral inflammatory infiltrate. Original magnification ×400.

increased number of $CD8^+$ cytotoxic lymphocytes in the tumor microenvironment also carry a favorable prognostic significance in HNSCC [10, 14, 29, 30].

Concerning the role of the tumor infiltrating B lymphocytes, opinions are still divided, highlighting both beneficial and unfavorable aspects regarding their antitumor immune effects and their prognostic impact [7, 17, 31]. An interesting finding is the close interconnection between B lymphocytes ($CD19^+$) and cytotoxic T cells ($CD8^+$) in the tumor microenvironment with a favorable prognostic impact in HNSCC [31]. The role of B cells within the tumor is complex. In other types of cancer, it was established that B cells promote tumor cell clearance through the release of specific immunoglobulins that enhance T cell-mediated response. Nevertheless, B cells

Table 3: Proportion of lymphocyte subtypes in tumor tissue and peripheral blood samples of HNSCC patients.

Variable (%)	Peripheral blood		Tumor tissue		p value
	Mean	SD	Mean	SD	
Helper T lymphocytes ($CD4^+$)	40.10	10.19	2.35	2.5	<0.0001#
Cytotoxic T lymphocytes ($CD8^+$)	29.10	7.39	52.72	10.87	0.0002#
B lymphocytes ($CD19^+$)	9.60	4.93	39.77	11.80	0.0020*
NK cells ($CD56^+$)	18.10	6.62	5.16	3.45	0.0003#

#Paired t-test; *Wilcoxon test.

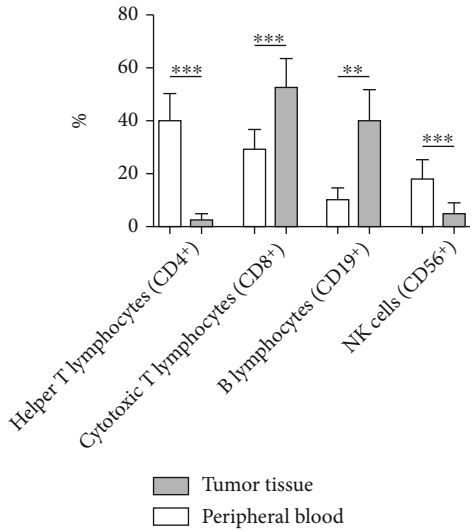

Figure 2: Percentual representation of immune cell populations in tumor tissue and peripheral blood samples of HNSCC patients. The error bars represent the standard deviation; $^{**}p < 0.01$; $^{***}p < 0.001$.

through their subpopulation (Bregs) can also suppress antitumor immune response by immunosuppressive cytokines which regulate T cells, NK cells, and myeloid-derived suppressor cells (MDSC). They also can secrete pathological antibodies or promote angiogenesis [32]. Our findings regarding the high percentage of B cells within the tumor site and their clustering suggest that in our tumor samples, there is an active immune response ongoing between the infiltrating immune cells. The lower percentage of circulating B cells accounts for their drainage toward the tumor tissue as part of the local immune response.

In our study, helper T lymphocytes ($CD4^+$) were the most important type of immune cells found in peripheral circulation. Helper T lymphocytes are leading actors in the initiation and modulation of the antitumor immune responses and are the main immune cell subtype present in peripheral blood [15, 33, 34]. However, a study comparing lymphocyte subtypes in HNSCC with healthy control subjects reported a decreased level of circulating helper T cells ($CD4^+$) in the test group [35]. Our results indicate a clear disproportion of $CD4^+$ T lymphocytes between circulation and tumor tissue that suggests several issues related to HNSCC: a reduced intratumoral cooperation between $CD4^+$ and $CD8^+$ cells, which has sustained tumor proliferation, and a decreased migration of $CD4^+$ lymphocytes towards the tumor site due to still unknown extracellular and/or intrinsic factors.

A high proportion of NK cells ($CD56^+$) was identified in the peripheral circulation of HNSCC patients. As NK cells ($CD56^+$) are effectors of the innate immune system with potent antitumor functions, finding a high percentage in the patient's circulation is a good indicator for HNSCC patients. Moreover, another study has indicated an increase in NK cell level after the treatment of HNSCC [36]. It is worth mentioning that alteration of their cytotoxic functions were associated with oral tumors, and inhibitory actions of NK cells were also emphasized in both peripheral circulation and tumor microenvironment in HNSCC [37, 38].

Our results showed significant differences between all investigated lymphocyte subtypes in the peripheral blood and the tumor tissue, bringing new evidence in a vast area of research, with complex mechanisms still not clearly understood [1–4, 7, 8]. In the scientific literature, there is little information about the differences between the distributions of immune cell populations in tumor tissue and peripheral blood from HNSCC patients, and most of them refer only to T lymphocytes.

An interesting study evaluating the subgroups of T lymphocytes and the immune regulatory mechanisms in untreated HNSCC patients [39] showed that T lymphocytes represent the vast majority of infiltrating lymphocytes in the tumor, with their proportion being higher in the tumor tissue than in the peripheral blood in HNSCC patients. The level of cytotoxic T lymphocytes ($CD8^+$) tended to be higher in the tumor than in the peripheral circulation; however, the difference was not statistically significant. In contrast, the percentage of $CD4^+$ T lymphocytes was significantly higher in peripheral blood than in tumor tissue. In addition, the study showed that the tumor microenvironment of HNSCC has a strong infiltration of T lymphocytes with an effector memory phenotype, with the regulatory T cells being significantly increased [39].

Other research has indicated different aspects than those revealed by our results. A correlation analysis of peripheral blood lymphocytes and tumor infiltrating lymphocytes in patients with oral squamous cell carcinoma found significant correlations regarding the level of total T lymphocytes ($CD3^+$), helper T lymphocytes ($CD4^+$), and cytotoxic T lymphocytes ($CD8^+$), suggesting that circulating T cell levels could be an indicator for the local T cell-mediated antitumor responses [38].

A comparative assessment of lymphocyte subtypes in tumor tissues, lymph nodes, and peripheral blood of patients

with HNSCC revealed significant differences regarding the local, regional, and systemic immune responses [24]. Thus, the level of $CD4^+$ T lymphocytes was higher in the peripheral circulation than in the tumor tissue, a result similar to our research. However, regarding the level of $CD8^+$ T lymphocytes, although an increasing trend was observed in the tumor compared to peripheral blood, the differences were not statistically significant. In addition, the same study revealed an altered cytotoxic activity of lymphocytes in the tumor microenvironment and a decreasing tendency of NK cells ($CD56^+$) in the tumor tissue compared to peripheral circulation, but again without reaching the threshold of statistical significance [24]. Acknowledging the limitations of our research regarding the low number of investigated patients, the differences from other studies could be explained by the different locations of the tumors, selection of patients in different stages of the disease, and inclusion in other studies of patients previously exposed to oncological treatment with radiotherapy and/or chemotherapy.

5. Conclusions

Our study revealed significant differences between the levels of lymphocyte subtypes in peripheral circulation and the tumor tissue of untreated HNSCC patients, suggesting that the local and systemic expressions of antitumor immune responses are different and that investigation of immune cell proportions in peripheral circulation has different cues that do not reflect the immune infiltrate pattern within the tumor microenvironment. Further studies are necessary to unveil the complex interplay involving local and systemic events in the immune system's fight against cancer.

Data Availability

The datasets used and/or analyzed during the present study are available from the corresponding author.

Ethical Approval

The study was approved by the Ethics Committee from "Carol Davila" Central Military Emergency Hospital, Bucharest (No. 25/November 27, 2017).

Consent

An informed consent form was signed from each subject.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

AC, SZ, CC (Constantin Caruntu), and MN were responsible for the research creation and design. AC, LM, MS, AM, CT, CC (Carolina Constantin), SZ, CC (Constantin Caruntu), and MN were responsible for data acquisition, analysis and interpretation of the data, statistical analysis, and manuscript drafting. AC, SZ, CC (Constantin Caruntu), and MN were responsible for the critical revision of the manuscript for important intellectual content. All authors have read and approved the final manuscript.

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