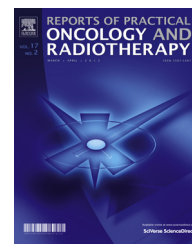


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## Review

## Tumor microenvironment – Unknown niche with powerful therapeutic potential



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## ABSTRACT

Head and neck squamous cell carcinomas (HNSCC) are in a group of cancers that are the most resistant to treatment. The survival rate of HNSCC patients has been still very low since last 20 years. The existence of relationship between oncogenic and surrounding cells is probably the reason for a poor response to treatment. Fibroblasts are an important element of tumor stroma which increases tumor cells ability to proliferate. Another highly resistance, tumorigenic and metastatic cell population in tumor microenvironment are cancer initiating cells (CICs). The population of cancer initiating cells can be found regardless of differentiation status of cancer and they seem to be crucial for HNSCC development.

In this review, we describe the current state of knowledge about HNSCC biological and physiological tumor microenvironment.

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## 1. Head and neck cancers

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide and represents 5% and up to 50% of all cancers in developed and developing countries, respectively.<sup>1,2</sup> According to the Polish National Cancer Registry ([www.onkologia.org.pl](http://www.onkologia.org.pl)), cancers of the oral cavity and lip account for approximately 4% in men and 1% in women of all cancers cases in Poland. The development of HNSCC is mainly caused by the carcinogens present in tobacco and alcohol as well as oncogenic viruses (HPV and EBV) and a wrong diet.<sup>3-5</sup>

The treatment method depends on the patient's status, clinical stage of the tumor, its localization and histological differentiation. Basic methods of HNSCC treatment are surgery, radiation and chemotherapy, used alone or in combination and recently also with targeted therapy agents. In the case of treatment involving conventional methods, the prognosis of HNSCC patients in advanced stages is largely unsatisfactory due to loco-regional recurrence.<sup>6-9</sup> The use of specific biomarkers, such as mRNA, lncRNA, miRNA or circulating RNA, is proposed as a good prognostic or predictive tool with clinical significance.<sup>10-17</sup> However, molecular characterization of patients' tumor is only used to a limited extent in standard diagnostic procedures.

## 2. Tumor microenvironment

Tumor microenvironment (TME) differs essentially from the environment of normal tissues. The TME is a complex of cellular components such as cancer-associated fibroblasts (CAFs), myofibroblasts, adipocytes, endothelial cells, epithelial cells, immune inflammatory cells as well as extracellular components which surround tumor cells.<sup>18,19</sup> TME does not only surround the tumor cells, it also actively contributes to tumor development and progression, drug resistance and metastasis.<sup>20,21</sup> As far as tumor progress is concerned, the microenvironment of cancer cells is activated through constantly existing paracrine communication and promotes further changes in the microenvironment and expansion of tumor.<sup>19-23</sup> Recent studies suggest that tumor microenvironment plays a crucial role not only in the progression but also in malignant transformation.<sup>20</sup> The exact role of selected TME components and their interactions with cancer cells are described below.

Cancer-associated fibroblasts (CAFs) are heterogeneous population of irreversibly activated fibroblasts. They play various roles and are an important element of tumor stroma which increases tumor ability to proliferate, phenotype changes, resistance to therapy as well as metastasis.<sup>23-25</sup> CAF secretes many factors (such as cytokines, chemokines, growth factors and other proteins) influencing both cancer cells and TME elements.<sup>24</sup> Many cancers have altered cellular receptors such as members of fibroblast growth factor receptors (FGFR) family: FGFR1/2/3/4. They are transmembrane kinase receptors (RTKs) involved in basic cell cycle processes, such as differentiation, proliferation, cell survival, adult-tissue homeostasis and tumorigenesis<sup>26</sup> as well as the formation of new blood vessels, wound repair and embryonic development.<sup>27</sup> FGFR

alterations can be divided into three main classes: gene amplification, gain-of-function coding mutation and gene fusion, with high impact in three most common malignancies worldwide.<sup>26</sup> Aberrations in FGFR pathways play a critical role in cancer resistance to therapy. Chae et al. reported that FGFR1 amplification occurs in 10–17% of HNSCC patients.<sup>28</sup> Additionally, Vairaktaris et al. demonstrated that a higher expression of FGFR1/2/3 contributes to early-stage cancer progression in HNSCC.<sup>29</sup> FGFR2/3 high expression was found in the majority of HNSCC cell lines.<sup>28</sup> Another interesting finding is that the reduction of FGFR3 level in HNSCC cell lines caused a 35% decrease of cell proliferation; furthermore – it caused their higher radiation sensitivity.<sup>30</sup>

Fibroblasts activation by tumor microenvironment, creating CAF phenotypes, depends on exogenous signals specific for a cancer type.<sup>31</sup> However, mechanisms for their activation in HNSCC microenvironment remain ambiguous.<sup>32</sup> Kellermann et al. revealed that oral fibroblasts transform to CAFs after co-culturing with oral squamous cell carcinoma (OSCC) cell lines. They demonstrated that cancer cell-derived TGF- $\beta$  induced trans-differentiation of human gingival fibroblasts into CAF-line cells.<sup>33</sup> Another group reported that TGF- $\beta$  and IL-1  $\beta$  might play a crucial role in CAF induction.<sup>34</sup>

In HNSCC, CAFs activation launches mechanisms involved in downregulation of tumor suppressor genes, such as p21 and CAV-1.<sup>32</sup> They may also represent a resistant stromal cell type engaged in tumor relapse. CAFs are involved in remodeling and reprogramming the extracellular matrix (ECM) and tumor microenvironment. In primary tumor it may enhance cancer cell invasion leading to metastasis. Metabolic reprogramming of CAFs can contribute to enhancing cancer cells adaptation influencing tumor progression. They also play a crucial role in the secondary tumor growth because CAFs may enhance metastasis by releasing growth factors and cytokines into the circulation. That stimulates the growth and invasiveness of cancer cells at a distant site. Moreover, CAFs have also direct or indirect pleiotropic immunomodulatory functions. However, the most crucial role of CAFs is their impact on cancer therapy response caused mostly by modulations in CAF-ECM pathways interactions.<sup>31</sup>

Myofibroblasts are one of the carcinoma-associated fibroblasts which help to preserve stemness of cancer initiating cells (CICs). Myofibroblasts secrete cytokines, growth factors, chemokines, hormones, inflammatory mediators, adhesion and ECM proteins. This kind of fibroblasts is found in the stroma of carcinomas, particularly in the invasive front of a tumor. Their activity causes a rise of cancer cells proliferation, migration and invasion. The presence of myofibroblasts in cancer is correlated with lymph node involvement, disease recurrence, advance clinical staging and lower survival rates of HNSCC patients.<sup>35,36</sup> The analysis of all CAFs functions may give new insight into TME biology and offer a novel approach in cancer therapy.<sup>37</sup>

Mesenchymal stem cells (MSCs), another element of TME, are plastic, multipotential adherent cells similar to fibroblasts, but with a unique phenotype.<sup>38</sup> They are characterized by strong tumor tropism.<sup>39</sup> Liotta et al. showed that the number of MSCs (CD90+ cells) is correlated positively with tumor size but negatively with tumor-infiltrating leukocytes (CD45+ cells). Tumor-MSCs are able to inhibit proliferation

and cytokine production of activated CD4+ and CD8+ T cells via IDO1 enzyme activity, causing impairment of anti-tumor immune response. Moreover, it was showed that MSCs can attract T-cells by CXCL10 chemokine.<sup>40</sup> MSCs produce pro- and anti-inflammatory cytokines, chemotaxis, angiogenesis and growth factors. *In vitro* co-culture study indicated that MSCs influence HNSCC cells morphology and proliferation probably via MSCs-secreted IL-6 and activation and phosphorylation of ERK1/2 expression in tumor cells.<sup>39</sup>

Other components of TME are endothelial cells which create perivascular niches in HNSCC. Krishnamurthy et al. indicated that most of CICs (80%) are located close to blood vessels in tumor mass. Endothelial cells secrete the factors which promote CICs-self renewal, proliferation and survival. It was also observed that the ablation of endothelial cells leads to the reduction of CICs.<sup>41</sup> The endothelial cells produce IL-6, CXCL8 and EGF which stimulate tumor cells. The reduction of secreted interleukins and epidermal growth factor causes the reduction of phosphorylation of STAT3, Akt and ERK in tumor cells and inhibits cell migration and anoikis.<sup>42</sup> Probably, the HNSCC cells stimulate endothelial cells by VEGF and induce Bcl-2 signaling pathway, which, in turn, activates endothelial cells to secrete IL-6, CXCL8 and EGF.<sup>42-44</sup>

Tumor-associated macrophages (TAMs) are also an element of TME. They are a major population of inflammatory cells infiltrating tumors. Macrophages switch their own phenotypes in response to specific microenvironmental elements of the tumor. The TME-mediated signals determine the state of macrophages – M1 or M2. The M1 macrophages reveal cytotoxic activity toward cancer cells and produce toxic mediators, e.g. reactive oxygen intermediates, nitric oxide and TNF- $\alpha$ .<sup>45</sup> The M2 macrophages show a poor antigen-presenting expense and act as anti-inflammatory factor by suppressing Th1 adaptive immunity.<sup>46</sup> In HNSCC, the M2 macrophages are reported as TAMs and can lead to cancer progression.<sup>32</sup>

When we talk about TME we should not only mention its biological, but also physiological aspects, such as oxygen tension. The areas of low oxygen tension (hypoxia) are created due to tumor growth, vascular disturbances and metabolic changes in solid cancers.<sup>47,48</sup> The phenotypes of different subpopulations of cancer cells and stromal cells are controlled through epigenetic mechanisms, which are regulated by hypoxia.<sup>49,50</sup>

It was shown that hypoxia causes an aggressive phenotype of cancer with high treatment resistance and poor clinical prognosis.<sup>51-55</sup> The specific hypoxic molecular panels were defined and they are supposed to be useful in clinical applications in HNSCC patients.<sup>56-60</sup> It should be noted that HPV infection does not seem to change the expression of hypoxia-related genes nor HNSCC cell lines response to irradiation in low oxygen conditions compared to control (normoxic) cells.<sup>61</sup>

The cellular response to chemo- and radiotherapy is tightly connected with the hypoxia-inducible factor (HIF), family of transcription factors, which regulates the expression of many genes associated with cancer cells adaptation and progression.<sup>62</sup> For example, stabilization of HIF-1 alpha by hypoxia (or other factors) enhances epithelial-to-mesenchymal (EMT) process in HNSCC cell lines *in vitro*.<sup>63,64</sup> It was showed that under hypoxic conditions the action of many drugs is overcome by changes in cell cycle (slower rate of cycle

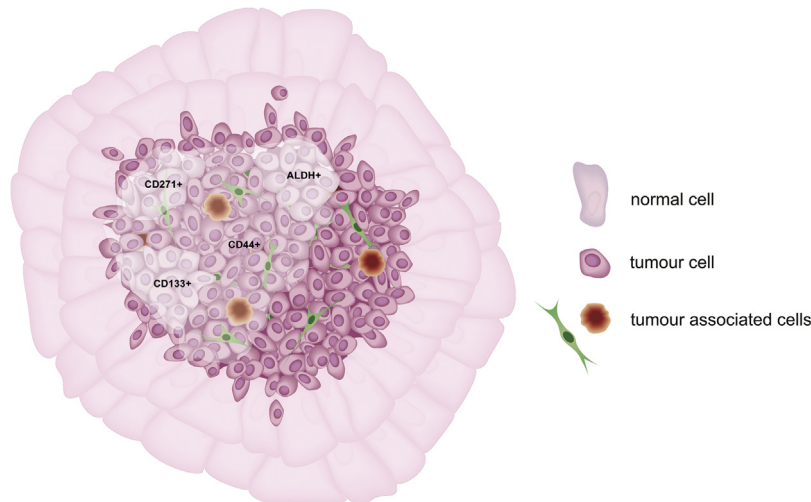
or G1 arrest),<sup>65</sup> up-regulation of drug reflux system<sup>66-68</sup> or changes in apoptosis.<sup>69,70</sup> Wiehceh et al. reported that hypoxia in some cases could increase cell sensitivity to anti-EGFR (cetuximab) exposure *in vitro*. Moreover, knock-down of HIF-1 alpha reverses this sensitivity.<sup>71</sup> It is supported by observation that the interaction between HIF-1 alpha and AKT signaling pathway depends on tumor type and its histological characterization. Under hypoxia condition, up-regulation of pAKT is induced by HIF-1 alpha only in some HNSCC lines. Similarly, only in some cases, inhibition of AKT leads to the reduction of HIF-1 alpha. Thus, the use of AKT inhibitors is not successful for all hypoxic HNSCC and it should be verified which patients could benefit from AKT inhibitor treatment.<sup>72</sup> However, Lu et al. indicated that cetuximab down-regulates the LDH-A (lactate dehydrogenase A) and inhibits glycolysis in HNSCC cell lines through down-regulation of HIF-1 alpha. The cetuximab-induced resistant cells express high level of HIF-1 alpha and were highly glycolytic. The inhibition of LDH-A can reverse the resistance to cetuximab.<sup>73</sup>

Many reports indicated the role of hypoxia in the regulation of radioresistance. Sasabe et al. noticed that over-expression of HIF-1 alpha causes inhibition of reactive oxygen species (ROS) in OSCC cell lines, which are important in response to irradiation.<sup>69</sup> Wozny et al. showed differences in cell survival after photon and carbon ion irradiations on different cell phenotypes in both normoxic and hypoxic conditions *in vitro*. The better *in vitro* therapeutic effect was connected with carbon ion irradiation method and cell phenotype marked as CICs. It seems, that HIF-1 alpha plays an important role in HNSCC resistance through its influence on ROS production. Probably, the use of HIF-1 alpha-knock-down adjuvant therapy with carbon ion irradiation method could effectively overcome cancer radioresistance and recurrence.<sup>74</sup> As mentioned above, HIF-1 alpha influences the EMT process, which, in turn, causes cell resistance to irradiation by expression of high levels of free radical-scavenging proteins or by suppressing p53-mediated apoptosis.<sup>75,76</sup> One of the possible methods to overcome hypoxic resistance of HNSCC is targeting STAT3 pathway by molecular inhibitors such as Stattic.<sup>77</sup> Moreover, the strong evidence of beneficial use of hypoxia modifiers in breaking radioresistance was shown by Overgaard in meta-analysis of 4805 HNSCC patients from 32 randomized clinical trials.<sup>48</sup>

To summarize, main cancer hallmarks, such as cell proliferation, apoptosis, immune response, metabolism, vascularization, genomic instability, invasion and metastasis are affected by cellular and non-cellular factors within tumor consisting of TME and should be taken into consideration under treatment strategies.

### 3. Cancer initiating cells (CICs)

A solid tumor contains not only one type of cancer cells, but various cancer cells with different phenotypes and many different types of surrounding cells (Fig. 1). There are cancer initiating cells (CICs) among them, with unique genetic and behavior characteristics. The CICs are found in specific locations within tumor mass named “niches” which are, probably, the best microenvironment for them. In HNSCC, CICs reside



**Fig. 1 – The HNSCC contains small population of pluripotent cancer cells named cancer initiating cells (or cancer stem cells). Cancer initiating cells are found among other cells populations characterized by expression of markers such as CD44, CD133, CD27, ALDH (marked on the diagram by clouds).**

close to blood vessels, where endothelial cell-secreted factors protect CICs against anoikis. Secreted VEGF enhances CICs' proliferation and survival via phosphorylation of AKT in PI3K-AKT signaling pathway.<sup>78</sup>

The specific population of CICs can be found regardless of the differentiation status of cancer, even in the absence of visible distinction of tissue architecture.<sup>79</sup> They are internal cell population characterized by expression of cell markers<sup>80,81</sup> (Table 1 and Fig. 1). It should be noted that the commonly used markers are not specific and can generate inconsistent results, denying CICs theory.<sup>82,83</sup> However, these results do not refute the theory as a whole but show the lack of specific detection methods of these cells.

CICs seem to be crucial for HNSCC development. It was observed that ALDH+ and CD133+ cells of oral leukoplakias have 4.17-fold and 2.86-fold increased risk of cancer transformation as compared to negative ones, respectively.<sup>84</sup> Moreover, Sun et al. indicated that the expression of CD133 can be used to predict risk of developing oral cancer from lichen planus.<sup>85</sup> The retrospective studies also indicated that oral leukoplakias with up-regulated ABCG2 and BMI-1 are associated with tumor development (3.24 and 4.03-fold increased risk, respectively), thus they could be used as a diagnostic marker.<sup>86</sup>

Some of HNSCCs are closely connected with HPV or EBV infections. Virus infection is one of the most important clinical factor in HNSCC and has also connection with CICs. It is well known that virus-related HNSCCs are less aggressive and HPV infection is a good prognostic factor. It was shown that HPV+ HNSCC (patients' samples as well as cell lines) have a lower rate of CICs compared to HPV negative-. Moreover, HPV- cells are more sensitive to radio-induced dedifferentiation than HPV+, which highlights a distinct mechanism of maintenance CICs population in cancers caused by virus infection.<sup>87</sup> Cai et al. showed that EBV-miR-BART7-3p targets PTEN and modulates PI3K/AKT/GSK-3beta pathway as well as influences the EMT process by changes in Snail and beta-Catenin

expression. This phenomenon affects cell migration ability and is one of the known factors of nasopharyngeal carcinoma (NPC) metastasis.<sup>88</sup>

The main feature of CICs is the ability to self-renewal from a single cell. Prince and colleagues showed that CD44+ cells isolated from tumors of HNSCC patients restored tumor from a small number of cells. However, CD44+ cells proliferate asymmetrically and differentiate into CD44-, which are the main component of tumor mass. CD44- cells never form a tumor alone. The CD44+ cells express Cytokeratin 5/14, a marker of normal squamous epithelial stem and progenitor cells. They also express BMI-1 protein that plays a role in self-renewal and tumorigenesis.<sup>79</sup>

The CICs have some traits of embryonic stem cells or induced pluripotent stem cells, such as expression of KLF4, SOX2, OCT3/4 and c-MYC genes. Their unique phenotype is supported by changes in many important signaling pathways, epigenetic changes or mutations.<sup>89-93</sup> Biological and clinical significance of these genes has been checked in HNSCC patients as well as in cell lines. Persistent KLF4 over-expression is detected only in some HNSCC patients and is correlated with a worse disease-specific survival, especially in a subgroup of advanced cancer. The up-regulation of KLF4 in SAS cell line leads to higher cell migration, invasion and higher resistance to some chemotherapeutic drugs. Moreover, KLF4 induces tumorigenicity of HNSCC cells injected to mice.<sup>94</sup> Expression of SOX2 is higher in HNSCC metastasized to lymph nodes than in non-metastasized cancers. The over-expression of SOX2 is associated with poor outcome, but correlation between SOX2 expression and tumor grading, T-classification, Ki-67 expression or only cell-specific expression is not observed. These findings are not consistent with CICs theory, but SOX2 is probably a commonly activated oncogene affecting early step of the tumorigenesis. SOX2 is mostly associated with HPV-negative tumors and its over-expression activates the anti-apoptotic Bcl-2 preventing apoptosis.<sup>95</sup> However, a meta-analysis of SOX2 revealed that it

**Table 1 – Characteristics of common markers used to describe the cells population with high probability of cancer initiating cells presence in HNSCC.**

Marker	Population characteristic	Ref.
CD271	<ul style="list-style-type: none"> <li>• Only CD271+/CD44+ population contains mostly tumorigenic cells</li> <li>• Loss of CD271 reduces cell proliferation via inhibition of G2-M transition and inhibition of Erk1/2 phosphorylation</li> </ul>	103
CD44	<ul style="list-style-type: none"> <li>• CD44+ cells are more tumorigenic than CD44–</li> <li>• CD44+ cells have primary cellular morphology and express Cytokeratin 5/14 (basal cell marker)</li> <li>• CD44– cells are differentiated squamous epithelial cells and express Involucrin (differentiation cell marker)</li> <li>• CD44+ cells express higher levels of BMI-1 protein than CD44–</li> <li>• There are several CD44 isoforms and only some of them can be used as CICs and radioresistance markers</li> </ul>	79,83
CD133	<ul style="list-style-type: none"> <li>• CD133+ cells are more invasive, with higher migration and clone-formation ability than CD133– cells; they possess differentiation capacity</li> <li>• CD133+ cells exhibit higher expression of anti-apoptosis genes, higher Bcl-2/Bax ratio and show dysregulated Hedgehog, Wnt signaling pathways and higher Bmi-1 expression</li> <li>• BMI-1 seems to be central master of CIC-phenotype in the CD133+ cells through the regulation of p16(INK4A) and p14(ARF)</li> <li>• CD133+ expression is positively associated with more advanced TNM stage, pathological grade and lymph node metastasis</li> <li>• CD133+ patients have shorter overall survival and disease-free survival</li> <li>• CD133 expression negatively correlates with KAI1/CD82 protein and both could be used as independent prognostic factors</li> <li>• CD133+ cells have higher proliferation ratio than CD133– and possess higher expression of Glut-1 important in glucose transport</li> <li>• CD133 gene is over-expressed in SP cells (CD133+ SP) while down-regulation of CD133 reduces number of SP and increases chemosensitivity</li> </ul>	104-110
CD24	<ul style="list-style-type: none"> <li>• CD24+ cells co- express OCT3/4 and CIP2A proteins</li> <li>• CD24+/CD44+ population is more invasive, more chemoresistant and creates larger tumor compared to CD24-/CD44+ population</li> </ul>	97,111
ALDH	<ul style="list-style-type: none"> <li>• ALDH+ cells are more tumorigenic than ALDH–; most of ALDH+ cells are CD44+</li> <li>• ALDH enzyme is probably required for cancer initiating cell activity</li> <li>• Expression of ALDH is observed in CD44+/CD24– cell population; CD44+/CD24-/ALDH+ subpopulation is more aggressive and resistant to therapy than other populations marked by these markers</li> <li>• CD44+/CD24-/ALDH+ subpopulation (as well as only ALDH+ cells) shows up-regulated stemness genes (OCT3/4, Nanog, SOX2, KLF4, BMI-1, Nestin) and the drug-resistant genes (MDR-1, MRP-1, ABCG2); CD44+/CD24-/ALDH1+ population has the most similar gene signature to mesenchymal stem cells (MSC) and MSC-related drift</li> <li>• EMT-related genes are activated in ALDH+ cells and Snail seems to be the most important regulator of CICs phenotype</li> </ul>	112,113
CD166	<ul style="list-style-type: none"> <li>• CD166+ cells have high capacity of sphere and tumor formation</li> <li>• Patients with high CD166 expression levels have poorer clinical outcome</li> <li>• CD166 expression is associated with tumor recurrence</li> <li>• CD166 enhanced phosphorylation of EGFR and (EGF)/EGFR pathway activation</li> <li>• Stimulation of CD166– cells by EGF causes increased cell tumorigenic ability <i>in vitro</i> and <i>in vivo</i></li> </ul>	114,115

is significantly connected with more differentiated, advanced and metastatic tumors and could be used as a prognostic factor.<sup>96</sup>

OCT3/4, another marker of CICs, positively regulates CIP2A expression. The co-expression of these two genes in HNSCC is linked with CD44+/CD24+ cell population, tumor poor differentiation, enhanced aggressiveness, radioresistance and reduced 5-year survival of patients.<sup>97</sup> Habu et al. showed that OCT3/4 and two other genes Nanog and ABCG2 were up-regulated in SP (side population) cells. SP cells are a class of CICs detected by low accumulation of Hoechst 33342 dye and characterized by high migration and invasion ability. It is not

surprising that the analysis of patients' samples showed that OCT3/4 can be used as a predictor of metastasis and advanced cancers.<sup>98</sup> Moreover, Nanog/OCT3/4/CD133 triple-positive oral carcinoma patients have a worse survival prognosis compared to the triple-negative group.<sup>99</sup> Tsai et al. proposed that OCT3/4 and Nanog could be used as potential predictive markers to distinguish resistant patients from those sensitive to cisplatin therapy.<sup>100</sup> However, this statement is not confirmed on a large group of patients.

Chang et al. observed that HNSCC cells can be divided into 3 groups depending on the level of reactive oxygen species (ROS): (i) ROS<sup>low</sup>, (ii) ROS<sup>high</sup> and (iii) ROS<sup>medi</sup> cells.

**Table 2 – Agents indicated as potentially useful in therapy of HNSCC based on CICs depletion and chemotherapy/radiotherapy.**

Agent	Description	Ref.
Salinomycin (livestock antibiotic)	Inhibition of cell viability and induction of apoptosis (elevation of Bax/Bcl-2 ratio); Synergistically action with cisplatin and paclitaxel causing higher cell mortality; Reduction of CICs – reduction of sphere formation capacity and suppression of CD44 and BMI-1 expression; Induction of EMT, phosphorylation of Akt and up-regulation of miR-328, miR-199a-3p and down-regulation of miR-203.	123
ABT-737 (BH3 mimetic small molecule inhibitor)	Enhancement of apoptosis and delay of tumor growth <i>in vivo</i> in combination with irradiation; Reduction of CICs (– SP+/CD44 <sup>high</sup> /ALDH <sup>high</sup> cells remain in the sub-G1 phase); cells are more sensitive to irradiation; increase the expression of Bcl-2 family members (except of Bak and PUMA).	124
Valproic acid (histone deacetylase inhibitor)	Reduction of CICs (reduction of sphere formation capacity, CD44+ population cells and expression of OCT3/4 and SOX2); Inhibition of tumor growth <i>in vitro</i> ; Enhancement of cisplatin action – increase of cell mortality by suppressing ABCG2 and ABCG6 transporters, activation of Bax and Caspase3 in CICs population.	125
Suberoylanilide hydroxamic acid (histone deacetylase inhibitor)	Inhibition of cell proliferation; Reduction of CICs – reduction of sphere formation capacity and Nanog expression; inhibition of tumor growth and metastasis ability <i>in vitro</i> ; Enhancement of cisplatin action in cisplatin resistance HPV+ and HPV– cell lines; Lack of additional toxicity <i>in vivo</i> .	126
Rapamycin (Sirolimus, mTOR inhibitor)	Inhibition of mTOR signaling and reduction of CICs – down-regulation of CD44 and SOX2 (no influence on OCT3/4); Reduction of tumor volume and weight; Reduction of tumor invasion by down-regulation of MMP-2 (no influence on MMP-9).	127
Curcumin (Diferuloylmethane) with cisplatin	Enhancement of CD133+ cells sensitivity to cisplatin; Reduction of cell colony formation; Decreasing expression of ABCG2 in CD133+ population.	128
Mesoporous silica nanoparticles (MSNs) with chemotherapeutic drug and siRNA against ABCG2	Combination of classical drug (5-FU, cisplatin or paclitaxel) with siRNA against ABCG2 loaded into nanocarrier; Enhancement of drug activity by blocking ABCG2 and breaking multidrug resistance; Enhancement CD133+ cells apoptosis; Reduction of tumor volume and weight.	129
Gene therapy (RNA restoration or RNA interference)	Restoration of IL-24 in CD133+ cells causing reduction of cell proliferation; Reduction of Snail expression improving sensitivity of ALDH+ cells to chemoradiotherapy.	113,130

This classification characterizes cells into groups with different chemoresistance, stemness and proliferation activity. The ROS<sup>low</sup> cells have features of CICs (CD133+, <sup>mem</sup>Grp78, Glut3, ALDH+) and their state may be supported by ROS-scavenging enzymes such as SOD2, CAT and PRDX3. The specific antioxidant phenotype probably allows CICs to protect their own genome and maintain the state of stemness. The microenvironment induces persistent ROS redox stress on CICs and causes generation of non-CICs populations in tumor mass.<sup>101</sup>

CICs are associated with cells ability to close and distant metastases. Some distinct cell populations with CICs features can be found circulating in blood and lymphatic vessels. They are referred as circulating tumor cells. Weller and colleagues observed cells in blood samples from HNSCC patients and distinguished three groups of cells: (i) mesenchymal, (ii) epithelial and (iii) a group with features of both types of these cells. Moreover, they detected circulating CD133+ cells subpopulations (probably CICs). It was shown that the number of epithelial-, mesenchymal- and stem cell-like circulating cells was decreased after the removal of tumor compared to the

number before the surgery. This indicates that tumor mass is the main source of these circulating cells. Patients had significantly shortened survival rate when mesenchymal-like circulating cells were still present after surgery.<sup>102</sup>

It should be noted that CICs formation is also affected by some different biological and physical factors. The influence of chemo-physical factors is clearly visible in 3D cell culture *in vitro* where changes in the culture method causes the maintenance and enrichment of CICs.<sup>116</sup>

#### 4. New therapeutic strategies

The CICs are able to survive after irradiation and chemical exposure.<sup>100,117</sup> Two of the most commonly used chemotherapeutic drugs in HNSCC treatment are cisplatin and 5-FU but it was proved that these drugs promote self-renewal and survival of CICs both *in vitro* and *in vivo*. They cause over-expression of BMI-1 as well as OCT3/4, CD44 and ALDH markers.<sup>120,121</sup> It was shown that cisplatin-resistant oral

squamous carcinoma cell line has more aggressive phenotype than resistant parental cell line and stemness markers (Nanog, OCT3/4, BMI-1, CD117, CD133) and drug resistant ABCG2 protein are over-expressed.<sup>100</sup> It should be noted that cisplatin can not overcome IL-6 induced STAT pathway that is involved in the maintaining of stemness.<sup>120</sup> Moreover, the CICs resistance to cisplatin may be regulated by ROS-scavenging enzymes. Treatment HNSCC ROS<sup>low</sup> cells with ROS-scavenging inhibitors chemo-sensitizes a tumor.<sup>101</sup> Yang et al. indicated that CD133+ cells are more resistant to chemotherapy, probably because of high expression of ABCG2.<sup>122</sup> Another study indicated significant correlation of high CD44 expression and positive lymph node with incomplete response to radio-chemotherapy and both of them could be used as independent predictive markers.<sup>117</sup>

The future therapies will target cancer cells and components of TME in the specific manner as well as reduce the number of CICs directly or indirectly. The depletion of cancer cells can be achieved by specific drugs (in most cases inhibitors), classical drugs with enhancement agent or specific cancer cell-related receptor, and by gene therapy–RNA interference.<sup>118,119</sup> There are many ongoing studies which are testing some stimulant agents with potential for therapeutic usage (Table 2). These “drugs” act as inhibitors for pro-cancer signaling pathways. Moreover, they may prevent the EMT process but also enhance chemotherapy effectiveness leading to cancer cell death.

## 5. Conclusions

Many different components have influence on cancer progression, invasiveness and metastasis. That provides evidence of the heterogeneity of cell types among the tumors and strong interaction between cancer cells and their environment. Each element is important on the way to the TME-targeting therapy in cancer cases. Cancer initiating cells rely on tumor microenvironment niche. Many researchers are trying to find a better solution for HNSCC patients by investigating signaling pathways existing in TME and understand CICs biology. The scientists are convinced that TME supports invasion of the tumor, its progression and metastasis. They also believe that TME may offer a broad spectrum of novel anti-cancer therapies, not only in HNSCC but also in other cancer types.

## Authors' contributions

TK and WP have contributed equally to this work.

## Conflict of interest

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

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