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The Transcription Factor NRF2 and its Interactions with the Steroid Receptors ERα, PRA and PRB in Epithelial Ovarian Cancer

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List of Abbreviations

β-TrCP	β-transducin-repeat-containing protein
(TP53) p53	Tumor protein p53
AhR	Aryl Hydrocarbon Receptor
AKR1C1	aldo- keto reductase family 1 member C1
AMPK	5' adenosine monophosphate-activated protein kinase
ARE	Antioxidant Response Element
BRAF	B-Raf (rapidly accelerated fibrosarcoma) proto-oncogene, serine/threonine kinase
BRCA1/BRCA2	Breast Cancer 1/2 gene/protein
cAMP	Cyclic adenosine monophosphate
c-MYC	cellular Myelocytomatosis
CUL1	E3 ubiquitin ligase
DJ-1	Protein deglycase
DNA	Deoxyribonucleic acid
E2	estrogen
EMT	epithelial–mesenchymal transition
EOC	Epithelial Ovarian Cancer
ER (α/β)	estrogen receptor (α/β)
ERE	estrogen-responsive elements
ERK	extracellular signal-regulated kinase
ESR1/ESR2	Estrogen receptor 1/2 genes
FDA	United States Food and Drug Administration
FIGO	International Federation of Gynaecology and Obstetrics
HBOC	hereditary breast ovarian cancer syndrome
HGSOC	High-grade serous ovarian carcinoma
HNPCC	hereditary non-polyposis colorectal carcinoma syndrome
HO-1	heme oxygenase 1
HRT	hormone replacement therapy
KEAP1	Kelch-like ECH-associated protein1/gene
KRAS	Kirsten Rat Sarcoma
Maf proteins	musculoaponeurotic fibrosarcoma proteins
MAPK	mitogen-activated protein kinase
mRNA	Messenger RNA (Ribonucleic acid)

NADPH	Nicotinamide adenine dinucleotide phosphate
NFE2L2	Nuclear Factor, Erythroid 2 like 2
NF-kB	nuclear factor k-light-chain- enhancer of activated B cells
NOTCH1	Notch homolog 1, translocation-associated
NRF2	Nuclear factor erythroid 2-related factor-2
p21	cyclin-dependent kinase inhibitor 1
p62	ubiquitin-binding protein
PARP-Inhibi-tors	Poly (ADP-ribose) Polymerase-Inhibitors
PCR	Polymerase chain reaction
PGR	progesterone receptor gene
PI3K-AKT	phosphoinositide 3-kinase (PI3K)– protein kinase B (Akt)
PKA	cyclic adenosine monophosphate (cAMP)/protein kinase A
PR (A/B)	progesterone receptor A/B
PRE	progesterone response elements
RAF kinase	Rapidly Accelerated Fibrosarcoma (serine/threonine-specific protein kinases)
ROS	reactive oxygen species
siRNA	Small interfering RNA (Ribonucleic acid)
SOD1	Cu/Zn superoxide dismutase
TLR	toll-like receptor
TNM	Classification of Malignant Tumors
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless-related integration site

List of Publications

Journal Articles

1. Czogalla, B.; Kahaly, M.; Mayr, D.; Schmoeckel, E.; Niesler, B.; Kolben, T.; Burges, A.; Mahner, S.; Jeschke, U.; Trillsch, F., **Interaction of ERalpha and NRF2 Impacts Survival in Ovarian Cancer Patients.** *International journal of molecular sciences* **2018,** 20, (1).

2. Czogalla, B.; Kahaly, M.; Mayr, D.; Schmoeckel, E.; Niesler, B.; Hester, A.; Zeder-Goss, C.; Kolben, T.; Burges, A.; Mahner, S.; Jeschke, U.; Trillsch, F., **Correlation of NRF2 and progesterone receptor and its effects on ovarian cancer biology.** *Cancer management and research* **2019,** 11, 7673-7684.

3. Deuster, E.; Hysenaj, I.; Kahaly, M.; Schmoeckel, E.; Mayr, D.; Beyer, S.; Kolben, T.; Hester, A.; Burges, A.; Mahner, S.; Jeschke, U.; Trillsch, F.; Czogalla, B., **Rupatadine as a useful therapy in ovarian cancer?: the impact of platelet-activating factor receptor on patients' survival time and its inhibition by Rupatadine.** Manuscript submitted for publication

Conference Posters and Abstracts

1. Kahaly, M.; Rahmeh, M.; Vogel, M.; Jeschke, U.; Mahner, S.; Czogalla, B., **Der Transkriptionsfaktor NFE2L2 und seine Signalwege in der Ätiologie des Ovarialkarzinoms.** *Geburtshilfe Frauenheilkd* **2017,** 77, (05), A39. Jahrestagung der Österreichischen Gesellschaft für Gynäkologie und Geburtshilfe (OEGGG) gemeinsam mit der Bayerischen Gesellschaft für Geburtshilfe und Frauenheilkunde e.V (BGGF) Congress Hofburg Wien, 14.–17.06.2017

2. Kahaly, M.; Rahmeh, M.; Mayr, D.; Niesler, B.; Burges, A.; Trillsch, F.; Mahner, S.; Jeschke, U.; Czogalla, B., **Combined Expression of Nuclear Factor Erythroid 2-Related Factor 2 and Estrogen Receptor Alpha is Prognostically Positive in Ovarian Cancer.** *Oncology Research and Treatment* **2018,** 41, (1), 1-221. 33. Deutscher Krebskongress, Berlin 21.-24.02.2018

Author Contribution to the Submitted Publications

Contribution to the First Submitted Publication

For the publication ‘Interaction of ERα and NRF2 Impacts Survival in Ovarian Cancer Patients’ the author Maja Kahaly (M.K.) performed all the experiments which had been conceived by the co-authors Udo Jeschke (U.J.) and Bastian Czogalla. (B.C.). M.K. performed the majority of the experiments during her scheduled full-time research semester ‘Modul 6’ in 2016 and continued the experiments during her medical studies in 2017 and 2018. M.K. and U.J. analyzed the data and M.K. presented extracts of the results at two scientific conferences in Vienna in 2017 and in Berlin in 2018. M.K. designed the figures and together with B.C. wrote the article. M.K. and B.C. share co-first authorship of the publication which was published in 2018.

Contribution to the Second Submitted Publication

For the second submitted publication ‘Correlation of NRF2 and progesterone receptor and its effects on ovarian cancer biology’ M.K. was involved in most of the experiments which took part in 2018 and 2019. M.K. performed the staining evaluation of the immunohistochemical staining of NRF2, PRA and PRB, contributed to the PCR and si-RNA assays and designed the corresponding figures. M.K. and U.J. analyzed the data. The first author B.C. wrote the article which was published in 2019 with the contribution of M.K. and M.K. is second author.

1. Introduction

1.1 Epithelial Ovarian Cancer

1.1.1 Epithelial Ovarian Cancer Epidemiology

Ovarian cancer is the seventh most frequent malignant tumour in females and the second most common reason of gynaecological cancer decease worldwide (1, 2). The majority of Ovarian Cancer consists of Epithelial Ovarian Cancer (EOC) (3). Collectively 7350 women were detected with Epithelial Ovarian Cancer in Germany in 2016 and EOC was the reason for 5.2% of all cancer-related deaths in German females in 2016 (4). Age-standardised ratios for EOC are consistent or decreasing in the majority of high-income countries, but they are increasing in numerous less wealthy countries (1). Insufficient screening methods and unspecific symptoms lead to late stage detection in the preponderance of cases with EOC. The mortality of ovarian cancer stays high relative to other cancers of the female reproductive organs and relative five-year survival is 43% for women with EOC in Germany (2, 4).

1.1.2 Epithelial Ovarian Cancer Risk Factors

“A family medical record of breast or ovarian cancer” marks the most influential risk factor for EOC (5). There are two hereditary tumour syndromes that can cause EOC. The hereditary breast ovarian cancer syndrome (HBOC) and the hereditary non-polyposis colorectal carcinoma syndrome (HNPCC) otherwise specified as ‘Lynch Syndrome’ (3). *Breast Cancer 1* and *2* (*BRCA1* and *BRCA2)* are the primary genes linked to HBOC which code for BRCA1 and BRCA2, essential “proteins in charge of the homologous recombination mending of double-strand DNA disruptions” (6). “Germline alterations of *BRCA1* and *BRCA2* are detected in 5-10% of breast cancer involved patients and in 10-18% of patients suffering from EOC” (7, 8). Next to *BRCA1* and *BRCA2*, several other genes have been discovered to be associated with the development of HBOC. Also alterations in the genes *ATM, CDH1, CHEK2, NBN, MLH1, MSH2, MSH6, PALB2, PMS2, RAD51C, RAD51D, TP53,* were connected to ovarian cancer evolvement (2-4, 9). Two-sided Salpingo-Oophorectomy is the utmost efficient method to decrease the mortality and risk of developing hereditary EOC (10). Other factors identified as potential risk factors for the development of EOC are nulliparity, endometriosis, obesity, age, post-menopausal hormone therapy and perineal talc application (3, 4, 11). Gravidity, the use of oral contraceptives and tubal ligation diminish the risk to develop EOC (2, 3).

1.1.3 Epithelial Ovarian Cancer Subtypes and Prognostic Markers

The large majority of ovarian cancers is caused by EOC (3). Nonepithelial ovarian carcinomas classify less than 5% of all ovarian malignancies. They comprise “germ cell neoplasms, sex cord-stromal neoplasms, small cell carcinomas” and ovarian sarcoma (5). EOC is classified in five major “subtypes; the high-grade serous ovarian carcinoma (HGSOC) and low-grade-serous ovarian carcinoma, the mucinous ovarian carcinoma (expansile and infiltrative subtype), the endometrioid ovarian carcinoma (high grade and low grade subtype) and the clear-cell ovarian carcinoma, differing regarding their phenotype, molecular background, aetiology and clinical outcome” (12-14). HGSOC is the most frequent subtype representing more than 50% of EOC cases (4) and is mostly detected in late cancer stages. The majority of high-grade ovarian carcinomas is assumed to originate in the distal fallopian tube (15, 16). 15-20% HGSOC cases are linked to inheritable alterations “in *BRCA1* and *BRCA2* genes or less frequent variations in other homologous recombination genes” (3).

The crucial prognostic marker for EOC is the extensiveness “of residual illness after primary debulking operation” (17). Other prognostic markers comprehend “the International Federation of Gynaecology and Obstetrics (FIGO) stage, the quantity of ascites, age of a patient and histological subtype” (17, 18).

1.1.4 Epithelial Ovarian Cancer Therapy

EOC therapy comprises initial debulking surgery by an experienced gynaecological oncologist which allows accurate staging using the FIGO and TNM classifications (18). Cytoreductive operation is succeeded by platinum-based monochemotherapy or combined with paclitaxel (4). Unfortunately, chemoresistance represents an important challenge in EOC treatment (19). Targeted therapy has been gradually introduced into EOC treatment in recent years. The humanized monoclonal antibody Bevacizumab which aims at the vascular endothelial growth factor (VEGF), has become an essential part in the therapy of woman with progressive stage and recurring disease (3, 4). Poly (ADP-ribose) polymerase (PARP)- Inhibitors target especially *BRCA1/BRCA2*-mutated cancers. PARPs are enzymes implicated “in base excision restore, crucial in the process of single-strand DNA breaks repair” (7). In suppressing the reparation of single-strand DNA breaks, PARP-Inhibitors confer ‘synthetic lethality’ to *BRCA1/BRCA2*-mutated cells (20). Recently, PARP-Inhibitors were authorized as first-line maintenance treatment for women suffering from advanced EOC, regardless of their *BRCA* status, after initial successful platinum-based chemotherapy (4, 7, 21-23). Immune Checkpoint Inhibitors, which have greatly improved the treatment of other tumour entities, like melanoma, are being tested in pre-clinical and clinical studies for EOC, until now with mixed results (24-26). Findings

of latest investigations suggest that the combination of immune checkpoint inhibitors with chemotherapeutics or PARP-Inhibitors are a promising approach for ovarian cancer treatment (25, 27, 28). Most of EOCs express the estrogen receptor (ER), however antiestrogen therapy was not favourable for patients with EOC (29). Local radiation therapy of the affected area is only used in selected cases of recurrent disease for symptom control (4).

1.2 NRF2

1.2.1 NRF2's Role in Physiology

Nuclear factor erythroid 2-related factor-2 (NRF2) is a transcription factor which is ubiquitously fabricated at low quantities and appertains "to the cap "n" collar group of transcription factors" (17, 30). NRF2 regulates genes with "antioxidant and cytoprotective" properties (31). The physiological role of activated NRF2 is to protect the cell from inordinate defect evoked "by metabolic, xenobiotic, and oxidative stress" (17, 32, 33). NRF2 is present in the cytoplasm in its inactive form bound by Kelch-like ECH-associated protein1 (Keap1), under physiological states. Keap1 controls the stability of NRF2 in accordance with the redox conditions. In a redox-balanced environment, Keap1 continually guides NRF2 for ubiquitination and successive decomposition by the 26S proteasome (30, 34). Whilst a cell is confronted with oxidative stress, cysteine fragments of Keap1 are oxidized involving a conformational alteration of the Keap1-NRF2 compound and enabling NRF2 to move to the nucleus (35). Within the nucleus, NRF2 connects along with small musculoaponeurotic fibrosarcoma (Maf) proteins, to antioxidant response elements (AREs) in the promotor area of target genes which induces the transcription of its downstream genes (31, 36, 37). Another NRF2 decomposition mechanism exists in the nucleus (38). β-transducin-repeat-containing protein (β-TrCP) builds a complex with "E3 ubiquitin ligase CUL1 and ubiquitinates NRF2" to inhibit dispensable "NRF2 overinduction provoked by Keap1 inactivation" (38, 39). NRF2 induces the transcription of numerous cytoprotective genes and its downstream genes are classified in distinct categories. These categories include genes which code for "intracellular redox-balancing proteins, phase I/II/III detoxifying enzymes", in addition to enzymes participating in the "lipid, heme and glucose metabolism" (32). Furthermore, NRF2 induces the transcription of genes coding for enzymes which play a role in the nicotinamide adenine dinucleotide phosphate (NADPH) generation and pentose synthesis, along with proteins regulating cellular autophagy, apoptosis and xenobiotic responses (32, 38).

The NRF2-Keap1-ARE pathway was discovered in 1999 (40). Since then, the regulatory mechanisms of NRF2 and its role in the cellular defence against oxidative stress have

been an important topic of research. In recent years, the dialogue and interaction among the NRF2-Keap1-ARE pathway and various influential networks were revealed, which confirmed the position of NRF2 at the centre of a complex regulatory network (30, 40-42). The transcription factor Aryl Hydrocarbon Receptor (AHR) for instance which is implicated “in the supervision of drug-metabolizing enzymes” (43), binds to the promotor region of the gene *Nuclear Factor, Erythroid 2 like 2 (NFE2L2*), which encodes for NRF2 and thus regulates *NFE2L2* gene transcription directly (37). Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) proteins, a widely studied group of transcription factors which play an essential part in a multitude of mechanisms, such as inflammation, immune response, apoptosis, development, and cell growth (37), interact with NRF2, too (44). Bidirectional crosstalk between NRF2 signalling and p53, an important tumour suppressor, and Notch homolog 1, translocation-associated (NOTCH1), a transmembrane receptor involved in the development of tissues, have been described as well (37, 42). Apart from its role in DNA repair mechanisms, BRCA1 also acts as an influential modulator of oxidative stress by regulating the Keap1-mediated NRF2 ubiquitination activity and therefore stabilizes NRF2 (44-46).

1.2.2 NRF2’s Role in Cancer

A dual role for NRF2 in cancer, chronic diseases and inflammation has been described (30). The controversial question if activating, or rather inhibiting NRF2 is a useful strategy for averting or treating cancer (47), has been an important field of research in recent years (47). Generally speaking, the role of activated NRF2 in carcinomas and their “microenvironment is intricate and determined by the cell type and context” (46). Several studies indicated the beneficial effects of NRF2 in the prevention of oxidative stress- or inflammation-associated carcinogenesis (48, 49), which is due to the ability of NRF2 to balance the intake, repartition, “metabolism and excretion of carcinogens”, together with its anti-inflammatory response (30). However, increased levels of NRF2 were detected in numerous malignancies (39). High and prolonged activation of NRF2 is linked to advancement, growth of metastases, angiogenesis and the resistance to radio- and chemotherapy in different cancers, including EOC (19, 39, 50-52).

Several mechanisms exist which lead to the elevated activeness of NRF2 in malignancies. Somatic mutations of the encoding gene for Keap1 (*KEAP1)* leading to the inactivation of Keap1 strongly induce NRF2 (38), as well as epigenetic silencing of *KEAP1* (50, 53). Different proteins can alter the NRF2-Keap1 interaction, too. The cyclin-dependent kinase inhibitor 1 (p21), the ubiquitin-binding protein p62 and Protein deglycase DJ-1 positively regulate NRF2 through various mechanisms and more proteins interacting with NRF2 and Keap1 are continuously discovered (32, 39). Furthermore, the tran-

scription of *NFE2L2* is upregulated by activated oncogenes, such as *Kirsten Rat Sarcoma* (*KRAS), "rapidly accelerated fibrosarcoma (B-Raf) proto-oncogene, serine/threonine kinase (BRAF)"* and *cellular Myelocytomatosis c-MYC* (54). Metabolic factors can increase NRF2 as well when Fumarate Hydratase, an enzyme engaged in the Krebs cycle, is mutated which contributes to the "accumulation of Fumarate" within the cell and therefore to the stabilization of NRF2 (50, 53). In *BRCA1* deficient cells, NRF2 activation is regulated by estrogen (E2) which promotes "antioxidant genes that shield the cell from reactive oxygen species (ROS) -induced death" (55).

1.2.3 NRF2's Role as a Pharmaceutical Target

Several molecules that reinforce the activity of NRF2 were investigated for the prevention of diseases in recent years (50). Numerous NRF2 enhancers are natural, "plant-derived phytochemicals" such as sulphoraphane or curcumin (50). NRF2 inducers have been shown to protect from critical damages "to the lung, kidney, brain, liver, eye and heart" (47, 56-62) that are provoked by different elements such as "cigarette smoke, hypoxia, ischaemia–reperfusion injury, and chemical toxins" (47, 61-64). Furthermore, NRF2 activating agents exhibited favourable effects in the prevention of chronic illnesses for instance diabetes, obesity and various neurodegenerative diseases. Dimethyl fumarate, a synthetic NRF2 activator was authorized by the United States Food and Drug Administration (FDA) for multiple sclerosis therapy in 2013 (30, 32, 38, 47).

The implication of NRF2 in cancer advancement and chemo- and radio resistance led to the discovery of various NRF2 inhibitors (65, 66). NRF2 inhibitors have been shown to sensitize former chemo- or radio resistant cancer cells to anti-tumour drugs and radiotherapy (53, 66), making them a useful addition in cancer therapy. In recent years, already established therapeutics have been repurposed for new indications especially for cancer treatment. Several well-known established drugs have been shown to act trough NRF2 signalling on cancer cells (67). Metformin, a biguanide applied in the therapy of type II diabetes, inhibits proliferation in various carcinoma cell lines by suppressing heme oxygenase 1 (HO-1) fabrication via the blocking of a "Rapidly Accelerated Fibrosarcoma (RAF)/ extracellular signal-regulated kinase (ERK)/" NRF2 signalling and "5' adenosine monophosphate-activated protein kinase (AMPK)–independent" pathways (67, 68). Metformin has also been demonstrated to chemo sensitize cancer cells from various cancer types, including ovarian cancer by downregulating NRF2 (69, 70). The tyrosine kinase inhibitor Apatinib, stimulates ROS-dependently "apoptosis and autophagy through the NRF2/HO-1 mechanism in EOC cells" (71).

1.3 Estrogens and Estrogen Receptors

Estrogens are steroid hormones derived from cholesterol. The most effective estrogen hormone in the circulation,17β-Estradiol, modulates many crucial physiological mechanisms comprising the growth and preservation “of reproductive organs and the” management “of cardiovascular, musculoskeletal, immune, and central nervous system homeostasis” (72, 73). As Estrogens influence many physiological processes, they are also involved in various diseases such as “obesity, metabolic disorder, numerous carcinomas, osteoporosis, lupus erythematosus, endometriosis, and uterine fibroids” (74). The biological effects of estrogens are exerted through the estrogen receptors (ER) α and β, which appertain to a large group of ligand-activated nuclear receptors and which are found on separate genes (*ESR1* and *ESR2*) (72, 74, 75). ERα is primarily detected “in reproductive tissues, the ovary (interstitium and theca cells), kidney, bone, white adipose tissue, and liver”, whereas ERβ is found “in the ovary (only in the granulosa cells), prostate, lung, gastrointestinal tract, bladder, hematopoietic cells, and the central nervous system” (33, 74, 76-79). ERα is also reported to be present in the mitochondria where it is concerned with the regulation of ROS and apoptosis (78). ERα and ERβ form dimers to regulate gene transactivation and ER dimers connect directly “to estrogen-responsive elements (EREs) in the managerial areas of estrogen target genes” (77). There exist two other genomic ER-guided transcriptional control procedures; the indirect attachment to other transcription factors (tethering) and the “ligand-independent receptor activation by growth factors” along with further signalling mechanisms within the cell (74, 78).

ERα interacts with a variety of proteins and pathways. ERα, bound to the DNA, together with protein conglomerates are implicated “in base excision restore, cell fate decision, and oxidative stress reaction” (80). The Cu/Zn superoxide dismutase (SOD1) is a protein affiliated to ERα and engaged in the management of oxidative stress (80) for which an interaction with NRF2 has been described (81). Estradiol also increases NRF2 protein concentration but not mRNA levels directly by generating oxidative stress (31, 82, 83). As already mentioned above, Estrogen regulates NRF2 activation in *BRCA1*-deficient cells (55) through stimulation of the phosphoinositide 3-kinase (PI3K)– protein kinase B (AKT) allowing *BRCA1*-deficient cells to survive (84). A dual role for Estradiol in the modulation of NRF2 has been reported; NRF2 is activated by the metabolites of Estradiol through the production of ROS (independent of ER), however Estradiol is able to suppress the NRF2 downstream genes through connecting to ERα (31, 85-87).

1.4 Progesterone and Progesterone Receptors

Progesterone is a steroid hormone derived from cholesterol and the precursor to androgens and estrogens (88). Progesterone plays a crucial role in normal physiology (89).

Progesterone is also named ‘the pregnancy hormone’, as it is indispensable ahead of and throughout pregnancy (88). Furthermore, progesterone is a mediator for female reproductive activity, “nerve restoral, adaptive immune system, brain damage rehabilitation, sleep apnoea, and nervous systems” (88, 90). Progesterone exerts its biological effects through progesterone receptors A and B (PRA, PRB), two isoforms which appertain to the group of nuclear hormone receptors (75, 88) and which are encoded by the gene *PGR*. PRs are found in numerous human tissues, for instance the female reproductive organs the “brain, pancreas, bone, testes, and tissues of the lower urinary tract” (89). PRA is crucial for normal ovarian function and nearly absent in ovarian carcinoma cells, whereas PRB is mostly expressed in ovarian carcinoma cells (91). Progesterone binds to the PR leading to receptor dimerization and DNA binding (89). The PR then binds to progesterone response elements (PREs) for gene transcription (75, 91), but can also bind to other DNA-bound transcription factors or growth factor receptors for gene expression and activation of downstream signalling cascades (91).

Interactions of progesterone and the PR with various networks including the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), mitogen-activated protein kinases (MAPK), toll-like receptors (TLRs)/NF-κB, and the PI-3K/Akt signalling pathway have been described (84, 90). Additionally, steroid receptors, including the PR and ER, participate in complexes and modulate activities of each other (91). Progesterone also interacts with p21 which is able to stabilize NRF2 (39, 91). Progesterone was also shown to activate NRF2 signalling directly and together they exert neuroprotective effects on patients with traumatic brain injury (90). Recent studies revealed that interaction of NRF2, aldo-keto reductase family 1 member C1 (AKR1C1) and PR might be the molecular mechanism for progestin (synthetic progesterone) resistance in endometrial cancer (92).

1.5 Steroid Hormones and Steroid Receptors in Epithelial Ovarian Cancer

Numerous therapies for estrogen associated diseases are linked to regulating the activities of ERα and ERβ (93). The expression of steroid hormone receptors is of “therapeutic and prognostic” interest in the management of “breast, endometrial or prostate cancer”, however investigations on EOC and ER or PR expression are scarce and provide discrepant results (29, 94). Estrogen and progesterone seem to have different roles in the prevention or induction of cancer, also depending on cancer types. In breast cancer cells, progesterone, together with estrogen, stimulates proliferative and pro-survival gene programs (91), on the other hand, progesterone protects against the evolvement of estro-

gen-induced endometrial cancer (88, 91). Several studies indicated the beneficial prognostic impact of ER and PR expression on disease-specific and progression free survival and proposed PR-B as independent prognostic marker for EOC (29, 94-100). As already mentioned above, hormone replacement therapy (HRT) including estrogen replacement therapy after menopause, is a risk factor for the evolvement of EOC (17), whereas pregnancy and oral contraceptive use decrease the possibility of developing EOC which is attributed to the high levels of progesterone (4, 16, 88, 101). Another study showed that estrogen led to “epithelial–mesenchymal transition (EMT)” and enhanced EOC cell migratory capability therefore increasing the metastatic potential (88). Progesterone, however, had “anti-proliferative and anti-metastasis” impacts in EOC and acted as an opponent to estrogen (88). This point of view is supported by recent studies which show that “progesterone averts HGSOC through promoting necroptosis of p53-deficient fallopian tube epithelial cells” (15) and by repressing Wnt/βcatenin signalling in serous ovarian cancer precursor lesions in fallopian tubes (16). In contrast, it was detected that estrogen enhances tumour growth by supporting the Wnt/βcatenin signalling in the precursor lesions (16). Additionally, it was discovered that the stroma surrounding epithelial tumours in the ovary, which present corresponding receptors (e.g. ER and PR) is activated to produce steroid hormones, which may stimulate further neoplastic growth (102). In contrast to the treatment of other gynaecological malignancies, antiestrogen treatment has not been shown to be successful in the therapy of EOC (29). Also, the efficacy of several antiprogestins was studied in clinical trials for breast cancer and gynaecological diseases (91). Therefore, the antiprogestin Mifepristone was studied in patients diagnosed with EOC but was not proven to be an effective instrument in the treatment of women with EOC (91, 103).

1.6 Intentions and Objectives of the Studies

Even though immense progress has been achieved in the diagnosis and therapy of many cancers in the last decades, the prognosis for women with EOC has hardly improved over time (4, 29). The majority of patients with EOC is detected with advanced cancer stages and cytoreductive surgery together with platinum-based chemotherapy have been the traditional therapeutic options for decades (4, 75). Until now, reliable prognostic markers and effective individual targeted treatments for patients with EOC, which take the heterogeneity of ovarian cancer subtypes into account, are scarce (29). With the latest initiation “of Poly (ADP-ribose) polymerase (PARP)-Inhibitors as maintenance” treatment after initial successful platinum-based chemotherapy for patients regardless their *BRCA1/BRCA2* status, a first step into the right direction of targeted therapies for EOC patients has been made (4).

The development of EOC appears to be associated with oxidative stress (31, 33). The transcription factor NRF2 has an essential function in the handling of oxidative stress in normal physiology by regulating a multitude of antioxidant and cytoprotective genes (30, 38). Both the lack of NRF2 and its overexpression have been linked to various pathologies. NRF2 seems to have a dual function in cancer since cancer preventive and cancer promoting properties of NRF2 have been described (32, 39, 48, 49, 53). Hence, the function of NRF2 in ovarian cancer has not been resolved (31, 52, 104-106).

The steroid hormones estrogen and progesterone are crucial for the evolution and function of the female reproductive system but exert their effects in a multitude of other physiological mechanisms through their receptors ERα, ERβ, PRA and PRB (72, 74, 77, 78, 93). The functions of steroid hormones and steroid receptors in different carcinoma types varies substantially and they appear to have both agonistic and antagonistic properties in the promotion or prevention of tumorigenesis. Oral contraceptives and pregnancy prevent the development of EOC whereas hormone replacement therapy was discovered to be a risk factor for it (75). Being an important part of cancer treatment regimens in breast or endometrial cancer, endocrine therapy for ovarian cancer had limited success in trials (4). Therefore, the prognostic significance of steroid hormones and the steroid receptors in EOC is still under debate (29, 88, 91, 94-97, 99, 107). Interactions between NRF2 and steroid receptors have been reported in other diseases (45, 55, 85-87, 108), but there exist only a few results on their interaction in ovarian cancer (83).

The first intention of the two submitted studies was to explore the prognostic function of NRF2 and the steroid receptors ERα, PRA and PRB in different ovarian cancer subtypes. We thus assessed “tissue specimen of 156 women who were operated on for EOC at the Department of Obstetrics and Gynaecology of the Ludwig-Maximillian’s-University in Munich between the years 1990 and 2002. Demographic and clinical information were acquired from the women’s records and follow up information from the Munich Cancer Registry” (17, 33). Immunohistochemistry was performed to detect NRF2, ERα, PRA and PRB staining in the nuclei and cytoplasms of the cells and staining was evaluated applying the semi-quantitative immunoreactive score (IR score, Remmele’s score). In the second of the enlisted publications, immunofluorescence staining was carried out to further investigate the existence of NRF2 and PRB in the EOC tissues. Subsequent to staining the tissue samples, statistical assessment was done with SPSS 25.0. We compared IR scores of NRF2 among distinct clinical and pathological subtypes, detected correlations amid results of immunohistochemical staining and determined survival times (17).

The second goal of the following studies was to further explore and compare the expression and correlation of *NFE2L2, ESR1* and *PGR* differentiating between subtypes and to

detect their potential interaction on a molecular level. We therefore performed polymerase chain reaction (PCR) analysis to detect expressions of *NFE2L2, ESR1* and *PGR* in the EOC "cell lines OVCAR3 (serous), ES-2 (clear cell), TOV112D (endometrioid) and UWB1.289 (serous, BRCA1 negative)" and compared them to their expressions in the benign ovarian cell line HOSEpiC (33). We then inhibited *NFE2L2* expression through small interfering RNA (siRNA) knockdown of *NFE2L2* to rate the impact of NRF2 on the genetic expression of *ESR1* and *PGR*. Messenger RNA "(mRNA) fabrication quantities of *NFE2L2, ESR1* and *PGR* in *NFE2L2* silenced cells were contrasted with *NFE2L2* comprising cells" to detect possible functional interactions (17).

1.7 Results and Evaluation of the Studies

In the two submitted studies we detected that NRF2 and PRA vary significantly in histologic subtypes of EOC and the level of NRF2 in the cytoplasm was linked to the expressions of ERα, PRA and PRB (17, 33). We demonstrated that "NRF2's cytoplasmic expression was significantly more present in women with low-grade histology" (17) and NRF2's expression in the cytoplasm alone, and combined with ERα, PRA and PRB expression corresponded to ameliorated overall survival (17, 33). These results correspond to a previous study suggesting that cytoplasmic NRF2, which corresponds to its inactive form, is beneficial for patients with EOC, whereas nuclear or activated NRF2 is linked to impaired overall survival (104). ERα, PRA and PRB expression could not be affirmed as independent prognostic factors for the studied cohort. In the molecular analysis of the first enlisted publication, it was revealed that the *NFE2L2* expression was doubled in EOC cell types OVCAR3 and UWB1.289 contrasted to the benign ovarian cell line HOSEpiC, whereas *ESR1* was significantly less expressed in all EOC cell types versus in the benign ovarian cell line HOSEpiC (33, 109). An augmented level of *ESR1* and *PGR* in the *NFE2L2* downregulated cells was detected confirming functional interactions of *NFE2L2, ESR1* and *PGR* on a molecular level (17, 33, 109). In conclusion, in the submitted publications the prognostic role and interactions of NRF2 and the steroid receptors ERα, PRA, and PRB in EOC were evaluated taking the different EOC subtypes into account. Further studies are warranted to investigate the NRF2-ER-PR-pathways and possible therapeutic perspectives in EOC.

2. Zusammenfassung

Das Ovarialkarzinom ist eine häufige gynäkologische Krebserkrankung der Frau. Die Prognose des Ovarialkarzinoms ist im Vergleich mit anderen gynäkologischen Tumoren schlecht. Da die Erkrankung erst spät zu spezifischen Symptomen führt und es bis heute keine verlässlichen Screening Methoden gibt, werden die meisten Patientinnen mit fortgeschrittenen Stadien diagnostiziert. Die zytoreduktive Operation in Kombination mit einer platinhaltigen Chemotherapie bilden die Standardtherapie in den meisten Fällen. An zielgerichteten Therapieansätzen, welche zwischen den Subtypen des Ovarialkarzinoms unterscheiden, und an subtypspezifischen prognostischen Markern mangelt es bislang. NF-E2-related factor-2 (NRF2) ist ein Transkriptionsfaktor, der eine Vielzahl von antioxidativen und zytoprotektiven Genen reguliert und in seiner inaktiven Form im Cytoplasma vorliegt. NRF2 galt lange als Tumorsuppressor, allerdings wurde in den letzten Jahren in verschiedenen Tumoren eine aberrante Expression von NRF2 festgestellt. Die Steroidhormone Östrogen und Progesteron und ihre Rezeptoren Östrogen Rezeptor α (Erα), Progesteron Rezeptor A und B (PRA, PRB) regulieren die Funktionen des weiblichen Reproduktionstraktes, sowie viele weitere physiologische Prozesse, sind aber auch relevant in der Entstehung von gynäkologischen Erkrankungen und Tumoren. Gegenstand der vorliegenden Dissertation war die Untersuchung des Transkriptionsfaktors NRF2, sowie der Steroidrezeptoren ERα, PRA und PRB in verschieden Subtypen des Ovarialkarzinoms. Das Ziel der beiden vorgelegten Publikationen war es, Rückschlüsse auf den prognostischen Wert der oben genannten Faktoren für das Ovarialkarzinom zu ziehen, sowie mögliche Interaktionen zwischen NRF2, ERα, PRA und PRB auf molekularer Ebene aufzudecken. Hierzu wurden in den beiden publizierten Artikeln die Proteinexpressionen von NRF2, ERα, PRA und PRB in 156 Tumorproben mittels Immunhistochemie bestimmt und anschließend statistisch aufgearbeitet. Dabei zeigten sich signifikante Unterschiede in den Expressionen von NRF2, PRA und PRB in den verschiedenen Subtypen. Außerdem korrelierten die zytoplasmatische Expression von NRF2, und somit seine inaktive Form, mit den Expressionen von ERα, PRA und PRB. In Kaplan-Meier Überlebenszeitanalysen konnten die hohe zytoplasmatische Expression von NRF2 und Expressionen von ERα, PRA und PRB mit einer verlängerten Überlebenszeit in Verbindung gebracht werden. Als unabhängige prognostische Marker konnten die untersuchten Faktoren allerdings nicht nachgewiesen werden. Um die Expression der kodierenden Gene von NRF2 (*NFE2L2)* und ERα (*ESR1)* auf molekularer Ebene zu erforschen, wurde in der ersten Veröffentlichung eine Polymerase-Kettenreaktion (PCR) Analyse durchgeführt, welche signifikante Unterschiede der Genexpressionen beider Gene zwischen den Karzinomzelllinien im Vergleich mit der benigne Ovarzelllinie feststellte. Darüber hinaus wurden in beiden Publikationen der Einfluss einer unterdrückten *NFE2L2* Expression mittels small interfering RNA (siRNA) auf die Expressionen von *ESR1* und

dem für PRA und B codierenden Gen (*PGR)* ermittelt. Es konnten somit Interaktionen zwischen *NFE2L2, ESR1* und *PGR* auf molekularer Ebene erwiesen werden.

3. Abstract

Ovarian cancer is a frequent gynaecological malignancy but its survival rates are poor in comparison to other gynaecological tumours. Most patients are diagnosed with advanced cancer stages as the disease leads to late unspecific symptoms and efficient screening methods are lacking. Cytoreductive surgery together with platinum-based chemotherapy compose the traditional therapeutic approach for most cases. Both targeted therapies, which differentiate between ovarian cancer subtypes, and subtype specific prognostic markers are scarce. The transcription factor NF-E2-related factor-2 (NRF2) regulates a multitude of antioxidant genes with cytoprotective properties and its inactive form is found in the cytoplasm. In recent years, aberrant concentrations of NRF2 in various tumours have been detected, although NRF2 has been considered as a tumour suppressor. Estrogen and progesterone are steroid hormones, which, together with their receptors Estrogen Receptor α (ERα), Progesterone Receptor A (PRA), Progesterone Receptor B (PRB), control the function of the female reproductive organs and many other physiological processes, as well as are implicated in the occurrence of gynaecological diseases and malignancies. The intention of the present dissertation was the investigation of the function of the transcription factor NRF2 and the steroid hormone receptors ERα, PRA and PRB in various epithelial ovarian cancer (EOC) subtypes. The goal of the two submitted publications was to examine the prognostic relevance of the aforementioned factors for EOC but also to detect potential interactions between NRF2, ERα, PRA and PRB on a molecular level. In the two published articles the protein expressions of NRF2, ERα, PRA and PRB were identified in 156 tumour tissue samples with immunohistochemistry and interpreted statistically subsequently. Significant differences in the expressions of NRF2, PRA and PRB were shown in ovarian cancer subtypes. The cytoplasmic expression of NRF2, therefore its inactive form, was linked to the expressions of ERα, PRA and PRB and Kaplan Meier calculations demonstrated longer survival times for women with strong cytoplasmic NRF2 expressions and expressions of ERα, PRA and PRB. The submitted publications did not confirm the aforementioned factors to be independent prognostic factors. In the first publication, polymerase chain reaction (PCR) analysis was conducted to investigate the presence of the encoding genes for NRF2 (*NFE2L2)* and ERα (*ESR1)* in different EOC cells. Significant differences in the gene expressions between the cancer cell lines and the benign cell line were identified. Small interfering RNA (siRNA) silencing of *NFE2L2* expression influenced the gene expressions of *ESR1* and the encoding gene for PRA and B (*PGR*), which prove the molecular interactions of *NFE2L2, ESR1* and *PGR* in both submitted papers.

4. Paper I

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Article

Interaction of ERα and NRF2 Impacts Survival in Ovarian Cancer Patients

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Abstract: Nuclear factor erythroid 2-related factor 2 (NRF2) regulates cytoprotective antioxidant processes. In this study, the prognostic potential of NRF2 and its interactions with the estrogen receptor α (ERα) in ovarian cancer cells was investigated. NRF2 and ERα protein expression in ovarian cancer tissue was analyzed as well as mRNA expression of NRF2 (*NFE2L2*) and ERα (*ESR1*) in four ovarian cancer and one benign cell line. *NFE2L2* silencing was carried out to evaluate a potential interplay between NRF2 and ERα. Cytoplasmic NRF2 expression as inactive form had significantly higher expression in patients with low-grade histology ($p = 0.03$). In the serous cancer subtype, high cytoplasmic NRF2 expression (overall survival (OS), median 50.6 vs. 29.3 months; $p = 0.04$) and high ERα expression (OS, median 74.5 vs. 27.1 months; $p = 0.002$) was associated with longer overall survival as well as combined expression of both inactive cytoplasmic NRF2 and ERα in the whole cohort (median 74.5 vs. 37.7 months; $p = 0.04$). Cytoplasmic NRF2 expression showed a positive correlation with ERα expression ($p = 0.004$). *NFE2L2* was found to be highly expressed in the ovarian cancer cell lines OVCAR3, UWB1.289, and TOV112D. Compared with the benign cell line HOSEpiC, *ESR1* expression was reduced in all ovary cancer cell lines (all $p < 0.001$). Silencing of *NFE2L2* induced a higher mRNA expression of *ESR1* in the *NFE2L2* downregulated cancer cell lines OVCAR3 ($p = 0.003$) and ES2 ($p < 0.001$), confirming genetic interactions of NRF2 and ERα. In this study, both inactive cytoplasmic NRF2 and high ERα expression were demonstrated to be associated with improved survival in ovarian cancer patients. Further understanding of interactions within the estradiol–ERα–NRF2 pathway could better predict the impact of endocrine therapy in ovarian cancer.

Keywords: estrogen receptor alpha; nuclear factor erythroid 2-related factor 2; ovarian cancer; immunohistochemistry

1. Introduction

Ovarian cancer is the eighth most frequent cause of cancer death among women and the most lethal gynecological malignancy [1]. Relative five-year survival is less than 50% for patients with epithelial ovarian carcinoma (EOC) [2]. Main reasons for poor prognosis are insufficient screening methods, late stage detection, and resistance to chemotherapy later in the clinical course. As most

patients have advanced stage disease, recommended therapy consists of cytoreductive surgery and platinum-based chemotherapy which might be combined with antiangiogenic bevacizumab. Residual disease after initial debulking surgery is the most important prognostic factor being influenced by treating physicians, while further clinical and pathological prognostic factors include the degree of differentiation, the International Federation of Gynecology and Obstetrics (FIGO) stage, and histological subtype [3–6]. With serous, mucinous, endometrioid, and clear cell histology, invasive EOC exhibits several histopathological subtypes that are phenotypically, molecularly, and etiologically distinct [7]. The association between tumor biomarker expression and survival varies substantially between subtypes and can be distinguished in overall analyses of all EOCs [8,9].

According to current investigations, the occurrence of EOC seems to be related to oxidative stress [9]. By activating the nuclear factor erythroid-2-related factor 2 (NRF2), a relevant regulator of antioxidant and cytoprotective genes, both healthy and tumor cells can cope with oxidative stress. NRF2 is ubiquitously expressed at low levels in all human organs. As NRF2 regulates a major cellular defense mechanism, tight regulation is crucial to maintain cellular homeostasis. High constitutive levels of NRF2 have been described in different tumors or cancer cell lines [10–14]. Overexpression of NRF2 might protect cancer cells from the cytotoxic effects of anticancer therapies, resulting in resistance for chemo- or radiotherapy [15,16].

So far, the role of estrogen in EOC is still debated [17]. While application of exogenous hormones for menopause-related symptoms could be associated with an increased risk of EOC [18], a protective effect of oral contraceptives has been described. The estrogen receptor (ER) is expressed in two isoforms, the ERα and ERß [19]. ERα mediates the effects of female steroid hormones on proliferation and apoptosis of EOC cells, and immunohistochemical assessment of ER status is routinely done for the clinical management of breast cancer [19]. Molecular and cell biological interactions between NRF2 and ERα have been reported so far [16,20]. The aryl hydrocarbon receptor and ERα differentially modulate NRF2 transactivation in MCF-7 breast cancer cells [16]. Furthermore, studies show an important crosstalk between NRF2 and ERα in neurophysiological processes [16,20].

To better understand these effects in EOC, we first assessed the prognostic influence of NRF2 and ERα in various subtypes of EOC. To understand the interaction of NRF2 and Erα on a molecular level, we investigated the expression and their correlation in vitro.

2. Results

2.1. NRF2/ERα Expression Correlates with Cinical and Pathological Data

Nuclear staining of NRF2 was technically successful in 145 of 156 cases (93%) with positive staining in 144 of 145 cases (99%). Cytoplasmic staining of NRF2 was evaluable with technically adequate staining in 139 of 156 cases (89%) (Figure 1 and Figures S1 and S2) and NRF2 expression was observed in all these 139 specimens (100%). Median (range) immunoreactivity scores (IRS) for NRF2 in nuclei and cytoplasm were 8 (2,12) and 8 (4,12), respectively.

NRF2 expression displayed correlations to clinical and pathological data (Table 1). NRF2 staining in both cytoplasm and nucleus was different between the histological subtypes ($p = 0.001$ and $p = 0.02$, respectively) with low nuclear NRF2 expression in serous, clear cell, and endometrioid histology and high expression in the mucinous subtype. In comparison, the strongest and weakest cytoplasmic NRF2 staining was found in the serous and clear cell subtypes, respectively. Cytoplasmic NRF2 expression had significantly higher expression in patients with low-grade histology ($p = 0.03$), and low nuclear NRF2 expression was associated with age ($p = 0.045$) (Table 1).

ERα staining was successfully performed in all 156 cases (100%), and ERα expression was observed in 70 of 156 (45%) specimens with a median (range) IRS of 4 (1,12) (Figure 1 and Figure S1). There was no significant difference in the ERα expression comparing all histological subtypes ($p = 0.21$). Analysis of clear cell and endometrioid ovarian cancer subtypes revealed nearly significant upregulation ($p = 0.05$). Analyzing the grading, there were no significant differences in general, and

low-graded patients showed significantly higher ERα expression compared to high-graded patients ($p = 0.028$). All other parameters, such as FIGO, lymph node involvement (pN), and distant metastasis (pM), showed no significant differences in the ERα expression. NRF2 cytoplasmic expression correlated with ERα expression ($p = 0.004$, Table 2 and Figures 1 and 2).

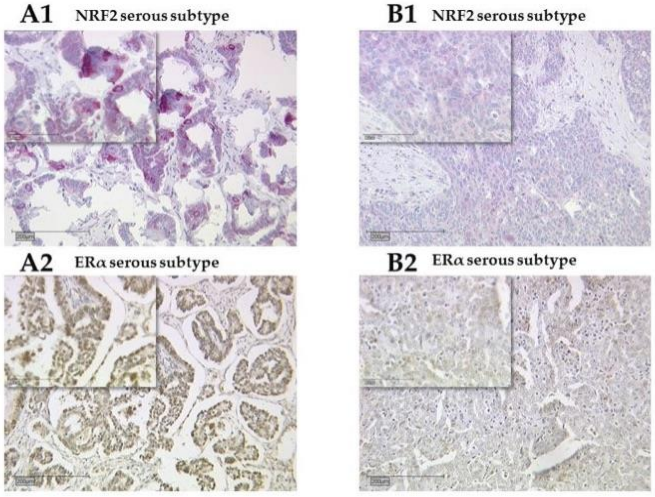

Figure 1. Detection of nuclear factor erythroid-2-related factor 2 (NRF2) (**A1, B1**) and estrogen receptor (ER)α (**A2, B2**) with immunohistochemistry. High (**A1**) and low (**B1**) cytoplasmic NRF2 stains in serous subtype correspond with high (**A2**) and low (**B2**) ERα stains, respectively. NRF2 shows faint staining in the nucleus in both cases (**A1, B1**).

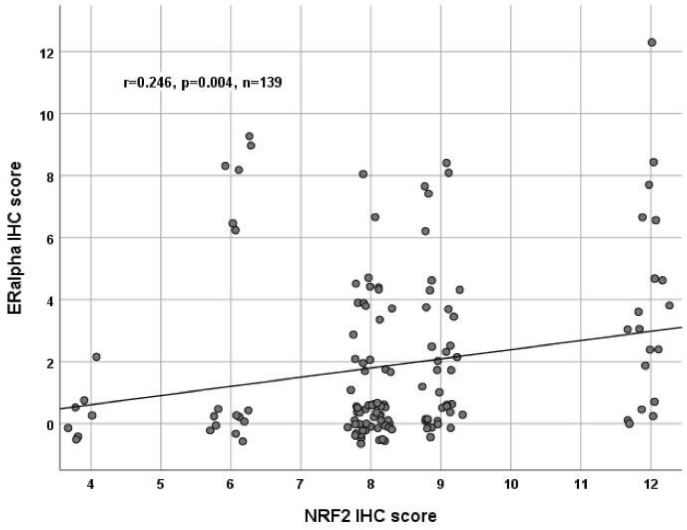

Figure 2. Correlation analysis of NRF2 and ERα in ovarian cancer tissue ($n = 139$). A significant correlation of cytoplasmic NRF2 expression with ERα expression was noted. For better visualization, dots have been jittered.

Table 1. Expression profile of NRF2 staining regarding clinical and pathological characteristics.

Parameters	*N*	Nuclear NRF2 Expression			*p*	*N*	Cytoplasmic NRF2 Expression			*p*
		Negative	Low	High			Negative	Low	High	
Histology										
Serous	103	0	87	16	0.02	98	0	54	44	0.001
Clear cell	11	1	7	3		11	0	11	0	
Endometrioid	20	0	18	2		19	0	12	7	
Mucinous	11	0	3	8		11	0	7	4	
Lymph node										
pN0/X	96	0	76	20	NS	93	0	59	34	NS
pN1	49	1	39	9		46	0	25	21	
Distant Metastasis										
pM0/X	141	1	112	28	NS	135	0	83	52	NS
pM1	4	0	3	1		4	0	1	3	
Grading										
Low	33	0	25	8	NS	33	0	16	17	0.03
High	100	1	83	16		95	0	64	31	
FIGO										
I/II	41	0	31	10	NS	40	0	24	16	NS
III/IV	99	0	81	18		94	0	56	38	
Age										
≤60 years	77	1	56	20	0.045	75	0	43	32	NS
>60 years	68	0	59	9		64	0	41	23	

Table 2. Correlation analysis.

Staining	*NRF2 Nucleus*	*NRF2 Cytoplasm*	*ERα*
NRF2 Nucleus			
cc	1.000	0.013	−0.019
p		0.88	0.82
n	146	138	146
NRF2 Cytoplasm			
cc	0.013	1.000	0.246
p	0.88		0.004
n	138	139	139
ERα			
cc	−0.019	0.246	1.000
p	0.82	0.004	
n	146	139	156

Immunoreactivity scores (IRS) of NRF2 and ERα staining in different compartments was correlated to each other using Spearman's correlation analysis. cc = correlation coefficient, *p* = two-tailed significance, *n* = number of patients.

2.2. High NRF2/ERα Expression is Associated with Improved Overall Survival

The median age of the patients was 58.7 (standard deviation (SD) of 31.4) years with a range of 31–88 years. Median overall survival of the EOC patients was 34.4 (SD 57.8) months. Cytoplasmic NRF2 expression in the serous cancer subtype was associated with longer overall survival (Figure 3, median 50.6 vs. 29.3 months; $p = 0.04$) as it was noted for ERα expression (Figure 3, median 74.5 vs. 27.1 months; $p = 0.002$). Improved OS was also seen for patients with combined and high expression of

both NRF2 and ERα in the cytoplasm comparing all histological subtypes (Figure 3, median 74.5 vs. 37.7 months; $p = 0.04$).

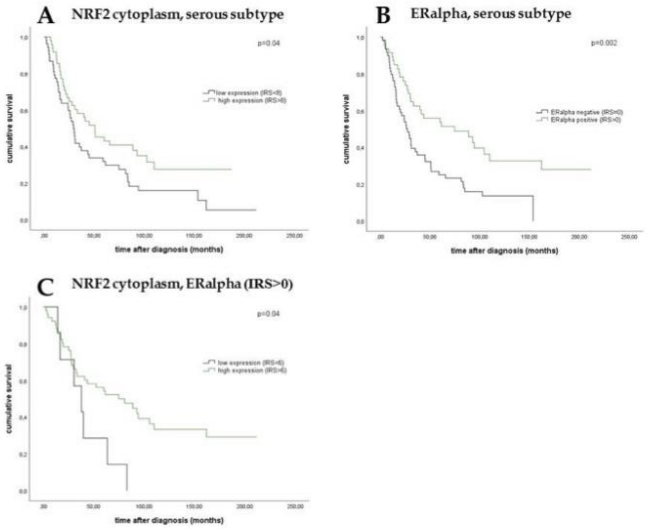

Figure 3. Kaplan–Meier estimates of NRF2 expression, ERα expression, and combined NRF2 and ERα expression were analyzed. In the serous subtype, patients with a high cytoplasmic expression of NRF2 showed a significantly increased overall survival compared with patients with a low cytoplasmic expression (**A**). In addition, high ERα expression was associated with significantly better overall survival in serous ovarian cancer compared with patients with a low ERα expression (**B**). Patients with combined high NRF2 expression in the cytoplasm and ERα expression in epithelial ovarian carcinoma (EOC) had significantly increased overall survival compared with those with low cytoplasmic expression and ERα expression (**C**).

2.3. Clinical and Pathological Parameters are Independent Prognostic Factors

Cancer grading, the FIGO classification, and patients' age were independent prognostic factors in the present cohort (Table 3). In contrast, prognostic impact of histological subtype, NRF2, and/or ERα staining/expression was not significant.

Table 3. Multivariate analysis.

Covariate	Coefficient (b_i)	[HR Exp(b_i)]	95% CI		*p*-Value
			Lower	Upper	
Histology (serous vs. other)	−0.108	0.898	0.678	1.188	0.45
Grade (low vs. high)	0.519	1.680	1.211	2.332	0.002
FIGO (I, II vs. III, IV)	0.722	2.058	1.421	2.979	0.000
Patients' age (≤60 vs. >60 years)	0.000	1.000	1.000	1.000	0.001
NRF2 cytoplasmic/ ERα	−0.166	0.847	0.531	1.351	0.49

2.4. Downregulation of NFE2L2 Increases ESR1 Expression, Confirming Their Genetic Interaction

Basal expressions of both *NFE2L2* and *ESR1* were analyzed by qPCR in all four EOC cell lines and compared with a benign ovarian cell line (HOSEpiC). As shown in Figure 4 and compared to HOSEpiC, *NFE2L2* expression increased 2-fold in both OVCAR3 ($p = 0.02$) and UWB1.289 ($p = 0.08$)

and was 1.5-fold elevated in the TOV112D ($p = 0.30$) cell lines. In comparison, *ESR1* expression was markedly reduced in all EOC cell lines compared to the benign ovarian cells (all $p < 0.001$).

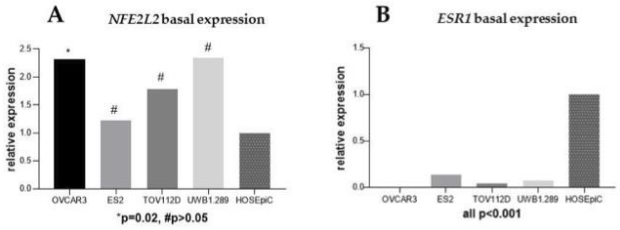

Figure 4. Basal gene expression of *NFE2L2* (**A**) and *ESR1* (**B**) in four ovarian cancer cell lines was compared to the expression in the benign ovarian cell line (HOSEpiC).

Following effective silencing of *NFE2L2* with siRNA to evaluate the impact on *ESR1* expression (Figure 5), an elevated expression of *ESR1* in the *NFE2L2* downregulated cancer cell lines OVCAR3 ($p = 0.003$) and ES2 was noted ($p < 0.001$).

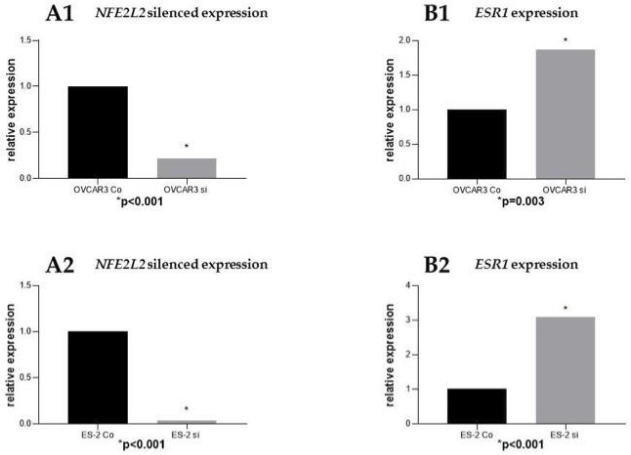

Figure 5. siRNA downregulation of *NFE2L2* in the ovarian cancer cell lines OVCAR3 (**A1**) and ES2 (**A2**). *ESR1* expression following *NFE2L2* downregulation in both cell lines (**B1, B2**).

3. Discussion

This cell and molecular biological experimental study reveals that NRF2 expression differs in histologic subtypes of EOC, with the strongest cytoplasmic expression in the serous subtype. Cytoplasmic NRF2 expression had significantly higher expression in patients with low-grade histology. Patients with higher cytoplasmic NRF2 expression in the serous type confirmed to have a significantly improved OS. Moreover, we could reveal that the combination of cytoplasmic NRF2 and ERα expression was associated with significantly longer OS. Molecular testing in cell lines exhibited that the *ESR1* gene was lower expressed in all four EOC cell lines, which could be upregulated by *NFE2L2* silencing in the subsequently *NFE2L2*-downregulated cancer cell lines.

NRF2 has been traditionally considered as a tumor suppressor because its cytoprotective functions are deemed to be the main cellular defense mechanism against exogenous and endogenous insults, including xenobiotic and oxidative stress [21,22]. Under homeostatic conditions, NRF2 activation

prevents excessive cellular damage produced by metabolic, xenobiotic, and oxidative stress [22]. NRF2 activation is thus important in cancer chemoprevention. Cancer chemoprevention mechanisms seem to be mediated through the Keap1–NRF2 pathway, and in experimental models, NRF2/Keap1 mutations are present at preneoplastic stages [23]. Further, NRF2-null mice are more prone to develop cancer in response to chemical and physical stimuli (nitrosamine, ultraviolet light, and aflatoxin) [17]. On the other hand, recent studies demonstrated that NRF2 hyperactivation may also create an environment favoring survival of normal as well as malignant cells, protecting them from apoptosis and senescence and against oxidative stress, chemotherapeutic agents, and radiotherapy [24,25]. Hence, the potential dual role of NRF2 in cancer may explain the described results below.

Our findings are in line with previous reports showing that nuclear or activated NRF2 expression is associated with upregulation of NRF2 target genes and poorer OS and disease-free survival (DFS), whereas patients with high cytoplasmic or inactive NRF2 expression displayed better OS and DFS [26]. Our evaluation of ER expression in the EOC tissue samples confirmed previous reports. In patients with EOC, the ER, especially ERα, is significantly associated with improved OS [8], grading, progression-free survival, and cause-specific survival, respectively [27]. There is a strong relationship between circulating sex hormones and female reproductive cancers (e.g., ovarian, breast, and endometrial cancers) [28]. Interestingly, estradiol may play a dual role in modulating NRF2 activity. On the one hand, its metabolites activate NRF2 via the generation of reactive oxygen species (ROS) (independent of the ER) [29]. While recent studies demonstrated that estradiol leads to an activation of NRF2 in a wide range of cell types [30,31], the estradiol effect was only noted on protein and not on mRNA levels, suggesting that the main effect of estradiol is based on NRF2 protein stabilization [32]. However, binding to ERα (dependent of ER) appears to be the mechanism for estradiol itself to inhibit the NRF2 downstream genes [9]. ERα, but not ERβ, interacts with NRF2 in an estradiol-dependent way and thereby represses NRF2-mediated transcription [33]. Thus, EOC patients with high tumor expression of ERα show a strong influence of the estradiol–ERα-dependent pathway, resulting in inactivated NRF2 and better survival rates. Otherwise, low ERα expression causes a dysbalance in favor of the estradiol–ERα-independent pathway with an activation of NRF2 (Figure 6). Studies show that other NRF2-associated factors also could play a crucial role in the above-described interaction. Glutathione S-transferase (GST), an NRF2 target gene, is modulated by miR-186 overexpression in OVCAR3 cells with consecutively increased sensitivity of ovarian cancer cells to paclitaxel [34]. Furthermore, it was described that the KEAP1–NRF2 pathway is important in ovarian cancer cell reaction to cigarette-smoke-induced ROS [35].

Endocrine therapy in EOC has been considered as a potential approach in subgroups of patients with a specific tumor biology that responds to this therapy [36]. Hereby, the rationale for endocrine treatment is based on the high ER/PR IHC expression as a predictive marker [37]. A present prospective study demonstrated evidence for the usefulness of letrozole as an aromatase inhibitor in serous EOC [38]. Under the conditions described above, treatment with aromatase inhibitors could cause a prognostically beneficial predominance of the ERα–NRF2-dependent pathway. As revealed in the present investigation, a putative functional association of endocrine therapy and NRF2 underlines the relationship of NRF2/ERα, as confirmed by significant correlation of expression. In addition to the mentioned approach, further therapeutic strategies as interference of DNA repair mechanisms are of great interest to overcome treatment burden [39–42].

The retrospective design, the relatively small number of tissue samples evaluated, and the semiquantitative scoring method may critically be regarded as limitations of the submitted work. The data are hypothesis generating and further prospective studies with a larger patient collective and standardized immunohistochemical and molecular methods are warranted to gain more detailed and better insight into this research field.

However, despite these drawbacks, our analysis indicates for the first time a putative molecular role of the estradiol–ERα–NRF2 pathway as a basis for a better understanding of endocrine therapy in EOC.

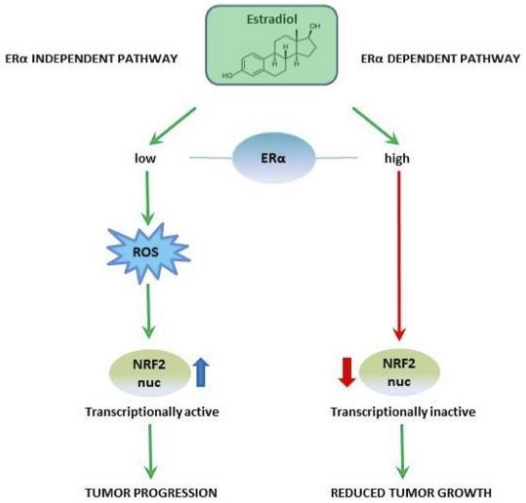

Figure 6. Summary of the hypothesized interaction within the estradiol–ERα–NRF2 pathway: High expression of ERα leads to an induction of the estradiol–ERα-dependent pathway, resulting in transcriptionally inactive NRF2 (low nuclear, high cytoplasmic expression) and consecutively less impact on tumor growth. In contrast, low ERα expression favors the estradiol–ERα-independent pathway, with activation of NRF2 (high nuclear, low cytoplasmic expression) causing tumor progression.

4. Materials and Methods

4.1. Ethical Approval

The current study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 227-09) on 30 September 2009. All tissue samples used for this study were obtained from material from the archives of LMU Munich, Department Gynecology and Obstetrics, Ludwig-Maximilians-University, Munich, Germany, initially used for pathological diagnostics. The diagnostic procedures were completed before the current study was performed. During the analysis, the observers were fully blinded to patients' data. The study was approved by the Ethics Committee of LMU Munich. All experiments were performed according to the standards of the Declaration of Helsinki (1975).

4.2. Patients and Specimens

Tissue samples of 156 patients who underwent surgery for EOC at the Department of Obstetrics and Gynecology, Ludwig-Maximillian's-University Munich from 1990 to 2002 were analyzed in this study. Clinical data was obtained from the patients' charts and follow up data from the Munich Cancer Registry. All samples had been formalin-fixated and paraffin-embedded (FFPE). Patients with benign or borderline tumors were excluded and no patients had adjuvant chemotherapy. Specialized pathologists for EOC examined and classified the samples for tumor grading—low (n = 38), high (n = 117)—and histological subtypes—serous (n = 110), endometrioid (n = 21), clear cell (n = 12), and mucinous (n = 13). Staging was performed using TNM and FIGO (WHO) classification: I (n = 35), II (n = 10,) III (n = 103), and IV (n = 3). Data on primary tumor extension were available in 155 cases—T1 (n = 40), T2 (n = 18), T3 (n = 93), and T4 (n = 4)—as well as data on lymph node involvement in 95 cases—N0 (n = 43), N1 (n = 52). Data on distant metastasis were available in nine cases—M0 (n = 3), M1 (n = 6).

4.3. Immunohistochemistry

Immunohistochemistry was performed as previously described by our lab [43]. For NRF2 staining, FFPE EOC samples were incubated with anti-NRF2 (Abcam, Cambridge, UK, rabbit, monoclonal, clone EP1808) at a final concentration of 5.93 µg/ml (1:100 dilution) for 1 h at room temperature. Afterwards, slides were incubated with isotype-matching MACH 3 Rabbit AP Polymer Detection (Biocare Medical, Pacheco, CA, USA, catalogue-number M3R533). The Permanent AP Red Kit (Zytomed Systems GmbH, Berlin, Germany, catalogue-number ZUC-001) was used as a chromogen. Slides were then counterstained with Gill´s hematoxylin (Vector Laboratories, Burlingame, CA, USA). System controls were included.

For the detection of ERα, resected EOC tissue samples were fixed in formalin and embedded in paraffin after surgery. ERα staining was performed by blocking slides with goat serum (1:100 dilution, Vectastain® ABC-Elite-Kit, Linaris, Dossenheim, Germany, catalogue-number PK-6101) for 30 min at room temperature. Subsequently, slides were incubated with anti-ERα primary antibody (1:400 dilutions, Abcam, Cambridge, UK, rabbit, monoclonal, clone EPR703(2)) for 16 h at 4 °C. Afterwards, slides were incubated with isotype-matching anti-rabbit IgG secondary antibody and avidin–biotin–peroxidase complex both for 30 min at room temperature, according to the Vectastain® ABC-Elite-Kit (Linaris, Dossenheim, Germany, catalogue-number PK-6101). All slides were washed twice in PBS for 2 min after every incubation step. 3,3′-Diaminobenzidine chromogen (DAB; Dako, Glostrup, Denmark, catalogue-number K3468) was used for visualization reaction. Slides were then counterstained with Mayer's acidic hematoxylin (Waldeck-Chroma, Münster, Germany, catalogue number 2E-038) and dehydrated in an ascending series of alcohol followed by xylol. System controls were included.

4.4. Staining Evaluation and Statistical Analysis

All EOC specimens were examined with a Leitz (Wetzlar, Germany) photomicroscope and specific NRF2 and ERα immunohistochemical staining reaction was observed in the nuclei and cytoplasm of the cells. The intensity and distribution pattern of NRF2 and ERα staining was rated using the semiquantitative immunoreactivity score (IRS, Remmele's score). To obtain the IRS result, the optional staining intensity (0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) and the percentage of positive stained cells (0 = no staining, 1 = <10% of the cells, 2 = 11%–50% of the cells, 3 = 51%–80% of the cells, and 4 = >81%) were multiplied. Nuclear and cytoplasmic NRF2 staining was successfully performed in 145 (93%) and 139 (89%) of 156 EOC tissue specimens, respectively. Cut-off points for the IRSs were selected for cytoplasmic and nuclear NRF2 staining considering the distribution pattern of IRSs in the collective. Nuclear and cytoplasmic NRF2 staining were regarded as negative with an IRS of 0–2, as low with IRS of 4–8, and as high with IRS of ≥9. ERα staining was successfully performed in all 156 (100%) EOC specimens. Cellular ERα staining was considered as negative with an IRS of 0 and as positive with an IRS of >0.

Statistical analysis was performed using SPSS 25.0 (v25, IBM, Armonk, New York). Distribution of clinical pathological variables was evaluated with the chi-squared test. The Mann–Whitney *U* test was used to compare IRSs of NRF2 between different clinical and pathological subgroups. Correlations between findings of immunohistochemical staining were calculated using Spearman's analysis. Survival times were analyzed by Kaplan–Meier (log-rank) estimates. To identify an appropriate cut-off, the ROC curve was drawn, which is considered as one of the most reliable methods for cut-off point selection. In this context, the ROC curve was a plot representing sensitivity on the y-axis and (1-specificity) on x-axis [44]. Consecutively, Youden's index, defined as the maximum (sensitivity+specificity-1) [45], was used to find the optimal cut-off maximizing the sum of sensitivity and specificity [46,47]. For multivariate analyses, a Cox regression model was applied, with *p*-values less than 0.05 considered to be significant. Ct values of each gene were obtained with qPCR and the relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ formula. Statistical data was acquired using Graph Pad Prism 7.03 (v7, La Jolla, CA, USA).

4.5. Cell Lines

The human ovarian cancer cell lines OVCAR3 (serous), ES-2 (clear cell), TOV112D (endometrioid), and UWB1.289 (serous, BRCA1 negative) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in culture in RPMI 1640 medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% FBS in a humidified incubator at 37 °C under 5% CO_2. The benign ovarian cell line HOSEpiC was purchased from ScienCell (Carlsbad, CA, USA). HOSEpiC cells were maintained in culture in Ovarian Epithelial Cell Medium (OEpiCM) (ScienCell, Carlsbad, CA, USA, catalogue-number 7311) in a humidified incubator at 37 °C under 5% CO_2.

4.6. PCR

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and 1 µg of RNA was converted into first-strand cDNA using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, Madison, WI, USA) according to the instructions of the manufacturer. The basal mRNA expressions of *NFE2L2* and *ESR1* were quantified by qPCR applying FastStart Essential DNA Probes Master and gene-specific primers (Roche, Basel, Switzerland). For normalization of expressions the housekeeping genes, *β-Actin* and *GAPDH* were used as reference controls. Basal expressions of *NFE2L2* and *ESR1* in the ovarian cancer cell lines were compared with their expressions in the benign ovarian cell lines.

4.7. siRNA

The specific siRNA for *NFE2L2* (Silencer Select Pre-designed and Custom Designed siRNA, Ambion, Carlsbad, CA, USA) was kindly provided by Beate Niesler (Department of Human Molecular Genetics, University of Heidelberg). Cells were transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) to silence the expression of *NFE2L2* in the cell lines. RNA isolation and mRNA quantification by qPCR was repeated as outlined above. mRNA expression levels of *NFE2L2* and *ESR1* in *NFE2L2*-downregulated cells were compared with *NFE2L2*-containing cells.

5. Conclusions

Here, *ESR1* expression was reduced in different ovarian cancer cells vs. benign cells in vitro (all $p < 0.001$). *NFE2L2* silencing showed a higher expression of *ESR1* in the *NFE2L2*-downregulated cancer cell lines OVCAR3 ($p = 0.003$) and ES2 ($p < 0.001$). In the serous cancer subtype, high cytoplasmic NRF2 expression (OS, median 50.6 vs. 29.3 months; $p = 0.04$) and high ERα expression (OS, median 74.5 vs. 27.1 months; $p = 0.002$) was associated with longer overall survival as well as combined expression of both inactive cytoplasmic NRF2 and ERα in the whole cohort (median 74.5 vs. 37.7 months; $p = 0.04$). Thus, interactions of NRF2 and ERα impact survival in ovarian cancer patients and may be important factors for the response to endocrine treatment strategies.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/1/112/s1.

Author Contributions: B.C. and U.J. conceived and designed the experiments; M.K. performed the experiments; M.K. and U.J. analyzed the data; D.M., E.S., and B.N. contributed reagents/materials/analysis tools; B.C., M.K., and F.T. wrote the paper. D.M., E.S., B.N., T.K., A.B., S.M., and U.J. critically reviewed the paper.

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5. Paper II

Cancer Management and Research

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ORIGINAL RESEARCH

Correlation of NRF2 and progesterone receptor and its effects on ovarian cancer biology

This article was published in the following Dove Press journal:
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Purpose: This study aimed to investigate the potential prognostic impact of nuclear factor erythroid 2-related factor 2 (NRF2) and progesterone receptor A (PRA)/progesterone receptor B (PRB) in ovarian cancer patients which might be the rationale for putative new treatment strategies.

Patients and methods: The presence of NRF2 and PRA/PRB was investigated in 156 ovarian cancer samples using immunohistochemistry (IHC). Staining of NRF2 and PRA/PRB was rated using the semi-quantitative immunoreactive score (IR score, Remmele's score) and correlated to clinical and pathological data. NRF2 and PRA/PRB expression were compared with respect to the overall survival (OS).

Results: NRF2 staining was different in both, the cytoplasm and nucleus between the histological subtypes (p=0.001 and p=0.02, respectively). There was a significant difference in the PRA expression comparing all histological subtypes (p=0.02). Histological subtypes showed no significant differences in the PRB expression. A strong correlation of cytoplasmic NRF2 and PRA expression was detected (cc=0.247, p=0.003) as well as of cytoplasmic NRF2 and PRB expression (cc=0.25, p=0.003), confirmed by immunofluorescence double staining. Cytoplasmic NRF2 expression was associated with a longer OS (median 50.6 vs 32.5 months; p=0.1) as it was seen for PRA expression (median 63.4 vs 33.1 months; p=0.08), although not statistically significant. In addition, high PRB expression (median 80.4 vs 32.5 months; p=0.04) and concurrent expression of cytoplasmic NRF2 and PRA were associated with a significantly longer OS (median 109.7 vs 30.6 months; p=0.02). The same relationship was also noted for NRF2 and PRB with improved OS for patients expressing both cytoplasmic NRF2 and PRB (median 153.5 vs 30.6 months; p=0.009). Silencing of *NFE2L2* induced higher mRNA expression of *PGR* in the cancer cell line OVCAR3 (p>0.05) confirming genetic interactions of NRF2 and PR.

Conclusion: In this study, the combination of cytoplasmic NRF2 and high PRA/PRB expression was demonstrated to be associated with improved overall survival in ovarian cancer patients. Further understanding of interactions within the NRF2/AKR1C1/PR pathway could open new additional therapeutic approaches.

Keywords: nuclear factor erythroid 2-related factor 2, progesterone receptor, ovarian cancer, immunohistochemistry

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Introduction

Ovarian cancer is one of the five most frequent cancer deaths among women with a five-year survival rate of less than 45%.[1,2] The non-specific symptoms combined with an insufficient screening method often lead to a diagnosis in advanced tumor stage with a consecutively impaired prognosis. Recommended therapeutic approaches include primary cytoreductive surgery and platinum-based chemotherapy with anti-angiogenic

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agents or PARP inhibitors. Most reliable prognostic markers include volume of residual disease after initial debulking surgery, the International Federation of Gynecology and Obstetrics (FIGO) stage, ascites volume, patient age, and histological subtype.[3–6] Epithelial ovarian carcinomas (EOC) are classified as serous, mucinous, endometrioid, and clear-cell histology, being distinguished in terms of phenotype, molecular background, and etiology.[7] Research to identify new molecular prognostic markers needs to take this heterogeneity of ovarian cancer into account. A better understanding of the differences between ovarian cancer subtypes appears crucial to enable new diagnostic and therapeutic approaches.

Current investigations attribute an important impact for the development of ovarian cancer to oxidative stress.[8] The nuclear factor erythroid-2-related factor 2 (NRF2) is a well-known regulator of antioxidant and cytoprotective genes mediating cellular coping of oxidative stress. Whereas NRF2 is ubiquitously expressed at low levels in all human organs, tight regulation of this major cellular defense mechanism is crucial to maintain cellular homeostasis. Different cancer entities and cell lines exhibit high constitutive levels of NRF2.[9–13] Our research group recently demonstrated that cytoplasmic NRF2 expression in its inactive cytoplasmic form is associated with improved survival in ovarian cancer patients.[14] Overexpression of NRF2 might protect cancer cells from the cytotoxic effects of anticancer therapies, resulting in resistance to chemo- and radiotherapy.[15,16]

Progesterone inhibits cell growth and metastasis in ovarian cancer cells and is considered as an established protective factor for the development of ovarian cancer as part of combined oral contraceptives.[17–20] The detailed molecular background of this mechanism has not yet been fully understood. The progesterone receptor (PR), a member of the steroid hormone receptor superfamily, is expressed in two isoforms, the PRA and PRB differing in their molecular weight. Studies show an up to date functional unknown dominant expression of PRB in ovarian carcinomas.[21–23] Progesterone receptor expression has been described to be associated with improved overall (OS) and progression-free survival (PFS) due to its putative anti-proliferative effect.[24–27] To our knowledge, interactions between NRF2 and PR are not well understood so far, but warrant further investigation based on the results of our present data.

This study aimed to investigate the potential prognostic impact of NRF2 and PR in ovarian cancer patients which might be the rationale for putative new treatment strategies.

Materials and methods

Patients and specimens

Tissue samples of 156 patients who underwent surgery for EOC at the Department of Obstetrics and Gynecology, Ludwig-Maximillian's-University Munich from 1990 to 2002, were analyzed in this study. Clinical data were obtained from the patient's charts and follow-up data from the Munich Cancer Registry. All samples had been formalin-fixated and paraffin-embedded (FFPE). Patients with benign or borderline tumors were excluded and no patients had neoadjuvant chemotherapy. Specialized pathologists for EOC examined and classified the samples for tumor grading: low (n=38), high (n=117), and histological subtypes: serous (n=110), endometrioid (n=21), clear cell (n=12), mucinous (n=13). Staging was performed using TNM and FIGO (WHO) classification: I (n=35), II (n=10,) III (n=103), IV (n=3). Data on primary tumor extension were available in 155 cases: T1 (n=40), T2 (n=18), T3 (n=93), T4 (n=4) as well as data on lymph node involvement in 95 cases N0 (n=43), N1 (n=52). Data on distant metastasis were available in nine cases M0 (n=3), M1 (n=6).

Ethical approval

This study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 227-09). All tissue samples used for this study were obtained from material from the archives of the Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Munich, Germany, initially used for pathological diagnostics. The diagnostic procedures were completed before the current study was performed. All patients´ data were fully anonymized, and the study was performed according to the standards set in the Declaration of Helsinki 1975. The ethics committee approved this consent process. During the analysis, the observers were fully blinded for patients' data.

Immunohistochemistry

Immunohistochemistry was performed as previously described by our lab.[28] For NRF2 staining, paraffin-embedded and formalin-fixed EOC samples were incubated with Anti-NRF2 (Abcam, Cambridge, UK, rabbit, monoclonal, clone EP1808) at a final concentration of 5.93 µg/mL (1:100 dilution) for 1 hr at room temperature. Afterward, slides were incubated with isotype-matching MACH 3 Rabbit AP Polymer Detection (Biocare Medical, Pacheco,

CA, USA, catalog-number M3R533). The Permanent AP Red Kit (Zytomed Systems GmbH, Berlin, Germany, catalog-number ZUC-001) was used as chromogen. Slides were then counterstained with Gill´s hematoxylin (Vector Laboratories, Burlingame, CA, USA). System controls were included.

For the detection of PR, paraffin-fixed tissue sections were dewaxed with xylol for 15 mins, then dehydrated in ascending concentrations of alcohol (70–100%). Afterward, they were exposed for epitope retrieval for 10 mins in a pressure cooker using sodium citrate buffer (pH 6.0) containing 0.1 M citric acid and 0.1 M sodium citrate in distilled water. After cooling, slides were washed in PBS twice. Endogenous peroxidase activity was quenched by dipping in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 mins. Non-specific binding of the primary antibodies was blocked by incubating the sections with "diluted normal serum" (10 mL PBS containing 150 µL horse serum; Vector Laboratories, CA) for 20 mins at room temperature. Then, slides were incubated with the primary antibodies (PRA: 1:250 dilutions, Sigma-Aldrich, St. Louis, MO, USA, rabbit, polyclonal, clone R04125; PRB: 1:50 dilutions, Novocastra Reagents, Wetzlar, Germany, mouse, monoclonal, clone SAN27) at room temperature for 60 mins. After washing with PBS, slides were incubated in diluted biotinylated anti-serum secondary antibody (10 mL PBS containing 50 µl horse serum, Vector Laboratories, CA) for 30 mins at room temperature. After incubation with the avidin-biotin-peroxidase complex (diluted in 10 mL PBS, Vector Laboratories, CA) for 30 mins and repeated PBS washing, visualization was conducted using substrate and chromagen 3,3′-diaminobenzidine (DAB; Dako, Glostrup, Denmark, catalog-number K3468) for 8–10 mins. Slides were then counterstained with Mayer's acidic hematoxylin (Waldeck-Chroma, Münster, Germany, catalog-number 2E-038) and dehydrated in an ascending series of alcohol followed by xylol. System controls were included.

Staining evaluation

All EOC specimens were examined with a Leitz (Wetzlar, Germany) photomicroscope and specific NRF2 and PR immunohistochemically staining reaction was observed in the nuclei and cytoplasm of the cells. The intensity and distribution pattern of NRF2 and PR staining were rated using the semi-quantitative immunoreactive score (IR score, Remmele's score). To obtain the IR score result, the optional staining intensity (0=no, 1=weak, 2=moderate, and 3=strong staining) and the percentage of positive-stained cells (0=no staining, 1=<10% of the cells, 2=11–50% of the cells, 3=51–80% of the cells, and 4≤81%) were multiplied. NRF2 staining was successfully performed in 145 (93%) of 156 EOC tissue specimens. Cut-off points for the IR scores were selected for the cytoplasmic and nuclear NRF2 staining considering the distribution pattern of IR scores in the collective. Nuclear and cytoplasmic NRF2 staining were regarded as negative with an IR score 0–2, as low with IRS 4–8, and as high with IRS >8. PRA and PRB stainings were successfully performed in all 156 (100%) EOC specimens. Cellular PRA and PRB stainings were considered as negative with an IR score 0 and as positive with IRS >0.

Immunofluorescence staining

Mouse anti-NRF2 IgGs were diluted at 1:200 with a diluting medium (Dako, Hamburg, Germany), while rabbit anti-PRB polyclonal IgGs were diluted at 1:200. After washing, slides were incubated with Cy2-/Cy3-labeled antibodies (Dianova, Hamburg, Germany) as fluorescent secondary antibodies for 30 mins at room temperature in darkness to avoid fluorescence quenching. Cy2-labeled secondary antibodies were used at a dilution of 1:100 and Cy3-labeled antibodies at a dilution of 1:500. Finally, the slides were embedded in mounting buffer containing 4′,6-diamino-2-phenylindole (DAPI, Vectastain, Vector Laboratories) for blue staining of the nucleus after washing and drying. Confocal laser scanning microscope images were acquired with Zeiss LSM 880 with Airyscan model for high-resolution visualization and analyzed with ZEN blue software.

Cell line

The human serous ovarian cancer cell line OVCAR3 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in culture in RPMI 1640 medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% FBS in a humified incubator at 37°C under 5% CO_2.

PCR

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and 1 µg RNA was converted into first-strand cDNA using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, Madison, WI, USA) according to the instructions of the manufacturer. The basal mRNA expressions of NFE2L2 and PGR were quantified by qPCR applying FastStart Essential DNA Probes Master and gene-specific primers

(Roche, Basel, Switzerland). For normalization of expressions the housekeeping genes *β-Actin* and *GAPDH* were used as reference controls.

si-RNA

The specific siRNA for *NFE2L2* (Silencer Select Predesigned and Custom Designed siRNA, Ambion, Carlsbad, CA, USA) was kindly provided by Beate Niesler (Department of Human Molecular Genetics, University of Heidelberg). Cells were transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) to silence the expression of *NFE2L2* in the cell line. RNA Isolation and mRNA quantification by qPCR was repeated as outlined earlier. mRNA expression levels of *NFE2L2* and *PGR* in *NFE2L2* downregulated cells were compared with NFE2L2 containing cells.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 (v25, IBM, Armonk, New York). Distribution of clinical pathological variables was evaluated with the Chi-Square test. Mann–Whitney U test was used to compare IR scores of NRF2/PR between different clinical and pathological subgroups. Correlations between findings of immunohistochemically staining were calculated using Spearman's analysis. Survival times were analyzed by Kaplan–Meier (log-rank) estimates. To identify an appropriate cut-off, the ROC curve was drawn which is considered as one of the most reliable methods for cut-off point selection. In this context, the ROC curve is a plot representing sensitivity on the *y*-axis and (1-specificity) on the *x*-axis.[29] Consecutively, Youden index, defined as the maximum (sensitivity+specificity-1),[30] was used to find the optimal cut-off maximizing the sum of sensitivity and specificity.[31,32] For multivariate analyses, a Cox-regression model was applied, with *P*-values less than 0.05 considered to be significant. Ct values of each gene were obtained with qPCR and the relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ formula. Statistical data were acquired using Graph Pad Prism 7.03 (v7, La Jolla, California).

Results

NRF2/PR expression correlates with clinical and pathological data

Clinicopathologic characteristics of the analyzed ovarian cancer patients are listed in Table 1. Nuclear staining of NRF2 was observed in 144 of 145 evaluable cases (99%), and cytoplasmic staining of NRF2 was observed in 139 (96%) of these 145 cases. Median (range) immunoreactivity scores (IRS) for NRF2 in nuclei and cytoplasm were 8 (2,12) and 8 (4,12), respectively.

NRF2 staining in both cytoplasm and nucleus was different between the histological subtypes (p=0.001 and p=0.02, respectively) with low nuclear NRF2 expression in serous, clear-cell, and endometrioid histology and high expression in mucinous subtype. In comparison, strongest and weakest cytoplasmic NRF2 staining were found in the serous and clear-cell subtypes, respectively. Cytoplasmic NRF2 expression was significantly higher expressed in patients with low-grade histology (p=0.03) and low nuclear NRF2 expression was associated with age (p=0.045).

All 156 cases could be successfully stained for PRA (100%) and PRA expression could be detected in 63 of 156 (40%) specimens with a median (range) IRS of 0 (0,12) and mean (range) IRS of 2 (0,12) (Figure 1). There was a significant difference in the PRA expression comparing all histological subtypes (p=0.02) with the highest expression in the serous subtype. All other

Table 1 Clinicopathologic characteristics of the ovarian cancer patients

Clinicopathologic parameters	N	Percentage
Histology		
Serous	110	70.5%
Clear cell	12	7.7%
Endometrioid	21	13.5%
Mucinous	13	8.3%
Lymph node		
pN0/X	104	66.7%
pN1	52	33.3%
Distant metastasis		
pM0/X	150	96.2%
pM1	6	3.8%
Grading		
Low	38	25.0%
High	117	75.0%
FIGO		
I	35	22.4%
II	10	6.4%
III	103	66.0%
IV	3	1.9%
Age		
≤60 years	83	53.2%
>60 years	73	46.8%

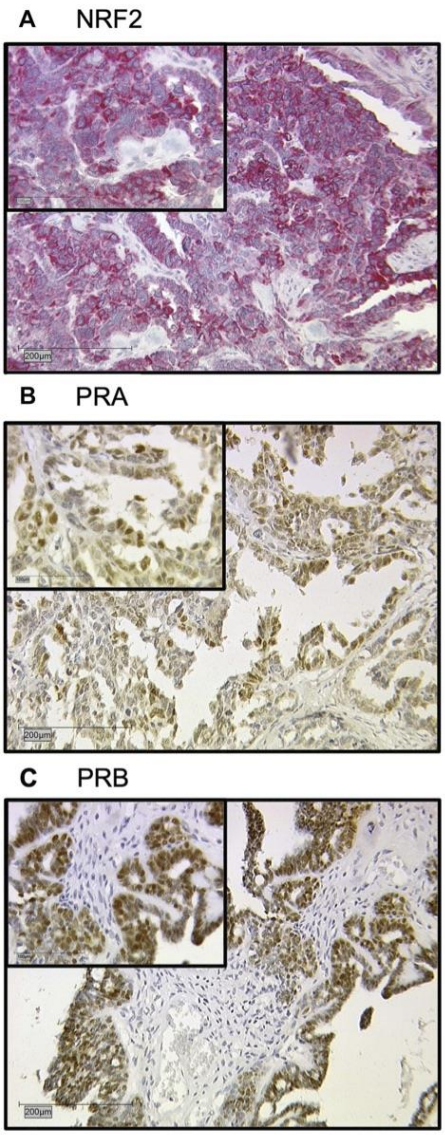

Figure 1 Detection of NRF2 and PRA/PRB with immunohistochemistry.
Notes: High cytoplasmic NRF2 staining (**A**) corresponds with high PRA (**B**) and high PRB (**C**) staining found in specimens from the same individual. NRF2 cytoplasmic expression correlated with PRA (cc=0.247, p=0.003) and PRB expression (cc=0.25, p=0.003).
Abbreviations: cc, correlation coefficient, p = two-tailed significance.

parameters like grading, FIGO, lymph node involvement (pN), and distant metastasis (pM) showed no significant differences in the PRA expression. Of note, a strong correlation of cytoplasmic NRF2 and PRA expression was detected (cc=0.247, p=0.003, Table 2 and Figures 1 and 3).

PRB staining was successfully performed in all 156 cases (100%) and PRB expression was observed in 63 of 156 (40%) specimens with a median (range) IRS of 0 (0,12) and mean (range) IRS of 2 (0,12) (Figure 1). Parameters like histological subtypes, grading, FIGO, lymph node involvement (pN), and distant metastasis (pM) showed no significant differences in the PRB expression. Again, NRF2 cytoplasmic expression was correlated with PRB expression (cc=0.25, p=0.003, Table 2 and Figures 1–3).

Table 2 Correlation analysis

Staining	NRF2 cytoplasm	PRA	PRB
NRF2 cytoplasm			
cc	1.000	0.247	0.25
p	.	0.003	0.003
n	146	142	144
PRA			
cc	0.247	1.000	0.622
p	0.003	.	0.0001
n	142	152	152
PRB			
cc	0.25	0.622	1.000
p	0.003	0.0001	.
n	144	152	154

Notes: IR-scores of NRF2 and PRA/PRB staining were correlated to each other using Spearman's correlation analysis.
Abbreviations: cc, correlation coefficient, *p*, two-tailed significance, n, number of patients.

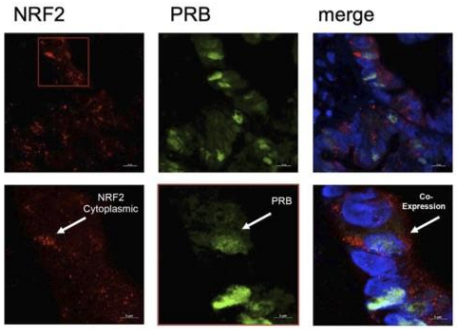

Figure 2 Double immunofluorescence of NRF2 and PRB.
Notes: Red stained cytoplasmic NRF2 expression, green stained PRB expression in ovarian cancer tissue. Co-expression of NRF2 and PRB + DAPI (triple filter excitation)

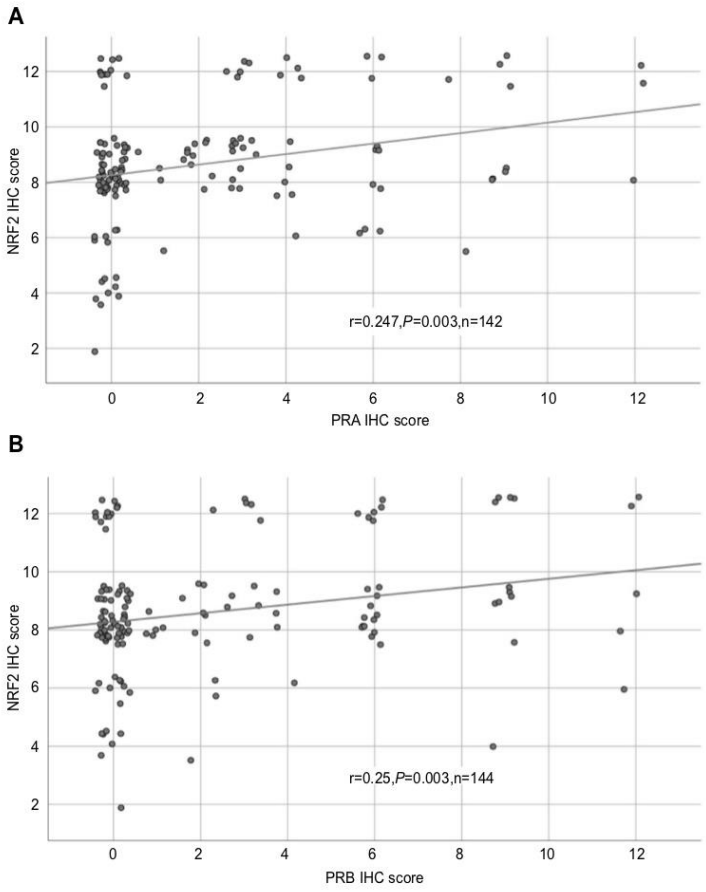

Figure 3 Correlation analysis of NRF2 and PRA/PRB.
Notes: Correlation analysis of NRF2 and PRA (**A**)/PRB (**B**) in ovarian cancer tissue. A significant correlation of cytoplasmic NRF2 expression with PRA/PRB expression was noted. For better visualization, dots have been jittered.
Abbreviations: r = correlation coefficient, p = two-tailed significance, n = number of patients.

High NRF2/PR expression is associated with improved overall survival

Median age of the patients was 58.7 (standard deviation [SD] 31.4) years with a range of 31–88 years. Median follow-up OS of the EOC patients was 34.4 (SD 57.8) months. Although not statistically significant, cytoplasmic NRF2 expression was associated with a longer OS (Figure 4, median 50.6 vs 32.5 months; p=0.1) as it was seen for PRA expression (Figure 4, median 63.4 vs 33.1 months; p=0.08). In addition, high PRB expression was associated with increased OS (Figure 4, median 80.4 vs 32.5 months; p=0.04).

Due to the biological relationship between NRF2 and PRA, concurrent expression of cytoplasmic NRF2 and PRA was evaluated revealing significantly longer OS for patients expressing both, NRF2 and PRA (Figure 4, median 109.7 vs 30.6 months; p=0.02). The same relationship was also noted between NRF2 and PRB with improved OS for patients with the combined expression of cytoplasmic

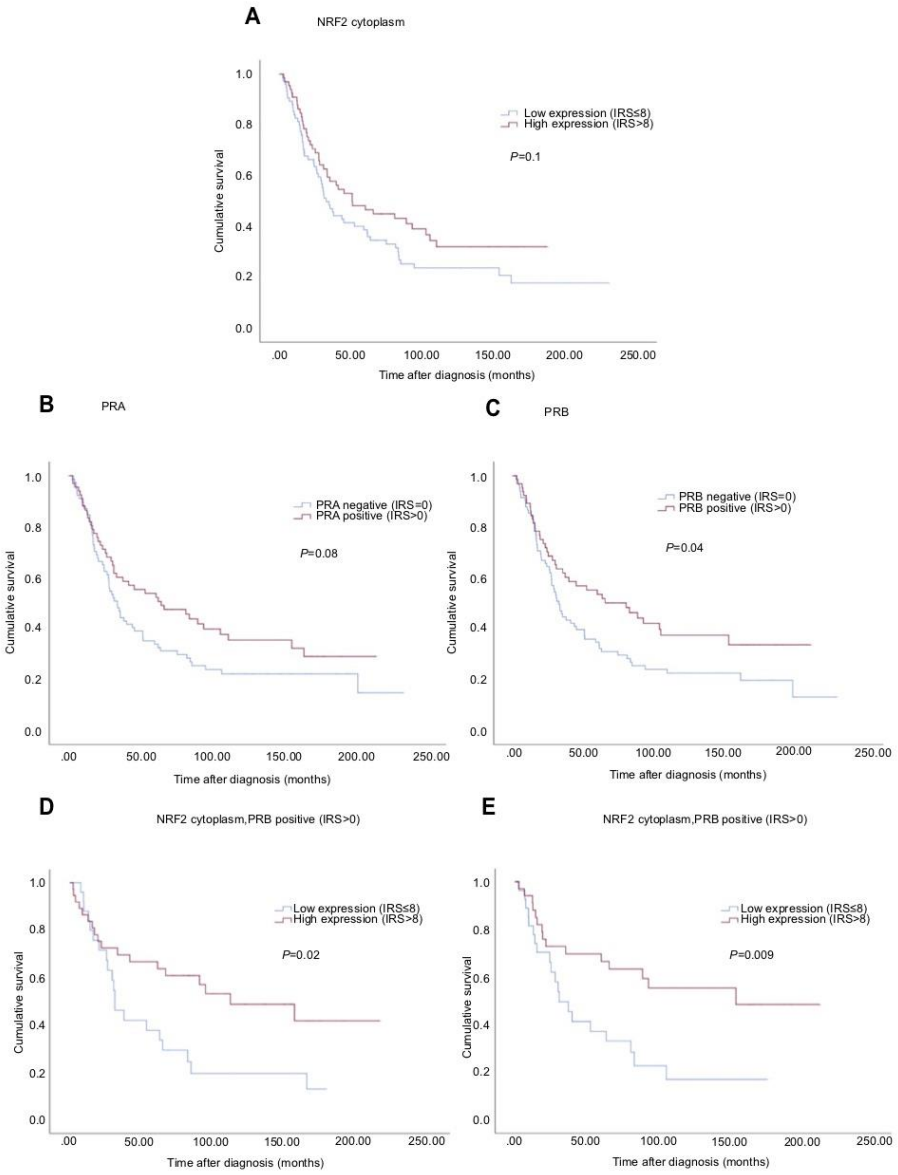

Figure 4 Kaplan–Meier estimates.

Notes: Kaplan–Meier estimates of NRF2 expression (**A**), PRA expression (**B**), PRB expression (**C**) and combined NRF2 and PRA/PRB (**D, E**) expression were analyzed. Although not statistically significant, cytoplasmic NRF2 expression was noted with a longer overall survival (**A**) as it was seen for PRA expression (**B**). High PRB expression was associated with increased overall survival (**C**). Patients with combined high NRF2 expression in the cytoplasm and PRA/PRB expression had significantly increased overall survival compared with those with low cytoplasmic expression (**D**/**E**).

NRF2 and PRB (Figure 4, median 153.5 vs 30.6 months; p=0.009).

Clinical and pathological parameters are independent prognostic factors

Cancer grading, the FIGO classification, and patient's age were independent prognostic factors in the present cohort (Table 3). In contrast, the prognostic impact of histological subtype as well as NRF2 and PRA/PRB expression were not confirmed to be of independent significance.

Downregulation of NFE2L2 influences PGR expression confirming their genetic interaction

Following effective silencing of *NFE2L2* with siRNA to evaluate the impact on *PGR* expression (Figure 5), an elevated expression of *PGR* in the *NFE2L2* downregulated cancer cell line OVCAR3 was noted, although not statistically significant (p=0.41).

Discussion

The present study investigating the expression patterns of NRF2 and PRA as well as PRB demonstrates that cytoplasmic NRF2 expression is significantly correlated with the expression of both PRA and PRB and that this correlation seems to be associated with a significant impact on OS of ovarian cancer patients. Silencing of *NFE2L2* induced a higher mRNA expression of *PGR* in the *NFE2L2* downregulated cancer cell line OVCAR3. Therefore, these results might corroborate a possible functional interaction between NRF2 and PR which merits further investigations.

As a main cellular defense mechanism against metabolic, xenobiotic, and oxidative stress, NRF2 has been generally regarded as a tumor suppressor.[33,34] NRF2 activation avoids excessive cellular damage under abovementioned conditions.[34] Thus, NRF2/Keap1 pathway is essential in cancer chemoprevention underlining NRF2/Keap1 mutations at pre-neoplastic stages in experimental models.[35] In comparison, recent studies revealed that

Table 3 Multivariate analysis

Covariate	Coefficient (b_i)	[HR Exp(b_i)]	95% CI		p-Value
			Lower	Upper	
Histology (serous vs other)	−0.124	0.883	0.678	1.188	0.35
Grade (low vs high)	0.472	1.604	1.158	2.138	0.002
FIGO (I, II vs III, IV)	0.679	1.972	1.550	3.096	0.000
Patients' age (≤60 vs >60 years)	0.008	1.008	1.003	1.013	0.001
NRF2 cytoplasmic/PRA	−0.090	0.914	0.382	2.332	0.85
NRF2 cytoplasmic/PRB	−0.422	0.656	0.276	1.639	0.35

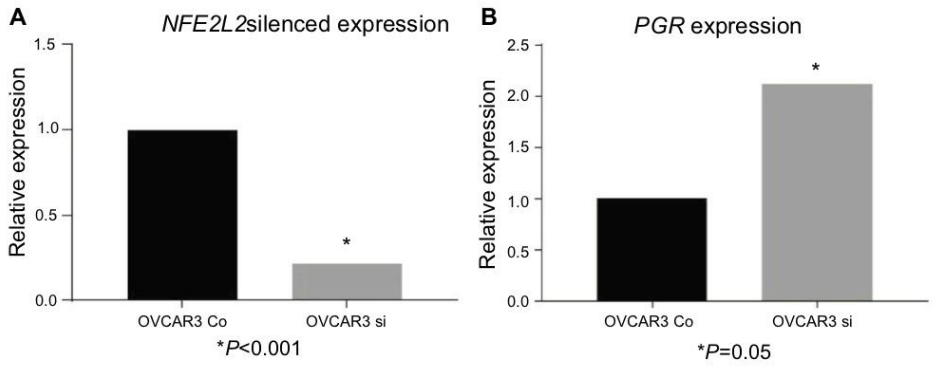

Figure 5 siRNA downregulation of *NFE2L2*.
Notes: siRNA downregulation of *NFE2L2* in the ovarian cancer cell line OVCAR3 (**A**) and the effect on *PGR* expression following *NFE2L2* downregulation (**B**).

NRF2 hyperactivation may facilitate conditions favoring the survival of normal as well as malignant cells, protecting them from apoptosis following oxidative stress by chemotherapeutic agents or radiotherapy.[36,37]

Hence, this potential dual role of NRF2 in cancer biology should be taken into account in interpretation of its molecular role. Studies revealed that nuclear or activated NRF2 expression is associated with upregulation of multiple target genes with negative prognostic effects leading to impaired overall as well as progression-free survival (PFS). In accordance, patients with high cytoplasmic NRF2 expression (inactive form of the transcription factor) displayed improved OS and PFS.[14,38]

Progesterone plays an anti-proliferative effect via its receptor and has hereby been reported to be associated with improved OS and PFS in ovarian cancer patients.[24–27] These findings are supported by studies showing that PR mediates apoptotic cell death.[39,40] Furthermore, upregulation of Forkhead-box transcription factor (FOXO1) through progestin activated PR causes cell cycle arrest by increasing of mediators of cell senescence.[40,41] A potential interaction between NRF2 and PR is not well understood yet, but can be conceived as a hypothesis-generating approach based on the presented evidence. NRF2 activates its target gene aldo-keto reductase family 1 member C1 (AKR1C1) amongst others via an antioxidant response element (ARE) in a specific promoter region. AKR1C1 converts progesterone to its inactive form, the 20-alpha-dihydroxyprogesterone (20-alpha-OHP).[42,43] Moreover, AKR1C1 can bind to the promoter region of PR and decreases hereby receptor activity.[44] In this study, silencing of *NFE2L2* induced a higher mRNA expression of *PGR* supporting this data. In endometrial cancer patients, concurrent NRF2/AKR1C1 overexpression was proposed to be part of the molecular mechanisms underlying progestin resistance.[45] Accordingly, increased expression of AKR1C1 is associated with the development of platinum resistance in human ovarian carcinoma cells as well as colon carcinoma cells.[46,47] In contrast, progesterone facilitates the toxicity of cisplatin in ovarian cancer cells and a preclinical murine xenograft model.[48] Taken together, the interaction of NRF2 and PR might represent a potential pathway significantly influencing platinum response being mediated by AKR1C1 in ovarian cancer which should be followed in future studies. NRF2/AKR1C1 expression can be downregulated by metformin treatment as described in endometrial and lung cancer cells.[45,49,50] Interestingly, recent studies show that metformin, usually applied in diabetic patients, prevents tumor growth, induces apoptosis and increases sensitivity to chemotherapy in ovarian cancer cells.[51–57] Mechanisms underlying these cellular effects include suppression of cancer stem cells, inhibition of epithelial-to-mesenchymal transition and interference with neoplastic cell metabolism.[58–62] Following promising data of epidemiological studies showing a favorable effect of metformin on ovarian cancer

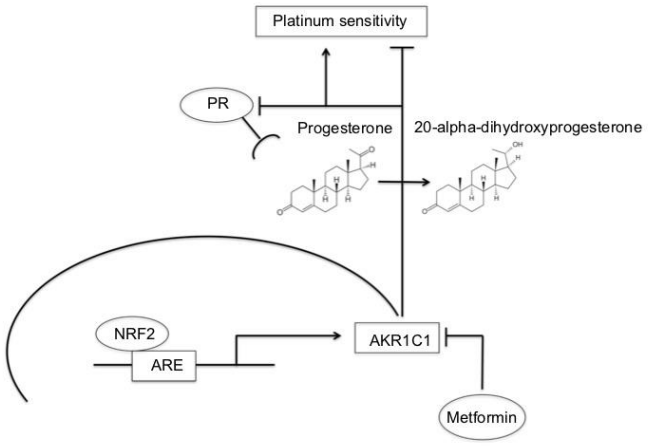

Figure 6 Summary of the hypothesized interaction within the NRF2/AKR1C1/PR pathway.
Notes: Activated NRF2 (high nuclear, low cytoplasmic expression) activates aldo-keto reductase family 1 member C1 (AKR1C1) via an antioxidant response element (ARE). AKR1C1 converts progesterone to its inactive form, the 20-alpha-dihydroxyprogesterone, and decreases PR receptor activity with consecutive platinum resistance. Metformin treatment counteracts this pathway, which may reverse the effects and consecutively lead to platinum re-sensitization.

incidence and survival, it was proposed that metformin should be followed as an additional approach in ovarian cancer treatment.[63–65] As metformin treatment is associated with an increased PR expression, these results further support future investigations of the above-described relationships in the NRF2/AKR1C1/PR pathway and their impact on ovarian cancer biology and the clinical behavior.

Conclusion

In summary, based on the results of the present study, we hypothesize that the interplay between NRF2/AKR1C1/PR might serve as an important pathway with significant impact on ovarian carcinogenesis elucidating additional therapeutic perspectives (Figure 6). With the rationale described earlier, metformin might have favorable effects on ovarian cancer biology and open new approaches to overcome platinum resistance which needs to be proved in future studies.

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Disclosure

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