Targeting the DNA repair pathway in pelvic epithelial cancer: a novel treatment strategy combining IGF-1R kinase and PARP inhibitors

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BRCA - Breast Cancer antigen; IGF-1- Insulin like frowth factor 1; IGF-1R- Insuin like growth factor 1 receptor; IGF-1RKi - Insulin like growth factor 1 receptor kinase inhibitor; PARP - poly(ADP-ribose) polymerase; RAD51- a family member of DNA repair protein.

ABSTRACT

Pelvic epithelial cancer (usually referred to as ovarian cancer) represents one-fourth of the malignancies of the female genital tract. Women carrying Breast Cancer Antigen *(BRCA)* germline mutations are at increased risk to develop pelvic epithelial cancer, and up to 50% of all patients with pelvic epithelial cancer show functional impairment of *BRCA1* [1]. These mutations in *BRCA1/2* genes exhibit impaired ability to repair double-stranded DNA breaks via homologous recombination (HR). Moreover, *BRCA1* mutated cancer cells were found to have over-expression of Insulin like growth factor-1 receptor (IGF-1R) levels [2, 3]. Previously, our lab reported that inhibition of IGF-1R results in growth inhibition and apoptosis of ovarian cancer cells [4]. In our current study, we assessed the correlation between IGF-1R inhibition and HR functionality of ovarian cancer cell. Interestingly, we observed the increased sensitivity of HR deficient cancer cells to IGF-1R kinase inhibitors (IGF-1Rki). Moreover, we assessed the effect of IGF-1Rki on HR DNA repair pathway and found the reduced expression of RAD51 both at protein and mRNA level. This data suggest that inhibiting the IGF-1R pathway suppresses HR. We further showed that IGF-1Rki and poly(ADP-ribose) polymerase (PARP) inhibitors act in synergy to inhibit cancer cells. This data offers a basis for future pre-clinical work

involving IGF-1Rki and PARP inhibitors in patients with cancer, providing new opportunities for the development of targeted personalized cancer therapy.

Re`sume`

Les cancers épithéliaux du petit bassin (habituellement appelé “cancer de l’ovaire” ; CEP) représentent un quart des cancers génitaux chez la femme. Les patientes porteusent d’une mutation germinale de BRCA ont un risque augmenté de développer un CEP, et jusqu’à 50% de toutes les patientes porteuse d’un CEP présente un déficit fonctionnel de BRCA1. Ces mutations des gènes BRCA1/2 sont responsables d’un défaut de réparation des lésions doubles brins de l’ADN lié à un déficit de fonctionnement du complexe de recombinaison homologue (HR). De plus, une hyper-expression de IGF-1R est retrouvée dans les cancers présentant des mutations de BRCA1. Notre équipe a montré précédemment que l’inhibition de IGF-1R dans certaines lignées cellulaires de cancer de l’ovaire entraine une inhibition de la prolifération et augmente l’apoptose. Dans notre étude, nous avons tout d’abord évalué la corrélation entre l’inhibition de IGF-1R et le fonctionnement du complexe HR dans les cellules cancéreuse. Ainsi, nous retrouvons une sensibilité accrue aux IGF-1Rki des cellules présentant un complexe HR déficient. Nous avons ensuite étudié l’effet d’IGF-1Rki sur la voie de réparation de l’ADN médiée par le complexe HR et nous avons retrouvé une diminution de l’expression de RAD51 en réponse à ce traitement, aussi bien au niveau du taux de protéine qu’au

niveau du taux d'ARN. Ces données suggèrent que l'inhibition de la voie de IGF-1R bloque le complexe HR. Nous avons également montré que les IFG-1Rki et les inhibiteurs de PARP ont une action synergétique sur les cellules cancéreuses. Ces données offrent une base intéressante pour l'élaboration d'autres études précliniques évaluant l'effet des IFG-1Rki et des inhibiteurs de PARP chez les patients atteints de cancer, et constitue une opportunité nouvelle dans le développement des thérapies ciblées personnalisées en cancérologie.

CHAPTER 1: INTRODUCTION

1. Pelvic Epithelial Cancer:

Pelvic epithelial cancer, referred to as "ovarian" cancer, is the most lethal of all gynecological cancers [5, 6]. A growing number of studies have revealed that these cancers can develop from multiple extra-ovarian origins, including fallopian tube [7-10]. Although data is emerging that the epithelial cancer cells migrate to the ovaries rather than originate in the ovary, we will use the term ovarian cancer throughout this thesis. Among the subtypes of ovarian cancer [11], epithelial tumors represent the largest and the most heterogeneous group. New histopathological, molecular, and genetic studies have recently provided a better classification and epithelial cancers are now categorized into type I and type II tumors. Type I ovarian cancers include low grade carcinomas and are usually indolent. They are often confined to the ovary at the time of diagnosis and are grouped together with low malignant potential neoplasms, also referred to as borderline tumors [11]. At the molecular level, Type 1 cancers are genetically stable having KRAS, BRAF, PIK3CA, PTEN, ERBB2, ARID1A and/or CTNNB1 mutations. On the other hand, Type II comprise high grade ovarian carcinomas, and are usually more aggressive and found at advanced stages.

Moreover, type II tumors are genetically highly unstable and the vast majority (95%) have Tp53 mutations, and close to 50% of the patients are found to have mutations, hypermethylation, or dysfunction of *BRCA1/2* gene [12-15].

Cure rates for ovarian cancer have remained unchanged over the past 30 years despite advances in chemotherapy regimens. New opportunities for treatment approaches using personalized targeted therapies are becoming available to improve outcome.

2. Insulin like growth factor (IGF)

IGF was first identified over 50 years ago, and consists of two ligands, IGF-1 and IGF-2, that interact with cell surface receptors called type 1 insulin-like growth factor receptor (IGF-1R) and type 2 insulin-like growth factor receptor (IGF-2R)[16]. Both IGF-1 and IGF-2 interact with IGF-1R, but IGF-1 shows a much higher affinity than IGF-2. IGF-1 is a small peptide consisting of 70 amino acids and its circulating concentration is mainly dependent on the production by the liver. The bioavailability of IGF ligands is controlled by a family of IGF binding proteins, regulating the ratio between free and protein-bound IGF. The primary action of IGF-1 is mediated by binding to its specific receptor, IGF-1R, which is present on many cell types in almost all tissues, including tumor cells [17].

IGF-1R is a heterotetrameric transmembrane receptor tyrosine kinase. It is composed of two extracellular α- subunits and two transmembrane β subunits. The α subunits have binding sites for IGF-1 and are linked by disulphide bonds. The β subunit has a short extracellular domain, a transmembrane domain, and an intracellular domain. The intracellular part contains a tyrosine kinase domain, which constitutes the signal transduction mechanism. Once the receptor is activated by the binding of IGF-1, it triggers a signalling transduction cascade modulating cellular functions. IGF signalling via the IGF-1R is essential for normal cellular function and has been shown to regulate cell proliferation and to inhibit cell death [18].

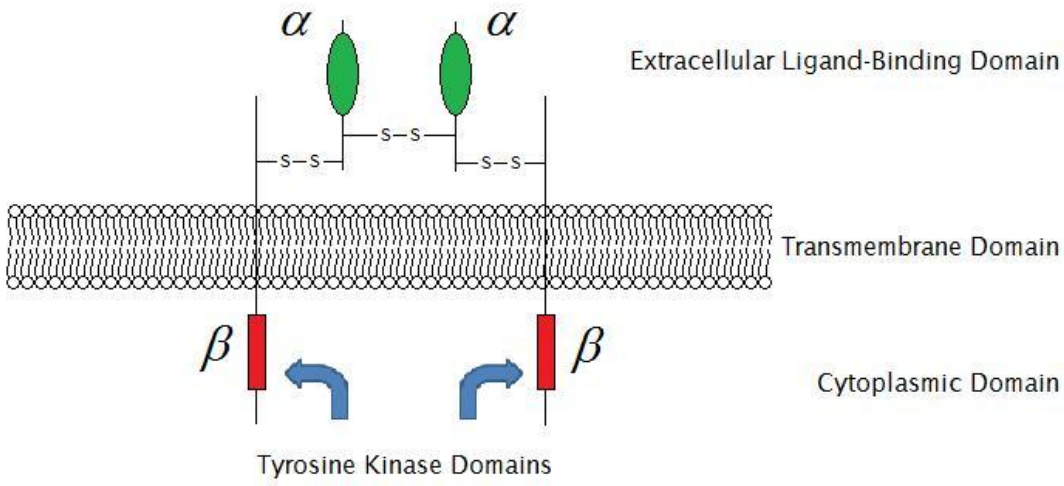

Fig. 1: Schematic diagram of the IGF-1R structure, (adapted from Wikipedia).

2.1 Role of IGF in human cancer:

The IGF signalling pathway influences oncogenesis, cancer progression, metastasis, and chemoresistance [19, 20]. In cancer, one often finds over-expression of the IGF-1R, or the establishment of autocrine or paracrine signalling loops [21]. It has been shown that IGF-1R, its ligands and IGF binding proteins are highly expressed not only in ovarian, but also in many other cancers: prostate [22], breast [23], colorectal [24], pancreatic cancers [25], melanoma [26], multiple myeloma [27], glioblastoma [28], mesothelioma [29], and childhood malignancies [30]. Moreover, it was reported that IGF-1R expression patterns in epithelial cells of normal benign breast biopsies were associated with an increased risk of subsequent breast cancer [23]. Further, studies have shown a correlation between circulating IGF-1 levels and cancer risk in some malignancies (breast, prostate and colorectal carcinomas, as well as lung, endometrial and bladder cancers) [31]. The IGF-I receptor pathway therefore represents a promising target for cancer therapy.

2.2 Role of IGF in ovarian cancer

The first study showing the expression of IGF-I mRNA in ovarian cancer cells and tissues, was published back in 1991 by Yee, D et al [32]. These authors also reported the expression of several insulin like growth factor binding proteins

(IGFBPs) and the IGF-IR by ovarian cancer cells. This study suggested that all necessary components for an IGF-I-mediated autocrine loop are present in ovarian cancer cells. Another study investigated the expression of IGF axis genes in relation to outcome using microarray profiles from 64 patients with advanced epithelial ovarian cancer. The study demonstrated that individual genes including IGF-I, IGF-IR and several genes downstream of the receptor were over-expressed in ovarian cancers associated with an unfavourable prognosis [33]. Several other studies also observed over-expression of IGF ligands and IGF-1R in ovarian cancer tissues [34-37]. Moreover, strong support for a role of IGF-I in ovarian cancer progression came from a study by Brokaw J. et al, which showed that high IGF-1 mRNA and protein expression in ovarian tumour tissue was independently associated with an increased risk of disease progression [38]. Further, two prospective studies reported a significant correlation between IGF-1 serum levels and ovarian cancer risk [39, 40]. In addition, IGF-I levels were found to be higher in cystic fluid from invasive malignant neoplasm compared to benign ovarian tumors [41]. These initial studies opened the door to a widespread area of research in ovarian cancer, indicating an involvement of the IGF pathway in ovarian tumorigenesis.

3. Development of inhibitors of IGF-1R

The IGF signalling pathway has become an attractive target for cancer therapy [42]. IGF-targeted therapies are mainly directed against the IGF-1R and are divided into two categories: small molecule inhibitors targeting the tyrosine kinase domain of the IGF-1R (IGF-1Rki), and monoclonal antibodies (mAb) directed at the IGF-1R, decreasing the binding of its natural ligands, IGF-1 and IGF-2 [21]. Currently, there are at least 30 drugs in development and over 60 clinical trials evaluating IGF-1R directed therapy as an anti-cancer treatment [43].

The majority of the small molecule IGF-1Rki can be given orally and their mechanism of action is to block the activation of the IGF-1R by direct binding to the ATP-binding pocket of the receptor [44]. Although highly effective, the receptor specificity is poor, and concurrent insulin receptor inhibition occurs. However, this lack of specificity may provide added benefit in the case of hybrid receptors (formed by IGF-1R and insulin receptor), which are over-expressed in certain tumor types, although their exact role in malignancy remains to be determined. The most common IGF-1Rki ATP competitive binding inhibitors; OSI-906, NVP-ADW742, NVP-AEW541, BMS-536924 and BMS-754807[45]. Several other tyrosine kinase inhibitors (tyrphostins, picropodophyllins, INSM-18, and BMS-754807) inhibit IGF-1R via non-ATP-competitive binding to the receptor. This is postulated to decrease side effects. Most of these IGF

inhibitors are well tolerated, with both general class-specific and individual drug-specific toxicities [46]. As predicted, hyperglycemia has emerged as a significant side effect, but most cases are grade 1 or 2 and are easily managed with oral hypoglycemics. Grade 3 or 4 reactions tended to occur in diabetic patients and were reversible. Not surprisingly, hyperglycemia was more severe in regimens including corticosteroids [47], an important consideration when combining IGF inhibitors with chemotherapy.

Monoclonal antibodies against IGF-1R seems attractive owing to their higher selectivity and lack of cross-reactivity with the insulin receptor. They bind the extracellular domain of the IGF-1R, block ligand binding, and cause receptor internalization and degradation [48]. Interestingly, despite the improved specificity of the IGF-1R-directed monoclonal antibodies, hyperglycemia was also observed in those patient cohorts as well. Given the longer half-lives of IGF-1R directed antibodies, hyperglycemia is more prevalent, and recently has raised concerns about toxicity. In combination with cytotoxic agents and other targeted therapies, toxicities are generally more frequent and severe [48].

3.1. Effect of IGF-1R inhibitors in human cancers

The influence of IGF-1R expression in cell transformation and cancer development has been evaluated in multiple studies, and IGF-1R inhibition was

found to inhibit the growth of a broad range of human tumor cell types [49], and induced the regression of endometrial and cervical tumor growth [50-52]. IGF-1R inhibition blocked IGF-1 induced phosphorylation of the IGF-1R [53, 54]. In addition, IGF-1R down-regulation resulted in sensitization of endometrial cancer cells to cisplatin [55], and IGF-1Rki treatment was reported to result in tumor regression in triple-negative breast cancers when combined with docetaxel [56].

Moreover, the role of IGF1-R inhibition in radiosensitization and DNA repair has been reported in several studies. Indeed, inhibition of IGF-1R by specific antibodies sensitized tumor cells to ionizing radiation in prostate and lung cancers [57, 58]. Another study tested the effect of IGF-1R siRNA transfection on the repair of radiation-induced Double Strand Break in prostate cancer cells. That study reported a role for IGF-1R in DSB repair, at least in part via HR, and supports the use of IGF-1R inhibitors with DNA damaging cancer treatments [59]. Similarly, we showed that BMS-536924 sensitizes ovarian cancer cells to the PARP inhibitor, 3-aminobezamide, possibly via the induction of DNA damage as indicated by the increased phosphorylation of histone H2AX [60]. Further, it was reported by Trojanek et al. 2003 that the signal from activated IGF-1R might be enhancing HR by a mechanism that controls the translocation of Rad51 to the sites of damaged DNA (nuclear foci). That study suggested a direct interaction between Rad51 and the major IGF-1R signalling molecule, insulin receptor

substrate 1 (IRS-1). The binding is direct and is negatively regulated by IGF-I-mediated IRS-1 tyrosine phosphorylation. Importantly, cells with low levels of the IGF-1R, or cells expressing an IGF-1R mutant that fails to phosphorylate IRS-1, retain Rad51 within the perinuclear compartment and show significantly less DNA repair by HR [61]. Similarly another study reported that IGF-1R contributes to radiation-induced DSB repair through the suppression of HR via IRS-1/Rad51 signal pathway [62]. Taken together these data suggest that IGF-1R inhibition can impact DNA damage repair pathways and thus sensitize tumor cells to therapies targeting DNA repair.

3.2. Role of IGF-1R inhibitors in ovarian cancer:

In the last couple of years, studies targeting IGF or insulin pathways in ovarian cancer mostly used small molecule IGF-1Rki. These inhibitors are in earlier phases of clinical development for ovarian cancer as compared to antibodies developed against IGF1-R. Currently, two phase II studies are evaluating the efficacy and safety of AMG-479 (IGF-1R monoclonal antibody) as a second line therapy in patients with recurrent platinum-sensitive ovarian cancer (NCT00719212) and in combination with standard chemotherapy agents as first line therapy (NCT00718523). Further, a phase I/II trial is studying intermittent and continuous therapy with OSI-906 (IGF-Rki) in combination with weekly

paclitaxel in patients with recurrent epithelial ovarian cancer (NCT00889382). Similarly, BMS-754807, an IGF-1Rki, is being evaluated with paclitaxel and carboplatin in a phase I study in patients with advanced or metastatic solid tumors, including ovarian cancer (NCT007938897), [clinicaltials.gov].

Previous studies by our lab using NVP-AEW541, another IGF-1Rki, demonstrated anti-proliferative activity in ovarian cancer in association with decreased activity of the IGF-1R downstream signalling pathway [4]. We demonstrated that this inhibition sensitized cells to the effect of cisplatin, an effect later described in other types of cancer cells [44]. Moreover, using this small-molecule inhibitor in a human ovarian cancer xenograft model (mice) also gave promising results . Indeed, the survival of these mice was improved by 60% when treated with an IGF-1R kinase inhibitor (NVP-AEW541) alone and up to 90% when combined with cisplatin [16]. Concurrently, our laboratory showed a dose and time-dependent growth inhibition of human epithelial ovarian cancer cell lines in response to BMS-536924 [60], a more recently developed IGF-1Rki. This effect was partly mediated by AKT and the ribosomal protein S6. This study further reinforced the concept that IGF-IR is a good therapeutic target in ovarian cancer.

4. Breast cancer susceptibility gene, *BRCA*

BRCA1/2 are human tumor suppressor genes inherited in an autosomal dominant fashion with incomplete penetrance. First, *BRCA1* was identified in 1994 on the long arm of chromosome 17, and in 1995, *BRCA2* on the long arm of chromosome 13 [63, 64]. *BRCA1/2* are tumor suppressor genes directly involved in the repair of DNA damage, transcription and cell cycle control. Initial evidence suggesting a role of BRCA1 in the repair of damaged DNA was derived from the observation that BRCA1 is hyperphosphorylated by an ataxia-telangiectasia mutated (ATM) kinase [65, 66], ATM-related kinase (ATR) [67] and checkpoint kinase 2 (CHK2) [68, 69], in response to DNA damage and relocated to sites of the replication forks [70, 71]. Subsequent studies demonstrated the involvement of BRCA1/2 in complexes that activate the repair of DSBs and initiate HR [72, 73], a preferred and potentially error free DNA repair pathway in which a chromatid that suffers a DSB uses neighboring undamaged sister chromatid as a template for DNA repair [74, 75]. These DSBs can be caused by a wide variety of external agents, such as UV lights, chemotherapy or ionizing radiation, and by endogenous processes; such as DNA replication or as a consequence of reactive oxygen species generated during oxidative metabolism.

During HR, as shown in Fig.2, DSBs need to be first extensively processed to allow for the search of the homologous undamaged template. First,

DSB are recognized by the MRN (Mre11-Rad50-Nbs1) complex. The MRN complex then recruits ATM kinase and phosphorylates it. The activated ATM then phosphorylates multiple substrates which leads to DSB resection forming ssDNA strands. BRCA1 is required for the recruitment of activated ATM to sites of DNA damage and cohesion phosphorylation by ATM [76]. BRCA1 has been implicated in the phosphorylation of H2AX as well as in the regulation of MRE11 activity [77, 78]. On the other hand, BRCA1/2 interacts with and regulates RAD51 localization and function [72, 73]. Co-localization of BRCA with Rad51 at sites of recombination and DNA damage-induced foci strongly suggests that BRCA1/2 have a role in both the detection and the repair of DSBs [72]. RAD51 then coats single-stranded DNA to form a nucleoprotein filament that invades and pairs with a homologous region in duplex DNA, and activates strand exchange to generate a crossover between the juxtaposed DNA [79, 80]. DSB repair by HR is deficient in cells having non-functional *BRCA1* [81, 82].

Intact DNA damage repair pathways are very critical for preventing the replication of damaged DNA templates and transmission of mutations to daughter cells. Therefore, defects in DNA damage repair will result in accumulation of genetic mutations, gene amplification, and chromosomal instability, which in turn contribute to malignant transformation and tumorigenesis[83]. Moreover, many of the genes in the DNA damage repair

pathway are often mutated and/or modified in cancer tissues. It is well known that patients who have loss-of-function of *BRCA1/2* genes are at increased risk of developing breast and ovarian cancer [84].

Further, several studies reported a mechanistic functional interaction between the BRCA1 and IGF-1 pathway. As reported previously by Maor et al [85], *BRCA1* gene expression might be regulated by the IGF-I signalling pathway. The same author showed that primary breast cancers bearing a mutation in the *BRCA1* gene have a significant elevation in IGF-I receptor levels compared with breast tumors having wild type *BRCA1* [2]. Another study [3] also suggested an increased intratumoral IGF-I protein expression in BRCA mutation carriers. Further, elevated IGF-I serum levels have been detected in women with a family history of breast cancer carrying a *BRCA1/2* mutation, compared with those without such a history [86]. Moreover, another study a reported strong association between serum levels of IGF-I and hereditary breast cancer risk, using a case-control approach in women belonging to high genetic risk families [87].

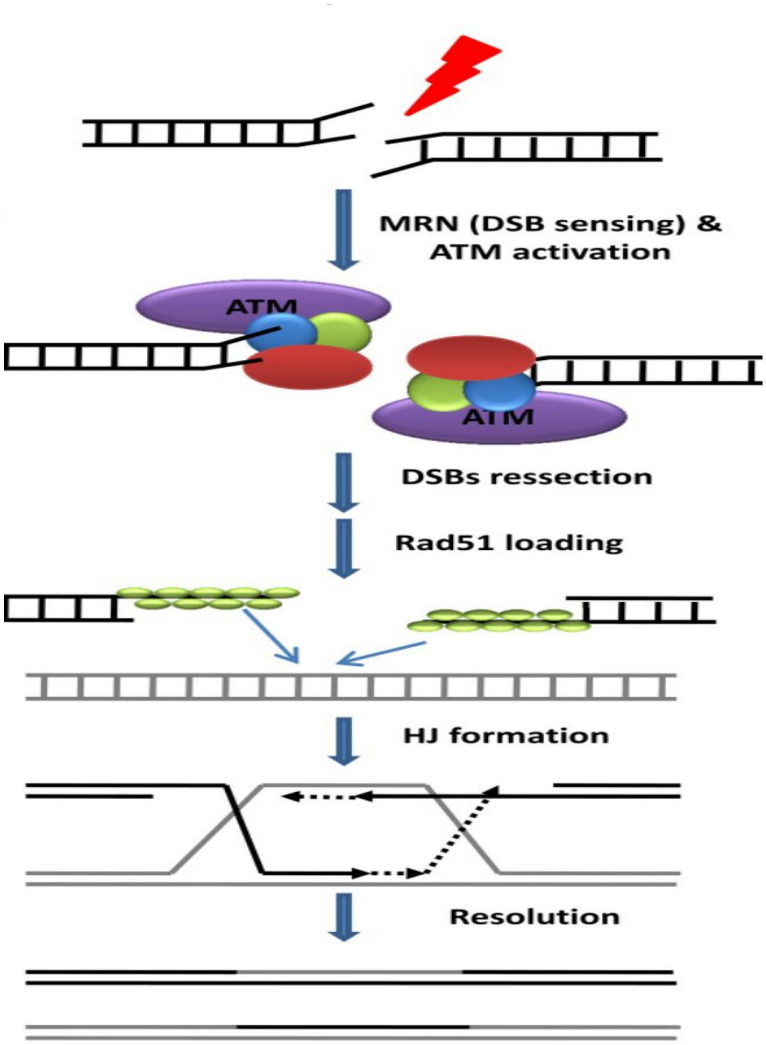

Fig. 2: DSBs repair mechanism; Homologous recombination.

The recruitment of ATM kinase to DSB via an interaction with MRN (Mre11-Rad50-Nbs1) complex. DSBs are resected forming ssDNA strands. These ssDNA regions attract Rad51 and other associated proteins, followed by invading the undamaged sister strands forming Homologous junction (HJ) structures . HR is completed by new DNA synthesis. Adapted from[88]

4.1 Role of BRCA in human cancer

Although it is well known that mutations in *BRCA1/2* increase the risk of breast and ovarian cancer (etiology equivalent to fallopian tube and peritoneal cancer), they also increase the risk of other cancers like, pancreatic and prostate cancer. Together, germline *BRCA1/2* mutations account for about 20-25% of hereditary breast cancers, 5-10% of all breast cancers, and around 15% of ovarian cancers overall [1, 89-92].

Moreover, recently the term "BRCAness" has been used to describe the phenotypic characteristics that some sporadic cancers share with tumors found in the setting of BRCA germline mutations. The term also reflects that this common biologic behaviour comes from molecular defects in the cellular machinery similar to the ones caused by BRCA mutation [93, 94]. This BRCAness pattern of biological and clinical behaviour seems to be the result of different epigenetic mechanisms of gene inactivation, resulting in the silencing of tumor suppressor genes [95]. The most studied epigenetic event is the aberrant methylation of cytosine residues in CpG dinucleotides. Although generally underrepresented in the genome, islands of these dinucleotides occur in the promoters of a substantial proportion of genes, where they are normally unmethylated. *Denovo* methylation of these CpG islands in cancer is associated with packaging of the surrounding DNA into densely packed histones, and

silencing of transcription. The aberrant methylation of the *BRCA1* promoter is found in 11-14% of sporadic breast cancers [96-98] and in 5-31% of ovarian cancers [96, 98-101]. In most of these cases, BRCA1 protein expression is undetectable, indicating complete silencing of gene expression as a consequence of methylation [97, 100, 101]. Further, *BRCA1/2* alterations of all kinds, including germline and somatic mutations, and promoter methylation have been reported in up to 82% of ovarian tumors [1, 102]. Also, up to 50% of sporadic ovarian cancers share a BRCAness phenotype [1]. This existence of a significant proportion of sporadic ovarian cancers with BRCA-like functional abnormalities raises the possibility of a wider application of treatment regimens designed for familial-BRCA tumors.

5. Poly ADP-ribose polymerase (PARP)

The discovery of PARP goes hand in hand with personalized anticancer therapy, representing the first personalized therapy targeting BRCAness tumor cells. PARP are a family of enzymes that have been implicated in a number of cellular pathways involving mainly DNA repair and programmed cell death. PARP activation is an immediate cellular response to metabolic, chemical, or radiation-induced DNA single strand break (SSB) damage. It catalyz the

cleavage of NAD+ into nicotinamide and ADP-ribose leading to the rapid consumption of NAD+. Moreover, it is an important component of the base excision DNA repair pathway, which is critical for the repair of SSBs [103].

Single strand DNA damage takes place when only one of the two strands of a double helix has a defect and the most common source of SSBs is oxidative attack by endogenous reactive oxygen species [104]. Major SSB repair pathways are the DNA mismatch repair (MMR), the nuclear excision repair (NER) and the base excision repair (BER) pathways. However, SSBs are predominantly repaired by the base excision repair (BER) pathway that removes the damaged nucleotide and replaces it with an undamaged nucleotide complementary to that found in the undamaged DNA strand utilizing a family of related enzymes termed PARP. PARP senses and binds to DNA breaks, leading to activation of catalytic activity and subsequent poly(ADP)ribosylation of PARP itself and other proteins involved in this pathway [105]. This modification then signals the recruitment of other components of DNA repair pathways, as shown in Fig. 3.

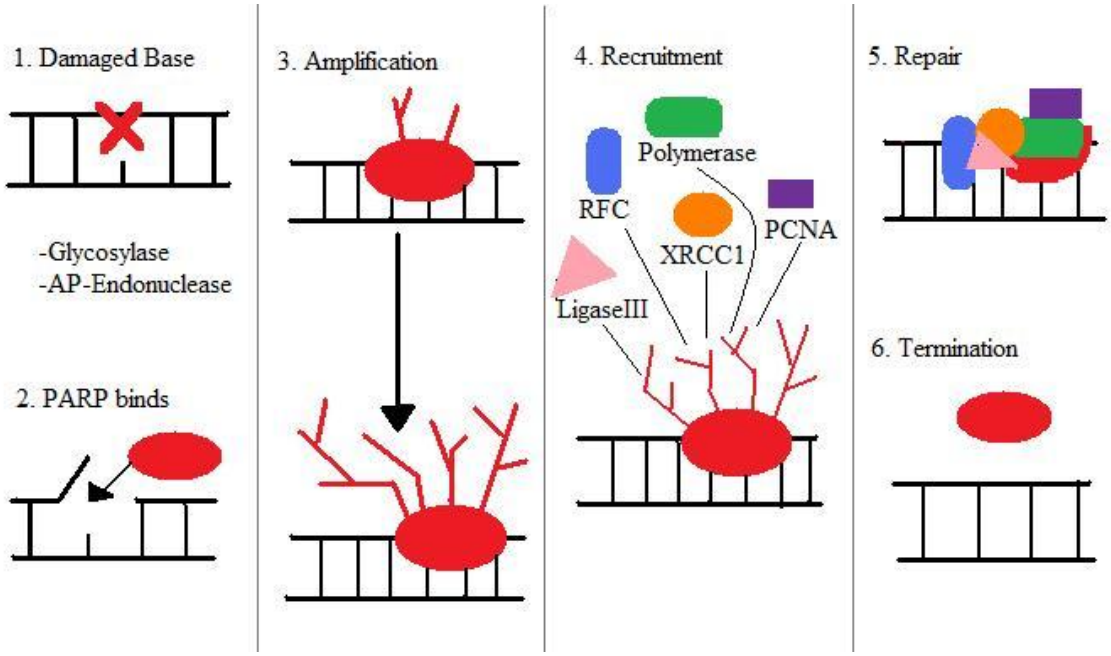

Fig. 3: Role of PARP in base excision repair.

Damage specific glycosylases recognize the presence of a faulty base and removes it. The enzyme PARP binds to the strand break. PARP then transfers ADP-ribose units from NAD+ to histones, and itself. This forms long and branched polymers of poly(ADP-ribose) on the PARP enzyme, that act as a signaling mechanism to recruit the BER machinery (including adaptor factor XRCC1, PCNA, RFC, ligase III and DNA polymerase β). The BER complex assembles at the site of damage and facilitates repair in a coordinated fashion (adapted from[105])

6. PARP inhibitors :

The initial impetus to the development of PARP inhibitors came from the need to develop tools to study the role of the enzyme. However, as the work has progressed, further applications for PARP inhibitors have been identified. Several academic investigators and pharmaceutical companies have had an active PARP

inhibitor development program and developed agents including niraparib (MK4827), rucaparib (AG014699,CO338) and NU1025 and several entered clinical investigations such as velaparib (ABT-888), BMN-673 and olaparib (AZD2281). It was proposed by Thomas Lindahl, as early as 1922, that these drugs inhibit the catalytic activity of PARP-1 but not its binding to the DNA and, since the enzyme needs to be poly(ADP-ribosyl)ated to dissociate from DNA, it can remain bound causing a physical obstruction to the repair of the break. That study showed that repair of nicked plasmid DNA by nuclear extracts was not dependent on PARP, but if PARP was present, NAD+ as the substrate was necessary for the repair, and binding of NAD+ could be inhibited by 3-aminobenzamide (nicotinamide analogue). This indicated that inactive PARP impeded DNA repair and that the presence of PARP poly(ADP-robosyl)ation was necessary for the repair to proceed. Similarly, it was reported recently that PARP inhibitors lead to the trapping of PARP enzyme at damaged DNA, forming cytotoxic PARP-DNA complexes [106, 107].

Moreover, recently the concept of synthetic lethality, which is a term to describe the situation whereby two pathway defects acting individually have little or no effect but when combined become lethal [108], has provided the new opportunities for the development of targeted therapies. This concept explains the selective killing of cancer cells with particular defects by some agents [109].

One of the best examples of targeted therapy taking advantage of this synthetic lethality is the development of PARP inhibitors [110]. Indeed, in cells with loss of function of *BRCA1/2*, which are therefore HR-deficient, inhibition of PARP activity leads to an accumulation of single strand breaks that are converted to DSB that can be repaired by HR in normal cells maintaining one copy of BRCA1/2, but cannot be repaired in the cancer cells lacking BRCA1/2. This results in increasingly high levels of genetic instability in the cancer cells and eventually their death without affecting normal cells, as shown in Fig 3. Further, preclinical and preliminary clinical evidence suggests a potentially broader scope for PARP inhibition. It was also shown to be synthetically lethal for cells lacking other proteins involved in HR besides *BRCA1/2,* such as RAD51, ATM, ATR [111]. This is important because, as mentioned earlier, HR seems to be defective in almost half of ovarian cancers [1] and in the future may lead to broadening of the patient population who may benefit from PARP inhibition [112, 113]. Moreover, HR defects are relatively common in tumors [114, 115] but not in normal tissues, so this is more likely to be a tumor-specific therapy.

Previous studies with PARP inhibitors have also demonstrated sensitization to ionizing radiation (IR) and a variety of cytotoxic drugs [116-118]. The rationale behind combinations of PARP inhibitors and chemotherapy is that PARP inhibitors disrupt base excision repair which is partly responsible for repair

of the damage caused by these chemotherapy agents, thus potentiating their action. Supporting that, it was reported that PARP null mice and cells derived from them are hypersensitive to ionizing radiation, DNA methylating agents and topoisomerase I poisons [116-118]. Moreover, potentiation of temozolomide (TMZ) anticancer activity by various PARP inhibitors has been investigated in a variety of in vivo models. In one study, it was reported that the PARP inhibitor velaparib enhanced TMZ cytotoxicity preferentially during S-phase, indicating that an accumulation of replication-associated DSBs were largely responsible for cell death [119]. Moreover, it was reported that velaparib increased topotecan cytotoxicity and cell cycle perturbations in a variety of human ovarian, leukemic and lung cancer cells [120]. Similarly, in models of pediatric cancer, ruparib (AG-014699) enhanced the anti-tumor activity of TMZ in neuroblastoma and medulloblastoma xenografts [121, 122]. Further, studies revealed that inhibition of PARP led to radiosensitization of mammalian cells [123]. Most published pre-clinical studies have been done with velaparib, which has been shown to significantly increase the anti-tumor activity of ionizing radiation in xenograft models of human colon, lung, and prostate cancer [124-126]. Also, MK4827 radiosensitized human lung and triple negative human breast carcinoma xenografts [127], and olaparib in combination with radiotherapy caused significant tumor regression of non-small cell lung carcinoma xenografts when

compared to radiotherapy alone [128]. Moreover, it was reported that PARP inhibitors selectively radiosensitize actively replicating S-phase cells [129]. It is proposed that the mechanism by which PARP inhibition increases ionizing radiation sensitivity is by inhibiting the repair of SSBs that convert to DSBs upon collision with replication forks in S-phase [130].

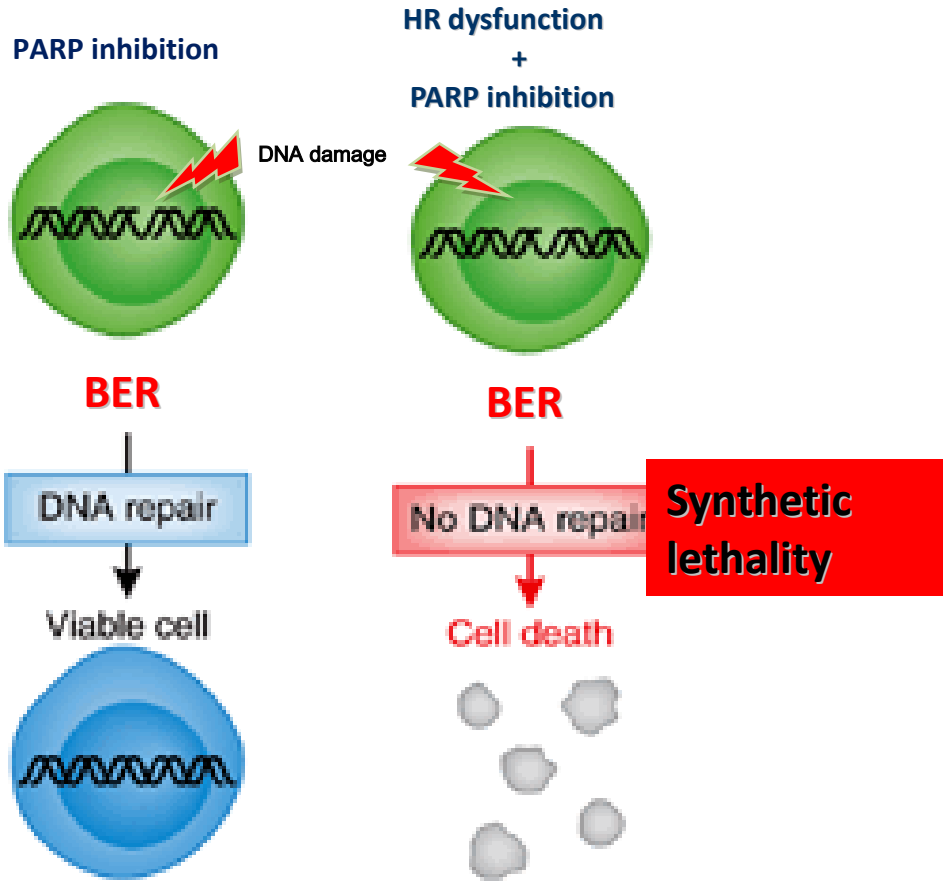

Fig. 4: Synthetic lethality of PARP inhibitors in HR deficient cell.

Cancer cells with proficient HR repair remains viable despite PARP inhibition via PARP inhibitors. Cells with HR deficiency will undergo apoptosis due to inefficient HR DNA repair.

6.1 PARP inhibition in ovarian cancer

PARP inhibitors were originally found to have anti-cancer activity both in vitro and in vivo in cancers with germline mutated BRCA genes [110, 131, 132], which has led to multiple clinical studies in breast and ovarian cancer. Table 1 summarizes multiple PARP inhibitors used in clinical studies for ovarian cancer. The first report of a clinical trial with PARP inhibitors as a single agent in *BRCA1/2* mutated cells was the pivotal phase I study of olaparib (AZD2281) [131]. Given these interesting preliminary data, a phase II international multicentre study showed a dose-response relationship to olaparib in patients with recurrent germline *BRCA1/2* mutated ovarian cancer [133]. Another phase II study evaluated the effect of olaparib as a single agent in patients with ovarian cancer and demonstrated that efficacy was observed with both germline BRCA as well as sporadic ovarian cancer without a known BRCA mutation, likely due to alternate HR DNA repair defects present in about 50% of ovarian cancer [1, 134], potentially expanding the patient population who could benefit from PARP

inhibition. Several other randomized phase II studies testing olaparib in recurrent ovarian cancer have been successfully completed or are ongoing, as listed in Table 2.

Study phase / Drug	Olaparib (AZD-2281)	Velaparib (ABT-888)	Rucaparib (AG 014699)	BMN673	INO1001
I	7	17	1	3	-
II	6	3	-	-	2
III	3	-	2	1	-
Total	17	20	3	4	2

Table 1: Clinical trials in phase I / II / III; PARP inhibitors,(www.clinicaltrails.gov).

Drug	Study phase	No. of patients	Response Evaluation Criteria in Solid Tumors (RECIST)	References
olaparib	2	57	Overall Response Rate (ORR) 400mg/d: 33% 100mg/d: 13%	Audeh MW et al, Lancet, 2010
olaparib	1	50	28% BRCA mutated	Fong PC et al, J Clin Oncol, 2010
olaparib	1	60	53% BRCA mutated	Fong PC et al, NEJM, 2009
olaparib	2	62	25-31% BRCA mutated	Kaye SB et al, JCO, 2012
olaparib	2	64	41% BRCA mutated 24% wt	Gelmon KA et al, Lancet Oncology, 2011
olaparib	2	265	Ola: 8.4 months Placebo:4.8 months	Lendermann J etal, NEJM, 2012
Niraaparib	1	100	50 or 33% BRCA mutated; 33 or 5% wt	Sandhu SK et al, Lancet Oncology, 2013
Olaparib + Carboplatin	1/1b	37	50% partial response	Lee, J-M et al, JNCI, 2014.
Olaparib	2	265	BRCAmut: Ola: 11.2 months Placebo: 4.3months	Ledermann J et al, Lancet Oncol, 2014.
Olaparib+ liposomal doxorubicin	1	28	ORR: 33%	Del Conte G et al, Br J Cancer, 2014.

Olaparib+ Cediranib	2	90	Ola: 52% Ola+ced: 81%	Liu JF, Lancet Oncol, 2014

Table 2: Clinical Trials; Olaparib

Rationale & Hypothesis:

The progressive elucidation of the molecular pathogenesis of cancer has fuelled the rational development of targeted drugs for defined patient populations, leading to the concept of personalized medicine. In ovarian cancer, it is estimated that up to 50% of patients have HR DNA repair deficiency [1]. We hypothesize that cancer cells harbouring HR deficiency could be more sensitive to the inhibition of IGF-1 signalling by IGF-1Rki for the following reasons:

1) Studies reported an overexpression of IGF-1R in *BRCA1/2* deficient cancer cells [3, 85],
2) IRS-1 (induced by IGF-1R activation) binds to Rad51, a BRCA dependent pivotal regulator of HR, in the cytoplasm [61]. Upon activation by ligand binding to the IGF-1R, IRS-1 is phosphorylated and releases Rad51, which translocates to the nucleus and participates in the HR. These data suggest a novel function for the IGF-1R/IRS-1 pathway that involves regulation of the intracellular trafficking of Rad51 to the site of damaged DNA, a crucial step in the process of DNA repair by HR.

By combining IGF-1Rki with PARP inhibitors we expect to see a decrease in both HR and single stranded break repair, leading to increased synthetic

lethality and expand the effectiveness of both the IGF-1Rki and the PARP inhibitors. We further will explore the mechanism through which IGF-1R is affecting HR DNA repair pathway both at protein and mRNA level.

CHAPTER 2

MANUSCRIPT

Deficient homologous recombination repair in “ovarian” cancer cells; increased sensitivity to insulin-like growth factor-1 receptor kinase inhibitor

(Submitted for publication)

Deficient homologous recombination repair in “ovarian” cancer cells; increased sensitivity to insulin-like growth factor-1 receptor kinase inhibitor

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Abstract

Background: Impairment of homologous recombination (HR) is found in almost 50% of ovarian cancers. Moreover, tumors with *BRCA1* mutations show increased expression of the Insulin-like growth factor type 1 receptor (IGF-1R). We previously have shown that inhibition of IGF-1R results in growth inhibition and apoptosis of ovarian tumor cells. In the current study, we aimed to investigate the correlation between HR and sensitivity to IGF-1R inhibition.

Methods:Using different ovarian cancer cellular models with known *BRCA1* status, we evaluated their HR functionality using RAD51 foci formation. The LC50 of IGF-1Rki in these cells was assessed and Western immunoblotting was performed to determine the expression of proteins involved in the IGF-1R pathway.

Results: Cells with non-functional *BRCA1* showed impaired HR function. These HR deficient cells have an over activation of the IGF-1R pathway, and a lower LC50 to inhibition of IGF-1R compared to HR proficient cells, suggesting a correlation between HR function and sensitivity to IGF-1R inhibitors in ovarian cancer cells.

Conclusion: These data indicate that the strategy of targeting IGF-1R might lead to improved personalized therapeutic regimens in cancer patients having HR deficiency.

Key Words: Ovarian cancer, BRCA1, Homologous recombination, Insulin-like growth factor 1.

Background

Pelvic epithelial cancer, referred to as “ovarian” cancer is the most lethal gynecologic malignancy. Although data is emerging that the origin of “ovarian” cancer is not in the ovary itself, we will continue using the term in this manuscript for simplicity [1,2]. 70-80% of ovarian cancer patients initially responds to standard treatments, but most relapse and ultimately die of the ovarian cancer. Having reached a stage of stagnation with conventional chemotherapeutic

agents, there is a need for new therapeutic modalities to overcome the chemoresistant cells.

Up to 50% of ovarian cancer patients show functional impairment of *BRCA1/2* and women carrying *BRCA1* germline mutations are often at increased risk to develop ovarian and breast cancer [3,4]. These mutations in *BRCA1/2* genes exhibit impaired cellular ability to repair double-stranded DNA breaks via the homologous recombination (HR) repair pathway, leading to reduced RAD51 foci formation following DNA damage [5,6].

BRCA1 has also been shown to directly affect the IGF-1 pathway [7]. Previous studies have suggested that *BRCA1/2* deficient breast cancer cells are associated with elevated expression of Insulin like growth factor-1 receptor (IGF-1R), possibly contributing to the tumorigenesis of these cancer cells [8-10]. Insulin and IGF-1R are widely expressed on normal and neoplastic cells, including ovarian tumors [11-16], and the role of the IGF-1 pathway in ovarian cancer etiology has been established [17]. High levels of free IGF-1 peptide in cytosol were reported to be independently associated with the ovarian cancer progression [16], and an IGF-1 autocrine loop was described in ovarian cancer cells [18,19]. Suppression of tumor growth and cellular proliferation were observed in a xenograft model using a small interfering RNA targeting IGF-1R in

ovarian cancer [20], emphasizing the major impact of lowering the IGF-1 pathway in ovarian tumorigenesis. Furthermore, inhibition of IGF-1R was shown to be associated with suppression of HR DNA repair pathway in prostate cancer cells [21] and it was reported that IGF-1R inhibitor sensitizes non-small cell lung cancer cells to radiation both *in vitro* and *in vivo*, and that this effect is likely attributable to the inhibition of DNA repair [22]. We found that there is decreased cell survival, increased apoptosis, increased sensitivity to cisplatin and inhibition of the IGF-1/AKT/mTOR/S6 pathway *in vitro* in ovarian cancer cell lines exposed to different small molecules Insulin Like Growth Factor-1 receptor inhibitors (IGF-1Rki) [17,18,23]. Hence in this study, we aim to evaluate if the HR functionality status of ovarian cancer cells modulates sensitivity to inhibition of the IGF-1R.

Materials And Methods:

Cells lines

The epithelial ovarian cancer cell lines SKOV3, UWB1.289(ATCC, Manassas, USA), IGROV1, A1847 (NCI) were used in this study. SKOV3, IGROV1 were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 10 μg/ml gentamicin; A1847 was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum(FBS), 2 mM glutamine, 10 μg/ml insulin and 10 μg/ml gentamicin and UWB1.289 was grown

in 50% MEGM medium (supplemented with hEGF, BPE, insulin, hydrocortisone), 50% RPMI-1640 (supplemented with 10% FBS), 2 mM glutamine) and 10μg/ml gentamicin. Each cell line was passaged every 5 to 7 days and maintained at 37˚C in a 5% CO_2, 95% air atmosphere incubator. According to published data, the *BRCA1* gene profile status of these cells is as follow: SKOV3 (wild type *BRCA1* gene); IGROV1 (heterozygous 280delA *BRCA1* mutation); A1847 (carrying methylated *BRCA1* gene); UWB1.289 (homozygous 2594delC *BRCA1* gene mutation) [24].

Patient tumor derived ovarian cancer cells labeled GOC31, GOC17, GOC15, GOC13, GNOV1, GOC23 were isolated in our laboratory from six surgical specimens, all from high grade (grade 3) stage 3/4 serous ovarian cancer. All patients participating in this study gave informed consent in accordance with the JGH ethics committee regulations (protocol # 03-041). Two of the epithelial cell lines (GOC23 and GNOV1) were derived from patients carrying the 5385insC *BRCA1* germline mutations. Presence of the mutations in these cell lines was confirmed by the JGH molecular pathology department. Primary cell lines were grown in OSE medium supplemented with 20% FBS and growth factors (insulin, EGFR, hydrocortisone, BPE). The cells were routinely passaged every 4 to 6 days. All cells were maintained at 37˚C in a 5% CO_2, 95%

air atmosphere incubator. All assays were performed in the respective cell medium.

Transfection

SKOV3 cells were seeded in 6- well flat-bottom cell culture plates at a density of $0.25x10^6$cells/well. Lipofectamine (Invitrogen, Burlington, Ontario, Canada) (1:1) was mixed with negative shRNA and *BRCA1* shRNA separately in RPMI-1640 with no FBS. Following 30 min of incubation at room temperature, both negative and BRCA1 shRNA were added to their respective wells. The cells were incubated at 37°C for 5 hr. Pools of stably transfected cells were selected using 2mg/ml puromycin for up to a week. The reduced levels of *BRCA1* mRNA and protein in transfected SKOV3 cell line was confirmed by PCR and western blot analysis (fig.1A,B).

Survival assays

The clonogenic assay was used to determine survival fraction of cells [25]. Briefly, 350-800 cells were plated in 6-well flat bottom cell culture plates in duplicates. Twenty-four hours after plating, cells were washed and a fresh medium was added in the presence or absence of increasing doses of BMS-536924; media containing the drug was refreshed on day 4. Colonies were fixed

and stained after 7 days of treatment with 1.5 ml of 6.0% glutaraldehyde and 0.5% crystal violet, and colonies were counted using the GelCount, Optronix. The surviving fraction (SF) of cells was calculated as follows: SF = number of colonies formed after treatment / number of cells seeded x PE, where plating efficiency (PE) = number of colonies formed in control / number of cells seeded [25].

The Alamar Blue assay was used to determine cell viability. Monolayers of 2000 cells were plated in 96-well flat-bottom cell culture plates in triplicates. Twenty-four hours after plating, when the cells had attached and reached ~40% confluency, cells were washed and a medium was replaced with a medium containing 1% FBS with increasing doses of BMS-536924 for the 72 hr. Controls included equal amounts of DMSO. Cell viability was assessed by visual inspection of the plates and by using the AlamarBlue colorimetric assay. AlamarBlue (Invitrogen, Burlington,Ontario) assay allows quantitative analysis of cell viability via the innate metabolic activity that results in a chemical reduction of AlamarBlue that changes from the oxidized (blue) form to the reduced (pink) form. After cells were treated, 6 µl of AlamarBlue was added into each well. When the color of the dye changed (approximately 4 h), plates were read in an ELISA plate reader at 2 different wavelengths, 562 nm and 620 nm to plot the graph. Percentage of reduced AlamarBlue was calculated using the following

equation: Reduced AlamarBlue % = A562 − A620 × Ro; where A562 and A620 are sample absorbencies minus the media blank; Ro=AO562/AO620 where AO562 is the absorbance of the oxidized form at 562 nm, and AO620 is the absorbance of the oxidized form at 620 nm.

Protein extraction and western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris•HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail tablet and phosphatase inhibitor tablet (PhosphoSTOP, Roche Diagnostics, Mannheim, Germany). Total protein content was measured according to Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Then protein lysates (50 ug) were resolved electrophoretically on denaturing SDS-polyacrylamide gels, and transferred to 0.45 nitrocellulose membranes. After blocking in 5% milk in PBST, membranes were probed with the following primary antibodies: anti-mouse BRCA1(Ab-1), anti-rabbit p-IRS1(S636/639), anti-rabbit p-AKT(S473), anti-rabbit p-S6(S240/244), anti-rabbit IGF-1 Receptor beta, anti-rabbit Beta-actin (Cell Signaling Technology) and anti-rabbit RAD51 (Santa Cruz Biotechnology). Immunobloted proteins were visualized using horseradish peroxidise (HRP)-conjugated secondary antibodies, and antigen-antibody complexes were detected using the ECL system.

Immunofluorescence analysis

Cells were seeded in 6-well plates at a density of 1 × 10^5 cells/35-mm dish and treated with cisplatin for 1hr and allowed to recover for ~6 hr. The cells were then washed in phosphate-buffered saline (PBS) and fixed using 4% paraformaldehyde. They were subsequently permeablized with 0.2% Triton-X 100 in PBS for 15 min. After blocking with 2% BSA/PBS for 1 h at room temperature, cells were incubated with primary antibodies: RAD51 (Santa Cruz Biotechnology, CA, USA; 1:200) in blocking buffer for 60 min at room temperature. Cells were then washed in PBS and incubated with AlexaFlour 488 chicken anti-rabbit IgG secondary antibody (Invitrogen, CA, USA; 1:500) for 30 min. Finally, cells were counterstained with 4`, 6-diamidino-2-phenylindole (DAPI) for 5 min before the final wash, and photographed (LEICA; DMI6000B).

Statistical analysis

Statistical analysis was performed using Prism. The % of positive Rad51 cells data before and after cisplatin treatment were assessed using the non-parametric two-tailed paired t-test. $P<0.05$ was considered statistically significant.

RESULTS:

HR function correlated with *BRCA1* protein levels in ovarian cancer cells.

We first wanted to assess the HR function in our cellular experimental models. To do so, we evaluated the RAD51 foci formation upon DNA damage with cisplatin by immunofluorescence as an indication of the cells ability to repair double strand DNA break [26]. As shown in fig. 2(A,B), cells transfected with shRNA *BRCA1* had less Rad51 foci in their nucleus after cisplatin treatment, suggesting a lower capacity of DNA repair through HR.

We observed a similar trend in cells with previously published *BRCA1* defects, i.e the A1847 and UWB1.289 cells [24]. It was reported that the *BRCA1* promoter in A1847 cells is hypermethylated, leading to a reduced protein expression (fig.1C). UWB1.289, on the other hand, carry a homozygous 2594delC *BRCA1* mutation, again leading to an absence of detectable levels of *BRCA1* protein (fig.1C). As expected, the levels of RAD51 foci formation in these cells was greatly reduced as compared to cells with higher *BRCA1* protein levels (fig.2C,D). Finally, we performed the similar experiment in patient`s tumor-derived cells. Among the six cell lines tested, three had no detectable *BRCA1* levels (fig.1D), similar to previous reports [27]. Again we observed, the cells that did not express *BRCA1* had a reduced RAD51 foci formation upon DNA damage,

suggesting a deficient HR functionality in these cells (fig.2E,F) [26,28].

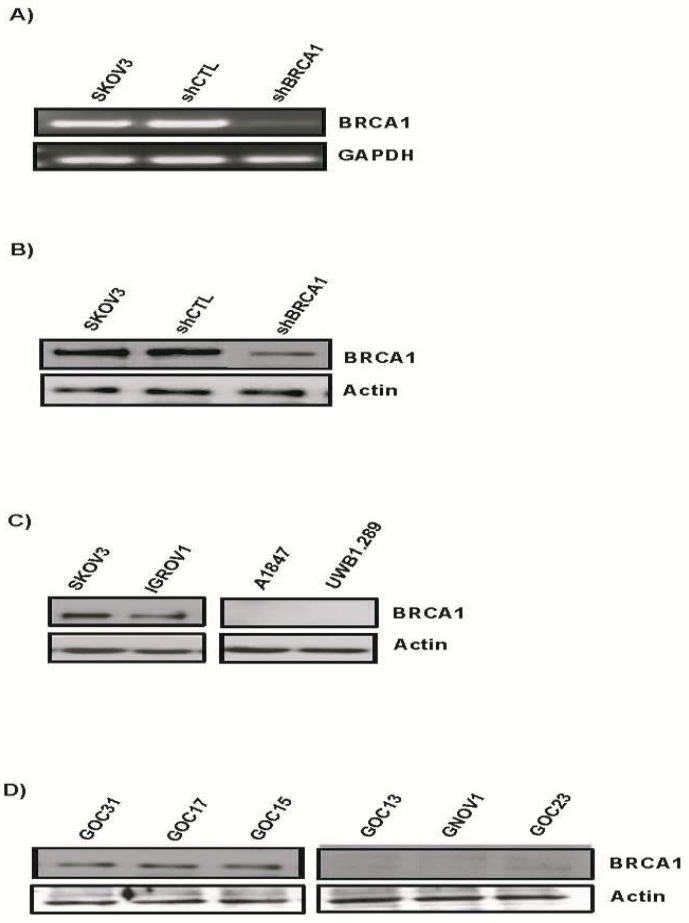

fig.1: Expression of BRCA1 in ovarian cancer cells.

Reduced expression of BRCA1 was observed in SKOV3 transfected with shBRCA1 at **A)** mRNA and **B)** protein level using PCR and western blot, respectively. **C)** Representative western blot out of two showing no BRCA1 protein expression in A1847 and UWB1.289 cells. **D)** Representative western blot out of three of BRCA1 protein expression from primary tumor cells isolated from 6 patients, as described in the materials & methods section, is shown.

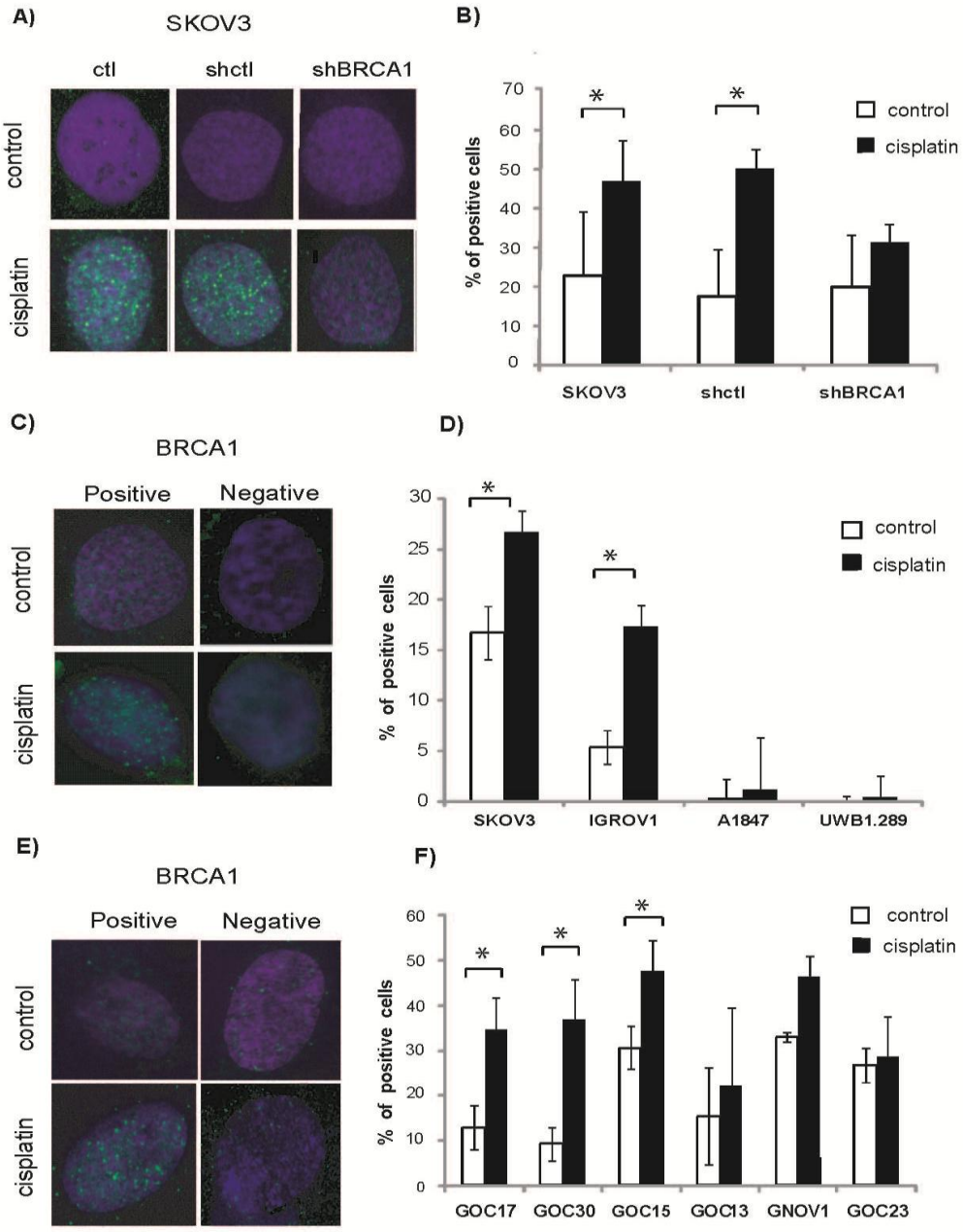

fig. 2: Reduced RAD51 foci formation in ovarian cancer cells with no BRCA1 expression.

A,C,E) Representative immunofluorescence staining of Rad51 in ovarian cancer cells expressing or not BRCA1 as determined by western blot. Cells were treated with 0.5ug/ml cisplatin for 1 hour, allowed to recover for 6 hours, and then fixed for immunofluorescence staining as described in the materials & methods section. Magnification 100X. **B, D, F)** Quantification of the percentage of cells with positive Rad51 foci. Cells with > 5 RAD51 foci / nucleus were considered positive. Results represent the average of three independent experiments *p<0.05.

Increased sensitivity of ovarian cancer cells bearing HR deficiency to an IGF-1Rki.

Using the above described cellular models, we sought to evaluate the sensitivity of these cells to an IGF-1Rki, using survival assays. In all the cells tested, we observed that cells with low or absent *BRCA1* protein with a concurrent deficient HR function, are more sensitive to IGF-1Rki (fig.3A). This is shown by a lower LC50 of IGF-1Rki in HR deficient primary cells as compared to cells capable of forming RAD51 foci (fig.3B). We next evaluated if a correlation existed between the LC50 of IGF-1Rki and the HR cellular function. Based on the RAD51 foci formation data, we plotted the LC50 against the difference between the % of RAD51 foci in cells treated with cisplatin and the % of RAD51 foci in cells without treatment. This x value was used as an indication of the HR functionality for each cell line. As shown in (fig.3C), a tendency towards a

positive correlation was observed between LC50 IGF-1Rki and HR functionality of cells.

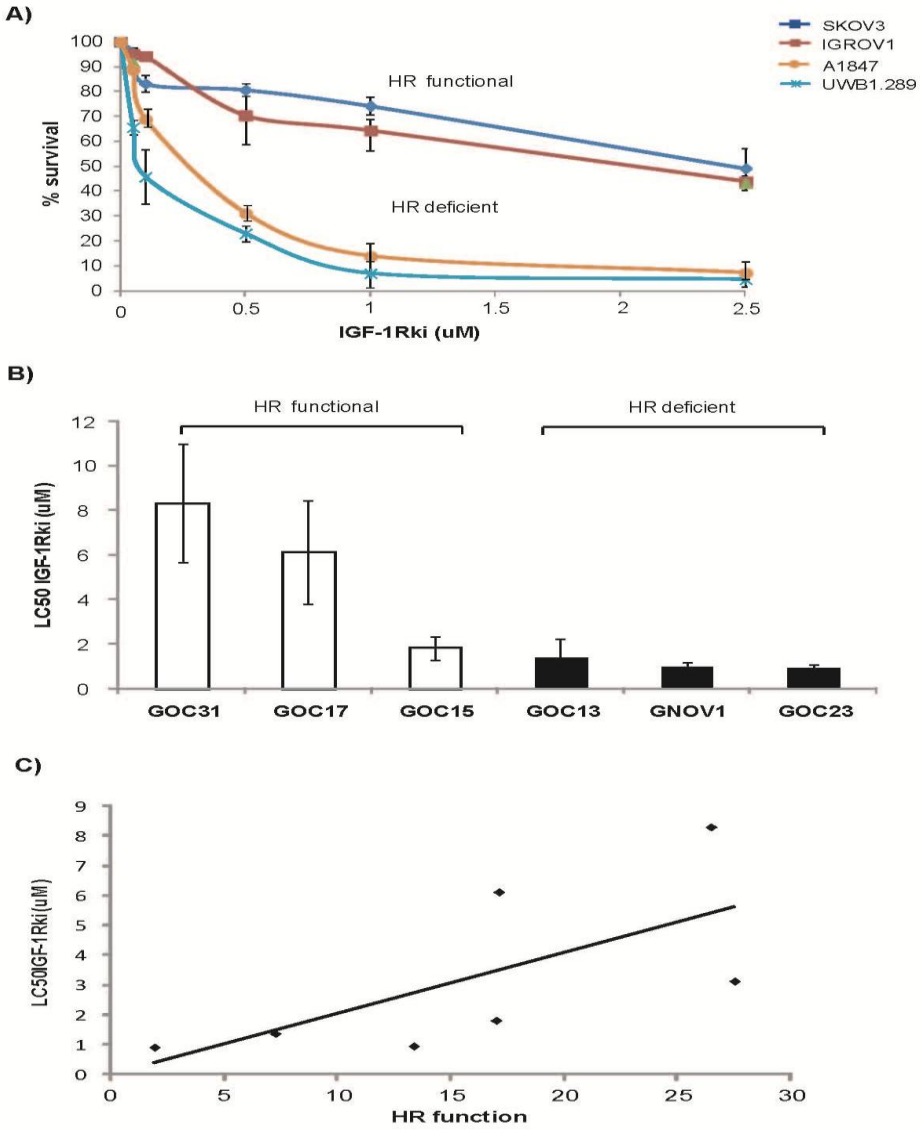

fig. 3: Increased sensitivity of HR deficient cells to IGF-1Rki (BMS-536924).

A) Clonogenic survival assay was performed in the presence of increasing doses of IGF-1Rki in HR deficient or functional cells. Results represent the average of four independent experiments. **B)** LC50 (μM) of IGF-1Rki in primary cells was determined using the Alamar blue survival assay as described in the

materials & methods section (n=3). **C)** Positive correlation was found between the LC50 of IGF-1Rki and the extent HR functionality. Results represent the average ± standard deviation of three independent experiments.

Overactivation of IGF-1R pathway in ovarian cancer cells with nonfunctional HR.

An interaction between the IGF-1R and the HR pathway has previously been suggested [38], therefore, we assessed if ovarian cancer cells with deficient HR express higher levels of downstream proteins of the IGF-1R pathway. As shown in (fig. 4A, B), higher levels of phosphorylated IRS-1, phospho-AKT, and phospho-S6 was observed in cells lacking BRCA1 protein expression.

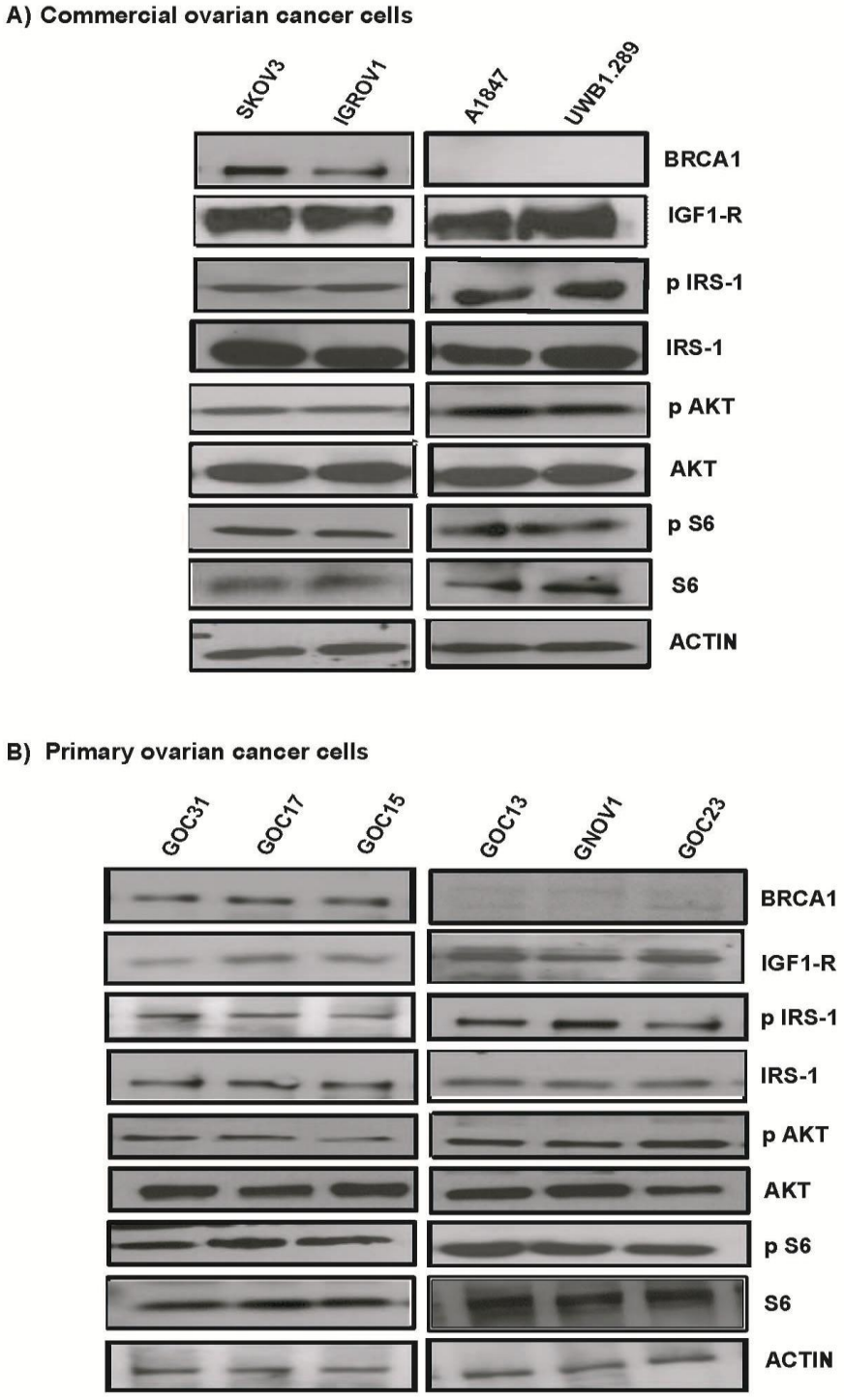

fig. 4: Over activation of the IGF-1 pathway in HR deficient cells.

Cellular lysates from A) ovarian cancer cell lines and B) patients-derived primary cells were subjected to Western blot analysis for the indicated proteins involved in the IGF-1R pathway. One representative blot out of three is shown.

Discussion:

The lifetime risk of ovarian cancer is greatly increased by *BRCA1/2* germline mutations [29,30]. These mutations account for 10-15 % of ovarian cancers overall, and 39% of women who inherit a harmful *BRCA1* mutation will develop ovarian cancer by age 70 years [31].

BRCA1 is a transcription factor involved in numerous cellular processes, including DNA damage repair, cell growth and apoptosis. *BRCA1* has also been shown to directly interact with IGF signaling such that variants in this pathway may modify risk of cancer in women carrying *BRCA* mutations [7]. Transcriptional suppression of the IGF-1R gene by *BRCA1* has also been reported in breast and endometrial cancer [32-34], and loss-of-function mutation of *BRCA1* leads to constitutive activation of the IGF-1R pathway in breast cancer [8-10]. The data presented in this manuscript further support these interactions, demonstrating for the first time enhanced protein levels of IGF-1R, p-IRS1(S636/639), p-AKT(Srr473) , p-S6 (Ser240/244) in *BRCA1* deficient ovarian cancer cells, and

suggesting an association between *BRCA1* mutations and IGF-1 pathway activation in ovarian cancer.

Consistent with the major role of the IGF-1 pathway in tumor cell proliferation [35,39] and the association of *BRCA1* and IGF-1R, we found a positive correlation between the LC50 of the IGF-1Rki and the HR functionality of ovarian cancer cells, suggesting that HR deficient ovarian cancer cells (mutated / methylated BRCA1) are more sensitive to IGF-IRki.

Despite the strong rationale around IGF-1R pathway inhibition, the promising preclinical data and its well tolerability, the clinical efficacy has been disappointing. Our study shows that cells with HR deficiency are more sensitive to IGF-1Rki. HR functionality can be assessed in ascites and tumor biopsy samples by RAD51 ionizing radiation induced foci assay, and represent a potentially useful way of functional diagnostic testing to identify patients with HR deficiency [26,28]. These patients harboring tumors with HR deficiency could then be selected to be treated with IGF-1Rki therapy, with a higher likelihood of responding.

Conclusion

Based on the published literature, this study is the first to evaluate the sensitivity of IGF-1Rki in ovarian cancer cells based on their HR functionality.

This study may offer a basis for further pre-clinical work involving IGF-1Rki in cancer patients, providing new opportunities for the development of targeted personalized cancer therapy.

List of abbreviations

BRCA1/2: Breast cancer 1/2, HR : homologous recombination, IGF-1: Insulin-like growth factor 1, IGF-1R: Insulin-like growth factor 1 receptor , IGF-1Rki: Insulin-like growth factor 1 receptor kinase inhibitor.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

OA carried out most of the experiments, analyzed the data, and drafted the manuscript. MCB did transfection studies. PN participated in western blot experiments. IL confirmed the diagnosis of patients included in the study. SI performed survival assays. AY isolated primary cells from tumor samples. MCB, AY and WHG designed and supervised the study and the writing in the manuscript.

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CHAPTER 3:

Supplementary Results

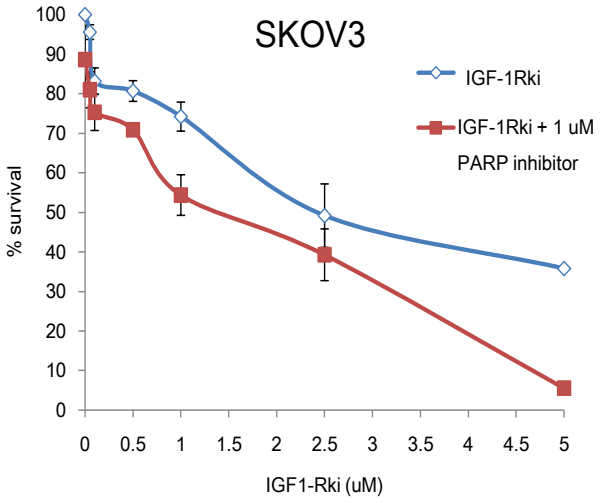
Combination Treatment of IGF-1Rki and PARP inhibitors.

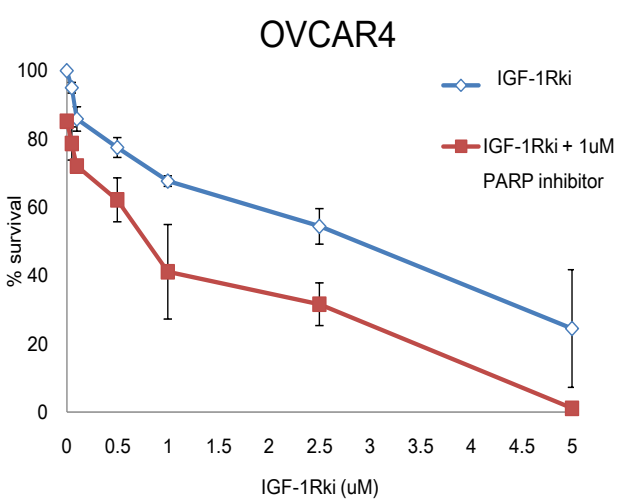
IGF-1R inhibition has suppressive effect on HR DNA repair [59], and we provide evidence in this work that IGF-1R inhibition by IGF-1Rki show increased cell death in HR deficient cells. Further, it was also reported that HR deficient cells are sensitive to inhibition of PARP enzymatic activity by PARP inhibitors [131]. Here in our study, we sought to evaluate the effect of impacting HR by IGF-1Rki and then combining IGF-1Rki with PARP inhibitors. We hypothesized that this interaction might lead to further synthetic lethality.

Using SKOV3 and OVCAR4 cells, we treated the cells with increasing doses (0.01-5μM) of IGF-1Rki (BMS-536924) and 1uM of PARP inhibitor (olaparib), alone and in combination. We found decreased % of survival of cells with combination treatment as compared to IGF-1Rki and PARP inhibitor alone. Further, to determine the nature of the interaction between IGF-1Rki and PARP inhibitor we used the multiple drug effects analysis method of Chou and Talalay [135]. This method quantitatively describes the interaction between two or more

drugs, with values less than 1 indicating synergistic interactions, values greater than 1 indicating antagonistic interactions, and values equal to 1 indicating additive interactions. In both cell lines tested, we observed synergy (Fig.S1). Our results provide the basis for further pre-clinical studies that may lead to improved personalized therapy using PARP inhibitors in cancer patients.

Fig. S1





SKOV3	R	I
1 uM (olaparib)	1.6667	0.8

OVCAR4	R	I
1 uM (olaparib)	10.545	0.2948

R	I
>1 SYNERGY	>1 antagonistic
1 - ADDITIVITY	1 - ADDITIVITY
1 <ANTAGONIST	< 1 synergistic

Fig. S1: Combination treatment of IGF-1Rki and PARP inhibitor in ovarian cancer cells. We have found decreased survival in SKOV3 and OVCAR4 cells in a clonogenic assay after combination treatment with IGF-1Rki and PARP inhibitor. The combination index revealed synergy based on the following formula: R= LC50 (d1) / LC50(d1+d2) ; I = [LC50(dI+d2) / LC50(d1)] + [(d2)/ LC50 (d2)] (d1 is IGF1-Rki and d2 is PARP inhibitor)

Effect of IGF-1Rki on HR DNA repair pathway

Our preliminary data and recently reported studies emphasize the role of IGF-1R inhibition in suppressing HR repair, but the mechanism is not fully understood. In the present study, we evaluate the effect of IGF-1Rki (BMS-536924) on HR both at protein and mRNA level.

a) Rad51 protein expression

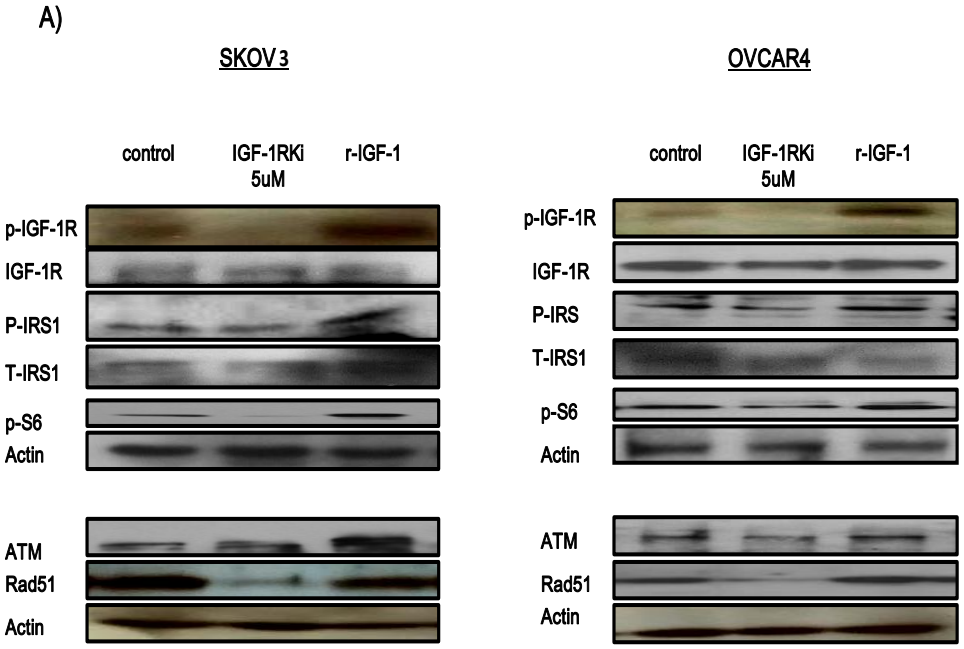
To determine the effect of IGF-1Rki on RAD51, an HR pathway protein, we treated SKOV3 and OVCAR4 cells with 5μM IGF1-Rki and 150ng r-IGF-1 for 24hrs. We observed a reduction of RAD51 protein expression by western analysis with IGF-1Rki, and an increase following r-IGF1 (Fig.S2A). Using IGF-1Rki, we also observed reduced protein expression of phosphorylated proteins of the IGF-1R pathway (p-IGF-1R, p-IRS1, p-

S6). The immunofluorescence RAD51 foci formation assay represents a useful method to test HR functionality [136, 137] We treated cells with 5uM IGF-1Rki for 24 hr followed by 1hr 1ug/ml cisplatin treatment, and 6hr recovery, and observed the reduction of RAD51 foci formation following IGF-1Rki in both cell lines (Fig S2B).

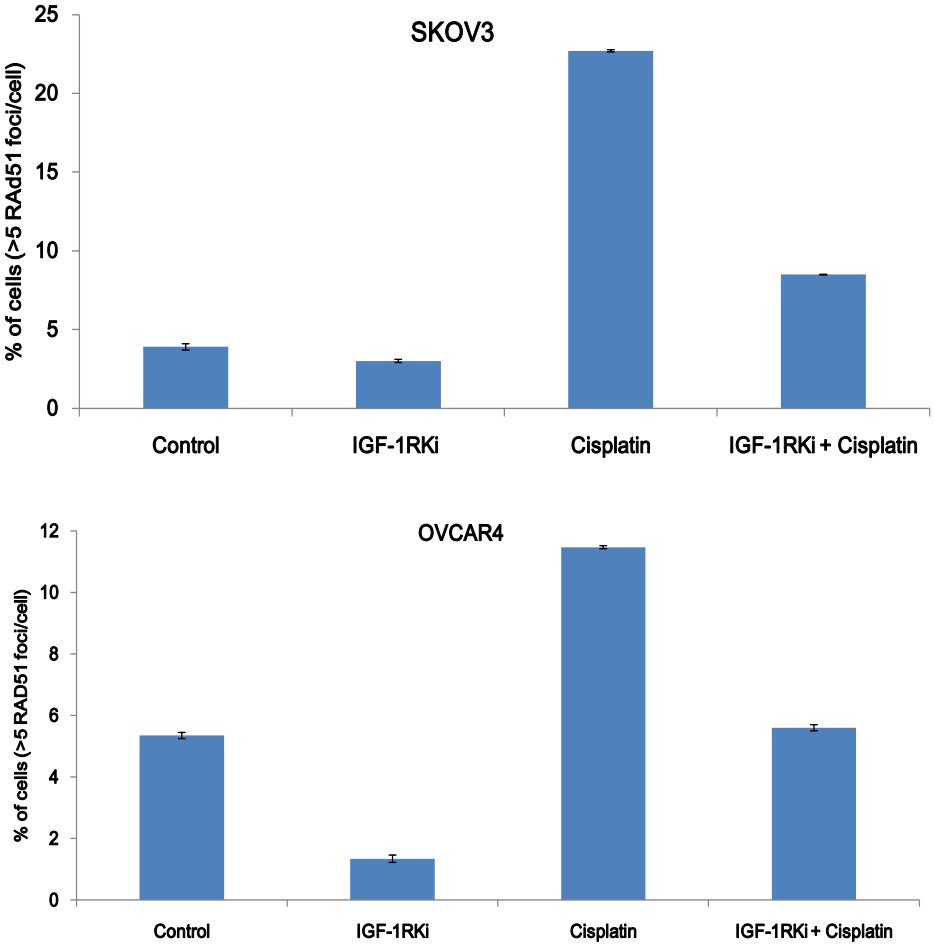
b) RAD51 mRNA expression

Next, we sought to evaluate the effect of IGF-1Rki on mRNA levels of RAD51 using qPCR at various times (12hr-24hr) and doses (1-5uM). r-IGF-1 (150ng) was used as a control. RAD51mRNA reduction was observed at 16 hr following IGF-1Rki (Fig S2C). Dose dependency was assessed at this timepoint, showing a reduction of RAD51 in function of increasing doses (Fig S2D).

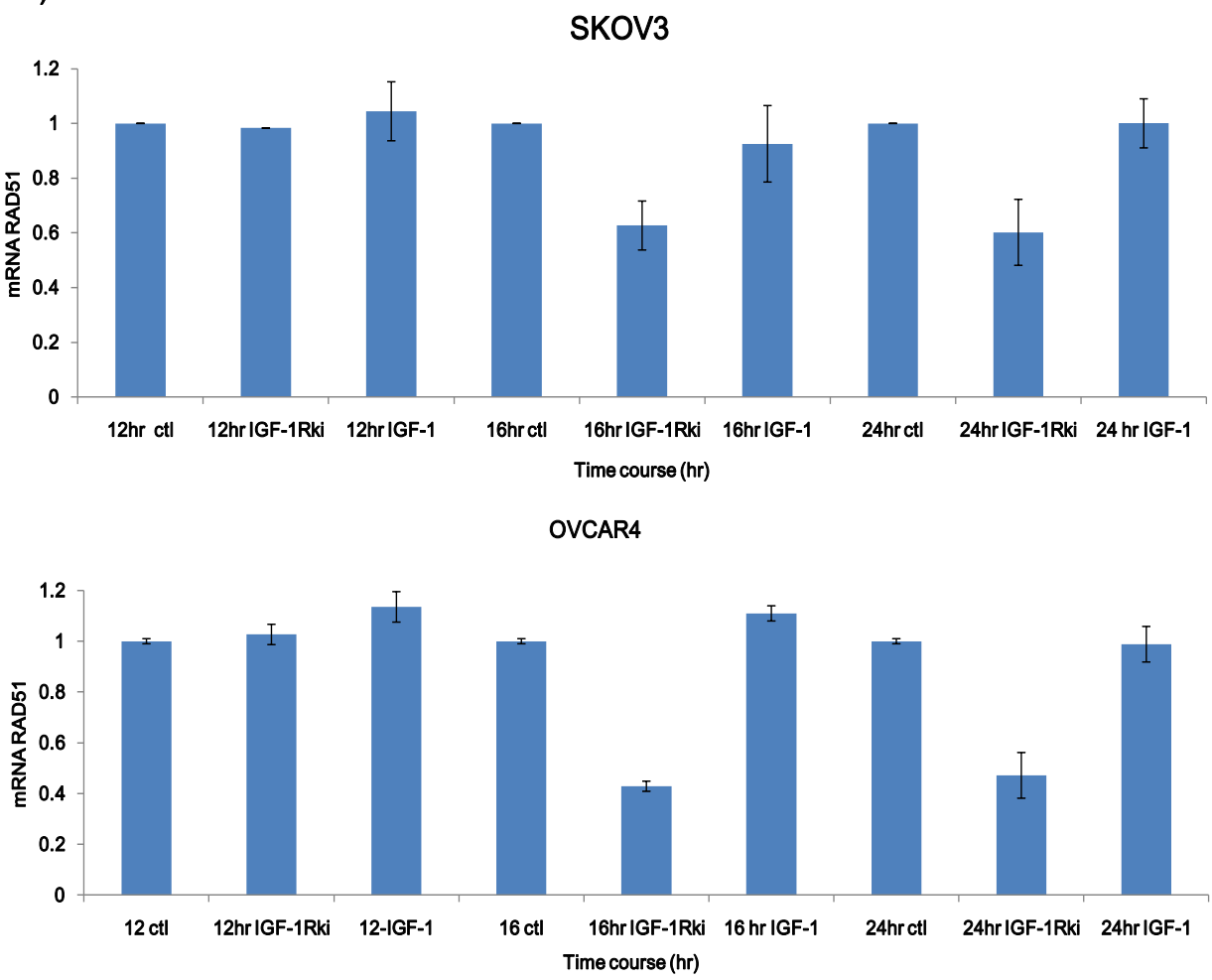
Fig.S2


A)
SKOV3
OVCAR4
control
IGF-1RKi 5uM
r-IGF-1
p-IGF-1R
IGF-1R
P-IRS1
T-IRS1
p-S6
Actin
ATM
Rad51
Actin
control
IGF-1RKi 5uM
r-IGF-1
p-IGF-1R
IGF-1R
P-IRS
T-IRS1
p-S6
Actin
ATM
Rad51
Actin

B)


SKOV3
% of cells (>5 RAd51 foci/cell)
25
20
15
10
5
0
Control
IGF-1RKi
Cisplatin
IGF-1RKi + Cisplatin
OVCAR4
% of cells (>5 RAD51 foci/cell)
12
10
8
6
4
2
0
Control
IGF-1RKi
Cisplatin
IGF-1RKi + Cisplatin

C)


SKOV3
mRNA RAD51
1.2
1
0.8
0.6
0.4
0.2
0
12hr ctl
12hr IGF-1Rki
12hr IGF-1
16hr ctl
16hr IGF-1Rki
16hr IGF-1
24hr ctl
24hr IGF-1Rki
24 hr IGF-1
Time course (hr)
OVCAR4
mRNA RAD51
1.2
1
0.8
0.6
0.4
0.2
0
12 ctl
12hr IGF-1Rki
12-IGF-1
16 ctl
16hr IGF-1Rki
16 hr IGF-1
24hr ctl
24hr IGF-1Rki
24hr IGF-1
Time course (hr)

D)

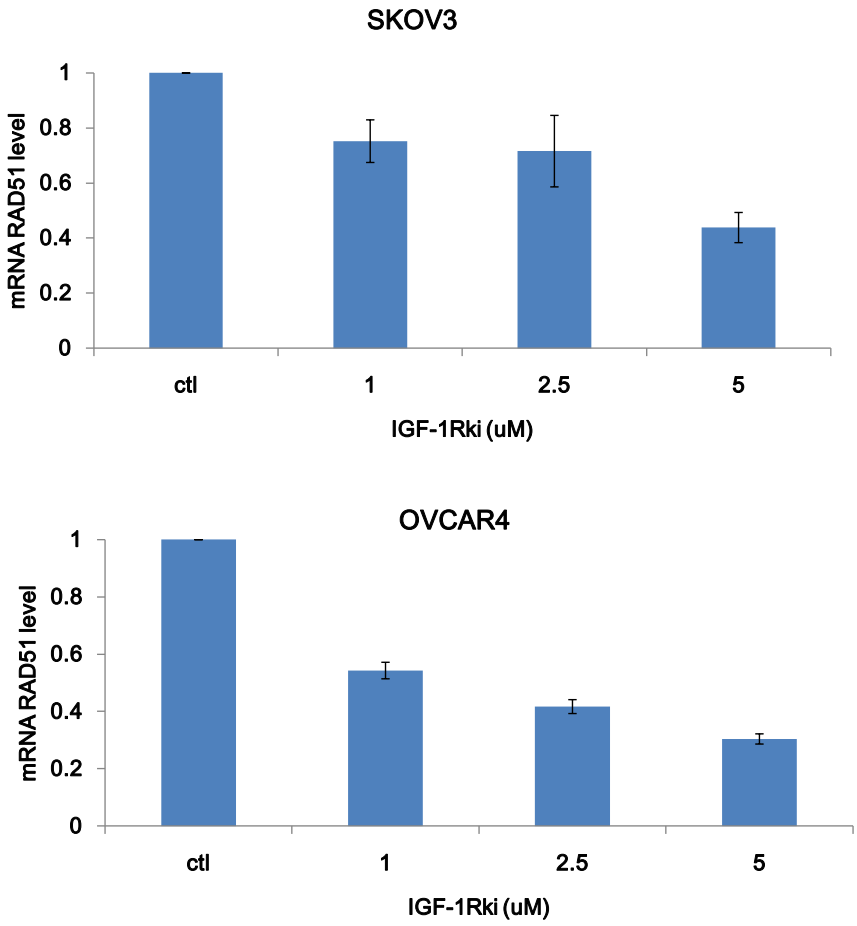


Fig. S2: IGF-1R inhibition decreased the RAD51 both at protein and mRNA level.

A) Treatment of SKOV3 and OVCAR4 cells with IGF-1Rki caused decreased protein expression of RAD51 and the phosphorylated proteins of HR and IGF-1R pathway, whereas r-IGF-1 increased expression of these proteins. **B)** Reduction of RAD51 foci formation using immunofloresence was observed in both cell lines, treated with IGF-1Rki for 24 hrs followed by 1ug/ml cisplatin for 1 hr (cells with >5 foci/nucleus were considered positive), n=3. **C)** and **D)** Decreased

expression of mRNA RAD51 was observed in cells treated with IGF-1Rki at 16 hr in a dose dependent manner. (n=3)

Conclusion and Future Directions

The present thesis investigated the potential interactions between the HR DNA repair and the IGF-1 pathways. Although IGF-1R is almost ubiquitously expressed by human tumors, sensitivity to anti-IGF-1R therapy varies widely between patients and cancer types. Here, we report that there is increased sensitivity of IGF-1Rki in HR deficient ovarian cancer cells, which is consistent with the findings of over activation of IGF-1R and its downstream pathway in HR deficient cells. Moreover, a functional diagnostic test to assess HR functionality in ascites and tumor biopsy samples by RAD51 ionizing radiation induced foci assay can be used to identify patients with HR deficiency [136, 137]. These patients, harboring tumors with HR deficiency, could then be selected to be treated with IGF-1Rki therapy, with a higher likelihood of responding.

Further, data are emerging showing that IGF-1Rki has a role in HR DNA repair pathway but still the mechanism through which IGF-1Rki has an effect on HR DNA repair pathway, is not fully known. Previously, it was reported that IRS-1 binds to Rad51 in the cytoplasm. Upon activation by ligand binding to the IGF-1R, IRS-1 is phosphorylated and releases RAD51, which translocates to the nucleus and participates in the HR. [61]. Here, we have reported that IGF-1Rki affects HR by reducing the expression of RAD51 at the mRNA and protein

level, adding to our understanding of the complex interactions at molecular levels between the IGF-1R and HR DNA repair pathway. Furthermore, preliminary data presented in this thesis demonstrates the synergistic effect of combining IGF-1RKi and PARP inhibitors in cancer cells. These findings serve as the basis for future preclinical studies which may lead to improved wider clinical applications of both IGF-1Rki and PARP inhibitors.

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