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Chapter 6

Squamous Cell Carcinoma: Biomarkers and Potential Therapeutic Targets

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Abstract

Squamous cell carcinoma (SCC) is the second most frequent non-melanoma skin cancer (NMSC) and carries with it a significant psychosocial and economic burden for both patients and health-care systems. Known risk factors for SCC include chronic ultraviolet (UV) exposure, chronic wounds and inflammation, exposure to certain chemicals and immunosuppression. The considerable risk of SCC recurrence and metastasis has driven the need for the discovery of new molecules that could explain the initiation and biological behavior of this type of NMSC. In this respect, proteomic research techniques have rapidly evolved and adapted in order to connect missing links and single out distinctive skin cancer biosignatures. Proteomic analysis of normal, dysplastic, and malignant keratinocytes appears to be promising in respect to SCC biomarker discovery, with the potential to aid in risk assessment, early detection, disease progression and development of novel targeted therapeutic agents. Identifying changes in the keratinocyte proteome pattern from normal to inflammatory and malignant cells will lead to the discovery of novel SCC biomarkers that could represent valuable tools for patient screening, diagnosis, management and follow-up.

Keywords: squamous cell carcinoma, keratinocytes, carcinogenesis, biomarkers, proteomics, diagnosis, therapy

1. Introduction

Squamous cell carcinoma (SCC) accounts for about 25% of non-melanoma skin cancers (NMSC) and together with basal cell carcinoma (BCC) (75%), it represents the most frequent

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skin malignancy worldwide [1, 2]. Particularly, in the last several decades, the risk of developing cutaneous squamous cell carcinoma (cSCC) has been increasing epidemically, reaching approximately 7–11% [3]. Clinically, cSCC shows up red patches, rough or scaly, that can bleed or crust with slow healing. The affected skin is usually the one that is most exposed to sunlight, and body regions such as the head, face, neck and dorsum of the hands can carry a significant risk of developing cSCC. Seldom, it occurs in genital areas. However, it is important to know that cSCC can also be found in scars or skin sores [4]. Although numerous risk factors for developing cSCC have been noted, one of the most significant etiological factors is ultraviolet (UV) light that is responsible for damaging DNA, followed by chemicals, ionizing agents, radiation, chronic skin ulceration, weakened immune system, HPV infection, smoking, light-colored skin, and male gender [4]. In almost 65% of cases, cSCC arises from premalignant conditions such as actinic keratosis [5]. Also, immunosuppression caused by organ transplant or chemotherapy targeting BRAF favors the development of cSCCs with RAS mutations, elevating steadily the incidence of skin cancer by over 65-fold [6].

Even though its mortality rate is relatively low, approximately 2.1%, cSCC has many subtypes that widely vary from harmless to aggressive skin tumors with important metastatic potential, from 2 to 10% [7]. Initially, it invades adjacent tissue, then the regional lymph nodes and ultimately it affects distant organs [8]. The localization of cSCC influences the risk of recurrence and dissemination; cSCC affecting the lips or ears was demonstrated to have a higher risk of invasion (10–25%) [9]. In addition, an up-to-date prospective study established that a primary skin tumor size above 2 cm has a 15% chance of recurrence and a 30% chance of metastasis. Also, histological features such as speed of tumor growth, tumor depth greater than 4 mm, poor differentiation, perineural and subcutaneous invasion is associated with aggressive cSCC, leading to significant morbidity and mortality [8, 10, 11].

Although the vast majority of cSCC usually respond well to conventional treatments including wide surgical excision, chemotherapy, targeted therapy and radiotherapy, none of them can ensure the cure. Hence, approximately 3–5% of cSCCs recur and almost 5% metastasize within 5 years [8]. In addition, approximately 5% of metastatic cSCCs are associated with very poor clinical outcomes. There are no therapies officially approved by the FDA with a specific indication for metastatic cSCC and so the development of new agents has been relatively deliberate, due to a limited knowledge of the molecular basis of this disease. Therefore, there is a high necessity of identifying the complete genomic portrait of cSCC represented by multiple genes with recurrent mutation, amplificatiotn, and deletion including several other alterations which are aimed at developing new biomarker-associated therapeutic targets [12].

2. UV-induced keratinocyte proteome alterations

UV radiation could be considered a “Dr. Jekyll and Mr. Hyde” factor being both beneficial by facilitating vitamin D and endorphins synthesis but also harmful in prolonged exposure of the skin working as a carcinogen [13]. As cellular DNA is the major target for UVB radiation

(290–320 nm), this range has an increased mutagenic and carcinogenic potential by comparison with UVA (320–400 nm), being the most harmful constituent of sunlight that reaches the Earth surface [14]. Chronic and excessive exposure to UV radiation conveys many health risks where, besides photoaging, genomic and proteomic alterations at skin level can lead to immunosuppression favorable to the most common forms of skin cancer, BCC, SCC and melanoma. Genetic factors such as polymorphisms of the melanocortin 1 receptor gene can also influence the skin's sensitivity to UV and enhance cancer risk [15].

The UV-derived effects on skin cells in the proteomic context have not commonly been approached in photobiology and, as a consequence, only a few studies could be retrieved in this domain [16]. Although UVA is about 20-times more abundant than UVB in incident sunlight, its damaging potential on cellular DNA is less dangerous than UVB. The UVA effects are mediated by reactive oxygen species (ROS) that induce oxidative stress affecting the proteome through oxidation of DNA repair proteins, thus inhibiting DNA repair [17].

Almost entirely, published studies refer to UVB effects as triggers of significant alterations in skin cell layers, especially in keratinocytes, the major cell type of the epidermis and the main defensive barrier against external threats. UVB could also raise the increased ROS level responsible for oxidative damage of nucleic acids and proteins. Normal human epithelial keratinocytes isolated from foreskin and subjected to UVB were tested by parallel proteomics approach for assessing the protein expression profile and also for identifying proteins modified through chemical oxidation. In UVB-irradiated keratinocytes, various proteins involved in cellular homeostasis such as cytoskeleton integrity, removal of damaged proteins or heat shock response were differentially regulated (e.g., prohibitin, integrin alpha-3, cytokeratin 5, proteasome subunit alpha type-6) while some specific proteins with roles in cell adhesion, intercellular interaction, and protein folding were carbonylated (e.g., Glucosidase 2 b subunit, GRP 78, actin-related protein 3, annexin 2). These protein alterations driven by UVB exposure could cause cell homeostasis deregulation and eventually trigger cellular senescence or carcinogenesis [18].

Although it was reported that keratinocytes are more resistant to UV than other cell types, recurrent exposures to UVB induce at keratinocyte level, a so-called alternative state of differentiation, noticeable even 64 h after exposure [19]. Thus, a 2D-DIGE proteomic profiling of this specific state revealed a 69 differentially abundant protein patterns belonging to differentiation and survival keratinocyte machinery. Specifically, upon UVB action, an increased expression of a protein called TRI partite Motif Protein 29 (TRIM29) was noticed, further confirmed by Western blot assay. TRIM29 protein protects against UVB exposure damaging effects, as knocking down the TRIM29 expression by RNA interference, the viability of keratinocytes declined. These findings suggest that TRIM29 protein contributes to the survival of differentiating keratinocytes by inducing an alternative differentiation status protecting cells from dying, owing to UVB exposure-related stress [20]. The enhanced expression of TRIM29 as keratinocytes "regenerator" should be associated *in vivo* with the altered expression of other key proteins (heat shock proteins, cytokeratin, and cytoskeletal proteins), inflammation process, epidermis remodeling, and immune response type, as these could be novel mechanisms of keratinocyte survival upon UV damage [16, 21].

3. Chemically induced keratinocyte proteome alterations

Chemically induced tumors in experimental models can mimic all the clinical cancer progress phases being useful in the evaluation of new drugs, studying biological context or in decoding molecular mechanisms responsible for tumor initiation and development. Among chemical carcinogens commonly applied in cancer models are the following: environmental contaminants, N-nitroso compounds, food additives, antineoplastic agents, natural and synthetic substances, etc.

Combining chemically induced cancer models with innovative molecular imaging techniques may help to advance new anticancer diagnostics and therapeutics protocols [22]. By studying skin carcinogenesis, phases of early alterations in the skin layers and of the mechanisms beneath are highlighted. These mouse models share common mechanisms with human skin tumorigenesis, and moreover, there are similarities in terms of genetic milieu caused by carcinogens and pro-inflammatory cytokines and chemokines that favor tumor progression [23].

Different carcinogens imprint different changes on skin cells, including on the keratinocyte proteome pattern. Mancozeb—*ethylene (bis)dithiocarbamate*—is a fungicide and a multipotent carcinogen whose underlying mechanism of action is mostly unknown. By a two-dimensional gel electrophoresis and mass spectrometry analysis, a proteomic profile of mice skin exposed to mancozeb (200 mg/kg body weight) was generated. By comparison with control samples, two significantly upregulated proteins were found, Calcyclin (S100A6) and Calgranulin-B (S100A9); these two proteins are well-known markers of keratinocyte differentiation and proliferation, suggesting their role in neoplastic alterations induced by Mancozeb. The same approach in human keratinocyte carcinogenesis model with HaCaT cells revealed that upregulation of S100A6 and S100A9 confirms the neoplastic potential of Mancozeb. The authors conclude that S100A6 and S100A9 modulate the ERK1/2 signaling pathway underlying in this way the Mancozeb-induced neoplastic potential in human skin [24], and thus, a certain proteome milieu prescribe keratinocyte behavior in a chemically triggered carcinogenesis.

4. Spotting differences between normal and inflammatory keratinocyte proteome pattern

The skin proteome has been the target of intense research in the last years, hence human epidermal keratinocytes, dermal fibroblasts, human epidermis, were characterized regarding their proteome pattern [25–28]. Ong et al. furthered these studies and published the specific proteomic markers in the normal skin and in the one subjected to inflammatory processes. In normal skin, there are several proteins that were reported as having high expression, such as carbonic anhydrase, HSP27, gelsolin, prostate binding protein, MnSOD, a1-antitrypsin, keratin 1 and keratin 10. On the contrary, in keloid scars, there is a low expression or even absence of carbonic anhydrase proving the maintenance of local inflammatory status of the skin. In this manner, the inflamed skin shows intense expression of the proliferative keratin 16 [29]. Other proteomic markers are found to be increased in the inflamed keratinocytes in

comparison to normal skin, such as S100A4 /A8 /A9 /A10 [30]. The over-expression of this protein family was found also in other inflammatory diseases as well as in psoriatic keratinocytes [31, 32]. Over-expression of mast cell proteins was also found in inflamed keratinocytes, namely activation proteins, mast cell b-tryptase, and so on [29]. Mast cell b-tryptase can further induce tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin-1b (IL-1b) upregulating and also collagen type I and fibronectin expression [33]. Another pro-inflammatory protein found overexpressed in inflammatory keratinocytes, macrophage migration inhibitory factor (MIF), can be involved in the amplification of the inflammatory responses developed during wound healing.

Components of the skin's extracellular matrix (ECM), like the small leucine-rich proteoglycan family members, asporin and decorin, are inhibited by direct binding of the transforming growth factor β (TGF-β) activity [34]. In inflamed keratinocytes, high asporin expression was reported and this overexpression is probably due to the inflammatory response in human dermal wounds [29].

Differences in the proteomic pattern between normal and inflammatory keratinocytes reside in several important classes of overexpressed proteins. These are appending to the inflammation, tumor suppression, and fibrosis processes. The dynamic expression of these proteins can be important in depicting the therapeutic target potential.

5. SCC progression and aggressiveness

Recent studies have compared non-advanced SCC to advanced SCC, in order to identify pathways that are activated in SCC progression. Considering the fact that SCC develops on skin areas exposed to sunlight, UV radiation is found to be responsible for activating signal transduction pathways in the processes of apoptosis, inflammation, proliferation, and differentiation, necessary for SCC development [2].

Molecular marker studies that reflect the initial changes in skin carcinogenesis have shown that the most important gene involved in the ultraviolet radiation effects is the p53 tumor suppressor gene, which plays an important role in apoptosis, cell proliferation, DNA differentiation and repairing process. Mutations of the p53 tumor suppressor gene result in the occurrence of other mutations in cascade with the loss of control of aberrant cell growth, leading to the formation of cancer cells [35, 36].

Also, several biomarkers, such as E-cadherin, Ki-67 and cyclin D1, have been shown to correlate with malignancy in NMSC [37]. Thus, designed to maintain the stability of epithelial tissues, E-cadherin is a Ca(2+) dependent intercellular adhesion molecule, whose downregulation is closely related to the increased potential for tumor invasiveness and metastasis. In SCC, a decrease in E-cadherin expression in the primary lesion is correlated with the development of regional lymph node metastases [38]. Ki-67 is a marker of the cell proliferation and a representative in fast and frequent recurrent aggressive tumors [39]. An important regulator of the cell cycle, cyclin D1 is a proto-oncogene which is essential in the development of skin cancer leading to the organization and abnormal differentiation of tissues [40].

It is known that main factors leading to tumor genesis are mutations in the tumor suppressor genes, such as the APC gene. Thus, mutations at this level lead to the synthesis of a non-functional APC protein that induces β-catenin destruction (the latter being a factor that activates transcription of oncogenes such as MYC and cyclin D1). In addition, 75% of patients diagnosed with cSCC were identified with mutations in NOTCH tumor suppressor genes [41].

Inhibition of squamous cell differentiation has been shown to be one of the most important mechanisms in the development of cSCC. Many therapeutic approaches have been proposed that have had at the forefront this mechanism and the molecules involved. Thus, S100 is a family of Ca-modulated proteins comprising the S100A7-psorasin-protein identified both in the keratinocytes in the psoriatic skin and in the various types of SCC (lung, oral cavity, bladder, skin) with an important role in metastasis and cellular differentiation [42–46]. Furthermore, the gene encoding the information required for the synthesis of this protein is located in the chromosome 1q21 containing the epidermal differentiation complex [47]. Recent studies have highlighted that overexpression of S100A7 is associated with increased cellular survival rates by decreasing cell differentiation, while poor expression of S100A7 correlates with significant cellular differentiation [48].

It has also been found that expression in tumor cells of a member of the signal transducer and activator of phosphorylated pSTAT3 transcription is closely correlated with the depth of tumor invasion and metastasis [49].

A fairly controversial issue has recently been the link between SCC and collagen VII, as a mortality of about 80% has been reported in patients with severe generalized recessive dystrophic epidermolysis bullosa (RDEB) associated with metastatic SCC. Currently, the link between SCC aggressiveness and collagen VII (Col 7) is being debated considering that mortality is high (more than 78%) in patients with severe generalized RDEB with metastatic squamous cell carcinoma. Mutations occurring in COL7AI, the gene encoding information for Col 7 synthesis, cause RDEB [50]. This disease is characterized by the fragility of skin and mucous membranes due to a decrease in Col 7 formation (the main component of the anchored fibrils) leading to formation of blisters and chronic skin trauma (a risk factor for SCC) [50]. There are many studies in progress that try to increase Col 7 synthesis by different methods but it has been observed that high levels of Col 7 are associated with activation of Phosphoinositide 3-kinase pathway which leads to an increase in SCC invasiveness, so there is no formal control of this process [51].

Moreover, tumor clinical factors such as size, anatomical location, tumor thickness, depth of invasion, histopathological subtypes, perineural invasion and inflammation [10, 37] correlate with an increased risk of developing metastatic lesions with significant impact on progression and aggressiveness of SCC. Immunocompromised patients have been shown to have a more aggressive course of SCC. In addition, there is evidence that age and sex can play a role in survival [52]. Although some of these factors provide a perspective on the prognosis and metastatic potential of SCC, they are less used in practice and have not been included in staging schemes [53].

6. Cancer stem cells in SCC

Cancer stem cells (CSC) represent a pluripotent population of tumor cells with self-renewal properties playing an important role in tumor initiation, growth and maintenance [54, 55].

There are many studies, both *in vitro* and *in vivo*, that investigate the involvement of epidermal stem cells in skin carcinogenesis, tumor invasion, metastasis [56, 57] as well as tumor recurrence [58, 59]. Post-initiation, CSCs can generate macroscopic tumors through self-renewal and processes leading to stem cell differentiation generating several cellular variants. Normal epithelial tissue continuously renews and is maintained through the action of proliferating stem cells. When their density decreases, stem cells generate proliferative colonies called holoclones. They bear different characteristics to the abortive colonies of differentiated cells, called paraclones [60]. It is worthwhile highlighting the limited ability of stem cells to renew, making them susceptible to carcinogenesis. This is relevant because it shows the impact of CSC in the development of SCC. It is therefore important to focus on any kind of CSC-related biomarkers that could provide insight into potential therapeutic SCC management schemes.

Cancer stem cells derive either through transformation of normal stem cells (NSCs) or through de-differentiation of tumor cells. Following initial transformations at the level of 17p (TP53) and 3p/9p (p16/FHIT), NSCs give rise to transformed transit-amplifying cells (TACs). These cells first multiply, then expand, and can lead to development of a neoplastic cell field. A further modification of the Rb 13q gene is followed by the inception of the main tumor. Tumor invasion can occur either in a monoclonal or polyclonal cancer inducing way. Thus, tumor CSCs can either spread through lateral migration (CD44h/ALDH1A1h) or they can plant and form a genetically similar tumor, as per the monoclonal model. On the other hand, successive modifications of normal stem cells in the epithelium can lead to the development of independent clones, as per the polyclonal model (**Figure 1**) [61].

Another important biomarker linked to proliferation and differentiation of skin cancers is CD133 [62, 63]. It is a transmembrane hematopoietic stem cell glycoprotein that correlates with an advanced stage of a poorly differentiated tumor, thus having a poor outcome in SCC [64–69]. Several studies have provided evidence that CD133+ CSCs exhibit resistance to apoptosis induced through action on TGF-β, or through tumor necrosis factor. This strengthens the conclusion that new therapeutic agents are required and they need to focus on CD133 being directed at stopping tumor recurrence and metastatic spread [70, 71].

An interesting aspect is that the phenotypic heterogeneity and plasticity of CSC has been associated with epithelial-to-mesenchymal transition (EMT), another important factor linked to both local and remote tumor invasiveness. Although directly responsible for many deaths caused by cancer, its role in SCC is still under debate. EMT is a process involved in embryogenesis and it is designed to create the mesoderm during gastrulation. This is a process through which epithelial cells acquire a migratory mesenchymal phenotype [71]. When the migratory mesenchymal cells mature, they may undergo a reverse process—mesenchymal-epithelial transition, to regain the epithelial phenotype. EMT and non-EMT CSC populations show a strong evidence of CD44 so much that they co-exist transitioning between the two phenotypic states through EMT and reverse mesenchymal-epithelial transition. To note, both cell types are present in oral squamous cell carcinoma (OSCC) generated cells. In addition, recent research that studied the expression of CD44 and epithelial-specific antigen (ESA) clarified that CD44(high)/ESA(low) EMT CSC has a mesenchymal phenotype, while CD44(high)/ESA(high) non-EMT-CSC has epithelial characteristics. To note, EMT CSC requires an ALDH + phenotype (aldehyde dehydrogenase 1) to evolve into non-EMT CSC and to develop metastasis [72].

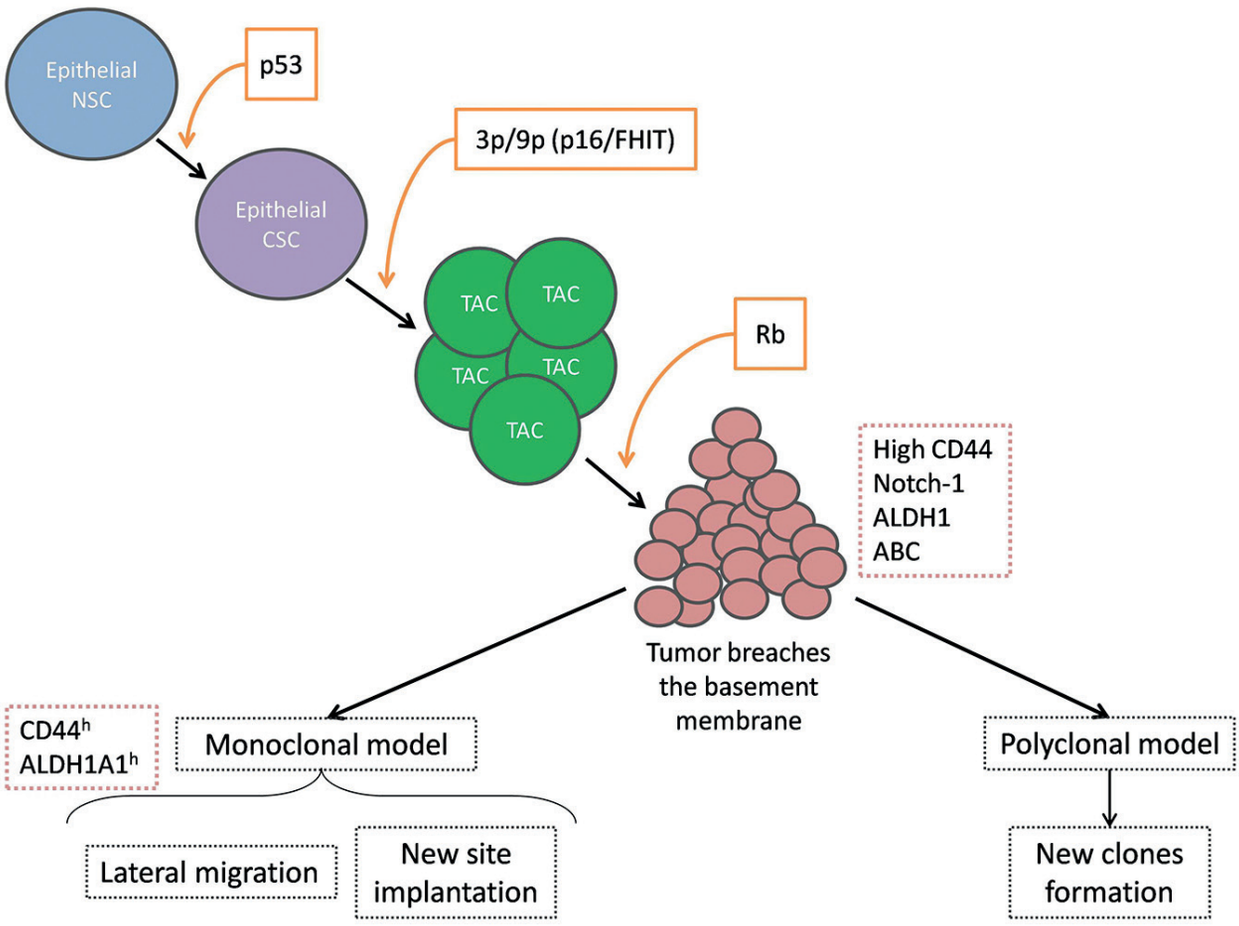


Figure 1. Model for cancer stem cell field onset the process is initiated by a carcinogenic injury producing an alteration (p53/p16/FHIT) in the epithelial normal stem cell (NSC). The cancer stem cell (CSC) will proliferate and form a patch of transit amplifying cells (TAC) which then extends to form a field. At this point, the cells are still in a dysplastic, premalignant stage. It is only after another assault (Rb), one of the field's cells forms the primary tumor. Field cancerization progression takes place through either the monoclonal or polyclonal models. In the monoclonal model, CSCs extend the field by lateral migration (CD44h/ALDH1A1h) or implant at a new site ultimately forming a genetically similar tumor. In the polyclonal model, multiple assaults to epithelial NSCs lead to the evolution of independent clones.

A large number of ALDH1 cells have been detected in lymph node metastases, more than the corresponding primary tumors—indicating the CSC capacity to complete metastatic cascade and to develop metastases.

It has also been shown that a CD44-regulated signaling pathway mediated by the phosphorylation of glycogen synthase kinase 3 (GSK3) can influence CSC phenotypes [73]. Hence, the inhibition of GSK3 reduces the expression of stem cell markers and upregulation of the differentiation markers found in the CD44(high)/ESA(high) cell fraction reversing from EMT and back to the epithelial CSC phenotype [74].

EMT's involvement in several types of cancers such as OSCC [74], breast cancer [75] and others is variable, affecting both tyrosine kinase receptors as well as Wnt signaling pathways [76].

It has been shown that cell lines derived from oral and dermal SCC contain a new population of CSC that influences EMT. It has also been established that EMT is involved not only in

therapeutic resistance but also in tumor recurrence [59, 77, 78] being associated with resistance to epithelial growth factor receptor (EGFR) inhibitors [79]. In epithelial tissues, some stromal signals may induce EMT, leading to downregulation of epithelial processes and upregulation of EMT-inducing transcription factors such as Twist and Snail [80, 81].

7. Current and future molecular therapeutic targets in cutaneous SCC

The high level of gene mutations in UV-exposed skin has hampered the search for novel hints of disease invasiveness and metastatic potential. In SCC, metastasis to regional lymph nodes occurs in roughly 5% of cases and is associated with significant morbidity. Clinical biomarkers of SCC metastasis are currently missing and histological assessment could be unreliable [82]. Cutaneous SCC typically manifests gradually, ranging from a precursor actinic keratosis (AK) to *in situ* SCC, invasive SCC, and finally metastatic SCC. Molecular inquiring into SCC could be done by different experimental models. Although the differences in skin structure amid mice and humans would limit somewhat the correlation with human disease, transgenic mice models have revealed that upregulation of the EGFR/Fyn/Src/Erk pathway acts critical for promoting SCC [83] or UVB-induced cutaneous neoplasia [84].

Latest studies associated omics approaches with humoral immune systems components in SCC involvement; thus recent approaches discern the expression of complement system components in SCC. SCC cell lines and human normal keratinocytes were profiled with an Affymetrix platform and then subjected to quantitative real-time PCR revealing upregulation of complement factor H (CFH) and factor H-like protein-1 (FHL-1) mRNA in cancer cell lines and were proven significantly higher in tumors compared to normal skin. Moreover, immunohistochemistry analysis of CFH and FHL-1 in invasive SCCs, *in situ* SCCs and premalignant lesions (actinic keratoses) showed a specific and stronger expression in SCCs compared with *in situ* carcinoma and actinic keratoses. Not surprisingly, it was found that the level expression of complement factor I (CFI) was higher in the aggressive transformed cell line (RT3) than in less tumorigenic HaCaT cell lines. In addition, by knocking down CFH and FHL-1 expression, proliferation and migration of SCC cells were inhibited, suggesting a role of CFH and FHL-1 in cSCC progression and spotting them as progression markers and potential therapeutic targets in skin SCCs [85, 86].

Huge costs related to skin cancers therapies in general, including SCC became another request for defining reliable biomarkers and better understanding a pathogenesis with significant public health impact. Host immune system influences SCC risk as its incidence is considerably higher in patients with compromised immunity. Very recent studies assign a role for HLA system in SCC risk. Unlike BCC and cutaneous melanoma, SCC often displays partial expression of HLA I proteins, also exhibiting aberrant surface expression of HLA II proteins as a defense mechanism for immune evasion. Analyzing allelic variation and cell-surface protein expression germline of HLA I and II antigens in SCC patients and healthy controls, it was suggested that HLA pattern differs between immunocompetent and immunosuppressed patients regarding the risk for developing SCC. This difference may be owed to some viruses (HIV, HPV) that potentiate tumorigenesis in immunosuppressed patients

[87, 88]. In immunosuppressed HPV-infected patients, it was reported a notable HLA I—SCC connection, probably due to the fact that HLA I processes and presents intracellular peptide antigens, including viral proteins, and thus HPV could be a co-factor of tumorigenesis [87].

Alterations in the composition of basement membrane and dermal extracellular matrix of premalignant lesions are early events in cSCC progression. An influx of inflammatory cells promotes the secretion of proteases, which in turn regulates the availability of growth factors, cytokines, and chemokines and thus influences the growth and invasion of cSCC. Later, the number of inflammatory cells increases with cSCC progression, and the expression of complement factors and inhibitors by tumor cells is induced (CFI, CFH, FHL-1) [89]. A fine interplay between matrix metalloproteinases (MMPs) and their inhibitors could settle the scene for discovering new targets and prognostic or monitoring predictors of the disease. As in cutaneous melanoma, where the role played by MMPs in the phenomenon of regression is an actual approach [90], in SCC, the cellular enzymatic portfolio is a good pool for emerging novel targets coupled to novel biomarkers. For instance, upregulation of MMP-7 expression has also been registered in cSCC, especially in the tumor invasive edge, and moreover activates heparin-binding epidermal growth factor-like growth factor (HB-EGF) promoting cellular proliferation [91] and thus suggesting a future therapeutic effect of HB-EGF antagonists in advanced cSCC [12].

Serine peptidase and their inhibitors (Serpins) are also considered useful for biomarker monitoringing of cSCC progression. Studies performed on serpin family gene expression levels in cSCC cell lines versus normal keratinocytes demonstrate a significantly raised Serpin-A1 expression correlated with the tumorigenic change of keratinocytes [92]. *In vivo* studies correlate Serpin-A1 expression with tumor progression in SCC tumor cells. By using a chemically induced skin carcinogenesis mouse model, as a valuable tool in completing cancer progression profile [23] it was checked the correlation of Serpin-A1 expression with progression of mouse skin SCC [92], suggesting that Serpin-A1 may serve as an useful biomarker for monitoring cSCC progression. Maspin is another member of serpin family—an inhibitor of mammary serine protease—reported as a tumor suppressor in various cancers. Real-time PCR and Western blotting analysis found that Maspin was downregulated in the cSCC tissues compared with the nearby normal tissues. Studies performed on A431 cell line revealed that overexpression of Maspin inhibits growth, cellular proliferation and enhances A431 cells apoptosis by increasing PARP and Bax expression, while decreasing Bcl-2 expression. Therefore, Maspin analysis may provide new insights in the diagnosis and therapy of cSCC [93].

New potential classes of agents for cSCC are also directed to counteract the metastatic feature of this tumor which represents a difficult challenge, knowing that metastatic cSCC has a mortality rate of over 70%. As a comprehensive chemotherapeutic approach in the metastatic form is still lacking, new molecular insights are to be done. Recently, expression of EGFR and nuclear active IκB kinase (IKK) was proved to have a role in metastatic prediction. Thus, a newer and more promising class of agents for metastatic cSCC therapy is represented by EGFR inhibitors. Other advances in finding novel treatments for metastatic cSCC are related to p53 studies, epigenetic approaches such as hypermethylation of specific genes, chromatin remodeling, and the RAS/RTK/PI3K pathway [94]. Molecules with well-established roles in

epithelial adhesion are currently studied regarding their metastatic involvement. Thus, collagen XVII, integrin $\alpha 6\beta 4$ and especially their binding partner laminin 332 are mainly recognized to promote invasion and metastasis in various tumors. By tissue microarray analysis, it was registered that $\gamma 2$ chain of laminin 332 has the highest expression in SCC samples, whereas the expression of collagen XVII and integrin $\beta 4$ greatly differs in SCC and precursors lesions (actinic keratosis and Bowen's disease) [95] and moreover, integrin $\beta 4$ knockdown would reduce the migration of keratinocytes and of malignant cells [96]. All these results suggest the contribution of collagen XVII, integrin $\alpha 6\beta 4$ and laminin 332 to SCC tumorigenesis through their variable expression patterns translated in different migrations and invasion features [97].

Thereby, the tumor microenvironment plays an important role in cSCC progression, offering a genuine reservoir for finding novel targets for both therapeutic purposes and risk assessments in cSCC.

8. Biomarkers of oral SCC

Despite recent advances in diagnosis and therapy, OSCC is still one of the most difficult malignancies to handle due to its great invasive potential both locally and at lymphatic level (in the cervical lymph nodes) [98]. Its occurrence varies across the world as it is closely linked to diet and lifestyle choices (alcohol and cigarettes). OSCC occurs as a result of squamous cells genetic mutations, the new cells developing multiplicative and invasive characteristics [99]. Its genetic heterogeneity can be later highlighted by the fact that many tumors, at a similar stage and location, present significant clinical differences and they can react very differently to treatment. Although the therapeutic strategies are in a permanent development, the survival rate of OSCC patients remains low. It has also been found that predicting treatment outcome using conventional clinical and histopathological parameters carry a low success rate.

It is clear that histopathology remains to this day the benchmark decision-making process as far as diagnosis and treatment are concerned. However, recent molecular studies have made significant progress in understanding and identification of those biomarkers best placed to predict OSCC aggression. Attempts have been made to refine histopathological analysis with immunohistochemistry; this detects gene composition at protein level and brings forward several prognostic tumor biomarkers associated with OSCC's clinical outcome. As such, tumor suppressor genes, oncogenes, angiogenic markers, cell adhesion molecules and cell proliferation markers have been discovered to be potential tools that could help to predict the outcome of OSCC patients [99]. Therapeutic management through molecular inhibition directed at those biomarkers associated with radiotherapy and/or adjuvant chemotherapy are promising treatments for OSCC patients.

EGFR is a transmembrane cell-surface receptor that binds to ligands such as EGF and TGF-α and is one of the most studied OSCC biomarkers. It triggers the activation of the protein-tyrosine kinase system, which acts as a regulator of the signaling process linked to cell multiplication and differentiation [100]. It plays a significant role in OSCC's resilience to radiotherapy.

According to Shiraki et al. [100], cyclin D1 and EGFR together correlate to low survival rates of OSCC patients. It is worth mentioning that despite being an oncogenic gene with a major role to play in tumor invasion, cyclin D1 (independent of EGFR) bears no pathological significance to OSCC.

Recent years have seen a shift toward therapy and prognosis, with a strong emphasis on those molecular biomarkers associated with tumor suppression and apoptosis, especially p53/p63 and Bcl-2 [101]. High levels of Bcl-2 have been proven to correlate to low survival rates of OSCC patients [102–104].

Another important factor in the carcinogenesis of human solid tumors is hypoxia; it is responsible for the adaptive modifications of malignant cells allowing them to survive [105, 106]. Unfortunately, little data is available to help scale its importance within the framework of OSCC prognosis. Antitumor therapy targeting angiogenic biomarkers has been a subject to many recent studies. This is due to the fact that angiogenic processes play a key role in the formation of neo-capillary networks and is essential to cancer growth, progression and metastasis [107]. Thus, the most important angiogenic biomarker involved in carcinogenesis and OSCC tumor dissemination is VEGF which plays a crucial role in the maintenance of tumor vasculature [108, 109].

Tumor invasion is based on several factors, including cellular interaction, requiring both matrix degradation enzymes (MMPs) and cell adhesion proteins (cadherins). MMP is a family of proteases expressed by invasive tumors and adjacent stroma. They were also associated with low survival rates in patients with OSCC without lymph node metastasis [110]. Cadherins are transmembrane glycoproteins with important functions in cell adhesion making them important in tumor invasion and metastasis [111].

9. Biomarkers in genital SCC

Vaginal squamous cell carcinoma (VaSCC) is a tumor with a relatively low occurrence rate of 1–2% of all gynecological malignancies [112], but it can occur in approximately 30% of cervical cancer cases [113, 114]. Despite the low number of studies concerning this type of cancer, epidemiological, virological and clinical-pathological data available show two distinct entities of this genital SCC. They develop through two etiopathogenic pathways: one is linked to HPV infection, while the second is HPV-independent. Available studies do not provide enough information on their significance to the final outcome and they require further investigation. However, it is known that most VaSCCs are closely related to HPV, emphasizing the idea that it shares a common pathway with cervical cancer [114].

As far as the biomarkers linked to genital SCC [23, 115–117] are concerned, their discovery is relevant due to their significant impact on early diagnosis and timely treatment. Numerous studies link p16 expression with a less aggressive form of vulvar SCC and a reduced death rate. On the other hand, patients exhibiting p53 mutation have a worse prognosis, frequent relapses, and greater associated mortality [118]. Other molecular markers with a negative impact on SCC patients are cofilin-1, galectin-7, and wee1 [119]. Moreover, it has been found

that lymphatic invasion and poor tumor differentiation correlate with downregulation of galactin-7 and wee1 [120, 121]. A very important role is played by cofilin which has major implications in carcinogenesis and vulvar SCC invasion [122]. This has turned it into a therapeutic option as it significantly reduces tumor progression. Also, other reports worthy to be considered indicate that downregulation of galectin-7 and high wee1 expressions have been correlated with an increased metastasis risk [120, 121].

Regarding treatment options, surgical resection is associated with a high mortality rate; therefore, attempts are being made to avoid and replace it with radiotherapy associated with chemotherapy [123, 124]. Due to limited options available, there is a real need for new targeted therapies being developed grounded on specific biomarkers.

SCC with penile localization (PSCC) has a relatively low incidence and is associated with poor hygiene, lack of circumcision, HPV infection, and tobacco use [122, 125–127]. Much the same as vulvar SCC, HPV infections play an important role. Starting with the HPV DNA incorporation step into the human genome, E6 and E7 genes deactivate tumor-suppressing genes. Due to the low occurrence rate of PSCC, there are not many studies looking at this type of cancer. These studies have found an increased concentration of Hsp70 [128]. Although not specific to PSCC and present in other types of cancer such as breast, colon, liver, and prostate cancer [129], it is believed to play a protective role for the tumor cells and is thus involved in carcinogenesis. A study looking at families of plaque molecules involved in the binding of filaments, desmosomes, and hemidesmosomes [130] has linked poor expression of plectin (a cytolinker of this family) with rapid cancer progression [131]. Since the diagnosis of inguinal metastases is currently the most important prognostic factor, the discovery of other biomarkers involved in a possible therapeutic management is imperative [128].

10. Conclusions

cSCC is associated with different trigger factors such as UV radiation, especially UVB which induces the alteration of skin layers and therefore the destruction of defensive barrier against external threats, but also the oxidative damage of nucleic acids and proteins through the increased levels of ROS. Therefore, an increased expression of TRIM29 is observed with the survival of differentiating keratinocytes. The chemical factors responsible for inducing SCC are also interfering in the keratinocyte differentiation and proliferation.

The exposure to UV radiation determine mutations of p53 tumor suppressor gene (responsible for apoptosis, cell proliferation, and DNA differentiation) together with the modifications of different biomarkers such as E-cadherin (a decrease in E cadherin expression in the primary lesion is correlated with the development of regional lymph node metastases), Ki-67 (associated with recurrent aggressive tumors) and cyclin D1 (a proto-oncogene which is essential in the development of skin cancer leading to the organization and abnormal differentiation of tissues). Furthermore, the expression of S100A7 which belongs to Ca^{2+}-modulated proteins S100 family is associated with increased survival rate, while its poor expression correlates with significant cell differentiation.

Another important role in carcinogenesis is attributed to cancer stem cells which derive from the transformation of normal cell or through the differentiation of tumor cells migrating through normal tissue. CD 133 is one of the most important biomarkers linked to proliferation and differentiation of skin cancers so that new therapeutic targets are needed to be focused on this transmembrane hematopoietic stem cell glycoprotein.

Regarding the molecular aspects of cutaneous SCC, studies have shown not only the high levels of complement factor H and factor H-like protein 1 mRNA in comparison with normal skin, but also stronger expression in SCC than *in situ* carcinoma or actinic keratosis. In addition, it was demonstrated that knocking down CFH and FHL-1 lead to the inhibition of proliferation and migrations of SCC cells, suggesting their importance as progression markers and potential therapeutic targets in skin SCC.

An important aspect in risk evaluation for SCC is the integrity of immune systems. The high incidence of this malignancy in patients with compromised immune system was observed, pointing out the role of HLA system which varies between immunocompetent and immunosuppressed patients.

Other biomarkers involved in SCC development and progression are MMPs, serine peptidase and their inhibitors (Serpin-A1 being associated with tumorigenic change of keratinocytes and tumor progression). The metastatic prediction is attributed to EGFR and nuclear active IκB kinase (IKK) expression, thus a promising cSCC therapy is represented by EGFR inhibitors.

Oral squamous cell carcinoma is associated with EGFR that not only activates the protein-tyrosine kinase system involved in cell multiplication and differentiation, but also plays an important role in OSCC resilience to radiotherapy. Others biomarkers associated with OSCC are p53/p63 and Bcl-2. Tumor invasion is correlated with both matrix degradation enzymes and cell adhesion proteins.

Genital squamous cell carcinoma is linked with p16 (in less aggressive form of vulvar SCC), p53 (weaker prognosis), cofilin-1, galectin-7 and weel. HPV infection plays an important role in SCC induction and evolution through the deactivation of tumor suppression genes by E6 and E7. Increased Hsp70 is also increased in this type of cancer.

Overall, skin cell carcinoma is one of the most frequent malignancy worldwide that even if it is easily treated and the cure rate is high, there are cases when metastasize can occur. An accurate clinical exam correlated with histological, immunohistochemical and proteomic investigation can establish the biomarkers involved in the development and evolution of this malignancy and reveal the appropriate treatment strategy for each patient.

Due to the fact that SCC is associated with frequent recurrence and sometimes metastasis, it is necessary to realize the study of biological transformation that occurs in these types of cancers. The discovery of various biomarkers can outline the occurrence, evolution and the prognosis of this keratinocyte-derived tumor.

It is important to focus on the analysis of normal, inflammatory and malignant keratinocyte proteome in order to determine novel biomarkers that are associated with the development and progression of SCC and therefore can be used in the early detection, risk assessment, tumor monitoring and also discovery of new therapies for these patients.

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