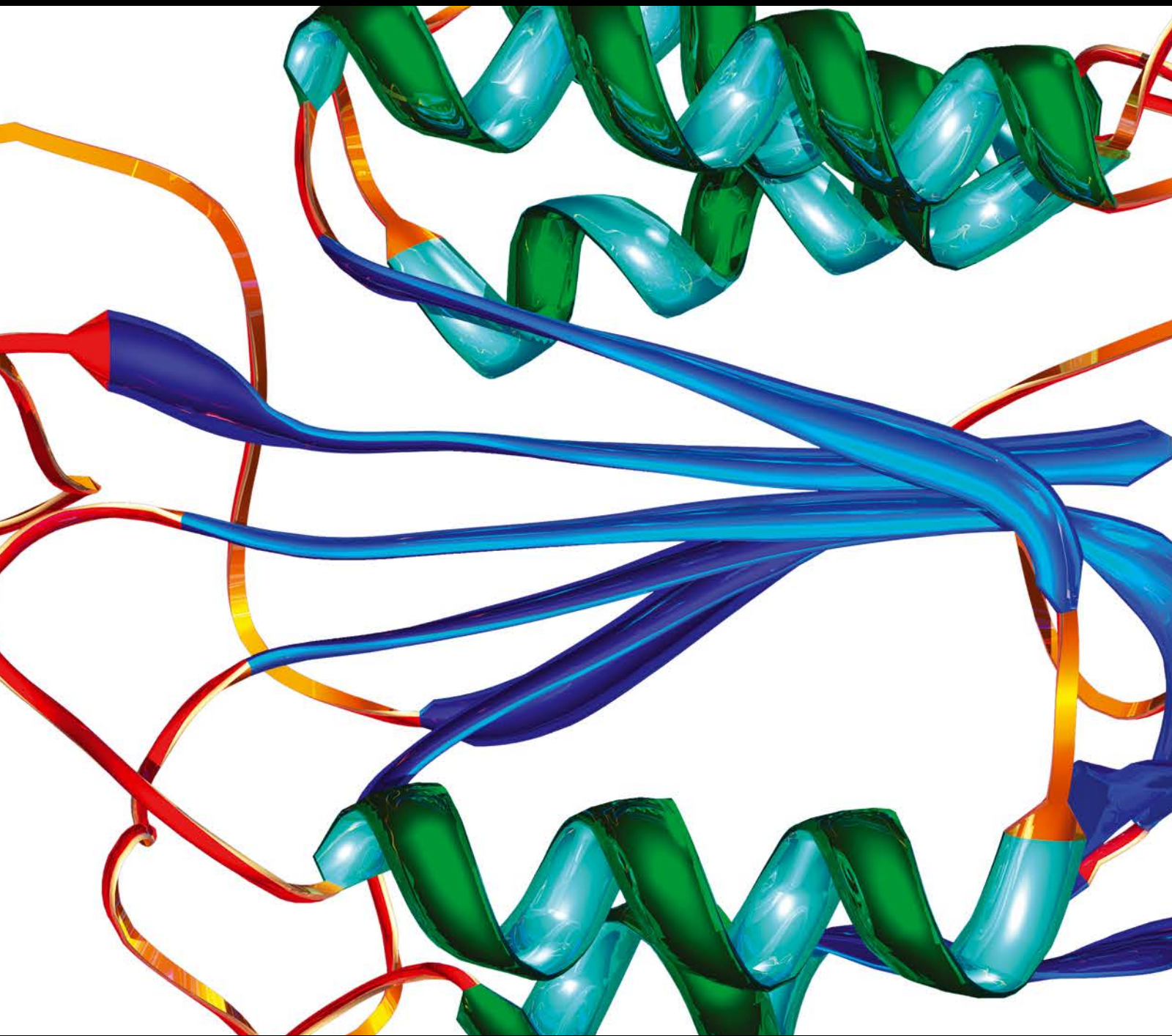
Disease Markers

Omics Landscape in Disease Biomarkers Discovery

Guest Editors: Monica Neagu, Caterina Longo, and Simone Ribero



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Editorial

Omics Landscape in Disease Biomarkers Discovery

Monica Neagu,[1,2] Caterina Longo,[3] and Simone Ribero[4]

[1] *Victor Babes National Institute of Pathology, Bucharest, Romania*
[2] *Faculty of Biology, University of Bucharest, Bucharest, Romania*
[3] *Skin Cancer Unit, Arcispedale Santa Maria Nuova-IRCCS, Reggio Emilia, Italy*
[4] *University of Turin, Turin, Italy*

Correspondence should be addressed to Monica Neagu; neagu.monica@gmail.com

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Biomarker discovery has an accelerated pace is taking advantage of ever growing omics technologies. In the last years we are facing the development of a myriad of technologies searching for the "golden biomarker." The omics family is constantly adding a new member, such as genomics, proteomics, peptidomics, newer ones, epigenomics, transcriptomics, metabolomics, lipidomics, pharmacogenomics, interactomics, and chronemics, just mentioning a few [1].

In the biomarker development, approaches are multidisciplinary, from basic to translational and clinical research. As biological processes are complex, for example, tumorigenesis, multitechnological approaches are crucial for protein/gene biomarkers discovery. Biomarker discovery goes through laborious individual phases, such as biomarker candidate identification, verification, and validation. In all these phases, various omics technologies take the lead. Depending on the development stage, when characterizing thousands to tens of thousands of proteins, the key technological players are mass spectrometry, multiplexed assays, and protein microarrays. For genomic studies, technological platforms of gene microarrays, next-generation sequencing, and mass spectrometry generate tumor-associated genes and potential biomarkers. Complementary omics approaches intended for biomarker candidates selection should focus on multiple proteomic/genomic/metabolomic targets. These multifaceted targets could better explain complex disease mechanisms [2].

The special issue is governed by three tumor pathologies, skin cancer, lung cancer, and colorectal cancer, while precancerous lesions like ulcerative colitis or particular inflammatory conditions like keratoconus, kidney disease, or microRNAs in pathological pregnancy are also tackled.

Hence from the large group of skin cancers papers, M. Lupu et al. review the genomic and the proteomic profile of basal cell carcinoma, the world's leading skin cancer. The paper analyzes the gene expression and proteomic profiling of both tumor cells and tumoral microenvironment. These patterns can represent novel predictive and prognostic biomarkers in BCC.

The second most frequent skin cancer squamous cells carcinoma is reviewed by V. Voiculescu et al. where environmental factor ultraviolet light, chronic scarring/inflammation, exposure to chemical compounds, and immune suppression can favour tumorigenesis. The paper shows that malignant keratinocyte proteome analysis is the pool for novel biomarker discovery future to be used in early detection, risk assessment, tumor monitoring, and development of targeted therapeutic strategies.

Another skin related paper focuses on melanoma development and G. Turcu et al. revised an interesting new possible biomarker, carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) with a definite role in melanoma metastasis. Knowing that melanoma progression is associated with cell adhesion molecules dysregulation, this molecule can further stratify high risk patients identifying subgroups that require close follow-up or more aggressive therapy.

Lung cancer was approached in two papers of this special issue. A. Quintanal-Villalonga et al. reviewed tyrosine kinase receptor with its therapeutical implications in lung cancer showing that in the "omics" landscape tyrosine kinase receptors gained their role in the different histological types of lung cancer. This receptor is highly involved in specific antitumoral therapies.

In the same field of lung cancer, A. Marrugal et al. reviewed the cytokines pattern due to their involvement in the inflammatory processes that can trigger and sustain tumorigenesis. Different proteomic techniques are presented in the continuous quest for biomarkers discovery in lung cancer. Within the future biomarker candidates, several cytokines are presented as important players in lung cancer that can stand for future therapy targets.

Colorectal cancer was another important subject presented in this special issue where J. Wei et al. presented their findings regarding hypermethylated markers. They have mapped DNA methylation profiling in colorectal cancer tissues. Over 1500 differentially methylated regions were found that proved to be different in colorectal cancer compared to normal tissue. Among these differentially methylated regions two genes (ADD2 and AKR1B1) are presented as potential screening markers of colorectal cancer.

Another original research paper within this issue is the one presented by C. Popp et al. regarding the specific expression profile of p53 and p21 in large bowel mucosa. The expression of p53 and p21 can be considered biomarker of inflammatory-related carcinogenesis in ulcerative colitis as a disease that slightly increases the risk of colorectal cancer in patients. The paper presents a one-year prospective observational study that shows results for future use of p53 as the most valuable tissue biomarker in ulcerative colitis surveillance, identifying higher risk for dysplasia patients.

Similarly, in the paper of C. Ionescu et al., inflammatory biomarkers for keratoconus are revised. Keratoconus, a degenerative disorder, results in decreased vision and irreversible tissue scarring and still has an unclear pathogenesis. Combination of inflammatory patterns and genetic and environmental factors are incriminated and studied in this type of special pathology. This paper revises biomarkers of inflammation and their signaling pathways that can indicate the etiopathogeny of this disease and that furnish new therapeutic possibilities.

Another pathology, chronic kidney disease-related biomarkers are presented by the original work of S. Mihai et al. Within the paper a panel of proteomic biomarkers, comprising inflammation markers (IL-6 and TNF-α) and mineral and bone disorder biomarkers (OPG, OPN, OCN, FGF-23, and Fetuin-A), was found to detect early chronic kidney disease.

Although a normal physiological condition, pregnancy can have a wide range of pathological conditions. D. Cretoiu et al. revise circulating microRNAs as potential biomarkers in pregnancy and show the significance of these circulating molecular markers from the implantation period to preeclampsia and their involvement in pathological processes like recurrent abortion and ectopic pregnancy.

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We would like to thank the authors who submitted their work for consideration to this special issue which would have not taken place without their efforts. Editors would like to thank the reviewers who thoroughly revised the papers and provided important suggestions that significantly improved the papers.

Monica Neagu
Caterina Longo
Simone Ribero

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Review Article

Proteomic Approaches to Biomarker Discovery in Cutaneous T-Cell Lymphoma

Alexandra Ion,[1] Iris Maria Popa,[2] Laura Maria Lucia Papagheorghe,[1] Cristina Lisievici,[1] Mihai Lupu,[1] Vlad Voiculescu,[1] Constantin Caruntu,[3,4] and Daniel Boda[5,6]

[1]*Department of Dermatology and Allergology, Elias Emergency University Hospital, 011461 Bucharest, Romania*
[2]*Department of Plastic and Reconstructive Surgery, "Bagdasar Arseni" Clinical Emergency Hospital, 041915 Bucharest, Romania*
[3]*Department of Physiology, "Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania*
[4]*Department of Dermatology, "Prof. N. C. Paulescu" National Institute of Diabetes, Nutrition and Metabolic Diseases, 020475 Bucharest, Romania*
[5]*Dermatology Research Laboratory, "Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania*
[6]*Department of Dermatology, Carol Medical Center, 020915 Bucharest, Romania*

Correspondence should be addressed to Alexandra Ion; ale_ion@ymail.com

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Cutaneous T-cell lymphoma (CTCL) is the most frequently encountered type of skin lymphoma in humans. CTCL encompasses multiple variants, but the most common types are mycosis fungoides (MF) and Sezary syndrome (SS). While most cases of MF run a mild course over a period of many years, other subtypes of CTCL are very aggressive. The rapidly expanding fields of proteomics and genomics have not only helped increase knowledge concerning the carcinogenesis and tumor biology of CTCL but also led to the discovery of novel markers for targeted therapy. Although multiple biomarkers linked to CTCL have been known for a relatively long time (e.g., CD25, CD45, CD45RA, and CD45R0), compared to other cancers (lymphoma, melanoma, colon carcinoma, head and neck cancer, renal cancer, and cutaneous B-cell lymphoma), information about the antigenicity of CTCL remains relatively limited and no dependable protein marker for CTCL has been discovered. Considering the aggressive nature of some types of CTCL, it is necessary to identify circulating molecules that can help in the early diagnosis, differentiation from inflammatory skin diseases (psoriasis, nummular eczema), and aid in predicting the prognosis and evolution of this pathology. This review aims to bring together some of the information concerning protein markers linked to CTCL, in an effort to further the understanding of the convolute processes involved in this complex pathology.

1. Introduction

Early stages of cutaneous T-cell lymphoma (CTCL) are frequently diagnosed as an indolent disease, usually with a long course of evolution. This type of primary lymphoma of the skin is the most frequent seen in humans. However, despite the evolution of medicine and its therapies, the specific cure is not easily found in some cases (15–20%) that have a high relapse rate [1].

The most frequent types of CTCL are mycosis fungoides (MF) and Sezary syndrome (SS). This disease is very complex, with a yet unknown pathogenesis. The development of the disease appears to be tightly connected with the variety of cytokines/chemokines [2]. Most cases of MF evolve over many years, with very slow progression. Early lesions of CTCL typically present as limited skin patches or plaques, called mycosis fungoides (MF), which can progress to tumor stage. In the tumor stage, the process may also involve extracutaneous sites, foremost lymph nodes, and, less frequently, bone marrow and visceral sites [3]. Somewhat different from MF, SS runs a much faster evolving clinical course, with malignant T cells present in the peripheral blood (PB). Patients usually present with lymphadenopathy, erythroderma, fever, and leukemic involvement [4].

CTCL is a type of skin cancer, which represents a significant percentage of all malignancies; therefore early diagnosis and targeted therapy represent the main direction of modern medicine [5]. CTCL, like other dermatological and oncological pathologies, has an important impact on the quality of life of the patient and his family, which is why understanding how CTCL develops may be useful in identifying methods of prevention and perfecting new therapeutic strategies [6, 7].

There are numerous studies that highlight the importance of proteomics as a tool for identifying, through noninvasive/minimally invasive procedures, biomarkers that may allow a more focused approach and management of patient [8–10]. In recent years, the medical community has given a great importance to proteomics, which presents numerous advantages such as the fact that, through it, we can identify molecular changes that sometimes may occur even before any other clinical or laboratory change commonly used for diagnostic; the biomarkers isolated can be used to establish early diagnosis, as well as monitoring and customizing therapy [10–14].

2. Cutaneous T-Cell Lymphoma (CTCL)

The increasing number of studies in proteomics and genomics has not only led to a better understanding of the carcinogenesis and tumor biology of CTCL but also led to the discovery of novel markers for targeted therapy. Some tailored target therapies for CTCL are chiefly based on the blockade/inhibition of certain receptors/proteins (IL-2R/CD25, CD4, CD30, and CD52), whose expression by cancer cells can be identified by techniques as immunohistochemistry [15, 16].

Well known diagnostic markers for CTCL include CD2, CD3, CD4, CD5, CD7, CD8, CD14, CD16/56, CD19, CD25, CD45, CD45RA, and CD45R0 [2]; although dissonant with cutaneous B-cell lymphomas and plasma cell disorders, no dependable protein marker for CTCL has been discovered.

Various molecules have been investigated as markers for CTCL, ranging from those involved in general cellular signaling processes, regulation of cellular proliferation, and apoptosis (Jun, Myc, c-myb, p53, STATs, bcl-2, Fas/CD95, and SOCS-3) to those contributing to disease immunopathology like the inhibitory MHC receptors (ILT2/CD85j), NK receptors (p140/KIR3DL2), and dendritic cell defects (CD40). As shown for other tumors, abnormal expression of these molecules influences disease prognosis [17].

It was noted that patients with CTCL had higher serum concentrations of sIL-2R, which can be correlated with lymph node size, large-cell transformation in the skin, or lymph node with increased severity. Regarding the large-cell transformation, it is important to mention that this process is responsible for the increasing concentration of sIL-2R in serum, considering that sIL-2R is produced in low quantities by tissue-based lymphoma cells [18]. Along with sIL-2R, it was demonstrated that patients with Sezary syndrome have elevated levels of β_2-microglobulin (β_2-MG) and neopterin, but only the latter may play a role in determining the prognosis in patients with nonleukemic CTCL [19]. However, studies have shown that these biomarkers have a low specificity for CTCL, as they may be found in elevated concentrations in other malignancies [20].

In a treatment response study [21] differentially expressed genes were significantly associated with treatment-responsive CTCL when compared to treatment-resistant disease. In patients with poor treatment response downregulated genes were involved in epidermal development, Wnt signaling pathway and frizzled signaling, and extracellular matrix pathway, while upregulated genes were those involved in immune response, T-cell activation, mitosis, and apoptosis [21].

Regulatory T cells (Tregs) have two subsets: "natural" Tregs (CD4+CD25+ T cells) which are selected in the thymus and "induced" Tregs which appear in periphery from CD4+ T cells. Although these two categories are very different in many aspects, it is important to mention that they both play a part in tumor immunity [22]. Studies have shown that a decrease in CD4+CD25+ T cells levels is correlated with a high immune response to self- and non-self-antigens [23]. In addition, the most important phenotypic features of this specialized helper T cell are the presence of CD25 component, α-chain of the IL-2 (IL-2Rα), and the transcription factor FoxP3 [24]. It was noted that increasing the levels of CD4+CD25+ T cells can prevent the development of autoimmune conditions, while a decrease in CD25+ T cells or loss of expression of FoxP3 in Tregs may induce a large number of autoimmune diseases [25, 26]. CD25 component is considered to be a stable one in "natural" Tregs and a transient one in "induced" Tregs, but it is important to mention that all Tregs express this marker. Once the exact mechanisms of Treg induction and differentiation are better understood treatment options can be improved [27].

Lymphoma, melanoma, colon carcinoma, head and neck cancer, and renal cancer have all benefited from ample studies on serologically defined antigens. Meanwhile, information about the antigenicity of CTCL remains relatively limited. Forgber et al. tested the sera of 87 cutaneous lymphoma patients and found 64 cases of serum reactivity against lymphoma cells, but the responses were relatively weak, restricted to a few antigens in each case, and heterogeneous. Fourteen different antigens were identified of which only one, vimentin, had been reported before [28].

Recently discovered by Krejsgaard et al. and confirmed in a meta-analysis study by Zhang et al. in 2012 [29], most malignant T cells exhibit ectopic expression of the B-lymphoid tyrosine kinase (Blk), a member of the Src kinase family. Moreover, gene knockdown experiments revealed that Blk promoted the proliferation of malignant T cells in CTCL patients [30] while another study suggested that murine Blk also has tumor-suppressive functions depending on the specific cellular context [29]. Peterson et al. [31] in a recent study of Blk in CTCL provided evidence that the active form of human Blk is able and sufficient to confer cytokine independence to cytokine-dependent lymphoid cells while promoting tumor growth in vivo and supporting growth of lymphoid cells in vitro.

Another recent study analyzed the importance of sCD26 (serum CD26) and TARC (thymus and activation-regulated

chemokine) levels in the diagnosis of CTLC. CD26 is a type II transmembrane glycoprotein with enzyme activity, expressed on different tissues such as epithelial cells, endothelial cells, natural killer cells, and a subset of T cells, which selectively removes the N-terminal dipeptide from peptides with proline or alanine in the penultimate position. The soluble form, sCD26, is thought to have the same functions as the membrane form. The sCD26 serum levels are significantly lower in patients with CTLC and psoriasis, while TARC serum levels are significantly higher in patients with atopic dermatitis and CTLC. The authors have shown that these two markers, combined, are helpful in the diagnosis of CTLC [32]. Pierson et al. demonstrated that a decreased CD26 expression is a useful diagnostic marker of peripheral blood involvement in SS and MF patients, but they have also emphasized that reactive T cells in tissue and body fluid specimens often show low levels of CD26 expression; thus this marker should be used in combination with others, in order to facilitate the diagnosis process [33].

Biopsies obtained from CTCL pointed out that the angiogenesis increases with the tumor stage and could play an important part in the pathophysiology and the progression of CTCL. Besides many other angiogenetic and angiostatic factors, VEGF is thought to play a central role in the tumor-associated angiogenesis, its expression being detected even in the early stages. Krejsgaard et al. have demonstrated that advanced stages of CTCL correlate with an increased expression of VEGF and with aberrant activation of its promoting pathways, JAK3 and JNKs. Thus, novel therapies based on the inhibition of these pathways or on the neutralization of VEGF may have an important role in the future and further studies should be conducted in this direction [34].

3. Mycosis Fungoides

Almost half of all primary cutaneous lymphomas, as classified by the WHO-EORTC, are represented by mycosis fungoides (MF) [2]. Mycosis fungoides displays a polymorphous clinical picture, varying from patch, plaque, to tumor-stage disease and rarely associates visceral involvement. Clinically discernable lymph node involvement occurs in about 20% and 50% of patients with plaque and tumor-stage MF, respectively [35], while circulating neoplastic cells can be detected even in patients presenting with limited disease [36, 37]. Unfortunately, early stage MF can prove particularly difficult to diagnose mainly due to its benign clinical aspect [38].

Cowen et al. [39] analyzed the possibility of using serum proteomics to distinguish between patients with tumor-stage (T3) mycosis fungoides, patients with psoriasis, and healthy patients. In their study, serum protein profiling successfully distinguished between MF, psoriasis, and healthy controls with acceptable accuracy using the SELDI-TOF Ciphergen MS-based data. Mycosis fungoides detection sensitivity in MF versus controls and MF versus psoriasis groups was 71.4% and 78.6%, respectively, while the specificity kept above 90% in both models. The authors convey the possibility of a unique signature of tumor-stage MF and that perhaps earlier stages of MF would not be as easy to distinguish from other nonneoplastic inflammatory diseases [39]. Moshkovskii et al. [40] attempted to estimate the probability of a correct MF diagnosis based on serum protein profiling using SELDI-TOF MS. The authors found that using their data, differentiation of MF from psoriasis had only 75% specificity and 65.2% sensitivity, an indication of low diagnostic value. However, when comparing protein peaks from MF versus normal controls they found a specificity of 77.7% and a sensitivity of 78.2% which, according to expert scale of AUC (area under curve), is considered to be good [41, 42]. The relatively low values found in MF versus psoriasis patients could be explained by the capacity of direct MS profiling to detect only major proteins, their modifications, and alterations in their serum levels [40].

Manganese superoxide dismutase (SOD2) is an enzyme which is encoded by the SOD2 gene on chromosome 6 and is a member of the superoxide dismutase (SOD) family, whose function is to transform toxic superoxide anion into hydrogen peroxide [43, 44]. Neoplastic cells have an anaerobic metabolism which puts them under intrinsic oxidative stress; due to this process, malignant cells rely on antioxidant enzymes aiming to eliminate reactive oxygen species, which may explain why in malignant lymphocytes in MF there is an overexpression of SOD2 (in epidermal keratinocytes as well as in dermal keratinocytes) [45]. Even though in a mouse model of T-cell lymphoma, SOD2 has a tumor suppressor effect, it was demonstrated that in HIV-infected HUT-78 cells, overexpression of SOD2 may have a tumor-supportive function due to the fact that it increases resistance to heat and radiation [46, 47].

S100A8 is found in the cytoplasm of neutrophils, macrophages, and endothelial cells as calprotectin (S100A8/A9) [48]. Lately it has been documented as a biomarker that is overexpressed in many types of cancers including MF lesions, where it is limited to the epidermis [45]. Even though the cause and role of its overexpression in MF remain unknown, S100A8 appears to be influenced by the hyperproliferative state in psoriasis; this process suggests a similar molecular mechanism in MF [49].

The 15-kDa cytosolic protein FABP5 is a member of the fatty acid binding protein family, involved in the uptake, transport and metabolism of fatty acids, and cellular signaling influencing differentiation and the regulation of cellular growth [50]. The overexpression of FABP5 in the lesional skin of MF patients could be linked to its role in the transport and metabolism of fatty acids in the epidermis, and in turn, the altered lipid metabolism may affect the proliferation and differentiation of keratinocytes [45].

Regarding the differential diagnosis, CD26 soluble serum levels and the expressions of TOX, Tplastin, TWIST, CD 158, and nkP46 have been taken in consideration. TOX is a small DNA binding protein which is regulated in the thymus during positive selection. It has an important role in the CD4+ T-cell development, but after that it is no longer expressed. Recently, some reports have shown that in MF, TOX is overexpressed in mature CD4+ lymphocytes. Furthermore, TOX is a direct target of miR-223, which is considerably reduced in MF/CTCL, leading to an important expression of TOX. Other targets of miR-223 are E2F1 and MEF2C. miRNA are small noncoding RNAs that target

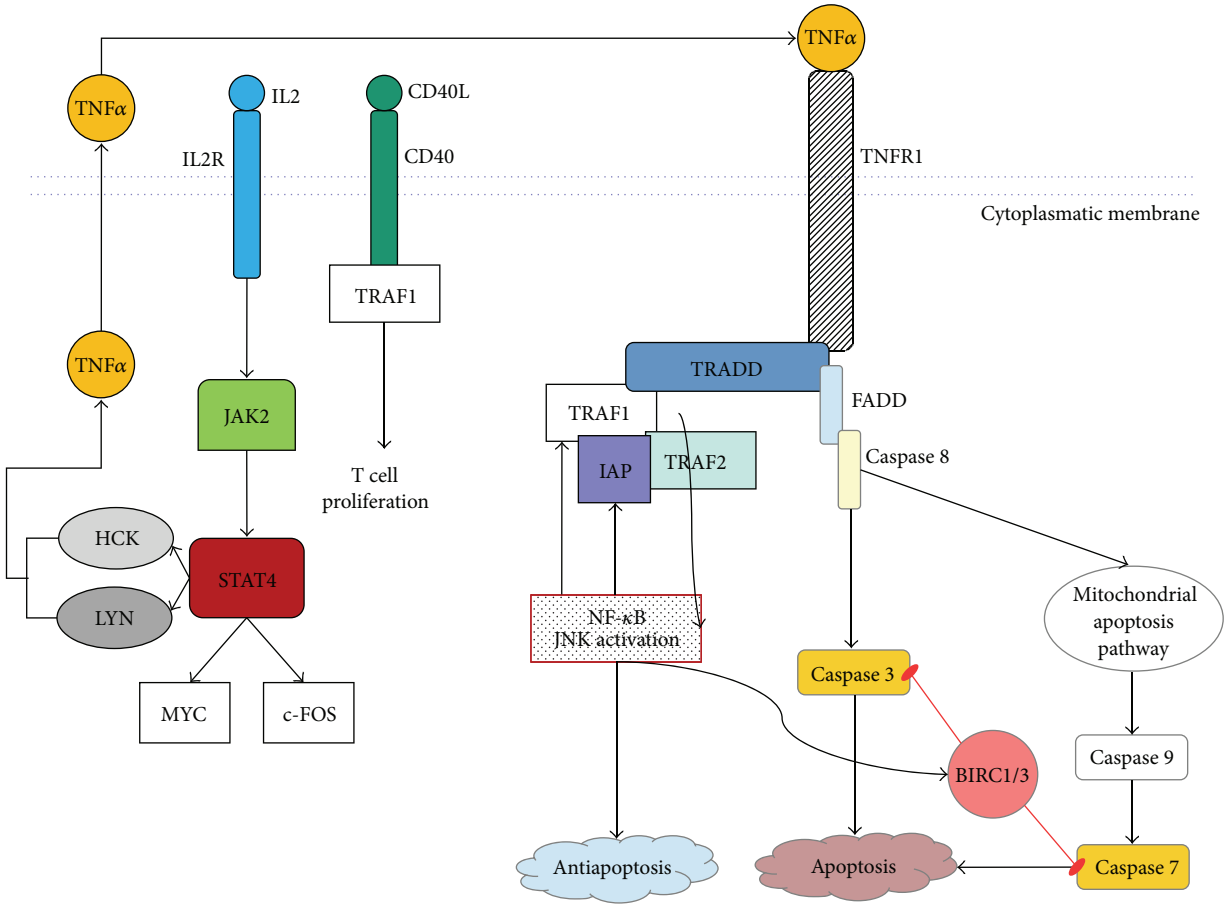

Figure 1: TNF signaling pathway anomalies through antiapoptotic signaling by TNFR1 helped by upregulation of TRAF1 and inhibition of proapoptotic TNFR1 signaling by BIRC1/3 caspase inactivation. Overexpression of CD40L could induce T-cell proliferation through CD40 receptor and TRAF1. IL2R overexpression leads to Jak2 and STAT4 activation which in turn induces oncogene c-MYC, LYN, and HCK expression. Meanwhile LYN and HCK take part in the feedback loop of antiapoptotic TNF signaling by producing endogenous TNF and thus stimulating TNFR1/2 antiapoptotic pathways.

mRNA, reducing its translation [51]. Morimura et al. have documented by immunohistochemical staining the TOX expression in the cutaneous lymphocytes from the T-cell infiltrated skin. According to their report, the TOX expression was limited to CD4+ in MF and in CD30+ cells in Lymphomatoid Papulosis (LyP). There was a great amount of TOX expressing cells among infiltrating lymphocytes in biopsies from SS, MF, LyP, primary cutaneous anaplastic large cell lymphoma (PCALCL), adult T-cell lymphoma/leukemia (ATLC), peripheral T-cell lymphoma, and not otherwise specified (PTCL, NOS). Massive infiltration of TOX cells was found in plaques MF, tumor MF, SS, LyP, and PCALC (>30% of infiltrating lymphocytes). A high specific nuclear staining of TOX was found in MF, SS, PTCL, and NOS. On the other hand, the tumor cells, in LyP, PCALCL, and ATLC, presented a weak nuclear staining of TOX. Moreover, the TOX expression was limited to CD4+ cells in MF, while the large anaplastic cells in LyP were positive for CD30+ and CD4+ [52].

Psoriasis, diabetic retinopathy, cardiovascular diseases, rheumatoid arthritis, and cancer are just a few of many diseases that have something in common: pathologic angiogenesis. According to some studies, this pathologic formation of blood vessels can be correlated with different forms of CTCL [53]. Through lymph node biopsies, increased capillary proliferation can be discovered in high-grade non-Hodgkin's lymphoma. The number of blood vessels did not correlate with the grade of the tumor in patients with small cell lymphocytic lymphoma. The progression of CTCL in MF was highlighted by the increased synthesis and expression of matrix metalloproteinases 2 and 9 [54].

Profiling of transcription factors in MF and SS patients has pointed out constitutively active NF-kB, STAT, and their respective signaling pathways as potential markers for target therapy. Several prototypic inhibitors of these targets and altered pathway components have also been identified [55].

Tracey et al. [56] have shown that there are abnormalities in the TNF signaling pathway in MF tumorigenesis (see Figure 1). Through a cDNA microarray-based approach, the authors [56] identified a total of 27 differentially expressed genes between MF and inflammatory dermatoses (20 upregulated and 7 downregulated in MF cases), including tumor

necrosis factor receptor (TNFR) and other oncogenes and apoptosis inhibitors. They designed a 6-gene "signature" (FJX1, STAT4, SYNE1, TRAF1, BIRC3, and Hs.127160) prediction model that may help to differentiate MF from inflammatory dermatoses. The model correctly identified 97% of cases in a blind test performed on 24 MF patients with low clinical stages [56]. cDNA microarray and quantitative RT-PCR expression analyses of peripheral blood samples, using a panel of genes (including STAT4, GATA-3, PLS3, CD1D, and TRAIL), have been shown to identify CTCL patients (who have at least 5% circulating tumor cells) with an overall accuracy of 90% [57, 58].

4. Sezary Syndrome

The metastatic potential of tumor cells is dependent on angiogenesis, which creates the conditions for tumor growth and progression [59, 60]. Angiopoietins represent a family of ligands for endothelium-specific tyrosine kinase Tie2 receptor. This family of proteins consists of 4 structurally similar members: Ang-1, Ang-2, Ang-3, and Ang-4 of which Ang-1 and Ang-2 have been identified to have angiogenetic properties, similar to VEGF [61–63]. Cells from MF skin lesions express Ang-2, but the serum levels of this protein are elevated only in patients with Sezary syndrome (SS), which may indicate that serum Ang-2 is produced by circulating tumor cells in SS [4, 64]. Kawaguchi et al. showed in a study published in 2014 that serum concentration of Ang-2 was increased, even in patients with MF, when the disease progressed, thus demonstrating that Ang-2 could be used as a disease activity marker [65].

TOX staining may also be useful in the differential diagnosis between SS and erythrodermic dermatitis. The first mentioned presented more than 50% nuclear staining of the infiltrating lymphocytes, while the last ones had a slightly dim nuclear staining of TOX (~25%). In addition, c-MYC did not have a significant contribution to the differential diagnosis [66]. Tplastin, a member of the fimbrin/plastin family expressed by epithelial tissues and nonhematopoietic mesenchymal cells, did not show a significantly higher expression in SS than in the benign erythrodermic inflammatory diseases (EID). In contrast, the detection of CD 158K/KIR3DL2 (a killer immunoglobulin-like receptor usually expressed by some circulating T CD8+ lymphocytes, NK cells, and recently some reports suggested that it might be expressed by some subsets of CD4+ T cells) transcripts using RT-PCR was significantly overexpressed in SS, in comparison with EID, and may be used as a diagnosis marker even in the early stages of SS [67].

In another study, Michel et al. have demonstrated for the first time that the combination of five biomarkers (PL53, TWIST, CD158K, KIR3DL2, and NKp46) using PCR has a high importance in the early diagnosis of SS [68]. In advanced MF/SS increased expression of Twist protein (a transcription factor which blocks p53 and inhibits c-MYC induced apoptosis, believed to promote the progression of solid tumors) was found through RT-PCR detection, but further studies are needed in order to correlate the high expression with the stages of these diseases [69]. NK46p belongs to natural cytotoxicity receptor (NCR) families of natural killer (NK) receptors. This receptor is frequently expressed by neoplastic cells in SS and it may be used as a diagnosis marker in the blood and the skin using RT-PCR [70].

5. Conclusions

Given the high prevalence of CTCL, it is imperative to determine specific biomarkers in order to distinguish between benign and aggressive prognostic courses. The diagnosis of CTCL requires a more holistic approach through which molecular findings are to be integrated with clinical, histological, and immunophenotypic data. Thus, future studies should be aimed at defining appropriate molecules with high sensitivity and specificity for the evaluation of disease treatment and prognosis. Moreover at-risk patients would benefit from diagnostic markers in order to prevent disease progression and late diagnosis, when appropriate therapies are of little efficiency. Establishing accurate protein markers would also be helpful for identifying target therapies.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

All the authors equally contributed to this work.

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Research Article

Proteomic Biomarkers Panel: New Insights in Chronic Kidney Disease

Simona Mihai,[1] Elena Codrici,[1] Ionela Daniela Popescu,[1] Ana-Maria Enciu,[1,2] Elena Rusu,[3,4] Diana Zilisteanu,[3,4] Radu Albulescu,[1,5] Gabriela Anton,[6] and Cristiana Tanase[1,7]

[1] *Victor Babes National Institute of Pathology, Biochemistry-Proteomics Department, Splaiul Independentei 99-101, Sector 5, 050096 Bucharest, Romania*
[2] *Cellular and Molecular Medicine Department, Carol Davila University of Medicine and Pharmacy, No. 8 B-dul Eroilor Sanitari, Sector 5, 050474 Bucharest, Romania*
[3] *Fundeni Clinic of Nephrology, Carol Davila University of Medicine and Pharmacy, Şoseaua Fundeni 258, Sector 2, 022328 Bucharest, Romania*
[4] *Fundeni Clinical Institute, Nephrology Department, Şoseaua Fundeni 258, Sector 2, 022328 Bucharest, Romania*
[5] *National Institute for Chemical Pharmaceutical R&D, Pharmaceutical Biotechnology Department, Calea Vitan 112, Sector 3, 031299 Bucharest, Romania*
[6] *Stefan S. Nicolau Institute of Virology, Molecular Virology Department, Şoseaua Mihai Bravu 285, Sector 3, 030304 Bucharest, Romania*
[7] *Faculty of Medicine, Titu Maiorescu University, Strada Dâmbovnicului 22, Sector 4, 040441 Bucharest, Romania*

Correspondence should be addressed to Cristiana Tanase; bioch@vbabes.ro

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Chronic kidney disease, despite being a "silent epidemic" disease, represents one of the main causes of mortality in general population, along with cardiovascular disease, which is the leading cause of poor prognosis for these patients. The specific objective of our study was to characterize the relationship between the inflammatory status, the bone disorders markers, and kidney failure in chronic kidney disease patient stages 2–4, in order to design a novel biomarker panel that improves early disease diagnosis and therapeutic response, thus being further integrated into clinical applications. A panel of proteomic biomarkers, assessed by xMAP array, which includes mediators of inflammation (IL-6, TNF-α) and mineral and bone disorder biomarkers (OPG, OPN, OCN, FGF-23, and Fetuin-A), was found to be more relevant than a single biomarker to detect early CKD stages. The association between inflammatory cytokines and bone disorders markers, IL-6, TNF-α, OPN, OPG, and FGF-23, reflects the severity of vascular changes in CKD and predicts disease progression. Proteomic xMAP analyses shed light on a new approach to clinical evaluation for CKD staging and prognosis.

1. Introduction

Nowadays, chronic kidney disease (CKD) represents a worldwide major public burden and its prevalence continues to rise [1].

Over the past century, CKD, despite being a "silent epidemic" disease, represents one of the main causes of mortality in general population, alongside neoplasia, cardiovascular diseases, malnutrition, and infection, in the context of epidemiology landscape. Moreover, in Europe, CKD stages 1–5 prevalence ranges from 3.3% to 17.3% [2, 3].

Cardiovascular disease remains one of the leading causes of CKD poor prognosis, since early stages of CKD are associated with higher risk of subsequent coronary heart disease [4].

According to several clinical studies, 50% of patients with CKD die of cardiovascular causes, such as advanced calcific arterial and valvular disease; nonetheless, the processes of

accelerated calcification in CKD remain poorly understood, and no therapies have been developed yet for disease prevention [5].

In CKD patients, screening for the presence of vascular calcification (VC) is suggested in current guidelines, since it is considered to be a cardiovascular risk marker and it is associated with a severalfold increase in morbidity and mortality risk, both in general population and in CKD, increasing steadily through the stages of CKD, peaking in CKD stage 5 patients.

Several proteins and factors are involved in passive and active processes that result in VC. In CKD population, various studies have identified circulating biomarkers that may be responsible for extraskeletal calcification and dysfunctions in mineral metabolism, which are features of CKD-mineral bone disorder (CKD-MBD) [6, 7].

Therefore, these observations have led to CKD-MBD study in association with cardiovascular diseases. These processes are interconnected and they have an important contribution to the morbidity and mortality rate of CKD patients [8, 9].

One of the main objectives in CKD therapy should be treating renal bone disease. The evaluation of CKD-MBD biochemical parameters (primarily phosphorus, calcium, parathyroid hormone, and vitamin D levels) as early as CKD stage 3, along with the assessment of bone status, should be considered in treatment decisions [10]. The prevalence of VC increases throughout the stages of CKD peaking in CKD stage 5 patients [7].

Cardiovascular calcification is an outstanding element of chronic inflammatory disorders associated with significant morbidity and mortality. Remarkably, CKD hastens atherosclerosis development and it has been demonstrated that CKD provokes excessive vascular inflammation and calcification [11].

Recent evidence also points towards alternative processes independent of osteogenic differentiation, including the release of matrix vesicles (e.g., secreted by macrophages) [5]. The understanding of the relationships between these mechanisms and signaling pathways could offer new mechanistic insight into the calcification process, and it may help lead to cardiovascular disease therapeutics in CKD patients [5, 11].

These data are also supported by genetic predisposition. Rutsch et al. [12] observed that 40–50% of coronary calcification cases can be attributed to genetics and several loci linked to coronary arterial calcification were identified [13, 14]. An implication of several single polymorphisms located at 9p21 locus near the cyclin genes has been suggested in the genesis of this pathology. These genes encode cyclins that may be broadly linked to cellular senescence and inflammation, though the accurate causative DNA sequences remain debatable [14, 15].

CKD is characterized by progressive loss of renal function, which results in reduced glomerular filtration rate (eGFR). Current clinical methods are accurate in diagnosing only advanced kidney dysfunction. In addition, there are no tools for predicting progression risk towards end-stage renal failure; thus, developing accurate biomarkers for prognosis of CKD progression constitutes a clinical challenge. Therefore, efforts are directed towards earlier detection and better prognosis, in order to allow better therapeutic interventions to slow down or even prevent the progression of the disease in the future. Omics approaches, including proteomics, provide novel insights into disease mechanisms. They may improve CKD management, providing stage-specific biomarkers [16–19].

In comparison with currently available markers, serum creatinine and urinary albumin, proteomic biomarkers may enable more accurate and earlier detection of renal pathology. Despite the "breaking point" being different in every patient, in some individuals serum creatinine levels remain normal despite loss of >50% of renal function; consequently, additional biomarkers of renal function are needed. Biomarkers that would facilitate the noninvasive differential diagnosis of kidney diseases, detect early onset of kidney disease, monitor responses to therapy, and predict progression to hard end points, such as end-stage renal disease (ESRD) or death [7, 20, 21], are needed, since they have potential for actual clinical implementation, which is an area to focus research on in the future [20, 22–25]. These biomarkers could prove very useful in terms of early detection and prognosis in CKD [7, 20, 26–31].

Considering the above-mentioned aspects, the specific objective of this study was to characterize the relationship between the inflammatory status and the indicators of kidney failure and bone disorders, in order to design a novel biomarkers panel that might improve early disease diagnosis and therapeutic response, thus being further integrated into clinical practice.

2. Materials and Methods

2.1. Patients and Samples

2.1.1. Study Population. We prospectively included 86 patients (28% female and 72% male; mean age 65) diagnosed with chronic kidney disease according to the KDIGO criteria, 20 with CKD stage 4 (35% female and 65% male; mean age 62), 52 with CKD stage 3 (33% female and 67% male; mean age 66), and 14 with CKD stage 2 (23% female and 77% male; mean age 65), hospitalized in Fundeni Clinic of Nephrology, Fundeni Clinical Institute, Bucharest, and 20 healthy controls. Before enrollment, written informed consent was obtained from all subjects, according to Helsinki Declaration and Ethics Committee that has approved the study. Patients with acute infection, known malignancy, acute heart failure, significant heart valvular disease, and chronic use of glucocorticoids and immunosuppressive agents were excluded.

2.1.2. Clinical and Laboratory Assessment. Clinical and anthropometric data were collected on the day of blood sampling: age, sex, weight, height, previous medical history, and concomitant treatment. Laboratory tests included hemoglobin, hematocrit, serum creatinine, urea, uric acid, glucose, total cholesterol, triglycerides, alkaline phosphatase,

phosphate, calcium, albumin, and fibrinogen. Estimated glomerular filtrate rate (eGFR) was calculated using CKD-EPI formula. Urinary protein excretion was measured from a 24 h urine sample. All blood samples were collected in the morning after an overnight fast and were stored at −80°C until being analyzed.

2.2. xMAP Array and ELISA Analysis. The xMAP array was performed according to the manufacturers' protocols, and the plates were analyzed using Luminex 200 system. Cytokine levels and bone metabolism analytes were determined using the Milliplex MAP Human Bone Magnetic Bead Panel Kit from Merck-Millipore, Billerica, MA, USA, with 6 analyte-specific bead sets (simultaneous quantification): proinflammatory cytokines IL-6 and TNF-α and bone metabolism and disorder biomarkers: Osteoprotegerin (OPG), Osteocalcin (OCN), Osteopontin (OPN), and Fibroblast Growth Factor 23 (FGF-23). Briefly, the beads, which were provided within each kit, were incubated with buffer, cytokine standards (included in the kit), or samples in a 96-well plate at 4°C overnight. All further incubations with detection antibodies and Streptavidin Phycoerythrin Conjugate (SAPE) were performed at room temperature in the dark with shaking at 800 rpm. Multiplex data acquisition and analysis were performed using xPONENT 3.1 software; the calibration curves were generated with a 5-parameter logistic fit.

Fetuin-A serum levels were assessed using Quantikine® ELISA Human Fetuin A Immunoassay kit, R&D Systems, Inc., USA, according to the manufacturer's instructions.

Duplicate samples were used for all specimens and the average concentrations were used for statistical analysis.

2.3. Statistical Analysis. Differences between CKD sample group and control were analyzed using Student's t-test. A two-tailed $p < 0.05$ was considered statistically significant. The chi-square test (χ^2; P) was used to determine the significance of the association between inflammatory cytokines, bone metabolism, and disorder biomarkers in CKD and control groups. Pearson correlation (r) was used to explore the association between different biomarkers expression, together with clinical parameters. The threshold values for the analyses were established in accordance with the mean values of the studied groups. Statistical analysis was performed using SPSS 19.0 software. Graphs were realized with GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

3. Results and Discussion

Given the fact that a large number of cytokines orchestrate the inflammatory response, the extent to which inflammation plays a role in increasing the risk of bone/mineral disorders in CKD remains unclear. Progressive renal failure in CKD contributes to abnormalities in mineral/bone metabolism—calcium, phosphorous, PTH, Vitamin D, and vascular calcifications [32].

3.1. Inflammation and CKD. Inflammation represents a hallmark of CKD and the degree to which inflammation is related to loss in kidney function, eGFR, remains an open question. Some studies revealed increased circulating levels of proinflammatory cytokines IL-6 and TNF-α in patients with kidney dysfunction [33]. Moreover, inflammation status in CKD seems to be correlated with CKD evolution and complications, like cardiovascular disease [33, 34].

Okada et al. study supported the assumption that IL-6 genetic variations may lead to CKD and the assessment of the genotypes involved could identify the risk of CKD development [35]. Barreto et al. also showed that IL-6 levels tend to rise as CKD progressed, with the increase becoming statistically significant in CKD stages 4 and 3 [36].

According to our data in this study, the mediators of the inflammatory response IL-6 and TNF-α have been overexpressed in all CKD groups (t-test; $p < 0.001$; Figures 1(a) and 1(c)). Statistical analysis (t-test) shows that IL-6 level was highest in CKD stage 4 ($p < 0.001$), being 11-fold higher than control, while, for CKD stages 3 and 2, the expression was also increased, 6-fold ($p < 0.001$) and 2-fold ($p = 0.005$), respectively, by comparison with control group (Figures 1(b) and 2).

In CKD stage 4, IL-6 enhanced expression was directly correlated with TNF-α ($r = 0.64$), OCN ($r = 0.67$), and OPN levels ($r = 0.59$) (Pearson correlation). In stage 2 of CKD, we have noticed a positive correlation between IL-6 and TNF-α ($r = 0.58$), OPG ($r = 0.63$), and OPN ($r = 0.52$), and, on the other hand, a negative correlation with Fetuin-A ($r = -0.5$) (Pearson correlation), as it is depicted in Tables 1, 2, and 3.

TNF-α displays an increased level in serum of more than 3.6-fold ($p < 0.001$) in CKD stage 4, recording a decrease with disease stage decrease as well, as follows: 2.8-fold ($p = 0.006$) in CKD stage 3 and 1.7-fold in CKD stage 2 ($p = 0.01$);

Table 1: Correlation between inflammatory cytokines and CKD biomarkers in stage 4 (Pearson correlation).

	IL-6	TNF-α	OPG	OCN	OPN	FGF-23	Fetuin-A
IL-6	1						
TNF-α	0.64	1					
OPG	−0.01	0.28	1				
OCN	0.67	0.69	0.21	1			
OPN	0.59	0.72	0.04	0.35	1		
FGF-23	0.24	−0.21	−0.36	0.06	0.07	1	
Fetuin-A	−0.37	−0.62	−0.31	−0.39	−0.67	0.23	1

Table 2: Correlation between inflammatory cytokines and CKD biomarkers in stage 3 (Pearson correlation).

	IL-6	TNF-α	OPG	OCN	OPN	FGF-23	Fetuin-A
IL-6	1						
TNF-α	0.147	1					
OPG	0.144	0.132	1				
OCN	0.240	−0.079	0.063	1			
OPN	0.173	0.106	0.53	0.083	1		
FGF-23	0.152	0.072	0.159	0.134	0.23	1	
Fetuin-A	0.196	−0.03	−0.048	0.072	0.015	−0.0006	1

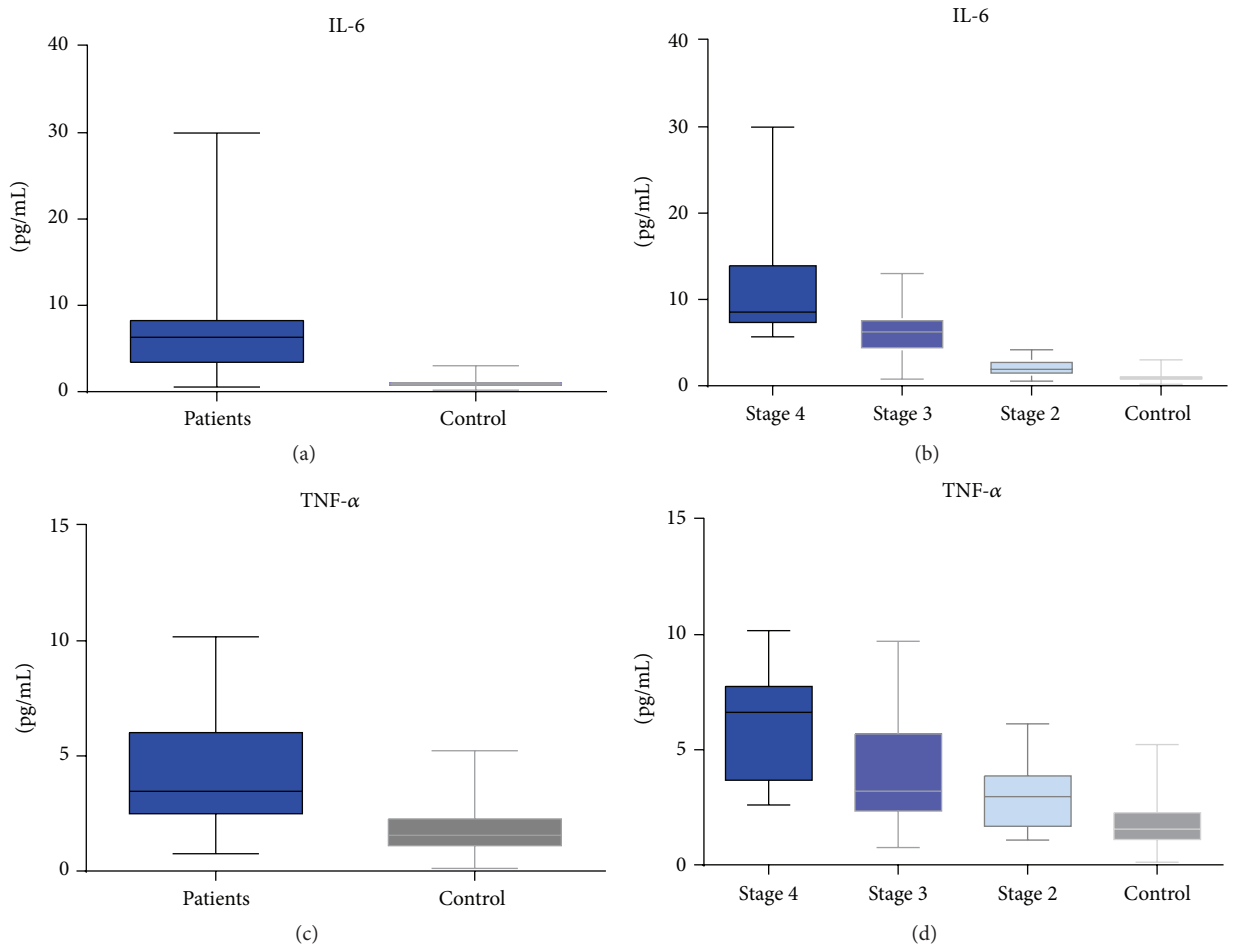

Figure 1: Serum levels of proinflammatory cytokines IL-6 and TNF-α, in CKD patients *versus* control, assessed by xMAP array.

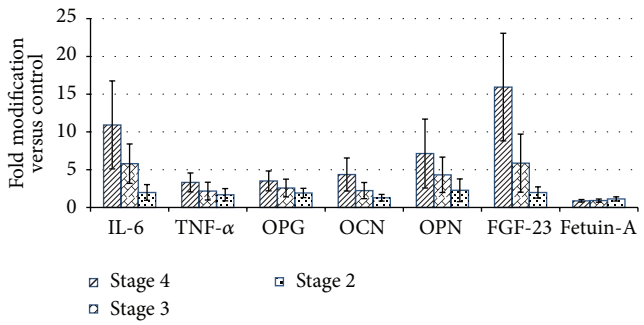

Figure 2: Modulation of serum biomarkers level in CKD stages. The data represent group averages of fold modification *versus* controls with standard deviations.

these results are also related to renal failure (eGFR). Details on expression of TNF-α are provided in Figures 1(d) and 2.

In CKD stage 4, TNF-α enhanced expression is positively correlated with OCN ($r = 0.69$) and with OPN ($r = 0.72$) and negatively correlated with Fetuin-A ($r = -0.62$). In CKD stage 2, TNF-α was positively correlated with OPN ($r = 0.51$), as it is shown in Tables 1, 2, and 3.

Table 3: Correlations between inflammatory cytokines and CKD biomarkers in stage 2 (Pearson correlation).

	IL-6	TNF-α	OPG	OCN	OPN	FGF-23	Fetuin-A
IL-6	1						
TNF-α	0.583	1					
OPG	0.638	0.375	1				
OCN	0.003	0.294	0.054	1			
OPN	0.525	0.511	0.011	0.286	1		
FGF-23	−0.125	0.136	−0.123	−0.2	0.334	1	
Fetuin-A	−0.503	−0.361	−0.655	−0.275	0.158	0.28	1

It has been shown that IL-6, a "bad" cytokine that could promote atherosclerosis [37], might be more helpful than TNF-α in CKD patients classification on stages [38].

Spoto et al. also concluded that inflammation is related to renal failure, with high IL-6 levels seen in CKD early stages exclusively; however, their data showed a negative correlation

between TNF-α levels and eGFR, indicating differences in the dynamics of the relationship between the above-mentioned cytokines and renal function [39]. Our data is in accordance with the increase of IL-6 in CKD, but we found a positive correlation between TNF-α and renal function.

The increased serum levels of IL-6 and TNF-α in CKD are in accordance with other studies, which mention that proinflammatory cytokines increase is linked to disease progression [40].

3.2. Markers of Mineral and Bone Disorders in CKD

3.2.1. Osteoprotegerin (OPG). OPG is considered a member of the TNF receptor family, considered to be correlated with the vascular dysfunction and further with cardiovascular disease, the common problem encountered in patients with CKD. Yilmaz et al. were among the first ones to mention the potential role of OPG in CKD patients stratification for cardiovascular risk, along with eGFR and FGF-23, in a CKD group not undergoing dialysis [41].

In this regard, according to another study, OPG increased expression could be linked to medial calcifications in aorta and renal arteries; thus its expression is recognized as a protective mechanism against vascular calcifications [6]. Thereby, the RANK/RANKL/OPG signaling pathway was found to be closely related to atherosclerosis progression [42].

Our data suggest a statistically significant increased expression of OPG in CKD patients group compared with control ($p < 0.001$, Figure 3(a)). The same results were found in a study by Demir et al. [43]. The upward trend of OPG levels is also maintained with regard to the distribution on CKD stages, as follows: 3.5-fold higher in CKD stage 4, 2.5-fold higher in CKD stage 3, and 2-fold higher in CKD stage 2 ($p < 0.001$ for all stages, Figures 3(b) and 2).

Despite the fact that several studies propose a direct involvement action of IL-6 in the increasing level of expression of OPG, we can conclude, based on our data, that the level of OPG in serum correlates with the expression level of IL-6 in patients of CKD stage 2 only ($r = 0.63$). Given the relatively small number of patients included in this study, further research is necessary to fully understand the therapeutic and biomarker potential of OPG in patients with kidney disease.

Morena et al. were among the first that also mentioned that increased OPG levels were correlated with the progression of coronary artery calcification (CAC) in a CKD nondialyzed group [44].

It was observed that OPG knockout mice develop severe calcifications, thus the potential protective role of OPG against vascular calcification being proposed [45].

Furthermore, Lewis et al. concluded that OPG might be a crucial biomarker in CKD stages 3–5 patients with poor long-term prognosis, based on their results showing that OPG high levels were correlated with the progress in renal dysfunction [46].

3.2.2. Osteocalcin (OCN). Considering the osteoblastic activity of OCN, this marker might be directly involved in bone-vascular axis [47, 48] and its systemic and local effects could be potentially related to bone remodeling, vascular calcification, and energy metabolism [49].

We found that OCN circulating levels were increased 4.6-fold in CKD stage 4 ($p < 0.01$) and 2-fold ($p < 0.01$) and 1.3-fold ($p = 0.05$) in CKD stages 3 and 2, respectively, thus revealing an overexpression of OCN in CKD patients *versus* control ($p < 0.001$) (Figures 3(c), 3(d), and 2). The OCN serum levels, in association with inflammatory markers IL-6 and TNF-α, showed significant correlation with regard to CKD stage 4 only ($r = 0.67$ and $r = 0.69$) (Tables 1, 2, and 3).

Other studies observed that OCN was inversely correlated to age and IL-6, in CKD hemodialysis patients [50].

Since the controversy still exists, further research and large clinical trials are needed to clearly explain the connections between the immune system and bone-vascular axis.

3.2.3. Osteopontin (OPN). In CKD patients, starting with early stages, Barreto et al. have noticed increased OPN levels compared with control and have also related a positive correlation of OPN with the inflammatory markers [4, 51].

In a univariate linear regression assessment, OPN was found to be directly correlated with inflammation markers like IL-6, C-reactive protein (CRP), and intact parathyroid hormone (iPTH), concluding that OPN could play an important role in the pathway where inflammation enhanced CKD poor prognosis [51].

Lorenzen et al. also found a possible link between OPN and inflammation markers (IL-6, CRP) in hypertensive patients [52].

Our results were in agreement with the above-mentioned studies and revealed significant differences between the control group and patients with CKD ($p < 0.001$), being increased more than 2-fold in CKD stage 2 ($p = 0.01$), rising at 4-fold in CKD stage 3 ($p < 0.001$) and 7-fold in CKD stage 4 ($p < 0.0001$) (Figures 3(e), 3(f), and 2).

The threshold values for the analyses were established in accordance with the mean values of the studied groups. A negative correlation was observed between OPN and Fetuin-A serum levels of CKD stage 4 patients ($r = -0.67$), and a positive correlation was found between OPN and IL-6 ($r = 0.59$) and TNF-α ($r = 0.72$). In CKD stage 3, a statistical correlation between OPN and OPG was observed ($r = 0.53$). A statistical correlation was also found with IL-6 ($r = 0.52$) and TNF-α ($r = 0.51$) in CKD stage 2 (Tables 1, 2, and 3).

3.2.4. Fibroblast Growth Factor 23 (FGF-23). FGF-23 is a phosphaturic hormone with elevated levels in early CKD stages, before mineral and bone disorders become obvious [53], and might be associated with endothelial dysfunction [54] and greater risk of congestive heart failure (CHF) and atherosclerotic events in patients with CKD stages 2–4 [55].

Nonetheless, in another study, FGF23 appears not to be an early marker of CKD, in elderly patients (age over 65) [56].

It is generally considered that CKD plays the most important role in increasing FGF-23 levels; in this view, FGF-23 high levels appeared to be independently linked to CKD prognosis [57, 58], although the mechanisms are poorly understood [59].

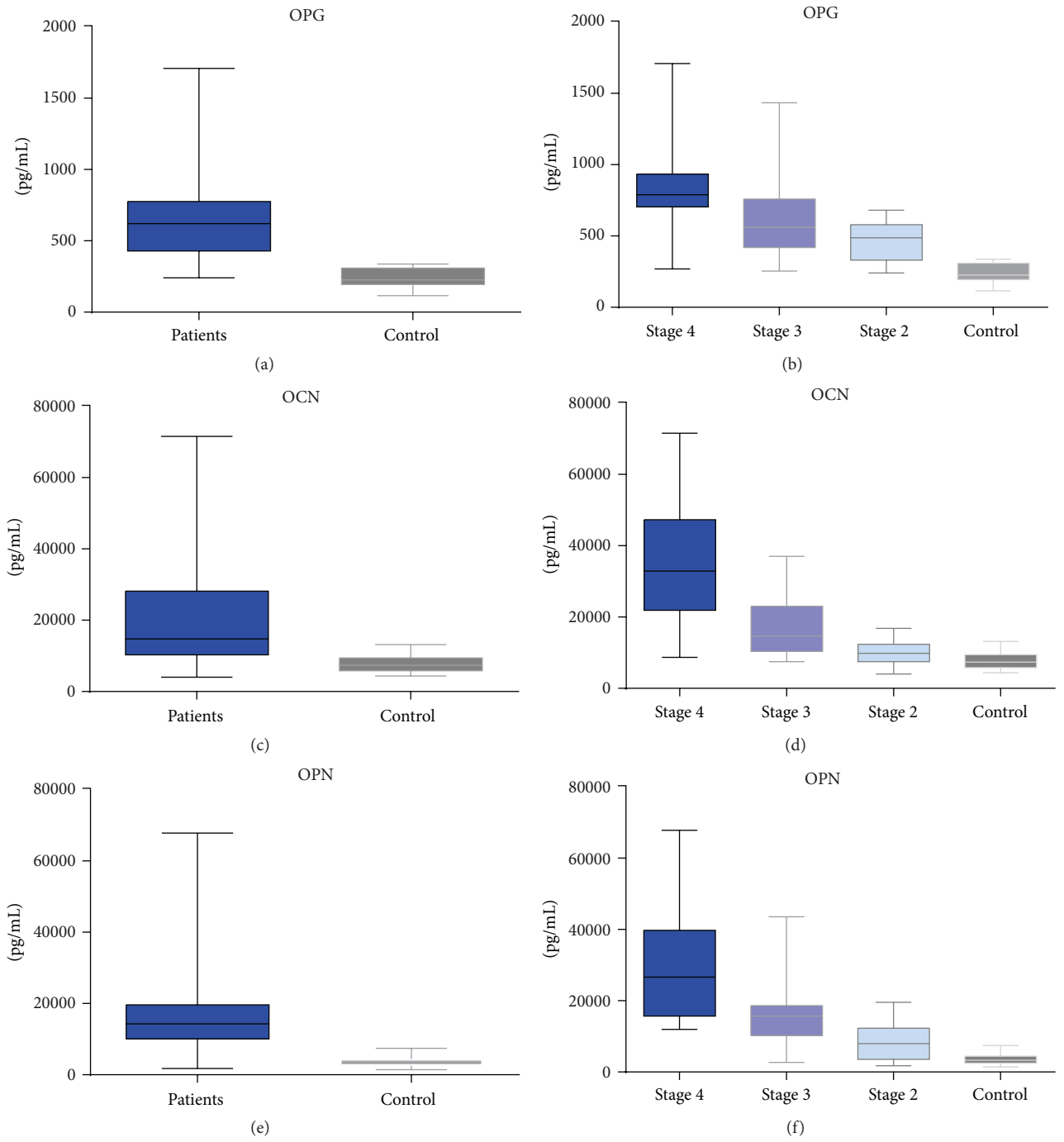

Figure 3: Serum level of OPG, OCN, and OPN in CKD patients compared with control, by xMAP array.

Desjardins et al. suggest that plasma FGF-23 could be considered an independent biomarker of vascular calcification in patients with CKD, starting from early stages [60].

We have found that FGF-23 levels were significantly enhanced in CKD patients ($p < 0.001$) (Figure 4(a)). Serum levels of FGF-23 showed a gradual increase, reaching the highest levels in patients with CKD stage 4 ($p < 0.0001$), being 16-fold higher than in the control group. According to previously analyzed biomarkers, serum FGF-23 levels still showed a significant increase of 6-fold ($p < 0.001$) in CKD stage 3 and 2-fold ($p < 0.001$) for CKD stage 2 (Figures 4(b) and 2). Although FGF-23 has been identified to be significantly overexpressed in CKD stages 2–4, there were no statistical correlations with the other multiplexed analyzed biomarkers (according to Pearson correlations).

3.2.5. Fetuin-A. Among the multiple players involved in vascular calcification pathogenesis, Fetuin-A is considered to

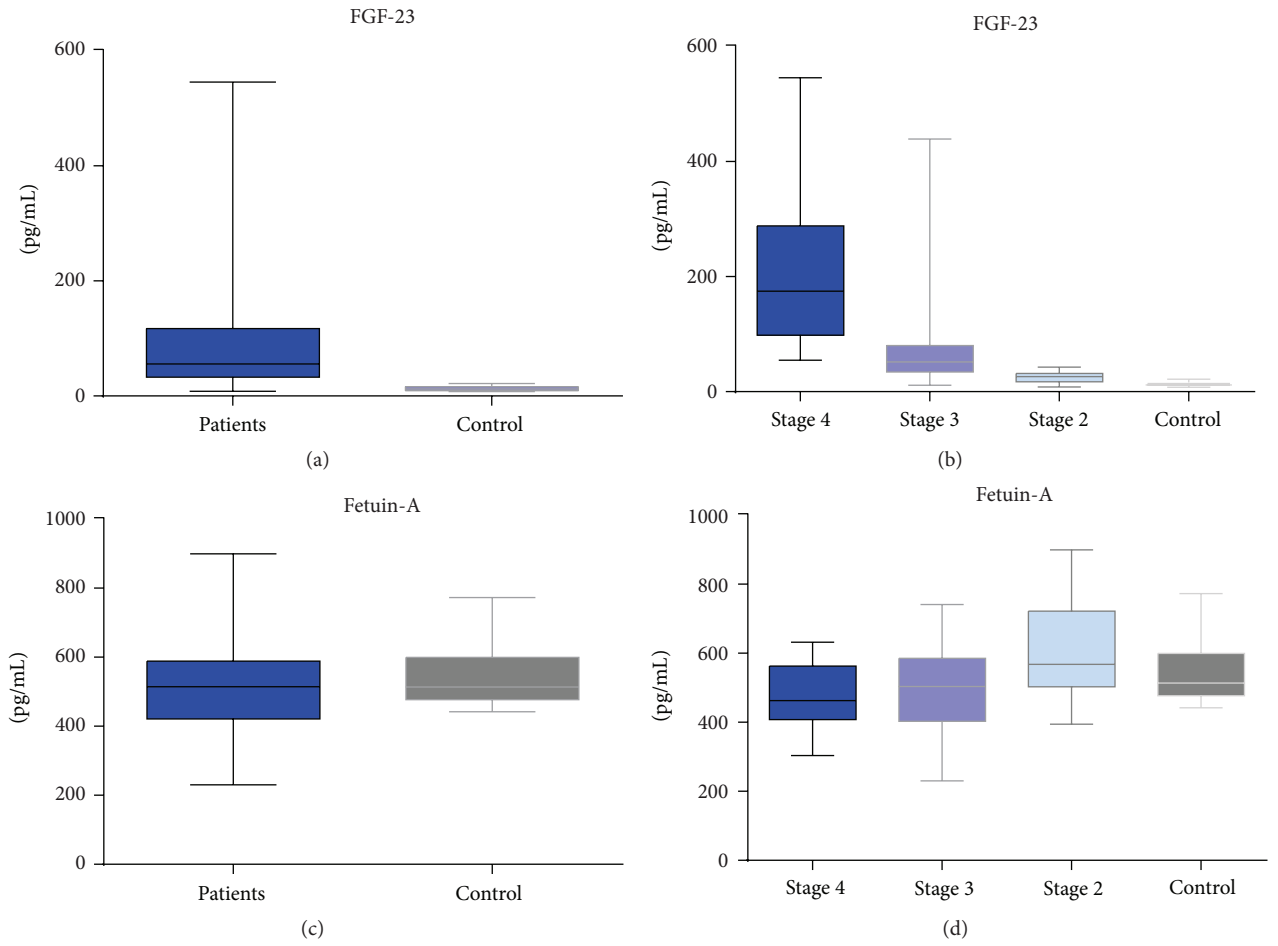

Figure 4: Serum level of FGF-23 and Fetuin-A in CKD patients compared with control, assessed by xMAP array.

be an inhibitory molecule; thus CKD patients are assumed to experience a Fetuin-A deficiency, which might be considered a common feature of this disease [61].

We have noticed that the level of Fetuin-A in CKD patients was decreased compared with control ($p = 0.15$). The highest decrease was found in CKD stage 4, being of 0.85-fold ($p = 0.02$), followed by stage 3 with 0.91-fold ($p = 0.05$) and stage 2 with 1.13-fold ($p = 0.13$) (t-test) (Figures 4(c), 4(d), and 2). Fetuin-A presented a negative correlation with TNF-α ($r = -0.61$) and OPN ($r = -0.67$) in CKD stage 4, while in CKD stage 2 Fetuin-A was negatively correlated with IL-6 ($r = -0.5$) and OPG ($r = -0.6$) (Tables 1, 2, and 3).

Smith et al. also reported an association between Fetuin-A decreased levels and inflammatory markers, also with procalcific cytokine, explaining the potential involvement of this biomarker in coronary calcification and aortic stiffness [62].

3.3. Correlations between CKD Markers and Inflammatory Status. We have observed a strong correlation between IL-6 and eGFR ($\chi^2 = 16.8$; $P < 0.01$), TNF-α ($\chi^2 = 7.9$; $P < 0.005$), OPN ($\chi^2 = 5.4$; $P < 0.02$), OPG ($\chi^2 = 8.28$; $P = 0.04$), and FGF-23 ($\chi^2 = 5$; $P = 0.02$). TNF-α was correlated with FGF-23 ($\chi^2 = 7.4$; $P = 0.006$) and Fetuin-A ($\chi^2 = 5.9$; $P = 0.001$). Strong correlations were also found between eGFR and OCN ($\chi^2 = 6.2$; $P = 0.01$) and FGF-23 ($\chi^2 = 19.9$; $P < 0.001$); also OCN correlated with OPN ($\chi^2 = 5.3$; $P = 0.02$) and FGF-23 ($\chi^2 = 6.9$; $P = 0.008$) in all CKD groups. The above-mentioned correlations, chi-square test (χ^2; P), between analyzed inflammatory mediators and mineral/bone disorders markers, alongside with eGFR, are shown in Table 4.

According to our results, we conclude that a crosstalk between bone, vasculature, and renal function exists in CKD, representing a major risk factor for cardiovascular morbidity and mortality.

In CKD early stage 2, an increased expression was observed for 6 out of the 7 analyzed biomarkers. From our data, circulating levels of IL-6, TNF-α, OPG, OCN, OPN, and FGF-23 were statistically increased ($P < 0.05$) in CKD stage 2, while Fetuin-A showed a slight alteration over control, but with no statistical significance ($P = 0.13$).

At a first glance, proteomic biomarkers offer the hope of improving the management of patients with CKD starting with early stages, yet more studies are needed to establish the diagnostic and prognostic value of these biomarkers.

Table 4: Correlations between inflammatory cytokines, bone and mineral disorder biomarkers, and eGFR in patients with CKD stages 2–4, not undergoing dialysis.

	IL-6			TNF-α			OPN			OPG			OCN			FGF-23			Fetuin-A		
	<6	≥6		<5	≥5		<6000	≥6000		<400	≥400		<12000	≥12000		<35	≥35		<400	≥400	
Gender																					
M	31	31	$P = 0.16$	39	23	$P = 0.29$	13	49	$P = 0.65$	10	52	$P = 0.17$	21	41	$P = 0.75$	20	42	$P = 0.29$	13	49	$P = 0.65$
F	8	16	$\chi^2 = 1.9$	18	6	$\chi^2 = 1.1$	4	20	$\chi^2 = 0.2$	7	17	$\chi^2 = 1.85$	9	15	$\chi^2 = 0.1$	5	19	$\chi^2 = 1.09$	4	20	$\chi^2 = 0.2$
Age																					
<60	10	12	$P = 0.99$	12	10	$P = 0.18$	7	15	$P = 0.09$	7	15	$P = 0.09$	9	13	$P = 0.5$	4	18	$P = 0.19$	7	15	$P = 0.09$
≥60	29	35	$\chi^2 = 0.0001$	45	19	$\chi^2 = 1.8$	10	54	$\chi^2 = 2.7$	10	54	$\chi^2 = 2.7$	21	43	$\chi^2 = 0.5$	21	43	$\chi^2 = 1.7$	10	54	$\chi^2 = 2.7$
eGFR																					
<60	27	47	$P < 0.001$	46	28	$P = 0.04$	12	62	$P = 0.04$	14	60	$P = 0.6$	22	52	$P = 0.01$	15	59	$P < 0.001$	17	64	$P = 0.07$
≥60	12	0	$\chi^2 = 16.8$	11	1	$\chi^2 = 4.02$	5	7	$\chi^2 = 4.2$	3	9	$\chi^2 = 0.2$	8	4	$\chi^2 = 6.2$	10	2	$\chi^2 = 19.9$	0	12	$\chi^2 = 3.08$
IL-6																					
<6				32	7	$P < 0.005$	12	27	$P < 0.02$	13	26	$P = 0.004$	18	21	$P = 0.04$	16	23	$P = 0.02$	7	32	$P = 0.7$
≥6				25	22	$\chi^2 = 7.9$	5	42	$\chi^2 = 5.4$	4	43	$\chi^2 = 8.28$	12	35	$\chi^2 = 4$	9	38	$\chi^2 = 5$	10	37	$\chi^2 = 0.2$
TNF-α																					
<5							19	38	$P = 0.03$	15	42	$P = 0.03$	22	35	$P = 0.3$	22	35	$P = 0.006$	7	50	$P = 0.01$
≥5							3	26	$\chi^2 = 4.6$	2	27	$\chi^2 = 4.6$	8	21	$\chi^2 = 1.02$	3	26	$\chi^2 = 7.4$	10	19	$\chi^2 = 5.9$
OPN																					
<6000										6	11	$P = 0.07$	10	7	$P = 0.02$	6	11	$P = 0.52$	3	14	$P = 0.8$
≥6000										11	58	$\chi^2 = 3.2$	20	49	$\chi^2 = 5.3$	19	50	$\chi^2 = 0.39$	14	55	$\chi^2 = 0.06$
OPG																					
<400													9	8	$P = 0.08$	4	13	$P = 0.57$	4	13	$P = 0.66$
≥400													21	48	$\chi^2 = 3.04$	21	48	$\chi^2 = 0.3$	13	56	$\chi^2 = 0.2$
OCN																					
<12000																14	16	$P = 0.008$	6	24	$P = 0.96$
≥12000																11	45	$\chi^2 = 6.9$	11	45	$\chi^2 = 0.001$
FGF-23																					
<35																			2	23	$P = 0.07$
≥35																			15	46	$\chi^2 = 3.07$
Fetuin-A																					
<400																					
≥400																					

4. Conclusions

The present study highlights the potential clinical utility of a multiplexed biomarker panel in CKD. Out of all analyzed candidate biomarkers, a panel which includes mediators of inflammation (IL-6, TNF-α) and mineral and bone disorder biomarkers (OPG, OPN, OCN, FGF-23, and Fetuin-A) was found to be more relevant than a single biomarker to detect patients in early CKD stages. We have noticed a positive correlation between the biomarkers panel of IL-6, OCN, and FGF-23 and renal failure progression (eGFR) in all CKD groups. The association between inflammatory cytokines and bone disorders markers, OPN, OPG, and FGF-23, reflects the severity of the vascular changes in CKD and predicts the disease progression. Proteomic xMAP analyses shed light on clinical evaluation for CKD staging and prognosis. Thus, new evidence has emerged within the relationship between bone and vascular pathology, especially in CKD patients, encouraging further investigations in the area.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

The authors contributed equally to this work.

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Review Article

CEACAM1: Expression and Role in Melanocyte Transformation

Gabriela Turcu,[1] Roxana Ioana Nedelcu,[2] Daniela Adriana Ion,[2] Alice Brînzea,[2] Mirela Daniela Cioplea,[3] Lucia Beatrice Jilaveanu,[4] and Sabina Andrada Zurac[3]

[1]*Colentina Clinical Hospital, 1st Dermatology Department, "Carol Davila" University of Medicine and Pharmacy, 020125 Bucharest, Romania*
[2]*Department of Pathophysiology II, Clinical Department No. 2, "Carol Davila" University of Medicine and Pharmacy, 021105 Bucharest, Romania*
[3]*Pathology Department, Colentina Clinical Hospital, "Carol Davila" University of Medicine and Pharmacy, 020125 Bucharest, Romania*
[4]*Department of Medicine, Section of Medical Oncology, Yale University School of Medicine, New Haven, CT 208028, USA*

Correspondence should be addressed to Roxana Ioana Nedelcu; roxanaioana.nedelcu@yahoo.com

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Metastases represent the main cause of death in melanoma patients. Despite the current optimized targeted therapy or immune checkpoint inhibitors the treatment of metastatic melanoma is unsatisfactory. Because of the poor prognosis of advanced melanoma there is an urgent need to identify new biomarkers to differentiate melanoma cells from normal melanocytes, to stratify patients according to their risk, and to identify subgroups of patients that require close follow-up or more aggressive therapy. Furthermore, melanoma progression has been associated with the dysregulation of cell adhesion molecules. We have reviewed the literature and have discussed the important role of the expression of the carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) in the development of melanoma. Thus, novel insights into CEACAM1 may lead to promising strategies in melanoma treatment, in monitoring melanoma patients, in assessing the response to immunotherapy, and in completing the standard immunohistochemical panel used in melanoma examination.

1. Introduction

Metastases represent the main cause of death in melanoma patients. Metastatic melanoma treatment is unsatisfactory, even with the current optimized targeted therapy (BRAF-oncogene inhibitors) or immune checkpoint inhibitors (anti-cytotoxic T-lymphocyte-associated antigen 4 (anti-CTLA4), anti-programmed death-1 protein (anti-PD1), and programmed death ligand-1 protein (anti-PDL1)). To date, lactate dehydrogenase (LDH) is the only serum biomarker used in clinical practice for melanoma; however, the sensitivity and specificity of LDH in predicting metastatic recurrence are low. Considering both the relative unsatisfactory potential of LDH as a biomarker and the poor prognosis of advanced melanoma, numerous attempts by our group and others have been made to identify new biomarkers to stratify patients according to their risk, thus identifying subgroups of patients that require closer follow-up or more aggressive therapy [1]. New blood biomarkers panels including CEACAM are tested, in order to complete laboratory and imaging recommendations for monitoring patients with melanoma [2]. Markel et al. report higher levels of serum CEACAM1 for melanoma patients compared with healthy donors [3].

Melanoma is an immunogenic tumor in which malignant melanocytes present high rate of DNA mutation and express tumoral antigens that can elicit multiple immune responses [4–9]. Dysregulation of cell adhesion molecules has been associated with disease progression in melanoma [10]. Thus, novel insights into carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) may lead to promising strategies in melanoma treatment.

2. CEACAM1 Structure

CEACAM1 (also referred to as C-CAM, biliary glycoprotein BGP, and CD66a) is a complex glycoprotein linked to the cell membrane by a carboxy-terminal transmembrane anchor; it belongs to the carcinoembryonic antigen (CEA) family of the immunoglobulin superfamily. So far, six other CEACAM glycoproteins have been described (CEACAM3–CEACAM8), all of them being characterized by several domains: an N-domain, a membrane IgV-related domain, and one/multiple IgC2-related domains. CEACAM1 N-domain may bind homophilically with CEACAM1 and heterophillically with CEA or other CEACAMs, thus acting as cell-cell adhesion molecule [11, 12].

CEACAM1 is encoded on chromosome 19q13.2 and is expressed on several types of cells such as epithelial cells (apical pole of epithelial cells of the gastrointestinal tract, esophageal glands, duodenal Brunner glands, intestinal and colonic superficial epithelial cells, epithelial cells of the gall bladder, bile ducts, pancreatic ducts, mammary ducts, epithelial cells of the endometrium, renal tubular epithelium, prostatic glands, extravillous (intermediate) trophoblast, etc.), endothelial cells, B and T lymphocytes, natural killer cells, and myeloid cells [13–19].

3. CEACAM1 General Biologic Functions

The expression of CEACAM1 mediates intercellular protein interactions and intracellular signaling during inflammation, microbial and viral infection, angiogenesis, cancer progression, and metastasis [20].

The role of CEACAM1 in inflammation is complex due to its alternate splicing and generation of various isoforms; in humans, eight transmembrane isoforms are present (including CEACAM1-3L, CEACAM1-4L, CEACAM1-3S, and CEACAM1-4S). In T lymphocytes CEACAM1 splice variants with long cytoplasmic tails associate with inhibitory function both *ex vivo* and *in vivo* by inhibiting the T-cell receptor CD3 complex. Moreover, these splice variants also enhance fibrinogen adhesion via Fc receptor and $\beta 2$ integrin [12, 15, 21–24]. CEACAM1 is involved in modulation of innate and adaptive immune responses, via its expression on B and T lymphocytes, natural killer cells, and myeloid cells. CEACAM1 has a role in T cell inhibition through direct T cell receptor cross-linking and binding of *Neisseria* opacity associated proteins; CEACAM1 inhibition of NK cells is independent of the major histocompatibility complex class I and dependent on the cytosolic tail [25].

CEACAM1 regulates angiogenesis by targeting several processes such as chemotaxis, endothelial cells proliferation, and tube formation; its biologic functions are additive to vascular endothelial growth factor (VEGF) effects, as demonstrated by CEACAM1 upregulation induced by VEGF as well as *in vitro* obstruction of VEGF-induced tube formation by CEACAM1. Several tissues overexpress CEACAM1 in endothelial cells such as normal or pathological nontumoral highly proliferating tissues (normal and/or pregnant endometrium, granulation tissue) or tumor tissues (such as solid human tumors) [19, 26].

CEACAM1 also intervenes in insulin action regulation increasing the clearance of insulin through the stimulation of receptor-mediated insulin endocytosis and degradation [27].

CEACAM1 is used by various pathogens to adhere to eukaryotic cells. For instance, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Escherichia coli*, and various species of *Neisseria and Salmonella* use the N-domain of CEACAM1 as a microbial receptor in human granulocytes and epithelial cells. Several viruses such as coronaviruses mouse hepatitis virus type 2 and severe acute respiratory syndrome coronavirus (SARS-CoV) use a soluble form of CEACAM1a to enter host cells [12, 28, 29].

Furthermore, CEACAM1 is also involved in apoptotic control by two mechanisms, the cleavage of CEACAM1-4L isoform intra- and extracellularly and caspase activation [30–32].

4. CEACAM1 Expression in Cancer

High expression levels of CEACAM1 have been detected in melanoma [33], adenocarcinomas [34], and small cell lung cancers [35] while lower levels of CEACAM1 have been detected in colon [36], prostate [37], and breast cancers [38].

In melanoma, through homophilic interactions, CEACAM1 inhibits natural killer (NK) cell activity and effector functions (such as cytotoxicity and interferon gamma (IFNγ) release) of tumor-infiltrating lymphocytes (TILs). Upregulation of CEACAM1, induced by IFNγ on melanoma cells that survive TIL-mediated attack, renders these cells even more resistant [16, 25, 39–41]. CEACAM expression in melanoma has often been noted in the invading part of the tumor [42] and has been associated with increased melanoma cell invasion and migration [43]. CEACAM also interferes with cell-matrix adhesion and enhances cell motility by modulating N-cadherin, an intercellular adhesion glycoprotein and epithelial-mesenchymal transition marker [44].

Recent studies evidenced the correlation between CEACAM1 expressions in melanoma cell lines and melanoma tissue with microphthalmia-associated transcription factor (MITF) [45]. MITF modulates proliferation and invasion in melanoma.

Mechanisms involved in CEACAM1 and MITF correlation in melanoma may involve sex determining region Y [SRY] related HMG-box 9 (SOX9); SOX9 is a transcription factor involved in chondrogenesis and sex determination in embryo [46]; its function in normal melanocytes is upregulation of melanin synthesis in melanocytic cells after ultraviolet B (shortwave) rays (UVB) exposure by increasing MITF, dopachrome tautomerase (tyrosine-related protein 2), and tyrosinase expression [47], while increased expression of SOX9 in melanoma inhibits tumor cell proliferation by binding p21 directly or via MITF [48, 49]. Whether SOX9 directly or indirectly regulates CEACAM1 expression is still a matter of debate with different authors obtaining opposing results, possibly due to differences between the types of cells studied (colon epithelium versus melanoma cells lines) and the differences of the roles fulfilled by CEACAM1 in colon carcinoma (tumor growth suppressor) and melanoma (tumor

aggressiveness promoter) [50–52]. Also, MITF binds the CEACAM1 promoter on an M-box motif, thus directly stimulating CEACAM1 expression [45].

Moreover, CEACAM1 induces Sox-2 overexpression, which in turn induces β-catenin expression, a mechanism involved in acquiring tumor aggressiveness. Sox-2 increases invasiveness of melanoma cells; it is upregulated by CEACAM-1L and has a similar progressive upregulation from 14% in nevi to 80% in metastatic melanoma [53–55].

5. CEACAM1 in Benign Melanocytic Lesions

CEACAM1 is not present on the surface of normal melanocytes [43]. There is a lack of data regarding CEACAM1 expression in melanocytic nevi. Gambichler et al. performed an immunohistochemical study, analyzing the expression of CEACAM1 in benign and malignant cutaneous tumors and in normal peritumoral skin: benign nevi (42 cases), dysplastic nevi (22 cases), thin superficial spreading melanomas (21 cases), and thick superficial spreading melanomas (21 cases). The results were reported as percentages of positively stained melanocytes in studied lesions. Median CEACAM1 expression was 1% in benign nevi, 9.6% in dysplastic nevi, 18% in thin superficial spreading melanomas (Breslow tumor thickness < 1 mm), and 74% in thick superficial spreading melanomas (Breslow tumor thickness > 1 mm) ($p < 0.0001$). Compared with benign nevi, median expression of CEACAM1 was significantly increased in both thin and thick superficial spreading melanomas and was not significantly increased in dysplastic nevi. Compared with dysplastic nevi, median expression of CEACAM1 was significantly increased in thick superficial spreading melanomas. In peritumoral skin, melanocytes were not immunohistochemically reactive [56].

CEA family expression was immunohistochemically assessed in different subtypes of melanocytic nevi: 5 junctional acquired nevi, 9 compound acquired nevi, 31 intradermal acquired nevi, 14 compound congenital nevi, 4 intradermal congenital nevi, and 20 blue nevi. The authors used a panel of monoclonal and polyclonal antibodies that recognize different epitopes of CEA and CEA-related molecules, including CEACAM1; none of the antibodies used in the study recognized only CEACAM1. The results of the study revealed an increased expression of CEA glycoprotein family in the various types of analyzed nevi, excepting blue nevi. The negative staining was also observed in normal melanocytes [57]. The carcinoembryonic antigen family is expressed in melanocytic nevi, in a similar pattern in acquired and congenital ones, and it is not present in blue nevi that represent neural crest melanocytes that failed to reach the epidermis during embryological migration [57].

In vitro investigation evidenced that invasion and migration of melanocytic cells that express CEACAM1 are enhanced. The authors studied melanocytic MEL6 cell line invasive capacity, after cells were transfected with CEACAM1. CEACAM1 transfected cells showed higher invasive and motility properties compared to untransfected cells [43].

6. CEACAM1 in Primary Melanoma

Gamblicher studied 42 cases of superficial spreading melanoma (SSM), using 2 different antibodies that revealed membranous and cytoplasmic staining of tumor cells. CEACAM1 expression was significantly higher in SSM compared to benign nevi, and the authors noticed a progressive increase from benign nevi, dysplastic nevi, and thin SSM to thick SSM. The authors highlighted a significant positive correlation between CEACAM1 expression and Breslow tumor thickness and Clark level of superficial spreading melanomas [56].

In 2002 Thies et al. evaluated 100 cases of primary melanoma and 40% expressed CEACAM1, the staining being more intense in the invasive part of the lesion. Out of the 40 cases of CEACAM1 positive melanomas, 28 had metastasized while out of the 60 cases of CEACAM1 negative melanomas, only 6 had metastasized. Vessel staining was identified in only 7 patients and there was no association between CEACAM1 expression in microvessels and prognosis [42].

In activated human tumor-infiltrating lymphocytes derived from patients with melanoma, all cells express CEACAM1, with inhibitory effects following homophilic interaction [41, 42].

The authors concluded that CEACAM1 expression in melanoma cells was an independent factor (regardless of the ulceration status, mitotic rate, and tumor thickness) for the risk of metastasis with a predictive value superior to that of tumor thickness. For patients with identical parameters in American Joint Committee on Cancer (AJCC) classification, CEACAM1 status provides a more accurate predictive estimation [42].

Studying the essential interplay between melanoma and tumor-infiltrating lymphocytes, Markel et al. coincubated melanoma cells with tumor-infiltrating cells *in vitro*. Results of the experiment showed a progressive increase in CEACAM1 expression on melanoma cells that resisted the attack of infiltrating lymphocytes. Increased CEACAM1 expression was dependent on the presence of interferon gamma [25].

7. CEACAM1 in Metastatic Melanoma

Ortenberg et al. showed that 89% of metastatic cutaneous melanoma lesions express CEACAM1, and its expression increases during tumor progression [53].

Khatib et al. first evaluated CEACAM1 expression in 79 cases of primary uveal melanomas and 21 metastases in the most frequently affected organ, the liver. CEACAM1 was identified in 45% of the primary uveal melanomas and in 81% of liver metastases, similar to CEACAM1 expression in cutaneous melanoma and its corresponding metastases. The presence of CEACAM1 on the primary tumor did not correlate with the development of metastases or survival [58].

In vivo studies showed that CEACAM1-L overexpression promotes xenograft tumorigenicity of melanoma [53]. CEACAM1 directly inhibits activated NK and T lymphocytes and this results in an increase of its expression in melanoma cells that survive an *in vitro* immune attack, which can further inhibit new immune cells [41, 42, 59, 60].

In vitro studies evidenced another possible mechanism responsible for these data: the expression of CEACAM1-L in melanocytic cells (CEACAM1 negative MEL6 melanocytic cell line) and melanoma cell (CEACAM1 negative MV3 melanoma cell line) increased the invasive and the migratory properties of melanocytic and melanoma cells, by interaction with integrins [43]. Cell migration was also modulated by interaction of CEACAM1-L and the protein filamin A, which reduce the migratory potential. The authors evidenced that CEACAM1-L, in addition to stimulation of migration, could also inhibit cell migration [61].

In a small series of 13 patients, Zippel et al. noticed a borderline significant increase in the membrane staining from primary lesions to lymph nodes and distant metastases [62].

In current practice immunohistochemical staining for S100, melanA, and human melanoma black 45-HMB45 is used to identify melanoma cells in clinically negative sentinel lymph nodes, with 10–20% improved rate of detection compared to hematoxylin and eosin (HE) staining alone. Immunohistochemical marker sensitivity and specificity for detection of melanoma cells in primary melanomas, sentinel lymph nodes, and distant metastases were studied by Thies et al. [33]. Expression of cell adhesion molecules CEACAM1 and L1 was analyzed and compared to expression of standard markers MelanA, S100, and HMB45 in 67 cases of primary melanomas, 40 cases of sentinel lymph nodes, 35 cases of distant metastases, and 12 cases of benign nevi. The authors compared the sensitivity of CEACAM1 and L1 markers to MelanA, S100, and HMB45 and describe results similar to other studies reporting an 87–93% sensitivity in primary melanomas and 60–95% in their corresponding metastatic lymph nodes [63]. The authors also compared two different antibodies for CEACAM1 and highlighted the superior sensitivity of monoclonal antibody 4D1/C2 to the commercial NCL-CD66a. Regarding the specificity of immunohistochemical markers for melanoma cells, a relevant observation was described; CEACAM1 and L1 are highly specific for melanoma cells, while MelanA, S100, and HMB45 are not. In this context, benign nevus cell inclusions in the capsule of sentinel lymph nodes could lead to false positive diagnosis of melanoma metastases [33].

Antibodies 4D1/C2 against CEACAM1 have a higher specificity and sensitivity for melanoma cells in lymphatic and hematogenous metastases and can be added to the standard panel of antibodies [33, 64].

In a study from 2012 Ortenberg et al. found positive staining for CEACAM1 in 89% of the metastatic melanoma and in CD8 lymphocytes surrounding the metastases [65]. The wide distribution of CEACAM1 in metastatic melanoma qualifies it for targeted therapy, alone or in combination.

The new immune checkpoint inhibitors, anti CTLA4 and anti PD1, are not tumor-specific and are sometimes associated with severe immunologic side effects.

In vitro and *in vivo* studies on melanoma xenografts show that antibodies built to target the extracellular portion of CEACAM1 and to block the N-domain of CEACAM1 (MRG1, a murine IgG1 monoclonal antibody against human CEACAM1) did not influence the proliferation rate but facilitate melanoma cell elimination by T cells and have no agonistic effect. Regarding the safety concerns about MRG1 antibody's effect on normal epithelial cells that express CEACAM1 or the effect of activated T cells on normal CEACAM1 positive cells and autoimmune events, it did not directly affect CEACAM1 positive cells and it did not induce nonspecific activation of T cells [53]. It is thought that concomitant blocking of CEACAM1 and immunotherapy with adoptive cellular transfer (ACT) might improve the clinical response [41].

Novel specific monoclonal antibodies for melanoma immunotherapy, based on functional blocking of CEACAM1, are now available from cCAM Biotherapeutics (CM24) and Agenus Inc. [65, 66].

A phase I trial (NCT02346955) is going on in 2 centers from the USA and 1 center from Israel and a primary outcome measure will be available after January 2018 [67]. Blocking of CEACAM1 may reverse the inhibitory action on the NK and activated T cells and enhance the antitumoral effect of the endogenous immune system.

8. CEACAM1 Serologic Results

CEACAM1 is normally identified in serum from healthy individuals, but at a low level. In melanoma patients, the level of CEACAM1 correlates with the amount of tumor cells that secret CEACAM1 [3].

Patients with melanoma also show a high percentage of CEACAM1 positive lymphocytes in the peripheral blood, which is not secreted and does not influence the total serum level of CEACAM1. In metastatic melanoma, the level of soluble CEACAM1 significantly correlated with LDH level [3].

Currently, LDH is the only serologic biomarker included in AJCC staging system as an important independent prognostic factor in advanced, stage IV melanoma, with a 92% specificity and 79% sensitivity [68, 69]. Egberts and collaborators found S100 calcium-binding protein B (S100B) evaluation superior to LDH in the identification of early distant metastasis [70]. The levels of both markers correlates with poor outcome, shorter disease-free and overall survival [71, 72].

Sivan et al. monitored CEACAM1, S100, and LDH serum level in 49 patients with regional or metastatic disease, prior to and after autologous vaccination [73]. Patients with evidence of disease showed significantly higher levels of CEACAM compared to patients with no evidence of disease or healthy volunteers, reflecting the disease burden. Kluger's group also showed that plasma CEACAM1 is elevated in patients with metastatic melanoma compared to healthy controls and early stage disease [2]. Serum levels of CEACAM1 correlated with S100, disease activity, and overall survival rates. The level of soluble CEACAM1 inversely correlated with time to death from melanoma in patients with active disease. As majority of the patients had normal LDH levels, CEACAM1 value facilitates a more precise prognostic prediction [60, 73].

9. CEACAM1 and the Risk of Tumor Recurrence

Another important issue for patients with stage I and II resected melanoma is the follow-up schedule, for which there is no general consensus. Melanoma cells can remain dormant for variable periods of time and valid biomarkers to detect recurrences are not yet available, so early surgical or systemic treatment is delayed. Kluger and colleagues proposed a multiplex, plasma-based protein biomarker panel that included CEACAM, ICAM-1 (intercellular adhesion molecule 1), osteopontin, MIA (melanoma inhibitory activity), GDF-15 (growth differentiation factor 15), TIMP-1 (tissue inhibitor of metalloproteinase 1), and S100B. With a sensitivity of 74%, once validated, this panel test can be used to monitor patients for melanoma recurrences and reduce the amount of unnecessary imaging in asymptomatic patients that have normal marker levels [2].

10. Conclusions

The reviewed articles highlighted the important role of CEACAM1 expression in the development of melanoma. CEACAM1 promotes melanoma progression: CEACAM1 enhances migration and invasion of melanoma cells; CEACAM1 impairs the antitumor immune responses of NK and T cells. Mechanisms by which CEACAM1 interacts with melanoma biology are incompletely elucidated. *In vitro* studies revealed the relation between CEACAM1 expression and melanoma cells behavior; increased expression of CEACAM1 in melanoma cell lines amplified the invading capacity of these cells; downregulation of CEACAM1 expression in melanoma cells decreased the expansion ability of cells. On the other hand, CEACAM1 expression modulates melanoma cell escape from immunologic attacks; when melanoma cells and tumor-infiltrating lymphocytes are coincubated *in vitro*, surviving melanoma cells increase CEACAM1 expression, which is dependent on constant presence of interferon gamma. CEACAM1 positive lymphocytes are more abundant in patients with melanoma; melanoma cells possibly transfer CEACAM1 to the attacking lymphocytes, affecting efficient immune reactions. In patients receiving immunotherapy for melanoma using adoptive cell transfer with tumor-infiltrating lymphocytes, downregulation of major histocompatibility complex (MHC) class I is often described; reduced surface expression of MHC class I molecules is seen in melanoma cells overexpressing CEACAM1. CEACAM1 expression correlates with poor prognosis of melanoma patients. Since melanoma is an immunogenic disease, CEACAM1 constitutes an attractive target for immunotherapy.

Correlation between serum CEACAM1 and melanoma progression and survival supports CEACAM1 as a standard biomarker in monitoring melanoma patients and assessing the response to immunotherapy.

Due to the high specificity and sensitivity of antibodies against CEACAM1 in melanoma metastases, it could enhance the standard immunohistochemical panel used in melanoma examination.

Reviewing the literature we have brought into focus a key molecule, CEACAM1, as one of the latest hallmarks of cancer involved in the main mechanisms responsible for increasing the invasiveness of melanocytes. Reports about CEACAM1 immunoexpression are scarce in the literature, but due to its promising potential for assessment, diagnosis, and targeted treatment, we consider that further investigation is warranted to advance current knowledge.

Abbreviation

anti-CTLA4:	Anti-cytotoxic T-lymphocyte-associated antigen 4
anti-PD1:	Anti-programmed death-1 protein
anti-PDL1:	Programmed death ligand-1 protein
LDH:	Lactate dehydrogenase
CEA:	Carcinoembryonic antigen
VEGF:	Vascular endothelial growth factor
IFNγ:	Interferon gamma
UVB:	Ultraviolet B (shortwave) rays
SOX-2:	Sex determining region Y-box 2
AJCC:	American Joint Committee on Cancer
HMB45:	Human melanoma black 45
S100B:	S100 calcium-binding protein B
MHC:	Major histocompatibility complex.

Competing Interests

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

Authors' Contributions

All the authors had equal contributions to the paper.

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Review Article

From Normal Skin to Squamous Cell Carcinoma: A Quest for Novel Biomarkers

Vlad Voiculescu,[1] Bogdan Calenic,[2] Mihaela Ghita,[3] Mihai Lupu,[1] Ana Caruntu,[4] Liliana Moraru,[4] Suzana Voiculescu,[5] Alexandra Ion,[1] Maria Greabu,[2] Nikolay Ishkitiev,[6] and Constantin Caruntu[3,5]

[1]*Department of Dermatology and Allergology, Elias Emergency University Hospital, Bucharest, Romania*
[2]*Department of Biochemistry, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*
[3]*Dermatology Research Laboratory, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*
[4]*Department of Oral and Maxillofacial Surgery, "Carol Davila" Central Military Emergency Hospital, Bucharest, Romania*
[5]*Department of Physiology, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*
[6]*Department of Medical Chemistry and Biochemistry, Faculty of Medicine, Medical University, Sofia, Bulgaria*

Correspondence should be addressed to Bogdan Calenic; bcalenic@yahoo.co.uk

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Squamous cells carcinoma (SCC) is the second most frequent of the keratinocyte-derived malignancies after basal cell carcinoma and is associated with a significant psychosocial and economic burden for both the patient himself and society. Reported risk factors for the malignant transformation of keratinocytes and development of SCC include ultraviolet light exposure, followed by chronic scarring and inflammation, exposure to chemical compounds (arsenic, insecticides, and pesticides), and immune-suppression. Despite various available treatment methods and recent advances in noninvasive or minimal invasive diagnostic techniques, the risk recurrence and metastasis are far from being negligible, even in patients with negative histological margins and lymph nodes. Analyzing normal, dysplastic, and malignant keratinocyte proteome holds special promise for novel biomarker discovery in SCC that could be used in the future for early detection, risk assessment, tumor monitoring, and development of targeted therapeutic strategies.

1. Introduction

Squamous cell carcinoma (SCC) is responsible for 20% of skin malignancies [1, 2]. Although most SCCs are curable, it was shown that 14% of them metastasize and of these unfortunate patients, 40% will eventually die; therefore they are responsible for the majority of deaths caused by nonmelanoma skin cancer [3–5]. Annually there are 400.000–600.000 new cases of cutaneous SCC (cSCC) diagnosed all over the world, more frequently among fair-skinned people. In USA and China studies show that 700.000 new cases of cSCC are diagnosed every year [6]. The incidence of skin cancer is growing with 5% every year in Central Europe and with 4% in regions with low sunlight exposure, such as Finland [7]. The major factor which influences the occurrence of skin abnormal cells which further evolve into cSCC is UV radiation, especially recreational sun exposure, which is in particular responsible for the increasing incidence of skin cancer in young people [8]. This is the reason why cSCC usually appears on the face and neck, sites that are frequently exposed to sunlight [9]. Other risk factors are exposure to carcinogenic chemicals, chronic skin ulceration, and immunosuppressive medication [1, 10, 11]. Actinic keratosis (AK) is a lesion that precedes cSCC, although not all AK progress into cSCC, which is represented by abnormal intraepidermal keratinocytes [1]. When these abnormal cells pass beyond the basement membrane we face an invasive cSCC, which has a greater risk of metastasis [12, 13]. Histopathological examination is considered the gold standard of diagnosis for SCC and other skin tumors, but noninvasive and minimal invasive diagnostic techniques have

gained increased attention in the past years, as they do not imply performing a skin biopsy [14, 15]. Although there are efficient methods of treatment available for cSCC, none of them can assure a complete healing, thereby 8% of cSCCs recur and 5% metastasize within 5 years [1]. This is the reason why there is a high necessity of identifying molecules that can help evaluate the risk of recurrence and metastasis from early stage [16]. It was noted that the risk of metastasis and recurrence varies depending on localization, so cSCC localized on the lips or ears is correlated with a higher risk of invasion (10–25%); initial tumor size, >2 cm, has 15% chances of recurrence and 30% chances of metastasis; histological features are, for example, the speed of tumor growth, tumor depth > 4 cm, poor differentiation, and perineural invasion [1, 3, 17].

Considering the increasing incidence in cSCC and the risk of metastasis and recurrence, even in patients with negative histological margins and lymph nodes, it is necessary to identify circulating molecules that can help predict the prognosis/evolution of this pathology [18–24]. Proteomics represents a field of molecular biology which studies the protein expression of an organism/cell. It is very well documented that DNA has the necessary information to synthesize the whole set of proteins that a cell needs to survive. Modifications in signalling pathways induce changes in gene expression and the result is the alteration in protein levels, which can be objectified through proteomics [25, 26]. In order to understand this mechanism and the way it correlates with different pathologies, clinical proteomics studies the characteristics of a specific protein (quantity, variation in time, and interactions) obtained from various biological fluid or tissue specimens. This tool may be useful in the diagnosis, prognosis, and therapy monitoring in various malignancies [27–33].

2. Proteome of Normal and Inflammatory Keratinocytes

Generally all epithelia play a paramount protective role for their underlying tissue, being the first line of defense against many harmful exogenous agents and at the same time acting as a permeable barrier that prevents loss of body fluids. Epithelia are usually composed of several layers of cells, each with their own specific phenotypes, being characterized by different degrees of differentiation according to their placement within the thickness of the tissue [34]. Keratinocytes constitute the most representative population of the epidermis and the proliferating cell in nonmelanoma skin cancers.

Studying the proteins present in normal keratinocytes and the changes of their pattern that occur in inflammation or carcinogenesis may lead to identification of new therapeutic targets or new biomarkers valuable in early diagnosis and prognosis of skin cancer [35].

A recent *in vitro* study [36] has identified 50 proteins considered specific to keratinocytes, most of them directly related to keratinocyte physiology. Some of these proteins, such as alpha-2 macroglobulin-like protein-1, alpha-2 macroglobulin-like protein 2, and interferon regulatory factor 6 (IRF-6) are involved in keratinocyte proliferation and differentiation. Another category of keratinocyte proteins that have attracted attention are dermokine and calmodulin-like protein 5, which are keratinocyte differentiation markers, and integrin beta 4, which plays a role in keratinocyte motility.

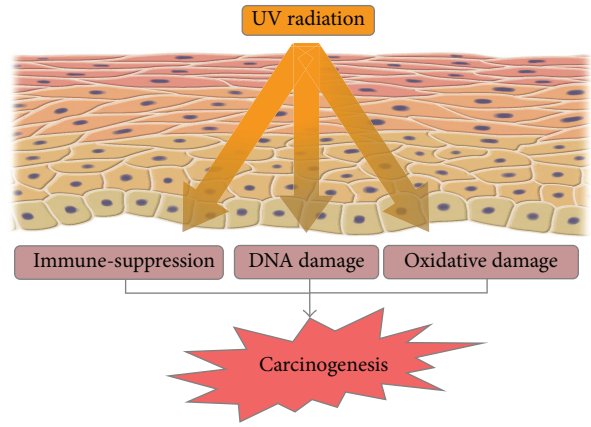

Figure 1: UV-induced skin carcinogenesis. UV radiation alters the normal immune responses, induces DNA damage and oxidative stress, and may lead to development of skin cancer.

The same study revealed a change in keratinocyte pattern of proteins in inflammatory conditions induced by stimulation with IL-1 beta. Thus, the level of proteins with roles in keratinocyte differentiation, such as alpha-2 macroglobulin-like protein-1, and the level of proteins involved in motility of keratinocytes such as integrin beta 4 were reduced. On the other hand, the presence of proinflammatory cytokines, such as IL-1F9 and IL-18, was observed. Moreover, stimulation with IL-1 beta increased the level of proteins involved in nuclear factor kappa B (NF-κB) communication pathway, in angiogenesis, and of those with antiapoptotic effect. Similar changes were found in epidermoid carcinoma cells suggesting an important role of inflammation in skin carcinogenesis [36].

3. Effects of Carcinogens on Keratinocyte Proteome

3.1. Ultraviolet Exposure Effects on Keratinocyte Proteome. UV exposure is one of the most important risk factors of skin cancer. Several studies using proteomic approaches have highlighted the alterations of protein expression induced by UV radiation on skin cells. UV exposure of skin induces suppression of cell-mediated immune responses, DNA damage, and formation of reactive oxygen species which can lead to oxidative stress and cellular damage (see Figure 1) [37, 38]. The first event after exposure to high doses of UV radiation is induction of keratinocyte apoptosis mediated by the p53/p21/bax/bcl-2 pathway and impairment of protein production, followed by hyperproliferation which may lead to subsequent epidermal hyperplasia [39, 40].

Chronic exposure to low doses of UV radiation also impacts the skin pattern of proteins by activation of different

cellular signalling pathways, such as the mitogen-activated protein kinases (MAPK) pathway, the phosphoinositide 3-kinase (PI-3K) pathway, and the nuclear factor NF-κB pathway, involved in modulation of cell growth, differentiation, proliferation, and motility. UV exposure increases expression of several matrix metalloproteinases (MMPs), such as MMP-1, MMP-3, and MMP-9, and the keratinocyte content of keratins 6, 16, and 17. On the other hand, it reduces type I collagen synthesis and impairs the transforming growth factor (TGF) beta communication pathway [38, 41–43]. Some of these changes may be associated not only with abnormal skin conditions, skin inflammation, but also with photoaging and skin carcinogenesis and the main challenge of future proteomic studies will be to identify a panel of biomarkers which allows differentiation between these various skin conditions.

3.2. Keratinocyte Proteomics in Chemically Induced Carcinogenesis. Exposure to carcinogenic chemicals is another factor that increases the risk of developing SCC. One of the main environmental factors with a strong link to skin carcinogenesis is arsenic [44]. Proteomic analysis of *in vitro* arsenic exposure of human keratinocytes showed a modified pattern of proteins with increased expression of several proteins such as heterogeneous nuclear ribonucleoprotein L isoform A, keratin 7, and keratin 9 [45] that can be associated with the development of premalignant lesions, or even SCC [46, 47]. On the other hand, in keratinocytes exposed to arsenic expression of involucrin was decreased, a similar pattern being previously highlighted in human cSCC [48].

Mouse models are the most commonly used animal models for the study of skin cancer, because in many aspects they mirror the mechanisms of human carcinogenesis [35].

However, there are numerous differences between distinct strains and different experimental models and there is hope that proteomic techniques will allow highlighting of the intimate mechanisms underlying these differences. Proteomic analysis in animals C57BL/6-resistant and DBA/2 sensitive, following 12-O-tetradecanoylphorbol-13-acetate (TPA) administration, demonstrated 19 different expressed proteins, such as S100 calcium binding proteins A8, A9, and A11 as well as parvalbumin α and annexin A1 [49]. After topical application of carcinogenic promoters chrysarobin and okadaic acid S100 proteins A8 and A9 were also elevated. Further research identified the majority of these proteins to be related to inflammation and more specifically to inflammatory networks that regulate and promote tumoral growth in skin such as TNFα and nuclear factor (NF)κB. Moreover, after TPA exposure DBA/2 mice but not C57BL/6 mice express mRNAs for a wide array of inflammatory proteins, such as TNF, Nfκb1, IL-22, and IL-1b, and chemokines such as Cxcl1, Cxcl2, and Cxcl5. Taken together, these results suggest that chemically induced carcinogenesis in murine models may be sustained by inflammatory genes activity [49]. Other proteins involved in skin carcinogenesis are cell surface markers such as tetraspanins, found on virtually all cell types [50]. CD markers are known to be expressed in several types of cancer; of these, CD151 has been shown to induce skin chemical carcinogenesis and to promote a fast development of SCC in mouse models. These results also match the results found in human SCC. CD151 is most often associated with activation of the transcription factor, signal transducer, and activator of transcription 3 (STAT3). The data suggests that CD151 may be used as a future antitumoral therapeutic target [51]. Other studies demonstrate that, in a murine carcinogenic model, DMBA-induced carcinogenesis in PKCα knockout mice tumor formation is suppressed but not tumor growth and progression [52]. Proteomics is a rapidly developing field that brings vital inputs in identifying and quantifying the proteome responsible for initiation and development of the carcinogenic process in skin [53].

4. Proteic and Other Potential Biomarkers of Squamous Cell Carcinoma

4.1. Cutaneous SCC

4.1.1. Inflammatory Markers. Inflammation is involved in many types of pathologies, from AK and Bowen's disease (BD) to cutaneous SCC (cSCC) and other kind of cancers; thus the involvement of inflammatory markers, such as the complement factor H (CFH) and FHL-1 (factor H-like protein-1) in the development of cSCC has attracted an increasing interest [60]. CFH is a soluble molecule that has a role in inhibiting one of the three pathways which activates the complement C3, the alternate pathway (which is continuously activated *in vivo*), and it also represents a cofactor for complement factor 1 in the inactivation process of C3b to iC3b [61, 62, 97]. CFH exists as two molecules with largely the same functions: CFH (150 kDa) and factor H-like protein-1 (45 kDa) [98, 99]. Studies show that as the cell progresses from AK to cSCC it has a higher rate of expression of CFH, FHL-1, and complement factor 1 in cSCC cells [60]. Also CFH facilitates proliferation and migration of cSCC cells; thus it is associated with negative prognosis in patients with CFH overexpression. In the study conducted by Riihilä et al. [60], CFH and FHL-1 expressions were analyzed *in vivo* through qPCR of RNA, 6 samples from cSCC lesions, and 11 samples from normal skin, concluding that in cSCC lesions the expression of these molecules was significantly higher than in normal skin [60]. Tissue samples from AK, BD, and cSCC were analyzed through immunohistochemistry showing that the expression of these inhibitors increases with the progression of the lesion but it is present even in early stages, which makes detection of CFH and FHL-1 very useful [18]. The study of Riihilä et al. showed that cSCC cells express C3 more than normal keratinocytes, which may be the reason why the inflammatory reaction is important in cSCC, C3 being the main component which activates all three pathways of complement cascade. It was also noted that inflammatory cytokines like IFN-γ, IL-1β, and TNF-α increased the expression of CFH by cSCC cells. In cultures a significant quantity of iC3 was present, reflecting that cSCC cells produce active CFH that helps this cell population escape the complement mediated cell destruction, having a very important role in cSCC progression. It was also demonstrated that complement factor I degrades C3b into

smaller molecules which facilitates CFH and FHL-1 activity [60, 100].

Serpin A1 or 1-antitrypsin is included in the serine peptidase inhibitors (Serpins) family which has a very large distribution in the human body and has various functions (coagulation, inflammation, and turnover of extracellular matrix). Serpins are divided into two groups: A which includes extracellular molecules and B formed by intracellular molecules [63, 64]. The value of Serpin A1 from cSCC samples was compared with the value from normal keratinocytes and it was noted that cSCC cells had a greater concentration and this result was correlated with the invasiveness and could be used for prognosis prediction. In addition, samples from AK were examined and it was observed that Serpin A1 was not as well expressed as in cSCC cells; this result pleads for the importance of Serpin A1 in detecting cSCC progression. It is well known that cSCC is accompanied by inflammation and studies show that inflammatory cytokines have tumor protective functions, theory supported by the fact that it was noted that the value of Serpin A1 is increased by TNF-α, IFN-γ, and IL-1β [113]. It has been demonstrated that Serpin A1 inhibits natural killer cell activity, stimulates malignant cell proliferation but not normal skin cell proliferation, and has an antiapoptotic effect (lung endothelial cells); therefore Serpin A1 has tumorigenic activity [101–103].

4.1.2. Early Markers of Skin Carcinogenesis. A factor which may promote tumor genesis is represented by the mutations in tumor suppressor gene. APC gene is such an example; mutations occurring in this gene conduct to the synthesis of a short nonfunctional APC protein. This gene was identified in patients with familial adenomatous polyposis (FAP) as well as in patients with sporadic colorectal carcinomas [104–106]. Loss of heterozygosity (LOH) is a molecular instrument which identifies loss of an allele, by comparing the same region on a chromosome from normal DNA (heterozygote) with one from tumor DNA. LOH of APC gene was identified in many types of cancer including oSCC [107]. Studies have shown that APC protein induces the destruction of β-catenin (which is the factor that activates the transcription of oncogenes as Myc and Cyclin D1) and plays a role in microtubule assembly (see Figure 2). It was observed that in normal epidermis APC localization was only cytoplasmic while in SCC samples (tumoral cells and normal surrounding tissue) APC staining was negative for cytoplasmic localization but the nuclear staining was positive, which can help conclude that APC protein is present in the nucleus of proliferating cells [65]. The fact that APC nuclear staining was found in apparently normal cells surrounding SCC may be proof that this tissue was exposed to genetic changes that modified the APC expression, but in order to assert that this result is not normal, skin samples from patients with SCC in non-sun-exposed sites and from age-matched individuals without skin cancer should be examined [108].

The development of cSCC is influenced by many other modifications induced by UV radiation such as the presence of melanocortin-1 receptor (associated with fair skin and red hair) which represents a risk factor for developing cSCC as well as melanoma [109], increased telomerase activity which may protect cSCC from apoptosis [110], and mutations of NOTCH genes, which are tumor suppressor genes identified in 75% of patients diagnosed with cSCC [56].

Studies of molecular markers reflecting initial changes in skin carcinogenesis showed that, in sun-exposed skin, in which AK or SCC develops, the main molecular mutation is of gene p53. Considering the fact that this mutation is found in AK as well as in SCC represents the proof that this alteration is produced early in the development of cancer (AK is considered a precursor of SCC) [111, 112]. The fact that this gene is inactivated creates the perfect conditions for simple and numerous mutations to appear; this is the reason why cSCC is considered to have the highest mutation rate.

4.1.3. Markers of Tumor Progression and Aggressiveness. Using reverse phase protein microarray (RPMA) samples from normal skin, AK, nonadvanced SCC, and advanced SCC were analyzed in order to identify which pathways were activated in the progression of SCC. The study showed that UV radiation activates numerous signal transduction pathways, such as p38, MAPK, and PI3K-AKT. These alterations may further influence apoptosis, proliferation, inflammation, and differentiation which may result in SCC development. It was demonstrated that in samples of skin from SCC and AK the percentage of phosphorylated AKT was significantly higher than in normal skin and in skin samples from metastatic SCC this protein value was the highest. The same results were obtained for mTOR (Ser2448), 4EBP1 (Ser65), 70S6K1 (Thr421), p70S6K1 (Thr421/Ser424), and S6 (Ser6) [55].

It appears that the inhibition of squamous cell differentiation is the most important mechanism that increases the invasiveness of cSCC; thus identifying molecules that can counteract this mechanism may help instate a more efficient treatment [63, 113]. S100 represents a family of calcium modulated proteins which include S100A7 (psoriasin) which was identified in keratinocytes harvested from psoriatic skin. It was noted that high concentration of S100A7 was found in various types of SCC (lung, oral cavity, bladder, and skin) which may indicate that this protein is a common biomarker for SCC [114] and it seems that this protein has an important role in metastasis [66–70]. It is believed that S100A7 may be involved in cell differentiation considering the fact that the more differentiated the cell population is, the higher the expression of S100A7 is. Also the gene that encodes the information necessary for the synthesis of this protein is located in chromosome 1q21 which contains the epidermal differentiation complex [115]. Studies have shown that S100A7 expression *in vivo* and *in vitro* can be influenced by induction and proliferation therefore S100A7 + cells switch to S100A7 − when the inducer is removed. It was noted that overexpression of S100A7 increased cell proliferation, survival rate, and tumor growth and cell differentiation was decreased, but when S100A7 expression was low cell differentiation markers increased while proliferation was inhibited [114].

The link between aggressive SCC and type VII collagen (Col7) is debated considering the fact that mortality is high (more than 78%) in patients with severe generalized recessive dystrophic epidermolysis bullosa (RDEB) from

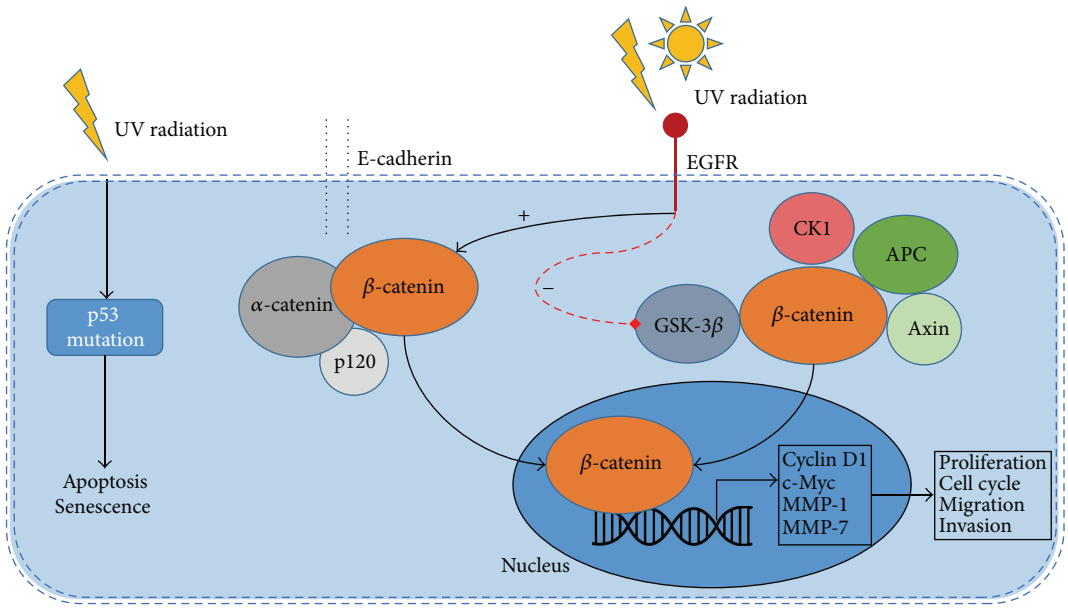

Figure 2: Dysregulation of cellular signalling in SCC. Aberrant activation of EGFR induces phosphorylation of β-catenin and GSK-3β, leading to uncoupling of β-catenin from both destruction complex (β-catenin/GSK-3β/APC/CK1/Axin) and E-cadherin/p120/α-catenin complex and translocation to the nucleus. Once translocated to the nucleus it influences gene transcription, including Cyclin D1, c-Myc, MMP-1, and MMP-7 (viable biomarkers for SCC) which have important roles in proliferation, cell cycle, migration, and invasion. The figure also shows one of the first events in SCC carcinogenesis, namely, the induction of tumor suppressor p53 mutations. *EGFR: epidermal growth factor receptor; GSK-3β: glycogen synthase kinase 3 beta; APC: adenomatous polyposis coli; CK1: casein kinase 1; MMP-1: matrix metalloproteinase 1; MMP-7: matrix metalloproteinase 7.

metastatic squamous cell carcinoma. Mutations occurring in COL7AI, the gene which encodes the information for Col7 synthesis, cause RDEB. This disease is characterized by skin and mucosal fragility due to a decrease in Col7 formation (the main component of anchoring fibrils) which leads to blister formation and chronic skin traumatisms (risk factor for SCC) [71]. Considering the fact that patients with dominant dystrophic epidermolysis bullosa (1 normal COL7A1 allele which means 50% normal Col 7 formation) develop SCC less than RDEB patients [71], scientists are trying to increase Col7 formation through divers methods (gene, protein, and cell therapy), but it was observed that high levels of Col7 are correlated with an important activation of the phosphoinositide 3-kinase pathway that leads to an increased invasion in SCC keratinocytes; therefore this kind of therapies should be applied with caution [116]. Matrix metalloproteinases are molecules implicated in maintaining homeostasis of many tissues including skin, by proteolysis of extracellular matrix. It was noted that MMP-7 has an increased concentration in cSCC (see Figure 2) but in cSCC that develops in patients with RDEB it has an even higher value which pleads for the aggressiveness of cSCC in this kind of patients [72–74].

It was discovered that SCC in mice is determined by Pam212, a keratinocyte cell population which does not have the ability of metastasizing, although cells that drift from Pam212 (LY lines) were found in lymph nodes metastases [117, 118]. Studies show that only LY lines can express keratin 8 (Krt8) and keratin 18 (Krt18) which are found in nonstratified epithelia but not in keratinocytes [119]. Cells from non-metastatic and metastatic transformed keratinocytes were analyzed and it was discovered that Krt8 and Krt18 were linked together forming filaments and they were also in high concentration in metastatic cells [75]. The high invasiveness and potential for metastasis were demonstrated *in vitro* and they seem to be very strong in the population of cells that express both Krt8 and Krt18. This idea is supported by a study which concluded that a cell population which highly expressed Krt18 became metastatic only after overexpressing exogenous Krt8 [120]. Other studies show that coordinated coexpression of these two keratins reduce the metastatic potential of the tumor cells [119, 121].

Tyrosine kinase receptor family contains a larger group of receptors named erythropoietin-producing hepatocellular (Eph) receptors divided into two smaller subclasses A and B. The molecule that serves as ligand is ephrins [122, 123]. It was demonstrated that EphA subfamily is tumor suppressors, considering the fact that low concentration of EphA1 was identified in nonmelanoma skin cancer and low levels of EphA2 favour the development of chemically induced skin cancer in mice [124]. In cSCC, EphB2 determines proliferation, migration, and invasion, thus becoming the object of possible targeted therapies [125]. Farshchian et al. [125] identified high levels of EphB2 in primary and metastatic cSCC cells through microarray, qRT-PCR, and next-generation sequencing. These receptors were found on the surface of cSCC cells (clustered and bound with their ligands) as well as in the cytoplasm [125, 126]. Cells from

Table 1: Available treatment options for skin SCC [54].

Nr. crt.	Type of cSCC	Therapy	Adjuvant
(1)	Low risk cSCC	Electrodessication Curettage	
(2)	Invasive cSCC	Surgical excision Mohs micrographic surgery	Radiation therapy provides good locoregional control and can also be used as primary therapy for lesions that cannot be surgically excised EGFR inhibitors
(3)	Metastatic cSCC		Chemotherapy
(4)	Prevention	Decreased UVR exposure Correct and early treatment for precancerous skin lesions	

normal cSCC, normal skin, and premalignant lesions were analyzed and it was shown that the concentration of EphB2 rises as normal cells progress to cancerous cells, which highlights that EphB2 overexpression is a process that starts early in cSCC development and has an important role in its invasiveness. To support this statement Farshchian et al. [125] showed that lowering EphB2 expression determined the inhibition of proliferation and migration of cSCC cells. These results identified EphB2 as a biomarker for cSCC progression and a potential therapeutic target [125].

4.1.4. Cancer Stem Cells Biomarkers. Cancer stem cells (CSC) represent a population of cells with the unique characteristic of being solely responsible for initiating and maintaining tumor growth [127, 128] (Tables 1, 2, and 3). Therefore, it is very important to identify any kind of biomarker related to CSC, which may provide vital information such as risk of metastasis, resistance to therapy, and recurrence. There are studies [96, 129] that investigated the role of CD133 (CSC biomarker), a transmembrane glycoprotein present in normal hematopoietic stem cells responsible for proliferation and differentiation in various types of cancers, including skin cancer, demonstrating that overexpression of this protein is correlated with poor prognosis [46, 76–81]. Samples of cSCC tissue were analyzed and it was observed that increased expression of CD133 was correlated with low differentiation and advanced tumor stage. Studies have shown that CSC CD133 + are resistant to apoptosis induced either by transforming growth factor β or by tumor necrosis factor and the self-renewal capacity of this cells is lost once CD133 is lost [130]. New treatment strategies that target CD133 would be useful for patients with high expression of this protein, who are at risk of developing cSCC with poor prognosis [131].

4.1.5. Molecular Therapeutic Targets. In the last decades cancer therapy studies have focused on targeted molecular treatments (monoclonal antibody, small molecule tyrosine kinase inhibitor); therefore scientists have developed a great interest for EGFR (epidermal growth factor receptor) which is a tyrosine kinase receptor and two of its most important ligands are epidermal growth factor and transforming growth factor-α and its roles are skin cell proliferation and differentiation, thus contributing to tumorigenesis [57–59]. Studies show that EGFR has high values in many types of cancer (oropharynx,

Table 2: Molecular pathways governing epidermal stem cells homeostasis and tumorigenesis.

Nr crt	Molecular pathway	Roles
(1)	p63	Proliferation, self-renewal, development, morphogenesis, tumorigenesis
(2)	SRF/MAL	Differentiation, development, cytoskeletal regulation
(3)	mTOR	Senescence, cell size, tumorigenesis oxidative stress [55]
(4)	p75	Apoptosis, communication, differentiation
(5)	Hippo	Organ size, antiproliferative, apoptosis maintenance, antitumorigenic
(6)	Notch	Differentiation, morphogenesis, suprabasal switch [56]
(7)	FOXM1	Proliferation, genome instability, tumorigenesis
(8)	p38 MAPK	Proliferation, wound healing, differentiation, cell migration, invasivity, tumorigenesis [55]
(9)	BMP	Proliferation, differentiation, plasticity, wound healing
(10)	TGFβ	Proliferation, immortalization, tumorigenesis [38, 41–44]
(11)	TGFα	Proliferation, hyperplasia, immortalization, tumorigenesis
(12)	EGFR	Proliferation, maintenance, tumorigenesis [57–59]
(13)	c-myc	Proliferation, differentiation, tumorigenesis
(14)	Shh	Development, morphogenesis, proliferation cell survival
(15)	Wnt	Proliferation, self-renewal, wound healing, morphogenesis, tumorigenesis

Table 3: cSCC biomarkers.

Nr. crt.	Biomarker	Roles
(1)	CFH FHL-1 Complement factor 1	(i) Inhibiting one of the three pathways that activate the complement C3 (ii) Facilitating progression and migration of cSCC cells [60–62]
(2)	Serpin A1	(i) Coagulation (ii) Inflammation (iii) Turnover of extracellular matrix (iv) Inhibiting natural killer cell activity (v) Stimulating malignant cell proliferation but not normal skin cell proliferation (vi) Antiapoptotic effect [63, 64]
(3)	APC	(i) Inducing the destruction of β-catenin (ii) Having a role in microtubule assembly [65]
(4)	Phosphorylated AKT, mTOR (Ser2448), 4EBP1 (Ser65), 70S6K1 (Thr421), p70S6K1 (Thr421/Ser424), S6 (Ser6)	(i) Influencing apoptosis, proliferation, inflammation, and differentiation [55]
(5)	S100A7	(i) Role in metastasis [66–70]
(6)	Col7A1	(i) Encoding the information for Col7 formation [71]
(7)	MMP-7	(i) Maintaining homeostasis of many tissues including skin, by proteolysis of extracellular matrix [72–74]
(8)	Krt8 Krt18	(i) Together they induce a higher rate of invasiveness in a cell population [75]
(9)	CD133	(i) Proliferation (ii) Differentiation [46, 76–81]
(10)	CYFRA 21-1	(i) Component of structural proteins involved in epithelial intermediary filaments formation [82–88]
(11)	mtDNA	(i) Mitochondrial functions [89, 90]
(12)	Hsp70	(i) it is presumed that it may help tumorous cells survive apoptosis and necrosis [91]
(13)	Plectin	(i) Cytolinker of plakins family which forms the links between filaments [92]
(14)	*Cofilin-I*	(i) Vulvar carcinogenesis (ii) Tumor progression [93]
(15)	*Galectin-7* *wee1*	(i) Invasiveness (ii) Poor tumor differentiation [94, 95]
(16)	EphB2	(i) Determining proliferation, migration, and invasion [96]

oesophagus, stomach, colorectal, pancreas, non-small cell carcinoma of the lung, and SCC) [132]. The mechanism explaining why EGFR has high expression in HNSCC is not completely elucidated, although several hypotheses including mutations in the receptor, high ligand levels, and increased mRNA transcription have been proposed (see Figure 2) [59]. However, the fact that monotherapy with EGFR inhibitors was not as successful as expected makes researchers believe that EGFR might not be the main component in the oncogenic process [133–135]. Studies show that only 47% of metastatic disease in cSCC overexpress EGFR which leads to the hypothesis that the metastatic cell population that does not overexpress EGFR may originate from another clone, hypothesis supported by the fact that a study on the use of EGFR inhibitor (gefitinib) in patients with metastatic cSCC showed that the therapy had no results [136].

4.2. Oral SCC. The incidence of *oral SCC* has a wide variability worldwide depending on food and lifestyle habits (alcohol and cigarette). In order to diagnose in an early stage this type of cancer, scientists tried to find new biomarkers that could provide the opportunity of predicting the prognosis. Therefore they studied cytokeratin 19, which is one of the 20 cytokeratin polypeptides discovered (they are structural proteins involved in epithelial intermediary filaments formation). Cytokeratin 19 is expressed by normal cells as well as by some cancerous cells like lung cancer cells [82, 137]. CYFRA 21-1 is the serum soluble component of this cytokeratin and

its high values was linked with high mortality in patients with lung cancer [138, 139]. Extrapolating, scientists observed that patients with HNSCC and high concentrations of CYFRA 21-1 had a poorer prognosis because it is considered that this molecule is released in the blood stream by metastatic tumor cells [82–88].

Another molecule that can help identify patients with a high mortality risk is CRP (inflammation marker), the low survival rate and cancer invasiveness being demonstrated for oSCC (inflammation provides the circumstances for proliferation and angiogenesis); also studies showed that elevated CRP was correlated with bone, skin, and lymph node invasion [140, 141]. Scientists tried to link these two biomarkers (CRP and CYFRA 21-1) in order to see if a prediction for poorer prognosis could be made before surgery and found that patients with increased concentrations of both of them were at a higher risk of developing distant metastases. As established above, tumor cells release CYFRA 21-1 into the blood stream; there they activate inflammatory cells that release inflammatory cytokines which in the end increases the CRP serum value [142].

In oral squamous cell carcinoma, measuring mitochondrial DNA (mtDNA) may be useful for postoperative monitoring considering the fact that an important number of patients with head and neck SCC (HNSCC) that had histological negative margins had mtDNA mutations [89, 90]. It is necessary to determine this molecule quantitatively, because it was demonstrated that even though there were no identifiable metastasis, high mtDNA values were detected in the organs and blood of mice injected with Sa3 cells (cutaneous squamous cell carcinoma cells). Uzawa et al. analyzed postoperative blood samples from 61 patients, and of those 16 had high mtDNA mutations which were correlated either with a local recurrence or with distant metastasis within the next 9 months after surgical treatment [143]. It is really important to note that mutations in mtDNA are identified only in tumoral tissue, and depending on the intensity it can be used as a prognostic predictor for patients with oSCC. Although low mutant mtDNA detection could not be correlated with the fact that the more the phenotype is differentiated, the better the prognosis is, this biomarker has a great potential of becoming a criteria of including a patient in high/low risk group even though histologically they are tumor-free [144].

4.3. Genital SCC. *Penile cancer* is a rare condition and its incidence was linked to lack of circumcision and hygiene, phimosis, HPV infection, and tobacco use [144–147]. Viral infection is a very important risk factor; HPV DNA incorporates itself in the human genome and induces an important expression of viral genes E6 and E7 which inactivates tumor suppressor genes. This type of cancer is another example of the necessity of reliable biomarker that can predict the prognosis, considering the fact that for the moment inguinal metastases are the most important prognostic factor [148]. Recently a study was conducted on 20 patients divided into 2 groups: group 1 composed of patients diagnosed with HPV and PSCC (penile squamous cell carcinoma) and group 2 (control group) containing samples of foreskins from patients with HPV and without tumors [148]. After analyzing the samples from the two groups the results showed that in group 1 the concentration of Hsp70 was very high and considering the fact that this protein was also found in high concentration in other types of cancer, it is presumed that it may help tumorous cells survive apoptosis and necrosis. This protective role was demonstrated by the fact that if/when an adenovirus expresses anti-Hsp70 it leads to an important tumor cell death in breast, colon, prostate, and liver cancer [91]. In group 1 component C3 of complement was not detected and a theory that may explain why this result occurred is the fact that viral proteins have the ability of counteracting the immune response; therefore viral infection has a protective role over the tumor cells providing them an environment favourable for their development [149]. Other molecules studied by [150] are plakins, which represent a family of molecules which form the links between filaments, desmosomes, and hemidesmosomes and plectin is a cytolinker of this family. Studies showed that defective expression of plectin induces genomic instability which creates favourable circumstances for cancer development and progression [92].

Vulvar SCC accounts for more than 90% of the malignant tumors with this localization [151]. Emerging evidence suggests the existence of two separate entities regarding the development of epidemiological, pathological, and clinical characteristics of vulvar SCC, namely, one associated with human papilloma virus infection (HPV) and a second independent of HPV. In trend with recent efforts for surrogate biomarker discovery in cancers [30–33, 35, 54, 152–155], research of vulvar SCC has demonstrated the importance of detecting differentially expressed proteins for early diagnosis and timely therapeutic intervention. In this regard, numerous studies indicate that *p16 expression* indicate a less aggressive variant of vulvar SCC, less likely to recur and with no related deaths. By contrast, patients with *p53 expression* had a poor prognosis and significantly increased local recurrence and disease-specific mortality [156]. Other molecular markers with negative impact in patients with vulvar SCC include *cofilin-I*, *galectin-7, and wee1*. *Cofilin-I* expression was found to be significantly increased in vulvar SCC compared with normal tissue and was suggested to be involved in vulvar carcinogenesis and subsequent tumor progression [93]. Downregulation of *galectin-7* and high *wee1* expressions was found to correlate with advanced clinical stage, poor tumor differentiation, and regional lymph node metastasis [94, 95]. Moreover, a gradual reduction into disappearance of estrogen-related receptor-α expression was observed from healthy vulva to precursor lesions and further to SCC [157]. Among these, *cofilin-I* was proposed as a potential target alone for therapeutic intervention as *cofilin-I* silencing by siRNA significantly reduced cell progression in vulvae SCC [93].

5. Conclusions

Considering the significant risk of recurrence and metastasis of SCC, there is a high necessity to discover novel molecules harvested from various biological samples that could explain the occurrence and evolution of this keratinocyte-derived

tumor. In this regard, protein-focused research based on high-throughput proteomic technologies has evolved rapidly to identify unique biosignature of skin cancer.

Analyzing differences between normal, inflammatory, and malignant keratinocyte proteome holds special promise for novel biomarker discovery in SCC that could be used in the future for early detection, risk assessment, and tumor monitoring. Furthermore, identification of novel potential biomarkers for SCC development and progression will aid the discovery of individualized targeted therapies for these patients.

Competing Interests

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

Authors' Contributions

All authors have equally contributed to the conception and preparation of the manuscript.

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Review Article

Gene Expression and Proteome Analysis as Sources of Biomarkers in Basal Cell Carcinoma

Mihai Lupu,[1] Constantin Caruntu,[2,3] Mihaela Adriana Ghita,[4] Vlad Voiculescu,[1] Suzana Voiculescu,[2] Adrian E. Rosca,[2] Ana Caruntu,[5] Liliana Moraru,[5] Iris Maria Popa,[6] Bogdan Calenic,[7] Maria Greabu,[7] and Daniela Elena Costea[8,9]

[1]*Department of Dermatology and Allergology, Elias Emergency University Hospital, 011461 Bucharest, Romania*
[2]*Department of Physiology, "Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania*
[3]*Department of Dermatology, "Prof. N. C. Paulescu" National Institute of Diabetes, Nutrition and Metabolic Diseases, 020475 Bucharest, Romania*
[4]*Dermatology Research Laboratory, "Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania*
[5]*Department of Oral and Maxillofacial Surgery, "Carol Davila" Central Military Emergency Hospital, 010825 Bucharest, Romania*
[6]*Department of Plastic and Reconstructive Surgery, "Bagdasar Arseni" Clinical Emergency Hospital, 041915 Bucharest, Romania*
[7]*Department of Biochemistry, Faculty of Dental Medicine, University of Medicine and Pharmacy "Carol Davila", 050474 Bucharest, Romania*
[8]*Gade Laboratory for Pathology and Centre for Cancer Biomarkers (CCBio), Department of Clinical Medicine, University of Bergen, 5021 Bergen, Norway*
[9]*Department of Pathology, Haukeland University Hospital, 5021 Bergen, Norway*

Correspondence should be addressed to Constantin Caruntu; costin.caruntu@gmail.com

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Basal cell carcinoma (BCC) is the world's leading skin cancer in terms of frequency at the moment and its incidence continues to rise each year, leading to profound negative psychosocial and economic consequences. UV exposure is the most important environmental factor in the development of BCC in genetically predisposed individuals, this being reflected by the anatomical distribution of lesions mainly on sun-exposed skin areas. Early diagnosis and prompt management are of crucial importance in order to prevent local tissue destruction and subsequent disfigurement. Although various noninvasive or minimal invasive techniques have demonstrated their utility in increasing diagnostic accuracy of BCC and progress has been made in its treatment options, recurrent, aggressive, and metastatic variants of BCC still pose significant challenge for the healthcare system. Analysis of gene expression and proteomic profiling of tumor cells and of tumoral microenvironment in various tissues strongly suggests that certain molecules involved in skin cancer pathogenic pathways might represent novel predictive and prognostic biomarkers in BCC.

1. Introduction

Basal cell carcinoma (BCC) is the most common skin cancer worldwide and its incidence is still rising with almost 10% each year worldwide [1, 2], thus representing a growing public health problem associated with negative psychosocial and economic consequences [3, 4].

These tumors that develop *de novo* have relatively uniform histology, and while not lethal they are locally invasive causing disfigurement and increasing morbidity due to frequent facial localization. Early diagnosis and prompt management are of crucial importance in order to prevent local tissue destruction or the occurrence of advanced disease. Although histopathological examination is considered the gold standard of diagnosis for BCC and other skin tumors, noninvasive and minimal invasive diagnostic tools have gained increased attention, as they do not imply performing a skin biopsy [5]. Among these novel optical imaging

techniques, dermoscopy and reflectance confocal microscopy allow a rapid, *in vivo*, noninvasive micromorphological evaluation of skin tumors and combining these techniques can increase the diagnostic accuracy in different subtypes of BCC [6–8].

Known risk factors for developing BCCs include Fitzpatrick type I phototype, freckling and sunburns in childhood, family history of skin cancer, iatrogenic immunosuppression, and internal or external exposure to carcinogenic chemicals, especially arsenic [9] and high cumulative exposure to UV light [10]. Among these, UV radiation is considered by most as the main carcinogen [11] and around 80% of BCCs occur on sun-exposed areas, mostly the head and neck.

Regional anatomical differences, such as type and density of hair follicles, could explain why the back of the hands, despite extensive sun exposure, is a rare location for these tumors.

Carcinogenic processes [12, 13] are likely the result of multifarious interactions between host genome and environmental factors. Because of its morphological similarities to the undifferentiated epidermal basal cells, BCC provides an excellent model for identifying differentially expressed genes when compared to normal cells [14]. Genomic and proteomic techniques have both the potential to deliver new biomarkers [15]. However, gene transcript levels do not always correlate with protein expression due to transcriptional/translational control and high-throughput proteomic technologies are preferred for the measurement of proteins associated with pathological states [16]. Moreover, in early stages of the neoplastic process, individual proteomic platforms or platform combinations are used to characterize the great number of intact and cleaved proteins that can separate patients from healthy subjects [17]. Further, novel soluble and/or tissue-specific biomarkers can be developed for diagnosis, prognosis, and therapy monitoring in various malignancies [18–20], including BCC and other nonmelanoma skin cancers [21].

2. Sporadic Basal Cell Carcinoma

Multiple signaling pathways are altered in carcinogenesis [22], and changes that lead to sonic hedgehog (SHH) patched 1 signaling pathway dysregulation have been recognized as essential events for sporadic BCC development [23] (see Figure 1). Gene expression alteration is an important event in tumor cells and mutations in the patched 1 gene are frequently present in BCCs [14].

Gli1 (glioma 1) transcription factor is an important downstream effector in the SHH pathway and has regularly been found upregulated in basal cell carcinomas [23].

GlI2 (glioma 2) is another key protein in BCC carcinogenesis and is also required for normal hair follicle development in the embryonic stages [24]. It has been proved that overexpression of GlI2 in the basal keratinocytes of transgenic mice leads to the development of multiple BCC-like tumors [25]. In addition, GLI2 has been regularly found upregulated in cancers presenting complex genomic alterations [26].

FOXM1, a Forkhead box protein, is a downstream molecule of SHH/Gli1 that has been found to be overexpressed in BCC and because of its role in cell proliferation it is thought to be one of the causes for aberrant SHH signaling in BCC tumorigenesis [14].

Lam et al. found another Forkhead box protein, FOXO3A, to be overexpressed in BCCs. FOXO3A, a transcription factor known for its involvement in cell-cycle arrest mediation, apoptosis, and DNA repair [27], also plays a key role in oxidative stress protection through upregulation of several antioxidants, such as SOD2 and catalase.

In a 2008 study Asplund et al. compared gene expressions of normal epidermal basal cells to those of BCC cells. The results revealed 201 upregulated and 160 downregulated genes in BCC cells compared to normal basal cells. Among them, they identified differentially expressed genes implicated in cell differentiation (aquaporin 3 and envoplakin), adhesion (claudin-1 and CD44), communication (desmoglein 2), and immune response (CD40 and MHC class II proteins). Consistent differences in immunoreactivity between tumor and control cells were found in half of gene products: claudin-1, cystatin A, CD44, calgranulin A (S100A8), prostaglandin-endoperoxide synthase 1 (COX-1), junctional adhesion molecule 3 (JAM3), envoplakin, and c-myc. Several members of the Wnt pathway were also found to be upregulated: Wnt receptor frizzled 8, β-catenin, Lef-1, and basonuclin-2. Desmoglein 2, a desmosomal cadherin strongly expressed in the bulge area and basal layer of the outer root sheath [28] and weakly in interfollicular epidermis [29], was also found to be overexpressed in BCC tumor cells. Several proteins were downregulated, namely, CD40, MHC class II molecules, Fc fragment of IgG binding protein, and immunoglobulin superfamily member 1 [14].

Zali [30] found that C3b, which has important roles in opsonisation and activation of the alternate pathway [31], had elevated expression levels in BCC cells when compared to controls, while transthyretin and ceruloplasmin had lower expressions. These results are supported by a previous study that found ceruloplasmin significantly decreased in BCC patients compared to healthy controls [32], probably owing to prolonged exposure to ultraviolet radiation. As no expression for aldolase C, FGG (Fibrinogen gamma chain), Prx-cis (periaxin), prothrombin, VDAC (voltage-dependent anion channels), and LRG (leucine-rich alpha-glycoprotein) was found in tumor cells, they were considered negative markers for BCC. In addition, the author underlines the necessity for further studies of these biomarkers in mucous secretions and blood [30].

3. Recurrent and Subsequent Basal Cell Carcinomas

Although various risk factors for increased incidence of recurrent BCC exist [33], none of them should be considered individually. Clinical risk factors for BCC recurrence include male sex, lesion topography (centrofacial region involving the inner canthus, nostrils, and periauricular area), tumor size (recurrence rate increases by 7% for each millimeter of increase in tumor diameter), photosensitivity, and over 60 years of age at first presentation [34, 35]. Aggressive-growth morphologic variants such as sclerosing, mixed (nodular and

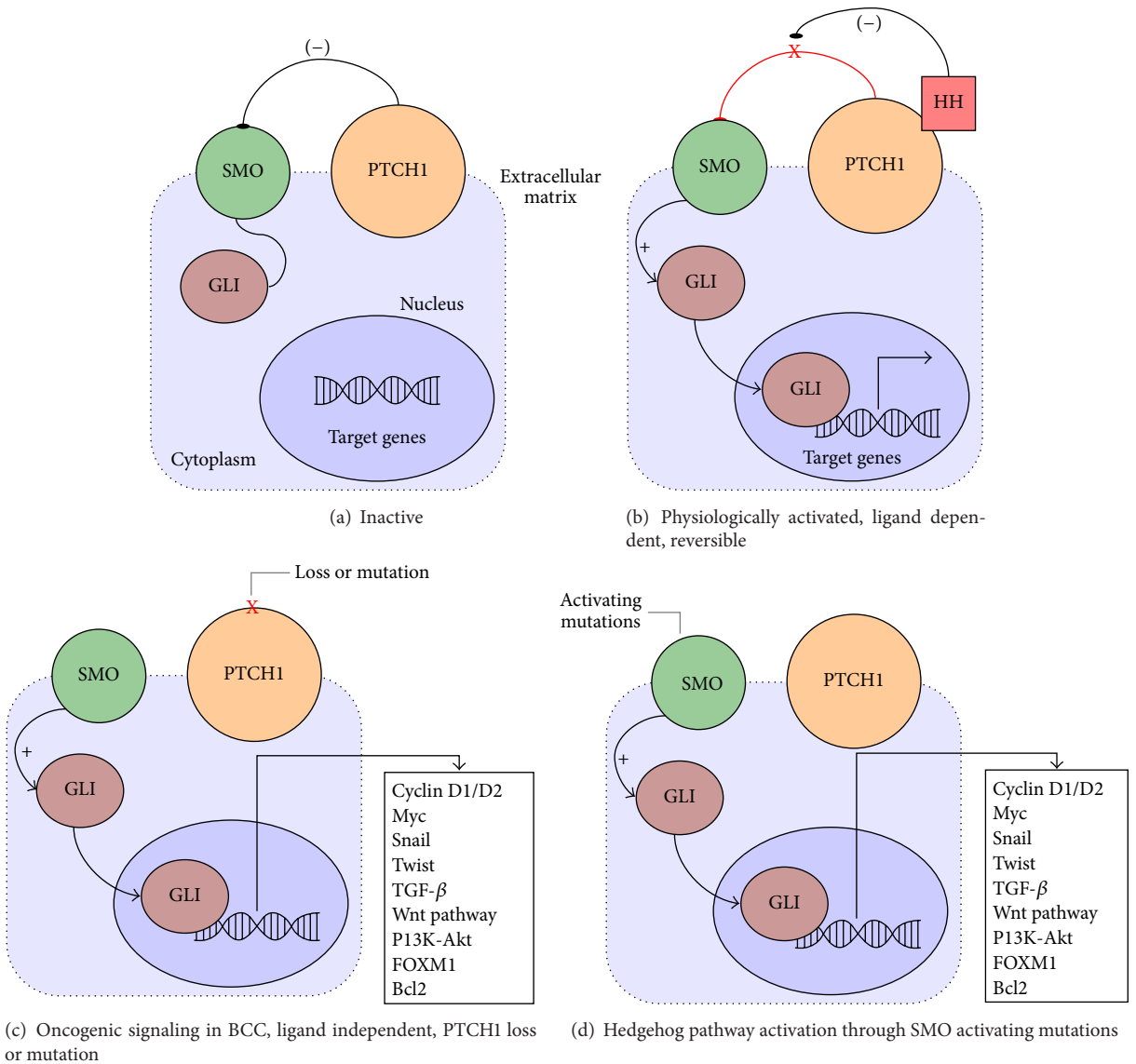

Figure 1: *Sonic hedgehog (SHH) signaling pathway*. (a) In the absence of the HH ligand, PTCH1 represses signaling through SMO causing GLI to remain inactive in the cytoplasm. (b) During physiological activation of the pathway the HH ligand binds to PTCH1, ending SMO suppression and causing activation and nucleus translocation of GLI thus influencing target genes expression. (c) Pathological activation of the SHH pathway through loss or mutational inactivation of PTCH1, suspending SMO inhibition in Gorlin-Goltz and sporadic BCCs (twist, FOXM1, Wnt pathway molecules, and others are viable biomarkers in BCC proteomic studies). (d) SMO activating mutations, found in sporadic BCCs, showing similar effects on the SHH pathway. HH: *sonic hedgehog* ligand; PTCH: *protein patched hedgehog* receptor, SMO: smoothened receptor, GLI: GLI factor, and Wnt: *wingless* signaling pathway.

sclerosing), and less commonly superficial multifocal type are considered by some [36, 37] to be histological risk factors.

A proteomic approach of potential predictive markers for BCC recurrence employed the study of cyclooxygenase-2, ezrin, and matrix metalloproteinase-9 [33].

One study found that overexpression of COX-2 plays a significant role in carcinogenesis through several mechanisms such as enhancement of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis, stimulation of invasion, and suppression of immune responses [38]. The promotion of tumor cell proliferation appears to result from cooperation between COX-2 and various cellular signaling pathways [39, 40]. Moreover, a connection between COX-2 overexpression and increased levels of vascular endothelial growth factor-A (VEGF-A), CD31 positive vessels, and regulators of apoptosis Mcl-1 and Bcl2 has been implied by previous studies [41]. El-Khalawany and Abou-Bakr found that 90.9% of recurrent BCCs (rBCC) expressed COX-2 compared to only 59.1% of nonrecurrent BCCs (nrBCC) [33]. Conversly, it was shown that COX-2 inhibition can mitigate tumor growth, decrease the expression cell proliferation markers, and promote cancerous cell apoptosis [39, 40].

Ezrin is a cytoplasmic peripheral membrane protein belonging to the ERM (ezrin, Radixin, and Moesin) protein family and acts as an intermediate between the plasma membrane and the actin cytoskeleton having a significant role in cell surface structure adhesion, migration, organization, tumor growth regulation and progression, and metastatic spread of numerous cancers [42, 43]. Higher frequencies for ezrin immunopositivity have been observed in rBCCs than in nrBCCs [33]. While Bagheri and collaborators found ezrin expression in 93% of the tested BCCs, no significant difference regarding ezrin expression was observed between different BCC subtypes. The authors did note a difference between the intensity of staining between BCC subtypes, with morpheaform BCC having a significantly higher intensity of staining compared to the nodular variant [44]. Some studies reported a higher ezrin expression in SCC compared with less aggressive tumors such as solar keratosis, keratoacanthoma, or Bowen's disease [42]. Moreover, in primary cutaneous melanomas ezrin expression was found to be associated with depth of invasion, progression, and metastasis [45]. These results are supported by a previous study [46] that found 92% positivity for ezrin expression in 25 BCCs. The authors, however, did not find any difference regarding positivity or staining pattern between BCCs and SCCs. They hypothesized that the intensity rather than the pattern of ezrin expression had a more probable impact on the tumor behavior but admitted that larger studies are needed for clarification.

Matrix metallopeptidase 9 (MMP-9), an enzyme also known as 92 kDa type IV collagenase, is a matrixin encoded by MMP-9 gene. Along with other molecules, it plays an important role in neutrophil migration across the basement membrane [47], angiogenesis, and neovascularization in tumoral tissues through recruitment of endothelial stem cells [48] and wound repair by stimulation collagen contraction [49]. MMP-9 was detected by ISH (*in situ* hybridization) in the stromal fibroblasts adjacent to tumor invasion sites in infiltrating basal cell and squamous cell carcinomas and in the eosinophils infiltrating the dermis in response to invasive BCC [50, 51] and another study found an increased expression of MMP-9 and MMP-2 in SCC versus BCC [52]. Dumas et al. considered the reduced expression of collagen IV accompanied by the increased expression of MMP-9 and MMP-2 could explain the increased aggressive behavior of SCC over BCC [52]. One study [33] did not find any statistically significant difference between MMP-9 expressions in recurrent versus nonrecurrent BCCs.

In a prospective study, Glaser et al. [53] measured the levels of mRNA for CD3e (a T-cell surface marker), CD25 (alpha chain of IL-2 receptor expressed on activated T-cells and B-cells), CD68 (marker for monocytes/macrophages), the cell surface glycoprotein ICAM-1 (intercellular adhesion molecule-1), and the cytokines interferon-gamma (IFN-γ) and IL-10 in BCC tumors from 138 patients. The median follow-up in this study was 26.6 months. It was revealed that subjects with initially low CD3e, CD25, CD68, and ICAM-1 mRNA levels had a significantly shorter tumor-free period ($p = 0.03$, $p = 0.02$, $p = 0.003$, and $p = 0.08$, resp.). It was also observed that nodular morphologies had lower gene expression levels compared to superficial or mixed tumors. The authors could not link IFN-γ mRNA levels to the risk of subsequent tumors [53]. This information shows that immune cell related gene expression in an initial BCC tumor could be used to predict subsequent BCC development. These results have been confirmed by other studies [54] which found elevated mRNA levels of IFN-γ, IL-2, and CD3e in regressing BCCs.

Hunt et al. conducted a study of primary BCCs with and without histological evidence of regression, proposing that some tumors induced immune responses capable of tumor disruption. They reported a significant increase in CD3 in tumors that present active regression compared to those showing no regression. They also found that expression of CD25 was greater in actively regressing BCCs compared to tumors that had no current or past regression [55].

These findings reinforce the importance of inflammatory and immune cells in tumor progression mediation.

4. Aggressive, Metastatic, and Giant Basal Cell Carcinoma

Numerous studies have employed proteomics in their attempt to characterize aggressive BCC and distinguish it from nonaggressive variants.

Ansarin et al. [56] found that elevated p53 protein expression could be considered a predictor of BCC aggressive behavior. However, another study [57] reported that even though differences in *p53* gene mutation frequency, types of mutations, and hot spots between aggressive and nonaggressive BCC exist, they do not clearly predict tumor behavior. Yu et al. [58], in a 2008 gene expression study, found that nodular and superficial BCCs demonstrate similar transcriptional profiles, but different from the morpheaform subtype, which shows a more diverse gene expression pattern, reflecting its invasive nature. However, Howell et al. [59] could not distinguish nodular from sclerosing BCC subtypes by their gene expression patterns.

As a common trait to all epithelial-derived tumors, BCC can express transcription factors like Snail and Twist 1 or mesenchymal markers like the cell adhesion molecule N-cadherin.

The basic helix-loop-helix (bHLH) transcription factor Twist 1 was initially identified in an experimental tumor model as a major regulator of epithelial to mesenchymal transition (EMT) [60]. It was also found to be significantly upregulated in patients with metastatic breast cancer when compared to early disease stages [61].

Epithelial to mesenchymal transition (EMT) is a complex process by which cells lose their epithelial traits and gain a mesenchymal-like phenotype. Numerous factors, such as transforming growth factor beta (TGF-β), epidermal growth factor (EGF), and Wnt-b signaling, have been described to promote the expression of transcription factors Twist 1 and Snail in epithelial cells, resulting in decreased expression of E-cadherin, upregulation of N-cadherin, vimentin, and fibronectin and the acquisition of morphological and functional characteristics of mesenchymal tissue cells [62].

In 2012, Majima et al. [63] present a case of morphoeic and multiple organ metastatic BCC exhibiting induction of Twist

1 and epithelial to mesenchymal conversion of cadherins in a 51-year-old Japanese male. Twist 1 expression was analyzed by immunohistochemistry on formalin-fixed paraffin-embedded sections of the tumor with representative sections from nodular BCCs serving as controls. Cells at the invasive front of the primary tumor proved to be positive for Twist 1, whilst cells from the tumor center were negative for this marker. Control cells from nonmetastatic nodular BCCs did not show nuclear Twist 1 expression. Additionally, cadherins (E-cadherin and N-cadherin) were assessed by double immunofluorescence stains that showed strongly expressed E-cadherin in nonmetastatic nodular BCC and very low expression of this epithelial marker in cells from the metastatic BCC. By comparison, while no N-cadherin expression could be detected in the control tumors, cells from the metastatic BCC were found to express N-cadherin at the invasive front. Cells from a metastasis showed high expression levels of Twist 1 and N-cadherin and notably decreased expression of E-cadherin. It has been previously shown that E-cadherin, a calcium-dependent cell adhesion molecule, plays a crucial role in tumor invasion suppression, and its loss of function is associated with increased tumor aggressiveness [64]. Pizarro reported reduced E-cadherin expression in infiltrative BCCs [65] that have also been previously shown to produce MMP-7 [66]. The authors consider Twist 1 a viable biomarker for either highly invasive or metastatic BCC [63]. Sasaki and collaborators found that in ESCC (esophageal squamous cell carcinoma) Twist or E-cadherin expression was correlated with tumor attributes, such as tumor stage, depth of invasion, lymphatic invasion, and regional and distant node metastasis, making their evaluation useful for determining prognosis [67].

Maspin, a protease inhibitor and a member of the serpin family, is the product of a tumor suppressor gene with an active role in apoptosis and inhibition of tumor invasion, metastasis, and angiogenesis [68]. Whereas Bagheri et al. [44] report expression of maspin in 74.4% of their samples of BCCs, they could not find a significant difference between BCC subtypes concerning maspin expression and intensity of staining. These results are in accordance with another study [69] in which maspin expression in BCC was found to be 87.5%. Although biased by the inclusion of 3 metatypical BCCs, Abdou et el. report a maspin expression in BCC of only 48% [70].

Rates for metastatic BCC are around the 0.55% mark, making it a very rare occurrence [71]. Defined by the AJCC (American Joint Committee on Cancer) as a tumor larger than 5 cm in diameter, giant BCC is also a very rare variant [72].

Alpha-smooth muscle (α-SMA) actin, an isoform typical of smooth muscle cells (SMC) encoded by the ACTA2 gene on chromosome 10q22-q24, is present in the skin in the arrector pili muscles, fibroblasts surrounding anagen hair follicles, myoepithelial cells of eccrine glands, perivascular pericytes, and vascular smooth muscle [73]. In several studies [74, 75] the expression of α-SMA was significantly higher in the stroma of aggressive BCCs when compared to nonaggressive BCCs. Adegboyega et al. implied that stromal expression of α-SMA was in fact restricted to aggressive tumors at the same time being highly predictive of aggressive behavior [74].

Recently, Motegi et al. studied the expression of Twist 1 and α-SMA in the stromal cells of a metastatic giant basal cell carcinoma. They concluded that although Twist 1 induced EMT of tumor cells might have been linked to distant organ metastases in their case, the presence of α-SMA in myofibroblasts surrounding BCC tumoral cell nests could certainly represent one of the trademarks of BCC aggressiveness [76].

Oh et al. suggested that membrane type-1 matrix metalloproteinase (MT1-MMP) and β-catenin could be considered biomarkers for high-risk BCC due to their important role in locally invasive and destructive growth tumor behaviour [77]. El-Bahrawy et al. noted that β-catenin is found mainly in the membrane of tumor cells of high-risk BCC and suggested that a molecular mechanism, other than the aberrant E-cadherin/catenin complex, is involved in these high-risk subtypes of BCC [78].

ER homeostasis in tumoral cells is dysregulated by either physiological or pathological stimuli, such as oxidative stress, DNA-damage, nutrient deficiency, calcium-depletion, certain growth factors, and oncogenic factors. Under such conditions, unfolded and misfolded proteins accumulate, leading to ER stress and the activation of ER-specific signaling pathways [79].

Endoplasmic reticulum protein 29 (ERp29) is a chaperone protein found in the lumen of the endoplasmic reticulum (ER) [80]. It is thought to play a role in protein processing and transport in the early secretory pathway. Erp29 is expressed at varying levels practically in every tissue, yet its precise role in the pathogenesis of neoplasia remains unknown [81].

One study pointed out the tumor suppressive role of ERp29 demonstrated by inhibition of tumor formation in mice xenografts. The authors also suggested that overexpression of ERp29 could indirectly result in activation of genes with tumor suppressive functions, like E-cadherin and spleen tyrosine kinase [82].

Cheretis et al. studied the implication of ERp29 in the pathogenesis of cutaneous BCC. The results revealed that 37.5% of all analyzed tumors expressed ERp29. Infiltrating carcinomas displayed more intense immunoreactivity compared to superficial variants which displayed less intense anti-ERp29 staining [81]. According to this study ERp29 is expressed in a subset of BCC in which the infiltrating morphologies exhibit the highest incidence of immunopositivity.

Following cell metastasis, disseminated mesenchymal cancer cells can be reversibly converted to an epithelial cell state by mesenchymal-epithelial transition (MET) [83]. Considering that ERp29 can drive MET in mesenchymal breast cancer cells [82], ERp29 may play an important role in promoting distant metastasis during disease progression in basal cell carcinoma.

5. Nevoid Basal Cell Carcinoma Syndrome (Gorlin-Goltz Syndrome)

The molecular mechanisms underlying the pathogenesis of multiple BCCs in Gorlin-Goltz syndrome (GS) differ considerably from those of sporadic BCC development. Patients

suffering from GS develop multiple BCCs at young ages and tumors are more often localized on non-UV-exposed skin [84]. Genetic anomalies of the PTCH1 gene in GS include nonsense, frameshift, in-frame, splice-site, interstitial, and missense mutations [85]. Immunohistochemical analysis with nonmutated site-targeting anti-PTCH1 antibody cannot differentiate GS associated from sporadic forms of BCC, because both show comparable patterns and intensity of staining [86].

A comparison of whole genome expression [87] between GS and healthy controls revealed a genomic signature which included several genes with known associations with tumor growth and invasiveness. The authors also report that the genotype of PTCH1+ fibroblasts from tumor-free skin of NBCCS patients was similar to that of BCC associated fibroblasts to the extent that NBCCS fibroblasts overexpressed mRNAs encoding MMP-1, MMP-3, and tenascin C, proproliferative factors such as fibroblast growth factor 7 (FGF-7) and stromal cell-derived factor 1 alpha. In addition, there was strong MMP-1 overexpression in PTCH1+ fibroblasts obtained from NBCCS patient compared to healthy donors [87].

Ponti et al. [88] analyzed profiles of fibroblast conditioned culture media of PTCH1+ and compared them to nonmutated fibroblasts. Statistically significant differences between two different types (missense versus nonsense) of PTCH1 mutations in the profiles of fibroblasts from conditioned media were revealed. Results confirmed previously documented [87] MMP-1 overexpression in PTCH1 mutated fibroblasts, thus confirming the relationship between PTCH1 mutation and MMP-1-related neoplastic transformation of epithelial cells.

Adding to these findings, matrix metalloproteinase-1 (MMP-1) was identified by Weiss et al. to be a downstream target of Twist 1. According to their study, MMP-1 may also enhance cellular motility and invasion of BCC by disrupting the basement membrane and degrading the stromal matrix [89].

MMP-1 can thus be considered a novel marker for target therapy in the context of NBCSS. Future studies of this particular proteomic signature could prove useful for the clinical, therapeutic, and prognostic evaluation of these patients.

6. Conclusions

In summary, it is apparent that gene expression alteration induced by different pathways in tumor cells due to the variation in the expression of other factors is an essential event in BCC carcinogenesis. Analyzing gene expression and proteomic profiles of tumor cells and its microenvironment in different tissue or fluid biological samples indicates new candidate molecules involved in skin cancer pathogenic pathways that might represent future predictive and prognostic biomarkers in BCC and other skin cancers.

Competing Interests

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

Authors' Contributions

All authors have equally contributed to the conception and preparation of the paper.

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Research Article

Expression Profile of p53 and p21 in Large Bowel Mucosa as Biomarkers of Inflammatory-Related Carcinogenesis in Ulcerative Colitis

Cristiana Popp,[1] Luciana Nichita,[1,2] Theodor Voiosu,[1,2] Alexandra Bastian,[1,2] Mirela Cioplea,[1] Gianina Micu,[1] Gabriel Pop,[1] Liana Sticlaru,[1] Andreea Bengus,[1] Andrei Voiosu,[1] and Radu Bogdan Mateescu[1,2]

[1]*Colentina University Hospital, 020125 Bucharest, Romania*
[2]*Carol Davila University of Medicine and Pharmacy, Bucharest, Romania*

Correspondence should be addressed to Cristiana Popp; brigaela@yahoo.com

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Ulcerative colitis (UC) is a chronic, relapsing inflammatory bowel disease that slightly increases the risk of colorectal cancer in patients with long-standing extended disease. Overexpression of p53 and p21 in colonic epithelia is usually detected in UC patients when no dysplasia is histologically seen and it is used by pathologists as a discriminator between regenerative changes and intraepithelial neoplasia, as well as a tissue biomarker useful to predict the risk of evolution toward malignancy. We present a one-year prospective observational study including a cohort of 45 patients with UC; p53 and p21 were evaluated in epithelial cells. p53 was positive in 74 samples revealed in 5% to 90% of epithelial cells, while 63 biopsies had strong positivity for p21 in 5% to 50% of epithelial cells. Architectural distortion was significantly correlated with p53 overexpression in epithelial cells. Thus, we consider that architectural distortion is a good substitute for p53 and p21 expression. We recommend use of p53 as the most valuable tissue biomarker in surveillance of UC patients, identifying the patients with higher risk for dysplasia. Association of p21 is also recommended for a better quantification of risk and for diminishing the false-negative results.

1. Introduction

Ulcerative colitis (UC) is a chronic, relapsing inflammatory bowel disease with increasing incidence and prevalence in Europe. Epidemiological studies are indicating that multiple characteristics of western way of life (high hygiene standard, overexposure to pollutants, and stress) are at least risk factors for this disease, suggesting that the number of cases will constantly increase in the next decades [1–3]. Also, the fact that UC has a low rate of mortality, but also is incurable for now, induces an ascending rate of prevalence for the disease.

Multiple studies are indicating that UC slightly increases the risk of colorectal cancer in patients with long-standing extended disease. Accepted risk of colorectal cancer is about 4/1000 per person year duration [4]. Also, the mortality of colorectal carcinoma in patients with UC is higher than that for sporadic cases [5–8]. These data, along with the fact that there is an increasing number of older patients, with long-standing disease, history of multiple flares, and increased risk for epithelial malignancy, are emphasizing the importance of proper surveillance of UC patients in order to prevent and/or early-diagnose intraepithelial and invasive neoplasia. Although there are insufficient data to sustain the importance of colonoscopic surveillance in preventing carcinoma in UC, cohort studies have demonstrated reduced risk of malignancy and improved survival in patients with UC undergoing routine colonoscopy at 2 to 3 years intervals, beginning 8 years after diagnosis [9]. Since intraepithelial neoplasia is difficult to identify with usual colonoscopic techniques, the diagnosis requires advanced endoscopic procedures or multiple biopsies (minimum 36 each time) [10]. Both alternatives are increasing the costs of this surveillance and have a significant risk of false-negative results. For this reason, studies of expression proteomics are needed to validate some

tissue biomarkers that can be used to evaluate progression toward malignancy in patients with UC.

Cause of carcinogenesis in UC is considered chronic inflammation of colonic mucosa with increased cell turnover and accelerated reepithelialization that leads, in the end, to a higher risk of errors in the cell cycle repair. UC flairs are characterized by a predominant neutrophilic infiltration with crypt abscesses and ulceration of the epithelium, on the background of chronic inflammation. Between flairs, usually there is a state of inactive mucosal inflammation characterized by predominance of lymphocytes. Over time, epithelial colonic cells suffer from genomic instability induced by oxidative stress, linked to chronic inflammation. Inflammatory infiltrate in UC generates oxygen radicals and nitrogen species that affect numerous metabolic processes involved in cell repair [11, 12].

Most important mutation occurs early in UC and involves p53 gene. Overexpression of p53 in colonic epithelia is usually detected in UC patients when no dysplasia is histologically seen and it is used by pathologists as a discriminator between regenerative changes and intraepithelial neoplasia, as well as a tissue biomarker useful to predict the risk of evolution toward malignancy. A high frequency of p53 mutations has been found in chronic UC patients with severe disease who were not diagnosed with cancer [13–15].

p21 oncoprotein expression is also persistently increased in epithelial cells in UC, in active phase, and also in remission, especially when it is associated with cryptic atrophy. This feature is considered to be the result of mutation in *ras* gene that plays an important role in UC-related carcinogenesis. Other studies linked p21 upregulation to p53 mutation, finding that is more frequent in UC-related carcinomas than in sporadic cases [16–18].

Colorectal carcinoma arising on the ground of UC is, commonly, the result of a multistep process including inflamed mucosa without dysplasia, then low-grade dysplasia, high-grade dysplasia, and finally invasive adenocarcinoma, although each of the premalignant lesions can evolve directly towards carcinoma, without the intermediate steps [19, 20].

UC-related carcinoma differs from sporadic one because, in most cases, preinvasive and invasive lesions are multifocal, small, and flat making detection more difficult. The promising role of expression proteomics is highlighted by the fact that genetic abnormalities such as alterations in the p53, bcl-2, and *K-ras* genes, and their tissue expression is still present [13, 21].

This study is evaluating the tissue expression of p53 and p21 in patients with UC, in order to identify the natural evolution of these biomarkers and their relationship with carcinogenesis. A proper understanding of the importance of these markers should allow a better stratification of UC patients according to their risk for dysplasia and invasive carcinoma, in order to personalize their treatment and surveillance.

2. Materials and Methods

This study is an observational prospective cohort study that included 45 consecutive patients with clinical, endoscopic, histologic, and imagistic diagnosis of UC in their first 6 years of the disease. Patients were enrolled for close clinical, biochemical, endoscopic, and histologic surveillance. All patients were treated according to national and international guidelines for the disease and pathologists were blinded to the therapeutical approach.

Criteria of Exclusion. Criteria of exclusion were as follows: malignancies (invasive or preinvasive) in the moment of enrolling and refusal of signing an informed consent.

All patients underwent a thorough medical history, complete clinical examination, and endoscopic examination (ileocolonoscopy with narrow band imaging and magnification chromoendoscopy with complete video and photo documentation). From each patient, multiple biopsies of normal and injured mucosa were taken, including samples of rectal mucosa and ileal mucosa, using EndoKit for proper orientation of mucosa. Usually from each patient 3 or 4 biopsies were performed in each visit (minimum of two biopsies from the large bowel, one from the most injured area and one from an area with normal appearance. In all cases one piece of rectum mucosa was harvested. Also, an ileal biopsy was taken in all cases). In 14 patients (5 in the first presentation and 9 in the second), additional biopsies were taken from areas suspected of dysplasia. All tissue fragments were immediately immersed in 10% buffered formalin and then routinely processed (24-hour fixation in 10% buffered formalin, one hour water rinse, dehydration in 3 baths of 80%, 90%, and 96% ethanol for 6 hours each, then two one-hour baths of absolute ethanol, then clearing with toluene for one hour at 58°C, and, finally, 3 one-hour baths of paraffin at 58°C). The samples were paraffin embedded and two pairs of 3 μm sections from two levels were obtained for each biopsy. The slides were stained routinely with hematoxylin-eosin and then examined and a histopathological diagnosis was formulated. If necessary, additional special stains were performed: periodic acid Schiff stain (PAS), Giemsa stain, Masson's trichrome stain, and Ziehl-Neelsen.

All of these procedures were repeated after a year (12 months) of follow-up for each patient included in our study.

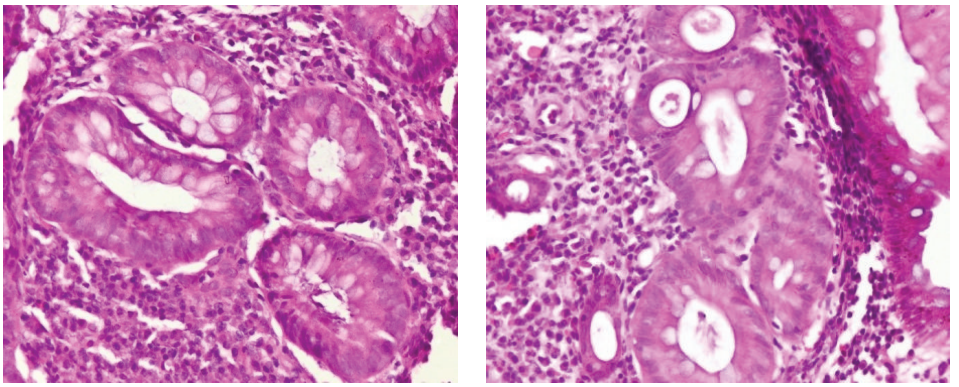
These activities were carried in perfect compliance with national and European research laws and professional deontology and were approved by Colentina University Hospital (CUH) ethical committee.

After histological diagnosis, all tissue samples from the first and from the second visit were prepared for being evaluated, in research settings, as follows.

First, manual tissue multiarray blocks were created. For each patient, the most significant tissue sample from each of the two visits (a sample with the most severe inflammation was used, including at least 10 crypts) was extracted from the paraffin block and then reembedded into a single recipient block, each including 6 samples from 3 patients.

From each multitissue block several histological slides were sectioned for routine stain (hematoxylin-eosin), special stains (PAS, Giemsa, and Masson's trichrome stain), and immunohistochemical tests for p53 and p21.

For immunohistochemistry we used indirect tristadial method, using peroxidase and alkaline phosphatase as



(a) (b)

Figure 1: Regenerative epithelial changes (a) versus low-grade dysplasia (b) in UC. Note in (a) preservation of crypt architecture as well as nuclear polarization. In (b) crypt is distorted and epithelial cells have lost nuclear polarity.

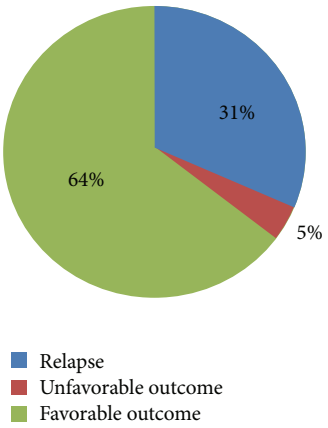


Figure 2: Patients' evolution during the study. Note that most of the patients had a favorable outcome.

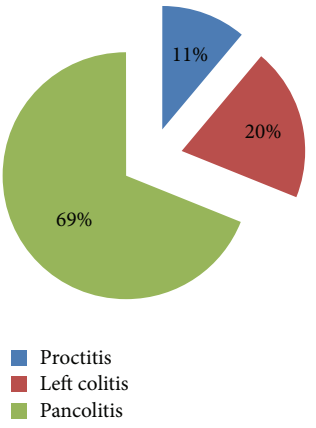


Figure 3: Disease extent at first presentation. Note that most patients had extensive disease, defined as pancolitis.

enzymes and DAB for peroxidase (brown)/AEC (red) and "fast red" and "fast blue" for alkaline phosphatase as substrates.

The sections were deparaffinized and hydrated on automatic stainer. Then, the following steps were made:

(1) Washing with TBS buffer, pH 7.4, 3 washes, 5 minutes each.

(2) Pretreatment: boiling in microwave oven in citrate buffer pH 8 or EDTA buffer pH 10, as specified for each antibody.

(3) Washing with TBS buffer, pH 7.4, 3 washes, 5 minutes each.

(4) Hydrogen Peroxide Block, LabVision, for 15 minutes.

(5) Washing with TBS buffer, pH 7.4, 3 washes, 5 minutes each.

(6) Blocking of nonspecific binding with Ultra V Block LabVision, 10 minutes.

(7) Washing with TBS buffer, pH 7.4, 3 washes, 5 minutes each.

(8) Primary antibody to each section for 1 hour in wet room.

(9) Washing with TBS buffer, pH 7.4, 3 washes, 5 minutes each.

(10) Secondary antibody biotinylate to each section 10 minutes (anti-mouse, Ultravision Detection Systems LabVision).

(11) Washing with TBS buffer, pH 7.4, 3 washes, 5 minutes each.

(12) Streptavidin Peroxidase LabVision, 10 minutes.

(13) Washing with TBS buffer, pH 7.4, 3 washes, 5 minutes each.

(14) Developing with DAB Plus Substrate System, LabVision.

(15) Washing with running water.

(16) Counterstain with hemalaum Meyer.

(17) Dehydration and drying.

(18) Mounting in Entellan.

Multiple histological and immunohistochemical parameters were evaluated and quantified by two independent fully trained pathologists, using preestablished scales.

Dysplasia was defined as "unequivocal neoplasia of the epithelium confined to the basement membrane, without invasion into the lamina propria" and was evaluated using the usual scale: negative for dysplasia, indefinite for dysplasia, low-grade dysplasia, high-grade dysplasia, and invasive carcinoma [22]. Consensus was reached in all cases by the initial two pathologists (Figure 1).

For p53 and p21 only intensely stained cells were quantified, results being expressed as percent from the epithelial cells examined on each section. If differences between the results of the two independent examinations were below 10 percentage points, the lowest value was taken. If the difference was higher, the slide was examined simultaneously with a third senior pathology and consensus was reached (lowest value that had complete agreement).

As control group we used 12 samples of normal colonic mucosa harvested from patients without inflammatory bowel disease (distant resection margins from sporadic colonic carcinomas). Each control sample underwent the same standardized procedures and was examined by the same two pathologists. Parameters identified were considered as normal counterparts and used as standard for evaluation of cohort samples.

Study Limitations. (a) The follow-up period (12 month) was somehow too short to assess the risk of development of cancer in these high-risk patients. Although, this study obtained some significant results correlating mucosal expression of p53 and p21 with other factors that indicate progression towards malignancy. (b) Patients included were in their first 6 years of the disease, when dysplasia is rare. But this study aimed to find subtle changes ratable before the occurrence of dysplasia, changes that can be used to identify patients with higher risk. (c) The studied group is pretty small, including only 45 patients, but this value is above the minimum needed for statistical significance, and all patients had a certain diagnosis of UC, and, also, all patients were submitted to a complex, multidisciplinary surveillance that offered significant data.

3. Results and Discussion

3.1. Data about the Cohort. The studied cohort included 45 patients, 31 men and 14 women. Only one patient had, on the second biopsy, an area of low-grade dysplasia, identified by chromoendoscopy and confirmed microscopically. No invasive carcinoma arose during this 12-month study.

Patients' evolution during the study was considered as follows:

(i) Favorable outcome: no relapse or complications during the study and a less severe clinical status (according Truelove & Witts classification) at the second presentation [23].

(ii) Unfavorable outcome: all patients that did not fulfill the above criteria.

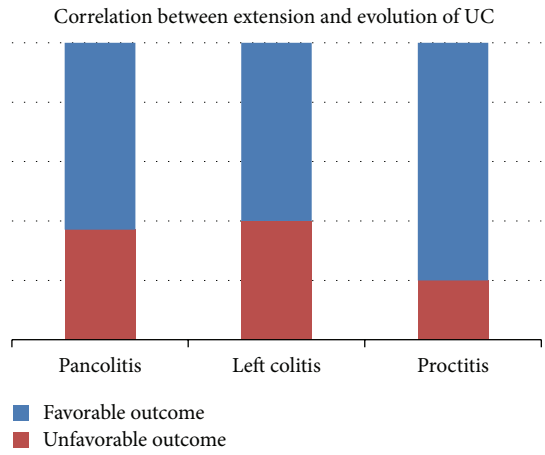


Figure 4: Correlation between patients' outcome and extension of the disease at first presentation.

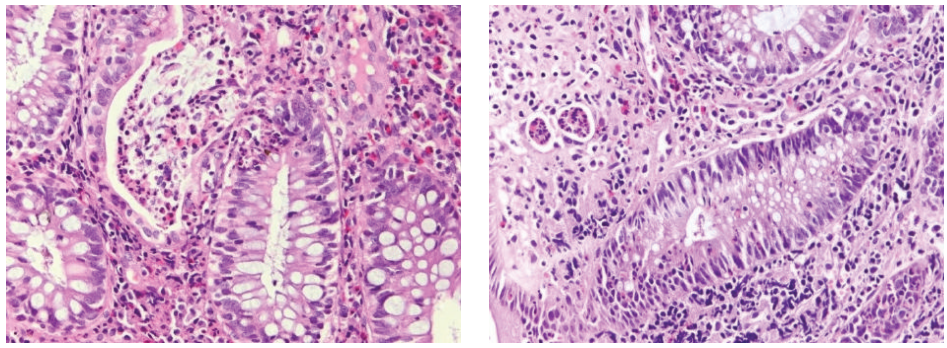
Thus, 16 patients (~35%) had unfavorable outcome, while 29 patients (~65%) had favorable outcome (Figure 2). From the first group, 14 patients had a relapse (defined as recurrence of significant clinical and endoscopic activity of the disease after more than 6 months of remission), relapse being the most frequent negative event that interferes with patients' outcome in UC [24]. The other two patients did not acquire remission during the study and had a more severe clinical and endoscopic score in the second evaluation. From the 14 patients with relapse, two had additional complications: one tuberculous ileocolitis and one low-grade dysplasia of the rectum.

The risk for relapse in patients with UC is about 70% per year in the lack of treatment, but, in treated patients, this risk is between 23 and 33%, similar to our study [25].

3.2. Extension of the Disease. One of the most significant parameters that influence the risk of dysplasia is the extension of the disease. Patients with pancolitis had a more severe outcome and a higher risk of dysplasia, while proctitis is associated with a better prognosis and a reduced rate of morbidity and malignancies [4, 26]. In our cohort, 31 patients, representing 68.69%, had pancolitis, while only 5 patients (11.11%) had proctitis and 9 patients (20%) had left colitis (Figure 3).

As expected, 15 patients with extensive disease (left colitis and pancolitis) had unfavorable outcome, while only 2 patients with proctitis had the same evolution (Figure 4). This correlation was statistically significant (Fisher two-tailed test $p = 0.036$). This situation has multiple explanations, the most important being the availability of topical medication which are more efficient and have a lower rate of drug resistance [26, 27].

3.3. Concordance of the Most Severe Affected Area. Also, an important impact on the risk of dysplasia has the duration of severe inflammation. It is more probable that an area of the mucosa is exposed to intensive oxidative stress for a prolonged period to develop DNA damage with neoplastic



(a) (b)

Figure 5: Severe inflammation in a patient with active ulcerative colitis. (a) Severe cryptitis with crypt destruction. (b) Cryptitis, crypt abscesses, and epithelial regenerative changes.

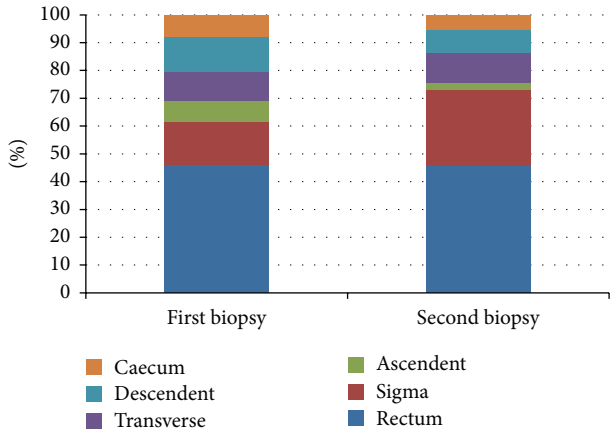


Figure 6: Presentation of the most affected area in the two evaluations. Rectum is always the most affected segment. Note a high concordance between the two biopsies, indicating the lesions are quite fixed in UC, regardless of patients' evolution.

changes. In our study, the concordance of the most severe affected area was as high as 78% (35 patients). Usually, in UC, rectum is the most affected segment, and our study confirmed this feature (Figure 5). From all patients, regardless of their evolution, 40 percent (18 patients) had the most inflamed area in both investigations in the rectum (Figure 6). In the late stages of the disease, rectum can be spared by the inflammation but remains the higher risk for dysplasia of local mucosa [28]. This high concordance indicates that in UC the same areas are usually affected, even in relapse after remission. These areas should be examined with additional attention in screening for dysplasia, since they are the most prone to having cellular mutations.

3.4. Evaluation of p53 and p21/Waf Expression. p53 and p21 are important proteins involved in normal settings, in avoidance of cellular mutations. p53 is a tumor suppressor, described as "the guardian of the genome," regulating gene expression to prevent mutations of the genome and inducing apoptosis in case of DNA damage repair failure. It is the most frequently abnormal protein in human cancer [29]. p21 is a cyclin-dependent kinase inhibitor, inducing growth arrest of cells with DNA damage, usually controlled by protein p53. Both are important biomarkers used to confirm dysplasia lesions. They also have prognostic significance; patients with significant and prolonged overexpression of p53 and p21 have a higher risk of developing dysplasia [30].

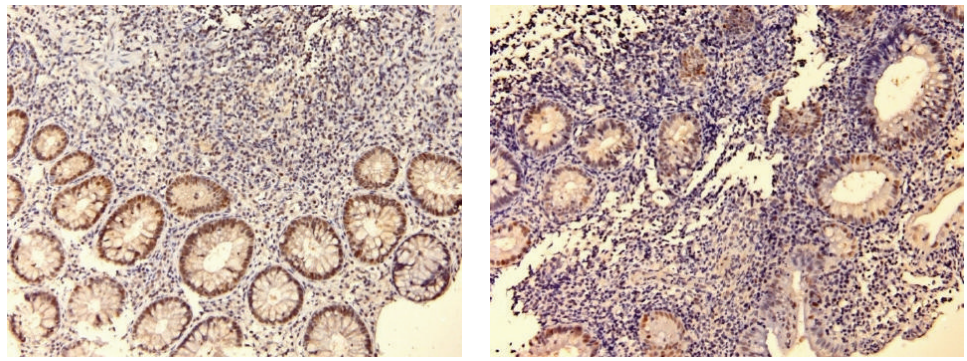
In our study, we quantified the percent of epithelial cells with strong positivity for p53 and p21 (indicating mutations of these proteins). p53 was negative in 16 biopsies (9 from the first presentation and 7 from the second one). The rest of 74 samples revealed intense positivity for p53 in 5% to 90% of epithelial cells (maximal value was identified in the area of low-grade dysplasia) (Figure 7).

Meanwhile, 27 biopsies were negative for p21 (18 from the first presentation and 9 from the second one). In the rest of samples, 63, we had strong positivity for p21 in 5% to 50% of epithelial cells. Expression and evolution of p53 and p21 did not correlate significantly with patients' outcome or risk of relapse (Figure 8).

Median percent of p53 positive cells was 17 for the first biopsy and 21 for the second one (no statistical significance). For p21, median percent of positive cells was 7.33 for the first biopsy and 10.67 for the second one (no statistical significance also) (Figure 9).

Correlated overexpression of p53 and p21 in epithelial cells in UC indicates accumulation of cellular mutation triggered by oxidative stress and build-up of toxic products in stromal microenvironment of colonic mucosa. Therefore, p53 and p21 are two members of expression proteomics family that respond to the need of pathologists and gastroenterologists to keep under control premalignant lesions of UC patients, identifying patients and areas with high risk of evolution towards malignancy, even before the apparition of unequivocal dysplasia.

Mitsuhashi et al. [31] identified a significant correlation between p53 and p21 expression and architectural distortion, concluding that DNA damages are accumulating in the same time with architectural abnormalities, both being results of oxidative on mucosal stroma and epithelium. We examined, also, the presence of this concordance. In our study,



(a) (b)

Figure 7: p53 (a) and p21 (b) positive in colonic epithelium in patients with UC.

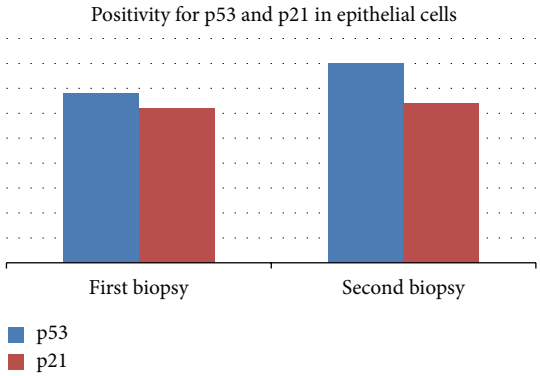


Figure 8: Evolution of p53 and p21 expression in epithelial cells in the 12 months of the study.

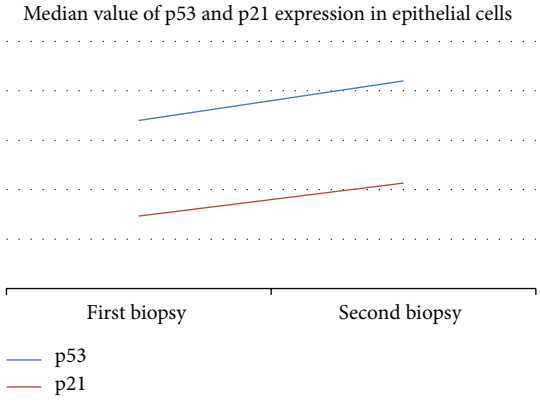


Figure 9: Evolution of median expression of p53 and p21.

architectural distortion was significantly correlated with p53 overexpression in epithelial cells (two-tailed t-test $p = 0.0251$) and, also, with p21 overexpression (two-tailed t-test $p = 0.0035$). Thus, we consider that architectural distortion is a good substitute for p53 and p21 expression and can be used instead of immunohistochemical analysis in samples without dysplasia. However, significant worsening of architectural distortion, especially in patients with long-standing disease, compels a more careful endoscopic examination of mucosa in order to identify areas of dysplasia and evaluation of p53 and p21 status of mucosa [17, 32].

4. Conclusions

Ulcerative colitis has a high but preventable risk of evolution towards colonic adenocarcinoma; therefore, prevention and early diagnosis of dysplasia are mandatory. p53 and p21 are tissue biomarkers not only suitable for routine surveillance, but also for stratification of UC patients in risk categories with a more personalized approach.

Despite being rare, dysplasia is a severe event in UC evolution, frequently imposing prophylactic colectomy. It also has an unknown risk to escape surveillance and to evolve silently towards invasive carcinoma [33]. Our data recommend association of p53 with p21 to increase value of p53 as tissue biomarker used for identification of patients with higher risk for dysplasia. Association of p21 is also recommended for a better quantification of risk and for diminishing the false-negative results.

Competing Interests

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

Authors' Contributions

Cristiana Popp, Luciana Nichita, Theodor Voiosu, and Alexandra Bastian have contributed equally to this study.

Acknowledgments

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Review Article

Inflammatory Biomarkers Profile as Microenvironmental Expression in Keratoconus

Catalina Ionescu,[1] Catalina Gabriela Corbu,[1,2] Cristiana Tanase,[3,4] Christian Jonescu-Cuypers,[5] Cristina Nicula,[6,7] Dana Dascalescu,[1] Miruna Cristea,[1] and Liliana-Mary Voinea[2,8]

[1]*Clinical Hospital of Ophthalmologic Emergencies (SCUO), Piața Alexandru Lahovari 1, Bucharest, Romania*
[2]*Division of Ophthalmology, Faculty of Medicine, "Carol Davila" University of Medicine and Pharmacy, Bulevardul Eroii Sanitari 8, 050474 Bucharest, Romania*
[3]*Biochemistry-Proteomics Department, Victor Babes National Institute of Pathology, Sector 5, 050096 Bucharest, Romania*
[4]*Faculty of Medicine, Titu Maiorescu University, Dâmbovnicului Street 22, 040441 Bucharest, Romania*
[5]*Department of Ophthalmology, Charité University Hospital, Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany*
[6]*Division of Ophthalmology, Faculty of Medicine, University of Medicine and Pharmacy "Iuliu Hatieganu" Cluj-Napoca, Victor Babes Street 8, 400012 Cluj-Napoca, Romania*
[7]*Clinical Emergency Hospital Cluj-Napoca, Clinicilor Street 3-5, 400006 Cluj-Napoca, Romania*
[8]*Department of Ophthalmology, University Emergency Hospital, Splaiul Independenței 169, 050098 Bucharest, Romania*

Correspondence should be addressed to Catalina Gabriela Corbu; carol_corbu@yahoo.com

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Keratoconus is a degenerative disorder with progressive stromal thinning and transformation of the normal corneal architecture towards ectasia that results in decreased vision due to irregular astigmatism and irreversible tissue scarring. The pathogenesis of keratoconus still remains unclear. Hypotheses that this condition has an inflammatory etiopathogenetic component apart from the genetic and environmental factors are beginning to escalate in the research domain. This paper covers the most relevant and recent published papers regarding the biomarkers of inflammation, their signaling pathway, and the potentially new therapeutic options in keratoconus.

1. Introduction

Keratoconus is a progressive, degenerative, usually bilateral disease of the cornea that leads to refractive errors (myopia and irregular astigmatism) with impaired visual acuity and conical corneal protrusion [1]. The progressive thinning of the stroma is the main condition responsible for corneal ectasia. Keratoconus affects typically adolescents at puberty and young adults until the fourth decade of life with the age between approximately 12 and 35 years, during which the disease can progress or spontaneously arrest [1, 2]. The pathophysiology of keratoconus is multifactorial and is still not completely understood. There are proofs that biochemical, biophysical, and genetic aspects play an important role in the etiology of this ectatic corneal disorder. The familial inheritance and the high correlation among monozygotic compared to dizygotic twins [1] show that keratoconus has also a genetic component. Its association with multiple systemic and ocular disorders such as Down syndrome, Leber congenital amaurosis, or Ehler-Danlos syndrome is another aspect that supports this [3]. An interplay between environmental and genetic factors is convincing for the development of the disease. Contact lens wear and eye rubbing are two of the most important exogenous environmental aspects that

induce mechanical changes causing corneal epithelial microtraumas that stimulate the expression of cellular inflammatory mediators [4].

Atopy is yet another studied risk factor that shows a correlation with keratoconus, although it is not well demonstrated whether the atopic ground itself or its effect (eye rubbing) is the one responsible for the effects on the cornea [5].

The most important aspect is the stromal degradation and its thinning, which has many hypotheses. Multiple studies relate the thinning to increased levels of proteolytic enzymes on the one hand and decreased levels of their inhibitors on the other hand [6].

The abnormal collagenolytic activity of the cells and the accelerated apoptosis of keratocytes induce a loss of extracellular matrix and redistribution of collagen fibrils. These actions result in stromal thinning and possible breaks in Bowman's layer with subsequent scarring [2, 7].

2. Histopathological Changes in an Injured Cornea

The corneal epithelium is a nonkeratinized, stratified, squamous 50 μm thick tissue composed of 6-7 cell layers. It is covered by a tear film, which is responsible for lubricating and protecting the surface from microbial pathogens and foreign bodies [9]. Bowman's membrane is an acellular, nonregenerating layer composed of types I, III, V, and VI collagen fibrils. The stroma represents approximately 85% of the corneal thickness. This substance is formed mainly out of type I collagen with special distribution and parallel orientation of the lamellae. The stroma consists of proteoglycans (keratin sulfate, dermatan sulfate) and keratocytes that play a key role in stabilizing the extracellular matrix. The endothelium is composed of a single layer of hexagonal cells that secretes Descemet's membrane towards the stroma. The most inner layer of the cornea acts as a barrier between the stroma and the aqueous humor and permits a sufficient ion flux to maintain the osmotic gradient [10].

In case of an epithelial injury, cell membranes extensions cover the edge of the wound. Fibronectin, an extracellular matrix protein that mediates the cell adhesion and migration, plays a key role in wound healing [9, 11].

Gelatinase B (metalloprotease-9) could be a factor in delaying the normal process of epithelial defect healing [12]. After a stromal injury, the stromal keratocytes are activated by enlarging their size and transforming into fibroblast-like cells. After this process, the keratocytes engage in apoptosis and myofibroblasts begin to remodel the stroma on a background of inflammation with increased levels of growth factors, cytokines, and matrix metalloproteases [4]. The response of an injured endothelium is the enlargement and migration of the adjacent cells [9].

3. Histopathology of Keratoconus

The major clinical signs of keratoconus are thinning of the corneal stroma (mainly inferiorly, which gives birth to the characteristic conical shape), Fleischer's ring (a complete or incomplete circumferentially iron deposit), Vogt's striae in the deep stroma, and Descemet's membrane, followed by possible stromal scars, visible nervous fibers, and ruptures in Descemet's layer in a more advanced stage of the disease [1]. In a histological study performed by Scroggs et al., "typical" keratoconus corneas presented an important thinning of the epithelium centrally, breaks localized in Bowman's layer, and morphological modifications of the superficial epithelial cells [13, 14].

The stromal collagen lamellae in keratoconic corneas tend to have specific orientation compared to normal subjects and are reduced in number. There is a direct correlation between stromal thickness and quantity of collagen fibrils [15]. It has been proven that the morphology and density of keratocytes are altered in the anterior stroma in contact lens wearers diagnosed with keratoconus [16].

4. Keratocytes' Dysfunction in Keratoconus

Pouliquen et al. showed that keratocytes of keratoconic eyes have fourfold more IL-1 receptors compared with normal subjects [17]. The effects of IL-1 are activation of collagenases, metalloproteinases, and overexpression of both keratinocyte growth factor and IL-6 [18, 19]. IL-1 stimulates the KC fibroblasts and triggers a massive production of prostaglandin E2 and, in contrast, a low collagen production [20]. Wilson et al. observed in vitro that IL-1 alpha and IL-1 beta induce cell apoptosis in the stroma, leading to altered tissue organization in keratoconic patients [4]. Transforming growth factor-beta 1 has the capacity of differentiating corneal keratocytes into myofibroblasts in order to stabilize the tissue. The other aspect is the upregulation of inflammatory cytokines activated by TGF-beta, resulting in cellular apoptosis. An alteration of this pathway could lead to corneal fibrosis. These facts explain the hypothesis that TGF-beta 1 could play a role in the scar formation in keratoconic corneas [21].

5. Biomarkers of Inflammatory Pathway in Keratoconus

The International Programme on Chemical Safety defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease". It is a promising field and an accessible and minimally invasive method for early diagnosis and disease progression obtained from body fluids or tissue biopsies. As an example for their importance, researchers have discovered new protein and cytokines biomarkers that are overexpressed in glioblastoma patients compared to healthy subjects [22, 23].

Biomarkers aim at the early detection of cancer and the development of personalized treatment. Thus, biomarkers are becoming a priority in oncology. However, biomarker panels have proven to make a difference in the approach of keratoconus, taking into account the perspective of an inflammatory rather than a noninflammatory disorder.

An important role in the immune system is played by the T helper cells that have regulatory immune characteristics by releasing T cell cytokines, activating both cytotoxic T cells and macrophages. Th cells can be categorized in effector,

memory, and regulatory T cells. While type 1 Th cells promote mostly the cellular immune system by stimulating the macrophages, type 2 Th cells are responsible for the humoral immune response through the proliferation of B cells, thus inducing the production of antibodies. Th 17 cell is yet another type of proinflammatory T cells and is involved in the production of interleukin-17, frequently associated with allergic responses. Among the proinflammatory mediators secreted by Th1 cells are interleukin-2, TNF (tumor necrosis factor), interferon-gamma, IL-12, and IL-15, while IL-4, IL-5, IL-9, IL-10, and IL-13 are released by Th2 cells [24].

Cytokines are glycoprotein molecules that are secreted by immune cells and can trigger an immune response through their complex interactions. These substances can initiate, amplify, or downregulate the immune response, as well as influencing cell proliferation and inflammatory processes, and are also called because of their actions inflammatory mediators [24].

T helper 1 cells are responsible for secreting interleukin-2 (IL-2) and interferon-gamma (IFN-gamma) that play an important role in the activation of macrophages and in the immune responses against intracellular pathogens. Th1 responses are connected to IL-12 that promotes the release of IFN-gamma and to IL-23 that enhances IL-17 release [25]. IL-4, IL-5, IL-10, and IL-13 are produced by T helper 2 cells that are closely related to allergic and antibody responses by triggering immune responses against extracellular pathogens. IL-6 is also produced by T helper 2 and stimulates both the cellular and humoral immune response. Th17 cells' role is the production of IL-17 family and of chemokine CCL20 that have a strong component in chronic tissue inflammation [26]. Interleukin-1 family contains 11 members that are mainly produced by monocytes, fibroblasts, and macrophages and expressed by B lymphocytes and have an important role in mediating the inflammation. IL-1 alpha, IL-1 beta, and the IL family of ligands and receptors are strongly connected in the process of apoptosis and necrosis in inflammation [27].

The most important aspect of these signaling inflammatory molecules is the balance between them with a positive or negative regulatory interaction. In acute or chronic diseases, Th 1 and Th 2 actions are amplified; thus, an increase or decrease of some cytokines can be detected and monitored. For example, IFN-gamma and IL-12 stimulate Th1 but inhibit proliferation of Th 17 cells [24]. Th2 (IL-4 and IL-10) has an inhibitory action on Th1 cells through a decreased production of IL-12 [28].

The cytokines have many characteristics: they are pleiotropic (different effects on different tissues) but also redundant (when different types of cytokines react in the same matter). These low molecular weight proteins have the ability on the one hand to antagonize each other and on the other hand to respond with a positive feedback to other cytokines [29].

Tumor necrosis factor-alpha is a transmembrane protein produced by macrophages, lymphoid cells, and fibroblasts in response to bacterial products, IL-1 or IL-6. TNF-alpha is considered a significant mediator of systemic and local inflammation. Recent studies show that the inflammatory activity of TNF and TNF ligand family is more important than their role in apoptosis [30].

Matrix metalloproteinases are a type of enzymes regulated by cytokines (IL-1, IL-6, and IL-7), TNF-alpha, growth factors, and hormones that play a deciding role in the degradation of extracellular matrix proteins and in cell proliferation and apoptosis. MMPs are inhibited by tissue inhibitor of metalloproteinases (TIMP) [31]. This complex (MMP-TIMP) is responsible for the integrity of the connective tissue and a normal wound healing after injuries [32].

6. Microenvironmental Changes and Cytokines Signaling in Keratoconus

Keratoconus was first described as a noninflammatory ectatic disease, a theory that is beginning to be contradicted by multiple studies that have brought strong evidences for sustaining the clause for the role of inflammation in the pathogenesis of the ectasia. The studies mentioned below will highlight the most relevant conclusions concerning the topic of inflammation in keratoconus.

In 2009, Lema et al. proved the possible inflammatory pathogenesis of keratoconus, showing an increased level of IL-6 and TNF-alpha in subclinical and keratoconic eyes, while MMP-9 was detected only in tears of the patients with the manifest disease [33].

Sorkhabi et al. demonstrated the marked presence of proinflammatory cytokines in the tear fluid in 42 subjects with keratoconus, such as IL-6, IL-1 beta, and interferon-gamma, and a decreased level of the anti-inflammatory IL-10 [34].

Few studies reflected the correlation between inflammatory mediators in the tear fluid and the severity of keratoconus. Kolozsvári et al. studied the correlation between inflammatory cytokines in the tear fluid and the severity of keratoconus. They revealed a significant positive association between CCL5 (chemokine ligand 5) and center/surround index as well as between IL-6 and maximum K value, yet a negative one between IL-13 and the severity of the disease. The researchers also found increased levels of nerve growth factor in keratoconic patients [35].

In the latest study, Pásztor et al. observed the association between cytokines and some Pentacam parameters and found strong positive correlations between CXCL8 and BAD-D (Belin-Ambrosio deviation index). MMP-9 levels were significantly increased in association with BAD-D and K2 keratometry values [36].

An intense proteolytic activity causes collagen denaturation that may accelerate the progression of the disease. Balasubramanian et al. studied the importance of proteolysis in the progression of keratoconus by comparing the total tear protein level, the protease, and inflammatory molecules in patients with keratoconus, patients after corneal collagen cross-linking, and normal subjects. The study showed increased levels of gelatinases and collagenases (1.9 times higher), as well as elevated MMPs, cytokines (MMP-1, MMP-3, MMP-7, MMP-13, and IL-6), and TNF-alpha and TNF-beta in the keratoconus group compared with the normal group [37]. In another study, the researchers observed a significant

increase in the tear film of keratoconic patients of cathepsin B level, a lysosomal protease capable of degrading extracellular matrix proteins. On the other side, there were downregulated levels of cystatin (a group of protease inhibitors) [38]. One year later, Balasubramanian et al. completed the previous study with a new hypothesis that eye rubbing modifies the tear levels of proteases and cytokines. A 60-second eye rubbing technique typically used by keratoconus patients had as a result a significant increase of MMP-13 in tears that has an important role in the apoptotic activity of the keratocytes. Also, TNF-alpha and IL-6 can also be found in atopic and vernal keratoconjunctivitis, suggesting that keratoconus could be related to allergies and to increased level of serum IgE [39].

Cheung et al. investigated the effect of injury in stromal cells in keratoconic corneas compared with normal subjects in order to determine whether there is a dysregulation in the reparative response. As a result, increased levels of IL1 alpha, TNF-alpha, and TGF-beta 1 were measured in keratoconic corneas without induced secondary injury compared with normal corneas and decreased levels of IL1 alpha, FGF-2, TNF-α, EGF, TGF-α, and PDGF were found in patients with keratoconus with secondary injury in relation to those without, which could emphasize the hypothesis of an ineffective wound healing in this ectatic disorder [40].

A presumed progression risk factor of keratoconus is the contact lens wear, especially rigid gas permeable contact lenses that induce the upregulation of IL-6, TNF-alpha, ICAM-1, and VCAM-1 in the tears of subjects with keratoconus [41].

Fodor et al. reported the theory of keratoconus' progression caused by contact lens wear that exacerbates the release of inflammatory mediators. The study observed increased levels of IL-6, MMP-9, and CXCL8 after contact lens wear and a decrease of nerve growth factor, TIMP1, and PAI1 (principle inhibitor of tissue plasminogen activator). The study revealed that this can alter the stromal structure through matrix degradation and proapoptotic effect [42].

In normal subjects, lactoferrin downregulates the expression of cytokines and proteinases. Lema et al. discovered in 2010 in patients with keratoconus the underexpression of lactoferrin, an antimicrobial and anti-inflammatory protein, that suggests a disruption of the protective barrier [43]. Chaerkady et al. made a complete proteome analysis of the cornea in keratoconus and observed overexpression of keratins, extracellular matrix proteoglycans, and types I, III, and V collagen fibrils, yet a downregulation of lactotransferrin, which plead in favor of a degenerative and inflammatory disease. They studied the corneal proteome in keratoconus and identified 932 proteins in the epithelium, respectively, 1,157 in the stroma, that brought to light the resemblance with other neurodegenerative disorders but also the inflammatory component and the importance of oxidative stress [44].

Pannebaker et al. published statistically significant increased levels of MMP-1 only in keratoconic eyes. They also studied tumor necrosis-related apoptosis-inducing ligand-R1 (TRAIL-R1) that had a decreased level in the gas permeable lens wearer keratoconus group and increased in the one without lenses. These results could suggest the alteration of the receptors in keratoconus [45].

Allergic states are correlated with increased levels of cytokines in the tears of patients with keratoconus. Weed et al. reviewed in their article the strong correlation between keratoconus and ocular allergy, as well as the positive association with atopy (asthma, eczema, and hay fever) [46]. Because allergy is a risk factor of keratoconus, Sharma et al. suggested that corneal topography should be a routine investigation in these patients [47]. Bawazeer et al. concluded that the most important risk factor of the disease is eye rubbing that could be induced by the itch of atopy [5].

Kolozsvári et al. analyzed the evolution of tear's biomarkers in keratoconic patients after corneal collagen cross-linking and reported that the levels of IL-6 and IL-8 decreased 1 year after CXL. The study revealed also a negative association between IL-6 and Th1, respectively, between MMP-13 and keratoconus index (KI) [48]. Analyses of the tear fluid showed abnormal levels of TH1, TH2, and TH17 cytokines, suggesting certain immune dysregulation in this disease as well [49].

Jun et al. analyzed the levels of Th1, Th2, and Th17 cell cytokines in the serum and tears of keratoconic patients in order to correlate a systemic inflammation with keratoconus. There were no significant levels of proinflammatory serum cytokines, in contrast to increased levels of IL-6 and IL-17 in the tear film of keratoconus patients. IL-4, Il-12, IL-13, and TNF-alpha were found to be downregulated in the keratoconus group [50].

More recent studies evidence the correlation between the progression of keratoconus and a systemic inflammation through a new measure called neutrophil-to-lymphocyte ratio (NLR). Karaca et al. found that the NLR was higher in the patients that presented progression opposed to the stationary and normal subjects [51].

There is a vicious circle between the proinflammatory cytokines, proteolytic enzymes, and inhibitors that are the ones responsible for the microenvironmental changes in keratoconus. This imbalance triggers the signaling of inflammatory pathways in the cornea inducing structural abnormalities that lead to progression of the disease (Figure 1) [8].

7. Enzymatic Profile in Keratoconus

MMP-9 is a gelatinase that belongs to the metalloproteinases family and is responsible for degrading the denatured collagen fibrils. Multiple studies have shown a positive correlation between MMP-9 and a multitude of diseases, such as keratoconus, herpetic keratitis, and Sjogren's syndrome, which all have an inflammatory component [52].

In keratoconus, MMPs are found to be overexpressed in every corneal structure, while TIMP levels are decreased, suggesting the hypothesis of tissue degradation. Lema et al. put the emphasis on subclinical keratoconus, comparing it to manifest keratoconus. They observed increased levels of MMP-9, tumor necrosis factor, and interleukin-6 in the tear film of patients with keratoconus and overexpression of IL-6 and TNF-alpha in the tears of subclinical keratoconic patients. As a conclusion of this study, they demonstrated the

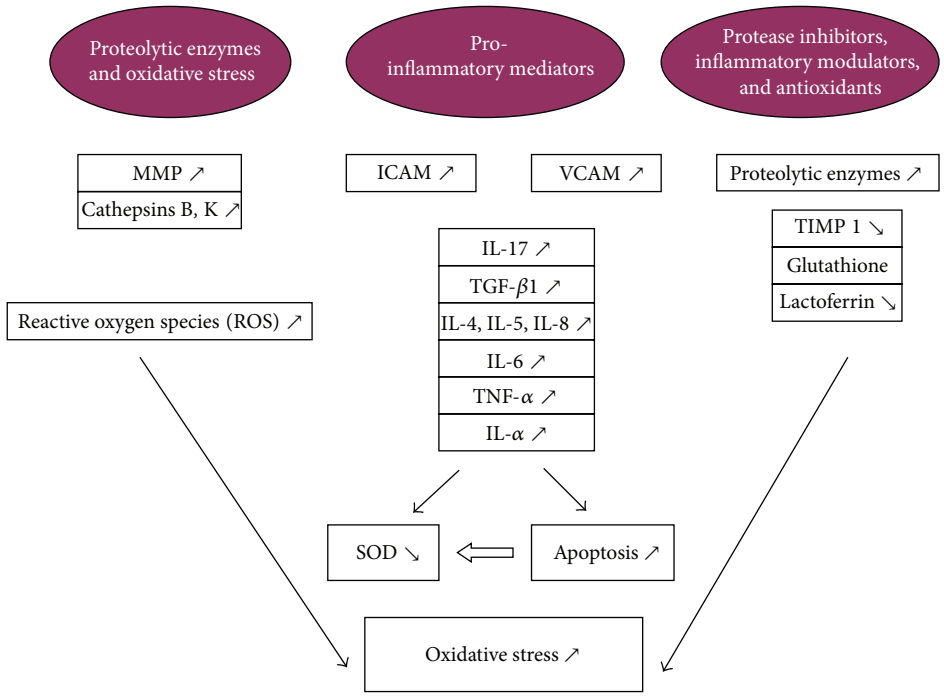

Figure 1: Adapted by Galvis et al. [8], illustrating the interplay between cytokines, proteases, and antioxidants and their complex multidirectional actions.

possibility of a significant progression risk towards bilateral disease [33]. Seppala et al. showed through immunohistochemical labeling that the extracellular matrix metalloproteinase inducer CD147 and MMP-1 are overexpressed in keratoconus, being responsible for degrading the fibronectin, membrane glycoproteins, and types I and III collagen [53].

Collier focused on immunohistochemistry and observed that MMP-14 had increased levels in the corneal epithelium and stroma. MMP-14 could overexpress MMP-2, thus activating the digestion of type IV collagen lamellae [54].

Mackiewicz et al. labeled corneal enzymes that have a potential of digesting collagen and observed first of all MMP-13 upregulation and a high presence of cathepsin K and human trypsin-2 in keratoconic patients compared to the control group. Once again, the importance of gelatinase A (MMP-2) and MMP-14 in corneal remodeling and the fact that increased levels of those enzymes could be a sign of deficient healing were ascertained [55].

8. Dysregulation of Oxidative Status in Keratoconus

A key role of the cornea is to neutralize free oxygen radicals and oxidants that are produced constantly by ultraviolet light and cellular metabolites. Oxidative stress begins to gain importance in the pathophysiology of glaucoma, age related macular degeneration, retinopathy of prematurity, and keratoconus [56, 57]. The main factors that protect the ocular tissue against oxidative damage are superoxide dismutase (protection against superoxide radicals), low molecular weight antioxidants such as ascorbic acid, ferritin, and glutathione, and high molecular weight antioxidants (catalase and glutathione peroxidase) [8, 57].

In order to predict the oxidative stress, the total oxidant and antioxidant status are measured, as well as the ratio OSI (oxidative stress index) between these two parameters [58]. Toprak et al. were the first ones to reveal that oxidative stress could be a predisposing factor for keratoconus. A higher OSI is an indicator of progression of keratoconus [59]. The balance between the formation of free radicals and their removal by antioxidants is altered in keratoconic corneas, which have a lower content in glutathione. Therefore, the final outcome is an accumulation of aldehydes and peroxynitrites that have a destructive, cytotoxic effect on the tissue [60, 61]. Olofsson and coworkers demonstrated that the upregulation of interleukin-1 alpha reduces the synthesis of superoxide dismutase and harms the normal antioxidant barrier [62]. Kenney et al. concluded that overexpression of cathepsins triggers the production of hydrogen peroxide. A decreased level of TIMP-1 that has antiapoptotic actions is responsible for the destruction of stromal architecture. TIPM-3 however has proapoptotic characteristics. This imbalanced ratio could shift the effect in favor of keratocytes apoptosis in keratoconic corneas [63].

9. Therapeutic Options in Keratoconus

Optical correction in early stages of the disease can be achieved with spectacles, with soft contact lenses, or in more advanced stages with rigid gas permeable, scleral, or hybrid lenses [64]. Another therapeutic minimal invasive option is collagen cross-linking that aims to halt the progression of

keratoconus by increasing the collagen fibrils' rigidity using riboflavin as photosensitizer and UVA light. Conventional protocol at 3 mW/cm^2 for 30 min (5.4 J/cm^2 energy dose) releases reactive oxygen species (ROS) that induces corneal stiffness. Another way is to shorten the duration of the procedure by increasing the intensity. Both are continuous light treatments. Recent studies emphasize the important role of tissue oxygenation, thus introducing the new protocol of pulsed light accelerated cross-linking [65].

Beside the minimal invasive therapy by corneal cross-linking, there are also surgical treatment modalities in keratoconus. In patients with advanced disease, it may be necessary to perform corneal transplantation, either a deep anterior lamellar keratoplasty (DALK) or penetrating keratoplasty (PK). DALK is a procedure based on a perfect dissection plane between the Descemet membrane and the deep stromal layer using basic salt solution or air in order to remove the corneal stroma below the Descemet membrane. In advanced stages of keratoconus is penetrating keratoplasty, which is indicated if Descemet membrane's or the corneal epithelium's integrity is altered. Intrastromal corneal ring segments (ICRSs) represent also an option in keratoconus. These are made of PMMA (polymethyl methacrylate) and are implanted in the deep corneal stroma in order to modify the corneal curvature [2, 66].

Kenney et al. proposed in his study the potential benefit of using ultraviolet light protection in order to prevent the oxidative mechanism that could have a negative impact on the progression of keratoconus [67]. Another hypothesis concerning new therapeutic management in keratoconus is the one suggested by Cheung et al., according to which riboflavin could have a constructive effect on the extracellular matrix and downregulate ROS [68].

Regarding the possible inflammatory etiology of the disease, Shetty et al. proposed Cyclosporine A, an immunosuppressant drug with strong anti-inflammatory characteristics, as a potential therapeutic option in keratoconus. In this study, 27 eyes with increased inflammatory biomarkers in the tear film were treated with topical Cyclosporin A. After 6 months, a clear downregulation of MMP-9, IL-6, and TNF-alpha was observed, as well as an important local flattening and reduction of corneal curvatures measured by corneal topography [69].

Priyadarsini et al. investigated the TGF-beta signaling in the pathogenesis of keratoconus on isolated human keratoconic cells from patients with advanced disease who underwent corneal transplantation. TGF-β1, TGF-β2, and TGF-β3 are TGF-β isoforms with a key role in extracellular matrix reorganization, keratocytes' differentiation to myofibroblasts, and activation of matrix metalloproteinases. While TGF-β1 and TGF-β2 have a profibrotic activity as a reaction to an injury, TGF-β3 is responsible for the antifibrotic effect. The study showed that TGF-β3 has the capacity to lower the levels of the key receptor TGF-βRII and as a result to ameliorate the profibrotic component of keratoconic human cells. Very important elements of the TGF-β pathway are the SMAD proteins, which are modified in keratoconus, thereby altering the signaling that could lead to accentuated fibrosis of corneal tissue in the process of wound healing. Priyadarsini et al. suggest that control and regulation of TGF-β receptor could be a new therapeutic option in the treatment of keratoconus [70].

10. Conclusion

The pathogenesis of keratoconus is still poorly understood. Until a few years ago, keratoconus has been defined as a degenerative, noninflammatory disease due to the absence of both corneal neovascularization and inflammatory cells infiltration. Mcmonnies explained in his study the consequences of eye rubbing in patients with keratoconus. The rubbing related corneal trauma could increase the corneal temperature, overexpress the levels of proinflammatory cytokines and proteinases in the tear film, and cause epithelial thinning with repercussions on every layer of the cornea [71].

In the future, the tear proteomics in keratoconus will be studied intensively to identify specific biomarkers for prevention or early diagnosis and new therapeutic options. We have now the information to state that keratoconus is a complex disease with a multitude of factors including genetic, environmental (external), and microenvironmental components.

We conclude that a key role in the pathogenesis of keratoconus is the altered balance between inflammatory cytokines, proteases, and proteases inhibitors, as well as free radicals and oxidants [8].

After reviewing the most relevant and recently published results, we emphasize the contribution of the altered signaling pathway of proinflammatory mediators in the pathogenesis of keratoconus and their role in the disease progression. The measured interleukins and metalloproteinases are biomarkers, although not sensitive nor specific for keratoconus. There are ongoing studies that try to identify a specific biomarker for early detection of the disease. In the future, such biomarkers could improve the therapeutic outcome [72].

Competing Interests

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

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Review Article

Tyrosine Kinase Receptor Landscape in Lung Cancer: Therapeutical Implications

A. Quintanal-Villalonga,[1,2] Luis Paz-Ares,[1,2] Irene Ferrer,[1,2] and S. Molina-Pinelo[1,2]

[1] *Medical Oncology Department, Hospital Universitario Doce de Octubre, 28041 Madrid, Spain*
[2] *Centro Nacional de Investigaciones Oncológicas (CNIO), 28029 Mardid, Spain*

Correspondence should be addressed to Irene Ferrer; iferrer@ext.cnio.es and S. Molina-Pinelo; pinelo_sonia@hotmail.com

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Lung cancer is a heterogeneous disease responsible for the most cases of cancer-related deaths. The majority of patients are clinically diagnosed at advanced stages, with a poor survival rate. For this reason, the identification of oncodrivers and novel biomarkers is decisive for the future clinical management of this pathology. The rise of high throughput technologies popularly referred to as "omics" has accelerated the discovery of new biomarkers and drivers for this pathology. Within them, tyrosine kinase receptors (TKRs) have proven to be of importance as diagnostic, prognostic, and predictive tools and, due to their molecular nature, as therapeutic targets. Along this review, the role of TKRs in the different lung cancer histologies, research on improvement of anti-TKR therapy, and the current approaches to manage anti-TKR resistance will be discussed.

1. Introduction

Lung cancer is responsible for most cases of cancer-related deaths [1, 2]. This pathology is a heterogeneous disease and can be histologically classified into two major different groups: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC accounts for 85% of the primary lung carcinomas [3] and yields the highest mortality rate of malignant tumors worldwide. Within this group of NSCLC we can find several subhistologic groups, of which the most common are adenocarcinoma (ADC) and squamous cell lung cancer (SCC). The majority of patients are clinically diagnosed at advanced stages, with a 5-year survival rate of 15% [4]. For this reason, the identification of oncodrivers, novel therapeutic targets, and clinically relevant predictive or prognostic biomarkers for this disease is of high importance.

The development of technology has made the analysis of high amounts of samples feasible through the so-called high throughput techniques. Regarding cancer, these techniques have allowed the identification of key biomarkers with translational relevance in lung cancer. Genomics, transcriptomics, miRNAomics, epigenomics, proteomics, metabolomics, lipidomics, glycomics, and many other "omics" techniques have been used to decipher the molecular pathogenesis of this disease. A proposed workflow for this aim through the use of the "omics" is shown in Figure 1. The first step would be the identification of candidate specific biomarkers, which will be differentially expressed among different experimental or clinical conditions. Different kind of biological samples, such as tumor tissue, cell lines, or biological fluids, can be used in this step. Then, the identified biomarkers must go through technical and biological validations that will confirm preliminary results. If a specific biomarker has the potential to be therapeutically targeted, clinical trials can be subsequently carried out to establish the security/efficacy of one certain drug against molecule target. Additionally, retrospective studies involving patient samples and clinical data can be carried out to support the role of biomarker.

The application of high throughput techniques in lung cancer has thus identified many gene alterations with a potential oncogenic role in this pathology. Many of these alterations take place in tyrosine kinase proteins, which integrate the so-called "kinome". Among them, the tyrosine kinase receptors (TKRs) (Table 1) are especially relevant in this pathology.

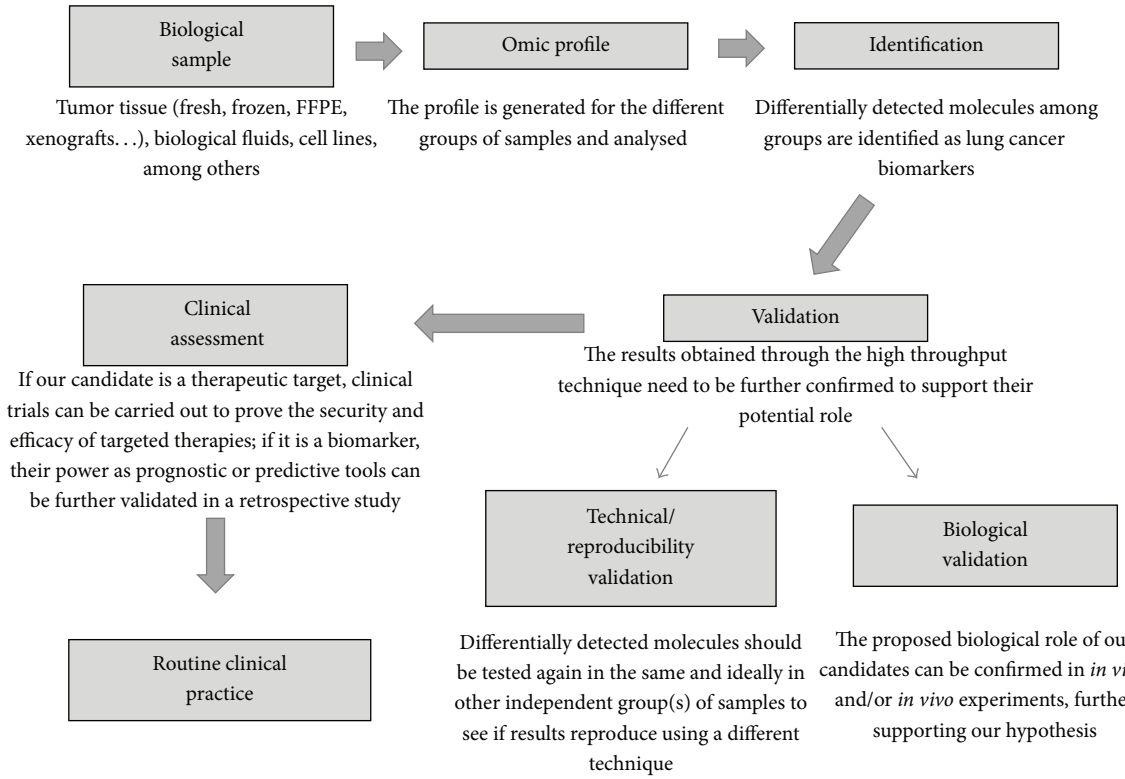

Figure 1: Workflow of the identification and validation of biomarkers and therapeutic targets through omics techniques.

These kinds of receptors have a common molecular structure, involving three modules with a different function: the extracellular domain, able to bind the receptor ligands; the transmembrane domain, which inserts the receptor in the plasma membrane; and the intracellular domain, which is the one with the tyrosine kinase activity [12]. Under physiological conditions, tyrosine kinase receptors bind to their ligands, which produce receptor dimerization and transactivation [13]. Transactivation occurs through the phosphorylation of concrete amino acid residues in each receptor, which allows the binding and activation of effectors, directly or indirectly through scaffold proteins. There are several cancer-related signalling pathways which are activated in TKR signalling, like PI3K/AKT, RAS/MAPK, STAT, or PLCγ1 [14]. The activation of these downstream effectors will at the end modify different aspects of cell behaviour, like proliferation, cell survival and metabolism, cell migration, and control of cell cycle, among others [13, 15]. The activation of TKRs depends thus on ligand binding upon normal conditions, and it is regulated through different feedback mechanisms. Some examples of these are the action of phosphatases which dephosphorylate and thus deactivate the receptor [13] or mechanisms involving receptor internalization and degradation [16]. However, different molecular mechanisms cause uncontrolled TKR signalling, leading to carcinogenesis. Some examples of those are mutations, gene amplification, and overexpression inducing ligand-independent receptor dimerization, or malfunctioning of TKR signalling regulation mechanisms [17]. Along this review, we will discuss the importance of TKRs in lung cancer and their relevance in the therapeutical management of this disease.

2. Importance of TKRs in Lung Cancer

Alterations in TKRs have been detected in every histological type of lung cancer (Table 1, Figure 2), with a potential role in the development of this disease.

2.1. TKRs in Lung Adenocarcinoma. There are well characterized lung cancer driver oncogenes, especially in ADC. In this lung cancer histology, mutations in KRAS and EGFR and ALK translocations account for the 15–25%, 10–35%, and 2–5% of cases, respectively, the two latter being TKRs [18]. Epidermal growth factor receptor (EGFR, HER1) is part of a family of four TKRs (HER1–4) involved in the pathway of epidermal growth factor (EGF). Some identified somatic activating mutations on this gene, like deletion del19E746-A750 and the point mutation L858R, were found to be a good prognostic biomarker. These mutations have been associated with a good response to EGFR-tyrosine kinase inhibitors (TKIs). Some of these inhibitors, such as erlotinib, afatinib, and gefitinib, have been approved for clinical use mainly as first/second treatment line for EGFR-mutated adenocarcinoma patients [19–21]. The most frequent mutations detected in EGFR are located in exons 19 and 21 and are present in 45% and 41% of EGFR-mutated tumors, respectively [22]. These mutations cause the constitutive

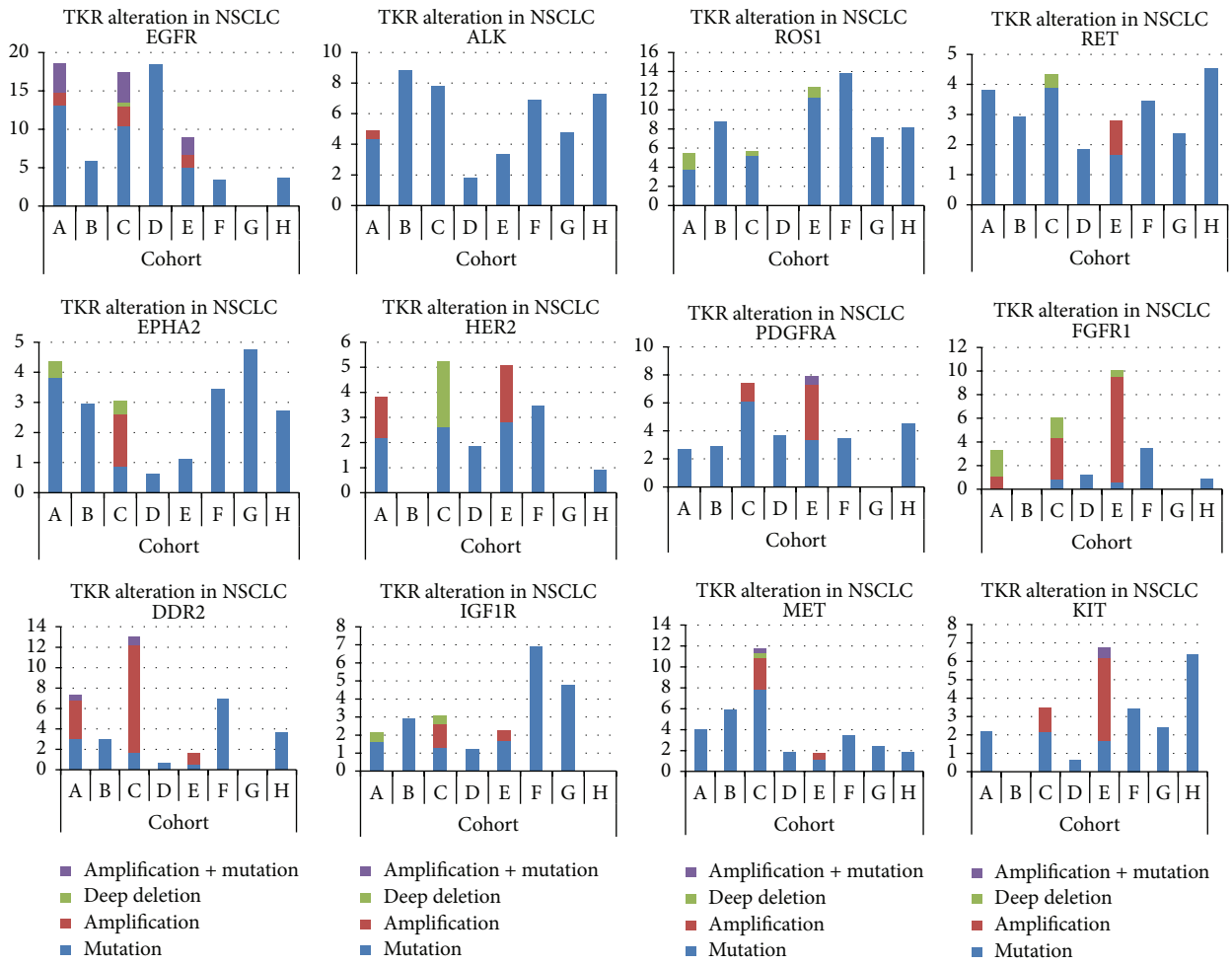

Figure 2: TKR alterations in lung cancer studies. Graphs showing the frequency of alterations in TKRs of relevance in the different lung cancer histologies found in different studies publicly available at http://www.cbioportal.org/. The different studies are designated by capital letters: (A) Imielinski et al., 2012 [5]; (B) MSKCC (Memorial Sloan Kettering Cancer Center) 2015; (C) TCGA, 2014 [6]; (D) Ding et al., 2008 [7]; (E) TCGA, 2012 [8]; (F) Peifer et al., 2012 [9]; (G) Rudin et al., 2012 [10]; and (H) George et al., Nature 2015 [11]. Only studies A, C, and E have information about copy number alterations. ADC: lung adenocarcinoma; SCC: lung squamous cell carcinoma; SCLC: small-cell lung cancer.

activation of the receptor resulting in uncontrolled EGFR signalling [23]. Independently from the role of EGFR in the membrane, where it activates its associated signalling pathways via ligand binding or as a cause of overexpression or mutation, EGFR is internalized to the nucleus. Once in the nucleus, EGFR is capable of acting as a coactivator for several oncogenes as Cyclin D1, nitric oxide synthase, Aurora Kinase A, c-Myc, and B-Myb [24]. Furthermore, nuclear EGFR promotes DNA replication and repair through its association to proliferating cell nuclear antigen (PCNA) [25] and DNA dependent protein kinase [26]. Interestingly, nuclear EGFR could also be involved in resistance to several cancer therapies like cetuximab, gefitinib, and even radiation and chemotherapy [27]. Other studies have focused on the potential role of EGFR as biomarker in noninvasive patient samples. Many of these have shown the feasibility and potential of EGFR mutation determination in circulating free DNA from peripheral blood samples. These studies show that there is a good correlation between tumor tissue and blood samples EGFR mutation status [28, 29]. Furthermore, circulating free DNA EGFR mutation status has been associated with clinical outcome to EGFR-TKI treatment [30, 31]. These works provide evidence that noninvasive samples can be used to detect EGFR activating mutations [32].

The second most important altered TKR in lung adenocarcinoma, ALK (Anaplastic Lymphoma Kinase), is a transmembrane TKR integrated in the insulin receptor superfamily. This gene is susceptible to suffer a rearrangement resulting in a fusion protein together with the echinoderm microtubule-like protein 4 gene (EML4), which is involved in the correct microtubule formation. This fusion protein consists of the intracellular tyrosine kinase domain of ALK and different truncations of EML4, resulting in different fusion protein variants [53, 54]. These gene rearrangements have been detected in NSCLC [55] and seem to be not mutually exclusive with EGFR and KRAS alterations [33,

Table 1: Summary of prevalence of TKR molecular alterations, divided by the main lung cancer histologies, and examples of TKIs available for every alteration.

Alteration	NSCLC		SCLC	TKI available
	ADC	SCC		
EGFR mutation	10–15% [33]	5% [34]	<5% [35]	Erlotinib, Afatinib, Gefitinib, AZD9291, rociletinib
EGFR vIII mutation	Very rare [36, 37]	—	5% [36, 37]	HKI-272
HER2 overexpression	5–9% [38]	—	3–5% [38]	Afatinib, Neratinib, Trastuzumab
HER2 mutation	2% [38]	—	1% [38]	
HER2 amplification	0,9% [38]	—	—	
FGFR1 amplification	1–3% [39]	4–6% [34]	20% [40, 41]	BGJ398, AZD4547, JNJ-42756493
FGFR rearrangement	Very rare [42]	—	1% [42]	
MET amplification	3–21% [43–45]	2% [43–45]	3–21% [43–45]	Crizotinib, Tivantinib
MET mutation	2% [38]	—	1% [38]	
DDR2 mutation	1% [46–48]	—	4% [46–48]	Dasatinib
ALK rearrangement	2–7% [33]	—	1% [49]	Crizotinib, alectinib, Ceritinib
ROS1 rearrangement	1,7% [50]	—	—	TAE684
RET rearrangement	0,9% [7, 51]	—	—	Vandetanib, ASP3026, Cabozantinib, Foretinib
KIT mutation	—	6% [34]	—	Axitinib, Imatinib
PDGFRA amplification	3.8% [52]	2% [34]	8.7% [32]	Crenolanib

NSCLC: non-small-cell lung cancer; ADC: adenocarcinoma; SCC: squamous cell carcinoma; SCLC: small-cell lung cancer.

49, 56]. Currently, there is a first-generation FDA approved therapy for locally advanced and metastatic NSCLC patients harbouring this rearrangement, called crizotinib [57, 58]. Furthermore, there is clinical evidence that patients treated with crizotinib show higher efficacy when compared to pemetrexed-plus-platinum chemotherapy [59]. The other most important alteration in lung cancer, KRAS mutation, does not occur in a TKR gene. However, there are studies showing the relevance of TKRs in KRAS-dependent NSCLC biology and therapy. One recent example of the involvement of TKRs in KRAS mutated lung adenocarcinoma is DDR1. DDR1 is a tyrosine kinase receptor that is activated by several types of collagen. In a recent work, DDR1 TKR was found to be overexpressed in hyperplastic tissue in a lung KRAS-mutant mouse tumor model. The genetic silencing and pharmacological inhibition of this gene impaired the tumor initiation and progression. Furthermore, in KRAS-mutant patient-derived lung xenografts treated with a combination of DDR1 and Notch signalling inhibitors, a similar efficacy as compared to standard chemotherapy was achieved [60].

Apart from these three oncodrivers, accounting for an important percentage of lung cancer cases, new molecular alterations in TKRs associated with oncogenicity have been recently described. One example of altered TKR is the ROS1 gene, which has been proved to be involved in lung cancer. This receptor belongs to the subfamily of tyrosine kinase insulin receptor genes. ROS1 fusions were detected as a potential oncodriver in a NSCLC cancer patient (with the CD74-ROS1 rearrangement) [61]. The ROS1 kinase domain in these fusion proteins is constitutively active and presents sensitivity *in vitro* to TKIs like TAE684 [62]. The analysis of the clinicopathological characteristics of a patient cohort showed that ROS1-positive patients, with an incidence of 1,7%, integrate a genetic subtype of NSCLC with similar characteristics to ALK-positive patients [63].

Another case of oncodriver TKR is RET, which is a tyrosine kinase receptor for the GNDF-family ligands (GFLs). A RET translocation (KIF5B-RET) was first identified by whole genome and transcriptome sequencing of tumor tissue from an adenocarcinoma patient in an advanced stage [64]. After that, several research groups have reported the presence of these fusions in patients who integrate a new molecular subset of lung cancer with similar characteristics to ALK-positive and ROS1-positive patients [65, 66]. Furthermore, the oncogenic potential of these fusions has been proved in NIH3T3 and Ba/F3 cells [65, 66]. Since their discovery, RET fusions have been reported in an increasing number of patients, comprising 1-2% of NSCLC patients, and they show mutual exclusivity with other known driver oncogenes [65].

Thanks to Next Generation Sequencing (NGS) and Fluorescence *In Situ* Hybridization (FISH) techniques, an oncogenic fusion involving another TKR, NTRK1, was identified in 3 ADC patients with no known oncogenic alterations in a work involving 91 ADC patients [67]. Furthermore, it has been reported that this TKR can be successfully targeted *in vitro*, as drugs like lestaurtinib, ARRY-470, and crizotinib have proved efficacy in Ba/F3 cells expressing NTRK1 fusion proteins [67].

Another TKR which has proved to be of relevance in lung ADC is ERBB2. ERBB2 (HER2) is part of the ERBB family of receptor tyrosine kinases, as EGFR. Constitutive activation of this TKR through amplification and mutation has been reported in NSCLC [68], where exon 20 insertions in this gene are common [69, 70]. *In vitro* experiments show that cell lines harbouring exon 20 insertions in this gene are sensitive to trastuzumab and to the EGFR/ERBB2 dual inhibitors afatinib and neratinib [50, 71, 72].

Furthermore, many works have been carried out to identify novel oncodrivers in adenocarcinoma with the help of high throughput technologies. In a collaborative work 188 human lung adenocarcinomas DNA samples were sequenced for 623 genes with a potential role in cancer. This analysis revealed more than 1000 somatic mutations which occurred preferably in 26 genes, 30% of which were TKRs. Two of those were ERBB3 and ERBB4, from the same receptor family of ERBB1 (EGFR) and ERBB2. In ERRB4, a total of 9 mutations were detected. From those, two were located on the protein kinase domain and five around the receptor ligand binding domain. In ERBB3, 3 mutations were found in the ligand binding domain. In another tyrosine kinase receptor from the ephrin family, EPHA3, 11 mutations were found in the extracellular and kinase domains. One of those mutations found in EPHA3 kinase domain, K761N, is located at a highly conserved position analogous to the mutation K641 in FGFR2. A significant number of mutations were also identified in VEGFR and FGFR family member, especially in KDR and FGFR4, where four and three tyrosine kinase domain mutations were found, respectively [7]. In another study, 20 cases of NSCLC patients with no previously identified EGFR mutations were selected for NGS. Mutations were found in MET, FGFR3, and ERBB4 and two previously undescribed EGFR mutations were reported. Furthermore, pathogenic mutations were also reported in VEGFR2, FGFR2, and RET [51].

2.2. TKRs in Lung Squamous Cell Carcinoma. As discussed in the latter paragraphs, during the last years, the most actionable TKR oncogenic mutations have been described in ADC. However, research on tumor biology of SCC has not resulted in as good results as in ADC at a therapeutical level so far. For that reason, efforts are currently being carried out to identify new oncogenes involved in the development of lung tumors of this histology. One of the most interesting TKRs in lung SCC is FGFR1. FGFR1 is part of the type 4 family of TKRs and has the ability to regulate proliferation via the MAPK and PI3K pathways, similarly to EGFR. A screen of SCC samples detected focal amplifications of the FGFR1 gene [73]. This alteration is characteristic of lung SCC, with 21% cases harbouring this amplification. FGFR1 has proven to be a potential oncogenic driver *in vitro*, where FGFR1-amplified cell lines have shown dependency on FGFR1 [73–75]. However, the response rates observed in FGFR therapy in SCC are not as promising as in EGFR or ALK-directed therapy in ADC. There is increasing evidence that this may be due to the lack of correlation between FGFR1 DNA amplification and mRNA and protein expression, so

that cell lines with low FGFR1 expression are insensitive to FGFR inhibitors even if they harbour FGFR1 amplification [38, 76]. FGFR-TKI-therapy is currently under development, with small molecule inhibitors like PD173074, which inhibit the growth of FGFR-amplified lung cancer cell lines and xenograft models [74, 75]. Another member of the FGFR family, FGFR2, has been identified as an interesting target in a subset of lung SCC patients. This gene is altered in 4-5% patients of NSCLC [77] and ongoing and recently completed clinical trials are going to test their potential role as therapeutical target in patients.

DDR2 is another receptor tyrosine kinase which has proven to be of relevance in SCC. This receptor binds to collagen in the extracellular matrix and regulates proliferation and migration. Mutations in DDR2 have been identified in this histologic subtype [78], suggesting a potential oncogenic role for this gene. Furthermore, an *in vitro* study has found out that reduced proliferation after DDR2 silencing or dasatinib treatment is produced in DDR2-mutant cell lines [78]. Several studies propose an incidence of DDR2 mutations of approximately 3-4% in SCC patients and although no specific anti-DDR2 therapy has been developed, ABL kinase inhibitors such as dasatinib or imatinib display activity against DDR2 [78–81]. Two studies have reported tumor shrinkage after treatment with dasatinib in SCC patients with the S768R DDR2 mutation [40, 78]. However, there is still some controversy about the role of DDR2 in tumorigenesis. This is because the DDR2 ligand, collagen, accumulates during lung tumor progression [39, 41]. However, collagen inhibits cancer cell growth through DDR2-dependent cell cycle arrest in some kinds of cancer [82, 83]. Furthermore, DDR2 mRNA levels are reduced in lung tumor as compared to matched nontumoral tissue [84]. All of this data suggests a possible context-dependent role for DDR2 in lung tumorigenesis, which needs to be further studied.

In the last years, many other TKRs are gaining attention in the study of the oncogenesis of SCC. One example is the insulin-like growth factor receptor 1 (IGF1R), which is involved in proliferation and inhibition of apoptosis [42, 46]. There is evidence of the oncogenic role of IGF1R in lung cancer, with especial relevance in SCC [47]. Several studies proposed that high-level expression of IGF-R1 is characteristic of SCC and can act as a prognostic indicator [48]. Another one is PDGFRA, a TKR from the family of the platelet-derived growth factor receptors involved in tumoral angiogenesis [85]. PDGFRA is frequently expressed in the tumor stroma, as well as in cancer cells, and its activation has been reported in 13% of NSCLC patients [61]. Alterations in this gene have been reported mainly in SCC [86]. On the other hand, a member of the Eph family of receptors, *EphA2,* was shown to be a relevance biomarker in SCC, where it promotes invasion, cell motility, and angiogenesis through the activation of *Src* [87, 88]. *EphA2* mutations are rare in NSCLC but are mainly present in SCC [89].

EGFR vIII, a mutated form of EGFR found in SCC, harbours deletion in exons 2–7. This EGFR variant is not present in normal tissues and causes uncontrolled cell growth in tumors [90]. Furthermore, there is *in vivo* evidence of the oncogenic role of EGFR vIII in a NSCLC murine model and of the efficacy of an EGFR inhibitor, HKI-272, in this model [91]. Several studies have detected this EGFR variant in 2–5% of SCC patients, but not in ADC [91, 92].

2.3. TKRs in Small-Cell Lung Cancer. The molecular pathology of small-cell lung cancer has not yet been addressed as much as in non-small-cell lung cancer. However, some molecular alterations with the potential to be oncodrivers in this lung cancer histology have been identified. In one recent study, DNA from 98 SCLC tumors was sequenced and analysed for genomic alterations. Mutations in EGFR (5% of cases) and KIT (6%) and amplification of FGFR1 (4%), EPHA3 (3%), PDGFRA (2%), and MET (2%) were detected, suggesting that these TKRs could have a role in lung SCLC oncogenesis [11, 93]. But probably the most studied TKR in this lung cancer histology is FGFR1. This FGFR has been suggested as an oncodriver in SCLC [11, 94]. High-level expression of FGFR1 has been found in SCLC patients as compared to healthy individuals. Elevated expression is associated with advanced stage and poorer overall and recurrence-free survival [95]. In another study involving an Asiatic SCLC patient cohort, FGFR1 amplification correlated with poorer disease-free survival to first-line chemotherapy [52]. Furthermore, there is *in vitro* and *in vivo* evidence that anti-FGFR therapy is effective in FGFR1 amplified SCLC [96, 97]. Other TKRs are often overexpressed in SCLC and could have protumorigenic effects in this lung cancer subtype. IGF-1R protein levels have been reported to be high in 95% of SCLC cell lines [98, 99]. VEGFR high levels have been reported in SCLC patients and related to higher tumor stage, disease progression, chemotherapy resistance, and poorer outcome [98].

2.4. Other TKRs in Lung Cancer. Some TKR alterations are not specific of one concrete lung cancer histology. The TKR MET has proved to be of relevance in NSCLC after the large scale molecular profiling work by The Cancer Genome Atlas (TCGA) in lung ADC [100]. MET alterations were found in 7% of tumors and were mutually exclusive with other known oncogenes, supporting the role of MET as an oncogene. The most common alterations for this gene are overexpression, amplification, and exon 14 skipping [36, 101, 102]. In one study involving lung cancer patient samples, they found a correlation between Notch-1 and c-MET coexpression and a poorer prognosis. They also found an association between MET expression and advanced stage [37]. Currently there are many MET-targeted drugs in clinical development, such as small molecule inhibitors, molecules which prevent the binding of MET to its ligand HGF, and monoclonal antibodies [34, 103]. However although some of these drugs have demonstrated high efficacy *in vitro,* clinical trials results have been disappointing [104, 105]. Nonetheless, further trials are currently in progress, aiming to get better results by a better patient selection [106]. Apart from MET, it has been recently reported that the VEGFR receptor family could have a prognostic potential in lung cancer. In a meta-analysis covering 74 studies with a total of 7631 patients, it was reported that VEGFR1 expression is an indicator of

poor prognosis in NSCLC. In this study, it was observed as well that combined high expression of VEGFR2 and VEGFA, or VEGFR3 and VEGFC, featured discrimination power as prognostic biomarkers [107]. In another study involving surgically resected NSCLC, different patterns of coexpression of HER family receptors have been associated with a shorter disease-free and overall survival [108].

3. Therapy Improvement through Biomarker Integration and Resistance Managing: EGFR Mutations and ALK Translocation

As commented before, acquired resistance to targeted therapy is a relevant problem in clinics. Besides, there are tumors potentially sensitive to a targeted therapy but which show innate resistance. For all these reasons, current efforts are focused on the managing and avoidance of these resistances, as well as on the improvement of eligibility criteria for TKI-therapy.

In the case of EGFR, 20–50% of patients with clinical or biological predictors of anti EGFR-therapy sensitivity do not respond [109]. This primary resistance is associated with EGFR exon 20 insertions [110]. And even if the patient responds to therapy, acquired resistance arises, due to molecular mechanisms like bypass signalling. This mechanism involves the reactivation of downstream signalling pathways via amplification of other TKRs (like MET or HER2) and mutations of downstream members of EGFR-signalling pathway (such as PIK3CA, KRAS, and BRAF) and even through ALK gene rearrangement [43, 44, 111]. Besides, to overcome sensitivity to EGFR-targeted therapies, some other tumors undergo a phenomenon similar to epithelial to mesenchymal transition (EMT), where the tumor can even suffer a change in histology, from NSCLC to SCLC [45, 112].

However, the most frequent cause of acquired EGFR-TKIs resistance, accounting for 50% of resistant cases, is a mutation in exon 20 of the EGFR gene, T790M [113]. Nonetheless, this mutation has been found as well in patients who have not received TKI-therapy [114, 115]. To overcome this resistance mechanism, second- and third-generation EGFR-TKIs have been developed and are currently under clinical trials [116]. The second-generation EGFR-TKIs, like dacomitinib, afatinib, and neratinib, display a higher affinity for the EGFR-tyrosine kinase domain [117]. They are pan-HER inhibitors and active against the T790M mutation. Unfortunately, second-generation TKIs show little activity in tumors which have acquired resistance to first-generation EGFR-TKIs [118, 119]. The third-generation EGFR-TKIs AZD9291 and rociletinib have proved efficacy against the T790M mutation *in vitro* [120] and in two Phase I-II clinical trials [121, 122]. Unfortunately, new generation EGFR-TKI would only postpone the inevitable, as new resistance mechanism will arise. In the case of AZD9291, a resistance mechanism occurring through the C797S mutation has been already identified [123].

Apart from second- and third-generation EGFR-TKIs, other treatment strategies are being developed to overcome acquired resistance. The switching to chemotherapy after resistance has appeared to be the most accepted approach, although there are several retrospective studies with inconsistent results to this respect [124, 125]. Another alternative therapy which is currently under clinical assessment is the combination of EGFR-TKIs and chemotherapy. Up to date, the results on the effectiveness of this combination therapy are not conclusive [126, 127], but ongoing clinical trials on this issue could clarify if this approach could be beneficial for patients with EGFR-TKI acquired resistance. Thanks to the identification of the molecular mechanisms leading to acquired resistance to TKIs, approaches with a more targeted design are being designed [128–132]. Many current research works bet on the combination of an EGFR-TKI with another molecularly targeted agent, for therapeutic tumor resensitization to anti-EGFR-therapy, with interesting preclinical results [133–135].

Furthermore, other more novel approaches aiming at EGFR-therapy resensitization have been recently proposed. A bispecific EGFR/MET antibody, called JNJ-61186372, has recently showed a potent inhibition of EGFR downstream effectors, resulting in tumor regression in NSCLC xenografts [136]. In another recent work, cetuximab delivery through a mesoporous silica nanoparticle (MP-SiO2 NP) suppressed progression of EGFR-therapy-resistant xenografts [137].

On the other hand, the expression of several lncRNAs has been associated with EGFR-targeted therapy resistance, suggesting a potential role for them as predictive and therapeutic biomarkers [138]. In addition, Park et al. found that a low EGFR/MET ratio was also predictive of poor response to anti-EGFR-therapy [139]. Another recent research work involving patients receiving erlotinib therapy has identified TGF-α and high soluble EGFR serum levels as negative and positive response predictive biomarkers to erlotinib, respectively [140].

Furthermore, the evaluation of circulating free DNA from liquid biopsies as a noninvasive method for resistance monitoring is currently under development, and promising results have been obtained for the detection of the T790M, c-MET amplification, and the C797S mutation [31, 141].

For ALK gene rearrangement targeted treatment, as in the case of EGFR-TKI treatment, acquired resistance arises in less than a year after the beginning of the treatment [142]. The best documented acquired resistance mechanisms to crizotinib-based therapy are mutations in the ALK gene [143–145]. These mutations represent the 28% of crizotinib-resistant cases. Some of them take place in the ATP-binding pocket of ALK and others occur distant to the ATP-binding site, but all of them finally reduce the ALK affinity for crizotinib [146]. Many other mechanisms of acquired resistance have been described for this therapy. One of those consists in the amplification of the ALK gene [146], which has been reported in the 18% of patients treated with crizotinib. Another mechanisms of reported crizotinib acquired resistance are KRAS mutations, amplification of KIT, and increased phosphorylation of EGFR [144, 146, 147]. Recently, it has been reported that NSCLC cells can acquire resistance to anti-ALK therapy through the activation of other receptor tyrosine kinases. In this work, two NSCLC cell lines with the ALK translocation were treated with alectinib, a potent and selective ALK inhibitor, and resistant clones were established.

In one of these cell lines, the translocation was lost and increased activation of IGF-1R and HER3 was detected, and when these two signalling pathways were inhibited, cells were resensitized against alectinib. The second alectinib resistant cell line showed MET activation [148]. To overcome these resistance mechanisms, a second-generation ALK inhibitor, ceritinib, has been developed. Ceritinib has been recently approved for patients with acquired resistance or intolerance to crizotinib [149]. However, ceritinib is only active against some of the ALK mutations [144, 146]. Furthermore, a new ALK-targeted drug, alectinib, showed higher potency than crizotinib. This drug was approved in Japan for treatment of recurrent ALK rearrangement NSCLC patients [150].

On the other hand, noninvasive detection of ALK rearrangements has proven to be feasible. In one work, the EML4-ALK translocation has been detected in circulating blood platelets. This is because platelets are able to sequester RNA released into the blood by tumor cells, and this is why the ALK translocation could be found in platelet RNA transcripts [151].

Currently, different novel therapeutic agents with improved characteristics are under evaluation [152] and, as in the case of EGFR, combination therapy approaches are gaining increasing interest in overcoming resistance [57, 153–155]. Again, some efforts are currently being made to understand the molecular biology of ALK therapy resistance, similarly to EGFR [156]. The better understanding of the molecular mechanisms underlying the sensitivity to or ineffectiveness of this therapy will help in the identification of novel predictive biomarkers and even new targets to address.

4. Research on Novel Targets

The recent discovery of genetic alterations on TKRs in patient samples has opened the door to research works aiming to find an appropriate and targeted therapy for subsets of patients with characterized oncogenic alterations.

For ROS1-fusion genes, *in vivo* models have been generated to test the efficacy of ROS1-targeted agents [157, 158]. Two transgenic mouse models have been produced, in which overexpression of CD74-ROS1 or SDC4-ROS1 fusion variants takes place in lung alveolar type II cells. In these transgenic models it was shown that these translocations have oncogenic potential *per se*, and that crizotinib and ASP3026 (an ALK/ROS1 inhibitor) are potentially efficacious therapies to target them [157]. Furthermore, acquired resistance mechanisms have already been identified for crizotinib in ROS1-rearranged patients, like the G2032R mutation [159]. Some TKIs, as cabozantinib and foretinib, seem to be effective against this resistance mutation [146, 160].

RET rearrangements have also been object of interest to study potential targeted therapies in lung adenocarcinoma. As in the case of ROS1, genetically engineered mouse models have been established. In one of these models, the KIF5B-RET fusion was exogenously expressed specifically in lung alveolar epithelial cells, generating multiple tumors in the lungs. In this model, vandetanib, a RET inhibitor approved to be used in thyroid carcinoma, showed antitumor efficacy [161]. This drug is currently under clinical assessment in a phase 2 trial involving NSCLC patients. The potential applicability of some other TKIs has been assessed in preclinical models with appealing results. Some examples are sunitinib and sorafenib, currently in clinical trials [65, 66]. Another example is dovitinib, which has shown *in vitro* and *in vivo* antitumor efficacy in a work involving a cell line harbouring the CCDC6-RET fusion variant and its xenografts. In this work, a mechanism of resistance to dovitinib through the activation of Src was also described, and the use of a Src inhibitor, saracatinib, was proposed to overcome this resistance [162].

In lung SCC, probably the TKR which has attracted the most attention is FGFR1. Regarding anti-FGFR therapy, there has been some controversy about predicting treatment effectiveness. There are some works in which *in vitro* and *in vivo* xenograft models have showed a correlation between efficacy of anti-FGFR therapy and FGFR1 amplification [73, 163]. However, a more recent work has proven that FGFR-TKI sensitivity depends on FGFR1 mRNA or protein expression levels and not on FGFR1 gene amplification [38]. Another member of the FGFR family, FGFR2, has gained interest in this histological subtype. Some preclinical models of FGFR2-driven lung SCC have been established. In one of those, a genetically engineered mouse model expressing a mutated variant of this gene proved to be oncogenic in a p53 deficient background. Furthermore, these FGFR2 mutant tumors were sensitive to FGFR inhibition [77]. Another TKR in which a lot of preclinical work has been developed is DDR2. DDR2 mutation has been associated with clinical response to dasatinib in SCC [40]. In addition, two acquired resistance mechanisms to dasatinib have already been described *in vitro*, that is, the DDR2 T654I mutation and NF1 loss [164]. Currently, novel DDR2 inhibitors with a higher selectivity are under development.

Regarding SCLC, several studies have investigated the role of FGFR1 in preclinical models [96, 97]. The FGFR inhibitor PD173074 appears to inhibit cell growth in several FGFR1-overexpressing cell lines and in cell line xenograft models, comparably to cisplatin treatment [97]. Currently, there are several FGFR TKIs under clinical assessment, some of which are more selective, like AZD4547 or BGJ398, and some of which are more promiscuous, like JNJ-42756493. However, preliminary results from clinical trials have not been very successful.

Certainly, the targeted therapy has shown promising results, but so far the appearance of resistances seems unavoidable. For this reason, many current efforts are focused on therapeutic approaches that delay the appearance of resistances. Ongoing work is assessing the effectiveness of many other options of combination, including a TKI against the same target, but with a different resistance profile [126, 165], and combination with immunotherapy [166], among others.

5. Directions of Future Research and Conclusions

Thanks to the omics techniques and their high throughput capacity of analysis, many alterations with potential involvement in lung cancer have been identified and validated in the

last years, leading to improvements in clinical practice. Many of these aberrations occur in TKRs, inducing a deregulated downstream signalling that leads to tumorigenesis. Due to the functional nature of TKRs, their action can be pharmacologically inhibited, making the TKRs very appealing for research in cancer. Indeed, addressing the TKRs has made very interesting achievements in lung cancer treatment, resulting in the development of targeted therapies that have provided a substantial benefit for patients eligible for those therapies. However, the benefit derived from any targeted therapy is unfortunately transient, due to the development of resistance to these therapies. Current and future research efforts will be focused on understanding the molecular nature of these resistances, aiming to find novel predictive biomarkers of therapy response and new therapeutic approaches that prevent, or at least delay, the appearance of resistances and tumor regression.

Competing Interests

The authors declare no competing interests.

Authors' Contributions

Irene Ferrer and S. Molina-Pinelo have contributed equally to this work.

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Review Article

Circulating MicroRNAs as Potential Molecular Biomarkers in Pathophysiological Evolution of Pregnancy

Dragos Cretoiu,[1,2] Jiahong Xu,[3] Junjie Xiao,[4] Nicolae Suciu,[5,6] and Sanda Maria Cretoiu[1,2]

[1] *Division of Cellular and Molecular Biology and Histology, Carol Davila University of Medicine and Pharmacy, 050474 Bucharest, Romania*
[2] *Victor Babeş National Institute of Pathology, 050096 Bucharest, Romania*
[3] *Department of Cardiology, Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China*
[4] *Regeneration and Ageing Lab, Experimental Center of Life Sciences, School of Life Science, Shanghai University, Shanghai 200444, China*
[5] *Department of Obstetrics and Gynecology, Polizu Clinical Hospital, Carol Davila University of Medicine and Pharmacy, 011062 Bucharest, Romania*
[6] *Alessandrescu-Rusescu National Institute of Mother and Child Health, 020395 Bucharest, Romania*

Correspondence should be addressed to Sanda Maria Cretoiu; sanda@cretoiu.ro

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MicroRNAs represent nonprotein coding small RNA molecules that are very stable to degradation and responsible for gene silencing in most eukaryotic cells. Increased evidence has been accumulating over the years about their potential value as biomarkers for several diseases. MicroRNAs were predicted to be involved in nearly all biological processes from development to oncogenesis. In this review, we address the importance of circulating microRNAs in different conditions associated with pregnancy starting with the implantation period to preeclampsia and we shortly describe the correlation between placental circulating miRNAs and pregnancy status. We also discuss the importance of microRNAs in recurrent abortion and ectopic pregnancy.

1. Introduction

MicroRNAs (miRNAs) are short, single-stranded RNA (19–25 nucleotide long) nonprotein coding genes able to recognize complementary messenger RNAs (mRNAs), acting as master gene regulators by repressing mRNA translation or by mRNA degradation (reviewed in extenso by [1, 2]). MicroRNAs proved to be involved in numerous biological processes from development to oncogenesis [3, 4].

In biomedical research, miRNAs are gaining more and more importance as novel biomarkers for diagnosis, prediction, prognosis, and reaction to therapy. Lately, it became evident that circulating miRNAs might be used as biomarkers for a great number of diseases and in fact represent the forthcoming expectation for a noninvasive diagnostic screening [5, 6]. Nowadays miRNA-bioinformatics tools and databases are used to cope with the huge amount of information since in humans approximately 3707 novel mature miRNAs were identified [7].

After the discovery of miRNAs in 1993 by Lee et al. [8], significant evidence has accumulated about the physiological relevance of miRNAs. Most of the research was focused on the role of intracellular miRNAs which have been shown to regulate genes involved in differentiation, proliferation, and apoptosis [9]. Over time, it has been shown that miRNAs are ubiquitously present in body fluids and might be the mechanism of genetic exchange between cells in a horizontal manner [10]. Circulating or extracellular miRNAs have been shown to be stable and protected from RNase degradation. This protection is achieved by inclusion either in various (lipo) protein complexes (e.g., HDL, Argonaute protein, and nucleophosmin 1) or in different types of extracellular vesicles [11–15].

Weber et al. divided body fluids into two categories depending on the method of harvesting, (a) without any

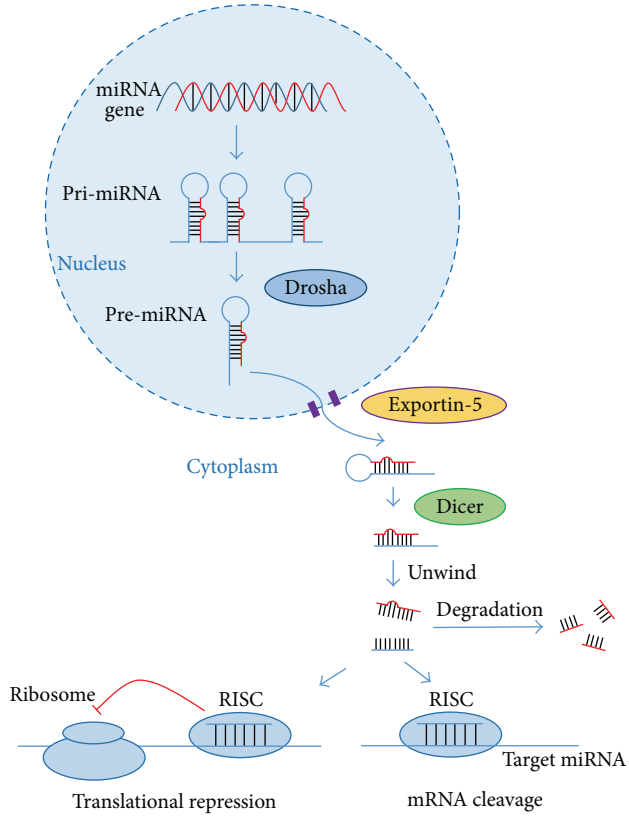

Figure 1: The biogenesis of microRNAs.

invasive means: breast milk, colostrum, saliva, seminal fluid, tears, and urine and (b) acquired by invasive procedures—amniotic fluid, cerebrospinal fluid, plasma, bronchial lavage, pleural fluid, and peritoneal fluid, and concluded that "the composition and concentrations of miRNAs are measurably different among them" [16]. Several circulating miRNAs were proposed as diagnostic biomarkers in human diseases and extensive reviews were written, to name but a few [17–21].

Women's reproductive medicine is also encouraged by the possibility to use circulating microRNA profiles (detection and quantification) for the evaluation of the ovarian function, placental function, uterine receptivity, pregnancy detection, embryonic development, and evolution/complication of pregnancy.

In this review, we will characterize the impact of circulating miRNAs as potential molecular biomarkers in the pathophysiological evolution of pregnancy.

2. The Biogenesis of MicroRNAs

miRNA production begins in the nucleus, with RNA polymerase II-mediated transcription. Using genomic DNA as template and generating a long primary miRNA (known as pri-miRNA, which, by folding, becomes a series of hairpin loops), the double-stranded RNA structure of hairpin loop in pri-miRNA can be easily cleaved by Drosha (a double-strand RNase III endonuclease), with the support from DGCR8/pasha [22]. After 70–90 nucleotides, hairpin structure premature miRNA emerges (known as pre-miRNA). The pre-miRNA can bind to nuclear export factor exportin-5 and be exported to the cytoplasm with GTP hydrolysis [23]. In the cytoplasm, pre-miRNA is cleaved by Dicer (a specific double-strand RNA endonuclease), yielding a miRNA:miRNA* duplex about 22 nucleotides in length [24] (Figure 1). In general, only one can recruit Argonaute in RNA-induced silencing complex (RISC) and work as RNA interference. Another is degraded by RISC.

3. miRNAs in Embryo-Endometrial Cross Talk at Implantation

A successful implantation depends essentially on timing and a dialogue between the free-floating blastocyst and the receptive endometrium and must be regarded as a multilevel, multiscale integrative approach [25]. It is common knowledge that autocrine, paracrine, and endocrine factors are working closely, coordinating their effects during embryo implantation. It has been suggested that, among this multitude of players, miRNAs might also contribute knowing that their expression throughout the menstrual cycle is sex hormone-dependent. This affirmation is supported by a study of Kuokkanen et al. who compared endometrial samples in the midsecretory phase and in the late proliferative phase. They found that the expression of miR-503 was significantly increased in the late proliferative-phase samples compared to the midsecretory phase samples while the expression level of

miR-210, miR-29B, miR-29C, miR-30B, miR-30D, miR193A-3P, miR-200C, and miR-31 was significantly decreased in the late proliferative phase versus midsecretory phase [26]. Several miRNAs were found to be differentially expressed in receptive versus prereceptive human endometria by Altmäe et al. [27]. They concluded that hsa-miR-30b, hsa-miR-30d, hsa-miR-494, and hsa-miR-923 might "play an important role in gene reprogramming at the time of endometrial receptivity" and "could serve as novel biomarkers of fertile receptive endometrium" in the future [27]. Moreover, a prospective analysis conducted on patients who received IVF treatment aimed to determine the effect of higher progesterone level on endometrial receptivity and found four downregulated miRNAs (hsa-miR-451, hsa-miR-424, hsa-miR-125b, and hsa-miR-30b) between normal and elevated progesterone groups that might explain the reduced pregnancy rate in patients with elevated progesterone [28].

There are several studies focusing on the importance of embryo-endometrial cross talk at implantation which seems to be mediated by exosomes released by the endometrium, but none is addressing humans (reviewed by [29]). Exosomes usually contain numerous lipids, proteins, mARNs, and miRNAs [30] and are now considered critical components of uterine luminal fluid [31]. Burns et al. evaluate exosomes in the uterine luminal fluid of sheep and found 81 conserved mature miRNAs emanating from the endometrial epithelia or derived from the conceptus trophectoderm and considered exosomes as essential players important for the establishment and maintenance of pregnancy [31, 32].

Another recent study assessed the role of miR-145 and its target IGF1R in early implantation and showed its involvement in embryo attachment by reducing the level of IGF1R in endometrium and also the importance of the finding in the improvement of pregnancy rates in women with recurrent implantation failure [33].

4. miRNAs in Recurrent Abortion

Recurrent abortion is defined as 2 or more consecutive pregnancy losses before the 20th gestational week or spontaneous abortion of a fetus weighing less than 500 g, affecting 1% to 2% of the reproductive age couples worldwide [34]. Recurrent abortion is extremely difficult to treat and novel therapeutic and diagnosis ways are highly needed [34].

Two variant alleles, namely, rs41275794 and rs12976445, in pri-miR-125a have been identified in recurrent abortion in a Chinese-Han population and these variant alleles would lead to the altered production of miR-125a. The decrease of miR-125a caused by these two variant alleles can cause increased LIFR and ERBB2, two target genes of miR-125a, playing critical roles in the embryo implantation and decidualization [35]. Moreover, the rs6505162 C>A in the miR-423 coding region was also identified to be associated with the occurrence of recurrent abortion. The A allele in the polymorphism rs6505162 could more effectively inhibit proliferation-associated 2 group 4 (PA2G4) than the C allele could [36]. Besides, in the Chinese population, a study in the Korean population has also been conducted. They found that miR-196a2CC, miR-499AG+GG, and the miR-196a2CC/miR-499AG+GG combination were associated with recurrent abortion in a Korean population [37].

Human leukocyte antigen- (HLA-) G confers fetal-maternal tolerance and plays an important role in successful pregnancy [38]. miR-133a was reported to be significantly increased in recurrent abortion villi with normal karyotype and HLA-G is a target gene of miR-133a [38]. In addition, miR-34a, miR-155, miR-141, miR-125a, and miR-125b were found to be increased in the recurrent abortion women, while miR-24 was decreased in decidual natural killer cells [39]. PI3K-Akt, MAPK, focal adhesion, T-cell receptor, estrogen, TGF-β, and actin cytoskeleton regulation signaling pathways were predicted to be regulated by these miRNAs [39]. Moreover, in the villi of recurrent abortion patients, miR-184, miR-187, and miR-125b-2 were upregulated, while miR-520f, miR-3175, and miR-4672 were downregulated [40]. In the decidua of recurrent abortion patients, miR-517c, miR-519a-1, miR-522, miR-520h, and miR-184 were increased [40]. However, the functional role of these aberrant miRNAs in recurrent abortion is unclear.

A recent work has reported the potential of using plasma miRNAs as biomarkers for recurrent abortion [41]. A total of 27 recurrent abortion patients and 28 normal early pregnancies patients were enrolled at 6–10 weeks of gestation. Based on miRNA microarrays and real-time quantitative reverse transcription polymerase chain reaction analysis, a total of 9 miRNAs were found to be increased while a total of 16 miRNAs were decreased [41]. Further studies confirmed that miR-320b, miR-146b-5p, miR-221-3p, and miR-559 were upregulated, while miR-101-3p was downregulated [41]. This study provides the idea that these circulating miRNAs might be biomarkers of recurrent abortion though the ROC curve has not been performed in the study and the results also need to be validated in an independent cohort.

5. miRNAs and Ectopic Pregnancy

Ectopic pregnancy (EP) is defined as conceptus implants outside the endometrial cavity [42, 43]. Although EP occurs in only about 1% to 2% of pregnant women, it is highly detrimental to patients usually leading to tubal rupture and death [44]. Current diagnosis of EP depends on transvaginal ultrasonography and measurement of serum human chorionic gonadotropin (hCG) and progesterone [45]. Owing to the fact that clinical ultrasonography is not always definitive and that serial hCG and/or progesterone assessment is associated with high false-positive and false-negative rates, searching for the novel noninvasive circulating biomarkers for detecting EP is highly important [46].

miRNAs are considered as potential biomarker candidates for multiple pregnancy-associated diseases [47, 48]. Previous studies demonstrated dysregulation of miRNA expressions in early embryonic tissues and in the fallopian tube of women with EP, including Lin28b, let-7, miR-132, miR-145, miR-149, miR-182, miR-196, miR-223, miR-424, and miR-451 [49–51]. However, a limited discovery was obtained with regard to circulating miRNAs as biomarkers for diagnosis of EP [52]. In a multicenter, retrospective, and case-control cohort study, serum levels of hCG, progesterone, and a group

of pregnancy-associated miRNAs were analyzed in women with EP, spontaneous abortion (SA), and viable intrauterine pregnancy (VIP) [53]. Data from this study demonstrate that concentrations of serum miR-517a, miR-519d, and miR-525-3p were significantly lower, while the concentration of serum miR-323-3p was higher, in women with EP and SA than in VIP. Among these miRNAs, circulating miR-323-3p has the highest sensitivity when used as a single marker. Furthermore, the combined hCG, progesterone, and miR-323-3p show even higher sensitivity and specificity when compared to each use alone, suggesting that miR-323-3p might be a useful biomarker to improve the diagnosis of EP [53]. In another independent population study, evidence was also gained that circulating level of miR-323-3p could distinguish EP cases from SA cases [54]. Further studies were needed to elucidate the underlying mechanisms by which miRNAs cause the clinical manifestations of EP.

6. Placental Circulating miRNAs and Pregnancy Status

Chim et al. showed high maternal plasma concentration of four placental miRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) which fell off in postdelivery plasma indicating a direct correlation with pregnancy status. Moreover, miR-141 concentration increased with gestational age [55]. This preliminary study is suggestive for the potential of miRNAs as molecular markers for pregnancy monitoring and diagnosis.

Kotlabova et al. demonstrated that seven placental specific microRNAs were present in maternal plasma, miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a, and might be pregnancy-associated microRNAs with diagnostic potential [56].

A comparative study determined plasma concentrations of cell-free, pregnancy-associated, placenta-specific microRNAs between nonlabor and labor groups (including 32 women) and found that miR-515-3p, miR-517a, miR-517c, and miR-518b placenta-specific miRNAs in the labor group were significantly higher than those in the nonlabor group before cesarean section and at 24 hours after delivery, respectively [57].

Miura et al. found that there is a direct correlation between maternal plasma levels of cell-free pregnancy-associated placenta-specific miR-515-3p, miR-517a, miR-517c, and miR-518b and placental weight [58]. Hasegawa et al. identified an association between placenta praevia and cell-free pregnancy-associated placenta-specific miRNAs in maternal plasma [59]. They found significantly higher plasma concentrations of cell-free miR-517a and significantly lower plasma concentrations of cell-free miR-518b in the placenta praevia group comparative with the control group and suggested that the circulating level of cell-free miR-517a may be a predictive marker for the risks of bleeding in late pregnancy and of massive hemorrhage at delivery [59].

These studies identified that a number of circulating miRNAs originating in placental trophoblast layer are a trailblazer in the field of identification of noninvasive markers for placental dysfunction. However, many challenges lie ahead before circulating miRNAs will answer actual clinical and therapeutic needs.

7. miRNAs in (Pre)Eclampsia

Preeclampsia is defined as a specific condition of late pregnancy, 2nd or 3rd trimester, affecting approximately 2–8% of all pregnancies worldwide and is characterized by maternal high blood pressure and high levels of protein in the urine [60]. Despite its gravity, there are no specific biomarkers predictive of the disorder and only a few studies have implicated an altered miRNA expression. The first study addressing miRNAs importance in preeclampsia dates in 2007 when Pineles et al. analyzed placentas from women with preeclampsia and small-for-gestational age and found different subsets of expressed microRNAs (miR-210, miR-182) in patients with preeclampsia [61]. The following studies support the involvement of placental miRNAs in the setting of preeclampsia and showed upregulated expression of miR-496 and lower expression of miR-15b, miR-181, miR-210, and miR-483–5p [62, 63]. Circulating miRNAs levels in plasma from severe preeclamptic pregnancies were first analyzed by Wu et al. who detected and validated seven elevated miRNAs, miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p, as potential markers for diagnosing preeclampsia [64]. Using the next generation sequencing platform of sequencing by oligo ligation detection (SOLiD) and RT-PCR for validation, Li et al. showed in their study that maternal plasma miR-141 and miR-29a were significantly overexpressed, while maternal plasma miR-144 was significantly underexpressed preeclamptic patients compared to normal control suggesting their potential use as preeclampsia biomarkers [65]. Xu et al. carried out a prospective cohort study at gestational weeks 15 to 18 and at term and found low circulating levels of miR-18a, miR-19b1, and miR-92a1 and high levels of miR-210 in preeclamptic patients comparative with normal controls [66]. Luque et al. assessed in a study of the usefulness of circulating microRNAs (miRNAs) as noninvasive molecular biomarkers for early prediction of preeclampsia [67].

A moderate negative correlation between miRNA-942 and the mean arterial pressure was noted and rather weak correlations between miR-143 and the ethnicity, parity, and the mean uterine artery Doppler pulsatility index was found, suggesting that circulating miRNAs have a minor predictive and functional pathophysiological relevance of early preeclampsia at first trimester of pregnancy [67]. Recently, Sandrim et al. compared circulating microRNAs expression profiles between preeclampsia and healthy pregnant women and found increased levels of miR-885-5p in plasma from preeclampsia women which was released into circulation mainly inside exosomes [68]. In another study, the same group also detected increased plasma levels of miR-195-5p in preeclamptic women [69].

As we can ascertain in all the above-mentioned studies, there are conflicting data and very few circulating miRNAs are overlapped. Although these data open perspectives for miRNAs as biomarkers for the prediction of preeclampsia, a large amount of work is required in the future which firstly

requires a standardization of methods/techniques used in miRNA profiling.

In conclusion, all these recent data support the evidence that indeed miRNAs are useful candidates in the prediction of the pathophysiological evolution of pregnancy and it is only a matter of time before some of these already described ones will be validated as diagnosis biomarkers.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Dragos Cretoiu and Jiahong Xu contributed equally to the paper.

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Research Article

Discovery and Validation of Hypermethylated Markers for Colorectal Cancer

Jiufeng Wei,[1,2] Guodong Li,[1,2] Shuwei Dang,[1,2] Yuhui Zhou,[1,2] Kai Zeng,[1,2] and Ming Liu[1,2]

[1]*Department of General Surgery, The Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, China*
[2]*Bio-Bank of Department of General Surgery, The Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, China*

Correspondence should be addressed to Ming Liu; mliu35@aliyun.com

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Colorectal carcinoma (CRC) is one of the most prevalent malignant tumors worldwide. Screening and early diagnosis are critical for the clinical management of this disease. DNA methylation changes have been regarded as promising biomarkers for CRC diagnosis. Here, we map DNA methylation profiling on CRC in six CRCs and paired normal samples using a 450 K bead array. Further analysis confirms the methylation status of candidates in two data sets from the Gene Expression Omnibus. Receiver operating characteristic (ROC) curves are calculated to determine the diagnostic performances. We identify 1549 differentially methylated regions (DMRs) showing differences in methylation between CRC and normal tissue. Two genes (*ADD2* and *AKR1B1*), related to the DMRs, are selected for further validation. ROC curves show that the areas under the curves of *ADD2* and *AKR1B1* are higher than that of *SEPT9*, which has been clinically used as a screening biomarker of CRC. Our data suggests that aberrant DNA methylation of *ADD2* and *AKR1B1* could be potential screening markers of CRC.

1. Introduction

Colorectal carcinoma (CRC) is one of the most prevalent malignant tumors worldwide. Global statistics showed that in 2012 alone, an estimated 1.36 million new cases were diagnosed with CRC, and approximately 694,000 people died from this disease [1]. Screening and early diagnosis are critical for the clinical management of CRC. Traditional screening tools include fecal occult blood test (FOBT) and colonoscopy. However, the effectiveness of FOBT is limited by the test performance, while colonoscopy is invasive, and it is therefore impractical to screen all patients for CRC in this manner. The identification of highly specific, noninvasive biomarkers is a top priority for screening and early diagnosis of CRC.

Aberrant DNA methylation is a well-recognized epigenetic feature of cancer, in general, and has been discovered in most tumors; it is thus gaining increasing attention as a potential biomarker [2–4]. Abnormally methylated genes can be used as biomarkers for early detection as well as tumor classification of CRC [5–7]. Some of these alterations have also been detected in stool or peripheral blood, suggesting that they can be candidates for noninvasive biomarkers of CRC. Epi proColon®, a blood-based assay for measuring methylated *SEPT9*, has become available for clinical application and has been approved by China and Europe. However, the sensitivity and specificity are still not satisfactory [5]. Novel biomarkers are needed to improve the accuracy of diagnosis of CRC.

In this study, the genome-wide methylation pattern of CRC was compared with adjacent normal tissues using the Illumina 450 K microarray, thus revealing aberrantly differentially methylated regions (DMRs) in CRC. Among the list of DMRs that we identified, potential biomarkers were validated in two independent data sets. We also established the sensitivity and specificity of the new molecular markers, which showed a higher area under the curve (AUC) than *SEPT9*. These biomarkers could improve the accuracy of CRC screening and diagnosis.

2. Materials and Methods

2.1. Subjects. Six pairs of CRC and adjacent normal tissues were obtained from the Bio-Bank of the Department of

Table 1: The list of top 10 DMRs by the P value.

Number	Start	End	Size	CHR	Arm	Gene	Feature	cgi	P value	Average deltaBeta
1	29520841	29521887	1047	6	p	NA	IGR	Shore	$2.17E-71$	0.297
2	130130327	130132504	2178	7	q	MESTIT1	Body	Shore	$4.20E-63$	0.118
3	33140275	33148582	8308	6	p	COL11A2	Body	Open sea	$5.36E-42$	−0.147
4	33130824	33138475	7652	6	p	COL11A2	3′UTR	Shore	$6.93E-40$	−0.134
5	27139876	27142774	2899	7	p	HOXA2	Body	Shelf	$3.18E-35$	0.232
6	133561800	133562545	746	6	q	EYA4	TSS1500	Shore	$2.09E-34$	0.306
7	31938678	31939388	711	6	p	STK19	TSS1500	Shore	$9.84E-32$	−0.149
8	32183994	32190096	6103	6	p	NOTCH4	Body	Open sea	$7.50E-31$	−0.151
9	78493049	78493778	730	13	q	EDNRB	5′UTR	Island	$9.47E-31$	0.296
10	30078146	30080891	2746	6	p	TRIM31	Body	Open sea	$1.47E-26$	−0.196

General Surgery of the Fourth Affiliated Hospital of Harbin Medical University. Inclusion criteria were no cancer other than CRC, no indications of heredity, and no radio- or chemotherapy prior to surgical resection. This study was approved by the Medical Research Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University, and informed consent was obtained. The diagnosis of CRC tissues was acquired from pathology reports. Fresh tissue samples were collected within 30 min after resection surgery, frozen in liquid nitrogen, and stored at −80°C. Clinicopathological characteristics of CRC patients are shown in Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2192853.

2.2. Human Methylation 450 K Microarray. Genomic DNA was extracted using standard phenol-chloroform techniques and quantified using Nanodrop 2000c. Genomic DNA from all samples was treated with an EZ Methylation Kit (Zymo Labs, Irvine, CA). Bisulfite-converted DNA (500 ng) was hybridized onto the Infinium Human Methylation 450 K BeadChip according to the manufacturer's standard protocol.

2.3. Differential Methylation Region Analysis. Infinium Methylation data were processed with the Methylation Module of the GenomeStudio software. Methylation levels of CpG sites were calculated as β-values (0-1). We removed unreliable probes that were detected with a P value > 0.05. In addition, CpG sites were removed on the X and Y chromosomes, containing single-nucleotide polymorphisms. The methylation data were deposited in the NCBI Gene Expression Omnibus (GEO): GSE75546. DMRs were analyzed using the ChAMP package, according to the instruction manual. To help identify regions of realistic length, the search was only conducted in regions where the distance between consecutive probes was less than 1 kb. The average β-values of the probes in the DMR were used as a representative of the DMR methylation levels. To screen the candidate DMR, the following criteria were used: β-difference > 0.4, β-value in normal tissue < 0.15, and P value < $1E-4$.

2.4. Data Set for Validation of Candidate Biomarkers. Methylation of candidate markers was evaluated in the data sets GSE48684 (147 samples containing CRC, adenoma, and normal tissues) and GSE68060 (118 samples containing CRC and normal tissues) from the GEO. The methylation status of these samples was determined using the same version of the 450 K methylation array.

2.5. Statistical Analysis. Statistical analysis was conducted using the GraphPad Prism 6 software (La Jolla, CA, USA) and MedCalc version 10.1.6 (MedCalc Software, Mariakerke, Belgium). The Mann-Whitney U test was used to compare methylation levels between CRC, adenoma, and normal tissue. All reported P values were two-sided, with $P < 0.05$ being considered statistically significant. ROC analysis was performed by MedCalc.

3. Results

3.1. DMRs in Tumors versus Adjacent Normal Tissues. To identify DMRs related to colorectal carcinogenesis, whole genome DNA methylation analysis was performed using the ChAMP package with the Illumina 450 K bead array. Through this method, 1549 DMRs were identified with significant methylation differences between the six pairs of CRCs and adjacent normal samples. The top ten DMRs, according to P values, are shown in Table 1. A gene-based variant of the region-level test was performed, revealing 629 DMRs located in the promoter region, 207 DMRs located in the 5′ Untranslated Regions (UTR), 117 DMRs located at the 1st Exon, 592 DMRs located in the gene body, 99 DMRs located in the 3′UTR, and 841 DMRs located in the intergenic region. A CpG island-based variant of the region-level test was conducted in the same manner, revealing 391 DMRs distributed in CpG islands, 585 DMRs distributed within the shores, 347 DMRs distributed in the shelves, and 1162 DMRs distributed in the open sea (Table S2).

We calculated the hypermethylated and hypomethylated DMRs as shown in Figure 1. Hypermethylated DMRs were mainly located in the promoter region and CpG islands (Figure 1(a)), while hypomethylated DMRs were mainly located in the intergenic region and open sea (Figure 1(b)). Interestingly, most of the hypermethylated DMRs were less than 400 bp, but most of the hypomethylated DMRs were greater than 1200 bp (Figure 1(c)).

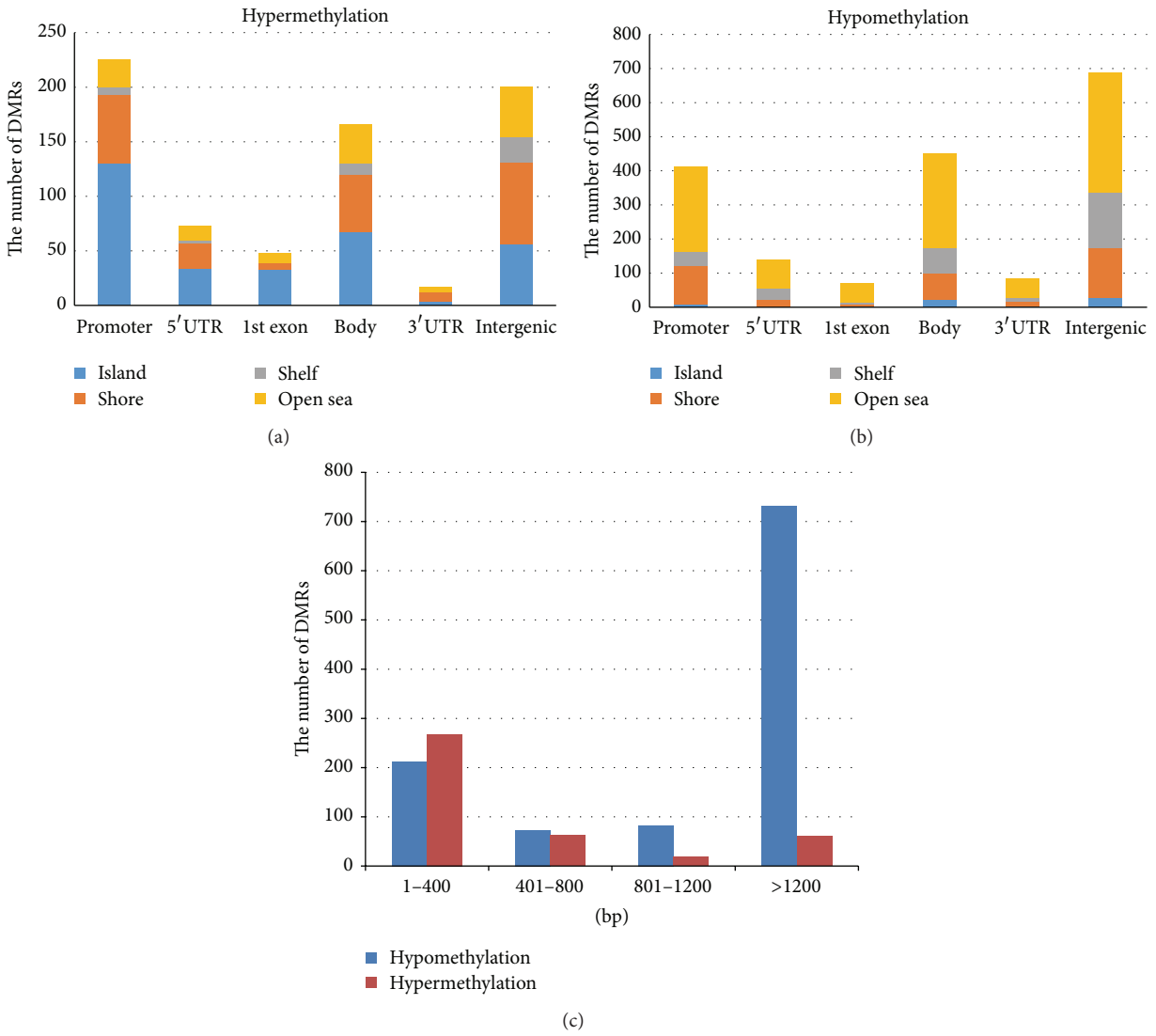

Figure 1: Distribution of DMRs in the human CRC genome. (a) Stacked bar charts showing the distribution of the hypermethylated DMRs over five gene categories: promoter, 5′UTR, 1st exon, gene body, 3′UTR, and intergenic regions. (b) Stacked bar charts showing the distribution of the hypomethylated DMRs over five gene categories: promoter, 5′UTR, 1st exon, gene body, 3′UTR, and intergenic regions. (c) Bar charts showing the distribution of the hypermethylated and hypomethylated DMRs considering four sizes: 1–400 bp, 401–800 bp, 801–1200 bp, and >1200 bp.

3.2. Identification of Candidate DNA Methylation Markers. To identify the candidate DNA methylation markers, the following criteria were used: β-difference > 0.4, β-value in normal tissue < 0.15, and P value < $1E-4$. Identification was restricted to hypermethylated DMRs as these can be easily transferred to clinical application with Methylation-Specific Polymerase Chain Reaction (MSP). After evaluating all DMRs, three DMRs were identified that met all the criteria. Information regarding these three DMRs is shown in Table 2. Of these three DMRs-related genes, SEPT9 has been clinically used as a screening biomarker. In the present study, SEPT9 was used as a reference.

3.3. In Silico Validation of Selected Candidates. To investigate the selected DNA methylation candidates, we used two independent data sets, namely, GSE48684 and GSE68060, from GEO. GSE48684 contained 41 normal tissues, 42 adenomas, and 64 CRCs. GSE68060 contained 36 normal tissues and 82 CRCs. The data set GSE48684 was generated from the 450 K methylation array. Technical and biological validation studies were conducted to demonstrate that the data were reproducible and robust [7]. The methylation levels of three DMRs in normal tissue, adenoma, and CRC in the two data sets are shown in Figure 2. In CRCs or adenomas, all candidates had significantly higher methylation levels compared to normal tissues (P < 0.0001). However, there were no significant differences in methylation levels of the three candidate DMRs between CRCs and adenomas.

3.4. Performance of Selected Aberrant DNA Methylation as Potential Diagnostic Markers. To determine whether these

Table 2: The list of candidate DMRs for validation.

Number	Start	End	Size	CHR	Arm	Gene	Feature	cgi	P value	Average deltaBeta
1	70995426	70995462	37	2	p	ADD2	TSS200	Shore	$2.22E-08$	0.41
2	75369210	75369237	28	17	q	SEPT9	TSS200	Island	$3.74E-05$	0.41
3	134144040	134144184	145	7	q	AKR1B1	TSS200	Island	$1.09E-07$	0.41

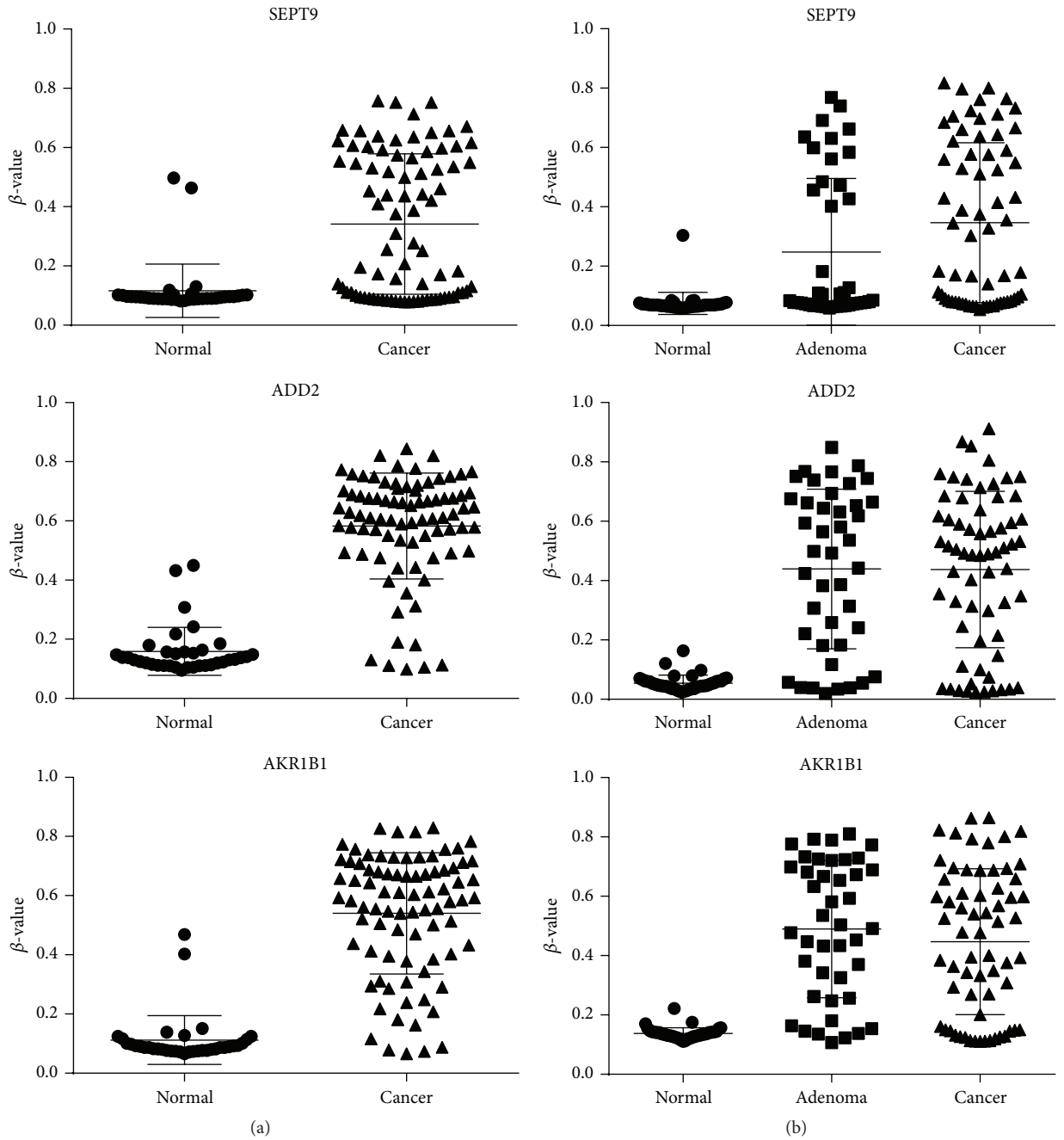

Figure 2: The different methylation levels of three genes in normal tissue, adenoma, and CRC. (a) The strip plot shows the different methylation levels of three genes (SEPT9, ADD2, and AKR1B1) in normal tissue and CRC with the data set GSE68060 from GEO. (b) The strip plot shows the different methylation level of three genes (SEPT9, ADD2, and AKR1B1) in normal tissue, adenoma, and CRC with the data set GSE48684 from GEO.

candidates could be potential biomarkers for use in diagnostics to distinguish between normal tissue and adenomas and CRC, we calculated AUC values for all three candidates in two data sets individually. In data set GSE48684, three markers had AUCs of 0.850 (*ADD2*, 95% CI: 0.767–0.912), 0.840 (*AKR1B1* 95% CI: 0.756–0.904), and 0.877 (*SEPT9*, 95% CI: 0.798–0.933) between normal tissues and CRCs (Figure 3(a)). The same three markers had AUCs of 0.862 (*ADD2*, 95% CI: 0.796–0.913), 0.874 (*AKR1B1*, 95% CI: 0.809–0.923), and 0.840 (*SEPT9*, 95% CI: 0.770–0.895) between normal tissues and adenomas + CRCs (Figure 3(b)). In data set GSE68060, the three markers showed AUCs of 0.982 (*ADD2*, 95% CI: 0.935–0.997), 0.954 (*AKR1B1* 95% CI: 0.895–0.985), and 0.752 (*SEPT9*, 95% CI: 0.659–0.831) between normal tissues and CRCs (Figure 3(c)). Two candidates, *ADD2* and *AKR1B1*, both showed better performances than *SEPT9*.

4. Discussion

Screening and early diagnosis is crucially important in the clinical management of CRC. Currently, colonoscopy and FOBT are the main approaches for CRC detection [8]. However, half of all CRCs are only detected at the advanced stages.

The widespread occurrence of modifications in CRC has major potential for being utilized as molecular markers, since alterations in DNA methylation in CRC was described by Fearon and Vogelstein over 20 years ago [9]. Compared with normal tissues, even adenomas showed apparent aberrant DNA methylation. Many aberrant DNA methylations have been reported as potential markers of CRC, such as *SEPT9*, *NDRG4*, and *VIM* [5, 10, 11]. To date, a blood-based assay named Epi proColon (Epigenomics AG, Berlin, Germany), which detects methylated *SEPT9*, has been applied clinically in several countries [12–14]. However, the sensitivity and specificity of *SEPT9* detection are still unsatisfactory. In a prospective clinical trial, sensitivity was 68% for all stages of CRC and 64% for CRC stages I–III, and much lower (22%) for advanced adenoma. In the present investigation, we performed a biomarker discovery and validation study to find new DNA methylation markers, which can be used for screening and diagnosing CRC.

Initially, we mapped the genome-wide methylation pattern of CRC compared with adjacent normal tissues using a 450 K bead chip and performed DMR analysis; this revealed that hypermethylation mainly occurred in CpG islands and promoter regions, while hypomethylation mainly occurred in the open sea and intergenic regions. These observations are in accordance with previous studies [15, 16]. We also found that most hypermethylated regions were short fragments (<400 bp), whereas most hypomethylated regions were long fragments (>1200 bp). These results suggest that hypermethylation occurs on a small scale and hypomethylation occurs on a large scale in CRC.

From the list of DMRs, we selected the candidates that most closely matched our criteria, which were set based on the premise that hypermethylation candidates are obviously better suited than hypomethylation ones for further clinical application. One of the candidates is *SEPT9*, which has already been applied clinically. The protein ADD2 is a subunit of adducin, a cytoskeletal protein, which caps and stabilizes the fast-growing end of actin filaments. ADD2 is usually expressed in the nervous system and erythroid tissues [17–19]. For the first time, the present study describes the hypermethylation in the promoter region of the *ADD2* gene in malignancy. The aberrant methylation of *AKR1B1* in CRC has been previously reported [20, 21]. However, the role of *AKR1B1* as a potential biomarker has not yet been demonstrated. Therefore, the two candidates (*ADD2*, *AKR1B1*) were compared with *SEPT9*, and the performances of *ADD2* and *AKR1B1* were further evaluated for their potential as biomarkers.

The Infinium Methylation 450 K bead array is the new generation of the Methylation 27 K bead array, which contains high density methylation probes with a distribution over the entire genome. Many investigations have demonstrated the accuracy and reproducibility of this technology and have shown that the results of the Infinium Methylation 450 K Bead Chip had a good positive correlation with bisulfite sequencing [22, 23]. In the present study, two independent data sets of the 450 K bead array from GEO were used for *in silico* validation. ROC curves were performed to determine the performance of the selected candidates. In the data set GSE48684, *ADD2* and *AKR1B1* have similar AUCs to *SEPT9* when CRCs are compared to normal tissues. Comparing adenoma + CRC with normal tissues, *ADD2* and *AKR1B1* have higher AUCs than *SEPT9*. When comparing CRC and normal tissues in GSE68060, *ADD2* and *AKR1B1* also have higher AUCs than *SEPT9*. These results suggest aberrant methylations of *ADD2* and *AKR1B1* may have better screening and diagnostic performances in the early detection of CRC than *SEPT9* alone. These findings should be confirmed by additional studies, for example, by carrying out tests in stools or blood.

5. Conclusions

In summary, we conducted investigations into the discovery of tissue biomarkers to identify DNA methylation markers associated with CRC and then replicated the findings in two independent sets from GEO. Further studies are required to confirm these results and understand the role of these genes in colorectal carcinogenesis.

Abbreviations

AUC: Area under curve
CRC: Colorectal carcinoma
DMR: Differentially methylated region
FOBT: Fecal occult blood testing
GEO: Gene Expression Omnibus
MSP: Methylation-Specific Polymerase Chain Reaction
ROC: Receiver operating characteristic
UTR: Untranslated Regions.

Disclosure

Guodong Li is a co-first author.

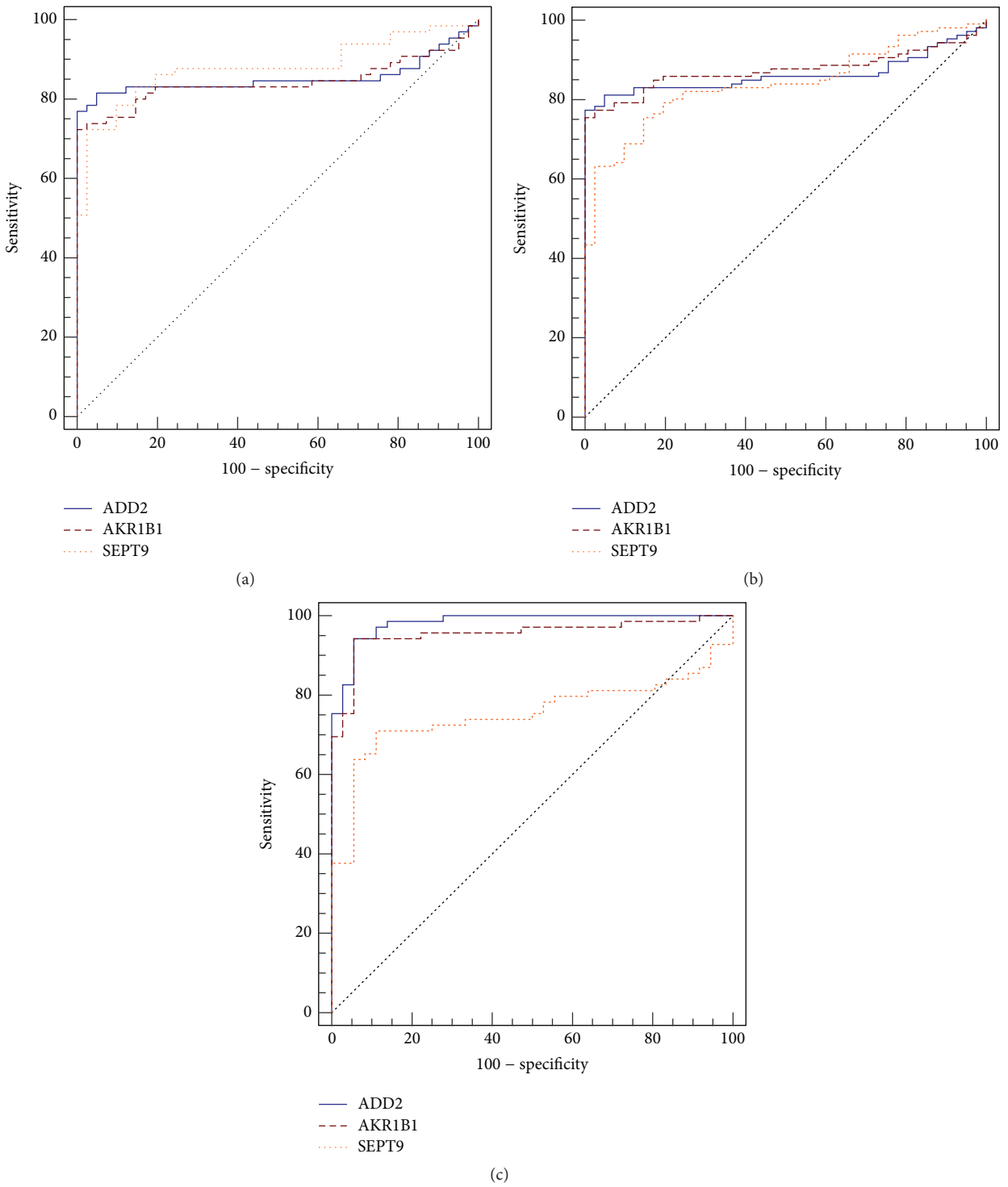

Figure 3: ROC curves of ADD2/AKR1B1/SEPT9 methylation using the data sets GSE48684 and GSE68060. (a) and (b) Receiver operating characteristic (ROC) curve showing accurate discrimination based on ADD2/AKR1B1/SEPT9 methylation using the data set GSE48684. (a) Between normal tissue and CRC tissue samples. (b) Between normal tissue and adenoma + CRC tissue samples. (c) ROC curve showing accurate discrimination based on ADD2/AKR1B1/SEPT9 methylation between normal tissue and CRC tissue samples using the data set GSE68060.

Competing Interests

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

Authors' Contributions

Jiufeng Wei and Guodong Li contributed equally as first authors.

Acknowledgments

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Review Article

Proteomic-Based Approaches for the Study of Cytokines in Lung Cancer

Ángela Marrugal, Laura Ojeda, Luis Paz-Ares, Sonia Molina-Pinelo, and Irene Ferrer

Medical Oncology Department, Hospital Universitario Doce de Octubre and Centro Nacional de Investigaciones Oncológicas (CNIO), 28041 Madrid, Spain

Correspondence should be addressed to Sonia Molina-Pinelo; pinelo_sonia@hotmail.com and Irene Ferrer; iferrer@ext.cnio.es

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Proteomic techniques are currently used to understand the biology of different human diseases, including studies of the cell signaling pathways implicated in cancer progression, which is important in knowing the roles of different proteins in tumor development. Due to its poor prognosis, proteomic approaches are focused on the identification of new biomarkers for the early diagnosis, prognosis, and targeted treatment of lung cancer. Cytokines are proteins involved in inflammatory processes and have been proposed as lung cancer biomarkers and therapeutic targets because it has been reported that some cytokines play important roles in tumor development, invasion, and metastasis. In this review, we aim to summarize the different proteomic techniques used to discover new lung cancer biomarkers and therapeutic targets. Several cytokines have been identified as important players in lung cancer using these techniques. We underline the most important cytokines that are useful as biomarkers and therapeutic targets. We also summarize some of the therapeutic strategies targeted for these cytokines in lung cancer.

1. Introduction

Lung cancer is one of the most frequent types of cancer worldwide, accounting for approximately 13% of the total cancer diagnoses in the most recent global statistics [1, 2]. Adenocarcinoma, squamous carcinoma, large cell carcinoma, and small cell carcinoma are the four most prominent histological types of lung cancer. The first three classes are collectively named Non-Small Cell Lung Cancer (NSCLC) and they represent 85% of lung cancer cases [3]. In particular, adenocarcinoma is the most often reported subtype of NSCLC in most countries [4].

Lung cancer is characterized by a poor prognosis, with a five-year survival rate of 15%, mainly due to an initial diagnosis at advanced stages of the disease [5]. For this reason, in addition to advances in treatment, the search for diagnostic strategies for early lung cancer detection is very important. Thus, the use of biomarkers is essential for early detection. A biomarker is a measurable indicator of a biological process. There are three different groups of protein biomarkers: diagnostic biomarkers, prognostic biomarkers, and biomarkers that predict the treatment response [6].

Proteomics is the systematic analysis of protein profiles in tissues or cells [2, 7] and is directly related to genomics because proteins are the final effectors of the genes in nearly all situations. Proteins are extremely dynamic molecules whose function is regulated by posttranslational modifications, degradation, and compartmentalization [8]. Therefore, the functional protein concentrations would not always be related to the differential expression of mRNAs. For these reasons, proteomics may contribute to improving our knowledge of cancer, in addition to genomics or transcriptomics [9, 10]. Thus, proteomics is a particularly appropriate tool to research lung cancer because proteomics approaches are much more objective and more precise than current methods of cancer diagnosis and patient stratification, which have been based on the study of tissue specimens by pathologists [10].

In the history of cancer studies, different hallmarks have been described that characterize tumor initiation, promotion, and invasion. Some of these hallmarks are related to tumor cells by sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death. Later, four new emerging hallmarks

were added from studies of the tumor microenvironment: evasion of immune destruction, tumor-promoted inflammation, genome instability and mutation, and deregulated cellular energetics [11]. Thus, taking the tumor microenvironment into account, inflammation is an important factor in the pathogenesis of cancer [12, 13]. Inflammatory cells can provide growth and survival factors, which contribute to several hallmarks of cancer. Similar to other tumors, it has been reported that chronic inflammation due to pulmonary disorders such as chronic obstructive pulmonary disease (COPD) significantly increases the patients' risk of developing lung cancer [14, 15]. Inflammation is regulated by the tumor microenvironment, which plays an important role in immune suppression or activation and in the epithelial-to-mesenchymal transition [16–18].

The main mediators of inflammation are cytokines, proteins that can be classified as proinflammatory and anti-inflammatory molecules, such as chemokines and growth factors [19–21]. These proteins can modulate different cellular responses, including inflammation, the immune response, apoptosis, and chemoattractant processes [22–25]. Characteristic cytokine patterns have been described in different cancer patients and are related to their prognosis. Therefore, some cytokines are good prognostic biomarkers of cancer [26–28].

We review the cytokines that are good biomarkers for the diagnosis, prognosis, and prediction of treatment responses in patients with lung cancer as well as the cytokines that could act as therapeutic targets and describe the therapeutic strategies based on these targets that are being used in clinic. In addition, we describe some proteomic techniques that are the best tools to study these important molecules. An in-depth analysis of the cytokine patterns using proteomics could provide important insights into clinical purposes.

2. Proteomics in Cancer Research

Proteins are the real functional players in cells and define their phenotype [29]. Thus, in some terms, they could provide more precise information about cancer than DNA or RNA. In fact, proteomic analyses also have the ability to quantify the effects of genetic abnormalities related to oncogenesis. Among these quantifiable changes, we highlight the differential expression of proteins encoded by genes with altered DNA copy numbers, splice variants, mutations, deletions, and insertions and regulation by microRNAs or epigenetics. Consequently, proteomics can improve our biological knowledge of cancer and help in the search for new potential therapeutic targets and biomarkers by connecting cancer phenotypes and genomic alterations [30]. The proteomic approaches that are currently used to study cancer and the samples used for this purpose are described next (Figure 1).

2.1. Proteomic Techniques Used in Cancer Research. Since the late 90s, the development of high-throughput platforms has allowed researchers to measure thousands of proteins and their modifications. Thus, proteomic assays have become essential tools to decisively detect the molecular patterns in malignant cells, which might be associated with disease evolution or the treatment response [31, 32]. In the last few years, the use of proteomic techniques in cancer research has produced great number of studies [5, 32–36]. The most frequently used proteomic techniques can be divided into gel-based or gel-free approaches, both of which are based on mass spectrometry (MS) and antibody-based techniques.

2.1.1. Gel-Based Techniques-Mass Spectrometry. These proteomic techniques employ two-dimensional gel electrophoresis (2D-PAGE), due to its relatively low cost and high applicability. In this assay, intact proteins are separated in two dimensions. Firstly, previously solubilized and denatured proteins are separated by their isoelectric point. Secondly, proteins are separated by their molecular weight to obtain different protein spots. Later, the resulting spots are analyzed and spots are picked and their peptides are digested for MS identification [32]. This technique allows researchers to study a large number of polypeptides in a single run and to evaluate different gels, making it possible to compare the spot patterns between different conditions, such as affected and unaffected patients. Unfortunately, this technique has limitations, which include low throughput, low sensitivity, and the need for large amounts of clinical material. Moreover, it is difficult to separate very low or very high molecular weight proteins, and there could be some variation among gels. However, progress in this approach has reduced gel-to-gel variation by marking the proteins with fluorescent dyes. Using 2D-DIGE, it is possible to evaluate different samples (such as test, control, and reference) in the same gel following the introduction of Cy3, Cy5, and Cy2 dyes immediately before 2D-PAGE [37].

2.1.2. Gel-Free Techniques-Mass Spectrometry. These methods provide high reproducibility by quantifying proteins in a gel-free setting, which decreases variability and allows researchers to measure complex, labelled, or label-free protein samples [38].

As an alternative to 2D electrophoresis, liquid chromatography separation (LC) can be coupled to MS (LC/MS) to identify the proteins contained in complex biological samples. In this workflow, the molecules resulting from the enzymatic digestion of the samples are separated in a liquid mobile phase by employing a solid stationary phase. Then, the amount of each peptide is quantified. These methods are known as shotgun proteomics and they principally use labelling (with a nonradioactive isotope) and nonlabelling approaches [33].

There are several isotope-based labelling approaches in which *in vivo* metabolic incorporation of the labels is essential. There are three principal techniques, ICAT, iTRAQ, and SILAC, according to the label used. Following trypsinization and subsequent MS analysis, these methods allow researchers to quantify and identify proteins from different samples at the same time. ICAT (isotope-coded affinity tag) employs chemical light or heavy reagents to label and compare pairs of samples. iTRAQ (isobaric tags for relative and absolute quantitation) can analyze up to 8 samples in the same experiment following labelling with fourplex or eightplex reagents [39, 40]. Currently, the most suitable method for quantitative

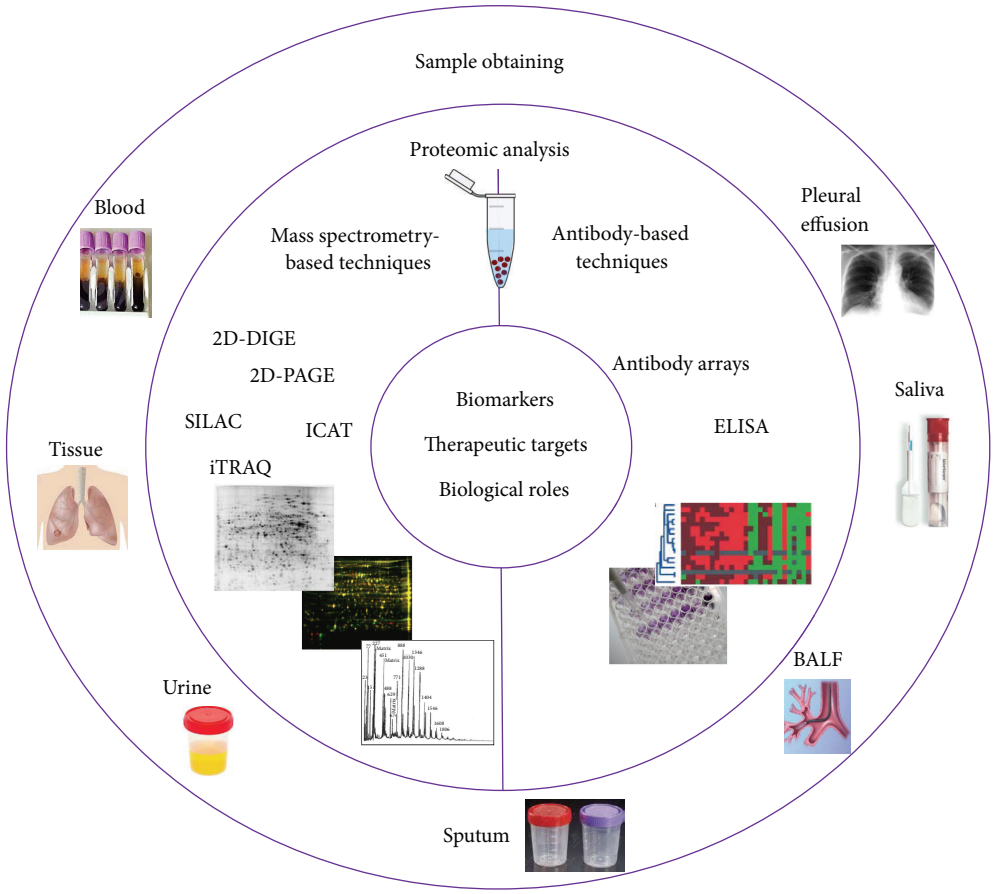

Figure 1: Workflow of the proteomic studies of cancer.

proteomics is SILAC (stable isotope labelling with amino acids in cell culture), mainly due to its robustness, reliability, and easy application [41]. This procedure differentiates two identical cell populations growing in distinct culture media. The "heavy" medium contains amino acids (usually arginine and lysine) substituted with stable isotopic nuclei (^{2}H, ^{13}C, and ^{15}N), whereas the "light" medium includes amino acids with the natural isotope. After a sufficient number of cell divisions, the whole proteome of the cell population growing in heavy medium is labelled, due to the incorporation of heavy amino acids into the newly synthesized proteins. Later, equivalent amounts of heavy and light samples are combined, digested, and analyzed by MS. The different signal intensities from both samples make it possible to quantitatively compare their relative abundances in the mixture, due to the specific masses of the heavy and light amino acids [42, 43]. At present, it is possible to compare up to five different samples in a single experiment based on mixtures of several isotopic forms of arginine and lysine [43]. Unfortunately, the clinical application of SILAC is limited because it cannot be used to directly label tissues or body fluids. However, the super-SILAC method has been recently developed to solve this problem. In this assay, a SILAC standard is generated to represent the clinical sample and is achieved using labelling and a combination of different cell lines to obtain a representation of the tissue or body fluid of interest. In the MS analysis, the SILAC standard is the heavy population, which will be compared to the light population from the clinical sample [44, 45]. Therefore, it is possible to differentiate histological subtypes of cancers and to search for biomarkers for use in other applications due to the precise quantification of human tumor proteomes [46].

The last step in all gel-free techniques consists of mass spectrometry analysis. MS has been widely used for protein and peptide sequencing and identification by measuring the molecular weights and charges (m/z ratio) of their ions. Firstly, the samples are ionized by an ion source. The main ionization methods employed are electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI), and surface-enhanced laser desorption ionization (SELDI), where the sample is introduced as spray, matrix, or chip, respectively. Later, the ionized samples are injected into a mass analyzer, where the ions are separated according to their m/z ratios. Time-of-flight (TOF), Fourier transform, and quadrupole-Q, linear quadrupole-LTQ, and Orbitrap ion traps are the methods that are most frequently utilized for this purpose. If a second mass analyzer is added (tandem mass spectrometry), both proteins and peptides could be identified. Thus, mass spectrometry techniques are commonly used for peptide and protein discovery as a real biomarker application [8, 47].

2.1.3. Antibody-Based Techniques. The search of proteomic profiles using antibodies facilitates the systematic examination of the cancer proteome and evaluation of cancer biomarkers [33].

Enzyme-linked immunosorbent assays (ELISAs) are one of the most frequently used methods to identify proteins in biological samples because they are a financially reasonable screening method that is easy to perform. In simple terms, an ELISA is performed in plates with a capture antibody, which specifically binds to the protein of interest, and a detection antibody linked to an enzyme. The enzyme can transform a substrate into a perceptible and quantifiable signal [48, 49]. Moreover, due to proteomic advances, the levels of many proteins, such as cytokines, can be determined at the same time using ELISA-based protein array technology. In this assay, peptides resulting from previously digested protein samples compete with their identical synthetic peptide (prebound to the ELISA plate) for a specific antibody [50]. This approach does not require the isolation and purification of the protein of interest, although its sequence is essential. Thus, in contrast to MS approaches, it is possible to identify proteins from a damaged or unpurified sample using ELISAs [10].

Antibody arrays are multiplex assays that are able to detect a large number of proteins and compare different groups of samples. In this assay, different antibodies are ordered onto a solid support to which the sample is added. Then, proteins can be detected by a laser scanner using a fluorescence signal. Finally, the binding pattern is correlated with the expression level of each protein [51].

2.2. Samples Used in Proteomic Studies. In biological proteomic studies, it is essential to choose the type and number of samples for a proper comparison. It is also important to use well-known model systems and controlled clinical samples. In addition, a large number of samples are needed to obtain statistical power. In cancer, researchers must consider the histological type of the tumor as well as its heterogeneity [52]. Different samples can be used in proteomic studies, including tissue, blood (serum or plasma), urine, and different fluids related to the tissue of interest, such as pleural effusions, sputum, or bronchoalveolar lavage fluid (BALF) for the lung [40, 53].

The majority of cancer research studies use paraffin-embedded, formalin-fixed, or fresh-frozen tissue samples. The limitations of these samples are related to their heterogeneity due to the inflammatory and stromal components and necrotic areas adjacent to islands of tumor cells. The use of tissue microarrays (TMAs) or laser capture microdissection to isolate tissue samples on microscope slides is required to solve this problem [54].

On the other hand, blood is an excellent sample for proteomic analysis due to the ease of obtaining a large amount of sample. Blood can be separated into plasma and serum, which is very useful because the depletion of abundant serum proteins is often necessary for the detection of tumor-specific markers [54]. It is also essential to separate proteins by their molecular weights and characteristics, such as ionic charges, modifications (phosphorylation or glycosylation), hydrophobicity, or hydrophilicity, by chromatographic methods to optimize the search for biomarker proteins in blood [10].

Another type of clinical sample that is particularly appropriate for analyses of tumor proteomes is pleural effusion (PE). PE is the fluid that accumulates in the presence of active disease. PE has a similar protein composition to plasma but is more enriched in tumor-derived proteins due to its proximity to the tumor. Therefore, PE is remarkably helpful in understanding tumor mechanisms and identifying cancer biomarker using proteomic techniques [34].

Urine has also been recognized as a potentially useful sample in nonurogenital diseases because it contains thousands of detectable proteins. These proteins are secreted in a mature and stable conformation. This point, together with the easy and noninvasive collection of a large volume of sample, makes urine a perfect biospecimen for the proteomic identification of cancer biomarkers [32].

Other proteomic studies are based on the proteins included in sputum [55, 56], BALF [5, 57], or saliva [37, 58]. These samples are often used to study nonmalignant conditions, although recent studies have employed them to search for potential lung cancer biomarkers. BALF is particularly useful for accessing cell populations that are in direct contact with lung tumors [57]. Saliva is a useful sample because of its easy accessibility and noninvasive collection and because it contains RNAs and a large amount of proteins [58]. Many of these proteins have been shown to be informative for the detection of oral and systematic diseases, such as lung cancer [37].

3. Cytokines as Biomarkers in Lung Cancer

Although several cytokines have been detected as powerful biomarkers, few are currently in clinical use because it is not easy to detect some proteins using noninvasive methods and their applicability may not always be very specific.

Here, we summarize the cytokines used as biomarkers in lung cancer, taking into account the different type of samples collected, blood, PE, BALF, lung tissue and sputum [6, 31, 59], and the type of information provided about the biomarker (Table 1). Several studies indicate the presence of different cytokines in samples of cancer patients compared to noncancer controls [26–28]. These cytokines are used as diagnostic biomarkers for the early detection and determination of the stage of the disease. Some examples of the cytokines detected in serum samples are IL-6, IL-2, IL-8, IL-10, IL-18, IL-13, IL-22, vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) [26, 27, 60–63]. Moreover, increased levels of IL-6, IL-8, IL-18, and VEGF have been detected in BALF [27]. IL-8 and VEGF are common lung diagnostic biomarkers that have been detected in sputum samples [56]. IL-6, IL-22, and VEGF have also been detected in pleural effusion and lung cancer tissue [64, 65]. Some of these cytokines are good biomarkers with both diagnostic and prognostic value and can predict treatment response. Focusing on prognosis, markers are important for predicting tumor progression. IL-6 overexpression is indicative of inferior survival outcomes

Table 1: Cytokines used as different types of lung cancer biomarkers.

Cytokine	Sample	Current purpose as a biomarker	Current well-known function in lung cancer
IL-6	Blood, BALF, and pleural effusion	Diagnostic, prognostic, and predicting the treatment response	Prooncogenic
IL-8	Blood, BALF, and sputum	Diagnostic and prognostic	Prooncogenic
VEGF	Blood, BALF, sputum, pleural effusion, and tissue	Diagnostic and prognostic	Prooncogenic
TNF-α	Blood	Predicting the treatment response	Prooncogenic and antitumor, depending on the context
IL-2	Blood	Prognostic and predicting the treatment response	Not yet determined
IL-18	Blood, BALF, and sputum	Diagnostic	Not yet determined
IL-10	Blood	Diagnostic	Prooncogenic and antitumor, depending on the context
IL-13	Blood	Diagnostic	Not yet determined
IL-22	Blood	Diagnostic and prognostic	Prooncogenic
IFN-γ	Blood	Diagnostic and prognostic	Not yet determined
IL-32	Tissue	Prognostic	Prooncogenic
IL-37	Tissue	Prognostic	Antitumor

in patients with NSCLC, and it is related to the acute phase response and cancer cachexia [66, 67]. Furthermore, high levels of IL-8 and VEGF are related to reduced survival of NSCLC patients [56, 61], and basal levels of VEGF and IL-22 in SCLC patients are associated with a poor prognosis [27, 64, 65, 68–70]. Another cytokine, IL-32, has been recently proposed as lung adenocarcinoma prognostic biomarker, as its overexpression in the tumor tissue correlates with a greater number of lymph node metastases [71]. Although most cytokines that are used as prognostic biomarkers have prooncogenic effects in lung cancer, some of them also have antitumor effects. In this sense, IL-37 is expressed at lower levels in the tumor tissues of patients with NSCLC, and it correlates with poorer overall survival compared to patients with high IL-37 expression [72]. Low IFN-γ levels are related to a shorter survival due to a lower lymphocyte count, indicating that some cytokines have important roles in the immune responses that protect against tumor formation [66].

On the other hand, as indicators of the treatment response, biomarkers can provide information about drug susceptibility, toxicity, and the clinical outcomes. The pleiotropic role of cytokines in the tumor microenvironment makes it difficult for cancer therapies to always be efficient. Cancer cells develop resistance to chemotherapy and targeted therapies through several mechanisms. In this sense, it has been shown that cancer cells can secrete cytokines that help them evade death induced by several anticancer drugs through the activation of tumor-promoting pathways and the induction of the secretion of other cytokines and growth factors. These molecules are also implicated in antiapoptotic mechanisms, vessel formation, tumor growth, and metastasis [73]. In lung cancer, it has been reported that patients with IL-6 overexpression have a poor response to chemotherapy [66, 67], which is important because IL-6 is administered in combination with cancer treatments because of its ability to induce platelet production [72]. In addition, although the increase in TNF-α expression does not have a demonstrated prognostic value, its overexpression in chemoresponsive patients has been used as a biomarker for predicting the treatment response because high levels of TNF-α indicate that the patients are sensitive to the treatments [60]. Moreover, the high levels of IL-2 are related to a good chemotherapeutic response in NSCLC patients [63].

Finally, it is known that some polymorphisms can act as genetic biomarkers. An association between some cytokine gene polymorphisms and the risk of developing lung cancer has been described. Variations in the cytokine protein levels resulting from polymorphisms have been investigated, and the conclusions in several meta-analyses are controversial [74–76]. Nevertheless, it has been reported that IL-10 and IL-6 polymorphisms increase the level of these proteins in serum, which correlates with higher number of cases of lung cancer. In the case of IL-10, the alleles IL-10-1082G, IL-10-819C, and IL-10-592 have been observed in lung cancer patients, suggesting a predictive value [74]. A recent study revealed that two IL-10 polymorphisms (-592C/A and -819C/T) show a significant association with the risk of developing lung cancer. In contrast, patients with the third polymorphism analyzed (-1082G/A) did not present susceptibility to this type of tumor [77]. Related to IL-6, several researchers agree that IL-6-174G/C polymorphism in the promoter region has prognostic value because NSCLC patients with G carrier genotypes (GG/CG) show lower overall survival compared with CC genotype carriers [76, 78].

4. The Roles of Cytokines in Lung Cancer

Cytokines can be secreted by tumor and stromal cells in the tumor microenvironment and they can function in an autocrine and/or paracrine manner. Although cytokines are important factors that preserve the correct function of

the organism, they can act as tumor-promoting or tumor-suppressor molecules in the context of neoplasia [28, 79]. Proteomic tools, such as ELISA or antibody arrays, are currently used to characterize the signaling pathways activated by cytokines in cancer. These approaches are making it possible to elucidate the roles of cytokines in lung cancer.

The increased levels of cytokines in cancer patients indicate their possible functional roles in tumor progression [61, 64, 70, 80–89]. Previously, we have described several cytokines that are used as lung cancer biomarkers (Table 1). Some of these cytokines (IL-6, IL-8, IL-10, IL-22, VEGF, TGF-β, and TNF-α) have also been studied to determine their role in lung cancer and are described in comparison to other cytokines next. Others, such as IL-2, IL-13, IL-18, and IFN-γ, require further in-depth study to determine their roles in lung cancer. Other cytokines that have not yet been shown to function as lung cancer biomarkers (TGF-β, IL-17, IL-32, IL-7, and IL-37) can play important roles in this disease, but further studies are required to determine whether they can act as biomarkers.

4.1. Prooncogenic Cytokines. There is evidence that some tumor cells may be able to use cytokines as autocrine growth factors and thereby promote tumor growth. Until now, most of the interest in cytokines in lung cancer has focused on IL-6, a proinflammatory cytokine that is upregulated in lung cancer patients and correlates with decreased cancer survival [61, 82, 83, 90–92]. It has been shown that IL-6 displays carcinogenic effects through the activation of the STAT3 pathway [84, 85, 93, 94]. STAT3 activation results in the secretion of malignant pleural effusion proteins and VEGF upregulation in patients' samples as well as increased cell colony formation in soft agar and tumor formation in nude mice [67, 85]. Moreover, the cell survival effect of STAT3 can limit the overall drug response to some lung cancer treatments, such as Erlotinib. Cells treated with Erlotinib exhibit changes in gene expression and the posttranslational regulation of secreted proteins, including IL-6, which is secreted from Erlotinib-treated cells at higher levels. Moreover, IL-6 triggers STAT3 activation, making the Erlotinib-treated cells more resistant to the treatment. STAT3 has well-known effects on cell growth, angiogenesis, immune system evasion, and the prevention of apoptosis [95].

Another prooncogenic cytokine is IL-22, which is a member of the IL-10 family. Its receptor (IL-22-R) is overexpressed in the lung of cancer patients and it is related to poor prognosis [64, 70]. It has been reported that IL-22 overexpression in lung cancer cells protects the cells from apoptosis by activating STAT3, Bcl-2, and Ccl-xL and inactivating ERK1/2 [64].

IL-8 is a proinflammatory chemokine that has autocrine and paracrine functions in lung cancer cells. It contributes to cancer progression, invasion, and metastasis because of its angiogenic and mitogenic properties [96]. IL-8 activates several oncogenic signaling pathways, such as the PI3-K, Ras/MAP-K and Jak/STAT pathways, which produce protumorigenic effects in many cancer types [97].

VEGF and its soluble receptors (VEGFR-2 and VEGFR-3) are expressed in some NSCLC cell lines [98]. VEGF and VEGFRs mediate angiogenesis, which has an important role in cancer progression because it modulates the chemotaxis and migration of endothelial cells [99]. Some signaling pathways that are commonly associated with cancer are activated by VEGF, such as the PI3-K, MAP-K, and STAT3 pathways [100].

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine involved in cancer progression through the PI3-K and MAP-K pathways [101]. TGF-β downregulates the epithelial marker E-cadherin and promotes the upregulation of N-cadherin and fibronectin, triggering the epithelial-to-mesenchymal transition and increasing the migratory potential of NSCLC cell lines [102].

IL-17 is another proinflammatory cytokine that is produced by T helper cells, which plays an important role in lung cancer development and the innate and adaptive immune responses in Lewis Lung Carcinoma (LLC) [86]. It has been reported that IL-17 promotes the expression of VEGF, MMP-2, MMP-3, and TNF-α, which are proangiogenic molecules. On the other hand, IL-17 increases the level of IL-6 and IL-8 in NSCLC cell lines and activates the STAT3 signaling pathway, mediating tumor angiogenesis [103, 104].

Finally, IL-32 plays an important role in the tumor microenvironment by inducing the secretion of inflammatory mediators, such as TNF-α, IL-1β, IL-6, IL-8, IL-18, and MIP-2, which are related to invasion and metastasis. In NSCLC, IL-32 transactivates the nuclear transcription factor NF-κB, which upregulates the expression of matrix metalloproteinases (MMP-2 and MMP-9), increasing the invasion of tumor cells [71].

4.2. Antitumor Cytokines. Although most cytokines have prooncogenic effects, there is some evidence regarding the antitumor roles of cytokines in lung cancer, which are related to inflammation and the immune system. IL-7 signaling is required to induce an immune response in a lymphopenic mouse model [105]. It has been shown that lymphopenia induces IL-7 secretion and the subsequent proliferation of T cells, antagonizing immune suppression [88, 106]; however, more studies are needed to clarify the detailed role of IL-7 in the induction of the antitumor effects.

IL-37 is a member of the IL-1 family and although it has been described as a suppressor of immune responses and inflammation, some studies have revealed that it has a protective role against cancer progression [107]. In this sense, it has been reported that lL-37 could play an inhibitory role in NSCLC as it has inhibitory effects on tumor growth *in vivo* by decreasing tumor angiogenesis. A high level of IL-37 correlates with a lower level of VEGF in lung cancer cell lines and reduced microvessel density in NSCLC patients [89].

4.3. Context-Dependent Cytokines. Some cytokines have two opposite effects in lung cancer progression, according to the molecular context, which is the case for IL-10 and TNF-α.

In some cases, IL-10 improves the metastatic capability of lung tumor cells by increasing the vascular density in the primary tumor and increasing the resistance of lung tumor cells to apoptosis by activating STAT3 pathway [87]. On the other hand, IL-10 has an important role in immunosuppression and

cell-mediated immunity because it induces the production of regulatory T cells, which can induce immunosuppression and reduce the number of IFN-γ secreting cells [108]. Furthermore, IL-10 secretion could result in the deactivation of macrophages, which are important promoters of tumor progression and neovascularization; IL-10 secretion could also decrease angiogenesis by downregulating VEGF [109].

TNF-α is another context-dependent cytokine. Although it has been reported that TNF-α decreases lung adenocarcinoma cell death [110] and promotes angiogenesis and invasion, there is a positive correlation between the TNF-α levels and the chemoresponse [60]. Doxorubicin treatments induce TNF-α expression [111]. Therefore, TNF-α can trigger cell apoptosis in the context of chemotherapy.

5. Cytokines in Lung Cancer Therapy

Immunotherapy, a tool for the treatment of malignancies that changes or stimulates the host immune system, has become a promising approach for cancer therapy [112, 113]. Traditionally, cytokine therapy has had a basic role in human cancer immunotherapy. In 1986, IFN-α (Peg-Intron®) was approved by the US Food and Drug Administration (FDA) for hairy cell leukemia therapy. IL-2 (Proleukin®) was approved by the FDA in 1992 and has been used as a single agent to promote endogenous antitumor immune responses for the treatment of metastatic renal cell carcinoma and metastatic melanoma [114]. In 1995, IFN-α was ratified as the first immunotherapy for adjuvant treatment of stage IIB/III melanoma [115].

Focusing on cytokine therapies, we can distinguish four options in lung cancer: decreasing cytokine expression in tumor cells, the use of cytokines as a treatment alone or the use of cytokines with other immunotherapies, and the use of endogenous cytokines to provide an advantage to immune system homeostasis [116].

On one hand, treatments based on a decrease in cytokine expression are commonly used. An excellent example of a treatment that reduces cytokine expression is Siltuximab (CNTO 328), an anti-IL-6-chimeric (murine-human) monoclonal antibody. Because IL-6 is involved in the pathophysiology of various solid tumors, such as lung cancer, the clinical use of this antibody has been analyzed in different contexts [117]. This treatment was evaluated in patients with EGFR-refractory or EGFR-resistant NSCLC, as well as in patients with other solid tumors, in Phase I/II study. However, the monotherapy of Siltuximab did not show clinical activity, although further studies with more patient samples should be performed [118]. In the same field, Belagenpumatucel Lucanix® is a whole-cell vaccine that decreases the expression of TGF-β2, which is its immune target. This cytokine leads to immunosuppression in lung cancer. Thus, its inhibition is related to a better prognosis in NSCLC patients [119]. Important results in Phase II trial in stage II–IV NSCLC patients showed a dose-dependent difference in survival for the groups treated with the higher doses of Belagenpumatucel-L [120]. Therefore, a placebo-controlled, randomized, Phase III trial in stage III or IV NSCLC patients was performed. When the overall survival of patients treated with the vaccine was compared, improved survival was observed in patients who were previously treated with chemo- or radiotherapy. These results are promising, although more studies are warranted [121].

On the other hand, the use of cytokines alone as a treatment is an excellent option. In some cases, TG4010, MUC-1 antigen-specific liposomal vaccine with the IL-2 gene [122], increases the levels of this cytokine and has been studied in different trials for lung adenocarcinoma. In combination with first-line chemotherapy, first Phase II trial in patients with advanced NSCLC showed the effectiveness of the treatment in patients with a normal number of activated natural killer cells, which improved their outcomes [123]. Based on these results, later Phase IIB trial showed that TG4010 enhanced the effect of chemotherapy in patients with advanced NSCLC [124], which led to Phase IIB/III trial. In these patients, the progression-free survival was improved when they were treated with TG4010 and chemotherapy [125]. Currently, Phase III part of the trial is ongoing.

As mentioned above, the third possibility of cytokine therapy in lung cancer is based on a combination of cytokine therapy with other immunotherapies. In this field, the most relevant clinical study includes the use of cytokines with Adoptive Cellular Therapy (ACT). This therapy consists of the transfusion of $\gamma\delta$ T cells, natural killer cells, or Cytokine-Induced Killer (CIK) cells to the patients. $\gamma\delta$ (Vγ9Vδ2) T cells are effector cells for immunotherapy that can secrete cytokines and display cytotoxic activity. Due to problems with the *in vivo* expansion of $\gamma\delta$ T cells, IL-2 has been required to stimulate their proliferation [126]. Based on this improvement, Phase I trial has been performed in patients with advanced or recurrent NSCLC and showed that the $\gamma\delta$ T cell treatments were viable and safe in this group of patients [127]. Similarly, cytokines are useful in NK cell therapy. IL-15 and hydrocortisone were used to activate and expand these cells *in vitro* and a clinical trial showed that allogeneic NK cells in combination with chemotherapy were safe and potentially clinically effective [128]. CIK cells are cytotoxic T lymphocytes with powerful antitumor activity that control and enhance the immune function of cancer patients [129]. Several studies have proved that CIK cells treatment improves the responses of NSCLC patients treated with chemotherapy, with a higher overall survival, clinical response rate, and T lymphocyte responses. To this end, supplementation with exogenous IL-2 or IFN-γ is required for the *in vitro* culture of CIK cells, suggesting the essential role of cytokines in this immunotherapy [130].

Finally, it should be noted that immune checkpoint inhibitors are promising lung cancer therapies that promote immunologic homeostasis through endogenous cytokines. The PD-1 (programmed death-1) signaling pathway is a receptor expressed on activated T cells, and its ligands, PD-L1 and PD-L2, are produced by stromal and cancer cells [131]. The activation of PD-1 following binding to its ligands promotes adaptive immune resistance [132]. PD-L1 overexpression has been noted in several cancer types. Therefore, monoclonal antibodies targeting PD-1 or PDL-1 have shown activity against these tumors [133, 134]. One of these monoclonal antibodies is Nivolumab, a human monoclonal IgG4-kappa antibody against PD-1. In randomized Phase III study, Nivolumab

promoted a superior overall survival, response rate, and progression-free survival for NSCLC patients compared to Docetaxel [135]. Nivolumab has obtained regulatory approval (FDA and EMA) as a first-line, standard, platinum-based chemotherapy for NSCLC progression. Another PD-1 blocking antibody is Pembrolizumab; its activity has been studied in Phase I trial and it showed antitumor activity in advanced NSCLC patients [136]. Therefore, Pembrolizumab has been approved by the FDA as therapy for advanced or metastatic NSCLC patients [137]. Based on this achievement, other studies, such as Phase II/III randomized KEYNOTE-001/010 trials, were performed. These studies focused on previously treated PD-L1-positive NSCLC patients. The trial showed an improved overall survival, progression-free survival, and response rate of the patients treated with Pembrolizumab compared to those treated with Docetaxel [138]. Further studies are ongoing, such as Phase III KEYNOTE-042 study, where Pembrolizumab is being compared to platinum-based chemotherapy in NSCLC patients expressing PD-L1 [137]. Based on these promising results, different studies of PD-1 inhibitors are ongoing. However, PD-L1 inhibition is also another excellent strategy. Atezolizumab, a human Ig-G1 antibody targeting PD-L1, has been analyzed in NSCLC patients. In this randomized Phase II trial, Atezolizumab produced an increase in the survival of previously treated patients compared to Docetaxel. The results are most obvious in patients with high expression of PD-L1 [139]. In addition to Atezolizumab, Avelumab and Durvalumab are PD-L1 blocking antibodies that are actually in Phase III trials in NSCLC patients [140].

6. Final Remarks

Cytokines are dynamic molecules that can regulate cellular functions and homeostasis in several types of tissues. In a neoplastic context, they can act as modulators of the initiation and progression of the disease due to their abilities to activate several signaling pathways implicated in tumor formation and metastasis. Their overexpression in several cancer types makes cytokines significant candidate biomarker molecules. Proteomics is the current tool used to study the whole proteome in several model systems. It will be useful to identify new biomarkers and study the effects of different proteins on cancer development. In this sense, proteomic approaches have been widely used to identify new cytokines related to lung cancer and they set the stage for the identification of new biomarkers and development of new treatments using these proteins as therapeutic targets. Due to the poor prognosis of lung cancer, it is important to continue to study the biology of this disease, and proteomic studies of cytokines have been widely used for this purpose. Further studies are needed to identify new biomarkers and to understand their roles in lung cancer, as they can act as new targets for the treatment of early stages of tumor progression.

Competing Interests

The authors declare that there are no competing interests.

Authors' Contributions

Ángela Marrugal and Laura Ojeda contributed equally to this work. Irene Ferrer and Sonia Molina-Pinelo contributed equally to this work.

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