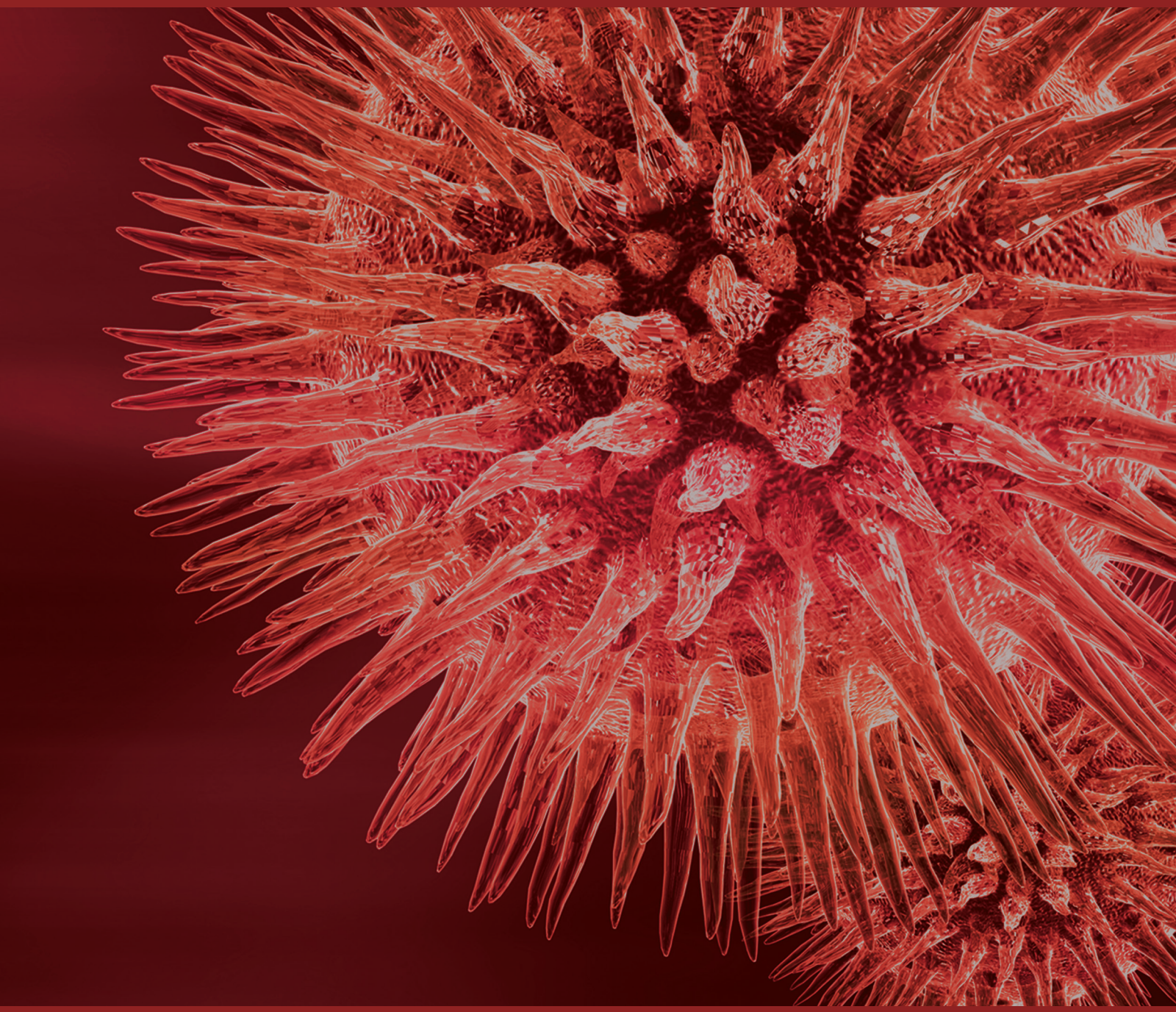
BioMed Research International

Optimal Use of Biomarkers in Oncology

Guest Editors: Ondrej Topolcan, Olive T. J. Wolfe, Vivian Barak, and Tomas Zima



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Editorial

Optimal Use of Biomarkers in Oncology

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The use of biomarkers in oncology has been much more extensive than in any other diseases for more than 30 years. Their use has evolved beyond the research laboratory to more routine clinical use as in diagnosis, monitoring of disease status, and treatment efficacy.

With the advances in molecular biology, other biomarkers than the "classical" serum markers are being developed for clinical use: gene mutations, microRNA, circulating tumor cells, circulating DNA, and others currently in research. Biomarkers such as these are currently being used for prediction: potential "at risk" for disease as well as response to therapy (predictive biomarkers), and for disease prognosis (prognostic biomarkers).

In oncological routine, the correlation of biomarker levels with the clinical status and medical imaging methods is of great significance. The purpose of the correlation is twofold: providing sequential and/or more specific diagnostics and keeping these procedures within affordable economical limits.

This special issue includes a number of reviews and research papers dealing with the above mentioned topics.

Reviews on Novel Biomarkers. The advances in molecular research and various "–omics" techniques have enabled us to study a number of potential novel cancer biomarkers. Studying gene expressions and molecular pathways may lead to the discovery of new prognostic biomarkers, markers of early diagnostics, or molecular targets for therapy.

E. Lastraioli et al. present a review on the expression of hERG1 potassium channels in different types of solid cancer (including breast, esophageal, gastric, colorectal, pancreatic, and other cancers). The authors, on the basis of publication review as well as their own findings, argue that hERG1 could be a novel biomarker and point out the overexpression of hERG1 in solid cancers, a feasible determination by immunohistochemistry and the potential of using them as therapy targets as the monoclonal antibody to block them is already available.

M. Rihacek et al. review B-cell activating factor (BAFF), a transmembrane protein, as a biomarker of malignant disease activity and prognostic factor in B-cell derived malignancies such as multiple myeloma. The authors report its proinflammatory properties and its contribution to cancer cachexia. Moreover, BAFF/BAFF-R signaling may be a promising target of future therapy in B-cell derived leukemias and lymphomas.

Cytoglobin, a protein of the globin family, is reviewed by T. Bholah et al. Thanks to the advances in molecular research, a possible role of this protein in cancer has been suggested. In their paper, the authors overview different functions of cytoglobin and provide a perspective on potential research areas that may elucidate its role as cancer biomarker.

The gene for gamma-glutamylcyclotransferase, an enzyme involved in glutathione metabolism, has recently been investigated and reported as an upregulated protein in various cancers. S. Kageyama et al. report an overview of the activities of GGCT in cancer cells.

Imaging Methods and Oncology. Imaging methods provide a useful tool in cancer diagnostics and management. Their

applications develop in reflection to new technologies and advances in molecular research.

M. Tolia et al. report the value of preirradiated in vivo magnetic resonance spectroscopy parameters in predicting recurrence free survival for patients with high grade gliomas, who undergone postoperative radiotherapy. R. Fusco et al. present the evaluation of the diagnostic value of an imaging protocol that combines dynamic contrast-enhanced MRI (DCE-MRI) and diffusion-weighted imaging (DWI) in patients with suspicious breast lesions. In addition, further research sought to determine if additional information provided by DWI could improve the diagnostic value of breast MRI.

Predictive Biomarkers and Risk Factors. J. H. Kim and S. K. Hong report a review, based on meta-analysis of PubMed publications, on potential novel biomarkers in active surveillance of low-risk prostate cancer. These include %[-2] proPSA and Prostate Health Index (PHI), PCA3, TMPRSS2:ERG, the genomic prostate score (GPS), a panel of four kallikrein markers, and the expression levels of different cell cycle progression (CCP) genes.

Y. Cao et al. review a meta-analysis of 25 independent epidemiological studies on the association between hormonal and reproductive factors and thyroid cancer risk. As the title of their paper suggests, reproductive factors but not hormonal factors are associated with thyroid cancer risk.

R. V. Liubota et al. report risk factors of the invasive breast cancer locoregional recurrence. The authors discovered that the scope of the surgical intervention (breast-conserving surgery (BCS) or radical mastectomy (RME)) does not essentially affect the recurrence appearance frequency or the recurrence-free period duration. However, in the BCS group, risk factors such as the presence of metastases in the regional lymph nodes or the hyperexpression Her/2neu presence increased the frequency of the locoregional breast cancer recurrence appearance.

Prognostic Biomarkers. M. Tolia et al. describe a study which aimed to identify whether or not the expression of serum baseline C-reactive protein (CRP) and albumin are related to overall survival in non-small-cell lung cancer (NSCLC). The study results suggested that, in fact, high pretreatment CRP and low albumin serum levels were promising independent prognostic factors of overall survival in NSCLC.

Biomarkers in Treatment Monitoring. Several indicators, including tumor markers, are used to monitor whether or not a particular cancer treatment is efficient with tolerable toxicity for patients. However, if a tumor marker has low sensitivity and/or specificity, adding another biomarker to the regimen could potentially enhance the treatment evaluation.

S. Kristiansen et al. present a study that focused on the association between hypermethylated DNA and the tumor markers CA 15-3, CEA, and TPA in serum during monitoring of patients with advanced breast cancer.

Disease Monitoring and Management. A combination of biomarkers or a set of tests provide a higher sensitivity and/or specificity than a unique biomarker or test. A. Pouliakis et al. report the evaluation of classification and regression trees for the triage of women at risk for cervical intraepithelial neoplasia. The computer-assisted algorithm was based on cytological HPV DNA typing, HPV mRNA detection, p16 immunocytochemical expression, and age and parous status. The authors proposed a methodology which could dramatically reduce the number of women that would require a colposcopy.

This special issue aims to prove the importance of biomarkers for the individualisation of the approach to an oncological patient and the improvement of his quality of life. Biomarkers in oncology are an important tool for diagnostics, prognosis, and therapy monitoring on condition that there is a clear goal for the biomarkers determination; biomarkers are monitored systematically and are interpreted in collaboration of the laboratory and the clinician. Single shot biomarker determination is useless and can lead to incorrect assumptions.

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Review Article

B-Cell Activating Factor as a Cancer Biomarker and Its Implications in Cancer-Related Cachexia

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B-cell activating factor (BAFF) is a cytokine and adipokine of the TNF ligand superfamily. The main biological function of BAFF in maintaining the maturation of B-cells to plasma cells has recently made it a target of the first FDA-approved selective BAFF antibody, belimumab, for the therapy of systemic lupus erythematosus. Concomitantly, the role of BAFF in cancer has been a subject of research since its discovery. Here we review BAFF as a biomarker of malignant disease activity and prognostic factor in B-cell derived malignancies such as multiple myeloma. Moreover, anti-BAFF therapy seems to be a promising approach in treatment of B-cell derived leukemias/lymphomas. In nonhematologic solid tumors, BAFF may contribute to cancer progression by mechanisms both dependent on and independent of BAFF's proinflammatory role. We also describe ongoing research into the pathophysiological link between BAFF and cancer-related cachexia. BAFF has been shown to contribute to inflammation and insulin resistance which are known to worsen cancer cachexia syndrome. Taking all the above together, BAFF is emerging as a biomarker of several malignancies and a possible hallmark of cancer cachexia.

1. The BAFF/BAFF-Receptor System Is Essential for B-Cell and Plasma Cell Development and Function

B-cell activating factor (BAFF, BLyS, TNFSF13B, TALL-1, and CD257) is a 285-amino-acid type II transmembrane protein that belongs to the superfamily of 19 known TNF ligands [1, 2]. Since its discovery, BAFF has been confirmed as a necessary element in B-cell proliferation and as a specific immunity response enhancer [3]. BAFF deficiency leads to almost complete loss of follicular and marginal zone B-cell production in murine secondary lymphoid organs [4]. BAFF neutralization by soluble receptor decoys blocks the Th1 to Th2 transition, thereby leading to inhibition of antigen-specific antibody production [4, 5]. BAFF also mediates immunoglobulin isotype switching in B-cells [6]. BAFF signaling is potentiated by BCR ligation [7] and enhances survival in B-cells via activation of NF-κB pathway.

Three receptors from the 29-member TNF receptor superfamily are now confirmed to interact with BAFF: BAFF-R, TACI, and BCMA [8] (Table 1). BAFF-R seems to be the most important receptor for BAFF, with a critical role in regulating B-cell survival [9]. Mice with a naturally occurring mutation in the BAFF-R locus (A/WySnJ mice) have a qualitatively similar phenotype to mice with BAFF deficiency, suggesting a unique role of BAFF-R in B-cell development that cannot be compensated by the two other BAFF receptors, TACI and BCMA [9, 10].

Unlike BAFF-R, TACI binds two ligands from the TNF superfamily: BAFF and APRIL [11]. The role of TACI in BAFF signaling is complex, as it induces both activation and inhibition of the NF-κB pathway. When ligated by TACI,

Table 1: Overview of BAFF receptors.

	Gene	Forms	Ligands	Affinity to BAFF (K_D)	Tissue expression	Function	Clinical relevance
BAFF-R (BAFF-receptor, TNFRSF13C, BLyS, BR3, CD268) [13]	22q13.2 3 exons [13]	Membrane bound, soluble (produced by decidual cells) [8, 14]	BAFF [8]	16 nmol·l^{-1} [8]	B-, T-cells [15, 16] mature and immature adipose tissue [17]	B-cell proliferation [18], T-cell proliferation [16]	BAFF-R is constitutively saturated in autoimmune and lymphoproliferative diseases [15, 19, 20]
TACI (transmembrane activator and calcium signal-modulating cyclophilin ligand, TNFRSF13B, CD267) [21]	17p11.2 5 exons [21]	Membrane bound [8]	BAFF, APRIL [8]	146 nmol·l^{-1} [22]	B-, T-cells immature adipose tissue [17]	T-cell activation [23] and humoral immunity response modulation [24, 25]	Mutations may result in common variable immunodeficiency [26, 27]
BCMA (B-cell maturation antigen, TNFRSF17) [28]	16p13.1 3 exons [28]	Membrane bound [8]	APRIL (BAFF) [8]	1600 nmol·l^{-1} [8]	B-cells immature [17]	Long-term plasma cell survival, B-cell antigen presentation [29]	Protection of multiple myeloma cells from apoptosis [30, 31]

BAFF has been shown to be a negative regulator of B-cell expansion. TACI$^{-/-}$ mice show B-cell hyperplasia and elevated levels of circulating antibodies, resulting in fatal autoimmune glomerulonephritis and splenomegaly [11, 12].

The primary ligand of BCMA is APRIL, although BAFF also binds to this receptor, albeit with low affinity [8]. A significant role for BCMA was determined in multiple myeloma. BCMA ligation provides survival signals for abnormal plasma cells to evade apoptosis [30, 32]. Notably, all three BAFF receptors activate NF-κB pathways via TRAF signaling molecules [8, 33, 34].

2. Cytokine and Adipokine BAFF Is Expressed Ubiquitously

BAFF is expressed primarily as a membrane bound protein but is also extensively cleaved to a soluble form [35, 36]. Soluble BAFF levels in blood are related closely to the number of circulating B-cells and the amount of BAFF receptors available for cleavage. The normal levels of soluble BAFF in healthy adults range from 0.3 to 2.25 ng/mL in peripheral blood. The cord blood of newborns contains significantly higher concentrations of BAFF, ranging from 0.6 to 4.5 ng/mL [37].

The homotrimeric soluble form of BAFF activates BAFF-R. Homotrimeric BAFF can undergo oligomerization that is required for activation of TACI [11]. The expression of BAFF is not related to a single tissue or a specific group of cells. BAFF is expressed on the surface of human myeloid lineage cells (monocytes), primary and secondary lymphoid organs (spleen, bone marrow, and lymph nodes), and various tissues that do not possess primary immune functions (e.g., low expression levels in heart and pancreas) [38]. Moreover, expression of BAFF and its receptors was confirmed in human adipose tissue cultures [17]. In a mouse model, BAFF expression was upregulated during adipocyte differentiation and under proinflammatory conditions (treatment with TNF-α) [39]. BAFF also negatively affects insulin sensitivity in murine visceral adipose tissue [40]. In light of these findings, BAFF, being a cytokine and member of the adipokine family, is considered an important player in many pathophysiological conditions, including inflammation, autoimmune disorders, primary immunodeficiencies [39, 41, 42], obesity, and diabetes [40, 43, 44]. Along with its connection to the apoptosis regulating NF-κB signaling pathway, the role of the BAFF ligand/receptor system in malignant diseases is steadily being elucidated [31, 45].

3. Expression of BAFF Is Regulated by Interferon and Estrogen Levels

Gamma interferon activation site (GAS) element was described in the promoter region of *BAFF* gene in human intestinal epithelial cells leading to IFN-γ-induced expression of BAFF via activation of JAK/STAT signaling [46]. This molecular mechanism of BAFF regulation was supported by correlation of IFN-γ and BAFF levels observed in various human immune system-related cells under physiological and malignant conditions [47–49]. Moreover, therapeutic IFN-β administration also increases BAFF levels *in vivo* [50, 51]. Hence, BAFF can be considered as a molecule that connects innate and specific immunity through its response to IFN-γ and IFN-β and its subsequent activation of B-cells.

Interestingly, BAFF expression is enhanced in the presence of elevated estrogen levels in mice with systemic lupus erythematosus [52] and estrogen-induced B-cell activation in lupus mice is blocked by the antiestrogenic activity of tamoxifen. Thus, estrogen-induced BAFF upregulation may

contribute to a higher incidence of autoimmune disorders in females [53].

4. BAFF Antiapoptotic and Proinflammatory Signaling Is Mediated by the NF-κB Pathway

NF-κB is an intracellular protein complex and the central member of a vital and pivotal signaling pathway [54] that plays a key role in immunity [55] and inflammation [56]. Various studies have presented NF-κB as an antiapoptotic and cell cycle control player in malignancies [57–59]. Owing to these qualities, the presence of inflammation and activated NF-κB signaling are risk factors in malignant transformation [60]. The molecular signaling of NF-κB starts with stimulation of receptors for proinflammatory cytokines [56] and certain members of the TNF receptor superfamily, including BAFF-R, TACI, and BCMA [61, 62]. BAFF-R-mediated activation of NF-κB goes through the noncanonical (alternative) signal pathway, whereas TACI and BCMA activate the canonical (classical) NF-κB pathway [8]. NF-κB has the ability to enhance recruitment of inflammatory cells [55] and the expression of proinflammatory cytokines such as IL-1β [63, 64], IL-2 [65], IL-6 [66, 67], and TNF-α [68]. Deficiency or mutations in the BAFF ligand/receptor system lead to inhibition of NF-κB, thus reducing its antiapoptotic and proinflammatory role [69–71].

5. BAFF Is a Biomarker of Disease Progression in Multiple Myeloma

Multiple myeloma (MM) is a malignant disease caused by aberrant proliferation of bone marrow plasma cells. Since BAFF is essential for the survival of B-cells and plays an important role in survival of plasma cells, particularly in early stages of their development, its role in the pathophysiology of multiple myeloma continues to be intensively studied [72, 73]. Serum levels of BAFF in MM patients were found to be significantly higher (6.0 ± 1.88 ng/mL) than in healthy controls (2.25 ± 0.71 ng/mL) in a study by Wang et al. [72] and elsewhere [74–76] and correlated with disease progression and intensity of plasma cell infiltration [76]. Patients with monoclonal gammopathy of unknown significance (MGUS) are reported to have significantly lower serum levels (3.24 ± 0.28 ng/mL) of BAFF and BAFF-R than MM patients [72].

Pretherapeutic, soluble BAFF levels positively correlate with TNF-α [72], IL-6 [75, 76], and other adverse markers of disease activity such as C-reactive protein and lactate dehydrogenase in MM patients [75, 76]. Posttreatment levels of BAFF correlate with IL-10, which also modulates apoptosis in B-cells [77], induces proliferation of MM cells [78, 79], and abolishes all-*trans*-retinoic acid inhibitory activity on MM cell growth [79]. Moreover, in the study of Lemancewicz et al., higher serum concentrations of BAFF predicted shorter progression-free survival [75]. Taken together, these clinical studies provide evidence of a strong correlation between BAFF and disease progression in MM.

6. BAFF/BAFF-R Signaling May Prove to Be a Promising Target of Future Therapy in B-Cell Derived Malignancies

Simultaneously with MM, the role of BAFF and its receptors was intensively studied in other B-cell derived malignancies such as certain subtypes of non-Hodgkin's lymphomas and precursor B-lineage acute lymphoblastic leukemia (B-ALL). Novak et al. found that BAFF levels corresponded with disease severity and clinical outcome and that elevated levels of BAFF correlated with aggressive phenotype of NHL in humans [80]. Similarly, increased BAFF expression profiles may contribute to *Helicobacter pylori*-independent tumor growth in MALT lymphoma [81]. Elevated levels of BAFF were also reported in other B-lineage lymphomas [82, 83], Hodgkin's lymphoma [84, 85], and B-ALL [82, 86]. Although there is only one FDA-approved anti-BAFF antibody, belimumab, which is used exclusively in rheumatology, new anti-BAFF antibodies are currently being tested for treatment of B-cell lymphomas [87].

In another setting, targeting BAFF-R in B-ALL with a novel humanized anti-BAFF-R antibody selectively kills chemotherapy-resistant precursor B-ALL cells [88]. The anti-BAFF-R antibody also significantly stimulates natural killer cell-mediated killing and macrophage phagocytosis of human ALL cells *in vitro* and decreases leukemia burden in murine bone marrow and spleen. Its therapeutic effects were augmented in combination with conventional chemotherapeutics [89]. BAFF-R might represent a promising therapeutic target because its expression is much higher in leukemic B-cells compared to healthy B-cells [90].

7. BAFF Levels Correlate with Disease Activity and Malignant Potential of Cancer Cells in Several Types of Nonhematologic Solid Tumors

Compared to MM and B-derived malignancies, a possible pathophysiological link between BAFF and solid tumors is not as obvious; however, BAFF expression has recently been studied in many types of solid tumors [91–95]. Neuroendocrine tumors (NET) usually express numerous biologically active mediators. Serum levels of BAFF in NET patients (1.195 ± 0.568 ng/mL) are significantly higher compared to healthy controls (0.666 ± 0.240 ng/mL) [94]. Patients in disease progression (1.503 ± 0.637 ng/mL) and patients with metastases (1.391 ± 0.724 ng/mL) have higher serum BAFF levels compared to those with stable disease (0.906 ± 0.273 ng/mL) [94].

BAFF plasma levels were further examined in solid childhood malignancies such as nephroblastoma (Wilms tumor), Ewing sarcoma, and rhabdomyosarcoma showing BAFF levels of 2.757 ± 3.332 ng/mL, 4.311 ± 4.750 ng/mL, and 6.593 ± 4.502 ng/mL, respectively, and these levels were higher compared to the childhood non-Hodgkin's lymphoma subgroup (2.376 ± 1.560 ng/mL) [95].

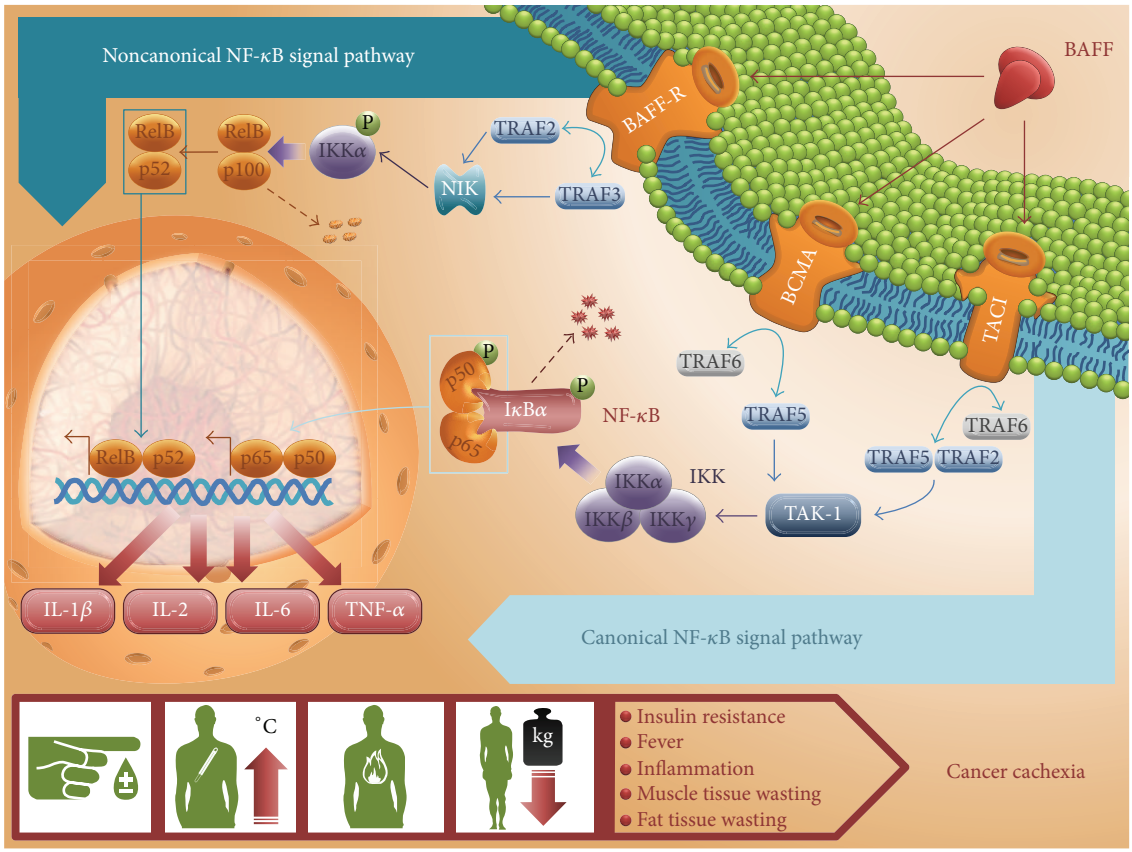

Figure 1: BAFF-induced activation of NF-κB signaling and increased expression of proinflammatory cytokines as procachectic mediators. BAFF interacts with three receptors from the TNF ligand/receptor superfamily, BAFF-R, TACI, and (with lower affinity) BCMA [8]. Upon activation, BCMA signal transduction goes through TNF receptor associated factors (TRAFs) 5 and 6 [96], whereas TACI signals through TRAF2, TRAF5, and TRAF6 [97]. TRAF2 and TRAF5 activate IκB kinase (IKK) via TAK-1 kinase (the canonical NF-κB pathway) [98]. Follow-up phosphorylation of NF-κB inhibitor alpha (IκBα) induces ubiquitination of IκBα and its proteasome degradation [99]. In this way, IκBα is released from the phosphorylated heterodimer p50-p65, and p50-p65 then migrates to the nucleus [99]. BAFF-R signaling starts with TRAF2 and TRAF3 degradation and accumulation of NF-κB inducing kinase (NIK) [100]. In this noncanonical NF-κB pathway, NIK phosphorylates inhibitor of NF-κB kinase alpha (IKKα) [101]. IKKα then induces cleavage of p100 protein in the p100-RelB complex into a p52-RelB complex which acts as a modulator of nuclear gene transcription [102]. Both canonical and noncanonical NF-κB pathways regulate the expression of genes encoding IL-1β [63, 64], IL-2 [65], IL-6 [66, 67], and TNF-α [68]. Proinflammatory cytokines participate in manifestation of cancer cachexia symptoms such as insulin resistance [103], fever [104], inflammation [105], and muscle [106–108] and fat tissue wasting [105, 109, 110].

8. BAFF May Contribute to Cancer Cachexia through Its Proinflammatory Properties and by Impairment of the Insulin Receptor Signaling Pathway

Involuntary weight loss is a complication that often follows many serious symptoms such as inanition (inadequate food availability or pathophysiologic conditions substantially decreasing the desire of food), anorexia (reduced food intake caused primarily by diminished appetite with high influence of CNS mechanisms), or cachexia (metabolic disorder of increased energy expenditure leading to a greater weight loss than that caused by reduced food intake alone) [111]. Cancer cachexia is a syndrome where tumors in host organisms play important roles in degrading certain host tissues by production of catabolic mediators [112]. The exact mechanism in which malignant diseases cause cachexia is not completely understood, but there is probably a role for inflammatory cytokines, such as TNF-α, various interleukins, and IFN-γ, as well as tumor-secreted proteolysis-inducing factor (PIF) and lipolysis mobilizing factor (LMF). Based on these findings, the ghrelin receptor agonist anamorelin hydrochloride has recently been introduced for therapy of cancer-induced cachexia (currently in phase III clinical trials for treatment of cancer cachexia in non-small-cell lung cancer) [113]. Ghrelin binds GHS receptors on T-cells and monocytes and inhibits proinflammatory cytokine expression (IL-1β, IL-6, and TNF-α). The mechanism of action of anamorelin in cancer cachexia is probably mediated by both a CNS-mediated increase in appetite and anti-inflammatory effects [114]. By inhibition of proinflammatory cytokines and inflammation, anamorelin acts indirectly against BAFF.

Proinflammatory cytokines target corresponding receptors on host inflammatory and tumor cells and activate

the NF-κB signaling pathway [61, 62]. Activation of NF-κB leads to production of even higher amount of cytokines in a positive feedback manner [61, 115]. Binding to its receptors, BAFF enhances NF-κB signaling that leads to increased production of proinflammatory cytokines and promotion of overall inflammation during malignancy (Figure 1) [63–68].

Another common complication arising from the altered metabolism in patients with cancer cachexia is insulin resistance [116]. Hamada et al. found that BAFF-treated mice exhibited increased blood glucose, insulin blood levels, and high expression of TNF-α, IL-6, and resistin with decreased expression of adiponectin in visceral adipose tissue, suggesting an impairment in insulin receptor signaling similar to that observed in type II diabetes mellitus and metabolic syndrome. That same study confirmed reduced activation of insulin receptor substrate (IRS-1) as a response to BAFF treatment [40]. BAFF-induced insulin resistance was later confirmed in another mouse model [117]. Insulin resistance augments cancer cachexia in patients with malignancy [112, 118] providing another link between BAFF and the development of cancer cachexia syndrome.

9. BAFF Signaling May Contribute to Cancer Progression and Cancer Cachexia Not Just via Its Proinflammatory Role

BAFF may contribute to cancer progression through the amplification of proinflammatory signaling. A causative role of BAFF in cancer and cancer cachexia independent of inflammation has been difficult to substantiate; interestingly however, Koizumi et al. have shown that *in vitro* incubation of tumor cells isolated from pancreatic ductal adenocarcinoma (PDAC) patients with human recombinant BAFF resulted in altered phenotype with increased invasiveness and motility. Downregulation of E-cadherin mRNA and significant upregulation of vimentin and Snail mRNAs were found in these cells. BAFF-induced alteration of epithelial-mesenchymal transition- (EMT-) related genes that support precancerous formations of pancreatic intraepithelial neoplasias and PDAC itself was confirmed on BAFF-R overexpressing cell clones [91]. Thus, BAFF may promote tumorigenesis indirectly by induction of inflammation in the tumor microenvironment and directly by induction of EMT.

Similar to BAFF's involvement in cancer progression, BAFF's involvement in cancer cachexia is difficult to distinguish from its proinflammatory effects. BAFF may contribute to cancer cachexia by affecting changes in NF-κB pathway-induced inflammation and through impairment of insulin sensitivity via reduction of adiponectin and possibly other adipokines maintaining glucose homeostasis.

Taken together, an increase in catabolic demands during inflammation and malignancy predispose to cancer cachexia development. BAFF may enhance the inflammatory background in cancer patients, providing a tantalizing link to involvement in cancer cachexia (Figure 2); however additional studies will be required to confirm such a link and potential avenue for therapeutic intervention.

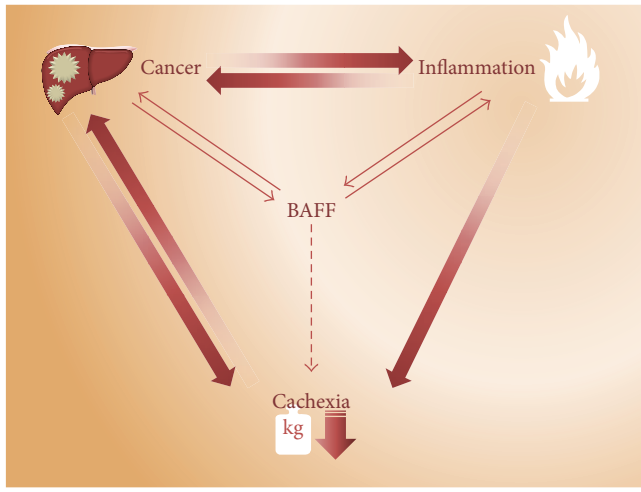

Figure 2: BAFF in cancer cachexia interplay. Outer arrows indicate well-described hallmarks of cancer cachexia. Cancer → inflammation: many types of cancer cells express cytokines that induce inflammation [119]. Inflammation → cancer: tumors often manifest on inflammatory background that supports transition of cells to malignant clones (e.g., hepatocellular carcinoma or PDAC as cited in the text). Cancer → cachexia: tumor tissue directly participates in the development of cancer cachexia by production of tumor specific factors like PIF and LMF [120, 121]. Cachexia → cancer: cachexia in cancer patients remains a significant cause of morbidity and mortality in cancer treatment [122]. Inflammation → cachexia: proinflammatory cytokines induce cachexia by increased catabolism with altered insulin sensitivity [119]. Inner arrows indicate established (solid line) and putative (dashed line) role of BAFF in pathophysiology of cancer cachexia. Cancer → BAFF: increased expression and serum levels of BAFF were demonstrated in many types of hematological and solid tumors making BAFF a possible new biomarker in malignancies. BAFF → cancer: BAFF has been found to augment manifestation of lymphoma and the formation of epithelial-mesenchymal transitions and pancreatic intraepithelial neoplasias. These events precede PDAC. (1) A TNF-independent role of BAFF in the pathophysiology of lymphomas was demonstrated in BAFF-Tg $TNF^{-/-}$ mice. More than 35% of BAFF-Tg $TNF^{-/-}$ mice had occurrence of various types of lymphomas within 1 year [123]. (2) BAFF-induced alteration of the epithelial-mesenchymal transition- (EMT-) related genes that support precancerous formation of pancreatic intraepithelial neoplasias and PDAC was confirmed on BAFF-R overexpressing cell clones [91]. Inflammation → BAFF: BAFF is produced by several proinflammatory cells. BAFF → inflammation: BAFF induces expression of proinflammatory cytokines by activation of NF-κB [124]. BAFF → cachexia: BAFF induces insulin resistance [40, 117] which has been associated with cancer cachexia [116, 118].

Conflict of Interests

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

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Clinical Study

Concordance of Hypermethylated DNA and the Tumor Markers CA 15-3, CEA, and TPA in Serum during Monitoring of Patients with Advanced Breast Cancer

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The serological protein tumor markers CA 15-3, CEA, and TPA are frequently used to monitor tumor burden among metastatic breast cancer patients. Breast cancer is associated with global DNA hypomethylation and hypermethylation of some promoter regions. No monitoring study has yet investigated the interrelationship between protein tumor markers, the global DNA hypomethylation, and hypermethylated genes in serum from patients with advanced disease. Twenty-nine patients with histologically proven advanced breast cancer received first-line chemotherapy with epirubicin. Samples were collected prior to each treatment and prospectively analyzed for CA 15-3, CEA, and TPA. The same samples were retrospectively analyzed for the concentration of hypermethylated *RASSF1A* and for global DNA hypomethylation using *LINE-1*. Among patients with elevated concentrations of the protein markers, concordance could be observed between serial changes of the hypermethylated *RASSF1A* gene and the protein markers. Among patients with lower concentrations, *RASSF1A* could only be detected periodically. There was discordance between changes of the hypomethylated *LINE-1* as compared to the protein markers. Circulating hypermethylated *RASSF1A* and protein markers may have similar kinetics during monitoring of tumor burden. Further investigations are needed to determine whether any of the hypermethylated DNA genes may provide predictive information during monitoring.

1. Introduction

Monitoring the treatment of metastatic breast cancer involves a wide array of assessments and the need for the clinician to integrate several different forms of information about the effectiveness of treatment and the acceptability of toxicity [1]. The information includes those from direct observations of the patient including patient reported symptoms; performance status; change in weight; physical examination; laboratory tests such as alkaline phosphatase, liver function, blood counts, and calcium; radiographic imaging; functional imaging; and, where appropriate, tumor biomarkers [1].

The tumor markers cancer antigen 15-3 (CA 15-3), carcinoembryonic antigen (CEA), and tissue polypeptide antigen (TPA) can optionally be used as a supplement to monitor the effect of the anticancer treatment in metastatic breast cancer [1]. The CA 15-3 assay is based on the monoclonal antibodies 115D8 and DF3 which are both raised against the human MUC1 protein [2, 3]. The CEA molecule is a glycoprotein involved in cell adhesion. CEA is a glycosylphosphatidylinositol cell surface anchored glycoprotein that is released into the bloodstream of cancer patients and healthy individuals [2, 3]. Being a secretory product both CA 15-3 and CEA are considered as serological markers of changing tumor burden in the individual patient; however, the exact mechanism of release from the cell membrane is unknown. TPA belongs to the cytoskeleton proteins circulating as a complex of soluble proteolytic polypeptide fragments of cytokeratins 8, 18, and 19 [2, 3]. Their release may indicate cell turnover and the information supplied by TPA may be distinctly different from

the information supplied by the markers of tumor burden CA 15-3 and CEA [2, 3].

The results of all clinical evaluations, that is, physical evaluations, laboratory tests, imaging, and serum biomarkers, generally are classified as response, continued response to treatment, stable disease, uncertainty regarding disease status, or progression of disease [1]. The clinicians typically must assess and balance multiple different forms of information to make a determination regarding whether disease is being controlled and the toxicity of treatment is acceptable [1]. Sometimes this information may be contradictory, and recent guidelines do not recommend the use of serum tumor biomarkers alone in metastatic breast monitoring for evaluating the response to anticancer therapy [1]. The average sensitivity for CA 15-3, CEA, and TPA is 70%, 55%, and 64%, respectively, for breast cancer at stage IV, and it drops to 35%, 25%, and 40% at stage III [2].

The significance of hypermethylation of tumor suppressor genes in carcinogenesis is being increasingly recognized as new serum biomarkers for monitoring metastatic breast cancer [5–7]. Some of the potential interesting hypermethylated genes associated with breast cancer have been reviewed recently [6–8]. Interestingly, the hypermethylated RAS association (RalGDS/AF-6) domain family member 1A gene (*RASSF1A*) has been reported to have clinical sensitivity of 67%–75% for stage IV breast cancer [9]. Lastly, the long interspersed nuclear elements (*LINE*) are a member of the autonomous retrotransposons encoding for a reverse transcriptase and are transcribed by a RNA polymerase II. This transposable element can change its position within the genome, and the *LINE-1* gene is one of the most abundant sequences in the human genome and makes up 17% of the human genome. *LINE-1* is often used as a surrogate for global hypomethylation, and quantification of *LINE1* in circulating DNA is suggested as a molecular biomarker of breast cancer [10]. Thus, hypermethylated *RASSF1A* and hypomethylation of *LINE-1* are candidates for clinical research studies of novel serological biomarkers for monitoring breast cancer.

So far, no studies have compared the kinetics of hyper- and hypomethylated DNA with the kinetics of CA 15-3, CEA, and TPA protein tumor markers during monitoring of advanced breast cancer [1]. In the present study, we have monitored the serial changes in the hypermethylated *RASSF1A* and global hypomethylation using *LINE-1* and compared with the changes in CA 15-3, CEA, and TPA concentrations.

2. Materials and Methods

2.1. Healthy Subjects. Women among the healthy staff at the Departments of Oncology and Clinical Chemistry, Her1ev Hospital, University of Copenhagen, Denmark, volunteered to participate in the study from 1990 to 1992 [11]. All subjects gave informed consent to their participation, and the study was approved by the regional Ethical Committee (KA 93076). All subjects stated they were free of disease at the time of the study, and none had any known chronic or recurrent illness or was taking any medication. The subjects continued their usual lifestyle during the period of the study. No investigations were performed to exclude asymptomatic breast cancer. Serum samples were stored at −80°C and later analyzed for hypermethylated *RASSF1A*.

Table 1: Distribution of metastasis before and after therapy among the 29 investigated patients.

	Start of therapy	End of therapy
Lung	31.0% (9/29)	24.1% (7/29)
Liver	3.4% (1/29)	13.8% (4/29)
Contralateral mamma	3.4% (1/29)	0.0% (0/29)
Bone	44.8% (13/29)	48.3% (14/29)
Intra-abdominal	3.4% (1/29)	3.4% (1/29)
Skin	20.7% (6/29)	10.3% (3/29)
Lymph node	48.3% (14/29)	20.7% (6/29)
Other locations	13.8% (4/29)	17.2% (5/29)
CNS	n.d.	3.4% (1/29)
Solitary location	20.7% (6/29)	6.9% (2/29)
Multiple location	75.9% (22/29)	69.0% (20/29)

2.2. Patients with Advanced Breast Cancer. The 29 investigated patients had histologically proven advanced progressive breast cancer with measurable or evaluable disease [4, 12]. They received epirubicin 70 mg/m^2 on days 1 and 8 every 4 weeks. Epirubicin was continued until progressive disease (PD) was noted or until a maximum cumulative dose of 1000 mg/m^2 had been administered. Pretreatment evaluation includes a complete history and physical examination, blood cell counts (hemoglobin, WBC, and platelets), serum chemistry profiles (creatinine, calcium, alkaline phosphatase, transaminase, and bilirubin), chest radiography, electrocardiography, ^{51}Cr-EDTA clearance, and bone scans. Areas of increased uptake on bone scans were further evaluated with roentgenograms to determine the nature of the abnormalities. Ultrasound scan of the liver was performed if the serum alkaline phosphatase or transaminase was elevated [12]. During treatment, history taking, physical examination, blood cell counts (hemoglobin content, leukocytes, and platelets), and routine biochemistry (sodium, potassium, creatinine, calcium, magnesium, alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase, and bilirubin) were repeated before each treatment cycle. Evaluable or measurable indicators were evaluated every second month, except for bone lesions, which were evaluated every third month [4]. The clinical study were carried out in the period from 1988–1991. In that period the clinical response evaluations were based on the criteria of the World Health Organization [13].

Table 2: Responses based on clinical evaluations and protein marker evaluations at the end of therapy among the 29 patients.

Evaluation	Complete response (CR)	Partial response (PR)	No change (NC)	Progressive disease (PD)
Clinical evaluation	24% (7/29)	14% (4/29)	31% (9/29)	31% (9/29)
CA 15-3 evaluation	14% (4/29)	24% (7/29)	48% (14/29)	14% (4/29)
CEA evaluation	10% (3/29)	17% (5/29)	59% (17/29)	14% (4/29)
TPA evaluation	14% (4/29)	7% (2/29)	55% (16/29)	24% (7/29)

Clinical response evaluation was performed by investigators without knowledge of the tumor marker data. Blood specimens for CA 15-3, CEA, and TPA analysis were sampled before each treatment cycle [4, 12]. Each specimen was analyzed for CA 15-3, CEA, and TPA. The specimens were analyzed consecutively, and each specimen from an individual patient was analyzed in a separate assay run. Changes in marker concentrations were evaluated by criteria as described by Sölétormos et al. [4]. Additional specimens were sampled whenever data for alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase, or calcium were requested outside the scheduled time points. The CA 15-3, CEA, and TPA concentrations were assessed by one investigator who had not participated in the clinical evaluation. At each sampling the serum specimen used for analysis of the protein tumor markers was saved in different aliquots at −80°C and used for the current analysis of hyper- and hypomethylated DNA. The study complied with the Helsinki II Declaration and was approved by the Scientific Ethics Committee of Copenhagen County (KA 89257, H-D-2009-048).

Serum DNA was isolated using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany) and subjected to sodium bisulfite conversion of nonmethylated cytosines (EpiTect Bisulfite kit from QIAGEN) and stored at −80°C. The probe and primer designs used for the hypermethylated *RASSF1A* and collagen 2 gene (*COL2A1*) have previously been reported [14, 15]. The primers targeted non-CpG-containing regions of *COL2A1*. The measured *COL2A1* concentration is therefore not sensitive to any potential methylation of CpG dinucleotide motifs and can therefore be used to measure the assay input of DNA. The *COL2A1* concentration was used to normalize the *RASSF1A* concentration. The PCR reaction was carried out with the 7500 Fast Real-Time PCR system (Applied Biosystem) using the TaqMan Genotyping Master mixture. The analytical coefficient of variance for the detection of hypermethylated *RASSF1A* gene was 10.9%.

The *LINE-1* gene was used as a surrogate for global hypomethylation by analyzing the concentration of methylated and unmethylated *LINE-1* by methylation-specific PCR [16]. A standard curve was prepared by using bisulfite-converted DNA from MCF7 breast cancer cells. The *LINE-1* amplicons were investigated by melt curve analysis and UV illumination of ethidium stained amplicons separated on 2% (wt./vol.) 1x TBE agarose gels. The percentage of methylated *LINE-1* was calculated using the formula: 100 × methylated reaction/(unmethylated reaction + methylated reaction). Relative % *LINE-1* methylation was investigated in serial samples obtained from six of the patients (total 71 serial serum samples). The analytical coefficient of variance for the *LINE-1* methylation-specific PCR method on 7500 Fast Real-Time system was 15.6%.

3. Results

Hypermethylated *RASSF1A* was not detected in serum samples obtained from eighteen healthy women with a mean age of 62.8 years (range 55–75). Thus, the clinical specificity of hypermethylated *RASSF1A* was 100%. The mean age of the twenty-nine patients was 49.6 years (range 34–67 year), and the mean length of the individual therapy period was 196 days (range 59–396 days) consisting of a mean number of 6.5 cycles per patient. The distribution of metastasis before start of therapy and at the end of therapy is shown in Table 1. The patients had metastasis at multiple locations (22 out of 29 patients), in the lymph node (14 out of 29) and bones (13 out of 29) before start of therapy. After the end of therapy, the majority of patients still had metastasis at multiple locations, bones, lymph nodes, lung, liver, and other sites. When comparing the status of metastasis before therapy with the status after therapy, there was a reduction in number of patients with metastasis in the lymph nodes, lung, skin, solitary locations, and multiple locations and an increase in number of patients with metastasis in the liver, bone, and other locations.

The percentage of biomarkers with below cut of level concentrations at the start of therapy was 41.4% (CA 15-3), 69.0% (CEA), and 24.1% (TPA). The clinical evaluations and protein marker evaluations at the end of therapy are shown in Table 2. In total, 422 serial serum samples were collected from the patients during therapy with a mean of 14.5 samples per patient. Hypermethylated *RASSF1A* was detected in all of the 29 patients at some time during monitoring and was detected in 45% of the serial samples. Thus, *RASSF1A* was only periodically detected in some patients during monitoring. The monitoring data for four representative patients, Patients A-D, are provided in Figures 1–4. The interrelationship between clinical evaluations and changes in serial concentrations of the protein tumor markers CA 15-3, CEA, TPA, and *RASSF1A* in samples from Patient A appears in Figure 1. Accordingly, Patient A presents with both clinical and protein tumor marker response of PR followed by PD. The clinical PR was based on an observation of a reduction of tumor size in the contralateral mamma, bones, and lymph nodes. The clinical PD was based on increased tumor burden at several sites. There was concordance between the changes of the hypermethylated *RASSF1A* with those of CA 15-3, CEA, and TPA as well as concordance with the clinical response evaluations (PR to PD). Figure 2 shows serial sets of data obtained

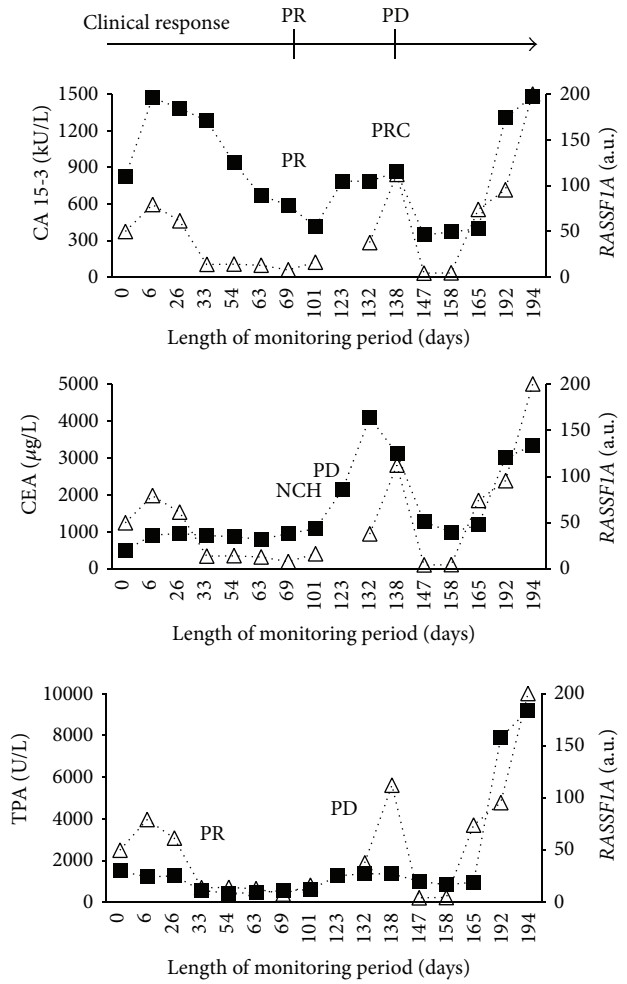

Figure 1: Monitoring Patient A with advanced breast cancer by measuring serial concentrations of CA 15-3, CEA, TPA, and hypermethylated *RASSF1A*. ■ denotes the respective protein biomarkers CA 15-3, CEA, and TPA. △ denotes the hypermethylated RAS association (RalGDS/AF-6) domain family member 1A gene (*RASSF1A*). The clinical response changed from a partial response (PR) to progressive disease (PD) during chemotherapy. Sixteen serial serum samples were investigated. The marker response was partial response (PR), partial response continued (PRC), no change high (NCH), and progressive disease (PD) for CA 15-3, CEA, and TPA according to previously reported assessment criteria [4].

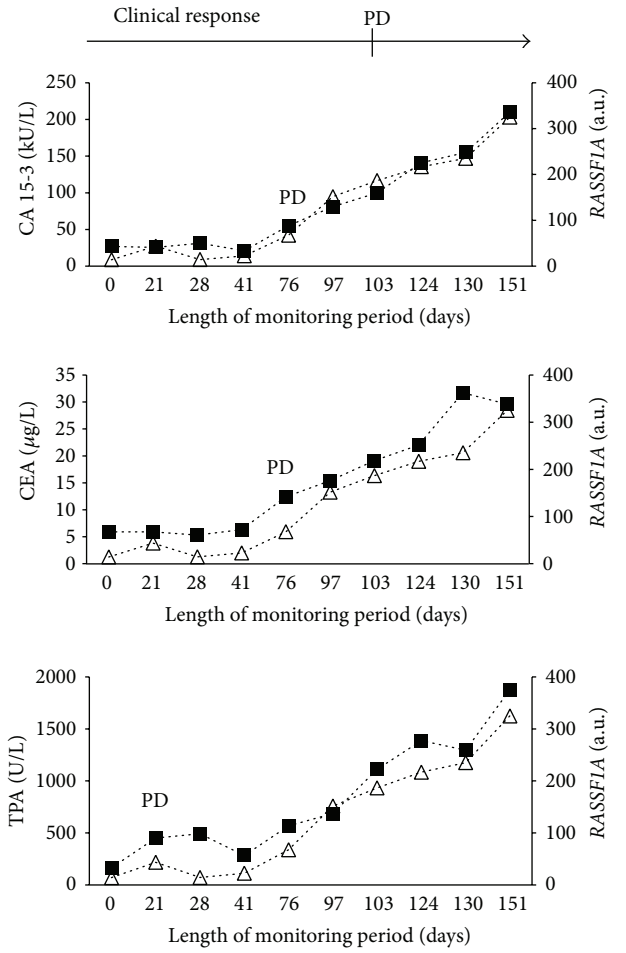

Figure 2: Monitoring Patient B with advanced breast cancer by measuring serial concentrations of CA 15-3, CEA, TPA, and hypermethylated *RASSF1A*. ■ denotes the respective protein biomarkers CA 15-3, CEA, and TPA. △ denotes the hypermethylated RAS association (RalGDS/AF-6) domain family member 1A gene (*RASSF1A*). The clinical response was progressive disease (PD) during chemotherapy. Ten serial serum samples were investigated. The marker response was progressive disease (PD) for CA 15-3, CEA, and TPA according to previously reported assessment criteria [4].

from Patient B who had clinical PD during treatment as well as PD of the three protein markers. The clinical PD was based on liver and bone metastases. There was concordance between the increments in the hypermethylated *RASSF1A* concentrations with the increments of the CA 15-3, CEA, and TPA concentrations as well as concordance with the clinical response evaluations (PD). Concordance between the change in *RASSF1A* and the protein markers was also observed in Patient C who had clinical response (PR) to the treatment as shown in Figure 3. The PR evaluation was based on a reduction in bone metastases. However, there was discordance between *LINE-1* hypomethylation and the three protein tumor markers among six patients. This is illustrated for CA 15-3 and *LINE-1* for one representative patient (Patient D) who had clinical PR based on reduction of bone metastases (Figure 4).

4. Discussion

In the present study, concordance of changes in serum concentrations of the hypermethylated *RASSF1A* with the tumor burden markers CA 15-3 and CEA and the tumor activity marker TPA has been demonstrated for the first time. Fackler et al. [9] also monitored circulating tumor DNA in metastatic breast cancer using a 10-gene panel of hypermethylated biomarkers including *RASSF1A*. They suggested that the concentration of the methylated genes in the panel correlated with the tumor burden as evaluated by the RECIST criteria

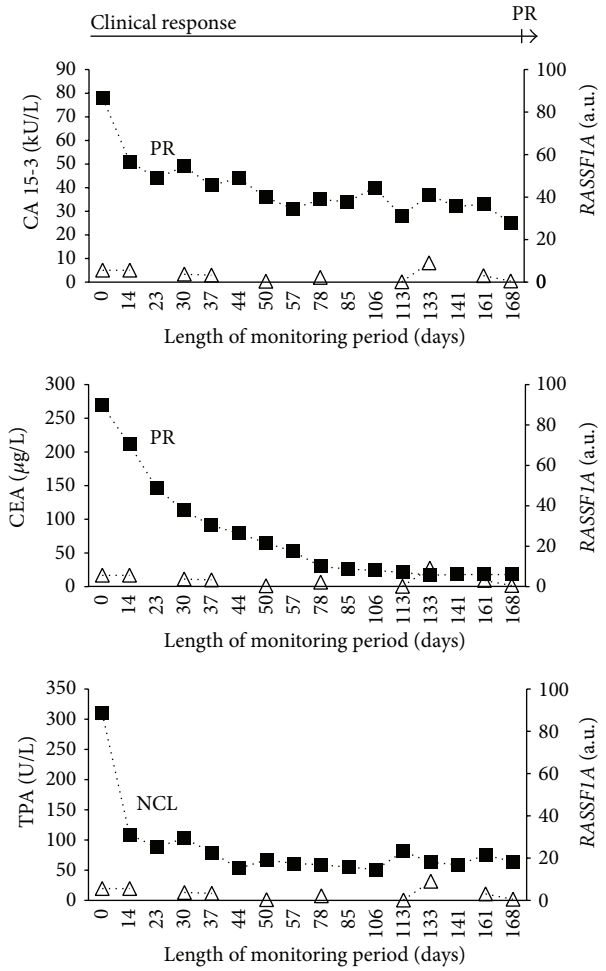

Figure 3: Monitoring Patient C with advanced breast cancer by measuring serial concentrations of CA 15-3, CEA, TPA, and hypermethylated *RASSF1A*. ■ denotes the respective protein biomarkers CA 15-3, CEA, and TPA. △ denotes the hypermethylated RAS association (RalGDS/AF-6) domain family member 1A gene (*RASSF1A*). The clinical response was a partial response (PR) during chemotherapy. Sixteen serial serum samples were investigated. The marker response was partial response (PR) for CA 15-3 and CEA. For TPA the response was no change low (NCL) according to previously reported assessment criteria [4]. *RASSF1A* was undetectable in six samples.

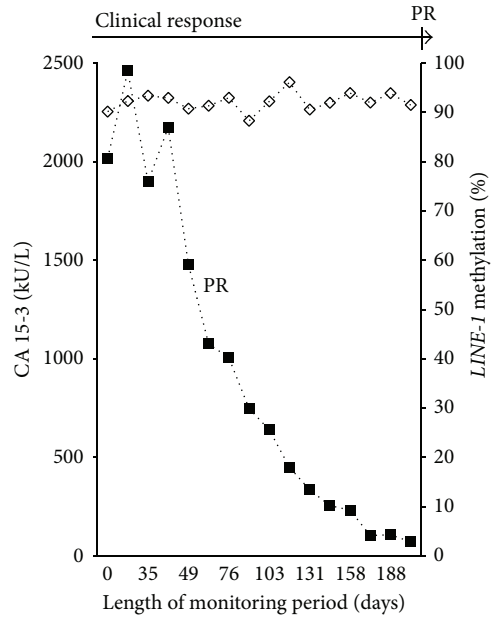

Figure 4: Monitoring Patient D with advanced breast cancer by measuring serial concentrations of CA 15-3 and hypomethylated *LINE-1*. ■ denotes the protein biomarker CA 15-3. ◊ denotes the hypomethylated long interspersed nuclear elements 1 (*LINE-1*). The clinical response was partial response (PR) during chemotherapy. Sixteen serial serum samples were investigated. The marker response was partial response (PR), according to previously reported assessment criteria [4].

[17]. However, the change in concentrations of the investigated genes was not compared with the kinetics of CA 15-3, CEA, and TPA [9].

In some samples, we observed that hypermethylated *RASSF1A* could not be detected. One example is illustrated by Patient C (Figure 3) where *RASSF1A* remained undetected in 6 out of the 16 serial serum samples. The CA 15-3 and TPA concentrations tended to be lower as compared with the concentrations obtained for Patient A and Patient B (Figures 1 and 2, resp.). This may indicate a relatively lower tumor burden and tumor activity in Patient C and suggests why *RASSF1A* was not detected among 6 of the 16 serial samples from Patient C (Figure 3).

The hypothesis of undetectable *RASSF1A* concentrations among patients with a small tumor burden is supported by our findings among 18 healthy females where presence of *RASSF1A* in the serum samples could not be demonstrated. The findings may support the view that there is no or alternatively there is a very low release of hypermethylated *RASSF1A* into the circulation among healthy individuals and among patients with low tumor burden or low activity of the tumor(s).

It may also be speculated that the periodically lack of detection of *RASSF1A* was due to errors in preparing the serum samples for PCR analysis, that is, poor recovery of DNA and incomplete conversion of the DNA fragments during incubation with sodium bisulfite. However, this is not a likely explanation since *COL2A1* was detectable in all sequentially serum samples. We also investigated whether the periodically lack of detection of *RASSF1A* in some patients could be due to rapid degradation of the sodium-bisulfite converted DNA. Time-course analysis of *APC* (adenomatous polyposis coli gene), *CCND2* (cyclin D2 gene), *CDKN2A* (cyclin-dependent kinase inhibitor 2A gene), *DAPK* (death-associated protein kinase 1 gene), *COL2A1*, and *RASSF1A* concentrations revealed no detectable temperature-dependent degradation of the bisulfite-converted DNA when stored for one day, 7 days, 30 days, and 60 days at 4°C, −20°C and −80°C (data not shown). Finally, thawing and immediately refreezing at −20°C 10 times did not result in any detectable change in the *COL2A1* concentration (data not shown). Taken

together, the stability study showed that the sodium bisulfite-converted DNA was stable, and the periodically lack of *RASSF1A* detection in some patients may be explained by *in situ* subdetectable concentrations.

In conclusion, circulating hypermethylated *RASSF1A* and protein cancer biomarkers may have similar kinetics during monitoring of tumor burden among patients with advanced breast cancer. However, further investigations are needed to determine whether any of the hypermethylated DNA genes may provide predictive information during monitoring.

Conflict of Interests

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

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Review Article

Gamma-Glutamylcyclotransferase: A Novel Target Molecule for Cancer Diagnosis and Treatment

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Gamma-glutamylcyclotransferase (GGCT) is one of the major enzymes involved in glutathione metabolism. However, its gene locus was unknown for many years. Recently, the gene for GGCT was found to be identical to C7orf24, which is registered as a hypothetical protein. Orthologs have been found in bacteria, plants, and nematodes as well as higher organisms, and the GGCT gene is highly preserved among a wide range of species. GGCT (C7orf24) was also reported as an upregulated protein in various cancers. Although the function of GGCT in cancer cells has not been determined, the following important activities have been reported: (1) high expression in various cancer tissues and cancer cell lines, (2) low expression in normal tissues, (3) inhibition of cancer cell proliferation via anti-GGCT RNAi, (4) inhibition of cancer cell invasion and migration via anti-GGCT RNAi, (5) an epigenetic transcriptional regulation in cancer cells, and (6) an antitumor effect in cancer-bearing xenograft mice. Therefore, GGCT is promising as a diagnostic marker and a therapeutic target for various cancers. This review summarizes these interesting findings.

1. Introduction

The existence of human gamma-glutamylcyclotransferase (GGCT) has been known since around 1970. However, its amino acid sequence and gene locus were unknown for many years [1–3]. Recently, Oakley et al. cloned cDNA encoding human GGCT and they found that GGCT is identical to the hypothetical protein C7orf24 (chromosome 7 open reading frame 24), which was previously registered as a putative open reading frame on the short arm of chromosome 7 (7p15-14) [4]. Although its function in cancer cells was unknown, several studies on C7orf24 had been previously reported by others [5–7].

Masuda et al. reported that C7orf24 is identical to cytochrome c-releasing factor (CRF21), which is a substance released into the cytoplasm when human leukemia cells U937 are treated with geranylgeraniol, an apoptosis inducer [5]. They presumed that CRF21 plays a critical role in apoptosis signaling because induction of cytochrome c release from mitochondria triggered apoptosis in HeLa cells overexpressing CRF21. Xu et al. identified 46 common cancer signature genes from a pooled DNA array database of previously reported human cancers and they reported that one of the highly expressed genes was C7orf24 [6]. We conducted proteome analysis to explore specific proteins that can be used as diagnostic markers for urothelial carcinoma, and C7orf24 was identified as a highly expressed protein in cancer tissues [7].

As mentioned above, a number of relationships between cancer and GGCT have been reported by some researchers and the role of this molecule in cancer has attracted attention. This minireview summarizes the role of GGCT in cancer cells and the possibility of using GGCT in cancer diagnosis and targeting treatment.

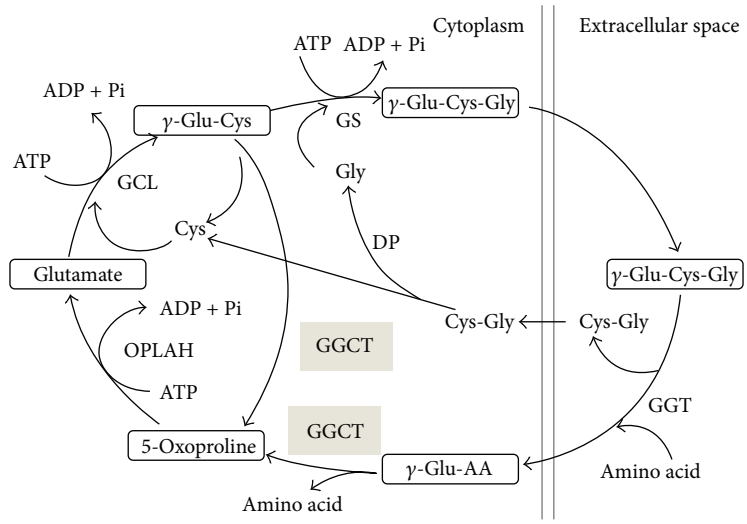

Figure 1: γ-glutamyl cycle. GGCT, gamma-glutamylcyclotransferase; GGT, gamma-glutamyltranspeptidase; GCL, glutamate cysteine ligase; GS, glutathione synthase; OPLAH, 5-oxoprolinase; γ-Glu-Cys-Gly, glutathione; DP, dipeptidase.

2. GGCT and Gamma-Glutamyl Cycle

GGCT is one of the major enzymes comprising the gamma-glutamyl cycle proposed by Orlowski et al. (Figure 1) [2]. GGCT catalyzes the reaction producing 5-oxoproline and free amino acids from gamma-glutamyl peptide taken into the cell. Orthologs of GGCT range from bacteria, plants, and nematodes to higher organisms, and the GGCT gene is highly preserved among a wide range of species.

Glutathione is a tripeptide consisting of glutamate, cysteine, and glycine. Glutathione is found in cells at a relatively high concentration of 0.5–10 mM and has a number of important functions such as an antioxidant and a detoxifying agent. Adequate amounts of glutamate, cysteine, and glycine are essential to maintain glutathione at proper levels [8]. Meister et al. proposed that the gamma-glutamyl cycle plays a role in active transport of several amino acids including glutamate, cysteine, and glycine [9, 10].

Although GGCT was isolated more than 40 years ago [1–3], its gene locus was unknown until identified by Oakley et al. in 2008 [4]. Nowadays, it has gradually been learned that GGCT is expressed at a high level in a number of cancers. However, the role that GGCT, as an enzyme in the gamma-glutamyl cycle, has in the activity of cancer cells is still not known. In addition to its enzymatic role, GGCT may be a multifunctional molecule that is involved in the growth of cancer. These issues need to be clarified in future studies.

3. Distribution and Intracellular Localization of GGCT in Normal Tissues

Several studies concerning the distribution of GGCT in normal tissues were reported. Oda et al. examined the mRNA expression of GGCT in rats [11]. They reported that relatively high levels of mRNA were expressed in the liver and kidney but were generally lower in other organs. Oakley et al. summarized the expression profiles derived from GGCT expressed sequence tags (ESTs) using a human EST database [4]. GGCT is widely expressed in many organs and the levels of expression were higher in bladder and salivary gland than in other organs. However, the reason for this remains unknown. Regarding GGCT protein expression, Gromov et al. performed an immunohistochemical analysis using tissue microarrays [12]. They examined more than 30 normal organ samples and reported weak to moderate immunoreactivity in a wide range of normal tissues. Amano et al. also studied the immunohistochemical expression of GGCT in many normal tissues [13]. They also reported that GGCT protein was detected in most normal human tissues and mainly in epithelial cells.

The intracellular distribution found by immunohistochemistry was in the cytoplasm and nucleus and these findings were almost consistent with a few other reports [12–14]. Cytoplasmic staining by immunohistochemistry is relatively homogeneous. However, nuclear reactivity is relatively heterogeneous. Azumi et al. analyzed the cellular localization of GGCT using full-length protein and its truncated mutants [14]. They produced the transfectants (NIH3T3) that express a GFP-GGCT fusion protein or mutants. They found that the region consisting of 61–120 amino acids is required for the full-length GGCT to anchor in the cytoplasm and they elucidated that deletion of this region allows GGCT to move to the nucleus. Although the role of GGCT in the nucleus has not been determined, the GGCT observed in the nucleus may represent a posttranslationally modified form or a splice variant [13].

4. High Expression of GGCT in Various Cancers

We conducted proteome analysis with two-dimensional electrophoresis in bladder cancer to search for diagnostic markers for urothelial carcinomas [15, 16]. Fifteen highly expressed proteins, including GGCT, were identified through

this analysis. The expression of GGCT in surgical specimens was examined by Western blot using our original monoclonal antibody, and high expression was observed in 64% and 10% of cancer and noncancerous tissues, respectively [7]. We also reported a high expression of GGCT in various human cancer cell lines other than urothelial carcinoma.

After our report, other groups reported similar results using clinical samples [12, 17, 18]. Gromov et al. conducted a large-scale proteome analysis in 123 cases of breast cancer, and they also found that GGCT was highly expressed in neoplastic as compared to normal tissues [12]. They studied the association of the expression of GGCT with the patient's outcome, and they showed that patients with high-level GGCT expression had a poor prognosis. In addition, they also studied the expression of GGCT in cancers other than breast cancer and reported that high expression of GGCT was observed in 58% of uterine cervical cancers, 38% of lung cancers, and 72% of colon cancers. Furthermore, they demonstrated that GGCT could be detected in the extracellular fluid of mammary glands and suggested the possibility of GGCT as a serum marker for breast cancer. Uejima et al. examined the expression of GGCT mRNA using 40 surgical specimens of osteosarcoma compared with normal human osteoblasts as a control. They reported a high expression (average 8.7 times higher than normal human osteoblasts) in all specimens [17]. Takemura et al. conducted an immunohistochemical examination with GGCT antibody in 200 specimens of esophageal lesions [18]. Increase of GGCT expression was observed in 87.5% of esophageal squamous cell carcinoma cases and 85.0% of high-grade intraepithelial neoplasia cases but remained at 17.5% in cases of low-grade intraepithelial neoplasia.

On the other hand, the results obtained by IHC were inconsistent in several types of cancers. Amano et al. examined 13 types of cancer specimens obtained by surgery (n = 30 in each cancer), and they reported significant decreased expressions were observed in renal and urothelial tumors [13]. They also reported that increased GGCT expression was not as high as in breast cancer. The reason for this discrepancy among different studies is not known. In these studies different anti-GGCT antibodies were used so their reactivity or sensitivity may not be uniform [12]. Further studies on this issue are needed.

Although some discrepant findings were observed, a high-level expression of GGCT protein was observed in a wide range of cancers. Accordingly, it is considered that GGCT has the potential to become a cancer biomarker.

5. Role of GGCT in Cancer Cells

Although the upregulation of GGCT in cancer cells has been reported, the role of GGCT in cancer cells is still unclear. Therefore, we performed gene transfection and knockdown experiments and demonstrated that cell proliferation is promoted by introducing the GGCT gene into NIH3T3 cells [7]. Furthermore, inhibition of cell-growth was observed by inhibiting GGCT expression with RNA interference in several types of cancer cell lines possessing high-level expressions of GGCT. In contrast, inhibition of cell proliferation was not observed in noncancer cells with very low levels of GGCT expression. Based on the above evidence, the function of GGCT related to cancer cell proliferation was suggested. Uejima et al. examined the activities of human osteosarcoma cells (HOS) using a Matrigel Invasion Chamber [17]. The migration and invasive capabilities as well as the proliferation of HOS cells were suppressed by the administration of small interfering RNA (siRNA). Furthermore, an increased expression of cell adhesion-related molecules, including integrin and cadherin, found by DNA microarray analysis of GGCT knockdown HOS cells was reported. Such results indicate the potential involvement of GGCT in not only the growth but also the invasion and metastasis of cancer.

On the other hand, we conducted a soft agar assay and focus-forming assay by introducing the GGCT gene into normal mouse fibroblasts. However, no significant changes were observed in comparison with controls [7]. Independently of our work, Azumi et al. also reported findings similar to our results by introducing the GGCT gene into HBL-100 cells, a breast cancer cell line with low-level GGCT expression [14]. Thus, it is assumed that the involvement of GGCT in malignant transformation was negated.

6. Molecular Regulation Mechanism of GGCT Expression in Cancer Cells

The mechanism of regulation of the GGCT gene was clarified in recent studies [19, 20]. Ohno et al. demonstrated that a region located at −371 to +14 bp of the 5′ end of GGCT is important for activation of GGCT transcription as a promoter in both cancer and noncancer cells. Sequencing analysis indicated that this region has a distinctive structure with three CCAAT boxes near the transcription start site. Moreover, a GC box exists upstream of these CCAAT boxes. NF-Y and Sp1 bind to the CCAAT boxes and GC box, respectively, to regulate positive transcription of the GGCT gene. Promoters having several NF-Y-binding CCAAT boxes were found in some genes related to the cell cycle. Accordingly, it was suggested that GGCT may also play a role in the cell cycle [19].

In addition, Ohno et al. reported a difference in GGCT gene expression between normal and cancer cells. They determined that the promoter of the GGCT gene has a stable heterochromatin structure in normal cells. In cancer cells on the other hand, the promoter has a euchromatin structure. Their report strongly suggested that high expression of GGCT in cancer cells is caused by a structural change in the chromatin of the GGCT gene associated with the oncogenic transformation of the cells [20].

7. Development of the GGCT-Targeting Cancer Therapy

We first reported that the administration of anti-GGCT siRNA inhibited cell proliferation in some cell lines, including bladder, prostate, lung, breast, and cervical cancers [7]. Similarly, other groups reported that the growth of cancer cells was inhibited by GGCT knockdown with RNAi *in vitro*.

Recently, we found the inhibitory efficacy of the combined use of docetaxel, a standard chemotherapeutic agent for prostate cancer, with anti-GGCT siRNA using prostate cancer cell lines. The combined use of docetaxel at IC_{50} (half maximal (50%) inhibitory concentration) with GGCT siRNA at a low concentration of 5 nM in each cell line resulted in an additional 25–41% inhibition of proliferation over coadministration of docetaxel and control siRNA (manuscript in submission). The combined use of a functional inhibitor of GGCT with chemotherapy may become a promising treatment.

A study of treatment targeting GGCT using an animal model was reported by Hama et al. [21]. They produced tumor-bearing mice by subcutaneous implantation of EBC-1, a lung squamous cell carcinoma cell line, and then administered anti-GGCT siRNA to the tumor using a needle-free jet injection and obtained significant tumor regression. We also attempted to examine intravenous administration of anti-GGCT siRNA conjugated with several carrier substances in tumor-bearing mice. However, no significant therapeutic effect was obtained (data not shown). Degradation of siRNA in the blood is a common problem in systemic administration and the delivery of siRNA to the tumor was very poor. The lack of an effective drug delivery system is considered to be the critical problem in cancer treatment by intravenous administration of siRNA. Recently, however, Ran et al. succeeded in systemic cancer treatment targeting GGCT in tumor-inoculated mice via a unique drug delivery system for intravenous administration of siRNA [22]. They established the PEGylated hyaluronic acid-modified liposomal delivery system and described significant antitumor effects in mice inoculated with drug-resistant MCF-7 cells (a breast cancer cell line). Surprisingly, they also reported that a systemic administration of anti-GGCT siRNA did not affect normal organs, such as kidney, heart, lung, liver, and spleen. GGCT is expected to be a target molecule for anticancer therapy, and the development of low molecular weight inhibitors is also needed.

Recently, Yoshiya et al. developed probes "LISA-4" and "LISA-101," which produce fluorescence as the result of an enzymatic reaction [23, 24]. The establishment of these new methods to measure GGCT activity would be very valuable to screen GGCT inhibitors.

8. Conclusion

It has only been a few years since the existence of GGCT was shown and accumulation of research results has not been sufficient. However, GGCT is a very attractive target molecule because its high expression has been observed in a wide range of cancer types. Inhibition of GGCT expression suppresses the proliferation of cancer cells, and mild adverse events are expected from a recent study. Further investigations are needed in the future.

Conflict of Interests

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

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Review Article

Reproductive Factors but Not Hormonal Factors Associated with Thyroid Cancer Risk: A Systematic Review and Meta-Analysis

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Many studies have investigated the association between hormonal and reproductive factors and thyroid cancer risk but provided contradictory and inconclusive findings. This review was aimed at precisely estimating this association by pooling all available epidemiological studies. 25 independent studies were retrieved after a comprehensive literature search in databases of PubMed and Embase. Overall, common hormonal factors including oral contraceptive and hormone replacement therapy did not alter the risk of thyroid cancer. Older age at menopause was associated with weakly increased risk of thyroid cancer in overall analysis (RR = 1.24, 95% CI 1.00–1.53, $P = 0.049$); however, longer duration of breast feeding was related to moderately reduced risk of thyroid cancer, suggested by pooled analysis in all cohort studies (RR = 0.7, 95% CI 0.51–0.95, $P = 0.021$). The pooled RR in hospital-based case-control studies implicated that parous women were more susceptible to thyroid cancer than nulliparous women (RR = 2.30, 95% CI 1.31–4.04, $P = 0.004$). The present meta-analysis suggests that older age at menopause and parity are risk factors for thyroid cancer, while longer duration of breast feeding plays a protective role against this cancer. Nevertheless, more relevant epidemiological studies are warranted to investigate roles of hormonal and reproductive factors in thyroid carcinogenesis.

1. Introduction

Thyroid cancer is the most common type of endocrine malignancy, which accounts for nearly 3% of all malignancies [1]. Despite low mortality rate, rates of local recurrence and distant metastases are high in thyroid cancer patients. The incidence of thyroid cancer has been increasing worldwide for the last five years, while the etiology remains largely unknown. Ionizing radiation is a well documented risk factor for thyroid cancer [2]. However, not all individuals exposed to radiation develop this disease, implicating some other unknown factors involved in thyroid carcinogenesis, such as hormone-related factors.

Gender discrepancy is well known in thyroid malignancies. Thyroid cancer occurs three times more frequently in women than in men, and the incidence decreases among postmenopausal women. It has been well established that female sex hormones, particularly estrogens, can influence the proliferation and invasion of thyroid cancer cells by recognizing corresponding hormonal receptors expressed in those cells, such as estrogen receptor alpha and beta [3–5]. It has been demonstrated that the secretion of thyroid stimulating hormone (TSH) increased during puberty, pregnancy, and oral contraceptive use [6]. Elevated TSH production can promote thyroid growth, while estrogens increase levels of TSH

in human body [7]. Therefore, regulation between TSH and estrogens may play a critical role in the development of thyroid disease, thyroid malignancies in particular. Taken together, it can be hypothesized that some hormonal and reproductive factors may confer modifying effects on thyroid carcinogenesis by influencing the signaling of sex hormones and their receptors in thyroid gland. Many epidemiological studies have investigated roles of hormonal and reproductive factors in the development of thyroid cancer, for instance, oral contraceptive use, hormone replacement therapy, menstrual factors, and fertility status [8–32]. Nevertheless, the precise association has not yet been fully elucidated due to conflicting and inconclusive findings in previous studies. We performed this meta-analysis by pooling all currently published studies to obtain a better estimation and provide important insights into the etiology of thyroid cancer.

2. Materials and Methods

2.1. Search Strategy. We searched studies on the association between hormonal and reproductive factors and thyroid cancer risk in PubMed and Embase databases from their inception up to September 10, 2014, using the following items: thyroid cancer, or thyroid carcinoma; and oral contraceptive, hormone replacement therapy, reproductive factors, menstrual factors, age at menarche, age at first birth, menopausal status, age at menopause, parity, pregnancy, reproductive history, or breast feeding; and incidence, or risk factor. References of relevant studies were also screened for additional papers. If studies were duplicated, only the most complete study was included.

2.2. Inclusion Criteria. The included studies must conform to the following inclusion criteria: (1) studies on the association of hormonal and reproductive factors with thyroid cancer risk; (2) cohort or case-control studies; (3) publications presenting odds ratios (ORs), relative risks (RRs), or hazard ratios (HRs) with 95% confidence intervals (95% CIs). Studies not associated with hormonal and reproductive factors and thyroid cancer risk, case-only, animal research, case reports, and duplicated studies were all excluded.

2.3. Data Extraction. Two investigators independently extracted data from each study by use of the following terms: name of first author, year of publication, study design, country of origins, sample size, study period, matching or adjusted factors, and RRs or HRs or ORs with 95% CIs for the estimation of thyroid cancer risk related to hormonal and reproductive factors. Disagreements were solved by discussion.

2.4. Statistical Analysis. Roles of hormonal and reproductive factors in thyroid cancer risk were assessed by calculating pooled RRs with 95% CIs by use of STATA 12.0 software (StataCorp, College Station, TX, USA). $P < 0.05$ was suggested to be statistically significant. The between-study heterogeneity was estimated by Cochran's Q and I^2 tests, and $P < 0.05$ and $I^2 > 50\%$ implicated obvious between-study heterogeneity [33, 34]. The random-effects model was used when the between-study heterogeneity was significant [35]; otherwise, the fixed-effects model was adopted [36]. Stratified analysis by study design (cohort studies, population-based case-control studies, and hospital-based case-control studies) was also performed. Sensitivity analysis by omission of each study was conducted for further analysis. Publication bias risk was evaluated by both Begg's funnel plots and Egger's test [37, 38].

3. Results

3.1. Identification and Characteristics of Studies Included into the Meta-Analysis. 112 studies were retrieved after a comprehensive literature in databases of PubMed and Embase. However, 87 studies were excluded due to irrelevance, reviews, animal research, and case reports. 25 independent studies on the association between hormonal and reproductive factors and thyroid cancer risk were finally included into our study [8–32]. Among the 25 studies, 13 were cohort studies, 10 were population-based case-control studies, and the other 2 were hospital-based case-control studies. Characteristics of all included studies were summarized in Table 1.

3.2. Association between Hormonal Factors and Thyroid Cancer Risk. The common hormonal factors including oral contraceptive and hormone replacement therapy did not modify the risk of thyroid cancer (for oral contraceptive: RR = 0.94, 95% CI 0.85–1.04, $P = 0.215$; for hormone replacement therapy: RR = 1.04, 95% CI 0.91–1.19, $P = 0.527$) (Table 2). Sensitivity analysis by sequential omission of each study confirmed the findings (data not shown).

Stratified analysis by study design showed that no significant relationship was observed between hormonal factors and thyroid cancer risk in cohort studies and studies in population-based case-control design (Table 2). We failed to perform stratified analysis in hospital-based case-control studies because of insufficient published studies.

3.3. Association between Reproductive Factors and Thyroid Cancer Risk. The pooled RRs revealed that older age at menopause was associated with weakly increased risk of thyroid cancer in overall analysis (RR = 1.24, 95% CI 1.00–1.53, $P = 0.049$) (Table 2; Figure 1), whereas longer duration of breast feeding was related to moderately reduced risk of thyroid cancer in cohort studies (RR = 0.7, 95% CI 0.51–0.95, $P = 0.021$) (Table 2; Figure 2). Stratified analysis in hospital-based case-control studies showed that more parity could increase the risk of thyroid cancer (RR = 2.30, 95% CI 1.31–4.04, $P = 0.004$) (Table 2; Figure 3). No significant relationship was observed between thyroid cancer risk and other common reproductive factors (Table 2). Sensitivity analysis did not materially alter the pooled results (data not shown).

3.4. Heterogeneity Analysis and Publication Bias Risk. No significant between-study heterogeneity was found in most comparisons of overall and stratified analyses, except for

Table 1: Characteristics of all epidemiological studies.

First author	Year	Origin	Time	Study design[§]	Hormonal or reproductive factors	Adjusted or matching criteria
Högnäs [13]	2014	Finland	1974–2010	Cohort study	Parity	Sex
Zamora-Ros [31]	2015	Europe	1992–2009	Cohort study	Oral contraceptive, hormone replacement therapy, age at menarche, parity, age at first birth, menopausal status, age at menopause, breastfeeding status, and duration of breastfeeding	Sex, center, and age at recruitment
Braganza [8]	2014	USA	1993–2009	Cohort study	Oral contraceptive, hormone replacement therapy, age at menarche, parity, age at first birth, and age at menopause	Sex, education, race, marital status, family history of thyroid cancer, baseline body mass index, and smoking
Islami [15]	2013	Iran	2003–2007	PCC	Parity	Age, sex, and neighborhood of residence
Kabat [16]	2012	USA	1993–2011	Cohort study	Oral contraceptive, hormone replacement therapy, age at menarche, parity, age at first birth, age at menopause, and duration of breastfeeding	Sex, age, education, height, history of goiter/nodules, pack-years, and alcohol intake
Schonfeld [27]	2011	USA	1995–2006	Cohort study	Oral contraceptive, hormone replacement therapy, age at menarche, parity, age at first birth, and age at menopause	Sex, smoking status, baseline body mass index, race, alcohol consumption, and education
Horn-Ross [14]	2011	USA	1995–2008	Cohort study	Oral contraceptive, hormone replacement therapy, age at menarche, parity, age at first birth, and menopausal status	Sex, ethnicity, family history of thyroid cancer, time since last pregnancy, smoking, alcohol consumption, physical inactivity, height, adolescent cycle length, time to regular menstruation, and age at menarche
Meinhold [18]	2010	USA	1983–2006	Cohort study	Oral contraceptive, hormone replacement therapy, age at menarche, parity, and menopausal status	Sex, birth year, smoking status, body mass index, number of personal radiographs to the head or neck, and cumulative occupational radiation dose
Pham [23]	2009	Japan	1988–1997	Cohort study	Hormone replacement therapy, age at menarche, parity, age at first birth, and age at menopause	Sex, age at baseline, body mass index, tobacco smoking status, education level, history of diabetes, and study area
Dorjgochoo [10]	2009	China	1996–2006	Cohort study	Oral contraceptive	Sex, education, age at menarche, number of live births, cumulative breast feeding months, body mass index, exercised regularly in past 5 years, smoking, menopausal status, first-degree family history of cancer, and other contraceptive methods
Hannibal [12]	2008	Denmark	1963–2000	Cohort study	Parity and age at first birth	Sex, parity, age at cohort entry and calendar year of cohort entry, and age at first live birth
Brindel [9]	2008	France	1981–2004	PCC	Age at menarche, parity, age at first birth, menopausal status, age at menopause, breastfeeding status, and duration of breastfeeding	Sex, educational level, height, body mass index, and interviewer
Wong [30]	2006	China	1989–1998	Cohort study	Oral contraceptive, age at menarche, parity, age at first birth, age at menopause, breastfeeding status, and duration of breastfeeding	Sex, age, number of live births, and age at first live delivery

Table 1: Continued.

First author	Year	Origin	Time	Study design[§]	Hormonal or reproductive factors	Adjusted or matching criteria
Truong [29]	2005	New Caledonia	1993–1999	PCC	Oral contraceptive, hormone replacement therapy, age at menarche, and parity	Sex, frequency, age, and residential area
Neale [21]	2005	Sweden	1961–1996	Cohort study	Age at first birth	Sex, date of birth of the mother, parity, and age at first birth
Navarro Silvera [20]	2005	Canada	1980–2000	Cohort study	Oral contraceptive, hormone replacement therapy, age at menarche, parity, age at first birth, and menopausal status	Sex, age at first live birth, study center and randomization group, parity, and HRT use
Zivaljevic [32]	2003	Serbia	1996–2000	HCC	Parity	Sex, age, place of residence, and time of hospitalization
Negri [22]	2002	Mixed	1974–1992	PCC	Age at menarche, parity, age at first birth, and menopausal status	Sex, age, ethnicity, and study center
Memon [19]	2002	Middle East	1981–1999	PCC	Oral contraceptive, hormone replacement therapy, age at menarche, parity, age at first birth, and age at menopause	Sex, year of birth, nationality, and district of residence.
Sakoda [26]	2002	USA	1992–1998	PCC	Age at menarche, menopausal status, and age at menopause	Sex, frequency, age, ethnicity, education level, history of goiter or nodules, history of radiation to the head or neck, and family history of proliferative thyroid disease
Rossing [24]	2000	USA	1988–1994	PCC	Age at menarche, parity, age at first birth, and duration of breastfeeding	Sex, age, county of residence, race, and relative weight at age 10
Mack [17]	1999	USA	1980–1983	PCC	Age at menarche, parity, age at first birth, menopausal status, breastfeeding status, and duration of breastfeeding	Sex, age, prior thyroid disease, and number of births
Takezaki [28]	1996	Japan	1988–1993	HCC	Age at menarche, parity, and age at first birth	Sex, age, and year of visit
Rossing [25]	1998	USA	1988–1994	PCC	Oral contraceptive and hormone replacement therapy	Sex, age, county of residence, race, and relative weight at age 10
Galanti [11]	1996	Sweden and Norway	1985–1993	PCC	Oral contraceptive, hormone replacement therapy, parity, age at first birth, and age at menopause	Sex, frequency, year and month of birth, type of menopause, and parity

[§]PCC: population-based case-control studies; HCC: hospital-based case-control studies.

the estimation of parity's effect on thyroid cancer risk (I^2 = 61.2%, $P < 0.001$). Stratified analysis by study design suggested that the main source of between-study heterogeneity resulted from studies in cohort design (I^2 = 70.6%, $P < 0.001$).

As suggested by Begg's funnel plots and Egger's test, there was no significant publication bias under all comparisons in the present meta-analysis (data not shown).

4. Discussion

Hormonal and reproductive factors have been implicated in the development of thyroid cancer, but the precise association and underlying molecular mechanisms have not yet been fully understood. A previous pooled analysis has investigated the association between female reproductive factors and thyroid cancer risk [39]. Unfortunately, only 17 epidemiological studies are included into the meta-analysis, which shows weak and equivocal association between some hormonal and menstrual cycle factors and thyroid cancer risk [39]. The present meta-analysis was based on 25 epidemiological studies on the association between hormonal and reproductive factors and thyroid cancer risk. No significant association was observed between thyroid cancer risk and common hormonal factors including oral contraceptive and hormone replacement therapy. Interestingly, older age at menopause might increase the risk of thyroid cancer, as suggested by the pooled RR in overall analysis (RR = 1.24, 95% CI 1.00–1.53, $P = 0.049$). Besides, longer duration of breast feeding was associated with moderately decreased risk of thyroid cancer, which had been suggested by the pooled analysis in cohort

Table 2: Summary results for the association between hormonal and reproductive factors and thyroid cancer risk.

Comparisons	[a]RR	[b]95% CI	[c]P value	Tests for heterogeneity	
				I^2 (%)	[d]P
All					
Oral contraceptive	0.94	0.85–1.04	0.215	36.0	0.095
Hormone replacement therapy	1.04	0.91–1.19	0.527	0	0.520
Age at menarche	1.08	0.97–1.19	0.142	0	0.486
Parity	1.10	0.94–1.28	0.234	61.2	<0.001
Age at first birth	1.07	0.95–2.20	0.255	14.8	0.281
Menopausal status	0.96	0.79–1.18	0.727	4.9	0.394
Age at menopause	1.24	1.00–1.53	0.049	26.4	0.201
Duration of breast feeding	0.84	0.65–1.08	0.178	38.3	0.150
Breast feeding	0.84	0.69–1.02	0.080	4.9	0.368
Cohort studies					
Oral contraceptive	0.96	0.86–1.07	0.448	39.5	0.104
Hormone replacement therapy	1.05	0.91–1.20	0.513	0	0.439
Age at menarche	1.01	0.90–1.14	0.808	16.1	0.299
Parity	1.00	0.78–1.28	0.993	70.6	<0.001
Age at first birth	1.04	0.92–1.18	0.530	0	0.571
Menopausal status	0.90	0.71–1.15	0.418	26.6	0.252
Age at menopause	1.3	1.04–1.63	0.022	2.5	0.400
Duration of breast feeding	0.70	0.51–0.95	0.021	0	0.661
Breast feeding	0.82	0.66–1.02	0.078	0	0.442
PCC					
Oral contraceptive	0.89	0.73–1.08	0.240	41.0	0.166
Hormone replacement therapy	1.01	0.68–1.50	0.962	6.0	0.363
Age at menarche	1.28	1.04–1.57	0.960	0	0.937
Parity	1.00	0.93–1.07	0.960	21.6	0.258
Age at first birth	1.35	0.97–1.88	0.072	42.3	0.123
Menopausal status	1.10	0.78–1.57	0.577	0	0.481
Age at menopause	0.82	0.43–1.56	0.554	44.0	0.147
Duration of breast feeding	1.24	0.80–1.93	0.332	28.2	0.248
Breast feeding	0.92	0.60–1.41	0.699	57.6	0.125
HCC					
Parity	2.30	1.31–4.04	0.004	0	0.787

[a]RR: relative risk; [b]95% CI: 95% confidence interval; [c]P: the value of P for pooled analysis; [d]P: the value of P for heterogeneity analysis.

studies (RR = 0.7, 95% CI 0.51–0.95, $P = 0.021$). Moreover, the pooled result in hospital-based case-control studies revealed that parous women were more susceptible to thyroid cancer than nulliparous women (RR = 2.30, 95% CI 1.31–4.04, $P = 0.004$). Additionally, other reproductive factors including age at menarche, age at first birth, menopausal status, age at menopause, and breast feeding status did not modify the risk of thyroid cancer.

The risk of thyroid cancer in women increases at the time of puberty and declines after menopause [7, 40], supporting the hypothesis that menstrual cycle factors are involved in thyroid carcinogenesis. Elevated risk of thyroid cancer was related to the menopausal status when compared with premenopausal status [9]. Nonetheless, no significant association between the menopausal status, age at first birth, age at menarche, and age at menopause and thyroid cancer risk was observed [16, 18, 23, 27, 31]. It was worthwhile to note that menopausal females due to surgical factors were more susceptible to thyroid cancer compared with natural menopause ones [31], which might reflect enhanced medical surveillance of women who underwent surgical interventions for gynecological diseases symptoms. Similarly, we failed to identify any appreciable relationship of menstrual factors with thyroid cancer susceptibility. The discrepancies and underlying mechanisms need to be further elucidated by more relevant studies in the future.

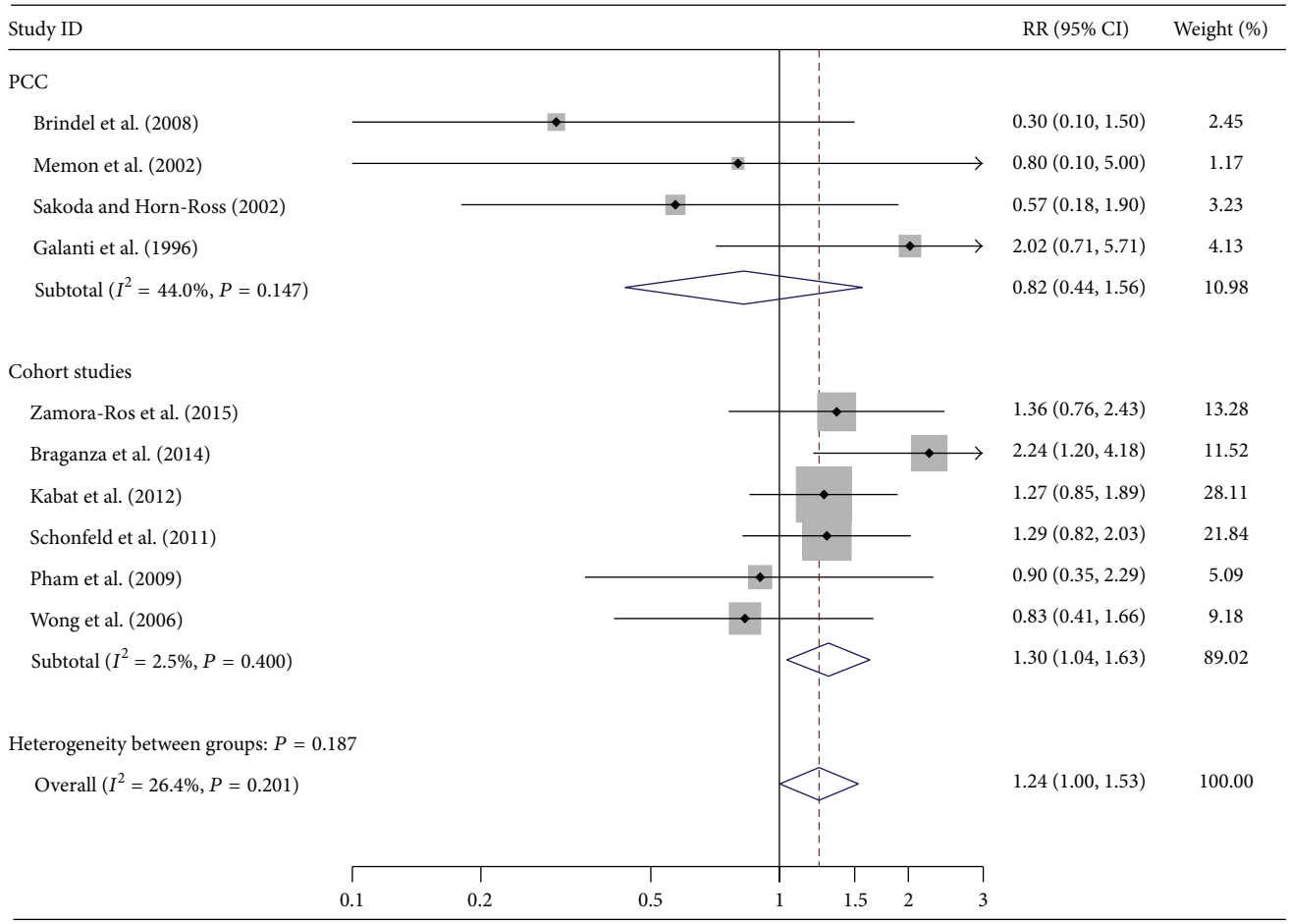

Figure 1: Forest plot for thyroid cancer risk related to age at menopause.

It has been well established that estrogen receptors are found in thyroid cancer tissue and confer effects on different molecular signaling pathways involved in the growth and function of thyroid [5, 41, 42]. Estrogens and estrogen receptors signals exert promoting effect on the growth of thyroid gland by enhancing levels of TSH [7]. A number of studies have suggested some hormonal related factors, for instance, oral contraceptive and hormone replacement therapy, which played different roles in the thyroid cancer risk [8, 23, 27, 31]. Oral contraceptive seemed to play a protective role against thyroid cancer while an increased risk of thyroid cancer was found with the use of hormone replacement therapy demonstrated by Zamora-Ros et al. [31]. Similar findings were elucidated in a Caucasian cohort study [27]. Conversely, no modifying effects of oral contraceptive and hormone replacement therapy on the development of thyroid cancer were in another independent cohort study [8, 23]. The contradictory findings may be attributed to different study design, use of contraceptive and hormone replacement therapy, ethnicity, and adjusted or matching criteria in individual epidemiological studies. Our study showed no appreciable roles of hormonal factors in thyroid carcinogenesis, as suggested by both overall analysis and stratified analysis according to study design. More future studies are warranted to further estimate the association between hormonal-related factors and thyroid cancer risk.

The effect of breastfeeding on thyroid cancer risk is still not clear. Kabat and colleagues demonstrated that duration of breastfeeding did not alter the susceptibility to thyroid cancer [16]. However, Mack et al. provided the evidence that longer duration of breastfeeding was negatively associated with the risk of thyroid cancer risk, suggesting a protective role of breastfeeding in thyroid carcinogenesis [17]. Similarly, the pooled RRs in all cohort studies implicated that longer duration of breast feeding was associated with moderately reduced risk of thyroid cancer. Nevertheless, there was no significant relationship between breastfeeding status and thyroid carcinogenesis in pooled analyses of total studies and population-based case-control ones. Thus, the moderate association might be a chance resulting from potential bias in the present meta-analysis. To better understand the role of breastfeeding status in the thyroid cancer development, more relevant studies with high quality are warranted.

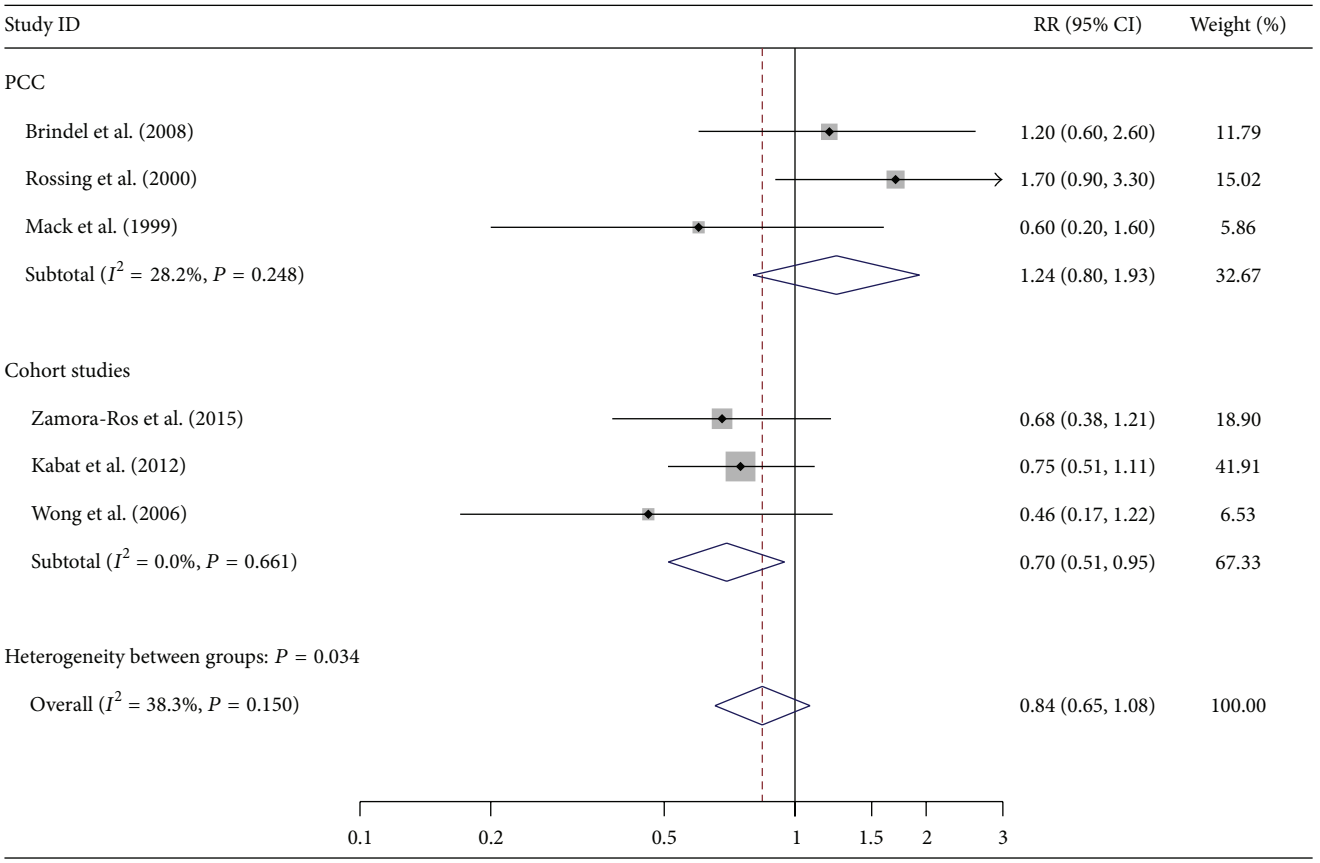

Figure 2: Forest plot for thyroid cancer risk related to duration of breastfeeding.

The status of parity conferred diverse effects on thyroid cancer risk in different populations. A recent study by Braganza et al. showed that parous women were at an elevated risk of thyroid cancer, as suggested by a recent epidemiological study [8]. Interestingly, for any given level of parity, there was about twofold increased risk of thyroid cancer among women with the age at last pregnancy larger than 30 years [19]. Unlike the findings mentioned above, no appreciable association was suggested between parity and the susceptibility to thyroid cancer among a Caucasian population [27]. In the current meta-analysis, significantly positive association was only demonstrated in the pooled analysis of two hospital-based case-control studies. Although age seemed to influence roles of reproductive factors in thyroid carcinogenesis, we failed to find appreciable association for age at first birth, age at menarche, and age at menopause. In addition, the pooled results, particularly in hospital-based case-control studies, must be interpreted with caution due to limited sample size and insufficient statistical power in current research.

Age is a main confounding factor for the association between hormonal and reproductive factors and thyroid cancer risk [19]. We failed to perform stratified analysis by age or other confounding factors, such as dosage and usage of hormonal drugs, the reason of menopause, number of live births, outcome of first pregnancy, and history of miscarriage, because of unavailable information about these items in single studies. Consequently, the association between hormonal and reproductive factors and thyroid cancer risk should be further investigated in view of above-mentioned confounding factors.

5. Conclusions

The current meta-analysis suggests that older age at menopause and parity are associated with increased risk of thyroid cancer, while longer duration of breast feeding plays a protective role against this cancer. In addition, the precise association needs further investigation by more epidemiological studies with sufficient statistical power in the future.

Conflict of Interests

The authors declare no conflict of interests.

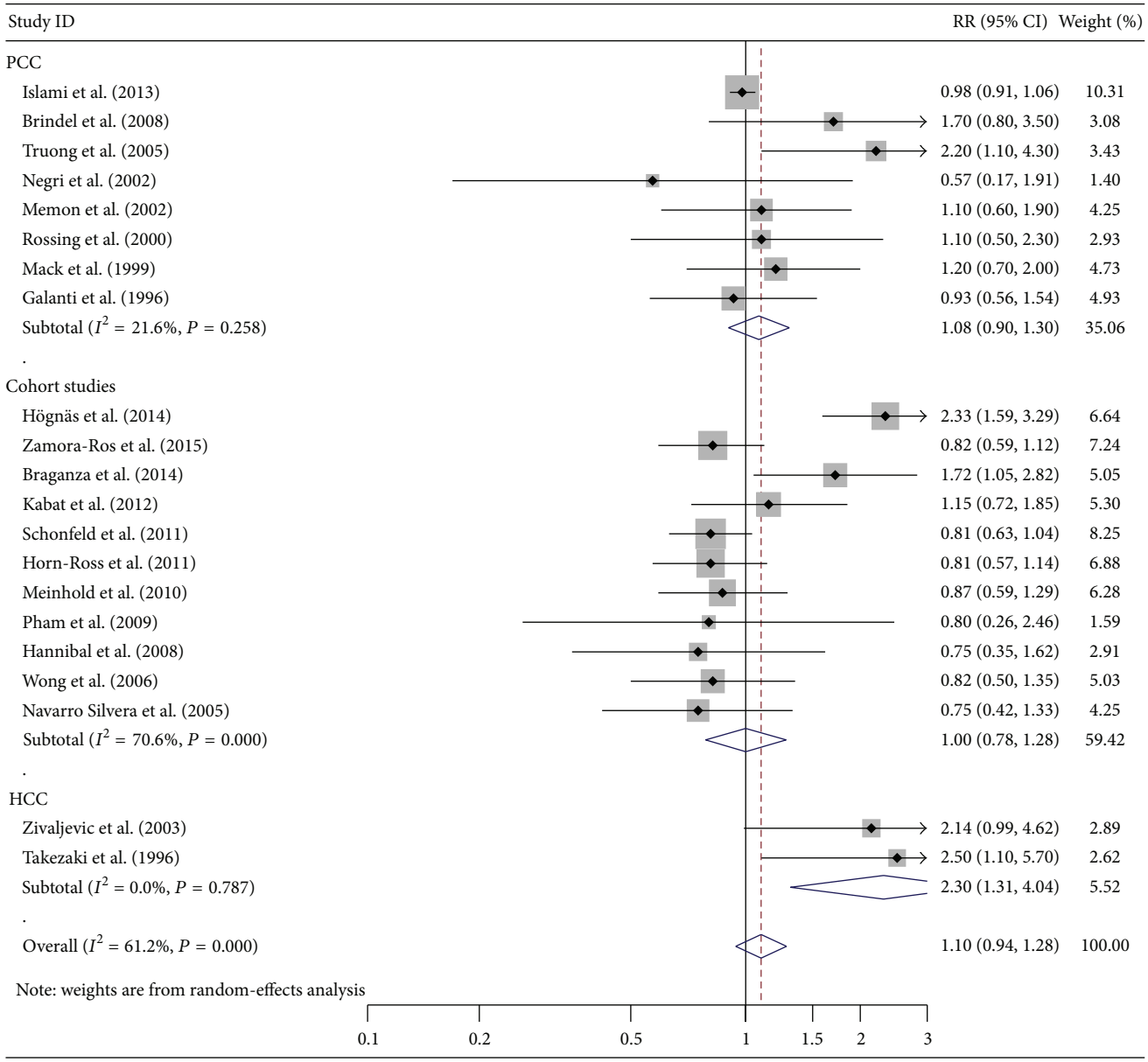

Figure 3: Forest plot for thyroid cancer risk related to parity.

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Research Article

Integration of DCE-MRI and DW-MRI Quantitative Parameters for Breast Lesion Classification

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Objective. The purpose of our study was to evaluate the diagnostic value of an imaging protocol combining dynamic contrast-enhanced MRI (DCE-MRI) and diffusion-weighted MRI (DW-MRI) in patients with suspicious breast lesions. *Materials and Methods*. A total of 31 breast lesions (15 malignant and 16 benign proved by histological examination) in 26 female patients were included in this study. For both DCE-MRI and DW-MRI model free and model based parameters were computed pixel by pixel on manually segmented ROIs. Statistical procedures included conventional linear analysis and more advanced techniques for classification of lesions in benign and malignant. *Results*. Our findings indicated no strong correlation between DCE-MRI and DW-MRI parameters. Results of classification analysis show that combining of DCE parameters or DW-MRI parameter, in comparison of single feature, does not yield a dramatic improvement of sensitivity and specificity of the two techniques alone. The best performance was obtained considering a full combination of all features. Moreover, the classification results combining all features are dominated by DCE-MRI features alone. *Conclusion*. The combination of DWI and DCE-MRI does not show a potential to dramatically increase the sensitivity and specificity of breast MRI. DCE-MRI alone gave the same performance as in combination with DW-MRI.

1. Introduction

Magnetic resonance imaging (MRI) applications such as dynamic contrast enhanced (DCE) and diffusion weighted imaging (DWI) have the potential to provide noninvasive digital biomarkers with good spatial resolution and reproducibility suitable for early detection of breast cancer and for therapy evaluation [1–9]. In general, DCE-MRI has shown high sensitivity for breast cancer detection (89–100%) [1–12], while DWI has shown utility in predicting suitable therapies and monitoring response [13].

DCE-MRI consists in the serial acquisition of images before and after the injection of intravenous contrast agent; it has been shown to give information about vascular permeability within the tumor [10, 11]. Different methods for DCE-MRI data analysis have been proposed, ranging from simple semiquantitative inspection of the time-intensity curves (TICs) to more sophisticated tracer kinetics modeling [14–18]. The different methods were designed to capture the biologically relevant components from the dynamic MR signal and to relate them to the underlying pathophysiological processes taking place in the tissue. In principle,

Table 1: Scan settings.

Settings	DCE-MRI	DW-MRI	Units
TR/TE/α	9, 8/4, 76/25	7700/129/90	ms/ms/deg
Pulse sequence	T1-weighted 3D FLASH	T2-weighted SPAIR	—
Plane	Coronal	Axial	—
FOV	185 × 370	183 × 360	mm^2
Matrix size	128 × 256	120 × 236	pixel
Pixel spacing	1.44 × 1.44	1.52 × 1.52	mm^2
Slice thickness	2	4	mm
Gap between slices	0	2	mm
Number of slices	80	24	—

the derivation of full-quantitative physiological data from DCE-MRI should rely on the application of appropriate tracer kinetics models to describe the distribution of contrast media following its systemic administration. However, the application of these techniques is still complex and they could not be widely available outside specialist centers. In response to this, many semiquantitative approaches for the classification of TIC shapes have been described and are now in relatively common use in clinical settings [18–26].

DW-MRI images are sensitive to water diffusion; preclinical and clinical data showed that it can reflect vessels structure [12, 13]. An approximated quantitative analysis of DW-MRI can be performed calculating the apparent diffusion coefficient (ADC) based on the relative signal intensity change of the tissue with increasing b values (see Section 2) or by using intravoxel incoherent motion (IVIM) [27–29] modeling for a more accurate quantitative analysis that has the potential to provide information about both the cellularity and perfusion of tumors. With the increasing awareness of the toxicity of MR contrast agents, DW-MRI could be considered a favorable alternative for deriving perfusion information without contrast agent injection [27–33].

At the time of writing only a few studies explored the correlation between these two methods and attempted a comparison in the case of breast cancer; moreover, it could be useful to assess their independence or complementarity.

The objective of the present study is to evaluate the correlation between DCE-MRI and DW-MRI data in breast cancer; moreover, we tried to establish if opportunely combining DCE and DW-MRI features for differentiation of benign and malignant breast lesions could improve performance.

2. Materials and Methods

2.1. Patients Characteristics. 31 breast lesions (15 malignant and 16 benign, proved by histological examination) in 26 female patients (mean age 37.2 ± 10.4 years, range 14–53 years) were included in this study. The malignant lesions included 9 infiltrating ductal carcinomas, 3 infiltrating ductal-lobular carcinomas, 1 infiltrating lobular carcinoma, and 2 ductal carcinomas in situ (DCIS). The benign lesions included 11 fibroadenomas and 5 fibrocystic dysplasias.

2.2. MR Protocol. Per each subject, DW-MRI and DCE-MRI data were acquired consecutively during the same session with a 1.5 T scanner (Magnetom Symphony, Siemens Medical System, Erlangen, Germany) equipped with breast dedicated coil. Scan settings are reported in Table 1.

DW-MRI data comprised 7 scans, each corresponding to a different b value (0, 50, 100, 150, 400, 800 and 1000 s/mm^2).

DCE-MRI data comprised 10 consecutive scans acquired with an interval between two successive scans of 56 s. The contrast agent bolus, 0.1 mL/kg body weight of Gd-DOTA (Dotarem,Guerbet, Roissy CdG Cedex, France), was injected at the start of the first postcontrast scan. An automatic injection system was used (Spectris Solaris EP MR, MEDRAD, Inc., Indianola, PA). The injection flow rate was 2 mL/s followed by a flush of 10 mL saline solution at the same rate.

2.3. Volumes Coregistration. A 3D linear interpolation was performed in order to align DCE and DW data on a common grid. Before alignment the voxel size of DCE and DW was 1.44 × 1.44 × 2 mm^3 and 1.52 × 1.52 × 6 mm^3, respectively. After the alignment the common spatial resolution was 1.5 × 1.5 × 6 mm. For the subsequent analysis only voxels included in both datasets were considered.

2.4. Region of Interest. Region of interests (ROIs) have been manually drawn by an expert radiologist on DCE images with virtual "fat-suppression" obtained subtracting the precontrast from the 5th postcontrast image. Per each patient only the slices including the lesion have been used. Voxels within ROIs were extracted from both DCE and DW realigned volume data. Features from DCE data and DW data have been computed.

2.5. DCE-MRI Features. Per each voxel, 20 features were extracted from DCE data: 17 were model free and 3 were model based.

2.5.1. Model Based Features. The time-course of contrast medium concentration is typically modelled using the extended Tofts model [22, 24]:

$$C_t\left(t, K_{\text{trans}}, k_{\text{ep}}\right) = C_p(t) \otimes K_{\text{trans}} \cdot e^{-k_{\text{ep}} \cdot t} + v_p \cdot C_p(t), \quad (1)$$

where $C_t(t)$ is the concentration of contrast medium within the tissue (voxel) of interest; $C_p(t)$ is the concentration of contrast medium within the plasma (also called arterial input function (AIF)); K_{trans} is the volume transfer constant

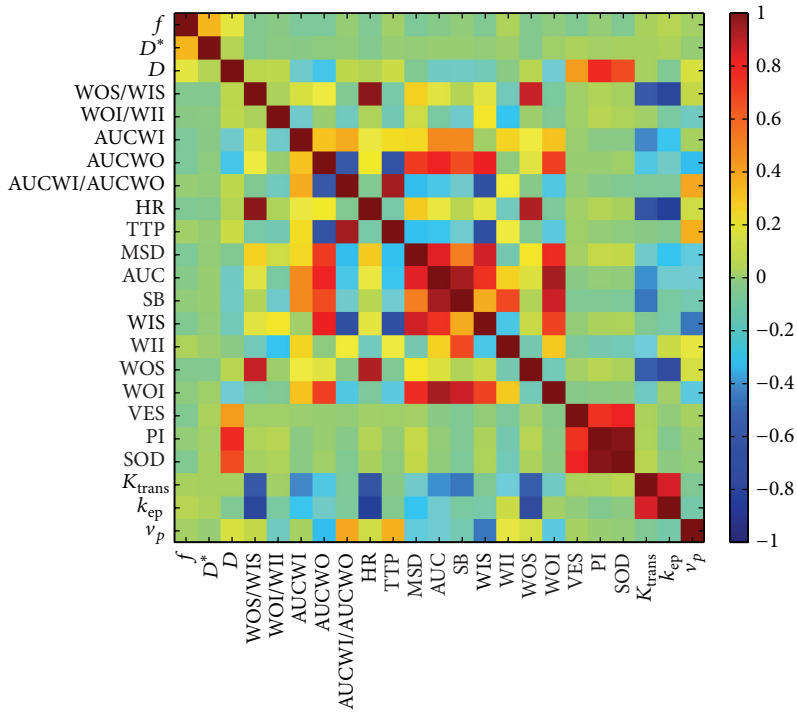

FIGURE 1: Per each couple of features the Spearman correlation coefficient (r) at a voxel-by-voxel level is reported in color code. Yellow to red colors indicate a positive correlation; cyan to blue colors indicate negative correlation. Most of DCE features are not correlated with DW features; a relatively strong positive correlation is observed between D and PI ($r = 0.70$) and between D and SOD ($r = 0.60$).

from plasma to extracellular-extravascular space (EES); k_{ep} is the diffusion rate constant from EES to plasma; v_p is the volume fraction occupied by plasma. These parameters can be related to the level of angiogenic activity. In particular, K_{trans} represents the vessel permeability and k_{ep} is linked to the duration of the wash-out phase [21]. We assumed the biexponential AIF proposed by Weinmann et al. [34]:

$$C_p(t) = d\left(a_1 \exp\left(-m_1 t\right) + a_2 \exp\left(-m_2 t\right)\right), \quad (2)$$

where d is the administered dose (mL/kg), $a_1 = 3.99$ kg/L, $a_2 = 4.78$ kg/L, $m_1 = 0.144\ \text{min}^{-1}$, and $m_2 = 0.0111\ \text{min}^{-1}$. Contrast medium concentration was calculated from the TIC using the approach suggested by Schabel et al. [35] with a fixed precontrast longitudinal relaxation time, $T_{1,0}$ of 820 ms, appropriate for breast parenchyma.

2.5.2. Model Free Features. For each voxel 17 TIC's shape descriptors were computed using an approach previously reported in [26]: basal signal (SB), maximum signal difference (MSD), the time to peck (TTP), the WI slope (WIS), the WO slope (WOS), the WI intercept (WII), the WO intercept (WOI), the WOS/WIS ratio, and the WOI/WII ratio are under curve (AUC), area under gadolinium curve in the wash-in phase (AUCWI), area under gadolinium curve in the wash-out phase (AUCWO), the AUCWO/AUCWI ratio, and the height ratio (HR).

Moreover in this study the perfusion index (PI) as proposed by [36], the sum of intensity difference (SOD) as proposed by [37], and variance of enhancement slope (VES) as proposed by [38] were calculated.

2.6. DW-MRI Features. Per each voxel, 3 features were extracted from DW data using the Intra Voxel Incoherent Motion (IVIM) model (Figure 1 step 7) [27–29].

DW-MR signal decay is most commonly analyzed using the monoexponential model [26, 27]:

$$\text{ADC} = \frac{\ln\left(S_0/S_b\right)}{b}, \quad (3)$$

where S_b is the MRI signal intensity with diffusion weighting b, S_0 is the nondiffusion-weighted signal intensity, and ADC is the apparent diffusion coefficient.

For a voxel with a large vascular fraction, the MRI data decay can deviate from a monoexponential form, in particular showing a fast decay in the range of low b values generated by the intravoxel incoherent motion (IVIM) effect [27, 28]. Thus, in addition to the monoexponential model, a biexponential model was used to estimate the IVIM-related parameters of pseudodiffusivity (D_p indicated also with D^*), perfusion fraction (f_p), and tissue diffusivity (D):

$$\frac{S_0}{S_b} = f_p \cdot \exp\left(-b \cdot D_p\right) + \left(1 - f_p\right) \cdot \exp\left(-b \cdot D\right). \quad (4)$$

The estimation of the three parameters in the biexponential model may often be ill-conditioned because of a limited number of samples, small perfusion fraction, and/or similar

Table 2: Summary of DCE and DW features.

ID	Symbol	Description	Units
1	f	Perfusion fraction	—
2	D^*	Pseudodiffusion coefficient	$mm^2\ s^{-1}$
3	D	Tissue diffusion coefficient	$mm^2\ s^{-1}$
4	WOS/WIS	Ratio between slopes of wash-out and wash-in phase (see below)	—
5	WOI/WII	Ratio between intercepts of wash-out and wash-in phase (see below)	—
6	AUCWI	Area under gadolinium curve in the wash-in phase	$s\ mmol\ L^{-1}$
7	AUCWO	Area under gadolinium curve in the wash-out phase	$s\ mmol\ L^{-1}$
8	AUCWI/AUCWO	Ratio between areas of wash-out and wash-in phase	—
9	HR	Height ratio	—
10	TTP	Time to peak	s
11	MSD	Maximum signal difference	—
12	AUC	Area under curve	—
13	SB	Basal signal	—
14	WIS	Wash-in slope	s^{-1}
15	WII	Wash-in intercept	—
16	WOS	Wash-out slope	s^{-1}
17	WOI	Wash-out intercept	—
18	VES	Variance of enhancement slope	s^{-1}
19	PI	Perfusion Index	—
20	SOD	Sum of intensity difference	—
21	K_{trans}	Volume transfer constant from plasma to extracellular-extravascular space	s^{-1}
22	k_{ep}	Diffusion rate constant from extracellular-extravascular space to plasma	s^{-1}
23	v_p	Plasma volume fraction	—

compartmental diffusivities, as found in other in vivo IVIM studies [29–33]. Thus, we performed a "two steps" analysis procedure as follows.

Typically, D_p is greater than D [28]; therefore, when the b value is significantly greater than $\sim 1/D_p$ (e.g., for $D_p \sim 10\,mm^2/ms$, $b > 100\,s/mm^2$), the contribution of the pseudodiffusion term to the signal decay becomes negligible. In this higher b value regime, (3) can be simplified to a monoexponential equation (4), where by D can be estimated:

$$S_{high} = S_0 \cdot \exp(-b \cdot D). \quad (5)$$

Therefore, D is determined from a monoexponential fit to data above a chosen threshold ($b > 200\,s/mm^2$, in this study). After determining D using (5), D_p, f_p can be estimated using a nonlinear fit of (4) to the entire dataset that minimizes the residual sum of squares.

2.7. Statistical Analysis. We performed two types of analysis: voxel-by-voxel and lesion-by-lesion analysis. It is expected that the results of the voxel-by-voxel analysis should quantify the possibility to automatically segment images using a combination of DCE and DW information. The results of the lesion-by-lesion analysis should indicate the capability to discriminate benign from malignant. Therefore, both voxel features and lesion features (per each lesion the median value along all the voxels for all features) have been calculated. Table 2 summarizes the 23 features.

In order to assess the correlation between DCE and DWI we made an analysis at a voxel-by-voxel level. Specifically, we computed the Spearman (nonparametric) correlation coefficient between each couple of features. It is expected that strongly correlated (or inversely correlated) features should show a high correlation coefficient (approximately 1 or −1).

Subsequently, we used both voxel-by-voxel and lesion-by-lesion analysis in order to assess the capability of the features to discriminate benign from malignant voxels or lesions. We applied a Linear Discriminant Analysis (LDA) [39] followed by a linear classifier in order to identify the best combination of features able to produce best classification results. ROC curves for classification were generated. The best linear classifiers were determined by maximizing the area under the ROC curves; best threshold was identified considering the unbalance of benign-malignant lesions [40, 41].

Finally, the performance of a simple algorithm for lesion classification based on voxel-by-voxel analysis was evaluated: a lesion was classified as malignant (benign) if the majority of voxel within that lesion is classified as malignant (benign). Classification based on DCE alone, DW alone, and combination of DCE and DW was compared. In the three cases, the percentage of correctly classified samples was computed for each lesion.

3. Results

Figure 1 shows the Spearman correlation coefficient (r) at a voxel-by-voxel level between all feature couples. The numbering of the features is as in Table 2. It can be seen that in

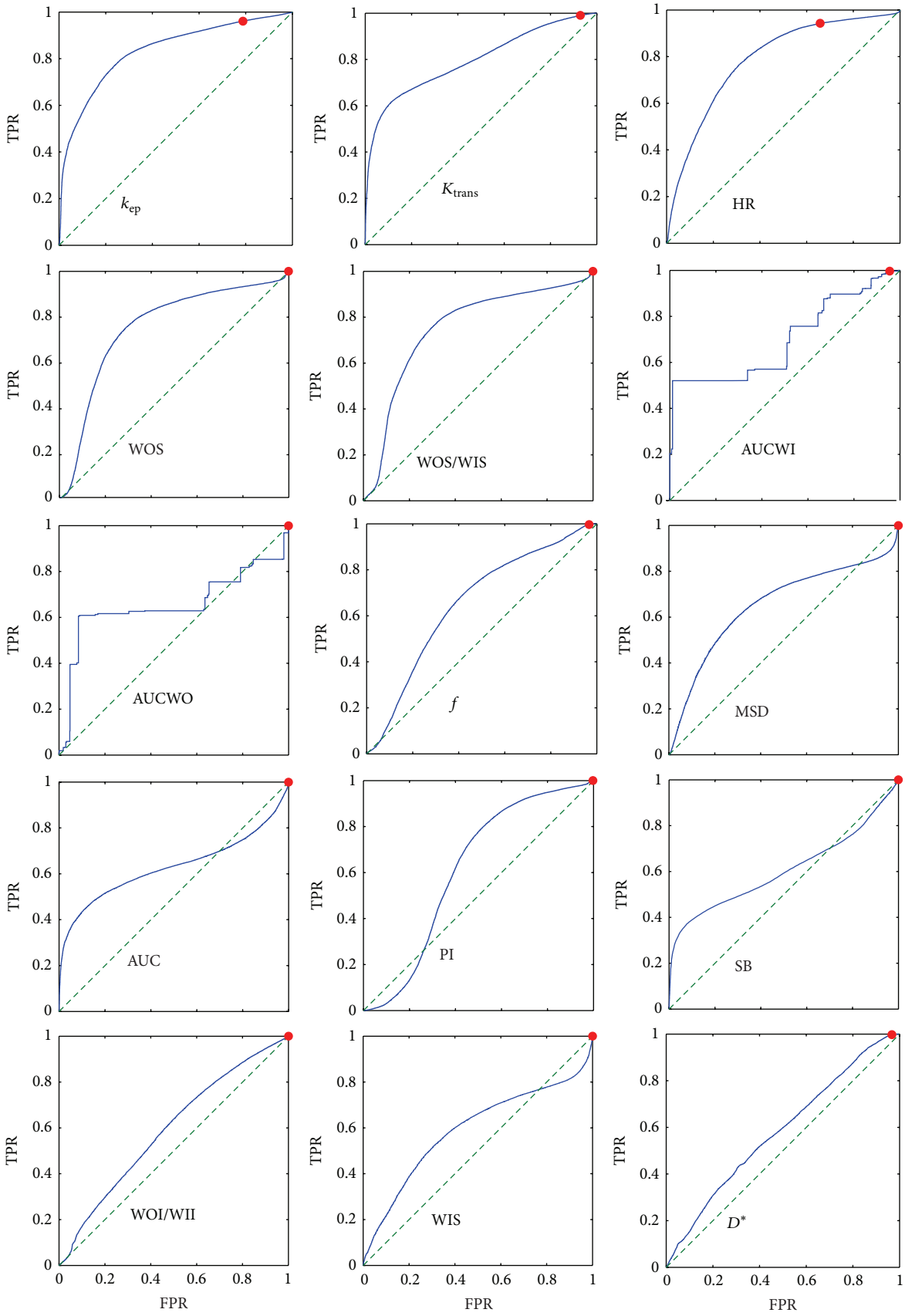

Figure 2: Continued.

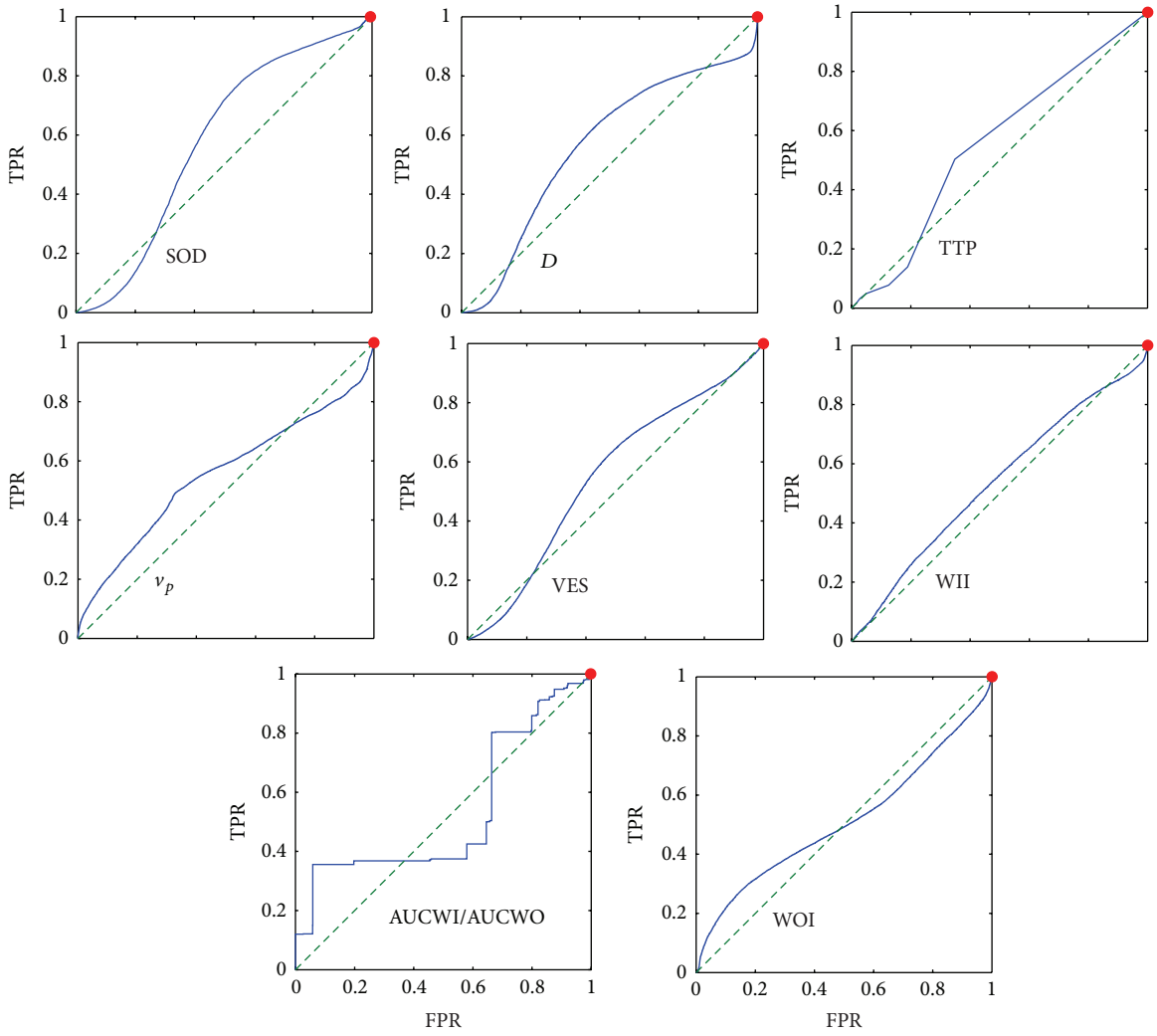

Figure 2: Receiver operating curves (ROCs) of single features in the case of voxel-by-voxel analysis. Per each feature (see Table 2) the ROC is reported in terms of true positive rate (TPR) and false positive rate (FPR). The plots have been aligned according to the area under curve (AUC) in row-wise descending order with the largest AUC at the top-left. The red dot indicates the best compromise between TRP/FPR considering the unbalance between benign-malignant subjects. FPR is generally very high except for k_{ep} and HR.

general, DCE and IVIM parameters show weak correlation except PI & D ($r = 0.70$) and SOD & D ($r = 0.60$).

Figures 2 and 3 show the receiver operating curves (ROCs) for single features in the case of voxel-by-voxel and lesion-by-lesion analysis, respectively. Within a specific ROC the best threshold is indicated by a red dot which has been evaluated considering the unbalance between benign and malignant lesions. The largest area under curve (AUC) with high sensitivity and good specificity has been obtained for k_{ep}, K_{trans} and HR, in the case of voxel-by-voxel analysis and for D^*, WIS, and f, in the case of lesion-by-lesion analysis (we discarded SB because of very low sensitivity).

Figures 4 and 5 report the results of the best combination of all features in the LDA analysis in the case of voxel-by-voxel and lesion-by-lesion analysis respectively. The best threshold is indicated by a red dot, which has been evaluated considering the unbalance between benign and malignant lesions.

Figure 6 shows the percentage of correctly classified samples by Linear Discriminant Analysis for each lesion. Using DCE features only showed the same behavior of combined DCE & DW. The number of misclassified patients using DCE only and DW only was the same (9 patients were misclassified in both cases).

Tables 3 and 4 report sensitivity and specificity of the parameters in the voxel-by-voxel analysis that provide the maximum area under the ROC (AUROC).

4. Discussion

The purpose of our study was to evaluate the diagnostic value of an imaging protocol that combines dynamic contrast-enhanced MRI (DCE-MRI) and diffusion-weighted imaging (DWI) in patients with suspicious breast lesions and to determine if additional information provided by DWI could improve the diagnostic value of breast MRI.

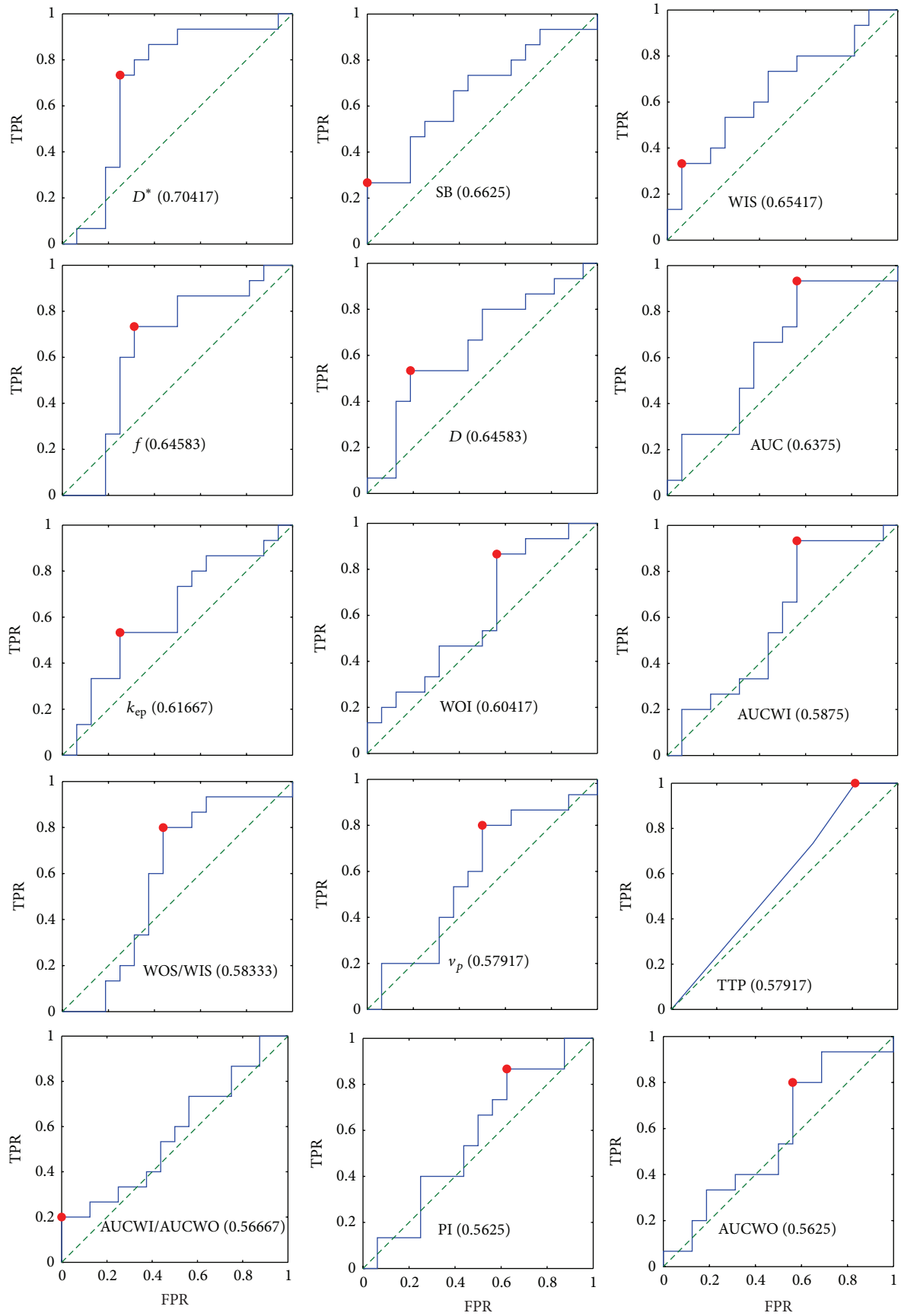

Figure 3: Continued.

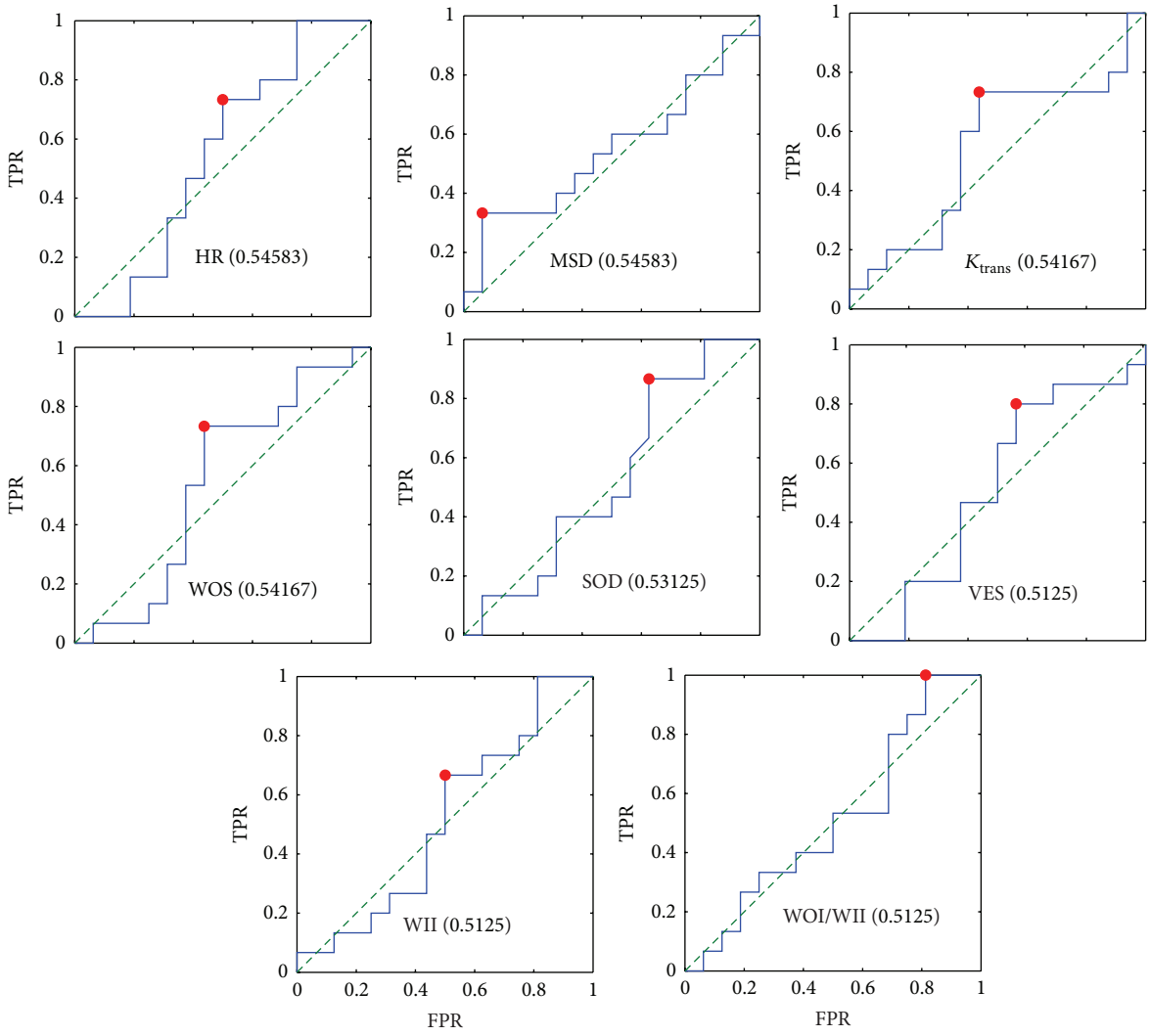

Figure 3: Receiver operating curves (ROCs) of single features in the case of lesion-by-lesion analysis. Per each feature (see Table 2) the ROC is reported in terms of true positive rate (TPR) and false positive rate (FPR). The plots have been aligned according to the area under curve (AUC) in row-wise descending order with the largest AUC at the top-left (per each feature the AUC is indicated in parenthesis). The red dot indicates the best value considering the unbalance between benign-malignant subjects.

Table 3: Sensitivity and specificity of the parameters in the pixel-by-pixel analysis that provide the maximum area under the ROC (AUROC).

Parameters	AUROC	Sensitivity	Specificity
k_{ep}	0.7	0.96	0.22
K_{trans}	0.66	0.99	0.18
HR	0.65	0.94	0.35

Table 4: Sensitivity and specificity of the parameters in the lesion-by-lesion analysis that provide the maximum area under the ROC (AUROC). We have discarded SB because of low sensitivity.

Parameters	AUROC	Sensitivity	Specificity
D^*	0.7	0.73	0.75
WIS	0.65	0.33	0.94
f	0.64	0.73	0.69

Our findings showed that no strong correlation was obtained between DCE-MRI and DW-MRI features (Figure 1). The largest values were obtained in correspondence of the pairs PI & D and SOD & D, probably because PI and SOD are the only two features that describe the whole trend of the time intensity curve course, which allow obtaining the best discrimination between the different types of lesion.

The ROC analysis showed that single features (both DCE and DW) do not have a good discriminative power (Figures 2 and 3). Moreover, the unbalance of our data does not allow determining a good threshold with both high sensitivity and specificity.

Results of Linear Discriminant Analysis (Figures 4 and 5) showed that the use of a combination of DCE and DW

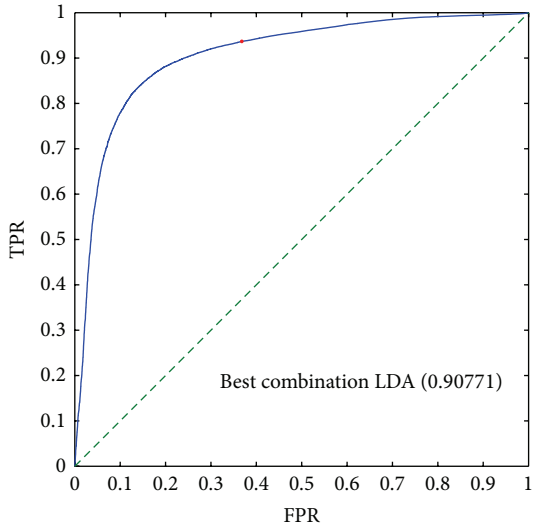

Figure 4: ROC analysis of the best linear combination of *all* features obtained using Linear Discriminant Analysis in the case of pixel-by-pixel analysis. The AUC is indicated in parenthesis. The red dot indicates the best point considering the unbalance between benign-malignant patients: the TPR is approximately 0.93 and the FPR is about 0.35.

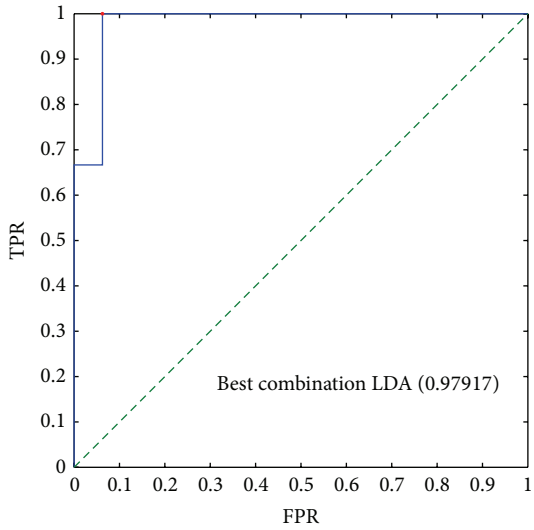

Figure 5: ROC analysis of the best linear combination of *all* features obtained using Linear Discriminant Analysis in the case of lesion-by-lesion analysis. The red dot indicates the best point considering the unbalance between benign-malignant patients. The AUC is reported in parenthesis. The TPR is 1 with FPR less than 0.1.

features, in comparison to single features, has the potential to improve sensitivity and specificity.

However, the potential of both the set of features cannot be achieved using simple classification algorithms such as the one proposed in Section 2.7. Further investigations toward to best way to combine the information from DCE and DW should be performed.

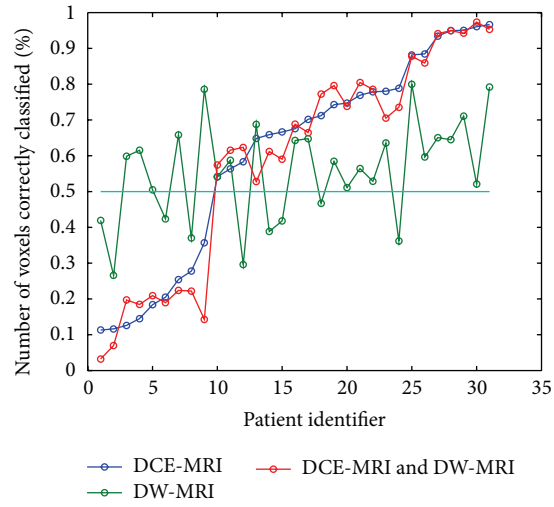

Figure 6: Result of a simple algorithm for classifying benign and malignant lesions: the voxels within a lesion can be classified as benign or malignant using the best combination of features in the pixel-by-pixel analysis: a lesion is classified as malignant if it has a percentage of malignant voxels higher than 50%. Per each patient, the percentage of the correctly classified voxels within the ROI is reported: if this percentage is higher than 50% than the lesion will be correctly identified. It can be seen that using DCE alone (blue line) only 9 lesions have been incorrectly classified. Using only DW we have again 9 lesions misclassified, but they are different from the previous ones. Moreover, the combination of DCE and DW produces the same results as DCE only.

According to our knowledge, no previous study in the literature tried to combine DCE and DW-MRI features including model free and model based parameters evaluated by DCE-MRI data and IVIM parameters evaluated by DW-MRI data, after automatic registration and preprocessing of two volumes, to assess the accuracy in differentiation of benign and malignant breast lesions and to evaluate the improvement of additional of DW-MRI parameters to DCE-MRI features in breast lesion classification.

Several authors in recent literature have combined DCE and DW-MRI data in breast cancer to different aims. Rahbar et al. [42] developed a model incorporating DCE and DW-MRI features, including semimodel free parameters and ADC, to differentiate high-nuclear-grade (HNG) from non-HNG ductal carcinoma in situ (DCIS) in vivo. Those preliminary findings suggested that DCE and DW-MR imaging features may aid in identifying patients with high risk DCIS. Kul et al. [43] evaluated the diagnostic value of an imaging protocol that combined DCE and DW-MRI in patients with suspicious breast lesions and to determine if additional information provided by DWI improves the diagnostic value of breast MRI. They concluded that the combination of DWI and DCE-MRI has the potential to increase the specificity of breast MRI. Partridge et al. [44] showed that ADC can improve the positive predictive value of breast MRI for lesions of varied types and sizes. Jena et al. [45] have tried to evaluate the combined effect of capillary permeability (K_{trans}) and tissue cellularity (ADC) on

the diagnostic accuracy for differentiating benign and malignant breast lesions by incorporating these parameters in routine clinical protocol for breast MRI. Wu et al. [46] reported in their study that the combined use of DW-MRI and CE-MRI has the potential to improve the diagnostic performance in monitoring neoadjuvant chemotherapy (NAC). Atuegwu et al. [47] presented a methodology for incorporating ADC and kinetic DCE-MRI features into a simple mathematical model of tumor growth to predict the tumor cellularity and early treatment response at NAC. In this contribution, results indicate how the integration of DW- and DCE-MRI data can improve specificity and positive predictive value to separate responder by nonresponder patients after one cycle of NAC. It is worth mentioning the recent work proposed by Cai et al. [48, 49]: they proposed a machine learning approach to combining diffusion-weighted imaging (DWI), morphology, and kinetic features from DCE-MRI in order to improve the discrimination power of malignant from benign breast masses. They examined seven features divided in four groups: morphological features, texture features, kinetic features, and one DWI feature (apparent diffusion coefficient). Together with the selected diagnostic features, various classical classification schemes were used to test their discrimination power through cross validation scheme. They concluded that multisided variables, which characterize the morphological, kinetic, pathological properties, and DWI measurement of ADC, could improve the discriminatory power of breast lesions.

However, some drawbacks must be underlined: in particular, they used a nonlinear classifier combining the seven features with support vector machine, Bayesian classifier, k-nearest neighbours, and logistic regression model, all approaches that determine a nonlinear manipulation of features; this latter is not easy to understand by radiologists with respect to a linear combination of features. Moreover, in our study, in contrast to Cai et al. [48, 49], we combined features including model free and model based parameters evaluated by DCE-MRI data and DW-MRI data.

A few remarks must be made: in our study model based parameters were used because they are more strongly related to physiological characteristics of the tissue (perfusion). We did not considered morphological features, as done in Cai et al. [48, 49], because we were interested only in functional aspects of the lesion: this could explain differences in results between our study and cited studies that included also morphological and textural features.

A major limitation of our study is the small size of the population: an increase is required to increase the power of the statistical tests and to detect statistical differences between the two groups (benign and malignant lesions).

Although not conclusive, our results seem to suggest that the combined use of DCE-MRI and DW-MRI does not provide a dramatic improvement compared to the use of DCE-MRI features alone, in the classification of breast lesions.

Conflict of Interests

The authors declare that they have no competing interests.

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Research Article

Risk Factors of the Invasive Breast Cancer Locoregional Recurrence

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Background. The aim of the research was to estimate the frequency of the locoregional breast cancer recurrence appearance, the recurrence-free period continuance, and the 3- and 5-year survival depending on the scope of the surgical intervention, menstrual profile, and histological and molecular-biologic characteristics of the primary tumor. *Patients and Methods*. Among 218 patients with a breast cancer, 99 patients had breast-conserving surgery (BCS) and 119 underwent radical mastectomy (RME); all patients had regional lymphatic nodes dissection. The size and the primary tumor differentiation degree, metastasis presence in the regional lymph nodes, ER expression, PR, and Her/2neu were assessed as the prognostics factors. *Results*. It was defined that the locoregional recurrence appearance frequency in patients with BCS turned out to be 13%, and in patients after RME it turned out to be 9%; the recurrence-free period continuance was 53 ± 8 months and 56 ± 10 months, respectively. *Conclusions*. The locoregional cancer recurrence frequency is higher in women with the menstrual function being preserved at the moment of the primary tumor detection than in postmenopausal patients and also in patients having the hyperexpression of the Her/2neu. The ipsilateral cancer recurrence decreases the 3-year survival by 7,1% and the 5-year one by 20,3%, respectively.

1. Introduction

According to the GLOBOCAN 2012 v1.0, in 2012, worldwide there were 14.1 million new cancer cases, 8.2 million cancer deaths, and 32.6 million people living with cancer (within 5 years of diagnosis). 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths, and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions. Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers), where it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), and it is the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer [1].

The successes in diagnostics and chemoradiotherapy of the breast cancer at the present stage of the oncology development lead to the reconsideration of the surgical treatment methods. At first, Maddox et al. [2] showed that the overall survival (OS) and disease-free survival (DFS) indices do not essentially differ among the patients who passed the radical mastectomy (RME) on Halsted and the modified mastectomy on Patey. In 1970-80, after comprehensive analysis, few clinical trials results in breast surgery prevailed the tendency to the elaboration of the breast-conserving surgery [3–9]. At the present time, to cure the BC, the modified radical mastectomies (Patey and Madden) or the breast-conserving surgery (lumpectomy and quadrantectomy) are often used. While choosing between RME and breast-conserving surgery (BCS), the main problem, both to the doctor and to the patient, is to reach the maximum cosmetic result at the minimum local recurrence risk. This is possible only with presence of the tumor occupying up to 25% of the breast size and upon the condition that the "clean" margin of excision is reached. The local recurrences after BCS require the subsequent surgical treatment (more frequently, mastectomy) neutralizing the reached cosmetic result, and being

the indices of the tumor aggression and a high degree of the distant metastasis availability [10–12]. For this reason, when treating the breast cancer, the determination of the recurrence risk factors should directly influence the surgical interference volume choosing process.

2. Patients and Methods

218 patients had been examined at the age of 31 to 92 (57 ± 1, 3) years, who passed BC treatment course at the oncological clinic of the National Medical University in Kiev Oncology Municipal Hospital in 2004–2009. Among them, 162 patients were treated from primary BC in 2004-2005, and 56 patients were treated from BC recurrence in 2004–2009. The patients were divided into 2 groups: to the first group ($n = 99$) the patients who underwent the BCS were referred (lumpectomy: 35 and quadrantectomy: 64, both with the regional lymphatic nodes dissection), and to the second group ($n = 119$) we referred the patients who passed RME (RME by Madden: 91 and RME by Patey-Dyson: 28). In their turn, groups 1 and 2 were divided into 2 subgroups. To group A were referred the patients without breast cancer recurrence and to group B the patients with the recurrence appearance. Patients were assigned to the BCS with an attempted margin of 1 cm of healthy tissue. Margins were routinely inked to assess the microscopic completeness of the lumpectomy. The criteria to choose for each patient BCS or RME are tumor size, extensive DCIS, tumor margins, tumor location, need for radiation, risk reduction, and individual needs and preferences. Reasons to avoid BCT include multiple tumors, extensive tumor, and contraindication for radiation.

To identify the 3- and 5-year survival, the patients were divided into the following groups: the main group consisting of 154 women who did not have the locoregional breast cancer recurrence during the supervision period and the second group consisting of patients who experienced the locoregional breast cancer recurrence. The same groups were used in the assessment of the menstrual function influence on the recurrence frequency.

The cuts of 4-5 μm in thickness were made of paraffin bricks (standard procedure of haematoxylin-eosin preparation) and placed on the glasses treated with poly-L-lysine. Then, the material was treated according to the standard procedure using the following antibodies: ER-clone 1D5, PgR-clone 636, and Her-2/neu-clone Cb11. The results interpretation was done by immunohistochemical reaction using the nuclear reaction qualitative assessment: the negative "–," the low positive "+," moderately positive "++," strongly positive "+++," and quantity of dyed tumor cells in %.

In making an assessment of Her-2/neu expression, the intensity of cytoplasmic basal membrane coloring was pointed out: the reactions "–" and "+," the absence of hyperexpression, and the reaction "+++," hyperexpression of Her-2/neu. The presence of the hyperexpression Her-2/neu in cases of "++" reaction is conducted with the help of hybridization method in situ using the fluorescent marker FISH (fluorescent in situ hybridization). The investigations were conducted at the pathohistological laboratory at Kiev Municipal Oncology Hospital.

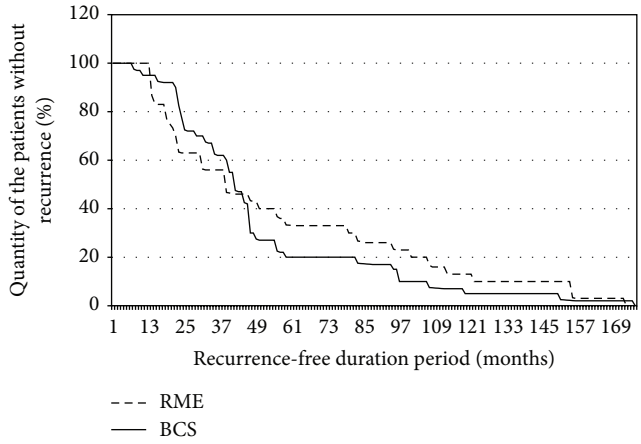

Figure 1: Recurrence-free duration period.

The patients received adjuvant systemic treatment and radiotherapy according of the St. Gallen International Breast Cancer Conference recommendations (2001–2005).

The histopathological diagnosis of breast recurrences was reviewed and compared with that of the initial tumor taking into account cytological, morphological/architectural, and stromal patterns, histological grade, and immunohistochemical staining (hormonal receptors, c-ErbB2). The majority of these parameters had to be similar for a given lesion to be declared a true recurrence.

3. Statistical Methods

In estimating the influence of the axillary node involvement, histological grade, tumor size, and immunohistochemical staining (hormonal receptors and c-ErbB2), the fourfold tables analysis method and the $2 \times K$ tables analysis were used.

The connection between the LR BC and the menstrual function condition of the patients at the primary tumor detection was defined via 2×2 tables' analysis.

The Kaplan-Mayer method was used to estimate the patient's survival rate [13].

4. Results

Table 1 shows clinicopathological information for the 218 enrolled patients. It was established that the frequency of the local recurrence appearance after BCS and RME (1 group) turned out to be 13%, and in patients after radical mastectomy (2 group) it turned out to be 9%. The recurrences frequency in this research corresponds to that of leading oncology hospitals: thus, according to the data from 6 prospective randomized researches [9–12, 14, 15], the recurrences frequency after the mastectomy ranges from 4 to 18%. The recurrence-free period duration in group 1 turned out to be at an average of 53 ± 8 months in the breast-conserving surgery group and 56 ± 10 months in the RME group. The minimum period of BC recurrence appearance after the BCS was 9 months (after RME, 10 months), and the maximum period was 177 months in group 1 against 174 months in the second one (Figure 1).

Table 1: Clinicopathological features for the examined patients.

Characteristic	Group 1 $n = 99$	Group 2 $n = 119$	p value
Age			
<50	42 (42%)	43 (36%)	
50–69 years	48 (48%)	70 (59%)	>0,05
≥70 years	9 (42%)	6 (5%)	
Menopausal status			
Premenopausal	67 (68%)	69 (58%)	>0,05
Postmenopausal	32 (32%)	74 (36%)	
Tumor size			
≥2 cm	73 (74%)	35 (29%)	<0,05
2,1–5 cm	26 (26%)	84 (71%)	
Tumor histological grade			
High (G1)	14 (14%)	7 (6%)	
Middle (G2)	73 (74%)	98 (82%)	>0,05
Low (G3)	10 (10%)	11 (9%)	
Undifferentiated (G4)	2 (2%)	3 (3%)	
Regional lymph nodes involved			
No	61 (62%)	69 (58%)	>0,05
Yes	38 (38%)	50 (42%)	
ER presence			
Negative	35 (35%)	46 (39%)	>0,05
Positive	64 (65%)	73 (61%)	
PR presence			
Negative	37 (37%)	51 (43%)	>0,05
Positive	62 (63%)	68 (57%)	
Her2/neu presence			
Negative	71 (72%)	99 (83%)	>0,05
Positive	28 (28%)	20 (17%)	
Breast cancer subtypes			
Luminal A	47 (48%)	73 (61%)	<0,05
Luminal B	17 (17%)	12 (10%)	
Her2-positive	11 (11%)	8 (7%)	>0,05
Triple negative	24 (24%)	26 (22%)	

Table 2: The primary tumor size.

Examined group	Tumor size		Total
	Till 2 cm	2–5 cm	
1 A group	56* (26%)	9* (4%)	65 (30%)
1 B group	27* (12%)	7* (3%)	34 (15%)
2 A group	23* (11%)	66* (30%)	89 (41%)
2 B group	12* (6%)	18* (8%)	30 (14%)
Total	118 (55%)	100 (45%)	218 (100%)

*The differences between groups are not statistically significant ($p > 0,05$).

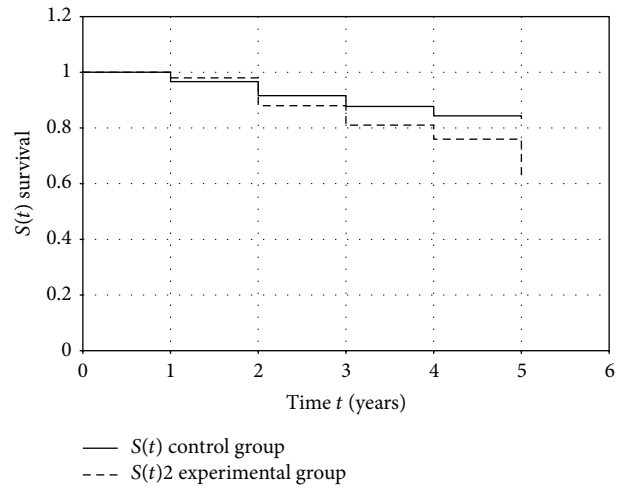

Figure 2: Patient survival curves according to the BC recurrence presence or absence.

The tumor size, axillary node involvement, and histological grade are the predictive factors of the disease run [14]. In the Tables 2, 3, and 4, the distribution of patients according to the tumor size, axillary node involvement, and histological grade, respectively, is shown. It was established that the tumor size, axillary node involvement after mastectomy, and histological grade do not influence the frequency of the BC recurrence at the level of significance (p) 0,05. But in the BCS group the axillary node involvement increases the frequency of the ipsilateral recurrence of the breast cancer.

The results of the immunohistochemical examination are shown in the Table 5. Evaluating the tumor receptor status used the $2 \times K$ table analysis method [13]. It was established that the primary tumor receptor status (ER, PR) in groups 1 and 2 and the expression degree Her/2neu after the RME do not influence the BC recurrence. In breast-conserving surgery group, the hyperexpression of Her/2neu increases the frequency of the locoregional breast cancer recurrence appearance.

The assessment procedure of Kaplan-Meyer was used to evaluate the overall patients' survival. Three-year survival rate in patients without BC recurrence was 87,6% and the 5-year survival rate was 82,8%. Three- and 5-year survival in patients with BC recurrence of the BC corresponded to 80,5% and 62,5%, respectively (Figure 2). The differences in 3-year survival between groups are not statistically significant ($p > 0,05$) and differences in 5-year survival between groups are statistically significant ($p < 0,05$).

5. Discussion

The era of conserving surgeries in breast cancer started in the 1970s. U. Veronesi in Milan Cancer Institute (Italy) and B. Fisher in The Abramson Cancer Center of the University of Pennsylvania (USA) proposed independently of each other to perform the conserving surgery in breast cancer. In 1969,

Table 3: The primary tumor histological grade.

Examined group	Tumor histological grade				Total
	High	Middle	Low	Undifferentiated	
1 A group	12* (5,5%)	44* (20%)	8* (4%)	1* (0,5%)	65 (30%)
1 B group	2* (1%)	29* (13%)	2* (1%)	1* (0,5%)	34 (15,5%)
2 A group	5* (2%)	73* (33,5%)	9* (4%)	2* (1%)	89 (40,5%)
2 B group	2* (1%)	25* (11,5%)	2* (1%)	1* (0,5%)	30 (14%)
Total	22 (9,5%)	170 (78%)	21 (10%)	5 (2,5%)	218 (100%)

*The differences between groups are not statistically significant ($p > 0,05$).

Table 4: The presence of the metastases in the regional lymph nodes.

Examined group	Axillary node involvement			Total
	The metastases in the regional lymph nodes are not present	The metastases in the regional lymph nodes are present in 1–3 nodes	The metastases in 4 and more regional lymph nodes	
1 A group	47** (22%)	11** (5%)	7** (3%)	65 (30%)
1 B group	14** (6,5%)	12** (5,5%)	8** (4%)	34 (16%)
2 A group	55* (25%)	18* (8%)	16* (7%)	89 (40%)
2 B group	14* (6,5%)	9* (4,5%)	7* (3%)	30 (14%)
Total	130 (60%)	50 (23%)	38 (17%)	218 (100%)

*The differences between groups are not statistically significant ($p > 0,05$).
**The differences between groups are statistically significant ($p < 0,05$).

Table 5: Immunohistochemical staining of the primary node.

Examined group	ER		PR		Her2/neu	
	Positive	Negative	Positive	Negative	Positive	Negative
1 A group	38* (39%)	22* (22%)	43* (43%)	16* (17%)	2** (2%)	58** (59%)
1 B group	26* (26%)	13* (13%)	20* (20%)	20* (20%)	26** (26%)	13** (13%)
2 A group	55* (46%)	26* (22%)	50* (42%)	31* (26%)	13* (11%)	68* (57%)
2 B group	18* (15%)	20* (17%)	18* (15%)	20* (17%)	7* (6%)	31* (26%)

*The differences between groups are not statistically significant ($p > 0,05$).
**The differences between groups are statistically significant ($p < 0,05$).

results of randomized studies to compare radical mastectomy with breast-conserving surgery, which was termed "quadrantectomy," were approved by the World Health Organization Committee of Investigators for Evaluation of Methods of Diagnosis and Treatment of Breast Cancer [15]. The recruitment of patients began at the Milan Cancer Institute in 1973, after the new procedure was standardized, and preliminary data showing that survival rates were equal after radical and breast-conserving surgery were published in 1977 and 1981 [5, 7]. In 1971, the National Surgical Adjuvant

Breast and Bowel Project (NSABP) initiated the B-04 study, a randomized clinical trial conducted to resolve controversy over the surgical management of breast cancer.

The afterward published results of the above investigations had not demonstrated the appreciable difference of the late fates. However, in patients after the BCS, the probability of the locoregional recurrence appearance is higher compared with the patients who have been undergone the radical mastectomy. The recurrence appearance requires another surgical intervention, oftener than the mastectomy which aligns the cosmetic an d esthetic effect reached at BCS. Besides, the appearance of the regional breast cancer decreases the 3- and 5-year survival.

The important thing in the clinical practice is the drawing distinctions between the real recurrences and the newly occurred ipsilateral tumors. This is due to the fact that the newly occurring breast cancer is susceptible to the X-ray therapy and standard schemes of the chemotherapy, whereas the recurrent tumor is chemo- and radio-resistant and requires the adjuvant treatment modification [9, 14, 15].

The causes of the ipsilateral recurrences development could be the following ones: tumor cells expansion along the muscle fiber, fascial plates, vessels, and nerve and perineural crevice tunics; the tumor could have plural rudiments (multicentricity and multifocality), which are the causes of underestimation of the process expansion; it could be rested in the edges the microscopic normal, but genetically changed cells, which will initiate the recurrent tumor development.

The presence of the established risk factors of breast cancer recurrence development in patients (the presence of metastases in the regional lymph nodes and the hyperexpression of Her/2neu in the primary tumor cells) requires more strict control of the adjuvant treatment; the main efforts must be directed to the early recurrence detection and the usage of the up-to-date methods of diagnostics (MRT, PET) and biopsy at the susceptible recurrent focuses.

6. Conclusion

(1) The surgical intervention volume does not affect the frequency of the locoregional breast cancer recurrence appearance.

(2) The recurrence-free period duration in patients who have been done the breast-conserving surgery does not essentially differ from the patients who have been undergone the mastectomy.

(3) In patients after the breast-conserving surgery, the presence of metastases in the regional lymphatic nodes and the hyperexpression Her/2neu in the primary tumor cells is associated with the higher risk of the locoregional recurrence.

(4) The regional breast cancer appearance decreases the 3-year survival by 7,1% and 5-year survival by 20,3%.

Conflict of Interests

The authors have declared no conflict of interests.

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Research Article

Prognostic Value of MRS Metabolites in Postoperative Irradiated High Grade Gliomas

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Purpose. We studied the prognostic significance of Magnetic Resonance Spectroscopy (MRS) in operated high grade gliomas. *Materials and Methods*. Twelve patients were treated with radiotherapy and Temozolomide. The MRS data were taken four weeks after operation (before radiotherapy) and every six months after the completion of RT. The N-acetyl aspartate, choline, creatine, and myo-inositol parameters were quantified, analyzed, and correlated to recurrence-free survival (RFS). *Results*. The median RFS was 26.06 months. RFS was significantly worse in elderly patients ($P = 0.001$) along with the higher choline/creatine ratios at either baseline ($P = 0.003$) or six months post Radiotherapy ($P = 0.042$). Median RFS was 23 months in high choline/creatine levels ≥2 at 6 months after radiotherapy and 11 months for those with <2 choline/creatine levels. There was a significant correlation of maximum difference of choline/creatine ratio with RFS (rho = 0.64, $P = 0.045$). *Conclusion*. Age and choline/creatine ratio are strong independent prognostic factors in high grade gliomas.

1. Introduction

High grade gliomas, anaplastic astrocytoma (grade III) and glioblastoma (grade IV) classified according to the World Health Organization (WHO), represent the commonest primary adult cerebral neoplasms. They account for approximately 7% and 54% of all gliomas [1]. Only 30% of patients survive more than 1 year and less than 5% beyond 5 years. The 5-year survival rate for anaplastic astrocytoma is 27% [2].

Magnetic Resonance Spectroscopy (MRS) can offer biochemical data from the different volumes of interest in tissues. It can provide metabolic information on tumor activity regarding rate of growth, heterogeneity, and extension and permits a noninvasive categorization of brain tumors. It is useful in grading tumors and can help in treatment strategies (as surgery, radiotherapy, chemotherapy, angiogenesis inhibitors, etc.), in radiation treatment planning delineation, in evaluating response to treatment, and in discriminating

tumor from radiation necrosis. MRS imaging enables the assessment of tissue metabolites such as choline (Cho), creatine (Cr), N-acetylaspartate (NAA), and myo-inositol (MI). Cho is a marker of phospholipid turnover and cellular density and it is increased in all gliomas [3]. N-Acetyl aspartate (NAA) is found in neuronal cell types and oligodendrocytes. Cho represents the precursor of phosphatidyl Cho (phospholipid of cell membrane). In malignant tumors there are higher levels of Cho because of the increased cell membrane turnover. The values of Cho are higher in grades III and IV than in grade II astrocytomas and it is relatively diminished when necrosis is present [4]. In brain tumors there are low levels of NAA and higher Cho levels because of the neuronal destruction, the high proliferation rate, and increased cell membrane turnover. Cr is a high-energy compound located in the mitochondria and serves as a marker for cellular energy metabolism. The ratios of Cho/NAA and Cho/Cr are higher in high grade gliomas, compared with low grade gliomas [5].

Many prognostic factors have been studied as influencing the outcome of these aggressive tumors [6–12]. Advanced age is inversely related to survival ($P < 0.0001$) [6]. Lobar tumor location, radiation therapy (RT) dose 5,000–6,000 cGy, Karnofsky performance status (KPS) at presentation ≥70, and a normal level of consciousness before biopsy are considered good prognostic factors [7]. Age ($P = 0.027$), log10 of epidermal growth factor receptor (EGFR) ($P = 0.025$), and labeling index (LI) measured by tritiated thymidine incorporation ($P = 0.0019$) were significant continuous variables and the survival was found to be shorter when the covariable increased [8]. Frontally located tumors were found to have longer median survival time and higher 1- and 2-year survival rates compared to tumors in other locations (101 versus 47 weeks, resp.; 76% and 44% versus 37% and 2.5%, resp.; $P = 0.00001$). Progression-free survival at 1 year was higher in the radically resected group than in the group that was biopsied (20% versus 0%, resp.; $P < 0.001$) [9]. Microvessel density of grade of 3+ or 4+ was found to correlate with shorter survival time than microvessel density grade of 1+ or 2+ ($P = 0.0022$) [10]. A statistically significant improvement in survival was associated with increasing total radiation dose to the tumor bed ($P < 0.001$) without additional benefit demonstrated for doses greater than 60 Gy [11]. Among patients with KPS ≥ 70 and age < 50 years, median survival was 57 weeks if the corpus callosum was involved (35% 2-year survival) and 105 weeks if the corpus callosum was not involved (56% 2-year survival) [12].

The aim of this study was to determine whether MRS can be used for prognosis of recurrence in postoperative irradiated high grade gliomas and to correlate MRS metabolites with RFS.

2. Materials and Methods

Twelve patients (six females and six males) with a diagnosis of high grade glioma participated in the present study. All participating patients firmed the informed consent and etic committee approval was not needed. The patients' characteristics are shown in Table 1. The median age was 51 years (range: 29–72 years). All patients presented with central nervous system symptoms and were assessed with brain MRI that demonstrated a lesion compatible with brain tumor. All patients underwent surgery and biopsy confirmed a high grade glioma grades III-IV, according to the World Health Organization (WHO) classification. Six patients were diagnosed with a glioma grade III and 6 with a glioblastoma multiforme. Patients were evaluated with MRS before the delivery of external beam three-dimensional conformal radiotherapy (3D-CRT). We excluded gliomas located in the brainstem and patients with Karnofsky performance status <80.

Table 1: Patient characteristics and descriptive statistics of MRS parameters.

		Range
Age (median)	51	29–72
Sex (male/female)	6/6	
RFS (median)	26.06	7–49
Cr (mean)	4.309 ± 1.251	1.5–6.5
NAA (mean)	3.919 ± 1.466	1.2–6.8
MI (mean)	6.377 ± 2.914	2.4–14.4
NAA/Cr (mean)	1.0423 ± 0.3798	0.4–2.04
MI/Cr (mean)	0.9026 ± 0.3309	0.45–1.88
Cho/Cr (mean)	1.8216 ± 1.148	0.73–5.0

Abbreviations: RFS: Relapse Free Survival Interval; Cho: choline; Cr: creatine; MI: myo-inositol; NAA: N-acetyl aspartate.

All patients underwent an MRS at baseline before the initialization of RT and six months after irradiation. All the MRI examinations were performed on a 1.5 Tesla system (General Electric, Signa HDxt, Winsconsin, USA). The MRI protocol included the following pulse sequences: axial T2 flair (TE: 112 ms, TR: 9002 ms, TI: 2250 ms, 5 mm slice thickness and 1.5 mm gap, and 320 × 224 matrix), coronal diffusion (TE: 100 ms, TR: 4500 ms, 5 mm slice thickness and 1.5 mm gap, and 128 × 128 matrix), and axial T2 multiecho (TE: varying, TR: 675 ms, 5 mm of slice thickness and 1.5 mm gap, and 256 × 160 matrix). These pulse sequences help towards the differentiation of the tumor as well as the placement of the single-voxel and the 3D slab (i.e., active tumor volume and not edema). The MRS pulse sequences were single-voxel PRESS at TE: 35 ms and 135 ms (of variable voxel sizes which were normalized for comparison reasons, TR: 1500 ms, NEX: 8) and 3-dimensional PRESS at TE: 135 (TR: 1000 ms, of variable thickness and spacing between patients with typical values of the order of 48.5 thickness and 8.1 mm spacing, 10 × 10 matrix, and NEX: 0.80).

Each patient underwent a virtual CT-simulation, in the supine position, using dedicated devices. Patients were fixed in a custom-designed immobilization device and were simulated and treated in the supine position. The patients were scanned with 5 mm slice thickness in simulation CT scan and the CT datasets were transferred to the Prosoma Virtual Simulation and Contouring System through the DICOM network. The following structures were delineated as organs at risk (OARs): optic chiasm, optic nerves, brainstem, eyes, and lenses. The Clinical Target Volume (CTV) was delineated

Table 2: Cox regression survival analysis or RFS with age and MRS parameters. All significant parameters from univariate analysis were entered into the multivariate analysis to create the final model (Wald Chi-square$_3$ = 25.03, $P < 0.001$).

Variables	HR (95% CI) Univariate	P	HR (95% CI) Multivariate	P
Cho	—	0.87	—	
Cr	—	0.70	—	
NAA	1.23 (1.08, 16.39)	0.045	—	
MI	—	0.81	—	
Cho/Cr (baseline)	2.94 (1.22, 9.51)	0.001	2.38 (1.17, 8.78)	0.003
Cho/Cr (6 months)	1.54 (1.03, 12.76)	0.021	1.29 (1.08, 11.33)	0.042
NAA/Cr	—	0.31	—	
MI/Cr	—	0.72	—	
Age	6.85 (2.57, 11.36)	0.001	5.44 (2.11, 12.26)	0.001

Abbreviations: DFS: Disease Free Survival Interval; Cho: choline; Cr: creatine; MI: myo-inositol; NAA: N-acetyl aspartate.

using preoperative and postoperative MRI and postoperative MRS. The surgical cavity, the areas of contrast enhancement, and T2 flair signal abnormality expanded by 2-3 cm for subdiagnostic microscopic infiltration constituted the CTV. A margin of 5 mm to the CTV was added to generate the Planning Target Volume (PTV). Contours were edited to exclude air, bone, and brain parenchyma if possible.

RT was administered within 4–6 weeks of the surgery. The patients were all treated with adjuvant fractionated external beam 3D-CRT. A total dose of 60 Gy was delivered in 30 daily fractions (2 Gy/fraction) [13]. Fields were reduced for the last phase of the treatment as boost. The prescription dose was defined for the 95% isodose of the PTV. In particular, 95% of the PTV should have been covered within 95%–110% of the prescribed dose. The treatment planning was performed in the Eclipse (Varian Medical Systems, United States) treatment planning system (TPS). The photon beam energies used were 6 MV, using a 2100C Varian linear accelerator. The beam arrangement consisted of beams, where the beam angles, apertures, weights, and dynamic wedges were optimized by standard, forward planning. Partial wedging or dynamic (multileaf collimator (MLC)) was employed to improve dose homogeneity.

The target volumes and organs at risk were elaborated with the beam's eye view (BEV) technique. For the treatment technique, histograms of the OARs were generated; a number of parameters, including mean, median, and maximum dose, were evaluated.

Concurrent (with RT) 75 mg/m^2/daily of temozolomide (TMZ) was administered. After RT TMZ dose was 150–200 mg/m^2 per day for 5 days of a 28-day cycle [13]. The dose was adjusted according to standard hematological toxicity criteria. The intention was to give patients at least 12 cycles (up to 24) of TMZ, and treatment was continued until we observed disease progression or unacceptable toxicity.

During radiation treatment the patients were monitored every week. Posttreatment management included adjuvant endocrine therapy according to the National Comprehensive Cancer Network Guidelines. After completion of treatment, the patients were evaluated by a radiation oncologist every 3 mo.

The duration of RFS for patients with high grade gliomas was measured from the time of diagnosis to the time of recurrence (viable tumor on magnetic resonance imaging). To evaluate prognostic values, we performed univariate and multivariate Cox-regression survival analysis in terms of MRS parameters such as Cho, Cr, NAA, MI, NAA/Cr, Cho/Cr, Cho/NAA, and MI/Cr. Briefly, all factors with $P < 0.05$ on univariate analysis were entered into the model and the model was refit in a stepwise fashion after the sequential removal of nonsignificant factors. The process was stopped when only significant ($P < 0.05$) factors remained. Entering the significant factors sequentially and checking for and possibly removing factors that became nonsignificant confirmed the model. The correlation of RFS with the maximum difference of Cho/Cr ratio was performed with the Spearman rho test. Kaplan Meier curve and log-rank test were performed for RFS related to either <2 or ≥2 of Cho/Cr ratio in two cases: for baseline and 6 months after RT Cho/Cr ratio. The median value of Cho/Cr ratio was chosen as the cut-point for the comparative analysis of RFS related to Cho/Cr ratio. Statistical significance was accepted at the $P < 0.05$ level. All the analysis was performed with the SPSS software v.10 (IL, USA).

3. Results

All patients completed their irradiation schedule. At the most recent follow-up, five patients were alive and seven were dead. Median RFS was 26.06 months. The median overall survival time was thirty-two months. The patients' characteristics along with the RFS and MRS parameters are summarized in Table 1. In univariate analysis, age, NAA, Cho/Cr (baseline), and Cho/Cr at 6 months after RT were significant prognostic factors for RFS. When the above factors were entered into the multivariate model, the NNA lost its prognostic value, while only age and Cho/Cr ratios at baseline and 6 months thereafter had a significant impact top RFS. The other MRS parameters had no significant impact on RFS. The Cox-regression survival analysis for RFS between age and the MRS findings is shown in Table 2. As shown in Figures 1 and 2

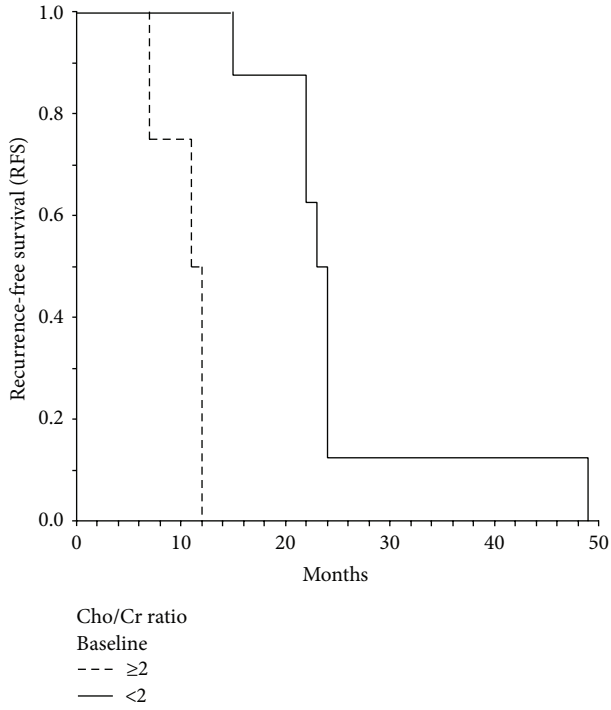

Figure 1: Kaplan-Meier curve for recurrence-free survival (RFS) in terms of Cho/Cr ratio ≥2 versus <2 (baseline). Median RFS 23 (SE = 1) versus 11 (SE = 2), log-rank $P = 0.0004$.

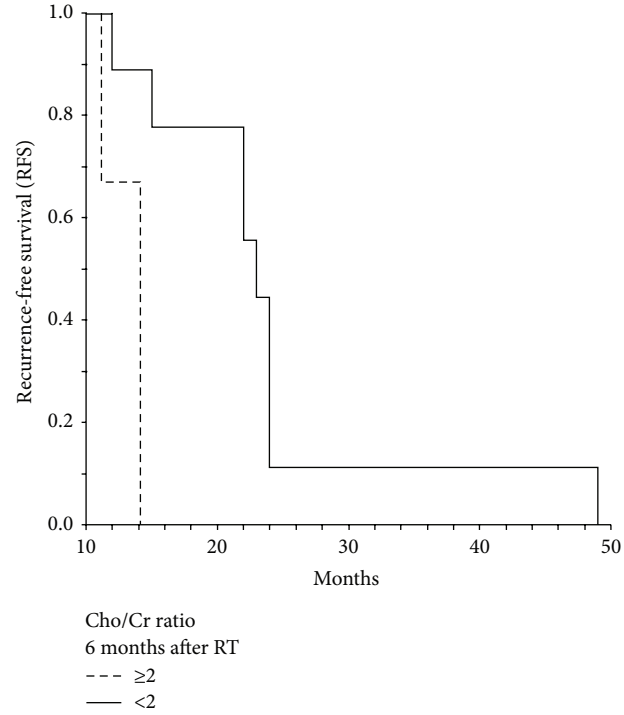

Figure 2: Kaplan-Meier curve for recurrence-free survival (RFS) in terms of Cho/Cr ratio ≥2 versus <2 (6 months after RT). Median RFS 23 (SE = 2) versus 11 (SE = 1), log-rank $P = 0.045$.

median RFS was 23 months for patients with high Cho/Cr levels ≥2 (at baseline and 6 months after RT) and 11 months for those with <2 Cho/Cr levels (log-rank test: $P = 0.0004$ and $P = 0.045$, resp.). There was a significant correlation of maximum difference of Cho/Cr ratio with RFS (rho = 0.64, $P = 0.045$), as shown in Figure 3.

4. Discussion

MRS is employed to obtain metabolic information regarding intracranial gliomas. It represents a useful tool that can allow improvement in neoplasia grading, biopsy/therapy guidance, and earlier evaluation of the response to therapy [14]. Regarding the assessment of brain tumours, conventional MRI deals with structural changes. However, recently developed advanced MRI techniques, such as diffusion weighted imaging (DWI), diffusion tensor imaging (DTI), and perfusion imaging, allow further studies of brain tumours delivering more reliable differential tumour diagnosis as well as tumour grading [15]. Briefly, in DWI studies, the majority of tumours demonstrate higher Apparent Diffusion Coefficients (ADC) values than in healthy brains [16]. Furthermore, DTI demonstrates the effects of tumours on the surrounding white matter tracts, who will be either displaced, or infiltrated, or destroyed. Fractional Anisotropy (F.A.) index has lower values in gliomas than in healthy brains due to abnormal brain architecture [15]. Perfusion MRI allows the study of blood supply in tumours via imaging of tumour vascularity either by IV contrast medium injection (Dynamic Susceptibility Contrast Imaging—DSCI, or Dynamic Contrast

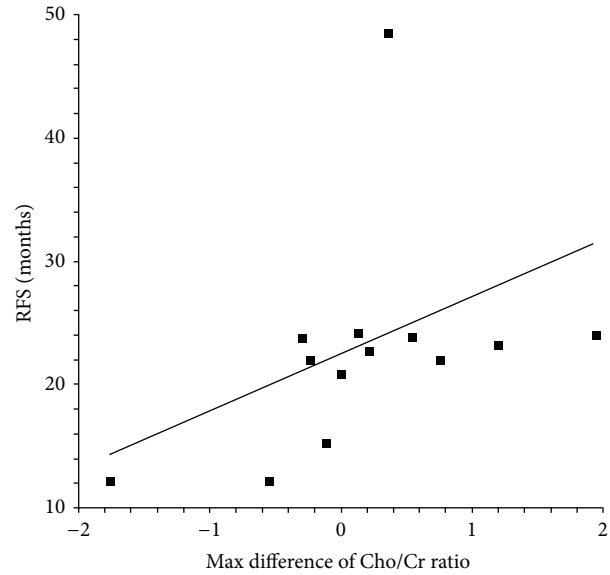

Figure 3: Recurrence-free survival (RFS) correlated with max difference of Cho/Cr ratio with baseline (Spearman rho = 0.64, $P = 0.045$).

Enhancement—DCE) or by endogenous contrast mechanism (Arterial Spin Labelling—ASL) [15].

The multivoxel MRS technique, the so-called chemical shift imaging, has advantages compared to the single voxel spectroscopy: it provides simultaneously spectra information over a wider volume of brain where early tumour diffusion

can be detected. Additionally, colour maps with respect to various metabolites can be displayed all over the selected 3D slab.

However, in case that Chemical Shift Imaging (CSI) is used then a number of issues have to be considered, such as, the long acquisition which can lead to patients' anxiety which is accompanied by head motion producing spectra deterioration, field inhomogeneities that produce large line widths deteriorating the spectra resolution, chemical shift misregistration and outer volume signal bleed [17]. In general, the single voxel technique is used for metabolites' quantification, whereas CSI is used for metabolites' spatial distribution [18].

Lee et al. [19] studied the pattern of failure of high grade astrocytomas treated with high dose conformal irradiation (70 or 80 Gy) and have shown that 89% of the patients failed with central or in-field recurrences, 8% had a marginal component to the recurrence, and 3% failed outside the high dose region. Oppitz et al. [20] evaluated the three-dimensional tumor regrowth relative to the treated volume which included the preoperative macroscopic tumor and a 2 cm margin. They found that the majority of tumor recurrences were located within the original 90% isodose. They suggested dose escalation to a more restricted volume. Narayana et al. [21] studied the utility of MRS and functional MRI (fMRI) in RT treatment planning. They evaluated 12 patients with MRS and functional imaging before irradiation. The Cho/Cr ratio maps were fused with the MR images and then transferred to treatment planning CT images for target volume delineation. They found that MRS volumes based on Cho/Cr ≥ 3 were 40% larger than MRI-T1 and functional MRI and assisted in beam orientation. Pirzkall et al. [22] studied the role of MRS in RT target delineation. They found that MRS optimized the target volume delineation and can improve local control. All their patients had MRI and MRS preoperatively. Target volumes were delineated on T1 + gadolinium images. MRS parameters were converted in a quantitative index and were displayed as three-dimensional contours. They found that MRS defined metabolically active gliomas extended beyond the T2 volume by approximately 3 cm. Balmaceda et al. [23] assessed the use of MRS in chemotherapy response of low grade gliomas. They found a significant correlation between increased Lac/Cr and Cho/Cr ratios during treatment and a decreased DFS. They concluded that Cho/Cr and Lac/Cr appeared to be reliable biomarkers of tumor progression. Zeng et al. [24] reported that MRS could discriminate postradiation injury from recurrence. They found higher Cho/NAA and Cho/Cr ratios and lower NAA/Cr ratios in recurrent tumors. The Cho/Cr and Cho/NAA ratios were lower in postradiation injury than in normal cerebral tissue. Weybright et al. [25] suggested that MRS parameters can help in diagnostic dilemmas regarding recurrent or residual tumor, treatment-related changes of posterior fossa, or brainstem tumors. They concluded that mean Cho/Cr ratios obtained in recurrent tumor, treatment-related changes, and normal white matter were 2.93, 1.62, and 0.97, respectively, mean Cho/NAA ratios were 4.34, 1.74, and 0.93, and mean NAA/Cr ratios were 0.74, 0.92, and 1.26, respectively. Alexander et al. [26] suggested that values of Cho during a RT course could indicate response to treatment. All patients were examined with MRS at diagnosis, at week 4 of treatment, and 2 months after RT. Patients who had >40% decrease in Cho levels between week 4 and after RT had a worse outcome ($P = 0.003$) and disease progression ($P = 0.012$). Czernicki et al. [27] performed MRS preoperatively and postoperatively at 6 months in high grade glioma patients. An increase in Cho/NAA and decrease in NAA/Cr ratios were associated with a shorter overall survival. In tumor recurrence Cho/NAA and Lac/Cr ratios increased and the NAA/Cr ratio decreased between the two evaluations. Quon et al. [28] performed MRS in high grade gliomas before RT, at week 4 of irradiation, and 2 months after treatment. They noted that a decrease of >40% in Cho levels from week 4 during RT to 2 months after RT had a statistically significant worse OS (9.1 months versus not reached, $P < 0.001$) and PFS (5.8 versus 19.8 months, $P = 0.0018$).

Our analysis indicates a significant correlation between postoperative Cho/Cr levels and prognosis in terms of recurrence-free survival. The survival of patients with high grade gliomas depends mainly on the intrinsic properties of tumor as potent malignancy and response to treatment. The present study examined a probable association between the postsurgery, pre- and post-RT-treatment MRS metabolite values, and disease-free survival. In our study age seemed to be related with a dismal prognosis, in accordance with the literature [29]. However, the most notable finding was that Cho/Cr ratio was associated with RFS.

5. Conclusion

This study has assessed the value of preirradiated in vivo MRS parameters in predicting RFS for patients with high grade gliomas, who undergone postoperative RT. With our findings we are sharing our experience concerning the fact that metabolic measures of residual tumor volume can be strongly related with survival, in terms of Cho/Cr ratio. Cho/Cr ratio is reproducible and can be easily incorporated into routine radiological examination, while it may optimize risk stratification and could be target for evaluating future therapies. The main weakness of the present study is the small number of patients. Future studies should further assess the parameters that have been identified in larger populations of patients and evaluate whether they can also be used to assess prognosis at other time points.

Conflict of Interests

All authors declare that they have no conflict of interests.

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Research Article

The Application of Classification and Regression Trees for the Triage of Women for Referral to Colposcopy and the Estimation of Risk for Cervical Intraepithelial Neoplasia: A Study Based on 1625 Cases with Incomplete Data from Molecular Tests

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Objective. Nowadays numerous ancillary techniques detecting HPV DNA and mRNA compete with cytology; however no perfect test exists; in this study we evaluated classification and regression trees (CARTs) for the production of triage rules and estimate the risk for cervical intraepithelial neoplasia (CIN) in cases with ASCUS+ in cytology. *Study Design*. We used 1625 cases. In contrast to other approaches we used missing data to increase the data volume, obtain more accurate results, and simulate real conditions in the everyday practice of gynecologic clinics and laboratories. The proposed CART was based on the cytological result, HPV DNA typing, HPV mRNA detection based on NASBA and flow cytometry, p16 immunocytochemical expression, and finally age and parous status. *Results*. Algorithms useful for the triage of women were produced; gynecologists could apply these in conjunction with available examination results and conclude to an estimation of the risk for a woman to harbor CIN expressed as a probability. *Conclusions*. The most important test was the cytological examination; however the CART handled cases with inadequate cytological outcome and increased the diagnostic accuracy by exploiting the results of ancillary techniques even if there were inadequate missing data. The CART performance was better than any other single test involved in this study.

1. Introduction

Cervical cancer (CC) is the third most common cancer and the fourth leading cause of cancer death in females worldwide [1]. More than 85% of these cases and deaths are in developing countries; this is due to lack of screening that may allow detection of precancerous and early stage cervical cancer. Despite the advances in screening, cervical cancer remains a serious problem of public health even in developed countries, due to the high percentage of detection failures [2].

CC is known to be caused almost always by human papillomavirus (HPV) infection which is the commonest sexually transmitted infection worldwide. About 100 types of HPV virus that can infect humans have been identified. Among them, 15 are oncogenic and can cause CC. Improved understanding of HPV infection and the natural history of cervical neoplasia have resulted in the addition of the HPV DNA test along with the Pap test and frequently a competing test.

Nowadays, ancillary techniques for CC screening are available. These include HPV DNA typing and mRNA identification of the viral E6/E7 oncogenes that are linked to oncogenic activation. Among them, mRNA typing with nucleic acid based amplification (NASBA) [3] and mRNA-Flow-FISH techniques in screening programs produced promising results in increasing PPV and reducing unnecessary recalls and referrals to colposcopy [4–7]. At the same time, it was reported that the immunocytochemical detection of p16 can increase the diagnostic accuracy of the Pap test [8].

Several published studies in the literature attempted to clarify the role of each technique as a unique test to substitute the Pap test [3–7, 9–17]. By the detailed analysis of these, it can be concluded that the performance measures of the methods under control differ significantly, affected by the disease incidence and the prevalence of HPV infection in the population study group; thus, application of a single method, even if it offers a level of protection, does not determine reliably the risk for individual women to harbor cervical intraepithelial neoplasia (CIN). However, from the meta-analysis of published studies [9–12] it is evident that the sensitivity of Pap test combined with the HPV DNA test is higher than the sensitivity of each individual method.

Computer science and artificial intelligence enabled the development of computer assisted systems for the support of clinical diagnosis or therapeutic and treatment decisions. Various classification techniques such as neural networks [18–29], discriminant analysis [18, 30–32], classification and regression trees (CARTs) [33–35], or genetic algorithms [36] have been used in medicine. The application of new molecular techniques that are nowadays used in the diagnostic cytology laboratory [37] improves the accuracy of the final diagnosis in comparison to that of cytology alone.

Among the various decision support techniques (CARTs) is an attractive statistical approach to extract knowledge from data as they are straightforward to construct and easily understandable by physicians. The application of these systems produces simple decision algorithms linked with probabilities that can be promising to define triage rules and perhaps give a better understanding of the disease.

The aim of this study was to investigate the potential role of CARTs applied on various diagnostic variables measured in the modern cytopathology laboratory and to build algorithms for the triage of individual cases. Special focus was given to design the study as pragmatic as possible: thus, (1) cases from two different parts of the country were selected, (2) a part of the cases were considered to be negative as no histological confirmation could be obtained due to ethical reasons, and (3) inadequate test results (i.e., missing data) were included as this is the cytopathology laboratory reality.

2. Materials and Methods

2.1. Involved Institutes and Ethics. Our study involved the 3rd Department of Obstetrics and Gynecology, the Department of Cytopathology and the 2nd Department of Pathology, all three hosted in "Attikon" University Hospital, Medical School of Athens University, and the Department of Obstetrics and Gynecology of University Hospital of Ioannina City. The study was approved by the University Hospital Ethics Boards and participating women signed an informed consent (ICON) form to allow use of their epidemiologic, diagnostic, and ancillary test data.

2.2. Cytology. All cytological and ancillary examinations were based on ThinPrep liquid based cytology (LBC) material obtained before colposcopical examination. The smears were routinely prepared for cytological examination and the remaining material in the ThinPrep vial was used for additional evaluation of biomarkers related to the HPV lifecycle. The smears were assessed by experienced cytopathologists. Histological material was obtained during colposcopy and/or during treatment by conization. The obtained histological samples were fixed and prepared according to standard histopathology protocols.

The cytological findings for each woman were formulated according to the revised Bethesda classification system (TBS2001 system) [38, 39].

2.3. Histological Confirmation. The histological diagnosis was the golden standard and was used as the target category of each woman. Punch biopsies were performed by experienced colposcopists (in practice for more than 10 years) as part of the study protocol. The three-tiered cervical intraepithelial neoplasia grading system was used for reporting histological diagnosis. Clinically negative (CN) cases were included in the study. These were defined as CN if the cytology, colposcopy, and the CLART Human Papillomavirus 2 HPV DNA test (see Section 2.4) were all negative. Despite the lack of histological biopsies due to ethical hurdles, these women were included and analyzed in a target category of less than CIN2. The correlation of the cytological results in relation to histology is presented in Table 1.

2.4. Molecular Tests. In relation to the HPV lifecycle biomarkers we used (a) HPV DNA typing using the CLART Human Papillomavirus 2 (GENOMICA) kit for the simultaneous detection of 35 different HPV genotypes by PCR

Table 1: Correlation of the cytological with the histological diagnosis.

	Histological result							Subtotal
	CN	Negative	CIN 1	CIN 2	CIN 3	SCC	ADENO-CA	
Cytology								
Inadequate	—	10	29	9	9	2	2	61
Negative	619	62	67	5	2	—	—	755
ASC-US	—	37	109	18	5	—	—	169
LGSIL	—	36	259	50	15	—	1	361
ASC-H	—	6	6	2	3	2	1	20
HGSIL	—	10	40	75	94	9	2	230
SCC	—	—	—	—	1	15	2	18
ADENO-CA	—	—	—	—	—	1	10	11
Total	619	161	510	159	129	29	18	1625

amplification of a fragment within the highly conserved L1 region of the virus [40]; (b) NASBA assays [41] (NucliSENS EasyQ HPV v1.0) that were used for the identification of E6/E7 mRNA of the HPV types: 16, 18, 31, 33, and 45; (c) the PermiFlow (Invirion Diagnostics, LLC, Oak Brook, IL) kit for the identification of E6/E7 mRNA expression of high risk HPV using flow cytometry [6]; and (d) the immunocytochemical expression of p16 using the CINtec Cytology Kit [42]. In addition to pure medical data, epidemiologic features were involved as well, specifically woman age and parous status.

Within the clinical laboratory, it is not infrequent that an ancillary test produces invalid results or the biological material that remains in the vial is not adequate to perform additional tests; therefore, it is not guaranteed that there are available sets of such data for all women participating in the study. Additionally parous details were not available for all women as such data often were not considered important and referral forms were incomplete.

2.5. Golden Standard. Our target was to classify each woman into one of the following categories: (a) <CIN2, which included the histologically negative and CIN-1 cases as well as the CN cases, and (b) ≥CIN2, which included the histological categories: CIN-2, CIN-3, SCC, and ADENO-CA.

2.6. Data Formulation. For each case, a vector of 50 variables was created (Table 2); this had the result of the cytological examination expressed according to the Bethesda system. Results of the HPV DNA test examination were expressed as 35 individual values (either positive or negative), one for each HPV DNA genotype; additionally, in relation to the found subtypes five other variables were investigated: the existence of high risk, low risk, or any type as well as the number of high risk and the number of low risk types that were identified. For the NASBA HPV mRNA typing, we used the result for each individual HPV type (16, 18, 31, 33, and 45). The result of the PermiFlow test was involved using two methods, either as a percentage or as positive or negative (the cut-off value to assign a flow cytometry result was 1.5%); in addition, the result of the immunocytochemical expression of p16 was included. Finally, two other variables were entered to the tree construction process: woman age and parous status. For all variables, if there were no data, the value was left blank or declared inadequate indicating that there was no valid result or there was not adequate material in the ThinPrep vial to perform additional examinations.

2.7. Statistical Techniques and Modeling. The CART model was created using IBM SPSS Statistics 19 for Windows (SPSS Inc., Chicago, USA). The CART algorithm is possible to be configured and use a specific feature at the first node of the tree; however, in this study CART was allowed to select as first test the test with the highest overall accuracy. To assess the performance, various statistical measures were extracted: specificity, sensitivity, positive and negative predictive value (PPV and NPV), false positive and false negative rates (FPR and NPR), and overall accuracy (OA).

3. Results

In total 1006 histologically confirmed cases (161 without evidence of CIN or malignancy, 510 CIN-1, 159 CIN-2, 129 CIN-3, and 47 cervical cancer cases (29 squamous cell carcinomas (SCC) and 18 adenocarcinomas (ADENO-CA))) were included in this study and additionally 619 CN cases. The correlation of the cytological versus the histological outcome of our material appears in Table 1. In our material, the percentage of valid data (i.e., after excluding inadequate, invalid, and unsatisfactory results) was for the cytological examination 96.25%, for ARRAYS 91.94%, for NASBA 67.75%, for flow cytometry 81.54%, and for p16 68.68%.

For the construction of the CART model, the CHAID algorithm was used; the CART architecture was 20-5-10; that is, each parent node was forced to 20 or more vectors, and each terminal node had more than 5 vectors, and the tree depth was not allowed to grow more than 10 levels. The system was pruned to obtain simpler forms, the significance level for splitting a node was set to 0.05, the chi-square statistic was based on the likelihood ratio and the significance values were adjusted using the Bonferroni method, resplitting of merged categories within one node was allowed, the age and flow

Table 2: Variables entered to the model.

Variable name	Description	Value range
Cytology	The result of the cytological examination expressed according to the Bethesda 2009 system	WNL, ASC-US, LGSIL, ASC-H, HGSIL, SCC, ADENO CA, and <blank> if there is no result
HPV DNA arrays A6, A11, A16, A18, A26, A31, A33, A35, A39, A40, A42, A43, A44, A45, A51, A52, A53, A54, A56, A58, A59, A61, A62, A66, A68, A70, A71, A72, A73, A81, A82, A83, A84, A85, and A89	The existence of individual subtypes according to the arrays examination	0 if the specific subtype is not found 1 if the specific subtype is found <blank> if there is no result
Has HR	Positive if one or more high risk subtypes were found during typing	Positive, negative, or missing
Has LR	Positive if one or more low risk subtypes were found during typing	Positive, negative, or missing
Has any type	Positive if one or more subtypes were found during typing	Positive, negative, or missing
No HR	The number of high risk subtypes that were found during typing	An integer or missing
No LR	The number of low risk subtypes that were found during typing	An integer or missing
N16, N18, N31, N33, and N45	The result of the E6/E7 mRNA test for the specific HPV subtype	0 if negative 1 if positive <blank> if there is no result
Flow	The result of the identification of E6/E7 mRNA expression of high risk HPV using flow cytometry technique	0 if negative (<1.5%) 1 if positive (>1.5%) <blank> if there is no result
Flow %	The result of E6/E7 mRNA expression of high risk HPV using flow cytometry technique expressed as a percentage	A number or <blank> if there is no result
p16	The result of the p16 immunocytochemical examination	0 if negative 1 if positive <blank> if there is no result
Age	The woman age at the time of examination	A positive number
Parous	Woman parous status	1 if she has born one or more children and 0 if not

percentage intervals were set to 10 (i.e., flow and age values had 10 levels), and finally the maximum number of iterations was 500.

The CART architecture appears in Figure 1. It is worth noting that the role of the cytological examination result is dominant and that characteristics, such as the woman's age and the majority of HPV subtypes as were identified by the HPV subtyping test, were not found significant to be included. An example of a method of tree usage in practice is as follows.

(i) The user starts from the top node and examines the value of the proposed characteristic, in our case the cytological examination outcome.

(ii) According to the value the user navigates to the appropriate node; in our case if the cytological examination is inadequate, we examine the flow result and a negative result leads the user to a probability for the case to be benign equal to 93.9% while if there is no result or the outcome of flow cytometry is positive then the user may examine the existence on HPV subtype 16 within the mRNA test, and interestingly a negative result provides a probability for malignancy equal to 93.8% as 15 out of the 16 cases with this profile were ≥CIN2 in histology.

(iii) During navigation to the tree, the user may choose to stay in the proposed node if he/she is satisfied by the risk danger (probability) otherwise may examine the next proposed feature to find a more accurate result.

(iv) The steps are repeated down to the terminal nodes if the user is not satisfied from the proposed risk levels from the previous parent nodes.

In a second example, the user may start with a negative cytological examination result; according to the lab performance, the probability for such a case to be positive is very small (0.9%). However, if more guarantees are required for this result, an HPV DNA test may be performed, a positive outcome on HPV subtype 16 reduces the probability of a case to be less than CIN-2 from 99.1% to 66.7% (4 out

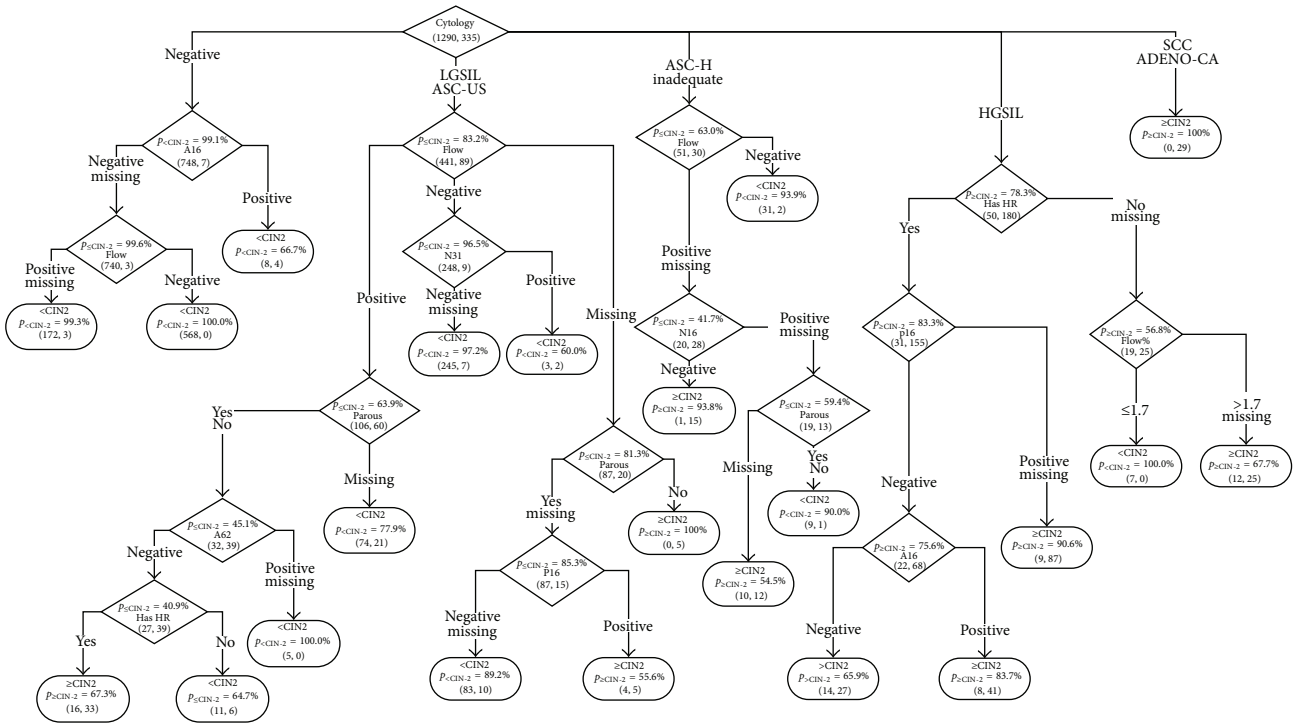

Figure 1: Structure of the CART model.

of the 12 cases with negative cytology and positive HPV DNA subtype 16 were finally found histologically CIN-2 or worst), and a negative or invalid result on test for HPV subtype 16 combined with a negative result for flow cytometry practically assures the woman that the probability to have a lesion worse than CIN-2 is negligible, as 568 cases with this profile in our material had less than CIN-2 and no case was found with equal or more than CIN-2 lesion.

A third example is related to the cases that are ASC-US or LGSIL in cytology; if the triage is based only on the cytological examination, all these women should be referred to colposcopy. However, in our material 441 cases were <CIN2 and 89 were ≥CIN2 (in total 530 women); thus, the probability to have <CIN2 is 83.2%, and if additional material is available in the vial, then a flow examination may be more helpful for the triage; in particular, a negative result in the flow examination gives a probability for <CIN2 equal to 96.5%, while a positive result indicates that this woman has more chances to harbor ≥CIN2 (46.1% Figure 1), and in such case these women could be immediately referred to colposcopy. This approach would allow reduction of referrals to colposcopy by 257, in more detail from 530 to 273; therefore, about half of the women (51.51%) could avoid immediate referral to colposcopy.

The assignment matrix of CART results appears in Table 3; actually the system classified 1216 out of the 1290 <CIN2 cases and 279 out of the 335 ≥CIN2 cases, and the statistics show that the model had sensitivity: 83.28%, specificity: 94.26%, PPV: 79.04%, NPV: 95.60%, FPR: 5.74%, FNR: 16.72%, and OA: 92.00%. In order to assess the CART performance in relation to the performance of each individual examination, the related statistics were extracted.

Table 3: CART results.

Actual category	Predicted category		Grand total
	<CIN 2	≥CIN 2	
<CIN 2	1216	74	**1290**
≥CIN 2	56	279	**335**
Grand total	**1272**	**353**	**1625**

Specifically in Table 4 are summarized the performance metrics of the CART model, of the cytological examination using the ASC-US cases as a cutoff (i.e., all cases that were ASC-US and above in cytology were considered positive and referred to colposcopy) and similarly using an ASC-H cytological outcome as cutoff. For the HPV DNA typing, three alternative methods were evaluated: a woman was considered to be at risk and referred to colposcopy if (1) any HPV type was found, (2) only if a high risk type was found, and (3) only if 16 or 18 subtypes were identified. Finally, in Table 4 are presented the performance metrics for mRNA test using NASBA or flow cytometry and p16; that is, a case was considered to be at risk if any subtype was found in the NASBA examination or if a flow cytometry result was positive or screening of a p16 slide gave a positive result.

Finally in order to allow a more detailed evaluation of the methodology, in Table 5 are summarized the results of the histological outcome (blocks of rows) along with the

Table 4: Performance of CART and individual examinations.

	CART	Cytology using cutoff ASC-US+	Cytology using cutoff ASC-H+	Arrays (positive if any type was found)	Arrays (positive if a high risk type was found)	Arrays (positive if subtype 16 or 18 was found)	NASBA (positive if any type was found)	Flow cytometry (positive if the result is positive >1.5%)	p16
Sensitivity	83.28%	97.76%	69.33%	87.71%	84.72%	52.82%	69.65%	88.19%	57.21%
Specificity	94.26%	59.79%	95.04%	70.49%	75.02%	90.28%	87.09%	79.60%	93.57%
PPV	79.04%	37.82%	77.78%	42.86%	46.11%	57.82%	62.15%	48.49%	69.68%
NPV	95.60%	99.07%	92.53%	95.79%	95.11%	88.35%	90.41%	96.87%	89.44%
FPR	5.74%	40.21%	4.96%	29.51%	24.98%	9.72%	12.91%	20.40%	6.43%
FNR	16.72%	2.24%	30.67%	12.29%	15.28%	47.18%	30.35%	11.81%	42.79%
OA	92.00%	67.39%	89.90%	73.96%	76.97%	82.73%	83.02%	81.13%	86.11%
Valid results	**1625**	**1564**	**1564**	**1494**	**1494**	**1494**	**1101**	**1325**	**1116**
% of valid results	**100.00%**	**96.25%**	**96.25%**	**91.94%**	**91.94%**	**91.94%**	**67.75%**	**81.54%**	**68.68%**

cytological categories to which the cases were assigned (rows) in combination with the CART model outcome (columns).

4. Discussion and Conclusions

Test Papanicolaou is viewed as the most successful CC test [43] if it is repeatedly applied. However, CC is not yet eliminated even in countries with well-organized cervical cancer screening programs. There are many available options for the application of biomarkers in the triage of abnormal cases [17, 44–51]; however, these are either highly sensitive or highly specific, but not both at the same time. Nowadays, there is no consensus for the optimal management of women with abnormal Pap smears and equally not infrequently women with negative cytology are found to have a high-grade lesion ≥CIN2 histologically. Women with an ASC-US result in cytology present more complex management problems. The widely accepted management options of such cases are either immediate referral to colposcopy or surveillance with repeated Pap tests. The first option can overload colposcopy clinics and may lead to overintervention and overtreatment due to subtle findings. Overtreatment commonly has negative psychological effects with increasing anxiety and may further increase the risk for long-term perinatal morbidity in subsequent pregnancies [52]. Conversely, repeat cytology with surveillance has an inherent risk of missing HGSILs, dependent on the laboratory performance, has the risk of poor compliance, and may inversely increase the psychological burden for women with cytological abnormalities that are not further assessed. It is clear that we need more accurate diagnostic tools in order to limit the number of unnecessary colposcopic referrals without compromising the detection of high-grade disease.

In our material the percentage of ≥CIN2 cases in the total of the cases given as ASC-US was 23/169 = 13.61%. Furthermore, the percentage of ≥CIN2 cases in the total of LgSIL cases was 66/361 = 18.28%. Both percentages are in agreement with those reported by other researchers [53] that range between 5–17% and 9–16%, respectively, in the published literature. On the other hand, the percentage of cases given in cytology as HGSIL and found histologically lower than CIN2 was 50/230 = 21.74%. This is also consistent with the rates published in the literature [54, 55], demonstrating an agreement across various study settings.

Exploitation of ancillary test data for improvement of cervical intraepithelial lesions is nowadays a hot research topic with important applications. Since 2010, the Hellenic Cervical Pathology (HeCPA) Study Group is working on innovative approaches that use advanced mathematical and computing tools for the exploitation of ancillary tests that are nowadays available. Up to now, preliminary results are presented in the literature [35, 56]. In our previously published study [35], we applied CART models based on a smaller dataset, using cases that had valid examination results for all the available ancillary tests. This approach had clearly the disadvantage of a reduced usable data volume and does not capture the real life situation, that is, missing values. In addition, parameters related to woman history and demographic data were not included and the probabilities for a woman to harbor CIN were not calculated for each tree part. In two other published reports [56, 57] by the same group, there were applied neural networks to solve the same problem; the disadvantage of these approaches was again the requirement to have complete data for each series and no risk estimation was performed. In this study, we exploited the CART ability to handle cases with missing data and therefore increase the power of the study. The probability for each individual part of the tree was extracted. We used additional information related to women and concluded that parous is an important factor. We also extracted knowledge from our dataset in the form of triage algorithms that not only could be useful to the decision-makers towards their requests for ancillary tests but also promote a scoring system classifying individual women as high, low, or middle risk.

According to our results, despite the multitude of features entered into the CART model (Table 2), the training algorithm identified as useful only a small number of those parameters and was finally included in the CART model (Figure 1). The major discriminating characteristic was the cytological diagnosis; in relation to typing, only the existence

Table 5: CART results in relation to the cytological outcome and the histological result.

Histology	Cytology	CART result			Grand total
		Correct	False negative	False positive	
CN	Negative	619			**619**
(CN) Total		**619**			**619**
Negative	Inadequate	10			**10**
	Negative	62			**62**
	ASC-US	36		1	**37**
	LGSIL	36			**36**
	ASC-H	3		3	**6**
	HGSIL	2		8	**10**
Negative total		**149**		**12**	**161**
CIN 1	Inadequate	25		4	**29**
	Negative	67			**67**
	ASC-US	107		2	**109**
	LGSIL	242		17	**259**
	ASC-H	2		4	**6**
	HGSIL	5		35	**40**
CIN 1 total		**448**		**62**	**510**
CIN 2	Inadequate	8	1		**9**
	Negative		5		**5**
	ASC-US	7	11		**18**
	LGSIL	26	24		**50**
	ASC-H	2			**2**
	HGSIL	75			**75**
CIN 2 total		**118**	**41**		**159**
CIN 3	Inadequate	8	1		**9**
	Negative		2		**2**
	ASC-US	2	3		**5**
	LGSIL	8	7		**15**
	ASC-H	3			**3**
	HGSIL	94			**94**
	SCC	1			**1**
CIN 3 total		**116**	**13**		**129**
SCC	Inadequate	2			**2**
	ASC-H	2			**2**
	HGSIL	9			**9**
	SCC	15			**15**
	ADENO-CA	1			**1**
SCC total		**29**			**29**
ADENO-CA	Inadequate				**2**
	LGSIL		1		**1**
	ASC-H		1		**1**
	HGSIL	2			**2**
	SCC	2			**2**
	ADENO-CA	10			**10**
ADENO-CA total		**16**	**2**		**18**
Grand total		**1495**	**56**	**74**	**1625**

of any high risk and of individual subtypes 16 and 62 was found important in our dataset and especially subtype 62 was a discriminating factor for a small number of cases. In relation to E6 and E7 expression, it was found that the flow cytometry results expressed both as positive/negative and as a percentage as well as the subtype 31 from the NASBA examination were important. Moreover, the immunocytochemical expression of p16 and parous data also appeared in the CART branches.

Based on the results, the proposed methodology had superior performance in relation to the overall accuracy (92.00%) than the majority of alternative methods (Table 4). There was marginal statistically significant difference only between CART and the cytology with ASC-H+ threshold ($\chi^2 = 4.027, P = 0.0448$). However, for all other comparisons, the differences in the overall accuracy were statistically significant, specifically CART against cytology with ASC-US+ threshold ($P < 0.0001$), arrays using any type ($P < 0.0001$), arrays using high risk subtypes ($P < 0.0001$), arrays for subtype 16 or 18 ($P < 0.0001$), NASBA for any type ($P < 0.0001$), and finally p16 ($P < 0.0001$). In relation to the comparison of the CART model and the cytological examination with threshold ASC-H+, the sensitivity of the CART model (83.28%) was significantly higher than cytology (69.33%); therefore, the proposed method had significantly ($P < 0.0001$) better performance than all other alternatives.

In relation to the false positive cases, the CART wrongly categorized 74 cases as positive (≥CIN2); from these 12 were negative and 62 CIN-1 in histology, although 50 of these cases were given as ASC-H or HGSIL in cytology (Table 5), 17 as LGSIL, 3 as ASC-US, and the remaining 4 as inadequate in cytology. No case was cytologically negative, as the cytological result is the primary characteristic that is considered as important by our methodology, and these results were expected. The FPR of the CART model was 5.74%, outperformed only by the cytological result with ASC-US+ cutoff (2.24%) but at the cost of specificity (94.26 versus 59.79%; see Table 4).

The analysis of false negative cases is more important; the CART model gave 56 false negative cases in total (14 ASC-US, 32 LGSIL, 2 inadequate, 7 negative in cytology, and 1 ASC-H; see Table 5). None of these cases had HGSIL or cancer as cytological result. The histological outcome of these cases was 41 CIN-2, 13 CIN-3, and 2 adenocarcinomas. The cytological result for the last 2 cases was ASCH and LGSIL and there was additionally colposcopical agreement.

In relation to the 61 samples that were inadequate in cytology (Table 5), 22 were ≥CIN2, among them 4 carcinomas (2 adenocarcinomas and 2 SCC), 9 CIN-2, and 9 CIN-3, and the remaining 39 cases were <CIN2; the CART model classified correctly 54 of them (35 <CIN2, 8 CIN-2, 8 CIN-3, 2 SCC, and 2 adenocarcinomas) and missed 6 cases (4 CIN-1, 1 CIN-2, and 1 CIN-3). It is worth noting that this decision was based only on biomarker data and parous status. Therefore, the number of women that would require a second cytological examination could be reduced dramatically.

Concluding the application of the proposed method gave encouraging results and not only could be helpful towards a better management of women for various findings during cytological examination but also provides a flexible technique for the estimation of ≥CIN2 risk. As a result, the proposed method provides a guide towards personalized management and therapeutic decisions, may reduce the overload of colposcopy clinics and unnecessary treatments, and identifies a higher percentage of women at risk of cervical cancer or precancerous lesions.

Conflict of Interests

All authors declare that there is no conflict of interests.

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Review Article

Cytoglobin as a Biomarker in Cancer: Potential Perspective for Diagnosis and Management

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The search for biomarkers to detect the earliest glimpse of cancer has been one of the primary objectives of cancer research initiatives. These endeavours, in spite of constant clinical challenges, are now more focused as early cancer detection provides increased opportunities for different interventions and therapies, with higher potential for improving patient survival and quality of life. With the progress of the omics technologies, proteomics and metabolomics are currently being used for identification of biomarkers. In this line, cytoglobin (Cygb), a ubiquitously found protein, has been actively reviewed for its functional role. Cytoglobin is dynamically responsive to a number of insults, namely, fibrosis, oxidative stress, and hypoxia. Recently, it has been reported that Cygb is downregulated in a number of malignancies and that an induced overexpression reduces the proliferative characteristics of cancer cells. Thus, the upregulation of cytoglobin can be indicative of a tumour suppressor ability. Nevertheless, without a comprehensive outlook of the molecular and functional role of the globin, it will be most unlikely to consider cytoglobin as a biomarker for early detection of cancer or as a therapeutic option. This review provides an overview of the proposed role of cytoglobin and explores its potential functional role as a biomarker for cancer and other diseases.

1. Introduction

Cytoglobin (Cygb) was discovered more than a decade ago in a proteomic screen of fibrotic liver by a group of researchers in Japan and was originally named STAP (Stellate Activating Protein) [1]. Since its discovery, many studies have been conducted to comprehend its functional role but the latter still remains presently poorly understood. Due to phylogenetic and structural similarities with other globins (myoglobin, haemoglobin, and neuroglobin), it was rapidly classified as a member of the globin family. This classification led researchers to suggest a putative role for Cygb as a respiratory protein similar to the other well characterised globins known to exist at the time, that is, haemoglobin (Hb) and myoglobin (Mb). Ubiquitously present in cells, Cygb appears to play a more universal role than that of Hb, Mb, and neuroglobin (Ngb, another recently identified globin), which are specifically found in red blood cells, muscle cells, and cells of the central nervous system, respectively. Interestingly, Cygb has been shown to exhibit many respiratory roles in normal cells including oxygen storage, reactive oxygen species (ROS) scavenging, terminal oxidase activity, and antifibrotic activities [2–7]. Its role in respiration has been reviewed, owing to its relationship with the globin family and also due to its upregulation in hypoxia [4, 8–10], with however no specific outcome to determine its exact role. More recently, Cygb has been reported to have some implications in cancer. In most cancer cells, Cygb expression is downregulated by hypermethylation, showing an epigenetic control [11, 12]. This downregulation in cancer cells prompts suggesting a possible

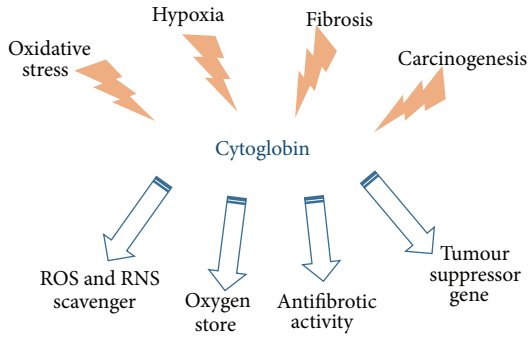

Figure 1: Potential functional roles of cytoglobin in response to insults. Such activities inspire further investigation for clinical applications.

role as a tumour suppressor gene (TSG) [13]. Conversely, in a few malignancies, Cygb is upregulated [14] where this stimulus is likely to be related to resistance to hypoxia. In line with on-going research in this field, this commentary paper is being proposed to debate the putative role of Cygb and to provide a perspective on potential research areas that may point out its role as a cancer biomarker (Figure 1).

2. Respiratory Functions of Cygb

Cygb is a globin protein, expressed in various tissues including liver, heart, stomach, lungs, spleen, and muscles, where its physiological function remains to be defined [1, 8, 13]. It is a 190-amino acid hexacoordinated hemeprotein of the globin family with a molecular weight of 20.9 kDa [10, 15]. Being quite similar to Mb in orientation, span, and primary structure, it has a distinct intramolecular disulphide bond and heme-coordination [4, 16–18]. Molecular phylogenetic studies point towards an ancient origin and highly conserved biological function for cytoglobin, supported by its slower amino acid mutation rate, compared to the other globins [19]. In the light of current available reports and also due to its part similarity to other globins (Mb and Ngb), several possible cellular functions of Cygb have been considered in line with respiratory activities. These include oxygen storage, terminal oxidase activity, and reactive oxygen species (ROS) scavenging [4, 20, 21]. Though the enzymatic activity of cytoglobin has been suggested, this remains controversial. There is limited evidence of catalase, peroxidase, and superoxide dismutase (SOD) activity due to very low reported quantitative levels [22].

The oxygen carrier and storage function of Cygb was suggested based on structural similarities with myoglobin and stimulation of its expression under hypoxic conditions [8, 15, 18]. It is hypothesized that Cygb acts as an oxygen source because of its high affinity for oxygen (about 1 Torr) and also due to its pH-dependent oxygen binding ability similar to Mb, though unlike Ngb [3, 23–25]. The cooperative binding of oxygen to Cygb-heme supports the ease of its loading and unloading over a narrow range of oxygen pressures/tension compared to noncooperative binding as in Ngb and Mb. However, the heme-heme interaction for cooperative binding remains still to be described to illustrate clearly its function in respiration [19, 24]. Nevertheless, the fact that Cygb is hexacoordinated unlike Mb and Hb strongly argues against a simple oxygen binding function. In the absence of exogenous ligands, Cygb takes up its original structure by binding Fe to the endogenous ligand, HisE7. Upon reduction of the hemeprotein, the affinity for exogenous ligands such as O_2, NO, and CO decreases. This makes binding of a forthcoming ligand even more difficult [16]. Thus the oxygen storage ability of Cygb is debatable. Additionally, its cellular localisation and level of cellular expression do not support specific reaction within mitochondria in a way analogous to Mb [26]. Consequently, it can be perceived that cytoglobin may probably act in a different manner to sustain respiratory activities in cells.

Indeed, it has been shown that Cygb's overexpression in anoxic and hypoxic conditions sustains survival of cells and its low expression in similar conditions led to increasing apoptosis of control cells [27–30]. Its gene contains a long CpG island upstream, with several transcription sites including hypoxia responsive elements (HRE) and hypoxia-inducible protein binding sites among others (HIF1, AP1, AP2, and SP1), thereby providing the basis of cytoglobin regulation in hypoxia [13, 31]. Regulation by HIF1α (hypoxia-inducible factor 1 α) shows significant increase in its expression [28, 32]. In the same line, it has been suggested that Cygb donates oxygen for hydroxylation of HIF1α to enhance its destabilisation in ischemic conditions. Thus the cell could bypass the sensing of hypoxia and apoptosis, ultimately leading to cell survival [19].

Furthermore, antiapoptotic pathway in ischemic conditions also involves cytochrome c (cyt c). In a study by Fago et al., Ngb was shown to be able to reduce the iron centre of cyt c preventing association with and activation of caspase 9 and could therefore be considered an "antiapoptotic" factor. Although at the moment there is no evidence that Cygb could function in an analogous manner this cannot be excluded and warrants further investigation. This hypothesis is further supported by similarities between the two proteins, being both hexacoordinated and having similar oxygen affinities [20]. A potential reaction of Cygb with mitochondria cyt c, as reported for Ngb [20], could also account for a respiratory or antiapoptotic activity. This further illustrates its respiratory functions in hypoxic conditions, without being directly involved with oxygen chemistry.

3. Cygb in Oxidative Stress

Together with upregulation of Cygb in hypoxia, Cygb is also overexpressed under conditions of oxidative stress (OS) [32, 33]. A significant increase in Cygb occurred in neuroblastoma cells when the latter were subjected to hydrogen peroxide (H_2O_2) [32]. During similar ROS insults, fibrotic cells such as hepatic stellate cells (HSCs) also upregulated their Cygb expression to neutralize the free radicals [34, 35]. In the same line, cytoglobin has hexacoordinated structure, similar to Ngb, and cyt c hemeproteins, which provide redox reaction abilities through their hemes [20]. These reports emphasize

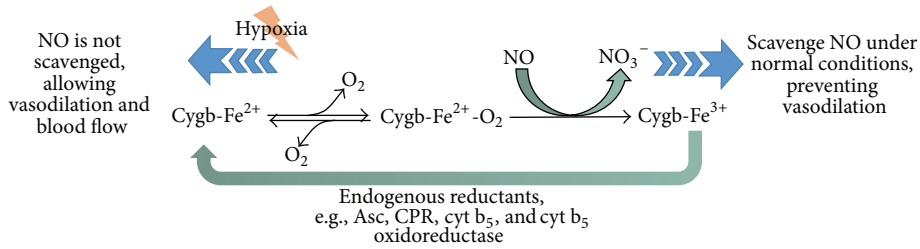

Figure 2: Illustrative diagram showing the regulation of NO level by cytoglobin.

the protective role of Cygb, more particularly its radical scavenging function, as evidenced by its ability to detoxify radicals via reactions with its heme group [36]. Cygb also shows lipid peroxidase activity through its heme group. Since products of lipid peroxidation are known to modulate cell signalling, the low levels of Cygb in cells might be liable to induce protective actions by means of the peroxidase activities in oxidative stress conditions [37]. However, the role of cytoglobin in OS is still to be discovered as the mechanisms of upregulation and protective role are still debatable. Recently, Verschoor and Singh have shown that an overexpression of Ets-1 has decreased ROS insults in cells. While an Ets-1 binding site is present at the promoter region of Cygb, no link was pursued in line with upregulation of Cygb [31, 38]. It can be anticipated that increase in Ets-1 has upregulated Cygb level in the cells which has consequently decreased the ROS levels, providing an inspiring target of research towards the signalling pathway for Cygb expression.

Cygb has been shown to be protective not only against ROS but also against RNS (Reactive Nitrogen Species) such as nitric oxide (NO), an endogenously present molecule known to cause cellular and DNA damage [2, 39]. The rate of metabolism of NO is O_2-dependent in both vascular and intervascular tissues, where Mb is deficient [40, 41]. Interestingly, Cygb has been reported to have a NO dioxygenase (NOD) activity similar to Mb and Ngb [2]. Though the level of Cygb in these cells is low (micromolar) [17, 19], it is noteworthy to reflect upon how Cygb could act as a NOD at this low level in dramatic inductions of NO in cells. This could be plausibly explained by considering that Cygb does not actually scavenge NO but regulates its levels in hypoxic conditions. Liu and colleagues [40] have reported that the rate of NO metabolism increases significantly in high O_2 level and decreases in low O_2 concentration. NO is a known endogenous molecule which has an important regulatory role in the dilation of blood vessels [42]. Therefore, Cygb can be suggested to indirectly regulate vascular tone in tissues under hypoxia by increasing the diameter of blood vessels, thereby alleviating the oxygen deficiency stress (Figure 2). This proposed mechanism can be depicted as follows: Cygb binds to oxygen at high PO_2 and donates its oxygen to scavenge NO to NO_3^-, forming met-Cygb (Fe^{3+}) [40]. In turn, the met-Cygb may be reduced by many endogenous reducing agents such as ascorbic acid, cytochrome P450 reductase (CPR), cytochrome b_5, and cytochrome b_5 oxidoreductase [40, 43]. The reduction of Cygb enables the recycling of the hemeprotein, powering an efficient regulation of NO even at a low expression level. Congruently, further analyses reveal that cellular reductants greatly increase the rate of NO metabolism in the presence of oxygen [40]. Following these reports, Cygb seems therefore to be an eligible candidate in regulating vascular tone in hypoxia. The underlying molecular mechanism of O_2-dependent NO regulation by Cygb and its intermediates (cygb-Fe^{3+}) remains however still uncertain and provides a potential perspective for future research.

4. Role in Carcinogenesis

Advances in the domain of molecular research have attributed another interesting function to Cygb: its ability to suppress tumour growth. Changes in Cygb expression have been shown to occur in many malignancies. Investigations on Cygb's tumour suppressing activity reported in 2006 have shown that most cancer cells have a reduced expression of Cygb, with a dramatic decrease (70%) of cytoglobin expression reported in tylosis with oesophageal cancer (TOC) [12, 44, 45]. Furthermore, studies have reported reduction in tumor growth with an overexpression of Cygb by the transfection of cytoglobin cDNA in non-small lung cancer cells and breast cancer cells [12]. In another study, knockdown of Cygb in glioma cells showed an increase in growth rate of the cells [46]. These reports strongly suggest a tumour suppressor activity of Cygb. Findings also show that NO and peroxynitrite ($ONOO^-$) produced during inflammatory responses can affect tumour suppressor proteins such as p53, mitogen-activated protein kinase pathways in dose-dependent manner to promote damage to DNA, and genetic library and cancer phenotypes of cells [39, 47]. Since in inflammation there is a global hypermethylation mostly in tumor suppressor genes [48], Cygb expression might also be affected in a similar manner. Furthermore, it has also been reported that Cygb's protective function against ROS was also extended to prevent cell death and ROS induced genetic damage in cells [49].

In the same line, loss of expression of cytoglobin in cancer cells occurs due to loss of heterozygosity (LOH) and also by epigenetic regulation via hypermethylation of CpG islands in the Cygb gene promoter region [44, 45, 50, 51]. In addition, differences in hypermethylation levels were observed in lung adenocarcinoma and lung squamous cell carcinoma [50] with however an unclear relationship between the quantitative

level of hypermethylation and stages of carcinogenesis [12]. The identification of a marker that assesses the possibility for cells to become cancerous and for cancerous cells to further develop remains of vital importance in cancer research. This link between the quantitative determinations of hypermethylation levels of Cygb promoter gene in different cancer cells could draw attention to the role of Cygb as a biomarker for carcinogenesis notably in determining the cancer level and the likelihood of cells to further developing phenotypes of cancer.

Also, studies conducted on Cygb-deficient mice have been shown to bridge the link between the role of the ROS scavenging and tumour repressing activities of Cygb, whereby susceptibility to tumorigenesis was increased in Cygb-deficient mice on treatment with DEN (*N*,*N*-diethylnitrosamine) [52]. Altogether, Cygb might exhibit both TSG and oncogene features. Contradicting most results, a small subset of malignant samples showed a high mRNA expression of Cygb [14], which is thought to arise from a different mechanistic pathway. Further investigations in this arena will certainly bring to light the eventual functional role of Cygb in cancer. In the same vein, the promising characteristics of Cygb may provide the basis for a prospective role in clinical treatments.

5. Role in Fibrosis

Cytoglobin, first named STAP (stellate cell activation-associated protein), was initially located in HSCs (hepatic stellate cells) during their activation upon liver fibrosis. The level of expression of Cygb was therein found to be elevated. In a functional point of view, expression of Cygb was suggested to support HSC in liver traumas where they are increasingly exposed to endogenous ROS. In that way, it was presumed to act as a potential ROS scavenger counteracting the increasing ROS insults [34]. Further evidences have led to suggesting a potential role of Cygb as an oxygen donor in the synthesis of collagen. Schmidt and colleagues have previously speculated the role of Cygb as an oxygen donor for the enzyme prolyl-4-hydroxylase in collagen synthesis, suggesting role of Cygb as a profibrotic globin [15]. In further support, Man et al. published reports with a view to elucidating the role of Cygb in collagen metabolism. In the study, liver tissues were subjected to hepatotoxin, CCl_4. 24 hrs after the exposure, Cygb was upregulated, followed by the upregulation of procollagen-α-1 expression 48 hrs later [35]. From these data, it is most probable that Cygb upregulation is associated with fibrosis, but the role of Cygb as profibrotic or antifibrotic globin remains still ambiguous.

In contrast to the above, accruing evidences point towards antifibrotic roles for Cygb. It was observed that Cygb upregulation in an induced fibrotic damaged kidney led to an improvement in the physiology of the organ [6]. Similar results were reported where transfection of Cygb gene in a CCl_4-induced fibrotic rat liver had resulted in physiologic remodelling and decreased fibrosis of the liver tissue. Cygb overexpression in these cells inhibited the anticipated upregulation of several fibrosis-associated components such as, procollagen-1, TGF-β1, TIMP-1 transcripts and α-SMA and TGF-β1 proteins [7]. Although the specific mechanism of inhibition was not elucidated, it was reported that antifibrotic activity originated from the heme group within Cygb [6]. There is evidently an association between Cygb and fibrosis, but the mechanism is largely unexplored. Elucidation of this association may potentially lead to development of Cygb-based therapies for fibrosis.

6. Future Perspectives

So finally, after more than a decade from the discovery of cytoglobin, where are we? Many studies in diverse arenas have been conducted to clarify the role of cytoglobin. Many putative functions have also been proposed. Along most of these suggested functions are contradicting comment and reports. Neither the oxygen storage ability nor the ROS/RNS scavenging function and not even the tumour suppressor activity of cytoglobin has been strongly accepted. This challenge is still on, as Cygb instigates increasing interest and curiosity as evidenced by very recent publications. As such, Chakraborty et al. are proposing a potential link between the proposed activities of Cygb: ROS scavenger, antifibrotic and anticancer abilities [53]. In the quest of unanswered questions, the antifibrotic activity of Cygb needs to be further exploited in line with preliminary data advocating its anticancer therapy. While, in tumor development and propagation, several biological and cellular changes occur, differential levels of hypermethylation of the CpG island of the Cygb promoter region have been reported. Detailed understanding of the level of hypermethylation at different stages of tumor development is of clinical significance to identify Cygb as a novel biomarker of cancer. Another point that requires elucidation is whether Cygb has a tumor suppressor or an oncogenic ability. Further molecular research is of notable importance to identify trigger switches responsible for the transition of Cygb's function in different biological insults (fibrosis, hypoxia, oxidative stress, and cancer). Recently, Cui et al. have reported a first compound, arundic acid, identified to upregulate Cygb. Similar investigations are needed to expand the molecular pathway of modulating the endogenous levels of Cygb [54]. These future investigations are strongly warranted with a view to strongly identifying Cygb as a potential biomarker, prognosis, and potential therapy for medical use.

Conflict of Interests

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

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Research Article

Prognostic Significance of Serum Inflammatory Response Markers in Newly Diagnosed Non-Small Cell Lung Cancer before Chemoirradiation

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Purpose. To identify whether the serum's baseline C-reactive protein (CRP) and albumin (Alb) levels related to clinicopathological parameters and overall survival (OS) in non-small cell lung cancer (NSCLC). *Methods*. In total, 100 consecutive patients (mean age = 68.38 ± 10.85 years) that underwent chemoradiotherapy were studied. Measurements of CRP and Alb were performed before any treatment. *Results*. Serum CRP levels were significantly associated with histological grade ($P < 0.001$), TNM stage ($P < 0.001$), PS ($P = 0.009$), and Alb ($P < 0.001$). Additionally CRP and Alb levels were found significantly associated with overall survival in univariate analysis (log-rank test, $P < 0.001$ and $P = 0.002$, resp.) and CRP remained significant after controlling for age, alcohol, performance status, and TNM stage, whereas albumin showed a borderline effect on the hazard rate ($P = 0.052$). *Conclusions*. CRP and Alb are both promising biomarkers in identification of NSCLC patients with poor prognosis and form a possible target for intensifying their therapies.

1. Introduction

Systemic inflammation increases cell proliferation because it promotes neoplastic risk [1]. Genetic events, as an intrinsic pathway, and inflammatory condition as an extrinsic pathway can predispose to neoplasia [2]. Cancer-related chronic inflammation affects DNA damage, continuous replication, sustained angiogenesis, apoptosis evasion, self-sufficiency in growth signaling, insensitivity to antigrowth signaling, and tissue invasion/metastasis [3].

Many different tumor-associated factors have been described and investigated for lung cancer. The identification of markers whose altered expression is correlated with OS differences might enclose the knowledge to distinguish those which could serve as indicators of the tumor's biological behavior. CRP and Alb are acute phase proteins and their

concentrations are related to the presence of an inflammation or neoplasm. CRP offers a reliable clinical information on the active inflammatory status due to its rapid variability [4, 5]. Alb serves as a splanchnic function indicator protein that in case of inflammation or hypothrepsia its synthesis is suppressed. Because of a tumor presence, a systemic inflammatory reaction is created and cytokines that induce acceleration of catabolism are released. Particularly interleukin-6 (IL-6) and interleukin-Ib (IL-Ib) decrease Kupffer cells' Alb production [4].

In this study, we employed nephelometric and photometric methods to evaluate serum CRP and Alb levels in NSCLC patients before chemoirradiation. Furthermore, we analyzed the correlation between CRP and Alb and variable clinicopathological features and patient prognosis.

2. Materials and Methods

All participating patients signed the informed consent. The inclusion criteria in the study were (a) PS according to the Zubrod Scale: 0–2, (b) newly diagnosed NSCLC (according to the TNM system), (c) no prior history of chemotherapy or RT, and (d) absence of acute inflammation signs.

A three-dimensional conformal radiotherapy (3DCRT) technique was used. The target volumes were defined according to ICRU Reports 50, 62 [6]. The organs at risk (OARs) and the dose constraints were determined by ICRU Report 62 and QUANTEC [6, 7]. A biologically equivalent dose equal to 60 Gy was delivered, in daily photon radiation fractions from Monday to Friday. Chemotherapy was administered according to the current NCCN (National Comprehensive Cancer Network) guidelines criteria [8, 9].

A peripheral blood sample was collected and centrifuged before starting any therapy. Serum CRP levels were measured using nephelometric method (Beckman Coulter, Image Immunochemistry System, USA), while serum Alb levels were determined using photometric method. Continuous data were presented as mean ± standard deviation, whereas categorical data were presented as absolute and relative frequency. Kolmogorov-Smirnov test evaluated the assumption of normality. To assess the differences of study parameters according to the levels of Alb and CRP, standard statistical procedures were used, as appropriate (Student's t-test for continuous data, Chi-square test for categorical data, and Fisher's Exact test for categorical data with limited number of frequencies). Survival curves were generated by Kaplan-Meier analysis and tested for significance using the Mantel-Cox log-rank test. Further on, Cox proportional regression analysis was used to identify potential independent prognostic marker. The SPSS statistical package (Version 20.0, IBM Corp.) was used to analyze the data. Significance level was set at $P = 0.05$ and Bonferroni-Holm correction was applied to compare differences between groups.

3. Results

The study sample consisted of 100 participants. The mean age of the total sample was estimated at 68.38 ± 10.85 years, ranging between 36 and 92 years. The cut point for albumin (median: 3.5, IQR: 0.775) and CRP (median: 23.1, IRQ: 48.93), range 25%–75% levels was according to the median value (3.5 and 23.1, resp.). Table 1 presents the distribution of the study variables according to the levels of Alb and CRP. There was no evidence for a possible association between Alb and presence of NEC ($P = 0.108$) and FIB ($P = 0.149$), smoking status ($P = 0.439$), ETOH consumption ($P = 0.275$), presence of INF ($P = 0.894$) and LVI ($P = 0.894$), performance status ($P = 0.036$), and TNM stage ($P = 0.012$) after P value adjustment with Bonferroni-Holm's correction. On the other hand, we found evidence that albumin levels are associated with patients age ($P = 0.002$). We also find evidence that CRP is strongly associated with histological grade ($P < 0.001$), TNM stage ($P < 0.001$), and OS ($P < 0.001$). The comparison of survival with Kaplan-Meier survival analysis between low Alb levels (blue line, $n = 52$) and high Alb levels (green line, $n = 48$) showed a statistically significant better prognosis for high levels of albumin ($P = 0.002$) (Figure 1). The median time for patients with lower albumin levels was 9.167 ± 0.821 (95% CI: 7.557–10.776) versus 13.267 ± 0.759 (95% CI: 11.779–14.754) for the patients with higher levels of albumin. Table 1 shows the distribution of 100 participants according to the Alb and CRP levels. Table 2 shows the results from the univariate Cox regression analysis examining the relationship between overall survival and Alb.

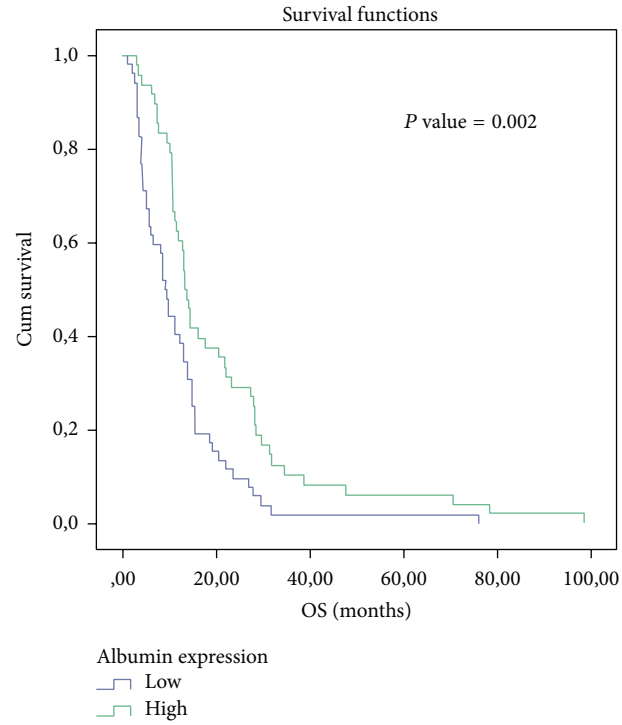

Figure 1: Kaplan-Meier survival analysis of Alb.

The comparison of survival with Kaplan-Meier survival analysis between low CRP levels (blue line, $n = 50$) and high CRP levels (green line, $n = 50$) demonstrated a statistically significant better prognosis for low CRP levels ($P < 0.001$) (Figure 2). The median time for patients with lower CRP

Table 1: Distribution of 100 participants according to the expression of Alb and CRP.

Variables	Alb expression		P value	CRP expression		P value
	Low expression ($N = 52$)	High expression ($N = 48$)		Low expression ($N = 50$)	High expression ($N = 50$)	
Age, years	71.58 ± 10.11	64.92 ± 10.66	**0.002**†	66.34 ± 9.52	70.42 ± 11.77	0.060†
PS			0.036††			0.009†††
0-1	38 (73.1)	43 (89.6)		46 (92.0)	35 (70.0)	
2–4	14 (26.9)	5 (10.4)		4 (8.0)	15 (30.0)	
Smoking			0.439†††			0.99†††
No	5 (9.6)	2 (4.2)		3 (6.0)	4 (8.0)	
Smokers	47 (90.4)	46 (95.8)		47 (94.0)	46 (92.0)	
ETOH			0.275††			0.99††
Social	24 (46.2)	17 (35.4)		20 (40.0)	21 (42.0)	
Heavy	28 (53.8)	31 (64.6)		30 (60.0)	29 (58.0)	
INF			0.894††			0.99††
No	47 (90.4)	43 (89.6)		45 (90.0)	45 (90.0)	
Yes	5 (9.6)	5 (10.4)		5 (10.0)	5 (10.0)	
LVI			0.894††			0.009†††
No	47 (90.4)	43 (89.6)		46 (92.0)	35 (70.0)	
Yes	5 (9.6)	5 (10.4)		4 (8.0)	15 (30.0)	
NEC			0.108††			0.68††
No	42 (80.8)	32 (66.7)		33 (66.0)	41 (82.0)	
Yes	10 (19.2)	5 (10.4)		17 (34.0)	9 (18.0)	
FIB			0.149†††			0.059†††
No	42 (80.8)	32 (66.7)		43 (86.0)	49 (98.0)	
Yes	10 (19.2)	5 (10.4)		7 (14.0)	1 (2.0)	
TNM stage			0.012††			<0.001††
I and II	8 (15.4)	18 (37.5)		21 (42.0)	5 (10.0)	
III and IV	44 (84.6)	30 (62.5)		29 (58.0)	45 (90.0)	
Histological grade			0.005††			<0.001††
I and II	20 (38.5)	32 (66.7)		39 (78.0)	13 (26.0)	
III and IV	32 (64.5)	16 (33.3)		11 (22.0)	37 (74.0)	
OS	12.09 ± 11.83	20.90 ± 19.04	0.007†	22.92 ± 9.73	19.85 ± 7.07	<0.001†

PS = performance status, ETH = alcohol, INF = inflammation, LVI = lymphovascular invasion, NEC = necrosis, FIB = fibrosis, and OS = overall survival. Data are presented as N (%) or mean ± standard deviation. Bonferroni-Holm correction was applied to compare differences between groups. $^{\dagger}P$ value derived from Student's t-test, $^{\dagger\dagger}P$ value derived from Chi-square test, and $^{\dagger\dagger\dagger}P$ value derived from Fisher's Exact test.

Table 2: Univariate Cox regression analysis examining the relationship between overall survival and Alb.

Variable	B	SE(B)	P value	Exp(B)
Alb (high/low expression)	−0.621	0.207	**0.003**	0.537

Table 3: Univariate Cox regression analysis examining the relationship between overall survival and CRP.

Variable	B	SE(B)	P value	Exp(B)
CRP (high/low expression)	1.055	0.218	<**0.001**	2.873

levels was 14.167 ± 2.220 (95% CI: 9.816–18.517) versus 8.133 ± 1.827 (95% CI: 4.553–11.714) for the patients with higher CRP levels. Table 3 presents the univariate Cox regression analysis examining the relationship between overall survival and CRP.

Table 4 shows a univariate analysis for all parameters. A multiple Cox regression analysis examined the relationship between overall survival and Alb, after adjustment for demographic, clinical, and histological parameters in the total sample of 100 participants. According to the findings, Alb seems to have a borderline effect on the hazard rate ($P = 0.052$). Other parameters that were found to be highly associated with the hazard rate were heavy drinkers with a hazard ratio of 1.767 versus nonsocial drinkers ($P = 0.017$), TNM stage with a hazard ratio of 2.506 ($P = 0.001$), and performance status with a hazard ratio 2.602 ($P = 0.001$).

Table 5 demonstrates a multiple Cox regression analysis examining the relationship between overall survival and CRP, after adjustment for demographic, clinical, and histological parameters in the total sample of 100 participants. According to the findings, CRP seems to have a significant effect on the hazard rate ($P = 0.002$). Other parameters that were found to

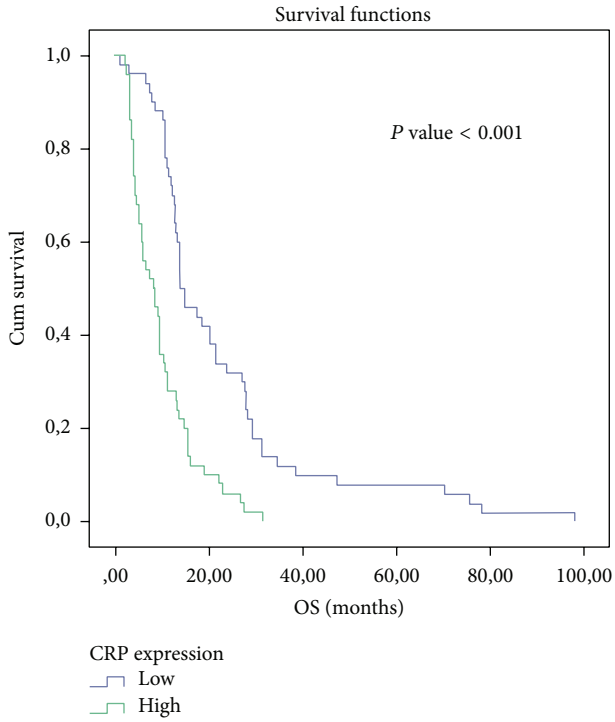

Figure 2: Kaplan-Meier survival analysis of CRP.

Table 4: Multiple Cox regression analysis examining the relationship between overall survival and Alb, after adjustment for demographic, clinical, and histological parameters in the total sample of 100 participants.

Variables	B	SE(B)	P value	Exp(B)
Alb (high/low expression)	−0.436	0.224	**0.052**	0.647
Age > 65 (yes/no)	0.386	0.228	0.091	1.471
ETH (heavy/social)	0.569	0.238	**0.017**	1.767
PS (0-1/2–4)	0.956	0.289	**0.001**	2.602
TNM stage (III and IV/I and II)	0.919	0.275	**0.001**	2.506

ETH = alcohol; PS = performance status.

Table 5: Multiple Cox regression analysis examining the relationship between overall survival and CRP, after adjustment for demographic, clinical, and histological parameters in the total sample of 100 participants.

Variables	B	SE(B)	P value	Exp(B)
CRP (high/low expression)	0.722	0.233	**0.002**	2.059
Age > 65 (yes/no)	0.354	0.230	0.123	1.425
ETH (heavy/social)	0.525	0.232	**0.024**	1.073
PS (0-1/2–4)	0.878	0.288	**0.002**	2.407
TNM stage (III and IV/I and II)	0.806	0.282	**0.004**	2.238

ETH = alcohol; PS = performance status.

be highly correlated with the hazard rate were heavy drinkers with a hazard ratio of 1.690 versus social drinkers ($P = 0.024$), TNM stage with a hazard ratio of 2.238 ($P = 0.004$), and performance status with a hazard ratio of 2.407 ($P = 0.002$).

4. Discussion

In the present study, we found a correlation between CRP and Alb with survival in NSCLC. CRP and Alb are easily obtainable biomarkers associated with the lung parenchyma lesion, caused by the tumor presence. The lower CRP baseline and the elevated Alb baseline values were correlated with better outcome in terms of OS (log-rank test $P < 0.001$ and $P = 0.002$, resp.). The combination of the increased CRP values and hypoalbuminemia may be due to one of the following: (a) patients' malnutrition (hypothrepsia) or (b) reactive response (tissue stress) due to the existence of cancer cells that activate the production of acute phase proteins [10]. McMillan et al. demonstrated that CRP may be a significant independent predictor of OS in advanced cancer patients ($P = 0.0002$) [11]. Siemes et al. [12] found that baseline CRP levels seemed to be a biomarker of chronic inflammation preceding lung cancer, even after subtracting a 5-year latent period (HR = 2.8; 95% CI = 1.6–4.9). Allin and Nordestgaard [13] demonstrated that individuals with CRP levels in the highest versus the lowest quintile had a 2-fold increased risk of lung cancer. Among individuals diagnosed with cancer, patients with a high baseline CRP (>3 mg/L) had an 80% greater risk of early death versus those with low CRP levels (<1 mg/L). Roxburgh and McMillan [14] showed that, in primary operable cancer, preoperative estimation of the systemic inflammatory response such as elevated CRP, hypoalbuminemia or increased white cell, neutrophil, and platelet counts predicted cancer OS regardless of the tumor stage. O'Dowd et al. [15] indicated that preoperative CRP more than 34 mg/L (HR = 1.65, 95% CI = 1.12–3.87, $P = 0.045$) retained independent significance of poor outcome in ninety-six lung cancer patients. CRP levels >10 mg/L had a median OS of 26.2 months versus 75.9 months of those patients with a CRP < or = 10 mg/L ($P < 0.05$). In our previous study [16] we found that CRP, Ferritin, and Alb were correlated with the acute complication of lung parenchyma radiation induced toxicity. CRP and Ferritin were elevated in the immediate postradiotherapy interval (after 2 months) compared to the preradiotherapy values ($P < 0.001$). The Alb levels were found to be lower ($P < 0.001$). Pine et al. [17] showed that the 10-year standardized absolute risk of lung cancer was the highest among current smokers with high IL-8 and CRP levels (absolute risk = 8.01%, 95% CI = 5.77% to 11.05%). Xu et al. [18] found that higher levels of CRP were associated with increasing lung cancer risk (OR = 2.11, 95% CI = 1.66–2.91, $P < 0.01$), suggesting that CRP could be used as surrogate biomarker of angiogenesis and prognosis in lung cancer. We observed a correlation between CRP and PS ($P = 0.009$), LVSI ($P = 0.009$), TNM stage ($P < 0.001$), and OS ($P < 0.001$). Accordingly Tulek et al. [19] also found that CRP levels were significantly elevated ($P = 0.001$) in NSCLC patients with poor PS. Higgins et al. [20] associated LVSI

with an increased risk of harboring regional lymphonodal involvement ($P < 0.001$). LVSI was also an adverse prognostic factor for the development of distant metastases ($P = 0.006$) and long-term survival ($P = 0.003$) in adenocarcinomas. The analysis of our data indicated significantly worse OS for lung cancer patients with hypoalbuminemia from survival analysis: log-rank test ($P = 0.002$), univariate Cox regression ($P = 0.003$), and multiple Cox regression (borderline $P = 0.052$). In multivariate analysis lower levels of Alb were linked with stage of disease ($P = 0.012$), the elderly ($P = 0.002$), and performance status ($P = 0.036$). Jin et al. [21] had already identified preoperative and postoperative hypoalbuminemia (<3.5 g/dL) as independent negative prognostic factors for recurrence ($P = 0.008$ and $P = 0.001$, resp.).

5. Conclusion

The present study provides evidence that higher pretreatment CRP and lower Alb serum levels are potential prognostic factors of OS. Our data could be useful to improve risk stratification and to develop better tailored treatment strategies in NSCLC patients.

Conflict of Interests

All authors declare that they have no conflict of interests.

Authors' Contribution

Maria Tolia and Nikolaos Tsoukalas contributed equally to this work.

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Review Article

Potential Utility of Novel Biomarkers in Active Surveillance of Low-Risk Prostate Cancer

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Active surveillance (AS) is now an accepted management strategy for men with low-risk localized prostate cancer (PCa). However, detecting disease progression in a patient selected for AS remains a challenge. It is crucial to know what will serve as the best parameter to correctly identify tumors that progress to a more aggressive phenotype so as not to miss the window of curability. Several biomarkers are now being actively investigated as novel tools to improve PCa risk assessments. To date, several serum, urinary, and tissue biomarkers have shown promising prognostic value. %[−2]proPSA and PHI showed improved predictive value for an unfavorable biopsy conversion at annual surveillance biopsy in the AS program. PCA3 and TMPRSS2:ERG had additional independent predictive value for the prediction of PCa detection and progression, although PCA3 was limited in predicting aggressive cancer. Other tissue biomarkers also showed promising ability to predict disease progression. Although several of these novel biomarkers have an improved predictive accuracy that is better than classical parameters, there is still a need for further well-designed, large, multicenter, prospective trials to avoid common bias and clinical validation.

1. Introduction

Active surveillance (AS) is now an accepted management strategy for men with low-risk localized prostate cancer (PCa), as the majority of men with such cancers are unlikely to die of PCa [1–3]. Nevertheless, as low-risk cancer does not mean complete absence of risk, the large majority of men with low-risk, early-stage disease undergo aggressive intervention with radical prostatectomy (RP) and/or radiation therapy (RT), despite their attendant long-term side effects and cost [4–6]. Detecting disease progression in a patient selected for AS remains a continuing challenge. It is a crucial issue to determine what will serve as the best parameter to correctly identify tumors that progress to a more aggressive phenotype in order not to miss the window of curability. Approximately one-third of the patients will be reclassified as a higher risk for progression and will be offered treatment during AS [7–10]. In most cases that are reclassified as higher risk, the reclassification is due to upgrading at the time of a repeat biopsy [7–10]. This upgrading is largely not time dependent, suggesting that it is due to more accurate sampling rather than true biologic progression [11]. Some patients with apparently low-risk disease actually harbor unfavorable disease due to inaccuracies in the currently used repeat biopsy protocols [8]. Nevertheless, current AS criteria may be too strict, thereby excluding some patients in whom expectant management would be appropriate and safe [12].

Currently, serial PSA measurements, digital rectal examination (DRE), and repeat prostate biopsies are being used for risk stratification of men with early-stage PCa in most AS cohorts. Although these tools have some predictive value, a substantial fraction of men that are expected to have low-risk disease are found to have more aggressive disease at prostatectomy. The role of PSA and PSA kinetics still remains contentious. The AS program at Johns Hopkins does not use PSA changes as a trigger for curative intervention [10]. Although, in the Toronto series, a PSA DT < 2 years has been used to prompt treatment, this group currently does not use PSA kinetics alone as a trigger for treatment, but rather to trigger either rebiopsy or multiparametric MRI

(MP-MRI) [13]. Furthermore, although morbidity is low [14], the discomfort, cost, and continued undersampling problem inherent in the prostate biopsy procedure advocate for the development of noninvasive tools capable of predicting disease progression more accurately and suitable for repeat measurements over time.

Accordingly, there is an unmet need for a noninvasive biomarker test that can provide a higher degree of specificity for detecting aggressive disease than the currently available clinical tools. Several biomarkers are now actively investigated as novel tools to improve patient selection and monitoring on AS for low-risk PCa.

2. Materials and Methods

2.1. Evidence Acquisition. We conducted a systematic review by the search of the PubMed database according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis statement guidelines (http://www.prisma-statement.org). Predefined search terms were used to identify articles published before August 5, 2014, for combinations of the following free search terms: "Biomarkers" and "Active Surveillance" or "Watchful Waiting" and "Risk Assessment" and "Prostatic Neoplasms."

2.2. Search Results. The literature search identified 39 original articles that were included for review: 12 on %[−2]proPSA and Prostate Health Index (PHI), 10 on PCA3, 7 on TMPRSS2:ERG, 2 on genomic prostate score (GPS), 5 on the panel of four kallikrein markers, and 3 on cell cycle progression (CCP) score.

3. PSA Isoform and Its Derivatives

Free PSA (fPSA) includes the subforms benign prostatic hyperplasia-associated PSA (BPSA), inactive PSA (iPSA), and proPSA [15–17]. BPSA and iPSA are associated with benign tissue, but proPSA is associated with cancer [15–17]. It is possible to detect three truncated forms of proPSA in serum, [−2], [−4], and [−5, −7], with [−2]proPSA being the most stable form. A [−2]proPSA assay showed the clinically acceptable analytical performance with excellent precision and reproducibility and had negligible interference with other PSA isoforms [18]. Development of the [−2]proPSA immunoassay by Beckman Coulter opens a new field of study for detecting PCa.

Several studies [19–25] have suggested that, in men with a total PSA (tPSA) between 2.5 and 10 ng/mL, %[−2]proPSA (the percentage of [−2]proPSA to fPSA) and Prostate Health Index (PHI; $([-2]\text{proPSA}/\text{fPSA}) \times (\text{tPSA})^{1/2}$) provide significantly better clinical performance for predicting PCa than total PSA or %fPSA and may be related to PCa aggressiveness, with higher levels of these tests being in patients with Gleason score ≥ 7 (Table 1).

In an observational, prospective, multicenter European study ($n = 646$) [22], [−2]proPSA, %[−2]proPSA, and PHI significantly increased the accuracy of the base multivariate model by 6.4%, 5.6%, and 6.4%, respectively ($P < 0.001$). At a PHI cut-off of 27.6, a total of 100 biopsies (15.5%) could have been avoided. Moreover, %[−2]proPSA and PHI were significantly correlated with Gleason score ($\rho = 0.245$; $P < 0.001$ and $\rho = 0.276$; $P < 0.001$, resp.).

Interestingly, the same group also reported that %[−2]proPSA and PHI are more accurate than tPSA, fPSA, and %fPSA for predicting PCa in men with a family history of PCa ($n = 158$) from the PROMEtheuS cohort [23]. At a %[−2]proPSA threshold of 1.20 and a PHI threshold of 25.5, 24.8% and 17.2% of prostate biopsies could have been avoided, respectively. Moreover, [−2]proPSA, %[−2]proPSA, and PHI were directly correlated with cancer aggressiveness in patients with PCa in this study.

In a recently published large multicenter study ($n = 1{,}362$) [24], %[−2]proPSA and PHI had better clinical performance for predicting PCa compared with other PSA derivatives (area under the curve (AUC); PHI = 0.74, %[−2]proPSA = 0.72, [−2]proPSA = 0.63, %fPSA = 0.61, and tPSA = 0.56, resp.). Significantly higher median values of %[−2]proPSA and PHI were observed for patients with a Gleason score ≥ 7 (%[−2]proPSA = 2.68 and PHI = 60) compared with a Gleason score < 7 (%[−2]proPSA = 2.34 and PHI = 53) ($P = 0.011$ and $P = 0.0018$, resp.).

In a meta-analysis by Filella and Giménez [26], measurement of %[−2]proPSA and PHI showed improved accuracy for detecting PCa compared with that of PSA or %fPSA, as well as a good relationship with cancer aggressiveness, particularly in the group of patients with PSA of 2–10 ng/mL.

Moreover, [−2]proPSA-based parameters including PHI appear to provide improved predictive value for biopsy reclassification during AS follow-up. Makarov et al. [27] evaluated the potential association of serum and tissue proPSA levels for predicting patients who will develop an unfavorable biopsy conversion (Gleason ≥7 or ≥ 3 positive cores or > 50% of any core involvement) on annual surveillance examination, using serum and prostatic biopsy samples from 71 men in a Johns Hopkins AS program. They found that the ratio of [−2]proPSA to %fPSA in serum was significantly higher at diagnosis in men developing unfavorable repeat biopsy compared to the favorable repeat biopsy group (0.87 ± 0.44 versus 0.65 ± 0.36 pg/mL; $P = 0.02$). In their extended investigation to incorporate PHI in this same cohort, [−2]proPSA/%fPSA ($P = 0.004$) and phi ($P = 0.003$) were also significant predictors of unfavorable biopsy conversion in a Cox regression analysis [28]. According to this study, PHI and [−2]proPSA/%fPSA, combined with biopsy tissue DNA content, improved accuracy to about 70% to predict unfavorable biopsy conversion at the repeat biopsy among men enrolled in an AS program.

Tosoian et al. [29] ($n = 167$) also reported that baseline and longitudinal %fPSA, %[−2]proPSA, [−2]proPSA/%fPSA, and PHI measurements are significantly associated with biopsy reclassification, and %[−2]proPSA and PHI provided the greatest predictive accuracy for high-grade cancer during AS follow-up. Recently, Hirama et al. [30] evaluated the predictive impact of baseline [−2]proPSA and related indices on the pathological reclassification at 1 yr in 67 of 134 candidates for AS. Multivariate logistic regression analysis revealed baseline %[−2]proPSA and PHI (both $P =$

Table 1: AUCs for PSA, %fPSA, %[−2]proPSA, and PHI, and relationship between %[−2]proPSA and PHI and Gleason score.

Reference	AUC PSA (95% CI)	AUC %fPSA (95% CI)	AUC %[−2]proPSA (95% CI)	AUC PHI (95% CI)	Relationship of %[−2]proPSA and GS	Relationship of PHI and GS
Catalona et al., 2011 [19] (n = 892)	0.525	0.648	Not reported	0.703	Not reported	The probability of GS ≥ 7 was 26.1% when PHI < 25 and 42.1% when PHI ≥ 55
Jansen et al., 2010 [20]						
Rotterdam (n = 405)	0.585 (0.535–0.634)	0.675 (0.627–0.721)	0.716 (0.669–0.759)	0.750 (0.704–0.791)	%[−2]proPSA discriminates GS ≥ 7 (with biopsy GS, P = 0.002; with pathologic GS, P = 0.09)	PHI discriminates GS ≥ 7 (with biopsy GS, P: <0.0001; with pathologic GS, P = 0.02)
Innsbruck (n = 351)	0.543 (0.473–0.594)	0.576 (0.523–0.629)	0.695 (0.644–0.743)	0.709 (0.658–0.756)	No (neither with biopsy or with pathologic GS)	No (neither with biopsy nor with pathologic GS)
Sokoll et al., 2010 [21] (n = 556)	0.58 (0.53–0.64)	0.66 (0.61–0.71)	0.70 (0.65–0.75)	0.76 (0.72–0.81)	%[−2]proPSA increased with increasing GS (P = 0.02)	Not reported
Lazzeri et al., 2013* [22] (n = 646)	0.50 (0.46–0.54)	0.64 (0.61–0.68)	0.67 (0.64–0.71)	0.67 (0.64–0.71)	Significant (Spearman r: 0.245; P < 0.001), and it did improve the prediction of GS ≥ 7 PCa in multivariable accuracy analysis by 7.3%	Significant (Spearman r: 0.276; P < 0.001), and it did improve the prediction of GS ≥ 7 PCa in multivariable accuracy analysis by 7.6%
Lazzeri et al., 2013** [23] (n = 158)	0.55 (0.47–0.63)	0.60 (0.52–0.68)	0.73 (0.66–0.80)	0.73 (0.66–0.80)	Significant (Spearman r: 0.366; P = 0.002), but it did not improve the prediction of GS ≥ 7 PCa in multivariable accuracy analysis	Significant (Spearman r: 0.464; P < 0.001), but it did not improve the prediction of GS ≥ 7 PCa in multivariable accuracy analysis
Stephan et al., 2013 [24] (n = 1,362)	0.56 (0.53–0.59)	0.61 (0.59–0.64)	0.72 (0.70–0.75)	0.74 (0.71–0.76)	Significantly higher median values of %[−2]proPSA were observed for patients with GS ≥ 7 (%[−2]proPSA = 2.68) compared with a GS < 7 (%[−2]proPSA = 2.34; P = 0.011)	Significantly higher median values of phi were observed for patients with GS ≥ 7 (PHI = 59.7) compared with a GS < 7 (PHI = 53.1; P = 0.002)
Ng et al., 2014 [25] (n = 230)	0.547 (0.421–0.674)	0.572 (0.437–0.708)	0.768 (0.660–0.876)	0.781 (0.675–0.887)	Not reported	Not reported

AUC: area under the curve; PCa: prostate cancer; PSA: prostate-specific antigen; %fPSA: percentage of free PSA to total PSA; %[−2]proPSA: percentage of [−2]proPSA to free PSA; PHI: Prostate Health Index; GS: Gleason score; CI: confidence interval.

*An observational, prospective, multicenter European cohort, the PROMEtheuS project.

**A nested case-control study from the same PROMEtheuS database.

0.008) to be the only independent predictive factors for pathological upgrading 1 yr after beginning AS. However, that study was limited by a short follow-up period (1 yr), because reclassification at short follow-up period during AS might be mostly due to more accurate sampling rather than true biologic progression.

Although studies evaluating the potential role of %[−2]proPSA and PHI in an AS program are currently scarce, we found improved predictive value for an unfavorable biopsy conversion at annual surveillance biopsy in the AS program. Additional validation is warranted to determine whether clinically useful thresholds can be defined and to better characterize the role of %[−2]proPSA and PHI in conjunction with other markers in monitoring patients enrolled in AS in the future.

4. PCA3

Prostate cancer antigen 3 (PCA3) is a prostate-specific noncoding mRNA detectable in urine and greatly overexpressed in PCa compared with benign tissue [31–33]. Measuring PSA mRNA allows for the standardization of the number of PCA3 RNA copies by calculating the ratio of PCA3 to PSA (PCA3 score). Despite its cost, PCA3 outperformed PSA and %fPSA for early detection of PCa [34]. In a meta-analysis of the clinical utility of urinary PCA3 for diagnosing PCa [35], sensitivity was 54–82% and specificity was 66–89%, with AUC of 0.66–0.87. Several studies [36–38] have investigated the correlations between PCA3 score and PCa aggressiveness features, including tumor volume, Gleason score, pT stage, and percentage of positive biopsy cores.

Marks et al. [36] ($n = 226$) demonstrated the superiority of PCA3 over PSA by using a third-generation PCA3 assay (Gene Probe Progensa) (AUC = 0.68 versus 0.52; $P = 0.008$). Using 35 as the most balanced PCA3 cut-off score resulted in sensitivity, specificity, and odds ratio (OR) of 58%, 72%, and 3.6, respectively. Unfortunately, the median PCA3 scores in patients with aggressive PCa (Gleason score <7 versus ≥7) were not significantly different [36]. Nakanishi et al. [37] ($n = 142$) found that PCA3 score was significantly correlated with tumor volume ($P = 0.008$) and an increasing PCA3 score was associated with a higher Gleason score ($P = 0.005$). In a receiver operating curve (ROC) analysis, the PCA3 score could discriminate low volume tumors (<0.5 cc) well with AUC of 0.757. Auprich et al. [38] ($n = 305$) also showed consistently that PCA3 scores were significantly lower in men with low volume tumors and insignificant PCa ($P < 0.001$).

Conversely, Hessels et al. [39] did not find a significant association between PCA3 score in urine sediment after DRE with any PCa prognostic parameter, including Gleason score, tumor volume, or stage. Similarly, Liss et al. [40] reported that PCA3 score did not correlate with adverse pathological features, including stage, Gleason score, or extraprostatic extension.

Based on the promising findings from several studies, urinary PCA3 was further evaluated for its ability to predict biopsy progression in men undergoing AS. Ploussard et al. [41] retrospectively evaluated the performance of PCA3 in men who met criteria for AS, but underwent immediate RP ($n = 106$). A high PCA3 score (≥25) was an important predictive factor for tumor volume ≥ 0.5 cm^3 and significant cancer, defined as nonorgan confined, or any Gleason pattern 4 or Gleason pattern 5, or tumor volume of at least 0.5 cm^3, in a multivariate analysis (OR, 5.4; $P = 0.01$ and OR, 12.7; $P = 0.003$, resp.). However, no relationship was observed between PCA3 score and disease stage ($P = 0.155$).

In the first evaluation of urine PCA3 in AS patients enrolled in the Johns Hopkins AS program ($n = 294$) [42], the PCA3 score was not significantly associated with biopsy reclassification ($P = 0.131$), or biopsy Gleason score ≥ 7 ($P = 0.304$), with minimal ability to discriminate unfavorable biopsy pathology (AUC = 0.589; $P = 0.076$). However, in the recently conducted multi-institutional Canary Prostate AS Study ($n = 387$) [43], PCA3 score was significantly associated with a higher biopsy Gleason score and tumor volume, assessed by the percentage of positive cores, in subsequent biopsies ($P < 0.01$ for all comparisons). Using log-transformed biomarker scores as continuous predictors, the OR for a Gleason score of ≥ 7 versus < 7 for PCA3 was 1.67 (95% confidence interval (CI): 1.10–2.52; $P = 0.02$).

In many studies, although PCA3 has clinical utility for detecting PCa, its contribution to prognostic prediction is still contentious. With respect to AS, prognostic value for predicting an unfavorable biopsy conversion at annual surveillance biopsy in the AS program could not be defined due to sparse data. Thus, its role in risk assessment during AS needs to be tested in larger studies with repeated PCA3 score measures.

5. TMPRSS2:ERG

TMPRSS2:ERG fusion is a rearrangement of the TMPRSS2 gene, an androgen-regulated transcriptional promoter, and the ERG oncogene, occurring in approximately half of Caucasian patients with PCa [44]. Similar to PCA3, a TMPRSS2:ERG rearrangement can be detected in urine after DRE [45] and can also be normalized to the amount of PSA mRNA to generate a TMPRSS2:ERG score. Hessels et al. [45] reported that detecting TMPRSS2:ERG fusion in urine has high specificity of 93% and 94% positive predictive value (PPV) for PCa detection. Moreover, a population-based study found that TMPRSS2:ERG gene fusion is associated with an increased cumulative incidence ratio of 2.7 for developing metastases and PCa-specific mortality [46].

Tomlins et al. [47] developed a clinical grade, quantitative TMPRSS2:ERG urine assay and measured TMPRSS2:ERG transcript levels in a large-scale multicenter study including a community biopsy cohort ($n = 471$), an academic biopsy cohort ($n = 623$), and prostatectomy cases ($n = 218$). TMPRSS2:ERG score was positively associated with direct markers of tumor volume, including number of positive cores and maximum percentage of positive cores, in both the academic biopsy cohort and the community biopsy cohort. TMPRSS2:ERG score was also significantly higher in men with high prostatectomy Gleason score (>6 versus 6) ($P = 0.009$) and was significantly associated with Gleason score upgrading and significant cancer ($P = 0.008$ and $P = 0.004$, resp.).

In the most recently published prospective multicenter study ($n = 443$) [48], both PCA3 and TMPRSS2:ERG had independent additional predictive value over the European Randomised Study of Screening for Prostate Cancer risk calculator (ERSPC-RC) parameters for predicting PCa in multivariate analyses (OR, 3.64; $P < 0.001$ and OR, 3.28; $P = 0.002$, resp.). The AUC increased incrementally from 0.799 (ERSPC-RC) to 0.833 (ERSPC-RC plus PCA3) to 0.842 (ERSPC plus PCA3 plus TMPRSS2:ERG) to predict PCa. Interestingly, in multivariate logistic regression analyses, only TMPRSS2:ERG added significant predictive value to the ERSPC-RC to predict biopsy Gleason score (OR, 7.16; $P < 0.001$) and clinical tumor stage (OR, 2.60; $P = 0.023$), whereas PCA3 did not.

In AS setting, within the above-mentioned multi-institutional Canary Prostate AS Study ($n = 387$) [43], TMPRSS2:ERG score was also significantly associated with higher biopsy Gleason score and tumor volume, assessed by the percentage of positive cores, in subsequent biopsies ($P < 0.01$ for all comparisons). Using log-transformed biomarker scores as continuous predictors, the OR for a Gleason score of ≥ 7 versus < 7 for TMPRSS2:ERG was 1.24 (95% CI, 1.01–1.53; $P = 0.05$). In a ROC curve analysis, the AUC for predicting a Gleason score ≥ 7 was 0.68 for PSA alone and 0.70 for the combination of both markers (PCA3 and TMPRSS2:ERG) and PSA, respectively. From their results, they showed that both markers are potential predictors to stratify the risk of having aggressive cancer for men on AS.

Whelan et al. [49] investigated secretion capacity biomarkers, including total RNA (TXNRD1 mRNA, PSA mRNA, TMPRSS2:ERG fusion mRNA, and PCA3 mRNA) and specimen volume in expressed prostatic secretion (EPS) specimens before RP from patients who were eligible for AS based on National Comprehensive Cancer Network (NCCN) guidelines ($n = 216$). Two high-performing models were identified, one featuring type III and IV TMPRSS2:ERG variants and another featuring two secretion capacity biomarkers. The AUCs of the TMPRSS2:ERG model and the secretion capacity model for detecting upstaging in the NCCN AS group were 0.80 and 0.79, respectively. Furthermore, the best performing model was associated with a reduced risk of upstaging and of both upstaging and Gleason upgrading by 7.8-fold and 5.2-fold, respectively. Interestingly, these results were supported by Berg et al. [50], who showed a significant association between ERG positivity at diagnosis and the risk of progression during AS (Cox hazard ratio (HR), 2.45; 95% CI, 1.62–3.72; $P < 0.0001$).

6. Oncotype DX Prostate Cancer Assay

The Oncotype DX Prostate Cancer Assay is a multigene RT-PCR expression assay that measures expression of 12 cancer-related genes representing four biological pathways and five reference genes in tumor tissue from formalin-fixed paraffin-embedded prostate needle biopsies. Gene expression is normalized by subtracting the aggregated expression of the reference genes and algorithmically combined to calculate the genomic prostate score (GPS) [51]. Some of the key challenges in developing this biopsy-based assay for PCa include the heterogeneous and multifocal nature of the disease and the very small amounts of tumor tissue available from diagnostic prostate needle biopsies.

In a clinical validation study presented at American Urological Association (AUA) annual meeting in 2013 [52], it was reported that GPS, assessed in diagnostic biopsy tissue, strongly predicted high-grade and/or pT3 disease after adjusting for Cancer of the Prostate Risk Assessment (CAPRA) score or other standard pretreatment factors in patients suitable for AS. In the most recently published validation study by Klein et al. ($n = 395$) [53], the biopsy-based 17-gene GPS improved the prediction of the presence or absence of adverse pathology, which may help men diagnosed with PCa decide between AS and immediate definitive treatment. In their study, GPS was strongly associated with clinical recurrence in the RP group ($n = 441$) (HR, 2.32; 95% CI, 1.81–3.00; $P < 0.001$). GPS predicted high-grade and high-stage disease in RP specimens (OR per 20 GPS units, 2.3; 95% CI, 1.5–3.7; $P < 0.001$ and 1.9; 95% CI, 1.3–3.0; $P = 0.003$, resp.) and high-grade and/or high-stage disease after adjusting for CAPRA score with OR of 2.1 (95% CI, 1.4–3.2; $P < 0.005$). Moreover, adding the GPS to the CAPRA score improved the AUC for favorable pathology to 0.67 from 0.63 with the CAPRA score alone. However, this improvement of AUC, as well as the decision-curve analysis, did not show a really perceptible benefit for clinical practice when adding the GPS to other clinical parameters. Moreover, they did not describe detailed information on the biopsy scheme used in the study.

Although GPS could provide additional prognostic information over the existing clinical risk-stratification tools, further validation studies are needed to provide robust evidence.

7. Other Potential Biomarkers

Several recent European studies [54–57] have indicated that a panel of four kallikrein markers, including tPSA, fPSA, intact PSA, and kallikrein-related peptidase 2 (hK2), can be used to improve the predictive accuracy of biopsy outcome and reduce unnecessary biopsies. Using data from the Sweden section of the ERSPC ($n = 740$), Vickers et al. [54] reported that a panel of four kallikrein markers showed significantly better predictive accuracy of biopsy outcome in previously unscreened men with elevated PSA compared with PSA alone (AUC from 0.68 to 0.83, $P < 0.0005$, and from 0.72 to 0.84, $P < 0.0005$, without DRE and with DRE, resp.). They estimated that using a 20% risk of prostate cancer as the threshold for biopsy would have reduced the number of biopsies by 424 (57%), while missing only a small number of cancers (31 of 152 low-grade cancers and three of 40 high-grade cancers). Furthermore, in men with a previous negative biopsy but persistently elevated PSA ($n = 925$), Gupta et al. [57] evaluated the performance characteristics of a panel of four kallikrein markers to determine the predictive value of repeat biopsy outcome in the Rotterdam section of the ERSPC. The full-kallikrein panel, incorporating age and DRE, had higher discriminative accuracy than PSA and DRE alone for predicting high-grade cancer (Gleason score ≥ 7) at biopsy with the AUC improving from 0.76 to 0.87

($P = 0.003$). Additionally, these markers had an improved ability to distinguish between pathologically insignificant and aggressive disease on pathologic examination of RP specimens in a recently published study from the Rotterdam section of the ERSPC ($n = 392$) [58]. Although the clinical model had good accuracy for predicting aggressive disease (AUC, 0.81), the panel of four kallikrein markers enhanced the base model, with an AUC of 0.84 ($P < 0.0005$). Moreover, this improvement was more remarkable in low- and very low-risk patients (AUC from 0.75 to 0.81 and from 0.72 to 0.81, resp.). Clinical application of the model incorporating these kallikrein markers would reduce rates of surgery by 135 of 1000 patients overall and 110 of 334 patients with pathologically insignificant disease [58].

The panel of four kallikrein markers was combined to generate the 4K score. The 4K score test was developed by OPKO Lab, a division of OPKO Health, and is being commercialized. Thus, it may soon be available for clinical settings. However, so far no evidence for the usefulness of the four kallikrein panel in AS programs has been presented.

The expression levels of different cell cycle progression (CCP) genes are highly correlated with cell proliferation, presumably reflecting the fraction of actively dividing cells within the sampled tissue [59]. The expression levels of these genes were known to be significantly associated with the risk of disease progression [60–62]. The CCP score (Prolaris) is calculated as the average expression level of 31 CCP genes, normalized to 15 housekeeper genes [60], which was developed as a clinical laboratory test by Myriad Genetics, Inc. The expression levels of these genes were measured using quantitative RT-PCR on RNA from formalin-fixed paraffin-embedded tumor samples.

Cuzick et al. [60] showed that CCP score provides a substantial amount of independent information regarding the risk of recurrence after RP (HR for a 1-unit increase in CCP score, 1.74; 95% CI, 1.39–2.17; $P = 3.3 \times 10^{-6}$) and the risk of death in conservatively managed PCa diagnosed by transurethral resection of the prostate (TURP) (HR, 2.57; 95% CI, 1.93–3.43; $P = 8.2 \times 10^{-11}$) in large retrospective cohorts ($n = 366$ and $n = 337$, resp.). The same group reported on the ability of the CCP score to predict death from PCa in a cohort of men with clinically localized disease diagnosed by a needle biopsy and managed conservatively ($n = 349$) [61]. In their subsequent study, the CCP score was the strongest independent predictor of cancer death outcome for conservatively managed patients (HR, 2.02; 95% CI, 1.62–2.53; $P < 10^{-9}$).

The CCP score also had significant prognostic accuracy in an academic RP cohort study for validation ($n = 413$) [62] after controlling for all available clinical and pathologic data. With or without adjusting for clinical variables, increasing CCP score was associated with markedly higher hazards for biochemical progression (HR, 2.1; 95% CI, 1.6–2.9; $P < 0.001$ in univariate analysis and HR, 1.7; 95% CI, 1.3–2.4; $P < 0.001$ with adjustment for CAPRA score, resp.). Moreover, the combined CAPRA and CCP score improved the concordance index for both the overall cohort and low-risk patients (0.77 versus 0.73 for CAPRA score alone).

Additional validation studies are under way using biopsy specimens from pre-RP and AS cohorts, which will help define the role of the CCP score in AS setting. However, there is still no definite evidence that histopathologic markers have clinical utility for patient selection and monitoring during AS.

8. Discussion

We have provided insight into the value of novel biomarkers that could be used for patient selection and follow-up on AS for low-risk PCa. Table 2 shows a summary of studies investigating the prognostic value of novel biomarkers in AS. Several of these novel biomarkers have the potential to improve the current practice of AS. In many series, MP-MRI showed promising results because of the very high negative predictive value (NPV) for significant PCa [63–66]. Thus, if validated, favorable MRI findings on a good-quality MP-MRI may be used for selection and follow-up of patients during AS and might obviate the need for repeat biopsies. In addition to promising imaging tools, several serum, urinary, and tissue biomarkers have been intensively investigated to determine their additional value for cancer detection and prognosis. However, a biomarker must demonstrate evidence of strong analytical validity, clinical validity, and clinical utility to enter wide clinical practice.

Many studies have shown that %[−2]proPSA and PHI are more accurate than the currently used PSA and other PSA derivatives for predicting the presence of PCa and aggressiveness, which might result in the avoidance of unnecessary biopsies without missing significant PCa. The results reported above show that %[−2]proPSA and PHI are particularly useful in patients with the PSA gray zone range of between 2.0 and 10.0 ng/mL, which might lead to reducing unnecessary biopsies in AS patients. Although studies evaluating their potential role in AS program are currently scarce, most results to date are promising. However, additional validation is warranted to determine whether clinically useful thresholds can be defined and to better characterize their role in conjunction with other biomarkers during monitoring patients in AS.

Although PCA3 has been reported to have clinical utility for detecting PCa in many studies, its contribution to prognostic prediction remains controversial. The consensus in most studies is that PCA3 is often correlated with insignificant PCa and tumor volume, yet, in the clinically significant cancers, there is no definite evidence for an association with histopathologic prognostic factors. Considering the heterogeneous character of the disease, combining PCA3 with other biomarkers might be a better option to improve diagnostic and prognostic accuracy instead of using a single prognostic variable.

TMPRSS2:ERG is highly specific for predicting clinically significant PCa on biopsy, despite the relatively low sensitivity. Robert et al. provided a rational basis for combining PCA3 and TMPRSS2:ERG in tissue samples [67]. After the first study on combining PCA3 and TMPRSS2:ERG reported by Hessels et al. [45], several studies [48, 68–70] showed better accuracy of the combination with TMPRSS2:ERG than PCA3 alone for the prediction of PCa detection and progression.

Table 2: Studies investigating the prognostic value of novel biomarkers in active surveillance.

Biomarkers	Reference	Year	n	Predictor variables	Study endpoint(s)	Results
%[−2]proPSA, PHI	Makarov et al. [27]	2009	71	[−2]proPSA/%fPSA	Biopsy progression	[−2]proPSA/%fPSA was significantly associated with unfavorable biopsy in repeat biopsy (Cox HR, 2.53; 95% CI, 1.18–5.41; $P = 0.02$).
	Isharwal et al. [28]	2011	71	[−2]proPSA/%fPSA, PHI, and biopsy tissue DNA content	Biopsy progression	PHI and [−2]proPSA/%fPSA showed improvement in the predictive accuracy (c-index, 0.6908 and 0.6884, resp.) for unfavorable biopsy conversion in the multivariate models including the biopsy tissue DNA content.
	Tosoian et al. [29]	2012	167	%fPSA, %[−2]proPSA, [−2]proPSA/%fPSA, and PHI	Biopsy progression	Baseline and longitudinal measurements of %fPSA, %[−2]proPSA, [−2]proPSA/%fPSA, and PHI demonstrated significant associations with biopsy reclassification, and %[−2]proPSA and PHI provided the greatest predictive accuracy for high-grade cancer.
	Hirama et al. [30]	2014	134	%[−2]proPSA, PHI	Biopsy progression	Baseline %[−2]proPSA and PHI were the only independent predictive factors for pathological upgrading in multivariate logistic regression analysis ($P = 0.008$ and $P = 0.008$, resp.).
PCA3	Ploussard et al. [41]	2011	106	PCA3 score	Prognostic pathologic findings in RP specimens	The risk of having a cancer ≥0.5 cm^3 and a significant PCa was increased by 3-fold in men with a PCA3 score of ≥25 compared with men with a PCA score of <25. In a multivariate analysis, a high PCA3 score (≥25) was an important predictive factor for tumor volume ≥0.5 cm^3 (OR: 5.4; $P = 0.010$) and significant PCa (OR: 12.7; $P = 0.003$).
	Tosoian et al. [42]	2010	294	PCA3 score	Biopsy progression	PCA3 alone could not be used to identify men with progression on biopsy (AUC, 0.589; 95% CI, 0.496–0.683; $P = 0.076$). After adjustment for age and date of diagnosis, PCA3 was not significantly associated with progression on biopsy ($P = 0.15$).
	Lin et al. [43]	2013	387	PCA3 score	Biopsy progression	PCA3 score was significantly associated with a higher biopsy Gleason score and tumor volume in subsequent biopsies ($P < 0.01$ for all comparisons). Using log-transformed biomarker scores as continuous predictors, the OR for a Gleason score of ≥7 versus <7 for PCA3 was 1.67 (95% CI: 1.10–2.52; $P = 0.02$)
TMPRSS2:ERG	Lin et al. [43]	2013	387	TMPRSS2:ERG	Biopsy progression	TMPRSS2:ERG score was significantly associated with a higher biopsy Gleason score and tumor volume in subsequent biopsies ($P < 0.01$ for all comparisons). Using log-transformed biomarker scores as continuous predictors, the OR for a Gleason score of ≥7 versus <7 for TMPRSS2:ERG was 1.24 (95% CI: 1.01–1.53; $P = 0.05$).
	Whelan et al. [49]	2013	216	TMPRSS2:ERG model Secretion capacity model (PSA, total EPS RNA, and total EPS volume)	Upgrading Upstaging in RP specimens	The AUCs of the TMPRSS2:ERG model and the secretion capacity models for detecting upstaging in the NCCN AS group were 0.80 and 0.79, respectively. TMPRSS2:ERG model was associated with a reduced risk of upstaging and of both upstaging and Gleason upgrading by 2.4-fold and 2.7-fold, respectively ($P = 0.1041$ and $P = 0.1576$, resp.).
	Berg et al. [50]	2014	265	ERG positivity	Clinical progression Biopsy progression PSA progression	The ERG-positive group showed significantly higher incidences of overall AS progression ($P < 0.0001$) and of the subgroups PSA progression ($P < 0.0001$) and biopsy progression ($P < 0.0001$). ERG positivity was a significant predictor of overall AS progression in multiple Cox regression (HR, 2.45; 95% CI, 1.62–3.72; $P < 0.0001$).

%[−2]proPSA: percentage of [−2]proPSA to free PSA; PHI: Prostate Health Index; %fPSA: percentage of free PSA to total PSA; HR: hazard ratio; CI: confidence interval; RP: radical prostatectomy; PCa: prostate cancer; OR: odds ratio; AUC: area under the curve; PSA: prostate-specific antigen; EPS: expressed prostatic secretion; NCCN: National Comprehensive Cancer Network; AS: active surveillance.

These encouraging results for combined biomarkers may help to improve the prediction of biopsy reclassification during AS and pathologic features at RP. However, additional multi-institutional studies on larger populations are needed to verify if these combined biomarkers improve the prediction of biopsy reclassification in AS patients.

The panel of four kallikrein markers also has good predictive accuracy for biopsy outcome and aggressive disease, and tissue biomarkers (i.e., Oncotype DX Prostate Cancer Assay, CCP score) show promising ability for predicting disease progression. There is a growing recognition that molecular biomarkers can complement conventional clinical and pathologic parameters to personalize the care of patients with cancer. However, incorporating these biomarkers into standard clinical practice requires a level of validation that is not often achieved. Unquestionably, these markers also need to be verified with a higher level of evidence for clinical validation and usefulness in AS programs in the future.

Nevertheless, the present systematic review of the literature on novel biomarkers has several limitations. First, most of the studies were retrospective, and different biopsy protocols were used, possibly causing significant heterogeneity. Further heterogeneity was found regarding study design (i.e., retrospective versus prospective, recruitment strategy) and population characteristics (i.e., age, race, and total PSA range). Second, the definition of clinical significance and disease progression was arbitrary. Third, most studies were limited to intermediate endpoints such as biopsy reclassification, treatment-free survival, or pathologic findings in RP specimens. No data are available with respect to longer term endpoints such as time to metastasis or prostate cancer-specific mortality.

The majority of biomarkers published during the last few years are still in the investigation or validation phase. Although several of these novel biomarkers showed improved predictive accuracy than that of classical parameters, there is still a need of a standard study design to avoid common bias and clinical validation. Further studies are required to define how these novel biomarkers could be used to select men that would most benefit from an AS program and how these markers could be incorporated into the follow-up schedule in AS patients.

9. Conclusions

Several biomarkers, which could be novel tools to improve PCa risk assessment, showed promising prognostic value. %[−2]proPSA and PHI showed improved predictive value for an unfavorable biopsy conversion at annual surveillance biopsy in the AS program. PCA3 and TMPRSS2:ERG had additional independent predictive value for the prediction of PCa detection and progression, although PCA3 was limited in predicting aggressive cancer. Nevertheless, both biomarkers improved the multivariate accuracy for predicting biopsy outcome when combined with each other. Other tissue biomarkers also showed promising ability to predict disease progression.

Implementing these promising novel biomarkers into clinical practice may not only increase the number of patients suitable for AS but also reduce the burden of monitoring during AS. However, there is a great need for further well-designed, large, multicenter, prospective studies, to validate the currently available biomarkers and identify an optimal combination of biomarkers and optimal thresholds for each biomarker.

Disclosure

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Conflict of Interests

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

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Review Article

hERG1 Potassium Channels: Novel Biomarkers in Human Solid Cancers

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Because of their high incidence and mortality solid cancers are a major health problem worldwide. Although several new biomarkers and potential targets for therapy have been identified through biomolecular research in the last years, the effects on patients' outcome are still unsatisfactory. Increasing evidence indicates that hERG1 potassium channels are overexpressed in human primary cancers of different origin and several associations between hERG1 expression and clinicopathological features and/or outcome are emerging. Aberrant hERG1 expression may be exploited either for early diagnosis (especially in those cancers where it is expressed in the initial steps of tumor progression) or for therapy purposes. Indeed, hERG1 blockage impairs tumor cell growth both *in vitro* and *in vivo* in preclinical mouse model. hERG1-based tumor therapy in humans, however, encounters the major hindrance of the potential cardiotoxicity that many hERG1 blockers exert. In this review we focus on recent advances in translational research in some of the most frequent human solid cancers (breast, endometrium, ovary, pancreas, esophagus, stomach, and colorectum) that have been shown to express hERG1 and that are a major health problem.

1. Introduction

A biomarker is defined as a biological molecule indicating atypical processes or disease that can be detected in tissues, blood, and other body fluids. Biomarkers can be used to evaluate the response to a particular treatment. In this view, oncology research greatly relies on biomarkers for diagnostic, prognostic, and predictive purposes.

In recent years, ion channels have been proven to be expressed in different human cancers where they regulate several cancer cell processes. In this view, ion channels could represent novel cancer biomarkers, once properly validated in the clinical setting.

Ion channels are pore-forming transmembrane proteins that regulate passive ion fluxes that are important for key cell processes (i.e., secretion, cell volume regulation). Ion channels are good potential markers because of their localization at the plasma membrane level. This fact makes their detection (e.g., by immunohistochemistry (IHC)) easy and their block with specific drugs and antibodies quick and tunable.

Among ion channels, those encoded by the ether-à-go-go related gene 1 (*hERG1* also named *KCNH2*) are often overexpressed in neoplastic cell lines and human primary cancers of different histogenesis (reviewed in [1]). hERG1 belongs to an evolutionarily conserved multigenic family of voltage-activated, outward rectifying K^+ channels, the EAG family. Physiologically, hERG1 channels are responsible for the potassium current (I_{Kr}) that mediates the rapid repolarizing phase following cardiac action potential.

The *KCNH2* gene (formerly indicated as *hERG1*) was cloned in 1994 from a human hippocampal cDNA library and it is localized on chromosome 7, in q35-36 position [2]. hERG1 channel is composed of 1159 amino acids, and both amino- and carboxy-terminals are located in the cytoplasm (Figure 1).

Functional hERG1 channels are tetramers, and each subunit is made of 6 transmembrane domains (S1–S6) and a long loop which constitutes the pore. Once assembled in the tetramers, the four loops contribute to form the aqueous pore

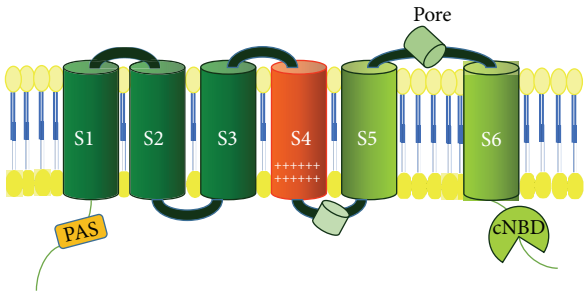

Figure 1: Structure of hERG1 potassium channel. PAS: PAS (acronym of Per Arnt Sim) domain; cNBD (cyclic nucleotide binding domain).

in the center of the structure. The fourth transmembrane segment (S4) contributes to form the voltage sensor domain (VSD), which sharply contributes to hERG1 biophysical properties [3].

In recent years, a progressively more defined picture is emerging, in which hERG1 channels are expressed in several types of human cancers [4] and regulate different cellular processes [5–8]. hERG1 channels are overexpressed in human primary cancers of different histogenesis such as endometrial [9], colorectal [10], esophageal [11], and pancreatic [12] adenocarcinomas and ovarian [13] and brain cancers [14] as well as leukemias [15, 16].

It has been shown that hERG1 is not expressed by the majority of normal nonexcitable tissues as well as hyperplastic lesions (adenomas) [9–11]. Data gathered in the last 15 years underlined that hERG1 channels are important modulators of apoptosis [17] and cell proliferation in leukemias [15, 16] and neuroblastomas [18]. However these tumors will not be discussed in the present review and we refer to a more focused review [19].

Cancers of the breast and reproductive system in females and tumors of the gastrointestinal tract in both sexes collectively represent a major health problem either for their high incidence or poor outcome. Pieces of evidence have been gathered that all the above-mentioned cancer types express high levels of hERG1 channels. Table 1 summarizes data gathered so far concerning hERG1 expression in cell lines and in solid human cancers.

From an epidemiologic point of view the above-mentioned solid cancers (breast, endometrium, ovary, esophagus, stomach, colorectum, and pancreas) represent a vast share of both incidence and mortality for cancer worldwide [20] (Figures 2(a) and 2(b)).

Five-year survival rates vary from 89.2% in women affected by breast cancer to 81.5% in women suffering from *corpus uteri* (endometrial) cancer and to 44.6% in women with ovarian cancer; in both sexes, 5-year survival rates vary from 64.7%, 28.3%, 17.5%, 16.8%, and 6.7% in patients with colorectal, gastric, esophageal, lung, and pancreatic cancer, respectively [21, 22].

The differences in survival are mainly represented by distinct biomolecular features as well as efficacy of prevention, diagnostic accuracy, and response to treatment. Nowadays, all these cancers require a multimodal approach that includes oncologists, surgeons, and radiotherapists, although the contribution of many other professionals is often of crucial importance.

The purpose of this paper is to review the recent advances in hERG1 research from cancers arising in breast, female reproductive system, and digestive tract.

2. Breast Cancer

Breast cancer (BC) is the most common malignancy among women worldwide and remains the primary cause of death from cancer in females [36]. Unfortunately, BC incidence is increasing everywhere and in less developed countries BC is becoming a major health issue [36–38]. On the other hand, mortality rates for BC are decreasing [36, 39] and it has been estimated that lung cancer instead of BC will become the first cause of death among women and in Europe in 2014, for the first time [39].

A better knowledge of biological features, screening protocols, and access to cutting edge therapies plays a key role in BC treatment. Fisher [40] dramatically changed the perception of BC, introducing the idea of a complex disease from the very beginning of the pathogenetic process, with different factors involved in the natural history of this cancer. Nowadays, not only the TNM stage but also the biological subtypes are crucial for BC clinical management. In order to get a more accurate prognosis and prediction of therapy benefits physicians should use accurate molecular technologies [41]. However, due to the high costs of such techniques, surrogate definitions of subtypes (i.e., hormones expression, proliferation index, and HER-2 expression) obtained through IHC have become a valuable approach for clinicians [42]. The choice of endocrine therapy, chemotherapy regimens, monoclonal antibodies, or kinase inhibitors is mostly driven by the above biomarkers. A striking example of it is the target therapy on HER-2 receptor employing the monoclonal antibody Trastuzumab. Such treatment has significantly changed survival rates in HER-2 positive BC [43]. Hormone-responsive and HER-2 positive cancers are candidate of a specific "biological therapy." On the contrary, triple negative cases, being devoid of any peculiar biomarker, can only be treated with strong chemotherapy regimens. Moving down this line, identification of biomarkers in BC is of utmost clinical importance either as prognostic tools or as possible therapeutic targets.

Ion channels could therefore represent novel biomarkers in BC. Indeed several studies have already been published addressing the expression of single ion channel types in BC. More recently an ion channel molecular profile was defined for BC, opening interesting perspectives in this field [44].

Long ago it was shown that hERG1 gene is expressed in BC cell lines [4]. More recently, it was shown that hERG1 hyperstimulation in SKBR3 and MDA-MB-231 cells might induce cell senescence [24]. In particular, the authors showed that the exposure to hERG1 channel agonist (NS1643) causes the cell cycle arrest in G0/G1 and induces cell senescence [24].

To our knowledge, no data have been gathered regarding hERG1 channel expression in primary BC so far. Through

Table 1: hERG1 expression and role in cell lines and in human solid tumors.

Tumor type	Cell lines	Human tumors
Breast cancer	hERG1 current is blocked by Tamoxifen [23]; induction of cell senescence [24]	—
Endometrial cancer	—	Overexpression [9]
Ovarian cancer	Expression [13]	Overexpression [13]; methylation and downregulation [25]
Esophageal cancer	—	Overexpression in EA and BE [11], ESCC [26]; malignant progression [11]
Gastric cancer	Cell proliferation [27]; apoptosis [28]	Grading, TNM stage, serosal, and venous invasion [29, 30]; Lauren's intestinal type, localization (fundus), low grading, and early stages (TNM I and II) [31]; in early stage (T1) HERG1 expression identified high-risk patients [31]
Colorectal cancer	Invasiveness [10]; chemosensitivity [32]; regulation of VEGF-A secretion [33]	++, correlation with invasive phenotype [10]; independent negative prognostic factor in stage I and II CRC [34]
Pancreatic cancer	Overexpression [12]	Lymphnode involvement, grading, and TNM stage I [12]
Lung cancer	Cell proliferation [35]	—

EA: esophageal adenocarcinoma; BE: Barrett's esophagus; ESCC: esophageal squamous cell carcinoma; TNM: tumor node metastasis; VEGF-A:vascular endothelial growth factor.

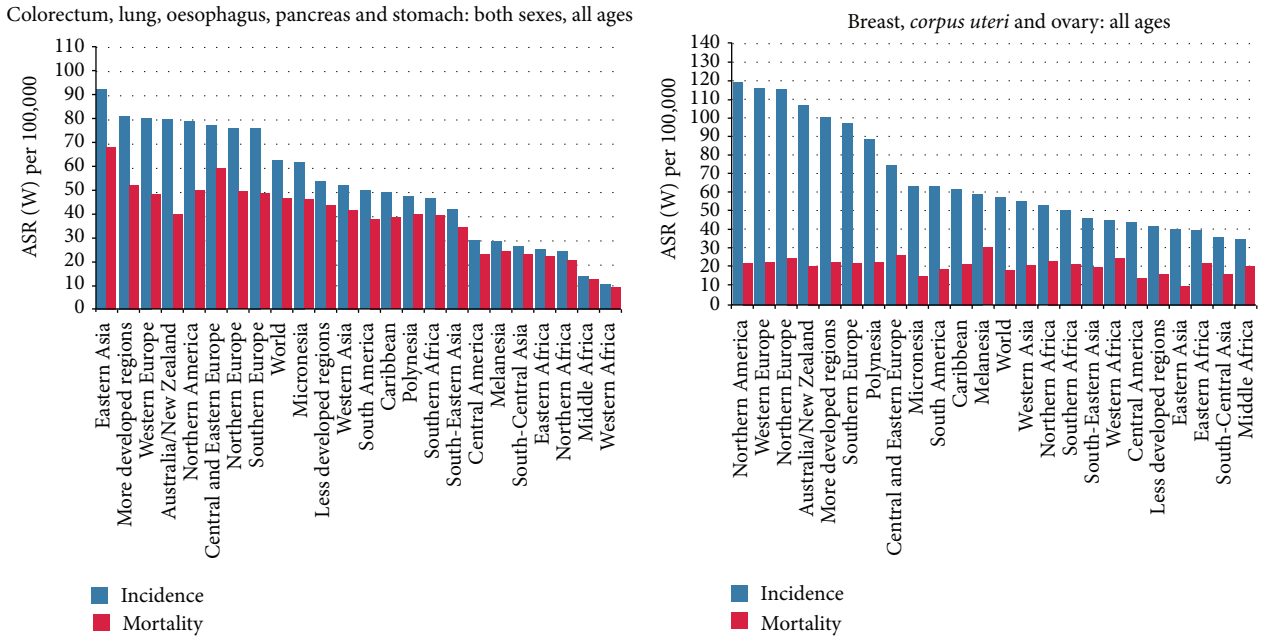

Figure 2: Incidence and mortality for the human solid tumors discussed in the present review. Colorectal, lung, esophageal, pancreatic, and gastric cancers in both sexes (a) and breast, *corpus uteri*, and ovarian cancers in females (b). Source: GLOBOCAN 2012.

IHC experiments we demonstrated that hERG1 is overexpressed in primary BC and correlates with clinicopathological parameters such as molecular subtype, grading, ER, and ki67 expression (Lastraioli et al., submitted to Br J Cancer). These findings might be useful in the clinical setting. It is worth recalling here that Tamoxifen (one of the most used drugs for BC treatment) was shown to block hERG1 currents [23], a fact that could explain the QT interval elongation observed in Tamoxifen-treated patients. Due to the high expression in BC and to the sensitivity to Tamoxifen it could be argued that hERG1 might serve as therapeutic targets and/or predictors of response to therapy, although further studies are surely needed.

3. Esophageal Cancer

Esophageal cancer represents the sixth leading cause of mortality from cancer worldwide, with a 5-year mortality of less than 15% [20, 22]. The unsatisfactory results are mainly related to late diagnosis and complex therapeutic approaches

that include combined regimens combining surgery (with high morbidity and mortality), radiotherapy, and chemotherapy. The current curative algorithm requires perioperative radiochemotherapy and demolitive surgery, although many patients presenting with inoperable locoregional advanced cancers or distant metastatic spread are proposed for palliative radiochemotherapy or support therapy alone [45]. Some new molecular therapies are currently incorporated into classical chemotherapy regimens, but results obtained so far are not satisfactory [46]. From a histopathological point of view, two types of cancer are the most frequent: esophageal squamous-cell carcinoma (ESCC) and esophageal adenocarcinoma (EA), with some differences in geographic prevalence and risk factors [47]. A well-known precursor lesion for EA is Barrett's esophagus (BE). BE is a condition where the normal squamous epithelium of the esophagus is replaced by columnar epithelium of intestinal appearance. BE is currently diagnosed based on the presence of goblet cells of the intestinal type within columnar epithelium [47]. There is currently no evidence that BE screening effectively reduces EA incidence and mortality; nevertheless in 2008 the American Gastroenterological Association Institute recommended performing screening and surveillance in patients with chronic gastroesophageal reflux disease symptoms. Although endoscopic surveillance might lead to early diagnosis, biomarkers would be more useful, as they would allow the measurement of distinct molecular alterations within the tissue. Among these alterations, the best characterized is p53. The immunohistochemical detection of p53 shows correlations with tumor progression and has been validated in big cohorts of patients. The technical easiness of detection makes it a promising biomarker to be translated into clinical practice (reviewed in [48]).

The expression and prognostic role of hERG1 was investigated in ESCC by means of IHC and RT-PCR [26]. *KCNH2* gene and hERG1 protein were found to be expressed in a high percentage of ESCC samples (with respect to normal esophageal squamous epithelium) but no correlations emerged with clinicopathological features. The authors showed that survival rates of hERG1-positive patients were shorter than hERG1-negative patients [26].

A similar approach was applied to the study of EA samples. In particular, the expression of hERG1 protein was detected in BE-derived adenocarcinomas [11].

In 2006, we published the results of a multicentric study [11] showing that hERG1 is overexpressed in the majority of BE samples (69%) while it is absent in normal esophageal mucosa as well as samples taken from patients affected by esophagitis. It was also shown that hERG1 expression is switched on at early stages of BE cancerogenesis and it is also highly expressed in dysplasias and BE-derived adenocarcinomas, thus characterizing both early and late steps of esophageal cancerogenesis [11].

hERG1 channel expression also shows a significant association with malignant progression towards adenocarcinoma, since 89% of BE patients who developed EA were positive for hERG1 protein expression [11]. On the whole, hERG1 channels might identify high-risk BE patients and could therefore be useful for endoscopic surveillance of BE patients in order to ensure a better follow-up and early EA diagnosis.

4. Gastric Cancer

Gastric cancer (GC) is the third commonest cause of specific death worldwide and 5-year survival is less than 30% [20, 21]. Many risk factors were investigated, including dietary regimen, smoking habits, alcohol consumption, genetic predisposition, and *Helicobacter pylori* chronic infection [48, 49]. The management of patients without distant metastases is pivoted on surgical resection, although recent clinical trials include perioperative chemotherapy or radiochemotherapy especially in cancer arising from the esophagogastric junction [48]. As many other gastrointestinal cancers, the multimodal management is guided by a correct preoperative TNM stage definition [45]. From a histopathological point of view, about 90% of GCs are classified as adenocarcinomas divided into two subtypes according to Lauren's classification, the intestinal and diffuse type showing different biological and etiological characteristics.

Recently, biomolecular patterns of GC were investigated, including E-cadherin, VEGF, and microsatellite instability [50]. The purpose of these studies was to develop new targeted therapies to improve the poor prognosis achieved by standard chemotherapy. To date, the only clinical trials available are those employing Trastuzumab (with chemotherapy) in HER2-positive advanced GC [51].

hERG1 channels have been proven to be expressed in GC cell lines and primary GCs. In GC cell lines it was shown that hERG1 regulates tumor proliferation [27] and that treatment with hERG1 specific blockers and siRNA impairs tumor growth [29, 52]. hERG1 expression was demonstrated also in primary GCs where it correlates with grading, TNM stage, and serosal and venous invasion [30, 31]. More recently, through an IHC-based study in a wide cohort of GC patients it was demonstrated that hERG1 channels are overexpressed in gastric adenocarcinomas, especially in those of Lauren intestinal type [31]. hERG1 expression also correlated with grading, TNM stage, and VEGF-A expression. Moreover, in GC cell lines it was shown that hERG1 modulates VEGF-A secretion through an AKT-dependent pathway [31]. Even more interestingly, by treating xenografted cancers with a combination of hERG1 blockers and Bevacizumab the effect was greater than that obtained with single-agent treatment [31].

Overall, data gathered so far are still contradicting, since Ding and colleagues proposed hERG1 as an independent prognostic factor [30] while in our series hERG1 identifies high-risk T1 patients [31] but is not an independent prognostic factor. It should be pointed out that the cohort analyzed in our study was bigger than the one analyzed by Ding and colleagues and it was composed of Italian patients, who display different characteristics than Asian patients. Moreover, a different antibody and scoring method was applied and this could account for differences in the obtained results.

In primary GC it was also demonstrated that hERG1 channels are expressed in the early stages of the disease (manuscript in preparation and [52]). By means of IHC we showed that hERG1 channels are expressed from the early steps of GC progression (gastric metaplasia) and that such expression is maintained during all the phases of the cancerogenic process [52].

Overall, the detection of hERG1 expression in gastric dysplastic lesions could therefore represent a novel prognostic marker of progression towards gastric adenocarcinoma of the intestinal histotype.

5. Colorectal Cancer

Colorectal cancer (CRC) is the fourth most common cause of death for cancer worldwide, with a 5-year survival rate higher than 60% taking into account CRC encompassing all the pathological stages [20, 21]. The prognosis of CRC patients has been consistently improving during the last decades due to many important developments in prevention, early diagnosis, and therapy. For example, the widespread screening colonoscopy has led to reduced cancer incidence (for benign or preneoplastic adenomas removal) and mortality (due to early diagnosis) [53]. TNM staging system is highly correlated with prognosis, with a 5-year survival of 90% for patients in earlier stages to less than 25% for those with metastatic disease [53]. The cornerstone of therapy is represented by en bloc surgical resection of tumor and regional nodes, although perioperative chemotherapy is mandatory in subjects with advanced disease and metastasis.

The most frequent histological subtype is adenocarcinoma, accounting for more than 95% of the cases and the molecular pathogenesis of colorectal cancer has been widely studied. The molecular sequence from adenoma to invasive cancer is well established, with the identification of misexpression and mutation of several genes (including rare inherited syndromes). Many molecular targets are currently used for prognostic and predictive purposes. In particular *k-ras* mutation profile is used to refine prognosis and to select patients who will benefit from treatment with anti-EGF-R antibodies. For therapy purposes, anti-VEGF-A antibodies have been employed in addition to standard chemotherapy agents. Despite all the efforts, the prognosis of patients with advanced stage disease has not been significantly improved [54, 55].

hERG1 protein is highly expressed in colorectal adenocarcinomas with respect to hyperplastic lesions of the colon [11] and in CRC cell lines [11, 33] and it was demonstrated that the protein is not expressed in small adenomas and sigma diverticulitis [56]. In CRC cell lines, a correlation between invasive phenotype and high hERG1 levels of expression has been shown [11] and proliferation assays demonstrated that treating the cells with a specific hERG1 blocker (E4031) reduced tumor growth [56]. The effects of a different hERG1 blocker (sparfloxacin, SPFX) were tested on colon cancer cells with a high hERG1 expression [57]. The authors showed that SPFX inhibits cell proliferation, migration, and apoptosis and a synergistic effect was observed treating the cells in combination with 5-FU [57].

In CRC cell lines it was also demonstrated that hERG1 channels modulate tumor progression by switching on a VEGF-A-dependent angiogenic pathway [33]. hERG1 expression was also evaluated in mouse models [58]. It was shown that colonic polyps of adenomatous polyposis coli ($Apc^{min/+}$) mice expressed the murine homolog of hERG1 (mERG1) and that treating the animals with a specific hERG1 blocker reverted polyps development [58]. Treating transgenic mice (overexpressing hERG1 in the colorectal mucosa) with a chemical carcinogen (Azoxymethane) resulted in an increased number of mucin-depleted foci and polyps [58].

Finally, in a cohort of primary nonmetastatic CRC samples it was shown that hERG1 expression was associated with Glut-1 (glucose transporter 1), VEGF-A, CA-IX (carbonic anhydrase IX), and EGF-R expression [34]. In a multivariate model, TNM, hERG1, and Glut-1 turned out to be prognostic factors [34]. Moreover, hERG1 presence associated with Glut-1 absence represents an independent negative prognostic factor in TNM I and II colorectal adenocarcinomas [34].

On the whole, data gathered so far in CRC cell lines, primary CRCs, and mouse models indicate that hERG1 has a role in CRC cancerogenesis that can be traced back to the regulation of VEGF-A signaling pathway [33, 34, 59]. Moreover, hERG1 has a prognostic value in CRC [47] and all these data stress the necessity of including hERG1 blocking therapeutic strategies in CRC treatment schedules.

6. Pancreatic Cancer

Pancreatic cancer (PC) is responsible for 6.8% of all cancer-related deaths [22]. PC incidence and mortality have been steady in the last 20 years, and despite recent efforts to optimize treatments, 5-year survival rate is still poor (6.7%) [22]. Several risk factors for PC have been described. 20% of PC is likely to be induced by cigarette smoking [21]. It has been shown that a family history of PC also increases the risk of developing PC [59] as well as a personal history of chronic pancreatitis, obesity, and diabetes [21]. From a histopathological point of view, 90% of PC is classified as ductal adenocarcinomas (PDAC), with the other histotypes accounting all together for the remaining 10%.

Unfortunately, currently there are no screening detection methods and the vast majority of PC is diagnosed when the disease has already spread beyond the pancreas. For these reasons, surgery and radiochemotherapy are used as treatment options but they can be curative only in a small percentage of patients. For PC patients presenting with advanced disease, chemotherapy with Erlotinib plus gemcitabine has been used, with a slight survival improvement.

Although recently several studies have been performed aimed at identifying novel prognostic and predictive molecular biomarkers, none of them can be included in routine clinical use yet [60].

Recently, it was demonstrated that hERG1 channels are highly expressed in primary PC and PC cell lines [12]. In PC samples, hERG1 expression was correlated with lymph node

involvement, grading, and TNM stage I [12]. The authors also showed that hERG1 is a target of miR-96 (microRNA-96, which is downregulated in PC) and that miR-96 overexpression regulates hERG1 expression hence significantly inhibiting PC cells malignant behavior.

We recently showed that hERG1 channels are overexpressed in human PC samples of the ductal type (PDAC) and correlate with EGF-R [61]. Moreover, blocking hERG1 in PDAC cells reduces cell growth and migration and we demonstrated that PDAC patients with high hERG1 expression had a worse prognosis.

7. Other Cancers

7.1. Endometrial Cancer. Endometrial cancer (EC) is nowadays the most common gynecologic malignancy and the most frequent among infiltrating tumors of the female genital tract, especially after menopause. About 75% of the cases affect *corpus uteri* and 15–20% of these show relapse and do not respond to systemic therapy [62]. Approximately 70 to 80% of EC patients have a localized disease that is treated by surgery alone; nevertheless roughly 30% of the patients will die from the disease. In this scenario, it appears clear that EC is a heterogeneous disease and novel biomarkers are urgently needed. This will allow better stratifying EC patients and ensuring the best treatment options. Biomolecular research has identified new targets for EC therapy such as mTOR (mammalian target of rapamycin, in particular for type I EC), p53 and HER-2 (human epidermal growth factor receptor 2, especially in type II EC), VEGF (vascular endothelial growth factor), and EGF-R (epidermal growth factor receptor) (reviewed in [63]). A recent report summarized data concerning new molecular markers in EC pointing out the relevance of several markers (p53, aneuploidy, HER-2, estrogen receptors, progesterone receptors, and Stathmin) [64].

The first paper demonstrating the expression of hERG1 potassium channels in human primary cancers was conducted on EC samples [9]. In such paper it was demonstrated that *hERG1* mRNA can be detected in human tissues by endpoint RT-PCR (Retrotranscription-Polymerase Chain Reaction) as well as by IHC and is more frequently expressed in human neoplastic tissues compared to normal endometrium and hyperplastic lesions [9]. Furthermore, patch clamp analysis indicated that functional hERG1 proteins are expressed on the cell surface of EC cells. This paper opened the way for further investigation of hERG1 expression in clinical samples, although the analysis was carried out on a small group of EC patients.

7.2. Ovarian Cancer. Ovarian cancer (OC) represents the leading cause of death among gynecologic malignancies, despite recent efforts in surgical and chemotherapy treatments. Currently, 5-year survival for OC is 44.6% [22]. The gold standard for OC treatment is surgery usually followed by chemotherapy [21]. Currently, there is no screening test for OC early detection although pelvic examination associated with transvaginal ultrasound and CA-125 (Cancer Antigen 125) evaluation in blood samples can be proposed to high-risk women, in particular those who have a family history positive for BC and OC.

Several molecules have been proposed as tumor markers for OC. The most used serum marker is CA-125 but many others have been tested as well (such as HE4 (human epididymal protein E4), Kallikreins, Osteopontin, Claudins, and VCAM-1 (vascular cell adhesion molecule 1)) (reviewed by [65]). Among epigenetic aberrations that might be used as biomarkers, the best characterized is *BRCA1* hypermethylation which leads to the absence of mRNA and protein and correlates with poor outcome [66]. Since VEGF-A and VEGFR-2 (vascular endothelial growth factor receptor 2) expression might be associated with reduced survival, a phase III clinical trial employing Bevacizumab in recurrent platinum-resistant OC patients (AURELIA Trial) was designed and such treatment resulted in a significant improvement of PFS (progression-free survival) and ORR (objective response rate) [67].

On the whole, despite the wide spectrum of serum biomarkers identified, unsatisfactory clinical results have been achieved. For these reasons, search for new biomarkers is mandatory.

A few studies have been performed to evaluate hERG1 expression and role in OC. A paper published in 2010 [13] demonstrated that hERG1 is expressed in OC cell lines and primary samples but no associations with survival emerged. More recently, a methylation profile for clear-cell OC was defined [25]. Among the nine genes investigated the authors included hERG1 potassium channel and they showed that the gene was methylated and hence its expression in the tumor tissue was lower, indicating epigenetic silencing. Those results, although obtained in a small set of OC samples, might indicate that that loss of hERG1 expression by methylation could represent a potential prognostic marker.

8. Concluding Remarks

Despite the improvements in surgical techniques and chemotherapy schedules, the treatment of solid cancers is still a big challenge for surgeons and oncologists. Therefore, targeted therapy represents the best opportunity for the treatment of patients not responding to classical approaches. Data gathered so far suggest that hERG1 channels could be used as biomarkers since they are frequently overexpressed in solid cancers and such expression associates with clinicopathological features in different tumors. A reliable monoclonal antibody for hERG1 protein is available and evaluation scores have been optimized in different solid cancer, thus making the detection of the channel easy for pathologists. Moreover, being a transmembrane protein, hERG1 is easily accessible and might be targeted by several small molecules that might be associated with the treatment with drugs already used in the clinical settings, contributing to lower costs of cancer patients' treatment. Moreover, anti-hERG1 antibody and its derivative scFv (Single Chain Variable Fragment) might be conjugated with drugs for treatment. Different strategies might be applied such as targeting specific conformations of hERG1 channels and using new molecular tools aimed at

decreasing hERG1 expression in tumor cells only to decrease channel expression in selected cancer types (for a more detailed review see [19, 68]).

Conflict of Interests

The authors declare they have no conflict of interests.

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