Aus der Klinik und Poliklinik für Frauenheilkunde und Geburtshilf

des Klinikums der Ludwig-Maximilians-Universität München

Vorstand/Direktor: Prof. Dr. med. Sven Mahner

Potential new biomarkers for breast cancer------ Vitamin D receptor (VDR) and BRCA1 proteins

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität zu München

vorgelegt von

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Jahr 2018

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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I dedicate this work to my family.

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1. Abbreviations

BC	Breast cancer
ER	Estrogen receptor
PR	Progesterone receptor
HER2(ErbB2)	Human epidermal growth factor receptor 2
SERMs	Selective ER modulators
TAM	Tamoxifen
SERDs	Selective ER down-regulators
AIs	Aromatase inhibitors
EGFR	The epidermal growth factor receptor
RTKs	Receptor tyrosine kinases
TNBCs	Triple-negative breast cancers
GEP	Gene expression profiling
TKIs	Tyrosine kinase inhibitors
uPA	Urokinase plasminogen activator
PAI-1	Plasminogen activator inhibitor 1
FFPE	Formalin-fixed, paraffin-embedded
qRT-PCR	Quantitative real-time reverse transcriptase polymerase chain reaction
RS	Recurrence score
IHC	Immunohistochemistry
EGTM	European Group on Tumor Markers
PPARs	Peroxisome proliferator-activated receptors
VDR	Vitamin D receptor
RXR	Retinoid X receptors
BRCA1	Breast cancer 1
NLS	Nuclear localization signals
SQ	Serine-glutamine
BRCT	C-terminal BRCA1
LOH	Loss of heterozygosity
IF	Immunofluorescence
CTCs	Circulating tumor cells
FDA	Food and Drug Administration

2. Publication list

Fluorescence analysis of vitamin d receptor status of circulating tumor cells (CTCs) in breast cancer: From cell models to metastatic patients

Zhang, X.; Hofmann, S.; Rack, B.; Harbeck, N.; Jeschke, U.; Sixou, S.

Int. J. Mol. Sci. 2017, 18(6), 1318; doi: 10.3390/ijms18061318

Impact of Etoposide on BRCA1 Expression in Various Breast Cancer Cell Lines

Zhang, X.; Hofmann, S.; Harbeck, N.; Jeschke, U.; Sixou, S.

Drugs R D. 2017 Sep 6. doi: 10.1007/s40268-017-0208-6.

Influence of vitamin D signaling on hormone receptor status and her2 expression in breast cancer

Zhang, X.; Harbeck, N.; Jeschke, U.; Doisneau-Sixou, S.J Cancer Res Clin Oncol. 2017 Jul; 143(7):1107-1122. doi: 10.1007/s00432-016-2325-y

Role of Plasminogen Activator Inhibitor Type 1 in Pathologies of Female Reproductive Diseases

Ye,Y; Vattai, A; **Zhang, X.**; Zhu, JY; Thaler, CJ; Mahner, S; Jeschke,U; Schönfeldt, V;

Int. J. Mol. Sci. 2017, 18(8), 1651; doi: 10.3390/ijms18081651

3. Confirmation of co-authors

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dean's Office
Medical Faculty

Cumulative Dissertation

Confirmation pursuant to § 4a Paras. 3 and 5 Doctoral Degree Regulations for Dr. med., Dr. med. dent. and Dr. rer. biol. hum.
and
pursuant to § 7 Para. 4 Doctoral Degree Regulations for Dr. rer. nat. at the Medical Faculty
Please note: for each published article, a separate "Cumulative Dissertation" form has to be submitted!

Xi Zhang
Name of doctoral candidate

Fluorescence Analysis of Vitamin D Receptor Status of Circulating Tumor Cells (CTCS) in Breast Cancer: From Cell Models to Metastatic Patients
Title of publication

International Journal of Molecular Science Vol. 18, Iss. 6: 1318, 2017
Journal (Name, issue, year, etc.)

I hereby confirm that none of the articles submitted for this doctoral degree have been the subject of another (current or completed) dissertation.

Signature of doctoral candidate

By signing, the following **co-authors** confirm that:

- the extent of their contributions (content-related and volume) in the publications submitted,
- their agreement to the submission of the publications, and
- the article in question is not the subject of another (current or completed) dissertation.

Name of co-author	Extent of contribution (content-related and volume)	Signature of co-author
1. Simone Hofmann	Assisted with the cell culture and techniques	
2. Brigitte Rack	Provided the concept and substantially contributed to manuscript editing	
3. Nadia Harbeck	Provided the concept and substantially contributed to manuscript editing	
4. Udo Jeschke	Conceived, designed the project and substantially contributed to manuscript editing	
5. Sophie Sixou	Supervised the research; Conceived, designed the project and substantially contributed to manuscript editing	

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Impact of Etoposide on BRCA1 Expression in Various Breast Cancer Cell Lines
Title of publication

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4. Sophie Sixou	Supervised the research; Conceived, designed the project and substantially contributed to manuscript editing	
5.		

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4. Introduction

4.1 An overview of breast cancer

Breast cancer (BC) is a significant global public health issue and the leading cause of death among women around the world. In 2012, it alone comprised 25% of all cancer cases and 15% of all cancer deaths among females [1], making it the most common female cancer. Female BC incidence rates vary in different districts, with 10-fold higher rates in Western Europe and United States than in Africa and Asia [2]. The international variations in incidence rates largely originate from differences in reproductive and hormonal factors and the availability of early detection services [3]. Although mortality rates decline in the historically higher-rate countries, they continue to increase in lower-rate countries, possibly due to changes in risk factors, as well as limited access to early detection and treatment [4, 5]. Conventional therapeutic options in BC are surgery (local), radiation therapy (locoregional), endocrine therapy or other targeted therapies and chemotherapy (systemic), often in combination or consecutive. Besides, recent and future therapy regimes are more and more focused on individual and personalized treatment options. Herein, a greater understanding of the underlying biology of BC has resulted in the development of novel therapeutics such as tyrosine kinase inhibitors (TKIs), inhibitors of intracellular signaling pathways, angiogenesis inhibitors, and agents that interfere with DNA repair [6, 7]. Of note, BC is a complex and extremely heterogeneous disease, with resistance to treatment, recurrences and/or metastases which are still puzzling clinicians in current BC therapy [8, 9].

4.2 Subtypes of tumors

The BC patient's and tumor's clinicopathological features determined traditional classification of BC, such as tumor size, lymph node involvement, histological grade, patient's age, estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2 or ErbB2) status. Tailored therapy based on these clinicopathological features, used in routine, may still have limitations. Therefore, these clinicopathological features will be completed by combining genomic information to estimate recurrence risk (prognostic value) and to predict therapeutic efficacy (predictive value) [10]. There has been extensive effort to unravel the molecular drivers of this disease,

which has led to the classification of BC into at least five intrinsic subtypes associated with patient survival, based on gene expression patterns: luminal A, luminal B, basal-like, Her-2 positive and normal breast-like [11-13]. Yet, this classification system has not been incorporated in clinic practice at the moment [14], and traditional biological features continue to provide an important guide to appropriate therapy for individual patients.

4.2.1 Biological subtypes of tumors

Based on tumor expression of the ER, PR and HER2, three broad phenotypes of BC are commonly used in clinical practice: ER positive, HER2 positive, and triple negative (TNBC, characterized by lack of expression of ER, PR, and HER2) [15].

4.2.1.1 ER positive BCs

The role of estrogen in breast carcinogenesis and cancer progression has been already clearly established as early as in 1896 since Beatson's first clinical observation of the antitumor effect of ovariectomy in a BC patient [16]. The effects of estrogen on proliferation are mainly mediated by their interaction with the ER. Estrogens drive the proliferation of mammary epithelial cells and therefore promote the growth of ER positive BC. Approximately 75% of BCs express nuclear staining of ER and 55% of PR [17, 18]. PR positivity can be considered as a surrogate for ER positivity due to the expression of the PR requiring functioning ER [15]. At the gene expression level, ER positive BC mainly composes Luminal A and Luminal B subtypes. ER positive tumors are more likely to be smaller, lymph node negative and low grade compared to ER negative tumors [18]. The hormone therapies used to treat ER positive BC are designed to antagonize the mitogenic effects of estrogens and include: selective ER modulators (SERMs) such as tamoxifen (TAM) that bind to ER and act as antagonists in the breast [19]; selective ER down-regulators (SERDs) such as fulvestrant that bind to and target ER for degradation in any tissues [20]; and aromatase inhibitors (AIs) that inhibit the activity of aromatase (CYP19A1) and consequently suppress the peripheral production of estrogen [21].

4.2.1.2 HER2 positive BCs

The epidermal growth factor receptor (EGFR) consists of four closely related receptor tyrosine kinases (RTKs): EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4) [22, 23]. This

family of receptors functions primarily through a process of signal transduction [24]: the ligand binding to the receptors induces homo- or hetero-dimerization, activates the kinase domain, then activates down-stream signaling such as the Ras/MAPK and PI3K/AKT signaling pathways [25]. Aberrant signal transduction through the EGFR family of RTKs, is a common feature of many types of solid tumors [26]. HER2, a transmembrane protein RTK, eventually hetero-dimerizing with ErbB3 [27] is an oncogenic driver of the growth of HER2-positive BC. Either E*erB-2* gene amplification or HER2 protein overexpression was independent of all other prognostic factors in BC [28]. At the gene expression level, most HER2 positive BC belong to HER2 enriched subtype whereas not all molecularly HER2 positive tumors are HER2 overexpressed and HER2 positive BC can be found in all intrinsic subtype [13]. In addition, HER2 positive BC are associated with younger age, high nuclear grade, more lymph node involvement and negative hormone receptor status [29, 30]. Approximately 15–20% of breast carcinomas are HER2-positive (HER2+), and half are ER negative [31, 32] which is generally considered to be a poor prognostic marker associated with more aggressive disease and a higher risk of metastasis. Nonetheless, the approval of newer HER2-targeted agents, e.g. trastuzumab, succeed in the last 10 years in improving the prognosis of these patients [33].

4.2.1.3 Triple-negative BCs (TNBCs)

Triple-negative breast cancers (TNBCs) represent 15-20% of all BCs [34], and are defined by a lack of ER, PR, and HER2 expression, resulting in limited treatment options. TNBC are more aggressive, affect younger women, than ER or HER2 positive BC, and are higher in incidence among patients with germline BRCA1 mutations [35, 36], or of African ancestry [37]. At the gene expression level, TNBCs overlap with the basal-like subtype, but not synonymous [38]. In addition, TNBC have demonstrated both a higher rate of recurrence and a worse clinical outcome compared to the other subtypes of BC, presenting larger tumors and more likely to be III grade [39]. Due to the lack of well-defined clinical targets, limited treatment options are offered and standard chemotherapy, combined or not with radiation therapy, is currently the only treatment option for women with TNBC, and there are no available preventive drugs [40].

4.2.2 Molecular subtypes of BCs

In recent decades, global gene expression profiling (GEP) studies of BC have provided a more established molecular classification system and identified distinct clusters or intrinsic subtypes based on the quantitative expression of several genes (transcriptome profiles). By using the expression of a subset (n = 496) of differentially expressed genes in GEP study, Perou and colleagues were able to identify two main clusters appeared to be related to ER expression, which allows to classify BC in 4 main classifications: luminal (with further differentiation in A and B), basal-like, HER2-positive and normal breast-like type [11, 12, 41]. The ER positive cluster was enriched with ER, ER-related genes and other genes characteristic of the luminal epithelial cells, herein this class was termed as 'luminal'. The luminal cluster was further stratified into subclasses with at least two distinct subclasses reported in following studies: luminal A and luminal B subtypes. Compared with luminal B tumors, luminal A tumors express higher levels of ER and GATA3 that regulates luminal epithelial cell differentiation in the mammary gland [42], whereas luminal B tumors more often express HER1, HER2, and/or cyclin E1. Most studies indicated that luminal B tumors were associated with a worse prognosis than tumors of the luminal A class [12, 35]. The other major cluster with ER negative shows three distinct subclasses termed 'HER2 positive', 'basal-like' and 'normal breast-like'. The HER2 subgroup is characterized by overexpression of HER2 and other genes concerning to the HER2 amplicon. The basal-like class is dominated by TNBCs, characterized by positive expression of genes typical of myoepithelial/basal epithelial cells, such as basal cytokeratin. The normal breast-like class displays a triple-negative phenotype but has gene expression similar to patterns found in normal breast tissue samples. These so-called "intrinsic BC subtypes" provide the basis of a molecular taxonomy of BC and they exhibit special molecular characteristics as well as different prognostic impact [43, 44].

As intrinsic subtypes are evolving, rarer subtypes, such as claudin low and molecular apocrine, have been identified [45-47]. To date, these subtypes have not been validated for clinical routine use [48].

4.3 Traditional and emerging biomarkers of BCs

Biomarkers play an essential role in the management of BC patients. The key point to significantly increase the survival of the BC patients is the development of specific biomarkers for prognosis and prediction then identify effective targeted therapies. Even though many therapies for BC including

chemotherapy, endocrine therapy, and target therapies have made a significant contribution to the decrease in BC mortality in the past two decades, resistance to treatment such as anti-estrogen agents is a major clinical problem in current BC treatment. For example, about 25% of the patients with ER positive tumors receiving 5 years adjuvant tamoxifen therapy develop recurrent disease within 10 years, and 30-40% of the patients in advanced stage with ER positive primary tumors do not respond to anti-estrogen therapy [49]. To overcome the therapy resistance and recurrence/metastasis process, deeper understanding of established biomarkers and further exploration of more specific new biomarkers for BC are necessary. A good candidate biomarker for monitoring cancer should be directly correlated with molecular mechanisms of carcinogenesis in pre-cancerous tissues, differentially expressed in various BC populations and reflect response to drug effect. Once a biomarker has been identified, targeted agents may be performed in clinical trials within a specifically enriched patient population incorporating the predictive biomarker of clinical benefit. Since 2007, international guidelines (*e.g.*, St. Gallen, ASCO) have recommended to additionally use validated protein or gene expression tests reflecting the intrinsic tumor characteristics to improve the clinical risk stratification to avoid under- or overtreatment based on clinicopathological parameters [50, 51]. Several markers have shown evidence of clinical usefulness and have been recommended for use in practice in BC patients including some established traditional markers like ER, PR, HER2, novel protein markers like urokinase plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI-1) and some molecular markers based on certain multigene assays (e.g. Oncotype DX, PAM50 and MammaPrint) [10].

4.3.1 Molecular markers assays

4.3.1.1 MammaPrint

MammaPrint ® (70-gene signature by Agendia, the Netherlands) was one of the first described mRNA-based multigene tests for predicting outcome in patients with BC [52]. The prognostic impact and guide to the administration of adjuvant chemotherapy of MammaPrint for both lymph node-negative and lymph node-positive patients (1–3 involved nodes) has been extensively recommended [53-56].

4.3.1.2 Oncotype DX

The Oncotype DX test (Oncotype DX™ Breast Cancer Assay by Genomic Health, USA) which is performed on paraffin-embedded and formalin-fixed (FFPE) breast tumor tissue is most advanced and widely used in clinical practice. This test analyses the expression of 21 genes (16 tumor-related and 5 housekeeping) at the mRNA level using a quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) [57, 58]. Oncotype DX recurrence score (RS) may help determine prognosis and aid decision-making with respect to administration of adjuvant chemotherapy in newly diagnosed ER-positive but HER2-negative BC patients with lymph node–negative invasive disease. In addition, Oncotype DX may be considered for identifying HER2-negative, ER-positive patients with 1–3 involved lymph nodes for treatment with adjuvant chemotherapy [53].

4.3.1.3 PAM50

Although the first definition of the intrinsic subtypes occurred almost two decades ago, an assay suited for diagnostic use was introduced recently: PAM50 (Prosigna™ Breast Cancer Prognostic Gene Signature Assay: Nanostring Technologies, USA). The test assigns each sample to the intrinsic subtype and predict the risk of relapse for ER positive patients, with the potential to be informative for identifying women who can benefit from adjuvant treatment [59, 60]

Besides three biomarkers mentioned above, there are increasing numbers of molecular markers that are already commercially available (e.g. Endopredict®, MapQuant Dx®, Breast Cancer Index and 76 gene Rotterdam signatures), or are in pre-clinical development or show promise from the research setting.

4.3.2 Protein markers assays

4.3.2.1 IHC4 assay

Besides ER, PR and HER2, Ki67 is a proliferative marker used to predict tumor growth rate, which has been shown to be a prognostic biomarker with predictive ability in the adjuvant context [61, 62]. Therefore, a combination of the 4 immunohistochemistry (IHC) based biomarkers (ER, PR, HER2 and Ki67) analyses could easily be performed on tumor biopsy tissues using FFPE tumor biopsy specimens, with a final algorithm calculating a risk score for distant recurrence, as a predictor of risk of distant recurrence in BC [63-66].

4.3.2.2 uPA/PAI-1 assay

With the ability to promote cancer progression, uPA and its inhibitor, PAI-1 are presently amongst the best validated prognostic biomarkers for BC. According to updated the European Group on Tumor Markers (EGTM), levels of uPA and PAI-1 protein levels may be combined with established factors for assessing prognosis and identifying ER-positive, HER2-negative and lymph node–negative BC patients that are unlikely to benefit from adjuvant chemotherapy. However, due to the requirement for fresh–frozen tissue by a validated ELISA measurement, uPA or PAI-1 not widely used for clinical purposes [10, 53, 67].

Of note, although massive time and money were invested into the development of new BC biomarkers, there are still only three biomarkers (ER/PR/HER2) for all BC patients predicting the benefit from corresponding therapies (endocrine therapy and anti-HER2 targeted therapy). In addition to these mandatory biomarkers, multianalyte tests, such as uPA/PAI-1, Oncotype DX, MammaPrint, Prosigna, may be performed in specific subgroups of BC patients [53].

4.3.3 Potential and promising biomarkers

4.3.3.1 Vitamin D receptor VDR

Besides classic steroid hormone receptors such as ER and PR, some nuclear receptors like retinoid and thyroid hormone receptors, peroxisome proliferator-activated receptors (PPARs) and vitamin D receptor (VDR) as members of the thyroid–retinoid receptor family of ligand-activated transcription factors, also exert profound and complex effects in the etiology of BC [68, 69]. VDR, which is found in normal breast tissue and in breast tumors [70], interacts with its ligand calcitriol, the active form of vitamin D, to modulate the normal mammary epithelial cell genome and subsequent phenotype [71, 72]. The human VDR (hVDR) gene is localized in chromosome 12q12-14, and consists of multiple promoter regions (A–C) followed by the coding region spanning exons 2 through 9 [73, 74]. Calcitriol, the most active vitamin D metabolite, acts similarly to classical steroid hormones: Ligand-bound VDR-calcitriol heterodimerizes with its cognate co-receptor retinoid X receptors (RXR), interacting with specific nucleotide sequences (hormone response elements) of target genes and functions via both genomic and nongenomic pathways to regulate around 60 target genes expression and produce a

variety of biological effects [75]. These target genes are involved in diverse molecular pathways, thereby resulting in a wide range of calcitriol-mediated anti-cancer actions *via* autocrine and paracrine including anti-proliferation, anti-inflammation, induction of apoptosis, stimulation of differentiation, inhibition of invasion, metastasis and angiogenesis on various malignant cells [76-78], including mammary cells [76]. Several epidemiologic, mechanistic and experimental data support the concept that optimal vitamin D status has a protective effect against development of BC [79-86], and that specific distinct signaling pathways can inhibit BC growth on 3 broad phenotypes (ER+/HER2+/TNBC) of BC through calcitriol [72]. For ER positive BC, VDR mediated pathways reduce estrogen synthesis and down-regulate ER levels, thus attenuating the stimulus of estrogen on BC cells, leading to significant inhibition of BC cells proliferation [87-89]. In HER2 positive BC, vitamin D-mediated VDR signaling reduced HER2-regulated downstream signaling in both *in vitro* and *in vivo* HER2-amplified mammary tumors, determined by down-regulation of the phosphorylation of HER2, AKT and ERK, and inhibited the expression of cyclin D1 as a downstream molecular target of cell proliferation. But the specific mechanism for the signaling suppression is still unknown [90, 91]. Recently, separate pre-clinic studies raised the possibility of developing novel VDR-targeted therapies for TNBC [92-95].

We therefore believe that VDR exploration is very relevant to evaluate its potential as a new prognostic biomarker and therapeutic target in BC.

4.3.3.2 Circulating tumor cells (CTCs)—'liquid biopsy'

To perform the therapeutic monitoring, frequent tumor inspect is needed, but it is not always possible due to tumor inaccessibility in cancer patients. 'Liquid biopsy' appears to be a promising approach to overcome this problem, providing the testing of circulating tumor cells (CTC) and/or tumor-specific circulating nucleic acids. CTCs circulate in the peripheral blood of patients with solid malignancies and are shed from an existing primary tumor or from metastatic lesions into the blood stream [14]. CTCs detected in BC patients are significantly associated with a poor outcome in both early and metastatic tumors [15–19]. In metastatic patients, several tumor lesions may potentially release CTCs which therefore comprehensively reflect tumor and metastasis characteristics. CTCs can be collected *via* a simple venipuncture; this 'liquid biopsy' achieves the repeatable and real-time monitoring of tumor cell

characteristics. It is a less invasive and cost-effective alternative to tissue biopsies [20], despite the fact that technical and conceptual advances are still necessary before this 'liquid biopsy' can be routinely used for the diagnosis, characterization, monitoring, and treatment optimization of cancer. CTCs are a promising marker, providing important predictive and prognostic information in both early and metastatic BC. They may help to assess the response to treatment and to detect early disease recurrence [21]. At the moment, the CellSearch® system for CTC enumeration is the only accepted standard by the Food and Drug Administration (FDA). Only a few studies have investigated HER2 and/or ER expression on CTCs, even though, as discussed ealier, HER2 and ER are currently the only validated predictive factors used for therapy decision making in BC [22]. In conclusion, the characterization of CTCs may be a major tool to support diagnosis, and should be included in clinical trials for the evaluation of new targeted therapies [23]. Detection of biomarkers changes in CTCs during treatment that resulted in resistance, is the promising way to shift the way of therapy as well as to find new therapeutic targets. In order to better predict disease progression and personalize treatment, new prognostic and predictive factors are needed. So far, studies on VDR status in CTCs are still lacking. Therefore, the evaluation of VDR expression on CTCs in BC patients could potentially help in individualizing BC Therapy.

4.3.3.3 BRCA1—tumor suppressor

Breast cancer 1 (*BRCA1*) is a susceptibility gene responsible for hereditary predisposition to BC. Since it was first found to encode a DNA repair enzyme involved in BC susceptibility in 1990 [96] and subsequently was successfully cloned in 1994 [97], *BRCA1* has received a great deal of attention in BC. It has been mapped to chromosome 17q21 containing 24 exons, encoding a pleiotropic full-length protein of 1863 amino acids in humans [97]. BRCA1 full-length form is the best-defined *BRCA1* gene products that contains multiple functional domains, including a highly conserved N-terminal RING domain, two nuclear localization signals (NLS) located in the exon 11, a serine-glutamine (SQ) cluster between amino acids 1280–1524 [98], and tandem C-terminal BRCA1 (BRCT) domains [99-101]. BRCA1 is a serine phosphoprotein regulated in a cell cycle-specific manner [102] and hyper phosphorylated in response to DNA damage [103-106]. As tumor suppressor, BRCA1 mediates many different molecular processes including double strand (DS) DNA breaks repair, transcriptional

activation, apoptosis, cell-cycle checkpoint control, and chromosomal remodeling, binds different functional proteins (c-myc, E2F, p53, RAD50, cyclins, CDKs, RNA polymerase etc.), and suppresses development of BC and ovarian cancers [107-110]. In addition, perhaps the most interesting among BRCA1 protein-protein interactions in mammary epithelial cells is the one with the ER [111].

Therefore, genomic sequencing of BRCA1 (and BRCA2) in women with a familial history of one or more incidences of early onset BC or ovarian cancer provides a powerful tool to detect disease predisposition. However, the genomic test is expensive and not be suitable for detection of sporadic cancers associated to somatic events. Overall, about 9.3% of female BC patients carry predisposing mutations [112]. Germline mutations of *BRCA1* and *BRCA2* are responsible for about 50% of hereditary BC [113, 114]; nevertheless these mutations account for only 3–8% of all BCs. Most BC are sporadic and occur in absence of BRCA1 mutations [115, 116]. In sporadic breast tumors, many researchers postulated that loss of heterozygosity (LOH) reduces BRCA1 mRNA and protein levels, induces incorrect subcellular localization [117-120] and impaired methylation of the BRCA1 promoter region [121-123]. These events lead to noticeable loss of BRCA1 function and provide evidence for BRCA1 tumor suppressor function in sporadic forms [124]. Then, besides BRCA mutated BC, sporadic cancers may exhibit a so-called 'BRCAness" feature, as they display a BRCA1 mutation phenotype without any mutation [125-128]. Nonetheless, BRCAness is generally associated with mutations of other genes of the same signaling pathway. Meanwhile, next to its involvement in the tumor suppressing process, BRCA1 is also considered a key player in establishing chemotherapy sensitivity and could thus be considered as a predictive factor for patient management [65]. In preclinical and clinical studies, the role of BRCA1 in response to DNA-damaging agents and other types of chemotherapy agents has only partly been elucidated [129, 130], suggesting that BRCA1 could be useful as a predictive marker of response to different types of chemotherapy agents [131]. To the best of our knowledge, numerous studies have investigated the clinic pathological value of BRCA1 protein level or of its subcellular localization in clearly defined breast carcinomas, including sporadic and BRCA1 mutated tumors. Nonetheless, in spite of the findings concerning BRCA1 expression, the clinical value of its subcellular localization is still controversial, mostly attributable to limited techniques and approaches [117, 132-150]. Therefore, the use of BRCA1 as a promising biomarker

should be examined more completely in prospective clinical trials, not only in BC but also in other cancers where BRCA1 seems to play a role in the development of the tumors such as ovarian, prostate, and non-small cell lung cancer.

4.4 Aim of the study

4.4.1 VDR expression in various BC cell models and status in CTCs from metastatic BC patients (Publication 1 and Review)

VDR expressed in normal breast tissue and breast tumors has been suggested as a new prognostic biomarker in BC. Besides, increasing evidence supports the view that the detection of CTCs predicts outcome in early and metastatic BC. Consequently, an evaluation of VDR expression in the CTCs of BC patients may allow optimization of their treatment. As an attempt to profile and subtype the CTCs of metastatic patients, in publication 1, we describe an innovative triple fluorescence technique that we developed to simultaneously visualize the presence of cytokeratin (CK), absence of CD45, and expression of VDR. We first characterized BC cell models, before validating the preclinical data in CTCs from 23 metastatic BC patients. The data could be published in Publication 1 [151]. In parallel, we published a Review summarizing the data of the literature demonstrating the involvement of VDR signaling pathway in BC [72].

4.4.2 BRCA1 expression induced by etoposide in various breast cancer cell lines (Publication 2)

BRCA1, as a tumor suppressor, exerts an effective influence on protecting DNA integrity to suppress the development of BC. BRCA1 expression is induced in response to DNA-damaging agents, like etoposide. Our study was designed to explore if cytoplasmic *vs* nuclear (phosphorylated) BRCA1 protein levels could be considered as a potential predictive marker for response to chemotherapy in both sporadic and hereditary BC. To address this issue, in Publication 2, we evaluated BRCA1 status using immunofluorescence (IF) in a panel of cultured breast cell lines with specific properties. In addition, we used etoposide, as DNA-damaging reagent, to validate its effect on BRCA1 protein regulation, and shed light on BRCA1 expression patterns in representative cell line models of the different BC types with or without etoposide treatment. The data were published in Publication 2 [152].

5. Summary

5.1 Publication 1:

Fluorescence Analysis of Vitamin D Receptor Status of Circulating Tumor Cells (CTCS) in Breast Cancer: from Cell Models to Metastatic Patients

The Vitamin D receptor (VDR) expressed in normal breast tissue and breast tumors has been suggested as a new prognostic biomarker in breast cancer (BC). Besides, increasing evidence supports the view that the detection of circulating tumor cells (CTCs) predicts outcome in early and metastatic BC. So far, studies on VDR status in CTCs are still lacking. Consequently, an evaluation of VDR expression in the CTCs of BC patients may allow optimization of their treatment. As an attempt to profile and subtype the CTCs of metastatic patients, we established an innovative fluorescence technique using nine BC cell lines to visualize, define, and compare their individual VDR status. Afterwards, we tested the CTC presence and VDR expression in blood samples (cytospins) collected from 23 metastatic BC patients. The results demonstrated major differences in the VDR levels among the nine cell lines. CTC analysis from patient blood samples was then performed with an individual assessment of VDR expression on each isolated tumor cell. We detected CTCs in 46% of the patients and demonstrated heterogeneities of the VDR status, aggregation and size with a total of 42 CTCs individually analyzed. However, due to the limited number of patients in this study, no correlation between VDR expression and BC subtype classification (according to ER/PR/HER2 expression) could be determined, but our data support the view that VDR evaluation is a potential new prognostic biomarker to help in the optimization of therapy management for BC patients.

5.2 Publication 2:

Evaluation of BRCA1 expression by etoposide in various breast cancer cell lines

Breast cancer 1 (BRCA1), as a tumor suppressor, exerts an effective influence on protecting DNA integrity to suppress the development of breast cancer (BC). BRCA1 expression is induced in response to DNA-damaging agents, like etoposide. Germline BRCA1 gene mutations are associated with development of hereditary BC. However, besides BRCA mutated BCs, some sporadic cancers may also exhibit a BRCA-like phenotype, displaying so-called ‘BRCAness”. This common phenotype may

respond to similar therapeutic approaches as BRCA-mutated tumors and may thus have important implications for the clinical management of these cancers. In order to determine whether and how etoposide regulates the protein levels of BRCA1 in breast cancer cells, we exposed a panel of 5 selected cell lines to etoposide, compared the results to untreated control cells and then stained the cells with the specific, reliable and reproducible MS110 antibody directed against phosphorylated Ser1423 BRCA1. By evaluating cytoplasmic BRCA1 protein levels, we were able to distinguish 3 aggressive BC subtypes with BRCAness characteristics. In addition, determination of early and late apoptosis helped to complete the analysis of BRCA1 functions in the DNA damage pathway of aggressive BC. In conclusion, our study suggested that cytoplasmic BRCA1 protein levels could be considered as a potential predictive marker for response to chemotherapy in both sporadic and hereditary BC. Tumors with either BRCAness phenotype or germline BRCA1 mutation are both aggressive BCs associated with poor prognosis and could both be subjected to targeted therapies against BRCA1 mutated BC in future clinical management strategies.

5.3 Annex Review:

Influence of Vitamin D Signaling on Hormone Receptor Status and HER2 Expression in Breast Cancer

Breast cancer is a significant global public health issue. It is the leading cause of death among women around the world, with an incidence increasing annually. In recent years, there has been more and more information in the literature regarding a protective role of vitamin D in cancer. Increasingly preclinical and clinical studies suggest that vitamin D optimal levels can reduce the risk of breast cancer development and regulate cancer-related pathways. In this review, we focus on the importance of Vitamin D in breast cancers, discussing especially the influence of Vitamin D signaling on estrogen receptor and human epidermal growth factor receptor 2 (HER-2), two major biomarkers of breast cancer today. We discuss the possibility of actual and future targeted therapeutic approaches for vitamin D signaling in breast cancer.

6. Zusammenfassung

6.1 Veröffentlichung 1: Fluoreszenzanalyse des Vitamin-D-Rezeptor-Status von zirkulierenden Tumorzellen (CTCS) bei Brustkrebs: von Zellmodellen zu metastasierten Patienten

Der Vitamin-D-Rezeptor (VDR), der in normalen Brustdrüsengewebe- und Brusttumoren exprimiert wird, wurde als neuer prognostischer Biomarker bei Brustkrebs (BC) vorgeschlagen. Außerdem unterstützten zunehmende Anzeichen dafür, die Auffassung, dass die Detektion von zirkulierenden Tumorzellen (CTCs) das Ergebnis in der frühem und metastatischen BC prognostiziert kann. Bisher fehlen Studien zum VDR-Status in zirkulierenden Tumorzellen (CTCs) noch. Folglich kann eine Bewertung der VDR-Expression in den CTCs von BC-Patienten eine Optimierung ihrer Behandlung ermöglichen. Als Versuch, die CTCs von metastatischen Patienten zu profilieren und zu unterteilen, haben wir eine innovative Fluoreszenztechnik etabliert, die neun BC-Zelllinien verwendet, um ihren individuellen VDR-Status zu visualisieren, zu definieren und zu vergleichen. Anschließend testeten wir die CTC-Präsenz und VDR-Expression in Blutproben (Zytospins), die von 23 metastatischen BC-Patienten gesammelt wurden. Die Ergebnisse zeigten große Unterschiede in den VDR-Levels unter den neun Zelllinien. Die CTC-Analyse von Patientenblutproben wurde dann mit einer individuellen Beurteilung der VDR-Expression auf jeder isolierten Tumorzelle durchgeführt. Wir haben festgestellt CTCs in 46% der Patienten und demonstrierte Heterogenität des VDR-Status, Aggregation und Größe mit insgesamt 42 CTCs individuell analysiert. Aufgrund der begrenzten Anzahl der Patienten in dieser Studie konnte jedoch keine Korrelation zwischen VDR-Expression und BC-Subtyp-Klassifikation (gemäß ER / PR / HER2-Expression) festgestellt werden, aber unsere Daten unterstützen die Ansicht, dass die VDR-Evaluation eine potenzielle neue vorhersagende Biomarker zur Unterstützung der Optimierung des Therapiemanagements für die BC-Patienten ist.

6.2 Veröffentlichung 2: Bewertung der BRCA1-Expression durch Etoposid in verschiedenen Brustkrebszelllinien

Breast cancer 1 (BRCA1), als Tumorsuppressor, übt einen wirksamen Einfluss auf den Schutz der DNA-Integrität aus, infolgedessen die Entwicklung des Brustkrebses (BC) unterdrückt. Die BRCA1-Expression wird als Reaktion auf DNA-schädigende Mittel, wie Etoposid, induziert. Germline

BRCA1-Gen-Mutationen sind mit der Entwicklung des erblichen BC assoziiert. Allerdings neben den BRCA-mutierten BCs können einige sporadische Krebsarten auch einen BRCA-ähnlichen Phänotyp zeigen, der sogenannte "BRCAness" aufweist. Dieser gemeinsame Phänotyp kann auf ähnliche therapeutische Ansätze wie BRCA-mutierte Tumore reagieren und somit wichtige Auswirkungen auf die klinische Behandlung dieser Krebsarten haben. Um zu bestimmen, ob und wie das Etoposid die Proteingehalte von BRCA1 in Brustkrebszellen reguliert, haben wir eine Gruppe von 5 ausgewählten Zelllinien gegenüber Etoposid exponiert, die Ergebnisse mit unbehandelten Kontrollzellen verglichen und dann die gefärbte Zellen mit dem spezifischen, zuverlässigen und reproduzierbaren MS110-Antikörper gegen phosphoryliertes Ser1423 BRCA1 gerichtet hat. Durch die Bewertung der zytoplasmatischen BRCA1-Proteinslevel konnten wir 3 aggressive BC-Subtypen mit BRCAness-Charakteristiken unterscheiden. Darüber hinaus hat die Bestimmung der frühen und späten Apoptose dazu beigetragen, die Analyse der BRCA1-Funktionen im DNA-Schadensweg von aggressivem BC abzuschließen. Abschließend stellte unsere Studie vor, dass die zytoplasmatische BRCA1-Proteinslevel als potentieller prädiktiver Marker für die Reaktion auf eine Chemotherapie sowohl im sporadischen als auch im hereditären BC in Betracht gezogen werden könnten. Tumoren mit entweder BRCAness-Phänotyp oder Keimbahn BRCA1-Mutation sind die beide aggressive BCs, die mit einer schlechten Prognose assoziiert sind und könnten beider in zukünftigen klinischen Management-Strategien einer gezielten Therapie gegen BRCA1 mutierte BC unterzogen werden .

6.3 Review von Annex: Einfluss der Vitamin-D-Signalisierung auf den Hormonrezeptorstatus und die HER2-Expression bei Brustkrebs

Brustkrebs ist ein bedeutendes globales Thema der öffentlichen Gesundheit. Es ist die führende Todesursache bei Frauen auf der ganzen Welt, mit einer jährlich steigenden Inzidenz. In den letzten Jahren gab es in der Literatur und Veröffentlichung immer mehr Informationen über eine schützende Rolle der Vitamin D bei Krebs. Die Zunehmende präklinische und klinische Studien deuten darauf hin, dass das optimale Level der Vitamin D das Risiko für die Brustkrebs-Entwicklung reduzieren und Krebs-bezogene Wege regulieren kann. In dieser Review konzentrieren wir uns auf die Bedeutung der Vitamin D bei Brustkrebs und diskutieren vor allem den Einfluss der Vitamin-D-Signalisierung auf den Östrogenrezeptor und menschlichen epidermalen Wachstumsfaktor Rezeptor 2 (HER-2), die zwei

wichtige Biomarker der Brustkrebs heutzutage sind. Wir diskutieren die Möglichkeit von aktuellen und zukünftiger zielgerichteten therapeutischer Ansätzen für die Vitamin-D-Signalisierung bei Brustkrebs.

7. Publication 1.

Int. J. Mol. Sci. 2017, 18(6), 1318; doi: 10.3390/ijms18061318

Fluorescence analysis of vitamin d receptor status of circulating tumor cells (CTCs) in breast cancer: From cell models to metastatic patients

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International Journal of
Molecular Sciences

MDPI

Article

Fluorescence Analysis of Vitamin D Receptor Status of Circulating Tumor Cells (CTCS) in Breast Cancer: From Cell Models to Metastatic Patients

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Received: 11 May 2017; Accepted: 16 June 2017; Published: 20 June 2017

Abstract: The Vitamin D receptor (VDR) expressed in normal breast tissue and breast tumors has been suggested as a new prognostic biomarker in breast cancer (BC). Besides, increasing evidence supports the view that the detection of circulating tumor cells (CTCs) predicts outcome in early and metastatic BC. Consequently, an evaluation of VDR expression in the CTCs of BC patients may allow optimization of their treatment. As an attempt to profile and subtype the CTCs of metastatic patients, we established an innovative fluorescence technique using nine BC cell lines to visualize, define, and compare their individual VDR status. Afterwards, we tested the CTC presence and VDR expression in blood samples (cytospins) collected from 23 metastatic BC patients. The results demonstrated major differences in the VDR levels among the nine cell lines, and VDR positive CTCs were detected in 46% of CTC-positive patients, with a total of 42 CTCs individually analyzed. Due to the limited number of patients in this study, no correlation between VDR expression and BC subtype classification (according to estrogen receptor (ER), progesterone receptor (PR) and HER2) could be determined, but our data support the view that VDR evaluation is a potential new prognostic biomarker to help in the optimization of therapy management for BC patients.

Keywords: vitamin D receptor; circulating tumor cells; breast cancer

1. Introduction

Breast cancer (BC) is a significant global public health issue and the leading cause of death among women around the world. Anti-cancer therapies including chemotherapy, endocrine therapy, and targeted therapy have significantly decreased BC mortality in the past 20 years. However, as BC is an extremely heterogeneous disease, resistance to treatment is a major clinical challenge for current BC management [1]. Therefore, the development of more specific biomarkers and recognition of new therapeutic targets would really contribute to solving the problems of therapy resistance and metastasis [2–4]. In recent years, an increasing number of clinical studies have suggested that an optimal vitamin D status has a protective effect against BC development and that high Vitamin D receptor (VDR) expression in breast tumors is associated with a better survival rate [5–8]. As one of the nuclear receptor (NR) members, VDR is found in both normal breast tissue and breast tumors [9,10]. As such, an analysis and understanding of the VDR pathway can probably provide a novel way for developing a new targeted therapy to escape resistance mechanisms. The group of NRs that are active as homodimers have been classified as type 1 NRs, whereas the NRs of the VDR group that bind as heterodimers are known as type 2 NRs. The type 1 group includes, among others, estrogen,

progesterone, and androgen receptors and the type 2 group contains VDR, retinoic acid receptors (RARs), retinoid X receptors (RXRs), and thyroid hormone receptors (THRs). Ligand-bound VDR-activated vitamin D_3 heterodimerizes with its cognate co-receptor RXR to control the expression of genes involved in its different functions [11]. Besides its classic functions to maintain extracellular calcium levels by regulating calcium absorption in the gut and bone turnover, the VDR–RXR heterodimer binds to vitamin D response elements with the positive or negative transcriptional regulation of gene expression involved in various molecular pathways. This results in a wide range of calcitriol-mediated anti-cancer actions in BC [12,13]. We therefore believe that VDR exploration is very relevant to evaluate its potential as a new prognostic biomarker and therapeutic target in BC.

Circulating tumor cells (CTCs) circulate in the peripheral blood of patients with solid malignancies and are shed from an existing primary tumor or from metastatic lesions into the blood stream [14]. CTCs detected in BC patients are significantly associated with a poor outcome in both early and metastatic tumors [15–19]. In metastatic patients, several tumor lesions may potentially release CTCs which therefore comprehensively reflect tumor and metastasis characteristics. CTCs can be collected via a simple venipuncture; this 'liquid biopsy' achieves the repeatable and real-time monitoring of tumor cell characteristics. It is a less invasive and cost-effective alternative to tissue biopsies [20], despite the fact that technical and conceptual advances are still necessary before this 'liquid biopsy' can be routinely used for the diagnosis, characterization, monitoring, and treatment optimization of cancer. CTCs are a promising marker, providing important predictive and prognostic information in both early and metastatic BC. They may help to assess the response to treatment and to detect early disease recurrence [21]. At the moment, the CellSearch® system for CTC enumeration is the only accepted standard by the Food and Drug Administration (FDA). Only a few studies have investigated human epidermal growth factor receptor 2 (HER2) and/or estrogen receptor (ER) expression on CTCs, even though HER2 and ER are currently the only validated predictive factors used for therapy decision making in BC [22]. In conclusion, the characterization of CTCs may be a major tool to support diagnosis, and should be included in clinical trials for the evaluation of new targeted therapies [23]. In order to better predict disease progression and personalize treatment, new prognostic and predictive factors are needed. So far, studies on VDR status in CTCs are still lacking. Therefore, the evaluation of VDR expression on CTCs in BC patients could potentially help in individualizing BC therapy.

In this study, we describe an innovative triple fluorescence technique that we developed to simultaneously visualize the presence of cytokeratin (CK), absence of CD45, and expression of VDR. We first characterized BC cell models, before validating the preclinical data in CTCs from 23 metastatic BC patients.

2. Results

2.1. Fluorescence Labeling of VDR, CK, and CD45 on MCF-7 Cells Mixed with Peripheral Blood Mononuclear Cells (PBMCs)

To mimic the physiological situation of identifying very few CTCs within numerous PBMCs, we first used MCF-7 and T47D cells as models for VDR-positive BC cells and mixed them with PBMCs from healthy donors (Figure 1). A 40× magnification allowed us to simultaneously visualize, in both cell lines, the ring-like appearance of CK in green and the nuclear staining of VDR in red labeling, as well as the blue ring-like appearance of CD45 in the PBMCs. The optimized staining protocol allowed us to screen for cancer cells expressing CK and no CD45 and distinguish them from the CD45 positive PBMCs. VDR expression could then be assessed in cancer cell lines.

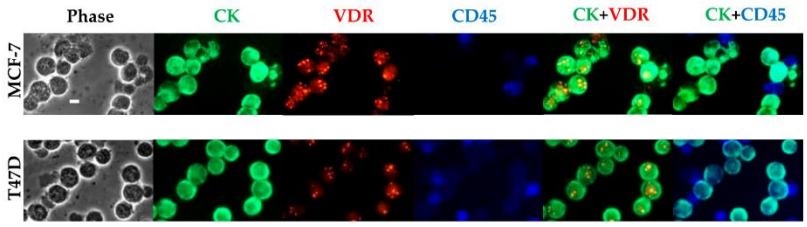

Figure 1. VDR expression detection in MCF-7 and T47D cells mixed with PBMCs. Fluorescence labeling of VDR (in red), CK (in green), and CD45 (in blue) was performed on a 10^6 MCF-7 and T47D cells/PBMCs mix (3/1). Original magnification, ×40. Scale bar (white bar in the upper left image), 10 µm. CK: cytokeratin; VDR: vitamin D receptor; PBMCs: peripheral blood mononuclear cells.

2.2. Fluorescence Labeling of VDR and CK with Parallel 4′-6-Diamidino-2-Phenylindole (DAPI) Staining in MCF-7 Cells and in a Panel of Eight other BC Cell Lines and One Endometrial Cancer Cell Line

To confirm VDR nuclear staining, the fluorescence labeling of VDR and CK with DAPI nuclear staining was then performed on MCF-7 cells (Figure 2). The previously described aspect of first nuclear foci of VDR was characterized [24,25]. It is noteworthy that not all MCF-7 cells exhibited the same intensity of VDR staining. Whereas some cells barely exhibited any fluorescence intensity (roughly 3%), most exhibited significant fluorescence and others expressed a particularly high intensity (around 4%).

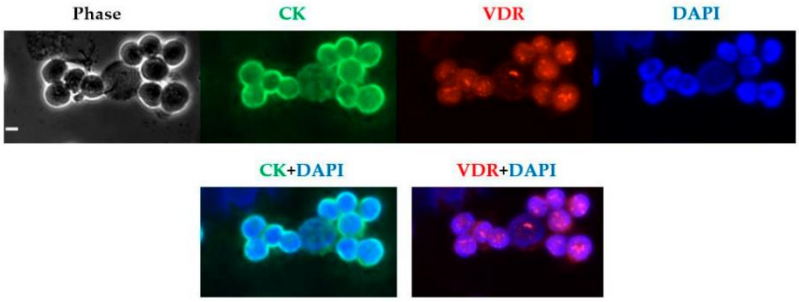

Figure 2. VDR expression detection in MCF-7 cells. Fluorescence labeling of VDR (in red) and CK (in green) with DAPI (in blue) nuclear staining was performed on 10^6 MCF-7 cells. Original magnification, ×40. Scale bar (white bar in the upper left image), 10 µm. DAPI: 4′-6-Diamidino-2-Phenylindole.

As presented in Figure 3, we then performed the labeling using this protocol on nine different human BC cell lines (MCF-7, T47D, Cama-1, ZR75, SK-Br-3, HCC 3153, HCC1937, MDA-MB-231, and MDA-MB-468) and one endometrial cancer cell line Ishikawa ERneg, to analyze the differences in VDR expression. For each cell with systematic CK labeling, we were able to characterize different VDR levels by evaluating the average intensity: low VDR: +, intermediate VDR: +~++ and high VDR: +++. Because VDR expression could exhibit clear differences within one cell line, like MCF-7, we assessed the average intensity observed for the majority of cells within each cell line. According to our results, all 10 cell lines then appeared as VDR positive. However, as shown in Table 1, we observed that the different cell lines expressed very different average levels of VDR. The following grading was obtained: high VDR +++ includes MDA-MB-231, MCF-7 and Cama-1; intermediate VDR +~++ includes T47D, HCC1937, ZR75 and SK-Br-3; low VDR + includes MDA-MB-468 and HCC 3153. In addition, Ishikawa ERneg belonged to intermediate VDR +~++.

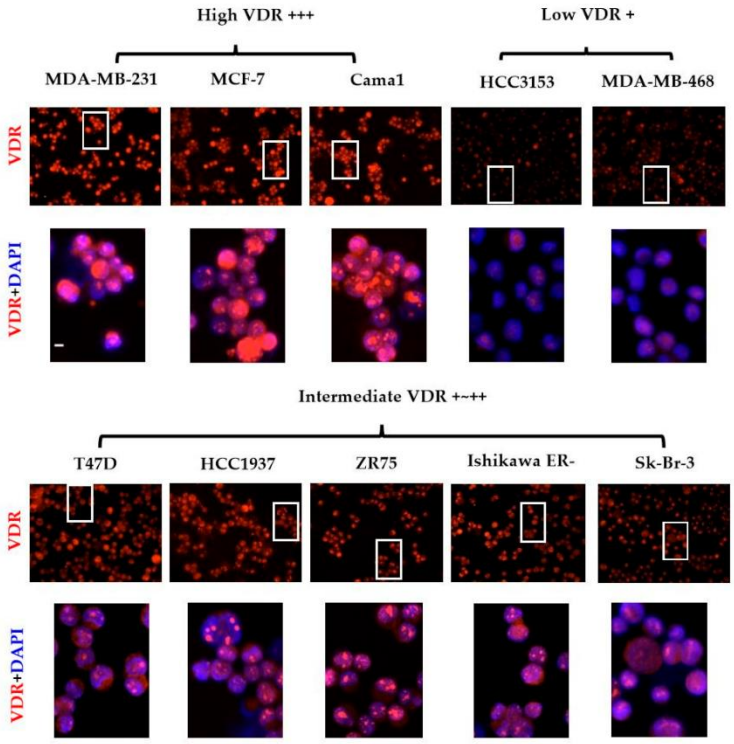

Figure 3. VDR expression in nine BC and one endometrial cell lines. Fluorescence labeling of VDR (in red), CK with DAPI (in blue) nuclear staining was performed on 10^6 cells from 10 cell lines. Various levels of VDR expression were observed, including low (+), average (+~++), or high (+++) levels. Photographs presented are representative of five independent reproducible experiments. Original magnification, ×40. Scale bar (white bar in the upper left image), 10 μm. BC: breast cancer.

Table 1. ER, progesterone receptor (PR), HER2, and VDR expression levels of the nine BC and one endometrial cell lines.

Cell Line	ER	PR	HER2	VDR Level
MDA-MB-231	−	−	−	high
MCF-7	+	+	−	high
Cama-1	+	+	−	high
T47D	+	+	−	intermediate
HCC1937	−	−	−	intermediate
ZR75	+	−	−	intermediate
SK-Br-3	−	−	+	intermediate
Ishikawa ERneg	−	−	−	intermediate
MDA-MB-468	−	−	−	low
HCC3153	−	−	−	low

BC: breast cancer; ER: estrogen receptor; PR: progesterone receptor; VDR: vitamin D receptor; −: negative; +: positive.

2.3. Patient Cohort

A cohort of 23 metastatic BC patients was included in the study. The clinicopathological data are shown in Table 2, with an average age of 64.9 years at the time of CTC analysis (median: 66 years; range: 46–82 years). Patients M10 and M25 had bilateral BC: While patient M10 had two ERα- and HER2 positive tumors, patient M25 had an ERα positive and HER2-negative tumor in the left breast and one ERα- and HER2-negative tumor in the right breast. Their tumors were considered as two independent primary tumors for statistical analysis. In total, 68.0% of the primary tumors were ERα positive (n = 17), 36.0% were HER2 positive (n = 9, with four patients both ERα and HER2 positive), and 12.0% were triple-negative (n = 3). At least 76.0% of the tumors were grade 2 or 3 at the time of primary diagnosis (n = 19). The first metastasis was diagnosed at an average of 3.5 years after primary diagnosis (median: 3 years; range: 0–10 years). CTC analysis was performed at an average of 9.8 years after primary diagnosis (median: 10 years; range: 4–16 years) and 6.3 years after the first metastasis (median: 5 year; range: 4–15 years).

Table 2. Patient characteristics and CTC presence.

Patient	Age *	Primary Tumor Status					Primary Diagnosis ~Metastasis (years) **	Primary Diagnosis ~CTC Analysis (years) ***	CTCs
		TNM	Grade	ERα	PR	HER2			
M1 [CTC]	56	pT2, pN2, M0	G2	+	+	−	5 and 10	14	>500
M2	62	cT4, N3, M1	G3	−	−	+	0	5	-
M4	69	cT3, pNx, pM1	G3	−	−	+	0 and 1	5	-
M6 [CTC]	49	ypTis, pN1, M0	G3	−	−	+	2	7	1
M7 [CTC]	75	pT2, pN1, M0	n.d.	+	−	− ****	7	16	3
M8 [CTC]	53	pT1b, pN0, M0	G2	+	+	−	8	16	5
M9 [CTC]	63	pT1c, pN0, M0	G2	+	+	−	8	16	1
M10 [CTC]	69	Left pT2, pN0, M0	G2	+	−	+	5 and 6	10	1
		Right pT1c, pNx, M0	G2	+	−	+			
M11 [CTC]	66	ypT2, pN3a, M0	G3	+	+	−	2 and 3	9	1
M12	63	ypT2, pN2, M0	G3	−	-	+	3	11	-
M13 [CTC]	77	pT2, pN1, M0	G2	+	+	−	2 and 6	11	1
M15	82	cT3, cN1, cM1	G2	+	+	+	0	5	-
M16 [CTC]	69	pT1c, pN0, M0	n.d.	+	+	−	10 and 12	16	16
M17 [CTC]	64	pT1c, pN0, M0	n.d.	+	+	+	6	13	1
M18 [CTC]	70	pT4b, pN1, pM1	G2	+	+	−	0	4	2
M19	69	pT2, N0, M0	G2	−	-	+	0	4	-
M20	61	pT3, pN3, M1	n.d.	+	−	−	0 and 1	5	-
M22	53	pT2, pN0, M0	G3	−	−	−	4	8	-
M25 [CTC]	73	Left pT1c, pN0, M0	G1	+	+	−	6	10	8
		Right pT2, pN0, M0	G2	−	−	−			
M26	46	ypT0, pN1a, M0	G2	+	+	−	1	5	-
M27 [CTC]	59	Left pT2, pN1, M1	G2	+	+	−	3	7	1
		Right pTis, pNx, M1	n.d.	n.d.	n.d.	n.d.			
M28 [CTC]	79	ypT3, pN1a, M0	G2	+	+	−	9	13	1
M29	67	pT1c, pN0, M0	G3	−	−	−	0, 2 and 5	15	-

n.d. indicates not determined; * indicates at time of CTC analysis; ** indicates time between primary diagnosis and metastasis (years); *** indicates time between primary diagnosis and CTC analysis (years); **** indicates no information for the primary tumor, but HER2-negative recurrence 10 years later (ERα-positive and PR-negative as the primary tumor); [CTC] indicates CTC positive patients. CTCs: circulating tumor cells, −: negative; +: positive; -: absence.

2.4. CTCs Screening and Enumeration

We found CTCs in 14 patients out of our 23 metastatic BC patients (60.8%). Patient M1 exhibited numerous CTCs which we estimated as more than 500 in the 1 million PBMCs that we analyzed on one cytospin. In addition, 42 CTCs were identified in the other 13 patients, with numbers ranging from one to 16 per patient and an average of 3.2 CTCs per patient (Table 3). Five patients had more than one CTC, with only one patient having more than 10 CTCs.

Table 3. Characteristics of the CTCs found in 14 patients.

Patients with CTCs	CTCs (n = 42 *)				Total
	Tiny CK pos		Normal CK pos		
	VDR		VDR		
	Neg	Pos +~++	Neg	Pos +~++	
M 1					>500
M 6			1		1
M 7	1		1	1	3
M 8	1		4		5
M 9	1				1
M 10	1				1
M 11				1	1
M 13	1				1
M 16	3	12	1		16
M 17				1	1
M 18	2				2
M 25	1		4	3	8
M 27				1	1
M 28	1				1
Total	12	12	11	7	42 *
% (n = 13)	28.6	28.6	26.2	16.6	100 *

* Indicates without taking into account the CTCs from patient M1. CK: cytokeratin, Pos: positive; Neg: negative.

2.5. VDR Status Determination in CTCs

As observed in the cancer cell line models, the strong CK staining allowed the screening of the CD45 negative CTCs (Figure 4). VDR staining was very high in some cases. Based on the cancer cell line controls, we classified two VDR staining statuses for the CTCs: positive if low, moderate, or high expression; or negative. The panels a and b in Figure 4 show the presence of both VDR positive and negative CTCs for the same patient, M25. Besides some VDR positive CTCs, we can see some CD45 positive cells that also expressed VDR (panel b). Similarly, for patient M16, both VDR positive and negative CTCs were seen (panels e and f versus c and d). For the same patient, M16, clear differences in the size of the CTCs occurred, with what we classified as tiny "CTCs" (panels d, e and f) of around a 5 µm diameter, compared to the so-called "normal" CTCs (panels c, around a 10–15 µm diameter).

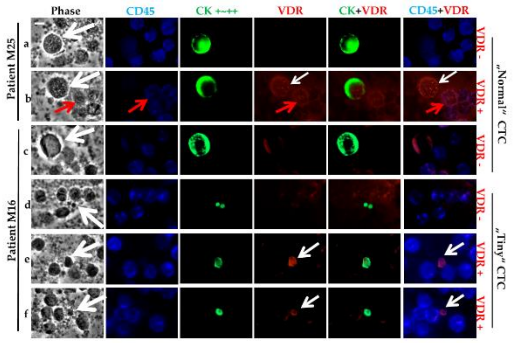

Figure 4. VDR status determination on CTCs of metastatic BC patients. Triple fluorescence labeling of CD45 (in blue), CK (in green), and VDR (in red) was performed on 10^6 PBMCs, with parallel phase analysis. CTCs (**with white arrows**) were classified as VDR+ or VDR-. For both patients M25 (**a**,**b**) or M16 (**c**–**f**), either status was observed with superimposed VDR and CK labeling. CTCs exhibit size heterogeneity for patient M16 ("Normal" or "Tiny" CTCs). VDR staining was also seen on PBMCs (**with red arrows**), with superimposed VDR and CD45 labeling. Original magnification, ×40. Scale bar (white bar in the upper left image), 10 µm.

For patient M1 (Table 3), no accurate quantification of the CTC number was possible, as more than 500 CTCs were identified within the 1 million PBMCs analyzed. This specific subtype of CTCs exhibited a regular size (around 10 µm) with positive or negative VDR expression. Of the remaining 13 patients with CTCs (Table 3), five had only one CTC that was VDR negative, and two patients had two or five CTCs that were all VDR negative. Altogether, seven patients out of 13 (53.8%) only had VDR negative CTCs, three patients (23.1%) had only one CTC that was VDR positive, and the last three patients (23.1%) had both VDR positive and negative CTCs.

Of the total 42 CTCs analyzed, 54.8% (n = 23) CTCs were classified as VDR negative and 45.2% (n = 19) as VDR positive.

We noticed that almost all patients exhibited round shaped CTCs, as expected after the cytospin preparation of the blood samples. Regarding the average size of the CTCs, eight patients had what we defined as "normal" CTCs (n = 18) with diameters ≥5 µm (as described above for panels a to c in Figure 4), whereas nine patients had tiny CTCs (n = 24) having a diameter <5 µm (panels d to f in Figure 4). The four patients with more than two CTCs had both tiny and normal size CTCs. Both populations of tiny and normal CTCs could equally express VDR or not express VDR. We noticed that 15 out of 16 CTCs from patient M16 were tiny CTCs. Of the total 42 CTCs, 24 (57.2%) were tiny and 18 (42.8%) were of a normal size.

2.6. Correlation between CTC Presence, VDR Status and the Primary Tumor Characteristics

No significant correlation was found between VDR expression on the CTCs and hormone receptor (HR) expression, HER2 status, or triple-negative status of the related primary tumors.

2.7. Specific CK Positive Cell Subtypes

Comparing the morphology of the total 42 CTCs analyzed and within the various CTCs of each individual patient, we observed striking heterogeneities not only in terms of VDR expression, but also with regards to cell size. Besides, we saw very atypical morphologies and fluorescence patterns for cells positive for CK and still negative for CD45. These cells could be CTCs, but we preferred not to include them in our analyses and thus did not report them in Tables 2 and 3 (Figure 5).

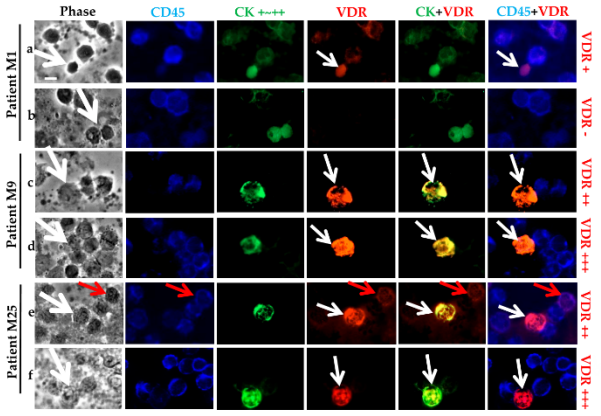

Figure 5. Particular subtypes of CK positive CD45 negative cells. Triple fluorescence labeling of CD45, CK, and VDR was performed on 10^6 PBMCs from patients, with parallel analysis. Distinct subtypes of CTCs (**with white arrow**) were observed: patient M1 (**a**,**b**) with more than 500 CTCs identified and patients M9 (**c**,**d**) and M25 (**e**,**f**) with superimposed VDR and CK labeling. VDR staining was also seen in PMBCs (**with red arrow**), with superimposed VDR and CD45 labeling. Original magnification, ×40. Scale bar (white bar in the upper left image), 10 µm.

First, the already described numerous CTCs from Patient M1, in which CK and VDR staining is faint and often superposed, can be considered as a distinct CTC subtype compared to the other CTCs (Figure 5, panel a,b). In some other patients, for example patients M9 and M25, the fluorescence labeling of VDR and CK performed a superposition (Figure 5, panel c–f). The phase contrast image of some CTCs showed a specific morphology, with a very faint aspect. As we first suspected them to be fragments or splitting cells, we systematically checked for their morphology and adjusted the exposure time for CK images in order to control and confirm the peripheral staining. We noticed a very high expression of VDR that forced us to decrease the exposure time five-fold (from 1000 ms for the previous pictures of other CTCs to 200 ms in that case) (Figure 5, panel d–f).

3. Discussion

VDR has been shown to be expressed in different tissues, as well as in BC cells; however, it has not yet been investigated in BC CTCs from archived specimens. We previously used various human carcinoma cell lines to develop a simple and efficient triple fluorescence technique for CTC receptor analysis, e.g., ER, HER2 [15], *N*-cadherin, and CD133 [26]. We tested the VDR expression in nine BC cell lines and CTCs from 23 archived metastatic BC cases.

The CellSearch® system, which is the only Food and Drug Administration (FDA) approved CTC enumeration method used for clinical purposes, classifies a CTC as a positive event if the nucleated cell is ≥4 μm, pan-CK positive, and CD45 negative [27]. More than a 90% expression of CK7, CK8, CK18, and CK19 was observed in breast carcinomas of all grades, thereby confirming their usefulness for BC identification [28]. Meanwhile, peripheral blood cells (PBCs) such as monocytes (i.e., PBMCs) only express very low mRNA levels of CK (18/19) [29]. Therefore, using a CK antibody combined with a CD45 antibody, a recognized white blood cell (WBC) marker, allows for the characterization of CTCs by CK positive and CD45 negative staining—a technique that has been the most frequently used so far [30]. An additional fluorescence channel is accessible for a user-defined detection of therapy relevant markers. The CellSearch® system (but also other techniques) allows for an analysis of markers such as ER [31], HER2 [15,32,33], epidermal growth factor receptor (EGFR) [34,35], and epithelial-mesenchymal transition (EMT) associated molecules such as *N*-cadherin [26]. Nevertheless, VDR has not yet been evaluated by this technology.

After optimization of the triple fluorescence protocol on BC cell lines, we observed that all nine tested cell lines were VDR positive, as already reported in other studies by Western blot analysis or other techniques [24,36–38]. We then characterized the expression heterogeneity among all of the cell lines. Limited publications can be found with regard to the intensity of VDR protein expression in BC cell lines, as most of them focus on mRNA expression [8,39–41]. According to our fluorescence analysis, we were able to divide the cell lines into three groups with distinct levels of VDR expression. Standardized identical values of exposure time for optimal pictures of VDR and CK expression were absolutely required for evaluation and comparison (namely 1000 ms for VDR and 2000 ms for CK). As already mentioned in the literature, T47D appeared to be among the high expressing cell lines (although not the highest) and MDA-MB-231 among the low expressing cell lines [36,40]. Besides these differences in the average fluorescence intensity for each cell line, we observed that even within one cell line, individual cells expressed variable VDR levels, thus explaining how mRNA expression cannot always be linked to protein expression. As an example, most MCF-7 cells exhibited a substantial level of VDR fluorescence, but the expression was not identical for all cells. Some cells barely exhibited any fluorescence intensity (2.1%) and others expressed a particularly high intensity (around 4.7%). We speculate that this heterogeneity within one cell line may rely on the cell cycle–related variations of VDR expression or on a wide variety of environmental factors such as cell adhesion and cell density [42,43]. Of note, given the individual heterogeneity of expression within one cell line, we characterized and graded the VDR level by semi-quantifying the average values of different cell lines as the majority of cells showed a consistently low, intermediate, or high intensity.

In order to perform VDR expression analysis on blood samples collected from a consecutive cohort of 23 metastatic BC patients, we first mimicked the CTCs analysis in blood by mixing cancer cell lines with PBMCs from healthy donors. PBMCs are identified as CD45 positive and CK negative,

and in our study, only a few of them expressed VDR. This observation is in accordance with the literature stating that VDR expression is controlled by immune signals [44]. It is noteworthy that normal human PBMCs may also express VDR and its target genes [45–47]. Using our triple fluorescence method, we detected VDR expression in 42 CTCs, present in 60.8% of our patient cohort with 1 million PBMCs analyzed in each case. Our cohort only consisted of metastatic patients as they have the highest probability of exhibiting CTCs. We first observed very atypical morphologies and fluorescence patterns with cells positive for CK and negative for CD45, and secondly with cells positive for CK and for CD45, which could be CTCs because dual CK and CD45 positive cells are occasionally found in humans [48,49]. Indeed, false-positive CK staining may also occur, as it is possible that antibodies against CK bind to hematopoietic cells through the Fc receptor [50]. We thus decided to exclude both of these groups from further analysis.

All of the studied BC cell lines expressed VDR from low to high levels. Based on the MCF-7 positive controls, we therefore grouped CTCs into two VDR status groups. We could demonstrate that 45.2% (n = 19) of the CTCs were VDR positive. In terms of VDR intensity, VDR staining in CTCs was relatively low compared to the high VDR expression observed in some BC cell lines. Optimal vitamin D status has a protective effect against BC development, but epidemiological and early clinical studies are inconsistent. Resistance to vitamin D develops or exists in many BC patients [51]. The anticancer role of vitamin D is mainly mediated by the VDR. Our hypothesis is that VDR may be abnormally (poorly) expressed in BC tissue and/or CTCs. It is known that VDR is lost during carcinogenesis and this may be the reason why tumors become insensitive to vitamin D [25]. Therefore, it would be very helpful to compare the VDR expression of CTCs with that of the corresponding primary tumor. Unfortunately, primary tumor tissue was not available for our cohort. In a previous study [7], Nina Ditch et al. analyzed the relationship between VDR expression in primary tumor tissue and survival in 82 BC patients. Patients with high VDR expression showed significantly better progression-free (PFS) and overall survival (OS) results than patients with moderate/negative VDR expression [7]. In the 13 CTC positive patients of our study, six (46.1%) had at least one VDR positive CTC, with three (23%) patients only having VDR positive CTCs. In contrast, 10 (76.9%) had at least one VDR negative CTC, including seven (53.8%) patients with only VDR negative CTCs. Therefore, we believe that VDR expression may be of clinical significance and that the CTC results need to be correlated to PFS or OS in a larger cohort of patients. Similar conclusions on the role of VDR expression as a prognostic marker have already been addressed in pancreatic cancer and gastric cancer [52,53]. In a large patient population, VDR expression on primary tumor tissue is inversely associated with more aggressive BC including a large tumor size, HR negativity, and triple-negative subtype ($p < 0.05$) [54]. A preclinical study suggested that calcitriol and inecalcitol, an epi-analogue of calcitriol, can inhibit BC cell line growth, especially in cells expressing ER and VDR [55]. This suggests that the VDR-mediated inhibition of ER-positive BC cells may be at least partly affected by the downregulation of ER [56–58]. Besides, in contrast to ER-positive cells, treatment with calcitriol was reported to induce the expression of ER in the ER-negative cell line. If confirmed in patients, this ability of calcitriol would have major implications for BC treatment [59]. In order to see whether it could be a potential biomarker, we correlated the VDR expression results observed in CTCs with the related clinicopathological parameters. Most likely due to the small number of patients and only one cytospin analyzed per patient, no significant association was found between VDR expression in CTCs and tumor subtype according to ER, PR, and HER2 status. In further larger studies, it will be essential to correlate VDR expression in CTCs (with at least duplicate cytospins for each patient) with ER, PR, and HER2 status, as well as the VDR itself of the primary tumors. Moreover, repeat analysis of VDR expression on CTCs during the course of disease and after treatments may give very relevant information. Another parameter to consider in parallel will be the serum level of the partially activated 25-hydroxyvitamin D, and consequently 1,25di-hydroxyvitamin D that are expected to be relatively low in BC patients. A large number of studies have concluded that low blood levels of vitamin D are associated with an increased BC incidence and decreased survival in BC [60]. Similar to postmenopausal patients with ER-positive tumors and extremely low serum estrogen levels, it

could be speculated that moderate or low levels of 25-hydroxyvitamin D may be sufficient to activate VDR.

The physiopathological significance of the observed CTC size heterogeneity is also a crucial point that may lead to further analyses [61,62]. The Cell Search® system classifies a CTC as a positive event if the nucleated cell is ≥4 μm [27]. We observed 24 (57.2%) CTCs that were "tiny" CTCs (around 5 μm, but higher than 4 μm) and 18 (42.8%) that had a normal size (≥5 μm). Prior publications by us and other research groups have already described and discussed the character of these "tiny" CTCs [15,26,61,63]. There are diverse explanations for "tiny" CTCs: Compared to the size of CTCs in patients with metastases or primary tumors, the size in dormancy candidates is smaller [64,65]. Furthermore, Marrinucci et al. found by DNA disruption analysis and microscopic images that the early apoptosis category of cells contains many CTCs that seem surprisingly small for carcinoma cells, suggesting that these small CTCs are undergoing cell death through apoptosis [66]. Stem cell-like CTCs which are smaller and more aggressive than other CTCs could be another possibility [67]. Similar findings were reported for disseminated tumor cells (DTCs) in bone marrow, where tumor cells with a stem cell-like phenotype were demonstrated [68]. CTCs that are in the process of EMT may be as deformable as WBCs to become more WBC-like and better adapt to the blood flow based on this size issue [69]. These findings likely explain some of the size heterogeneity we observed. However, further studies regarding the exact significance of these "tiny" CTCs in cancer research have not been able to give clear answers yet [70]. Of note, the technical issue of CTC enrichment methods also has to be considered when the methods rely on a size filtration of CTCs from PBMCs [71].

Some specific atypical subtypes of cells were also observed in our study. For example, patient M1's had more than 500 CTCs in the 1 million PBCMs analyzed, and CK and VDR staining was faint and often superposed. For some CTCs in patients M9 and M25, the fluorescence labeling of CK and VDR performed a superposition with a very high expression of VDR and the phase contrast image of some CTCs shows a specific morphology with a very faint aspect. We speculate that these specific subtypes derive from some stressed, evolving cells that may be going through the apoptosis process: When CTCs are shed from solid tumors, half of these CTCs perish within 2.4 h in vivo [65]. CTCs impaired through apoptotic events exhibit membrane perforation that triggers the leakage of intracellular components, including not only electrolytes or small molecules, but also DNA and chromatin. The technical steps of blood drawing and sample processing can induce the additional stress or degradation of CTCs by various factors. These parameters include the selected purification and analysis techniques, such as temperature shock, fluidic turbulence, shear force, or surface tension. As a result of the various harsh conditions, it is expected that fragmented cells, cellular debris, microparticles, and clump-like aggregates next to "normal" CTCs will be observed [72]. Although we did not include these CTCs in our analysis, they can clearly play a role in the dissemination and metastasis process [26,73–76].

4. Materials and Methods

4.1. Cell Culture and Cytospin Preparation

The human adenocarcinoma cell lines MCF-7, T47D, ZR-75-1, and MDA-MB-231, and an endometrial cancer cell line Ishikawa (Heraklio) 02 ER- (Ishikawa ERneg), were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom) and the Cama-1 (HTB-21), SK-BR-3 (HTB-30), HCC1937 (CRL-2336), and MDA-MB-468 (HTB-132) cell lines from the American Type Culture Collection (ATCC, Rockville, MD). The HCC 3153 cell line was kindly provided by Adi F. Gazdar (Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). Cryopreservation of cell cultures ranged from passages 1 to 10. Cells were used during up to 20 passages. Cells were grown routinely in Dulbecco's modified Eagle's medium (Biochrom, Berlin, Germany), supplemented with 10% FBS (PAA, Pasching, Austria).

For cytospin preparation, trypsinized cells were centrifuged (700 g, 10 min, 4 °C) and resuspended in phosphate-buffered saline (PBS; Biochrom, Berlin, Germany). Then, 1 million cells were spread on each cytospin and centrifuged (45 g, 5 min, room temperature). Cytospins were

allowed to dry overnight at room temperature and then stored at −80 °C. They were prepared with either 1 million adenocarcinoma cells or mixed with PBMCs from healthy volunteer donors, as indicated in the legends.

4.2. Triple Fluorescence Labeling of CK, VDR, and CD45 with Parallel Phase Analysis

According to the optimized procedure (see Supplementary Material), cytospins were thawed and immediately fixed in 3.7% neutral buffered formalin (Fischar, Saarbrücken, Germany) in PBS for 15 min at room temperature and permeabilized in cold (−20 °C) methanol (Sigma-Aldrich, Steinheim, Germany) for 2 min. After washing in PBS, Ultra V Blocking medium (Thermo Scientific, Fremont, CA, USA) was used for 15 min. This blocking step and all of the following steps were performed in a humidified chamber at room temperature. All antibodies were diluted in Dako Antibody Diluent with Background Reducing Components (Dako, Carpinteria, CA, USA).

As previously described [77], we selected a two-step protocol. Cells were first incubated for 45 min with a monoclonal mouse anti-human VDR antibody (clone 2F4, mouse IgG2a, MCA3543Z, Serotec, Puchheim, Germany) efficiently used in other studies on BC [7,40], washed in PBS, incubated for 30 min with a goat anti-mouse IgG-Fab fragment labeled with Cy3 (Jackson ImmunoResearch, Suffolk, UK), and washed in PBS.

Cells were then incubated for 45 min with a monoclonal rabbit anti-human CD45 antibody (D9M8I, 13917, Cell signaling, Leiden, The Netherlands) and a monoclonal mouse anti-human cytokeratin antibody (Igg1 A45 B/B3, Glycotope, Berlin, Germany), washed in PBS, incubated for 30 min with a goat anti-rabbit IgG labeled with Coumarin-AMCA (Jackson ImmunoResearch) and a goat anti-mouse IgG labeled with DyLight488 (Jackson ImmunoResearch), and washed in PBS.

After drying (30 min, at room temperature), the slides were mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) before manual analysis with a computerized fluorescence microscope Axioskop (Carl Zeiss Micro Imaging GmbH, Göttingen, Germany) for phase and fluorescence, with 40× magnification. An AxioCam MR camera and AxioVision software (version AxioVision LE 4.8, Göttingen, Germany) were used to capture, analyze, and save high-resolution images for the three fluorescence channels, considered independently or in combination. Criteria for CK and CD45 positivity were the ring-like appearance (cytoplasm staining in periphery) and we considered that the VDR positivity was always high, average, and low for specific punctuated staining of the nucleus with a low background and no cytoplasmic or peripheral staining. We followed the criteria already described for the identification of CK and CD45 positive CTCs by immunofluorescence [78] and the consensus recommendations for standardized tumor cell detection [79].

Definite threshold values of exposure time for VDR, CD45, and CK fluorochromes were determined, on the basis of the analysis of the cancer cell lines, and were systematically used for the patient analysis described below.

4.3. Fluorescence Labeling of VDR and Cytokeratin (CK) with Parallel 4'-6-Diamidino-2-Phenylindole (DAPI) Analysis

Cytospins were thawed, immediately fixed, permeabilized, and blocked as described above.

Cells were incubated as described above, first with a monoclonal mouse anti-human VDR and related secondary anti-mouse IgG-Fab fragment labeled with Cy3, and then with the monoclonal mouse anti-human cytokeratin antibody and the related goat anti-mouse IgG labeled with DyLight488, without the anti-CD45.

After drying (30 min, at room temperature), the slides could be mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) before manual analysis with a computerized fluorescence microscope Axioskop (Carl Zeiss Micro Imaging GmbH, Göttingen, Germany) with 40× magnification.

4.4. Patient Cohort

This analysis was performed at the Department of Obstetrics and Gynecology, Ludwig Maximilian University (Munich, Germany). Twenty-nine metastatic BC patients were recruited between May 2010 to July 2012; with six exclusions (patient M5 with a cancer of unknown primary origin (CUP) syndrome and patient M3, M5, M14, M21, and M23 with an insufficient blood sample). Table 2 describes the characteristics of the final cohort of 23 patients and their primary tumors. Written consent forms were collected from the patients using protocols approved by the institutional ethics committee (approval number 148-12; 12.05.2012, Ethikkommission bei der Ludwig-Maximilians-Universität, Munich). The phenotype of the primary tumor was routinely assessed at the time of diagnosis by immunohistochemical staining and potentially by FISH, Fluorescence In Situ Hybridization (for most HER2 ++ patients) in the original Department of Pathology and collected from the patient files. ERα status was classified by an evaluation of the percentage of tumor-stained cells and staining intensity, allowing for an assessment of an Immunoreactive Score (% score × intensity score). HER2-negative assessment include 0 or + staining and ++ staining with FISH-negative amplification and HER2-positive assessments include ++ with FISH amplification or +++ staining. The ERα and HER2 status were indicated in Table 2, if available. Because the HER2 status of the primary tumor of patient P7 was not determined in 1999, at the time of diagnosis, we considered the HER2-negative status of the local recurrence assessed in 2009, with ERα positivity and PR negativity recorded for the primary tumor.

4.5. Blood Sampling, Ficoll and Cytospin Preparation

Fifteen milliliters of blood from each patient was collected by needle aspiration and placed in EDTA tubes. The blood was processed by a modified Ficoll protocol, with Ficoll-Hypaque (Pharmacia, Erlangten, Germany) density gradient centrifugation (density 1.007 g/mol) at 900× *g* for 30 min [80]; the mononucleated cells or PBMCs, were counted and centrifuged (700 g, 10 min at 4 °C), and then 1 million cells were spread out on each cytospin and centrifuged (45× *g*, 5 min, room temperature), before being processed as described above.

4.6. CTC Analysis by Triple Fluorescence

The triple fluorescence labeling of VDR, CK, and CD45 with parallel phase analysis was performed on the cytospins prepared from patient blood, as described above. The preparation of BC cell lines MCF-7 was mixed with PBMCs from healthy donors, in which MCF-7 cells served as a positive control for CK and VDR stainings and as a negative control for CD45; Mixed PBMCs served as a positive control for CD45 staining and as a negative control for CK. In each batch of patient samples we analyzed, one MCF-7 mixed with the PBMCs control slide was systematically performed. The screening of CTCs through CK staining was performed using a 20× magnification to get an optimal sensitivity, and the VDR and CD45 expressions were then assessed using a 40× magnification. For each patient, one cytospin was analyzed (1 million cells per patient) and each slide was evaluated by two independent investigators, and three in doubtful cases (X. Zhang, S. Sixou and U. Jeschke). For one patient (4.3%), the evaluation of the two observers differed for either the CTC detection or VDR positivity. These cases were re-evaluated by the three observers together. After the re-evaluation, the observers came to the same result. The concordance before the re-evaluation was 95.7%. The analysis was always performed within 72 h after the labeling procedure. Each observed CTC was recorded with at least one picture for each channel of analysis.

4.7. Statistical Analysis

A Fisher exact probability test was used to evaluate the relationship between the receptor status of the primary tumor and the VDR expression of the CTC for the 14 CTC-positive patients. $p < 0.05$ was considered statistically significant.

5. Conclusions

In this study, we demonstrated the evaluation of VDR expression from BC cell models to CTCs of metastatic BC patients. CTCs are a promising marker, providing important predictive and prognostic information in both early and metastatic BC. To the best of our knowledge, this work is the first study about VDR status on CTCs from BC patients. This preliminary study gives a direction for further VDR exploration, suggesting that prospective larger studies should be performed in the future. This will help elucidate VDR profiling in BC, including a parallel analysis of vitamin D and its receptor in CTCs and the corresponding primary tumors. Eventually, VDR may serve as a new prognostic biomarker in BC and a promising target for innovative BC therapies.

Supplementary Materials: can be found at www.mdpi.com/1422-0067/18/6/1318/s1.

Acknowledgments: We would like to thank all of the patients for participating in this study and donating their blood samples for research purposes, M. Alunni-Fabbroni for helpful discussions, C. Kuhn and L. Majunke for technical advices, and the China scholarship council (CSC). Sophie Sixou was fully supported by the University Paul Sabatier in Toulouse (France).

Author Contributions: Sophie Sixou and Udo Jeschke conceived and designed the project. Xi Zhang wrote the paper and performed most experiments. Simone Hofmann assisted with the cell culture. Sophie Sixou contributed to the manuscript writing and editing. Nadia Harbeck, Udo Jeschke, and Brigitte Rack provided the concept and substantially contributed to manuscript editing. Sophie Sixou supervised the research. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

BC	Breast cancer
CTC	Circulating tumor cell
CK	Cytokeratin
EMT	Epithelial–mesenchymal transition
ER	Estrogen receptor
FDA	Food and drug administration
FISH	Fluorescence in situ hybridization
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor
NR	Nuclear receptor
OS	Overall survival
PBC	Peripheral blood cell
PBMC	Peripheral blood mononuclear cell
PFS	Progression free survival
PR	Progesterone receptor
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
THR	Thyroid hormone receptor
VDRE	Vitamin D response element
VDR	Vitamin D receptor
WBC	White blood cell

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8. Publication 2

Drugs R D. 2017 Sep 6. doi: 10.1007/s40268-017-0208-6.

Impact of Etoposide on BRCA1 Expression in Various Breast Cancer Cell Lines

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Drugs R D
DOI 10.1007/s40268-017-0208-6

ORIGINAL RESEARCH ARTICLE

Impact of Etoposide on *BRCA1* Expression in Various Breast Cancer Cell Lines

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Abstract Breast cancer 1 (BRCA1), as a tumor suppressor, exerts an effective influence on protecting DNA integrity to suppress the development of breast cancer (BC). BRCA1 expression is induced in response to DNA-damaging agents such as etoposide. Germline *BRCA1* gene mutations are associated with development of hereditary BC. However, besides *BRCA*-mutated BCs, some sporadic cancers may also exhibit a BRCA-like phenotype, displaying so-called 'BRCAness'. This common phenotype may respond to similar therapeutic approaches as *BRCA*-mutated tumors and may thus have important implications for the clinical management of these cancers. In order to determine whether and how etoposide regulates the protein levels of BRCA1 in BC cells, we exposed a panel of five selected cell lines to etoposide, compared the results to untreated control cells, and then stained the cells with the specific, reliable, and reproducible MS110 antibody directed against phosphorylated Ser1423 BRCA1. By evaluating cytoplasmic BRCA1 protein levels, we were able to distinguish three aggressive BC subtypes with BRCAness characteristics. In addition, determination of early and late apoptosis helped to complete the analysis of BRCA1 functions in the DNA damage pathway of aggressive BC. In conclusion, our study suggested that high cytoplasmic BRCA1 protein levels could be considered as a potential predictive marker for response to chemotherapy in both sporadic and hereditary BC. Tumors with either BRCAness phenotype or germline *BRCA1* mutation are both aggressive BCs associated with poor prognosis and could both be subjected to targeted therapies against *BRCA1*-mutated BC in future clinical management strategies.

Key Points

Treatment of breast cancer (BC) cells with etoposide markedly enhanced both the cytoplasmic breast cancer 1 (BRCA1) and nuclear phosphorylated BRCA1 protein levels in BRCAness phenotype breast cells.

The function of BRCA1 in the DNA damage pathway of aggressive BC cells may link to apoptosis.

Cytoplasmic BRCA1 expression has potential to be a predictive biomarker in response to chemotherapy in BC.

Electronic supplementary material The online version of this article (doi:10.1007/s40268-017-0208-6) contains supplementary material, which is available to authorized users.

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Published online: 06 September 2017

1 Background

Breast cancer (BC) is the leading cause of death among women diagnosed with cancer worldwide [1]. In 2012, it alone comprised 25% of all cancer cases and 15% of all cancer deaths among females [2], making it the most

common female cancer. However, BC is a complex and extremely heterogeneous disease [3]. Thus, a deep understanding of its biology and of certain prognostic factors is of great significance in predicting disease outcome and developing new target therapeutic strategies. Breast cancer 1 (*BRCA1*) is a susceptibility gene responsible for hereditary predisposition to BC. Since it was first found to encode a DNA repair enzyme involved in BC susceptibility in 1990 [4], and subsequently was successfully cloned in 1994 [5], *BRCA1* has received a great deal of attention in BC. It has been mapped to chromosome 17q21 containing 24 exons, encoding a pleiotropic full-length protein of 1863 amino acids in humans [5]. BRCA1 full-length form is the best-defined *BRCA1* gene product that contains multiple functional domains, including a highly conserved N-terminal RING domain, two nuclear localization signals located in the exon 11, a serine-glutamine (SQ) cluster between amino acids 1280–1524 [6], and tandem C-terminal BRCA1 (BRCT) domains [7–9]. BRCA1 is a serine phosphoprotein that is regulated in a cell cycle-specific manner [10] and hyper-phosphorylated in response to DNA damage [11–14]. As a tumor suppressor, BRCA1 mediates many different molecular processes including repair of double-strand DNA breaks, transcriptional activation, apoptosis, cell-cycle checkpoint control, and chromosomal remodeling, binds different functional proteins (c-myc, E2F, p53, RAD50, cyclins, CDKs, RNA polymerase, etc.), and suppresses development of BC and ovarian cancers [15–18].

Therefore, genomic sequencing of *BRCA1* (and *BRCA2*) in women with a familial history of one or more incidences of early-onset BC or ovarian cancer provides a powerful tool to detect disease predisposition. However, the genomic test is expensive and not suitable for detection of sporadic cancers associated with somatic events. Overall, about 9.3% of female BC patients carry predisposing mutations [19]. Germline mutations of *BRCA1* and *BRCA2* are responsible for about 50% of hereditary BC [20, 21]; nevertheless, these mutations account for only 3–8% of all BCs. Most BCs are sporadic and occur in absence of *BRCA1* mutations [22, 23]. In sporadic breast tumors, many researchers have postulated that loss of heterozygosity (LOH) reduces *BRCA1* messenger RNA (mRNA) and protein levels, induces incorrect subcellular localization [24–27], and impairs methylation of the *BRCA1* promoter region [28–30]. These events lead to noticeable loss of BRCA1 function and provide evidence for a BRCA1 tumor suppressor function in sporadic forms [31]. Besides *BRCA*-mutated BC, sporadic cancers may exhibit a so-called 'BRCAness' feature, as they display a *BRCA1* mutation phenotype without any mutation [32–35]. Nonetheless, BRCAness is generally associated with mutations of other genes of the same signaling pathway. In addition to its involvement in the tumor-suppressing process, BRCA1 is also considered a key player in establishing chemotherapy sensitivity and could thus be considered a predictive factor for patient management [36]. In preclinical and clinical studies, the role of BRCA1 in response to DNA-damaging agents and other types of chemotherapy agents has only partly been elucidated [37, 38]. To the best of our knowledge, numerous studies have investigated the clinic pathological value of the BRCA1 protein level or of its subcellular localization in clearly defined breast carcinomas, including sporadic and *BRCA1*-mutated tumors. Nonetheless, in spite of the findings concerning BRCA1 expression, the clinical value of its subcellular localization is still controversial, mostly due to limited techniques and approaches [24, 39–57].

To address this issue, we evaluated BRCA1 nuclear and cytoplasmic expression using immunofluorescence in a panel of cultured breast cell lines with specific properties. In addition, we used etoposide, as a DNA-damaging reagent, to validate its effect on BRCA1 protein regulation, and shed light on BRCA1 expression patterns in representative cell line models of the different BC types with or without etoposide treatment.

2 Methods

2.1 Cell Culture and Etoposide Treatment

The human adenocarcinoma cell lines MCF-7 and MDA-MB-231, both with the *BRCA1* wild-type gene, were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). The human breast epithelial cell line MCF10A and ductal carcinoma cell line HCC1937 (the latter with *BRCA1* mutation 5382insC [58, 59]) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human breast ductal carcinoma cell line HCC3153 with *BRCA1* mutation (943ins10) [58] was kindly provided by Adi F. Gazdar (Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). Cryopreservation of cell cultures ranged from passages 1 to 10. Cells were used during up to 20 passages. To minimize the heterogeneity that arises from different cultured conditions, and in agreement with our own and literature data [60, 61], all cell lines were incubated routinely in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany), supplemented with 10% FCS (Fetal calf serum) (PAA, Pasching, Austria), in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. A 50 mM etoposide (Sigma-Aldrich, Saint Louis, MO, USA) solution was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, MO, USA) as a stock solution for treatment. In

preliminary experiments (data not shown), we used different dilutions (25, 50, 75, and 100 μM) and incubation times (6, 12, 24, and 48 h). As a result of this optimization procedure, we used 100 μM of etoposide for 48 h as unique treatment for the five cell lines. Hence, cells were treated using a 1:500 dilution of the stock solution (etoposide 100 μM) and vehicle (DMSO 100 μM) was used as control in all experiments. For immunofluorescence and apoptosis assays, 5×10^5 cells were grown on slides (ThermoFisher Scientific, Braunschweig, Germany) overnight to 70–80% confluency, and then treated in 10% FCS with etoposide solution 100 μM for 48 h.

2.2 Fluorescence Labeling of Breast Cancer 1 (BRCA1) or Phosphorylated BRCA1 with Parallel 4′-6-Diamidino-2-Phenylindole (DAPI) Analysis

After 48 h of treatment, culture slides were washed in PBS (phosphate-buffered saline) (Fischer, Saarbrücken, Germany), then immediately fixed in 3.7% neutral buffered formalin (Fischer, Saarbrücken, Germany) in PBS for 15 min at room temperature and permeabilized in cold (− 20 °C) methanol (Sigma-Aldrich, Steinheim, Germany) for 2 min. After washing in PBS, Ultra V Blocking medium (ThermoFisher Scientific, Fremont, CA, USA) was used for 15 min. This blocking step and all the following steps were performed in a humidified chamber at room temperature. Both antibodies were diluted in Dako Antibody Diluent with Background Reducing Components (Dako, Carpinteria, CA, USA). Cells slides were incubated for 1 h with either a monoclonal mouse anti-human BRCA1 antibody (1:200 dilution) (MS110, ab16780, Abcam, Cambridge, UK) or a polyclonal rabbit anti-human phosphorylated BRCA1 (1:200 dilution) (phospho S1423, ab47325, Abcam, Cambridge, UK), washed in PBS, incubated for 30 min with a secondary either goat anti-mouse or anti-rabbit IgG labeled with DyLight488 (Jackson ImmunoResearch, West Grove, PA, USA), and washed in PBS. After drying (30 min, at room temperature), the slides could be mounted with Vectashield Mounting Medium with 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) before manual analysis with a computerized fluorescence microscope Axioskop (Carl Zeiss Micro Imaging GmbH, Göttingen, Germany) for phase and fluorescence, with 40× magnification. An AxioCam MR camera and AxioVision software were used to capture, analyze, and save high-resolution images for two fluorescence channels, considered independently or in combination (Carl Zeiss Microscopy, Göttingen, Germany). Definite threshold values of exposure time for BRCA1 were determined. The percentage of cells expressing no (−), low (+), average (++), or high (+++) levels of BRCA1 in cytoplasm (BRCA1) or nuclei (phosphorylated BRCA1) were calculated by analyzing 1500 cells in each slide. Three independent experiments were systematically performed to calculate the mean values and standard error (SE).

2.3 WST-1 Cell Viability Assay

After 48 h of treatment, cell viability was evaluated using the WST-1 reagent (Roche, Mannheim, Germany), based on the enzymatic cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondria dehydrogenases present in viable cells. Cells (1×10^4/well) were plated in 96-well plates in DMEM medium containing 10% FCS. 24 h later, cells were treated or not in 10% FCS with 100 μmol of etoposide. After 48 h, WST-1 reagent was added to the medium according to the manufacturer's instructions. After 30 min, the absorbance of the samples was measured using the microplate reader (MRX, DYNEX Technologies, Denkendorf, Germany) at 450 nm wavelength. The relative cell viability percentage in each cell line was calculated by comparison to that of the control group. Each condition was performed three times in each experiment and for each cell line, and three independent experiments were then performed to calculate the mean values and SE.

2.4 In Situ Nick-Translation (ISNT) Apoptosis Assay

After 48 h treatment, the in situ nick-translation (ISNT) technique was used to stain DNA fragmentation and apoptotic bodies in the cells for late apoptosis detection [62]. Slides were washed in PBS, then immediately fixed in acetone (Sigma-Aldrich, Steinheim, Germany) for 10 min. After rinsing with distilled water, the endogenous peroxidase was quenched with 0.3% hydrogen peroxide for 10 min. After being rinsed in distilled water again, the slides were equilibrated in nick buffer (0.1 M Tris, 0.1 M $MgCl_2$, 0.75% β-mercaptoethanol, 2 mg/mL bovine serum albumin [BSA]) at room temperature for 10 min. ISNT was then carried out by incubating the slides with deoxynucleotides (dNTPs) (1:50 dilution) (ThermoFisher Scientific, Fremont, CA, USA) and biotinylated 14-deoxyadenosine triphosphate (dATP) (1:20 dilution) (ThermoFisher Scientific, Fremont, CA, USA) diluted in nick buffer for 50 min at 37 °C. Terminating buffer (0.3 M sodium chloride and 0.03 M sodium citrate) was used to rinse the chamber slides at room temperature for 15 min. After washing in PBS and 1% FCS PBS for 10 min each, slides were incubated with extravidin–peroxidase (Sigma, Steinheim, Germany) at room temperature for 30 min. AEC-substrate (Dako, Glostrup, Denmark) was used for color development. Afterwards, the slides were counterstained with Mayer's hemalum (Merck, Darmstadt,

Germany), then immediately mounted with Aquatex (Merck, Darmstadt, Germany) before manual analysis with a Diaplan light microscope (Leitz, Wetzlar, Germany), with 10× and 40× magnifications. The late apoptosis ISNT was calculated by analyzing 1500 cells in each slide. Three independent experiments were systematically performed to calculate the mean values and SE.

2.5 M30 Cyto Death Apoptosis Assay

The M30 cyto Death assay was developed to detect caspase-cleaved Cytokeratin 18, which is one of the earliest apoptosis markers in epithelial cells [63, 64]. After treatment, cells were immediately fixed in pure methanol at − 20 °C for 30 min, washed in washing buffer (0.1% PBS-Tween) and blocked. Afterwards, cells were incubated with a mouse monoclonal antibody (1:25 dilution) (clone M30, Roche, Mannheim, Germany) overnight at 4 °C in a humidified chamber and then with a secondary goat anti-mouse IgG labeled with DyLight488. After drying (30 min at room temperature), the slides were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) before manual analysis with a computerized fluorescence microscope Axioskop (Carl Zeiss Micro Imaging GmbH, Göttingen, Germany) with 40× magnification. The early apoptosis by M30 cyto Death staining was calculated by analyzing 1500 cells in each slide. Three independent experiments were systematically performed to calculate the mean values and SE.

2.6 Statistical Analysis

IBM SPSS® Statistics for Windows, Version 24.0 (IBM, Ehningen, Germany) was used for collection, processing, and statistical data analysis. The student's *t* test was performed for comparison between control and treated group in each cell line. p values ≤ 0.05 were considered statistically significant.

3 Results

3.1 High Cytoplasmic BRCA1 Protein Levels in Aggressive Breast Cancer (BC) Cell Lines

To gain insights into the importance of BRCA1 expression, we characterized and compared five representative breast cell lines with or without etoposide treatment. BRCA1 protein levels were investigated by immunofluorescence in the human breast normal cell line MCF10A and in four human BC cell lines: MCF-7 (wild-type *BRCA1*), MDA-MB-231 (wild-type *BRCA1*, but 'BRCAness' phenotype), HCC1937, and HCC3153 (both *BRCA1* mutated). *BRCA1* mutations in the HCC1937 and HCC3153 cells were in exons 20 and 11, respectively, and the mutated BRCA1 still includes the epitope of the MS110 antibody, with truncation sites far away from the N-terminal end [58, 65]. Staining results are presented in Fig. 1a. The original 40× magnification shows that in control cells, BRCA1 was expressed in the nucleus as well as in the cytoplasm. The enlarged pictures show higher BRCA1 protein levels in the cytoplasm compared with the nuclei of each cell line. For etoposide-treated cells, original magnifications and enlargements demonstrate higher nuclear and cytoplasmic BRCA1 protein levels than in controls, with a more dramatic effect in cytoplasm. Because of this obvious visual difference, we concentrated on solely analyzing BRCA1 cytoplasmic staining to better clarify and quantify the etoposide effect. We counted 1500 cells in each cell slide and evaluated the intensity of BRCA1 cytoplasmic protein levels (no [−], low [+], average [++], and high [+++]) among all cell lines with or without etoposide treatment (Electronic Supplementary Material Table 1 for all data; Fig. 1b for cytoplasmic high expressions). It is noteworthy that within each cell line, cells did not exhibit the same intensity of BRCA1 cytoplasmic staining. Moreover, very few cells exhibit no fluorescence intensity at all (3.3% in untreated MCF-10A and 7% in untreated MCF-7). In the control groups, all five cell lines were found with predominantly low or average protein levels: 71.4 and 80.0% of cells expressing low BRCA1 cytoplasmic staining in MCF-10A and MCF-7 cells; 81, 92.4, and 84.9% of cells expressing low or average staining in MDA-MB-231, HCC1937 and HCC3153 cells, respectively. In the untreated cells, a certain percentage of the population expressed only high levels of cytoplasmic BRCA1 in the MDA-MB-231, HCC1937, and HCC3153 cells (19.1, 7.6, and 15.1%, respectively).

After etoposide treatment, all cell lines showed stronger BRCA1 cytoplasmic staining; in particular, the same MDA-MB-231, HCC1937, and HCC3153 cells expressed high of cytoplasmic BRCA1 levels with 80.4% ($p = 0.005$), 70.6% ($p = 0.002$), and 80.7% ($p = 0.01$), respectively, thus demonstrating a significant rise in the highest protein levels in the entire population (only 1.4% of the HCC1937 still expressed a low cytoplasmic expression, but no cells in the MDA-MB-231 and HCC3153). Besides, only 2.3% ($p = 0.02$) and 11% ($p = 0.003$) of the MCF-10A and MDA-MB-231 cells reached such high cytoplasmic expression, but 50.8% ($p = 0.05$) of the MCF-10A cells and 67.9% ($p = 0.009$) of the MCF-7 cells now expressed intermediate intensities, demonstrating the same action of etoposide—still significant, but to a lower extent than in the three other cell lines. In summary, high cytoplasmic BRCA1 expression characterizes only a minority

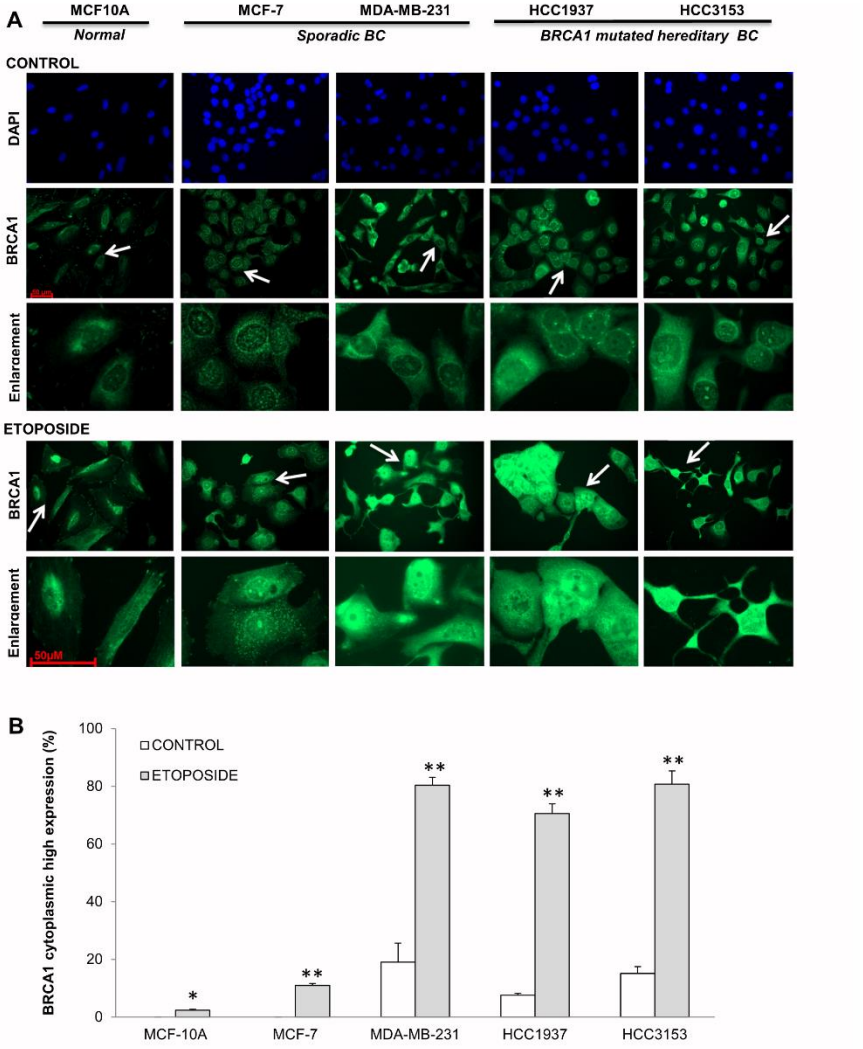

Fig. 1 BRCA1 expression in control and etoposide-treated breast cancer cell lines. Breast cancer cell lines were treated (ETOPOSIDE) or not (CONTROL) with 100 µM of etoposide for 48 h, then immunostained with BRCA1 antibody. **a** Immunofluorescence labelling of BRCA1 (*green*) was performed together with DAPI nuclear staining (*blue*). *White arrows* indicate enlargement parts. Original magnification before enlargement, ×40. *Scale bar* 50 µm. **b** The percentage of cells exhibiting high BRCA1 cytoplasmic staining after analysis of 1500 cells for each experiment (mean value and standard error, $n = 3$). The correlation is statistically significant for $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, or $^{***}p \leq 0.001$. *BC* breast cancer, *BRCA1* breast cancer 1, *DAPI* 4′-6-diamidino-2-phenylindole

of cells in the three more aggressive untreated cell lines (MDA-MB-231, HCC1937, and HCC3153) and etoposide treatment induces a dramatic increase of these cytoplasmic protein levels in all cell lines. For the less aggressive, hormone-dependent model of BC (MCF-7 cells) and for the normal breast cells (MCF-10A model), this specific high cytoplasmic BRCA1 expression only appears in a minority of the etoposide-treated cells.

3.2 High Nuclear Phosphorylated BRCA1 Protein Levels in Aggressive Etoposide-Treated BC Cell Lines

Phosphorylation of BRCA1 is regulated during the cell cycle and in response to DNA damage. We then studied phosphorylated BRCA1 expression, for the five cell lines and in the conditions described in Sect. 3.1 (Fig. 2a). We clearly observed that, in contrast to BRCA1 expression, the phosphorylated BRCA1 staining was all nuclear, with basal protein levels in all cells of the five untreated cell lines. We then semi-quantified the nuclear protein levels of phosphorylated BRCA1, according to the various intensities (again low [+], average [++], or high [+++]), as presented in Electronic Supplementary Material Table 2 for all data and in Fig. 2b for nuclear high expressions. Untreated cells expressed predominantly low/average levels of phosphorylated BRCA1: 100% of the MCF10A, 94.6% of the MCF-7, 91.7% of the MDA-MB-231, 98.5% of the HCC1937, and 88.1% of the HCC3153. Although very rare in any untreated cell line, the high protein levels of nuclear phosphorylated BRCA1, were nonetheless slightly increased in all cell lines after etoposide treatment to 3.2% ($p = 0.04$) of the MCF-10A, 8.4% ($p = 0.12$) of the MCF-7, most notably and significantly in 71.5% ($p = 0.007$) of the MDA-MB-231, 70.8% ($p = 0.001$) of the HCC1937, and 70.4% ($p = 0.003$) of the HCC3153. MCF-10A and MCF-7 cells still exhibited significant low nuclear phosphorylated BRCA1 staining (61 and 46.3%, respectively). In summary, high nuclear protein levels of phosphorylated BRCA1 predominantly characterize the three more aggressive cell lines (MDA-MB-231, HCC1937, and HCC3153) after etoposide treatment.

3.3 Effect of Etoposide on Cell Viability of Breast Cancer Cell Lines

To further investigate the effect of etoposide, cell viability was determined by WST-1 assay. As demonstrated in Fig. 3, etoposide inhibited the viability of all five cell lines at a concentration of 100 µM. Nonetheless, a significant minor effect was observed on the normal breast cell model MCF-10A (87.4% viability; $p = 0.05$) compared to dramatic effects on all the BC cell lines: 35.9% ($p = 0.004$) MCF-7, 22.6% ($p = 0.0001$) MDA-MB-231, 33.2% ($p = 0.005$) HCC1937, and 30.4% ($p = 0.03$) HCC3153.

3.4 Effect of Etoposide on Late and Early Apoptosis

We then wanted to correlate the viability results to apoptosis and performed in parallel assays for late apoptosis analysis by ISNT and for early apoptosis by M30 staining using conditions already described (Fig. 4a, b, respectively). The rate of late apoptosis (Fig. 4c) detected in the untreated and etoposide-treated MCF10A cells had a similar mean value of 0.5 and 0.6% ($p = 0.6$), respectively, demonstrating that etoposide did not significantly stimulate apoptosis of the normal breast cell model MCF-10A. The normal rate of apoptosis in the untreated MCF-7, MDA-MB-231, HCC1937, and HCC3153 had minimal means of 1, 0.9, 1, and 1.1%, respectively, while exposure to etoposide significantly increased apoptosis in MCF-7, and to a higher extent in MDA-MB-231, HCC1937, and HCC3153 to 2.4% ($p = 0.009$), 4.3% ($p = 0.005$), 3.3% ($p = 0.01$), and 3.1% ($p = 0.006$), respectively.

The rates of early apoptosis were found to be very similar to those of late apoptosis (Fig. 4d). The normal breast model, MCF10A cells, control or treated, again had a similar mean value of 0.8 and 0.9% ($p = 0.74$), respectively. Besides, the normal rates of apoptosis in the four untreated BC cell lines were confirmed to be very low, inferior to 2%, whereas they were significantly elevated to 2.7% ($p = 0.0005$), 6.5% ($p = 0.004$), 6.4% ($p = 0.008$), and 7.0% ($p = 0.001$) after etoposide treatment (MCF-7, MDA-MB-231, HCC1937, and HCC3153, respectively).

4 Discussion

Since the 1990s, the importance of BRCA1 expression and of its subcellular localization as a marker in sporadic BC has been under debate. Chen et al. [49] first reported that BRCA1 was found in the nuclei of epithelial cells, and detected mainly in the cytoplasm of malignant mammary cells. In contrast, Scully et al. [50] showed that BRCA1 was located predominantly in the nuclei of both normal and malignant cells, whereas Jensen et al. [51] contradicted this by stating that BRCA1 was observed in cytoplasm and cell membrane. Following this, there has been a slow stepwise progression in the understanding of the subcellular distribution of BRCA1, often hampered by technical problems attributable to cross-reactivity and low specificity of certain BRCA1 antibodies. In recent years, advanced technologies and approaches enabled to detect more phosphorylated than non-phosphorylated forms of BRCA1 in nuclear and mitochondrial genomes than in cytoplasm [66]. This demonstrated that BRCA1, as a shuttle protein, shuttles

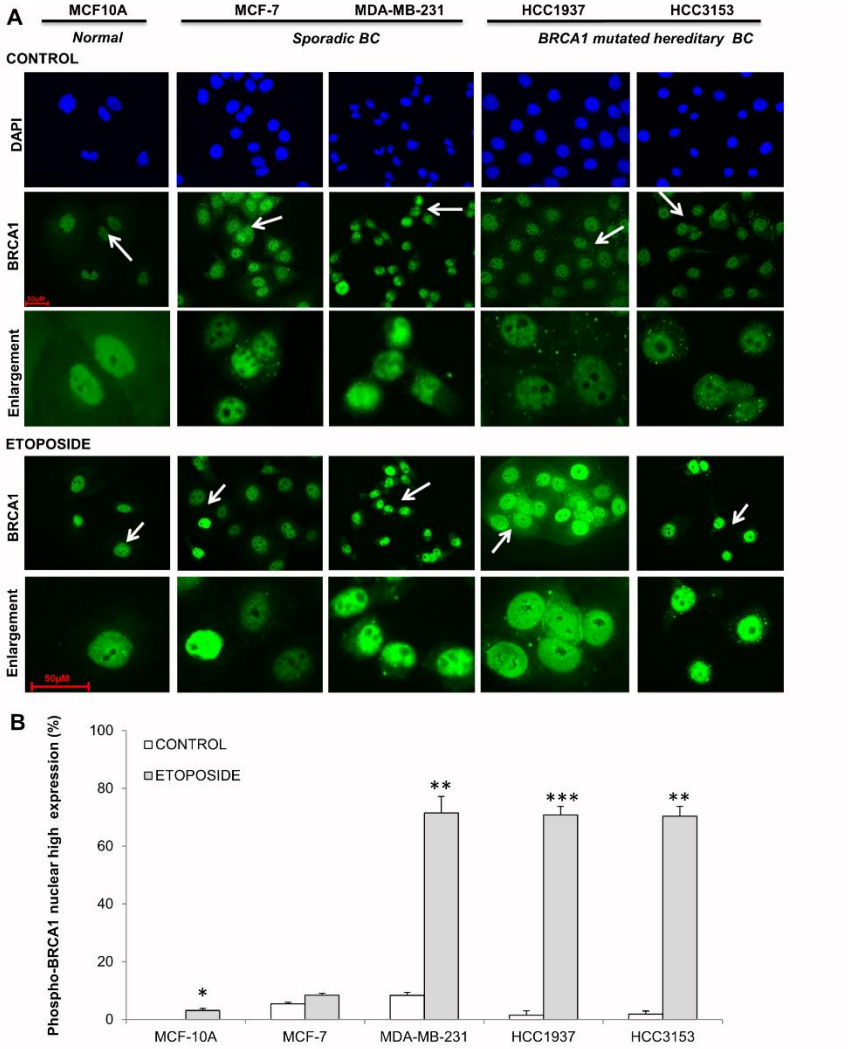

Fig. 2 Phosphorylated BRCA1 expression in control and etoposide-treated breast cancer cell lines. Breast cancer cell lines were treated (ETOPOSIDE) or not (CONTROL) with 100 µM etoposide for 48 h, then immunostained with phosphorylated BRCA1 antibody. **a** Immunofluorescence labelling of phosphorylated BRCA1 (*green*) was performed together with DAPI nuclear staining (*blue*). *White arrows* indicate enlargement parts. Original magnification before enlargement, ×40. *Scale bar* 50 µm. **b** The percentage of cells exhibiting high BRCA1 nuclear staining after the analysis of 1500 cells for each experiment (mean value and standard error, $n = 3$). The correlation is statistically significant for $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, or $^{***}p \leq 0.001$. *BC* breast cancer, *BRCA1* breast cancer 1, *DAPI* 4′-6-diamidino-2-phenylindole

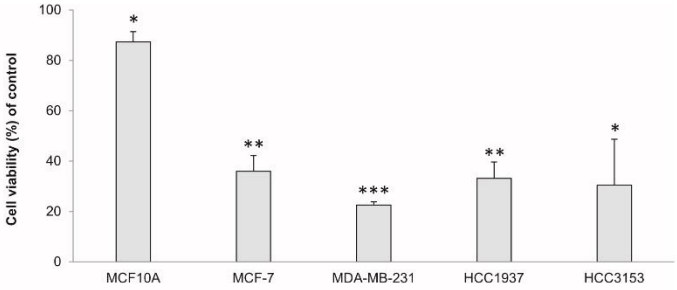

Fig. 3 Viability of etoposide-treated breast cancer cell lines. Breast cancer cell lines were treated (ETOPOSIDE) or not (CONTROL) with 100 μM etoposide for 48 h, then cell viability was analyzed by WST-1. The quantitative assessment of viability is presented as the mean value and standard error ($n = 3$). The correlation is statistically significant for $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, or $^{***}p \leq 0.001$

between specific sites within the nucleus and cytoplasm, including DNA repair foci, centrosomes, and mitochondria, and uses its different transport sequences to form distinct protein complexes with various protective roles [67, 68]. However, little is known about how BRCA1 shuttling between the nucleus and cytoplasm is controlled [69]. The specificity of the antibodies selected for BRCA1 detection is also a key point to explore. Wilson et al. [24] first tried to comprehensively characterize 19 anti-BRCA1 antibodies, suggesting that the monoclonal antibody MS110 (Ab-1), targeting the 304 first amino acids from the N-terminal end of BRCA1, is highly specific and allows evaluation of BRCA1 localization and relative protein levels in normal and malignant human breast and ovarian tissues. Perez-Valles et al. [70] demonstrated that this MS110 antibody gives the most accurate, reliable, and reproducible results in familial and sporadic non-BRCA1 associated breast carcinomas among a four-antibody panel. Using the same MS110 antibody, Milner et al. [71] proposed the measurement of nuclear BRCA1 expression by immunohistochemistry (IHC) on breast and ovarian tumor tissue sections, as patient selection biomarker by focusing exclusively on cells in the S/G2 phase where BRCA1 protein staining is expected. Wei et al. [72] aimed to investigate the associations of BRCA1 nuclear expression and clinic pathological characteristics in young Chinese BC patients, and Mylona et al. [47] applied IHC on sporadic BC patients to explore a different prognostic significance of BRCA1 protein, according to its subcellular distribution. In this study, we further investigated BRCA1 protein levels, by selecting five representative mammary cell lines: MCF-10A, a human normal breast epithelial cell line, which is a widely used in vitro model for studying normal breast cell function and transformation, in spite of some controversies [73], MCF-7 and MDA-MB-231, sporadic BC models, and HCC1937 and HCC3153, *BRCA1*-mutated BC cell models. Of note, the MCF-7 cell line is a model of non-aggressive hormone-dependent cancer cells (luminal A), whereas MDA-MB-231, HCC1937, and HCC3153 belong to aggressive triple-negative BC (TNBC) [74–76]. Regarding the MDA-MB-231 cell line, it shares many features with *BRCA1*-mutated tumors [77] and is associated to the BRCAness phenotype, defined as a phenocopy of *BRCA1* or *BRCA2* mutations, initially different from *BRCA1* mutations [32]. We selected the widely used antibody MS110 [24, 70–72, 78, 79] and demonstrated BRCA1 protein levels in both the nucleus and cytoplasm of the five normal and cancerous subtypes, which is consistent with other reports [47, 68, 80–83]. In this article, we wanted to detect whether BRCA1 protein expression—irrespective of *BRCA1* gene mutation—could differentiate BC subtypes: normal/sporadic/*BRCA1*-mutated or aggressive/non-aggressive. Some sporadic BC cell lines have no mutation of the *BRCA1* gene, such as MDA-MB-231, but nonetheless exhibit BRCAness. Consequently, we aimed to define the relationship between BRCA1 expression and different types BC cell lines. As all cell lines were observed to express predominantly null, low, or average protein levels of BRCA1, with heterogeneous expressions within each cell line, it made it difficult to differentiate BC subtypes using either nuclear or cytoplasmic BRCA1 protein levels. Nonetheless, it is noteworthy that 7–19% of cells expressed high levels of cytoplasmic BRCA1 only in the three more aggressive TNBC cell lines.

Etoposide, as topoisomerase II poison, induces double- and single-strand breaks in DNA [84]. This plant alkaloid is an oral drug used eventually in anthracycline and taxane pre-treated metastatic BC [85, 86] or may be useful in combination with new targeted therapy such as anti-vascular endothelial growth factor (VEGF), histone deacetylase, and DNA damage response (DDR) inhibition treatments [87–90]. In HeLa cervix carcinoma cells and SK-OV-3 ovarian cancer cells, BRCA1 mRNA levels were increased by etoposide treatment [91, 92], while BRCA1 expression displayed only a minimal increase in MCF-7 nuclei [93]. Using the conditions we optimized (100 μM concentration and 48 h duration), our data demonstrate that etoposide treatment induced higher cytoplasmic BRCA1

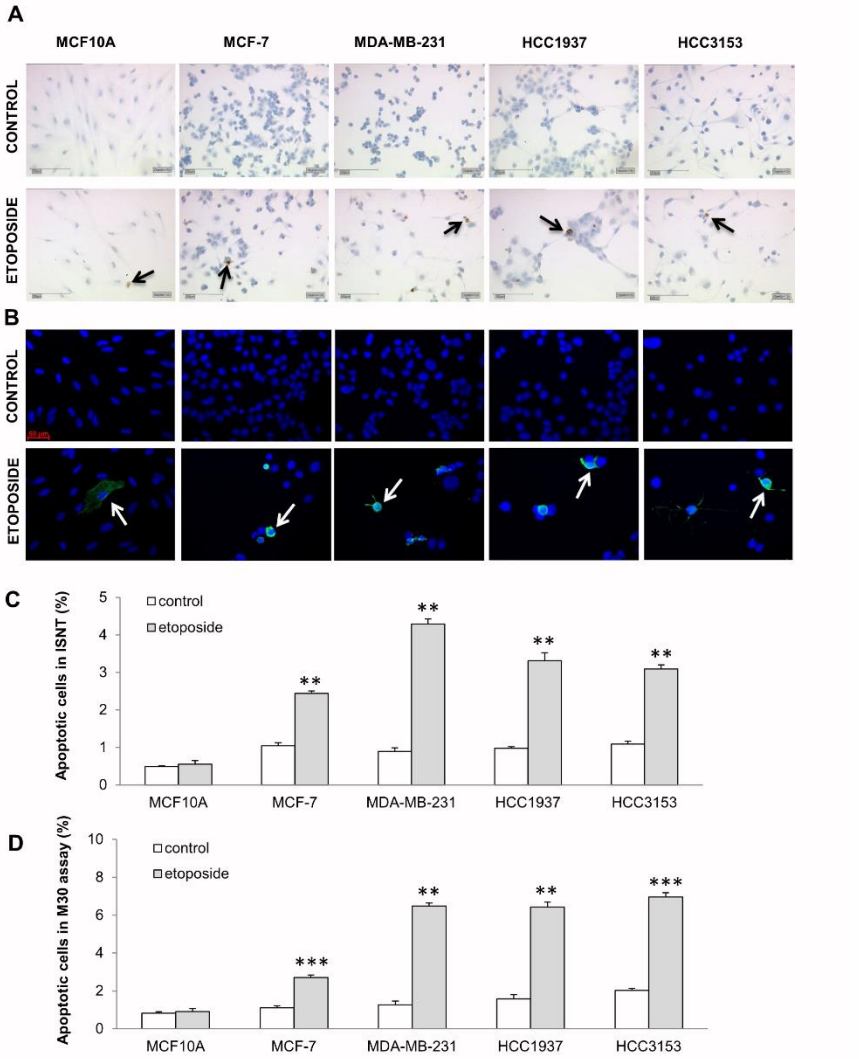

Fig. 4 Late and early apoptosis in etoposide-treated breast cancer cell lines. Breast cancer cell lines were treated (ETOPOSIDE) or not (CONTROL) with 100 µM etoposide for 48 h, then apoptosis was detected by in situ nick translation (ISNT) assay for late apoptosis (**a**) and M30 cyto Death assay for early apoptosis (**b**). Apoptotic cells were stained *brown* in (**a**) (*black arrows*) and *green* in (*b*) (*white arrows*). The related percentages of apoptotic cells are presented after the analysis of 1500 cells for each experiment in (**c**) and (**d**), respectively (mean value and standard error, $n = 3$). The correlation is statistically significant for $^{**}p \leq 0.01$ or $^{***}p \leq 0.001$

levels in the five breast models, with more than 70% of cells expressing high cytoplasmic levels of BRCA1 in the three aggressive *BRCA1*-deficient or -mutated cell lines, MDA-MB-231, HCC1937, and HCC3153. In comparison, only 2 and 11% of the MCF10A and MCF-7 cells expressed these high cytoplasmic levels of BRCA1: BRCA1 cytoplasmic protein levels increased essentially from low to average intensities in most cells of these non-tumorigenic MCF-10A and luminal A type MCF-7 models. Thereby, we could distinguish even better the three aggressive TNBC *BRCA1*-deficient or -mutated cell lines from the normal and luminal subtypes according to BRCA1 cytoplasmic protein levels after using etoposide. Cytoplasmic expression of BRCA1 could be explained by two probable mechanisms: cytoplasmic retention and nuclear export. BRCA1 is trapped in the cytoplasm following overexpression of the anti-apoptotic factor Bcl-2, which redirects BRCA1 to mitochondria and endoplasmic reticulum [94]. In addition, it is notable that HCC1937 has a phosphatase and tensin homolog on chromosome 10 (PTEN) deletion, and the PTEN inactivation causes an increase in cellular PIP3 levels subsequently activating PI3 K/AKT signaling. This causes an increased expression of several genes for cell growth, cell survival, and cell migration, including BRCA1. AKT1 kinase was also reported to suppress homologous recombination (HR)-mediated DNA repair through the cytoplasmic retention of BRCA1 and Rad51 [95, 96]. Meanwhile, the nuclear export of BRCA1 was directly linked to p53-independent pro-apoptotic activity [97, 98]. BRCA1 and p53 are both tumor suppressors, which are involved in many cellular processes. BRCA1 has been reported to bind directly to p53, thereby enhancing p53-mediated transcriptional activation [99–101]. Nuclear run-on experiments and luciferase reporter assays demonstrate that the changes in BRCA1 expression are mainly due to transcriptional repression induced by p53 [102]. Nuclear export of BRCA1 occurred in response to ionizing radiation DNA damage in cells with functional p53 but in cells lacking wild-type p53 BRCA1 was retained in the nucleus [69]. Compared to p53 wild-type MCF-7 and MCF10A, both HCC1937 and MDA-MB-231 are p53 mutants, while, to our knowledge, the p53 status of HCC3153 is unknown, although its protein level is negative [103]. In our study, MCF-7 and MCF10A demonstrated an increase of cytoplasmic BRCA1 expression after treatment, which is consistent with the former study. But due to an abnormal BRCA1 and p53 status, the other three cell lines showed much stronger cytoplasmic expressions before treatment. Fedier et al. [104] reported that BRCA1 deficiency in p53-null cells was associated with increased sensitivity to the topoisomerase II poisons etoposide, which could be a mechanism to explain our observations. A study claimed to observe a correlation between cytoplasmic localized BRCA1 and activation of the intrinsic caspase cleavage pathway, in particular after DNA damage [105, 106]. As mentioned earlier, p53, PTEN status, and other tumor suppressors that are also crucial for therapy outcome might have functional interplay with BRCA1 and thus lead to BRCA1 expression alteration and cellular shuttling. To date, the actual mechanism by which cytoplasmic-localized BRCA1 elicits cell death is not fully understood but may be a reason for the increased rate of apoptosis shown in the following apoptosis assay.

As BRCA1 is a serine phosphoprotein regulated in response to DNA damage [11], it has been reported that DNA damage induces both nuclear redistribution of BRCA1, which may also explain increased cytoplasmic staining and an increased phosphorylation of the protein through DNA damage-activated kinases [14, 107, 108]. Several phosphorylation sites have been identified under these conditions, including Ser-1423 [109–111]. We used phospho-Ser-specific antibodies recognizing the Serine in position 1423 of BRCA1 to further explore the regulation of BRCA1 phosphorylation in non-treated and etoposide-treated cells. Our study demonstrated that phosphorylated BRCA1 was mainly located in the nuclei, before and after treatment. BRCA1 being a serine phosphoprotein regulated in a cell cycle-specific manner, its phosphorylation starts when cells enter S-phase. Phosphorylated BRCA1 then accumulates in the nucleus where it functions in the cellular response to DNA damage and regulates specific processes including cell cycle checkpoint activation, DNA repair, and chromatin remodeling. Coene et al. [66] also support a universal role for BRCA1 in the maintenance of genome integrity in nucleus. In addition, DNA damage also induces an increased phosphorylation of the protein through DNA damage-activated kinases. Our results reasonably demonstrate the same trend as a low or medium basal nuclear expression of phosphorylated BRCA1 characterized all non-treated cell lines, with no cell line exhibiting high levels of phosphorylated BRCA1. As expected, etoposide treatment moderately increased the percentage of normal and luminal A cells expressing high nuclear levels of phosphorylated BRCA1 (reaching 3.2 and 8.4%, respectively). In contrast, more than 70% of the TNBC, *BRCA1*-deficient or -mutated, cells expressed high nuclear phosphorylated BRCA1. This extremely elevated expression may be the result of the inefficiency of the mutated or deficient *BRCA1* in these cell lines. These results obtained by immunofluorescence for BRCA1 protein levels and phosphorylation status in five different cell lines confirm preliminary data we generated using immunocytochemistry colorimetric, non-fluorescent staining (data not shown). However, samples are pre-treated differently according to the protein analysis technique and this may profoundly influence the ability of a given

antibody to bind specifically to its target [112]. So in the future, the results and conclusions of our study will have to be extended using alternate protein analysis technique as western-blot. Moreover, manipulation of BRCA1 expression using RNA interference may demonstrate the importance of BRCA1 for prediction of response to DNA-damaging drug.

Our data suggest that etoposide could induce apoptosis, as we observed an obvious reduction, 60–80%, in the four BC cell populations compared to control cells, whereas the normal breast cells exhibited only a slight decrease. We confirmed that etoposide did induce early and late apoptosis among the four BC cell lines, around a two-fold increase for the MCF-7 and three- to five-fold increases in the three aggressive TNBC cell lines. This higher apoptosis induction rate in the *BRCA1*-deficient/-mutated cells may relate to the higher expression of cytoplasmic BRCA1 and of nuclear phosphorylated BRCA1. All the results we generated strongly suggest that these three aggressive TNBC cell lines might share some identical pathways related to BRCA1 during DNA damage repair. The elevated expression of (phosphorylated) BRCA1 in cytoplasm or nucleus, before or after treatment, may be associated with the prognosis and further studies are needed to develop this approach as diagnostic assay in BC. In the near future, (phosphorylated) BRCA1 could be first analyzed in the tumors of a large cohort of patients with different BRCA1 status. Unlike the two *BRCA1*-mutated HCC cell lines, MDA-MB-231 is a model of sporadic BC without *BRCA1* mutation. But as a member of basal-like BCs (BLBCs), MDA-MB-231 shares many features with *BRCA1*-mutated tumors [77]. In the meantime, three-quarters of *BRCA1*-associated tumors are BLBCs [113]. Dysfunctions of the BRCA1 pathway detected in BLBCs mainly regards the impairment of double-strand break (DSB) repair through HR, leading to genomic instability. The hallmark of BLBCs is the 'BRCAness' [32]; previously, the concept of BRCAness referred to the fact that sporadic tumors characterized by reduced or absent BRCA1 expression share the same phenotype of familial BRCA cancers [35]. Over 20 years, a reassessment of the concept of BRCAness was required and nowadays it describes the situation in which an HR repair (HRR) defect exists in a tumor in the absence of a germline *BRCA1* or *BRCA2* mutation [33]. BRCAness is then a common characteristic for MDA-MB-231, HCC1937, and HCC3153. Since the role of *BRCA1* in DNA repair is mainly related to the HR, the new proposed biomarker (cytoplasmic BRCA1) should be compared to the classical (Rad-51 foci in cyclin-A positive cells) or even novel HR assays [114, 115].

There is limited information on *BRCA2* mutations in the discussed cell lines. Distribution of histologic types of *BRCA1*-associated BCs differs from sporadic BCs in various aspects: having distinct morphology, being more often medullary-like, being triple negative, and showing a 'basal' phenotype; but *BRCA2*-associated BCs do not appear to exhibit a specific pathologic phenotype [18, 116]. In *BRCA1*-mutant tumors, the capability of DNA damage repair is decreased, which makes tumor cells more sensitive to DNA-damaging drugs than normal BC cell lines [117]. Consistent with the HRR defect, tumors with BRCAness might also share therapeutic vulnerabilities with germline *BRCA1* or *BRCA2* mutation tumors, such as sensitivity to platinum-based drugs and then Poly (ADP-ribose) polymerase inhibitor (PARPi) [118]. It was recently suggested that inhibition of the DDR (cell cycle arrest and DNA repair) could increase the efficacy of conventional DNA-damaging agents. In particular, like PARPi, which targets the DDR in specific tumor cells, it can selectively kill tumor cells carrying *BRCA* mutations but not normal cells [119].

5 Conclusion

To date, BRCA1 protein measurement evaluated as a potential diagnostic and prognostic biomarker for BC has never reached a consensus. In our study, with etoposide induction, we can better distinguish BRCA1-associated BC cell line representative subtypes by evaluating cytoplasmic BRCA1 protein level. Meanwhile, our results also show that the increased sensitivity of BRCA1-deficient cells to etoposide may be due to the specific DSB created by topoisomerase II. However, a larger set of BC cell lines with specific sensitivity to various DNA damage agents and different levels of cytoplasmic BRCA1 should be characterized to confirm our hypothesis using other accurate and reliable technologies. Therefore, we suggest that cytoplasmic BRCA1 protein levels level could be considered and further explored as a potential predictive marker for response chemotherapy in both sporadic and hereditary BC. Although this evaluation could not specifically help in guiding treatment, we intend to analyze tumor samples through further collaboration with clinicians in the future. Our results also raise several issues concerning the functions of BRCA1 in the DNA damage pathway and biochemical details of signaling conferred by nuclear phosphorylated BRCA1. BRCAness phenotype and germline *BRCA1* or *BRCA2* mutation tumors are both aggressive BCs with a poor prognosis which could share common clinical management strategies. Many targeted therapies have been developed against *BRCA1*-mutated BC, of which PARPi are most promising drugs.

Acknowledgements We would like to thank C. Kuhn for technical advice and the China Scholarship Council (CSC) for a scholarship to Xi Zhang's.

Authors Contributions SS and UJ conceived and designed the project. XZ wrote the paper and performed most experiments. SH assisted with cell culture. SS contributed to manuscript writing and editing. NH and UJ conceived the topic and contributed to manuscript editing. SS supervised the research. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest Xi Zhang, Simone Hofmann, Nadia Harbeck, Udo Jeschke, and Sophie Sixou declare that they have no conflict of interest.

Funding Sophie Sixou's salary was supported by the University Paul Sabatier in Toulouse (France).

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

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10. Acknowledgements

First of all, I would like to express my appreciation and thanks to Prof. Dr. med. Nadia Harbeck for giving me this precious opportunity to work in the Department of breast center, Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, as a doctoral student.

I would like to express my sincere thanks to Prof. Dr. Udo Jeschke for his support, advice and trust in my work. I will not have my publication without his supports. His enthusiasm for scientific research motivated me and from his excellent scientific knowledge I could learn a lot in the last years.

My most special and sincere thanks must go to Prof. Sophie Sixou who is always supportive of me throughout my time in Munich. She provided me lots of help and advices both in my academic and daily life. I cannot make it without her supervision on my thesis and publications.

Special thanks go out to all my other colleagues Kuhn Christina, Simone Hoffman, Zhu junyan, Ye Yao and Shao Wanting. Thank you for your invaluable help and support whenever I needed and all the jokes that greatly enriched the everyday life in the lab! Moreover, I would like to acknowledge the excellent technical assistance of Ms Kuhn Christina and Ms Simone Hoffman, who taught me all the basic experiment techniques and are always available to help. I am so grateful being a member of such a supportive and considerate working group.

I would like to thank to my family for their support and faith in me during any time in my life and to my friends for motivating me and having enjoyable times out of the lab.

I give my special appreciation to my husband Yang Shiqing for his support and company.

My work in LMU was supported by scholarship China Scholarship council.