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ORIGINAL PAPER

PARP inhibition induces BAX/BAK-independent synthetic lethality of BRCA1-deficient non-small cell lung cancer

Ian Paul,[1] Kienan I Savage,[1] Jaine K Blayney,[2] Elisabeth Lamers,[1] Kathy Gately,[4] Keith Kerr,[6] Michael Sheaff,[5] Kenneth Arthur,[3] Derek J Richard,[7] Peter W Hamilton,[1] Jacqueline A James,[1] Kenneth J O'Byrne,[2] D Paul Harkin,[1] Jennifer E Quinn[1]* and Dean A Fennell[1,2]*

[1] *Centre for Cancer Research and Cell Biology, Queen's University Belfast, UK*
[2] *Centre for Biomedical Informatics, Queen's University Belfast, UK*
[3] *Tissue Core Technology Unit, Queen's University Belfast, UK*
[4] *Thoracic Oncology Research Group, St James' Hospital, Dublin, Ireland*
[5] *Department of Pathology, Barts and the London NHS Trust, London, UK*
[6] *Department of Pathology, University of Aberdeen, UK*
[7] *Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia*

**Correspondence to: Dean A Fennell and Jennifer E. Quinn, Queens University Belfast, Centre for Cancer Research and Cell Biology, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK. e-mail: d.fennell@qub.ac.uk; j.e.quinn@qub.ac.uk*

Abstract

Evasion of apoptosis contributes to both tumourigenesis and drug resistance in non-small cell lung carcinoma (NSCLC). The pro-apoptotic BCL-2 family proteins BAX and BAK are critical regulators of mitochondrial apoptosis. New strategies for targeting NSCLC in a mitochondria-independent manner should bypass this common mechanism of apoptosis block. *BRCA1* mutation frequency in lung cancer is low; however, decreased *BRCA1* mRNA and protein expression levels have been reported in a significant proportion of lung adenocarcinomas. *BRCA1* mutation/deficiency confers a defect in homologous recombination DNA repair that has been exploited by synthetic lethality through inhibition of PARP (PARPi) in breast and ovarian cells; however, it is not known whether this same synthetic lethal mechanism exists in NSCLC cells. Additionally, it is unknown whether the mitochondrial apoptotic pathway is required for BRCA1/PARPi-mediated synthetic lethality. Here we demonstrate that silencing of BRCA1 expression by RNA interference sensitizes NSCLC cells to PARP inhibition. Importantly, this sensitivity was not attenuated in cells harbouring mitochondrial apoptosis block induced by co-depletion of BAX and BAK. Furthermore, we demonstrate that BRCA1 inhibition cannot override platinum resistance, which is often mediated by loss of mitochondrial apoptosis signalling, but can still sensitize to PARP inhibition. Finally we demonstrate the existence of a *BRCA1*-deficient subgroup (11–19%) of NSCLC patients by analysing BRCA1 protein levels using immunohistochemistry in two independent primary NSCLC cohorts. Taken together, the existence of *BRCA1*-immunodeficient NSCLC suggests that this molecular subgroup could be effectively targeted by PARP inhibitors in the clinic and that PARP inhibitors could be used for the treatment of *BRCA1*-immunodeficient, platinum-resistant tumours.

Keywords: apoptosis; BAX; BAK; non-small cell lung cancer; BRCA1; predictive biomarker; PARP inhibitor; synthetic lethality

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Introduction

Synthetic lethality represents a novel therapeutic strategy in the treatment of cancer, as exemplified by the recent advent of poly(ADP-ribose) polymerase (PARP) inhibition in *BRCA1/2*-mutated tumours. Inhibition of PARP, a single-strand (ssDNA) break-repair protein, leads to an accumulation of DNA double-strand breaks, specifically in S-phase cells. Normally these breaks are repaired via homologous recombination (HR), a process requiring functional *BRCA1/2* [1]. However, the dual loss of both PARP-mediated ssDNA repair upon PARP inhibition and defective HR, in *BRCA*-deficient cells, results in the accumulation of DNA damage and ultimately cell death [2–4]. Currently it is unknown whether synthetic lethality due to inhibition of PARP-mediated DNA repair in HR-defective tumours requires mitochondrial apoptosis. The underlying mechanisms involved in cell killing, involving the accumulation of catastrophic DNA damage, would be predicted to induce cell death independently of the canonical mitochondrial death pathway. However, this hypothesis has not yet been fully investigated.

In contrast to breast and ovarian cancer, the mutation frequency of *BRCA1* in non-small cell lung cancer (NSCLC) is low [5]. However, reduced *BRCA1* mRNA and protein expression levels have been reported in both sporadic breast and ovarian cancer, indicating that these tumours may exhibit a 'BRCAness' phenotype [6]. Similarly, reduced *BRCA1* mRNA and protein expression levels are observed in up to 44% of NSCLC adenocarcinomas through a number of mechanisms, such as promoter hypermethylation, and altered mechanisms of transcriptional regulation, such as hypoxia-induced E2F-mediated BRCA1 repression [7–9].

Taron and colleagues [10] were the first to report that NSCLC patients with reduced *BRCA1* mRNA expression levels derived a significantly greater overall survival benefit following platinum-based chemotherapy in comparison to patients with high *BRCA1*. Similarly, low levels of *BRCA1* mRNA expression were correlated with prolonged survival in ovarian cancer [11]. Indeed, the correlation between *BRCA1* mRNA expression and clinical benefit following platinum chemotherapy in these retrospective studies has underpinned attempts to customize chemotherapy in NSCLC based on *BRCA1* mRNA expression levels [12,13].

More recently, absent/low BRCA1 protein expression, measured by immunohistochemistry, has been reported to predict for clinical outcome following chemotherapy in patients with ovarian cancer [14]. In support of this, multiple *in vitro* studies provide evidence that BRCA1-deficient breast and ovarian cancer cells lines display enhanced sensitivity to platinum agents [11,15]. Currently, there is also significant preclinical evidence to suggest that BRCA1-deficiency results in enhanced response to PARP inhibitors, such as olaparib [2,3,16]. A recent phase I clinical trial has reported significant anti-tumour activity in *BRCA1* germline mutation carriers following treatment with PARP inhibitor [4]. Phase II studies have provided positive proof of concept of the efficacy and tolerability of olaparib in *BRCA*-mutated advanced ovarian cancer and *BRCA1*/*BRCA2*-mutated breast cancer [17,18]. The preclinical efficacy of PARP inhibitors in NSCLC has yet to be investigated.

Defects in apoptosis underpin drug resistance and is a hallmark of cancer [19,20]. Mitochondria are key regulators of apoptosis, with the multidomain proapoptotic BCL2 family members, BAX and BAK, being essential for the mitochondrial apoptosis pathway to function. Knockout of BAX and BAK results in multidrug resistance, highlighting their essential role in apoptosis [21]. Of significance, the pro-survival proteins BCl-X and MCL-1 are amongst the most commonly amplified in the cancer genome and provide another mechanism through which tumours can acquire defects in apoptosis [22].

Overcoming resistance to chemotherapy in NSCLC is one of the major issues in the management of NSCLC patients. Therefore, developing therapeutic strategies that bypass the mitochondrial apoptosis pathway could help to overcome drug resistance, representing a major advance in lung cancer therapy. In the current study, we demonstrate that *BRCA1*-silencing in NSCLC cells results in synthetic lethality following treatment with the PARP inhibitor, olaparib. We also demonstrate that this mode of cell death is independent of the mitochondrial apoptosis pathway. Additionally, we demonstrate that siRNA-mediated depletion of *BRCA1* in platinum-resistant NSCLC cell lines results in enhanced sensitivity to PARP inhibition. Finally, we identify a significant subgroup of *BRCA1*-immunodeficient NSCLC patients who display reduced or absent BRCA1 protein expression.

Materials and methods

Cell culture

ATCC NCI-H1975 and NCI-H460 NSCLC cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml penicillin–streptomycin (Life Technology Inc). NCI-H157 NSCLC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1 mM sodium pyruvate and 50 μg/ml penicillin–streptomycin. All cell lines were maintained in a 5% CO_2 incubator at 37 °C.

Generation of BAX- and BAK-silenced NSCLC cell lines

Plasmids encoding shRNA sequences to BAX, BAK and non-targeting (NT) shRNA (SA Biosciences) were transfected into H460 NSCLC cells, using FuGene 6 reagent (Roche) according to the manufacturer's protocol. BAX plasmids with G418 antibiotic resistance selection marker, BAK plasmids with puromycin antibiotic resistance selection marker and separate NT plasmids bearing either G418 or puromycin antibiotic resistance selection markers were used. The cells were transfected in six-well plates and incubated with either 0.4 mg/ml G418 (Sigma Aldrich) or 2 μg/ml puromycin (Calbiochem). Discrete colonies were selected and screened by western blotting. Cells stably expressing shRNA targeting BAX were then retransfected with shRNA, targeting BAK to generate clones expressing stable knockdown of both BAX and BAK.

siRNA transfections

Cells were reversely transfected with 15 nM of either a BRCA1-siRNA oligonucleotide sequence or a scrambled control siRNA. All siRNA transfections were performed using Lipofectamine RNAi Max reagent (Invitrogen) according to the manufacturer's instructions. Two independent BRCA1 siRNA oligonucleotides

were used in all experiments. BRCA1-siRNA1, as previously published [23] and BRCA1-siRNA2 were purchased from Qiagen (BRCA1_15). Clonogenic assays and western blotting were then performed as described below.

BRCA1 over-expression

A549 cells were transfected with a flag-tagged BRCA1 construct (Fl4-BRCA1), a kind gift from Dr Richard Baer, Institute for Cancer Genetics and Department of Pathology, Columbia University, using electroporation with the Nucleofector Kit T (Amaxa Biosystems, MD, USA) according to the manufacturer's protocol. Clonogenic assays and western blotting were then performed as described below.

Western blotting

H157, H460 or H1975 cells were scraped into medium and pelleted by centrifugation at 1500 rcf relative centrifugal force at 4 °C for 5 min. Whole-cell lysates were obtained by snap-freezing cells in RIPA lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X, 0.1% SDS and 1 mini-complete protease inhibitor tablet/10 ml (Roche Diagnostics, Indianapolis, IN, USA)]. Cell or mitochondrial lysate (25 μg) was loaded and resolved by SDS–PAGE gel, as previously described [24]. The primary antibodies used were: anti-BRCA1 AB-1 (MS110; Calbiochem); anti-α tubulin (Abcam, Cambridge, MA, USA); anti-SMAC and anti-cytochrome *c* (Calbiochem); and anti-BAX, anti-BAK, anti-cleaved caspase-9 (Cell Signalling Technology, Danvers, MA, USA). The secondary antibodies used were goat anti-mouse HRP (DAKO P0447) and goat anti-rabbit HRP (DAKO P0448).

Mitochondrial isolation

Mitochondria were isolated from H460 stably expressing shRNA to BAX and BAK and treated with peptides indicated in the text, as described previously [24].

Caspase 3/7 activity assay

The Caspase-Glo 3/7 assay was obtained from Promega (Southampton, UK) and was performed according to the manufacturer's instructions.

Clonogenic assays

Transiently transfected H460, H1975 and H157 cells were trypsinized 48 h post-transfection and seeded in six-well plates at a density of 2000 cells/well. Following transfection with either BRCA1 or scrambled-control siRNA oligonucleotides, cells plates were treated 24 h later with serial concentrations of the commercially available PARP inhibitor Olaparib-AZD2281 (Axon Medchem). The cells were allowed to grow for approximately 8–10 days.

DNA damage repair assays

Cells transfected with control and BRCA1 siRNAs were plated onto glass coverslips 48 h post-transfection and allowed to attach for 24 h. The cells were then treated with 1 μM PARPi (Olaparib-AZD2281) for 12 h. Following the removal of PARPi, the cells were washed thoroughly in fresh medium and allowed to recover in fresh medium for 12 h to allow DNA repair to occur. The cells were fixed in 4% paraformaldehyde/PBS and then permeabilized in 0.2% Triton X-100/PBS, then blocked in 3% BSA/PBS and stained with α-γ-H2AX (Millipore 05–636) diluted 1 : 10 000 in 3% BSA/PBS. Following several PBS washes, the cells were stained with anti-mouse AlexaFluor 486 (Invitrogen). Coverslips were then mounted with Vectashield containing DAPI (Vector Labs) and the γ-H2AX foci were counted and imaged using an epi-fluorescence microscope.

Flow cytometry

Cells were seeded 48 h post-transfection with siRNA, as described, at a density of 1×10^5 cells/well in six-well plates. The cells were treated after 24 h and allowed to grow for a further 48 h. Following treatment with cisplatin, DNA content was evaluated by propidium iodide staining of the cells, using a FacScaliber Flow Cytometer (Becton Dickinson), in the FL-2 channel to determine the percentage of cells with DNA content < 2 N.

Tumour samples

BRCA1 protein expression levels on human tissues were assessed using immunohistochemistry (IHC). Two tissue microarrays (TMAs) were constructed, consisting of two independent cohorts of surgically-resected primary NSCLC tissue blocks with associated pathological evaluation and clinical follow-up. A total of 302 tumours were examined, with 113 tumours on the Belfast TMA and 189 tumours on the Dublin TMA. Appropriate ethical approval was obtained from the local research ethics committees to carry out this work.

BRCA1 immunohistochemistry (IHC)

TMA sections were deparaffinized by sequential incubations in xylene, followed by rehydration in ethanol. Endogenous peroxidise activity was blocked by incubating with 3% hydrogen peroxide solution for 10 min. Antigen retrieval was achieved by incubating with TRIS–EDTA buffer, pH 9.0, in a pressure cooker for 3 min at full steam. The slides were washed, followed by application of the primary antibody, BRCA1 Ab-1 (MS110; Calbiochem) at a 1 : 200 dilution in TRIS-buffered saline (TBS), pH 7.4, and incubation overnight at 4 °C. This clone has been reported previously to be highly sensitive and specific for the detection of BRCA1 in FFPE tissue using IHC [25,26]. Following TBS washes, an anti-mouse horseradish peroxidase-conjugated secondary antibody

was applied (DAKO EnVision HRP labelled polymer) for 30 min. The sections were washed in TBS and 3,3′-diaminobenzidine (DAKO), applied to detect primary antibody binding. The reaction was observed until stained to satisfaction and the slides were washed using distilled water. The slides were counterstained with Mayer's haematoxylin for 30 s, then ammonia water, before serial incubations in ethanol and xylene. All BRCA1 immunohistochemistry was performed within the Tissue Core Technology Unit at the Centre for Cancer Research and Cell Biology at Queen's University Belfast.

BRCA1 scoring

The BRCA1 IHC scoring of TMA slides was carried out using the PathXL™ TMA Toolbox (i-Path Diagnostics, UK), where pathologists could remotely access on-line, high-resolution whole-slide scans of the TMA, step through each core and keep track of progress using TMA navigation software, and visually score tissue IHC positivity using an on-screen interface, where these were stored centrally for review and analysis. Scoring of each TMA core was performed independently by two lung pathologists. BRCA1 scoring was determined using a binary approach and based on two previous reports [14,27]. Specifically, *BRCA1*-immunodeficent NSCLC tumours were classified as demonstrating either absent or very low levels of BRCA1 expression, with $< 10\%$ of cells exhibiting nuclear BRCA1 staining. However, *BRCA1*-immunopositive tumours were defined as those demonstrating $> 20\%$ of cells with nuclear staining. Only when the pathologists reached a consensus on IHC scores were they used. Where a consensus agreement could not be reached, patients were labelled 'no consensus'.

BRCA1 IHC scores were combined with associated clinicopathological and survival data for further analysis. Progression-free survival analysis was carried out using Kaplan–Meier/log-rank test pairwise comparison testing; $p < 0.05$ was deemed significant. Statistical analysis GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com) was used for all statistical analyses.

Results

PARP inhibition mediates synthetic lethality in BRCA1-silenced NSCLC

Previous preclinical reports have indicated that reduced BRCA1 expression confers sensitivity to cisplatin and other DNA-damaging agents [15]. We therefore investigated whether siRNA-mediated depletion of BRCA1 conferred a similar phenotype in NSCLC cell lines. Accordingly, we confirmed abrogation of BRCA1 expression using siRNA and correlated this with a significant increase in cisplatin-induced apoptosis in both the H460 (SC 9.2%, siBRCA1 17.6%) and H157 (SC 2.6%, siBRCA1 6.9%) NSCLC cell lines (Figure 1A).

We then proceeded to correlate BRCA1 expression with response to PARP inhibition, using clonogenic assays. Subsequently, siRNA-mediated inhibition of *BRCA1* was confirmed and resulted in a significant reduction in colony formation following serial treatments with PARP inhibitor, as compared to scrambled control cells, in all three NSCLC cell lines (Figure 1B). Specifically, H157 NSCLC cells demonstrated almost a log-fold reduction in IC_{50} following *BRCA1*-siRNA silencing (SC 2.07 μM versus BRCA1 0.227 μM). In addition, H1975 cells demonstrated a seven-fold reduction in IC_{50} following *BRCA1*-siRNA (SC 1.39 μM versus BRCA1 0.193 μM) and H460 cells demonstrated a four-fold reduction in IC_{50} following *BRCA1* silencing (SC 1.06 μM versus BRCA1 0.25 μM), as shown in Figure 1B(i–iii).

In breast cancer cells, synthetic lethality of PARP inhibition (PARPi) with *BRCA1* deficiency is due to the combined defect in ssDNA repair and HR-mediated DNA repair, leading to the accumulation of DNA damage. In order to determine whether PARP inhibitor-mediated death is associated with defective DNA repair in NSCLC cells, we examined DNA repair efficiency by monitoring the presence of DNA damage-induced, γ-H2AX foci generated in response to PARP inhibition and 12 h after the removal of PARPi. DNA damage was assessed by counting γ-H2AX foci, which are well-characterized markers of DNA double-strand breaks [28]. PARP inhibition induced DNA damage equally in both control and *BRCA1*-siRNA transfected cells in both the H460 and H157 NSCLC cell lines (Figure 2A, B). In contrast, 12 h after the removal of PARPi, approximately 70% of *BRCA1*-depleted cells retained unrepaired DNA damage, in comparison to control cells, 90% of which had repaired PARPi-induced DNA damage (Figure 2A–D). This suggests that *BRCA1*-siRNA-depleted NSCLC cells are unable to repair PARPi-mediated DNA damage and likely die due to the accumulation of DNA damage.

Following the observed effect of siRNA-mediated *BRCA1* depletion and response to PARPi in NSCLC cell lines, we subsequently performed a rescue strategy using the A549 NSCLC cell line, which expresses relatively low levels of BRCA1. Transient over-expression of BRCA1 in A549 cells resulted in a reduction in response to PARPi as measured by clonogenic assay. Following treatment with an IC_{50} dose of PARPi, A549 cells over-expressing BRCA1 exhibited a statistically significant increase ($p = 0.029$) in the cellular surviving fraction (71.2%) as compared to empty vector control transfected cells (47.8%) (data not shown).

Co-silencing of BAX/BAK results in mitochondrial apoptosis block

We then proceeded to examine whether mitochondrial apoptotic signalling was required for PARPi-induced synthetic lethality in *BRCA1*-siRNA-silenced NSCLC

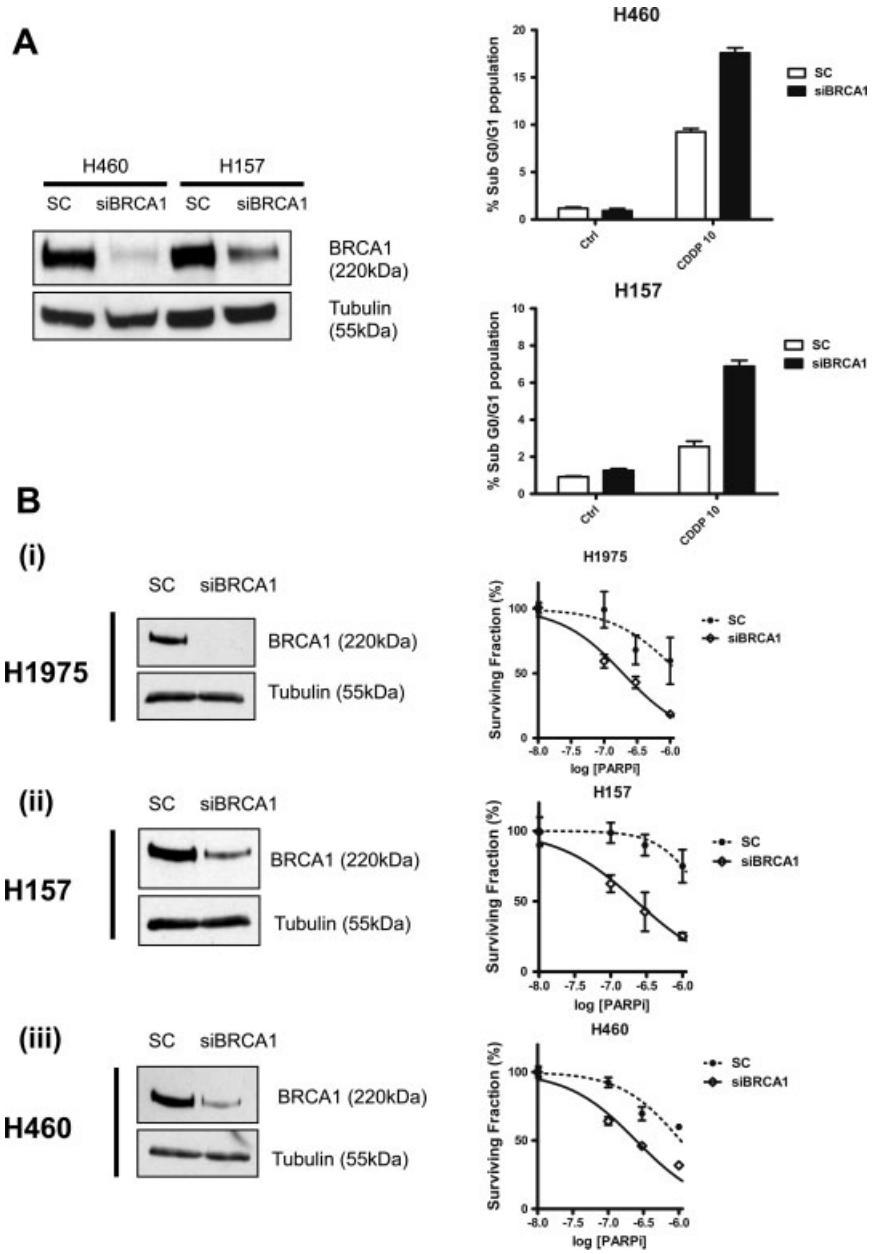

Figure 1. BRCA1 silencing confers sensitivity to cisplatin and synthetic lethality following inhibition of PARP. (A) (left) western blot demonstrating siRNA mediated silencing of BRCA1 in H460 and H157 NSCLC cells, 48 h following transfection with scrambled (SC) or BRCA1 (siBRCA1) targeted siRNA; (right) apoptotic cells, indicated by sub-G_0/G_1 fraction, as assessed by flow cytometry of PI-stained H460 and H157 cells, transfected as above, following 48 h treatment with 10 μM cisplatin. (B) siRNA-mediated silencing of BRCA1 confers sensitivity to PARP inhibition, resulting in a marked reduction in colony formation in H1975, H157 and H460 NSCLC cell lines. Clonogenic survival curves of H1975, H157 and H460 NSCLC cells transfected with scrambled (SC) or BRCA1 (siBRCA1) targeted siRNA after 8–10 days of continuous exposure to PARP inhibitor.

cells. In order to do this, we stably transfected H460 cells with short hairpin RNAs (shRNAs) simultaneously targeting both BAX and BAK ($H460^{shBAX/BAK}$) proteins, which are required for mitochondrial apoptotic signalling. Six clones were generated (sh-BAX/BAK1A–C and sh-BAX/BAK4A, C and F), expressing differing short hairpin RNA sequences targeting BAX and BAK, compared to control cells expressing two non-targeting shRNAs (sh-NTNT) (Figure 3A).

Several tests were performed to confirm the functionality of the mitochondrial apoptotic block in shBAX/BAK cells. Treatment with staurosporine induced strong accumulation of cleaved caspase 9 (p37) in the sh-NTNT clones, but no cleaved caspase 9 appeared in the sh-BAX/BAK clones (Figure 3B). The sh-BAX/BAK clones also exhibited resistance to pro-apoptotic peptides corresponding to the BH3 domain of the pro-apoptotic protein BID ($BH3^{BID}$). Isolated mitochondria from shBAX/BAK clones demonstrated the expected failure to release cytochrome *c* and SMAC into the supernatant fraction following incubation with $BH3^{BID}$ (Figure 3C). Finally, we confirmed the functionality of the sh-BAX/BAK cells by demonstrating significant attenuation in caspase-3 activity in response

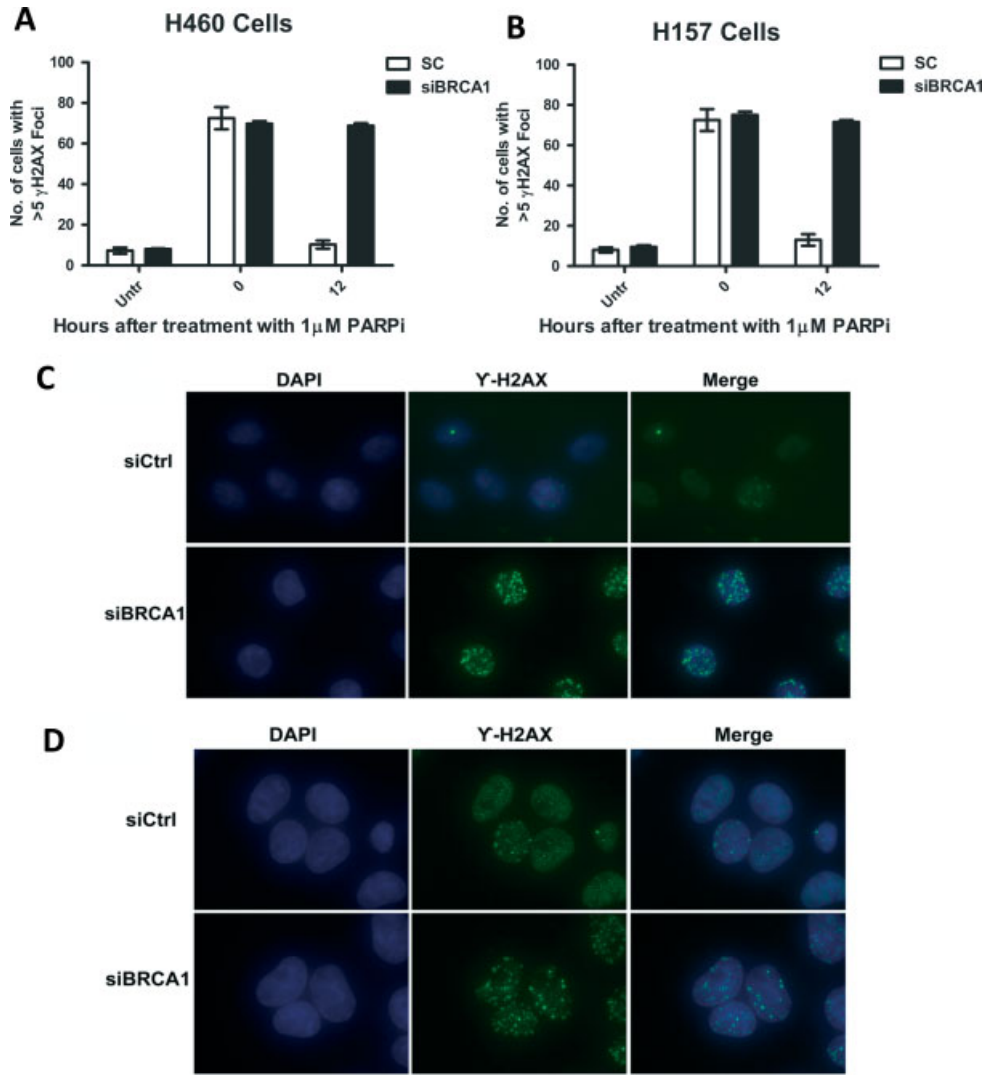

Figure 2. BRCA1 silencing leads to accumulation of PARPi-induced DNA damage. H460 (A) and H157 (B) cells were treated with vehicle (Untr) or 1 μM PARP inhibitor (PARPi) for 12 h to induce DNA damage. PARPi was then removed and the cells were fixed at 0 and 12 h following recovery. DNA damage was assessed by γ-H2AX staining and cells containing more than five DNA damage foci were scored. (C) Representative images of H460 cells stained with γ-H2AX, indicating DNA damage foci remaining 12 h after removal of PARPi inhibitor. (D) Representative images of H157 cells stained with γ-H2AX, indicating DNA damage foci remaining 12 h after removal of PARPi inhibitor.

to treatment with R_8BID, as compared to sh-NTNT cells (Figure 3D).

PARPi-induced synthetic lethality in *BRCA1* NSCLC cells occurs independently of BAX/BAK-mediated mitochondrial apoptosis

Despite the presence of mitochondrial apoptosis block in H460 sh-BAX/BAK clones, siRNA-mediated inhibition of *BRCA1* followed by PARP inhibition significantly reduced colony formation compared to scrambled control cells in all three H460 stably transfected clones, sh-NTNT (IC_{50}: SC, 1.55 μM; BRCA1, 0.33 μM), sh-BAX/BAK1C (IC_{50}: SC, 1.68 μM; BRCA1, 0.37 μM) and sh-BAX/BAK4F (IC_{50}: SC, 1.32 μM; BRCA1, 0.19 μM). These results indicate that PARPi-induced synthetic lethality in *BRCA1*-siRNA-depleted NSCLC cell line models is independent of mitochondrial apoptosis signalling (Figure 4A–D).

PARP inhibitor-induced synthetic lethality bypasses cisplatin resistance

Cisplatin resistance is a major problem in the clinic and is associated with alterations in DNA repair and a block of downstream mitochondrial apoptosis signalling [24,29]. However, due to the finding that *BRCA1* silencing-mediated PARPi sensitivity appears to be independent of the mitochondrial apoptosis pathway, we hypothesized that PARPi synthetic lethality would remain functional in cisplatin-resistant cells. We therefore explored whether NSCLC cells selected for cisplatin resistance, and harbouring defective mitochondrial signalling, would exhibit cross-resistance to PARP inhibitor-induced synthetic lethality mediated by *BRCA1* silencing [24]. Cisplatin-resistant cells were derived from H460 cells as described previously [24] and were used to address this hypothesis.

We confirmed that siRNA-mediated *BRCA1* silencing resulted in sensitization of parental H460 cells

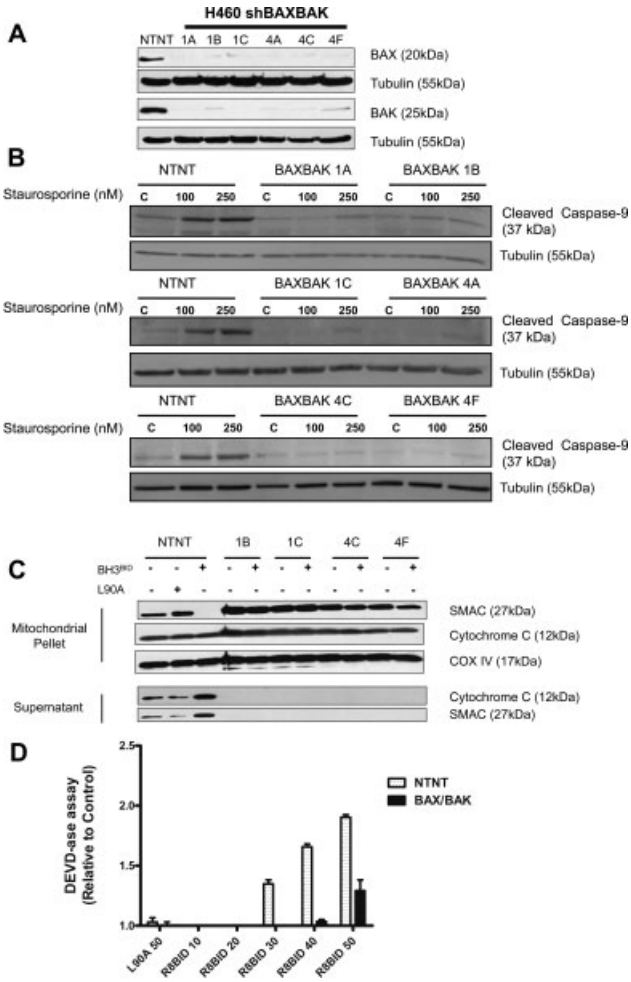

Figure 3. Mitochondrial apoptosis block induced by simultaneous silencing of BAX and BAK. (A) Western blot showing the relative levels of BAX and BAK expression in H460 cells stably expressing targeted shRNAs (sh-BAX/BAK) versus non-targeted control shRNAs (NTNT). (B) Western blot for cleaved caspase 9 in sh-NTNT and sh-BAX/BAK clones following 24 h treatment with 100 nM and 250 nM staurosporine. Caspase 9 cleavage occurs in sh-NTNT but not sh-BAX/BAK clones. (C) Western blot showing SMAC and cytochrome *c* content in the isolated mitochondrial fraction and supernatant following incubation with 30 μM $BH3^{BID}$ for 30 min in H460 sh-BAX/BAK clones compared to non-targeting control (sh-NTNT). SMAC and cytochrome *c* is not released in response to $BH3^{BID}$ in sh-BAX/BAK clones, indicating failure of mitochondrial outer membrane permeabilization (MOMP). (D) Luminescent DEVD-ase assay measures caspase-3-like activity in sh-NTNT and sh-BAX/BAK cells 24 h after R8BID treatment, shown relative to time-matched control for each cell line. The increase in Casp3 activity in the NTNT control is attenuated in sh-BAX/BAK cells.

to platinum, as expected. However, *BRCA1* silencing failed to induce sensitivity to platinum in the resistant H460CISPR cells, as measured by the degree of apoptosis [sub-G_0/G_1 population; Figure 5A(ii)]. In contrast, siRNA-mediated inhibition of *BRCA1* in the H460CISPR cells resulted in significant sensitization following PARP inhibition, similar to that observed for the parental H460 cells (siBRCA1–H460CISPR PARPi $IC_{50} = 0.63$ μM: siBRCA1–H460 PAR PARP $IC_{50} = 0.25$ μM) [Figure 5A(iii)]. Taken together, these data demonstrate that siRNA-mediated abrogation of BRCA1 expression does not restore platinum sensitivity to platinum-resistant cells but does result in enhanced response sensitization to PARP inhibition.

A significant proportion of primary NSCLCs are *BRCA1*-immunodeficient

Our current *in vitro* data indicate that *BRCA1*-deficient NSCLC cell line models are highly sensitive to the PARP inhibitor olaparib. In addition, *BRCA1*-mutated breast cancer patients are reported to respond well to treatment with PARP inhibitors [2–4]. Taken together, these data suggest that *BRCA1*-deficient NSCLC patients may also derive benefit from PARP inhibitor therapy. Currently, there is evidence of the existence of a subgroup of NSCLC patients with reduced *BRCA1* mRNA expression levels who benefit from platinum-based chemotherapy [10]. However, there is currently no method routinely used in the clinic for identifying this subgroup of *BRCA1*-deficient NSCLC patients.

We therefore set out to independently verify this subgroup of NSCLC patients in two separate cohorts, using an IHC-based approach. BRCA1 IHC was conducted on two independent TMAs representing two independent cohorts of primary NSCLC patients. The Belfast cohort represented 113 patients and the Dublin cohort represented 189 patients.

In the Dublin cohort, 19% of primary NSCLC specimens were scored as *BRCA1*-immunodeficient, whereas in the Belfast cohort 11% of patients were scored as *BRCA1*-immunodeficient (Figure 5B, C). In both cohorts, BRCA1-immunodeficiency occurred in both squamous and non-squamous NSCLC. Combining data from both cohorts, BRCA1-immunodeficiency was relatively more frequent in non-squamous tumours, with 26.2% of these tumours classified as BRCA1-immunodeficient compared to only 8.7% of squamous tumours. (Table 1). Loss of BRCA1 expression was not found to be prognostically significant based on analysis of progression-free survival data (not shown).

Discussion

Non-small cell lung cancer is the most prevalent malignancy worldwide and is associated with 27% of cancer deaths in the UK. The rapid lethality of NSCLC is associated with significant *de novo* drug resistance, which has accounted for a therapeutic plateau over the last few decades [30]. In recent years, our understanding of how to treat NSCLC has undergone a paradigm shift, initiated by the identification of somatic activating mutations in the epidermal growth factor receptor (EGFR) [31,32]. Similarly, ELK4–ALK translocation [33] is associated with extreme preclinical sensitivity to respective ALK kinase inhibition in both preclinical and clinical settings. Additional molecular subclasses with associated somatic gene alterations have been discovered, predominantly in lung adenocarcinomas, and include mutations of *BRAF* [34], *HER2*

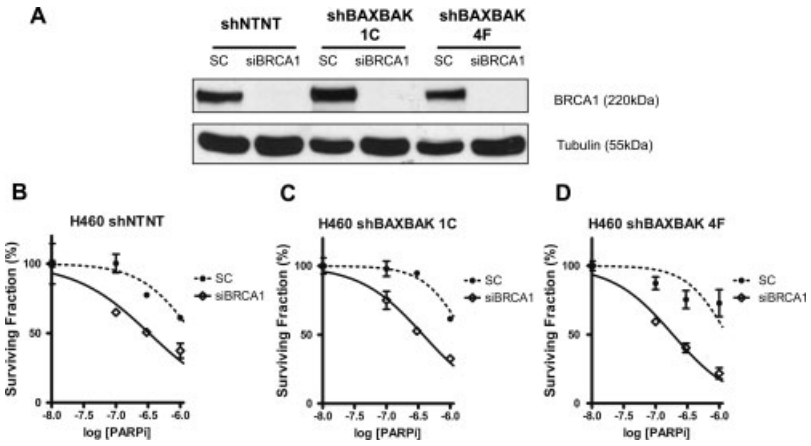

Figure 4. Synthetic lethal killing bypasses the mitochondrial apoptosis block induced by BAX and BAK silencing. (A) Western blot showing siRNA-mediated silencing of *BRCA1* in BAX/BAK-silenced H460 cells. Cells were transfected for 48 h with the indicated siRNA. (B–D) Clonogenic dose response assays for shNTNT, shBAXBAK 1C and shBAXBAK 4F cells transfected with control (SC) and *BRCA1* targeting siRNAs (siBRCA1). PARP inhibition induces an equivalent degree of synthetic lethality, measured by clonogeneic survival, in *BRCA1*-silenced BAX/BAK-depleted cells in comparison to NTNT control cells.

[35] and *PIK3CA* [36]. Continued identification of new molecular subclasses within NSCLC is paramount in improving the effectiveness of treatment of this disease.

Apoptosis block contributes to drug resistance [19]. The BCL-2 family of proteins constitute key regulators of mitochondrial apoptosis, with pro-apoptotic versus pro-survival members engaged in a rheostat-like functional interaction that regulates the propensity of tumour cells to initiate mitochondrial outer membrane permeabilization and apoptosis. The pro-survival proteins BCL-X and MCL-1 represent two of the most commonly amplified genes in the cancer genome [37]. Targeting the pro-survival members of the BCL-2 family to derepress the pro-apoptotic machinery is one strategy that has been investigated extensively over several years, culminating in the discovery of bona fide BH3 peptidomimetics, such as ABT-263 [38]. Identifying efficient methods of overcoming mitochondrial apoptosis block, eg by BH3 peptidomimetics, has been an intensively researched therapeutic strategy that has yet to become a mode of treatment. A complementary strategy, however, could be to bypass the mitochondrial apoptosis pathway altogether, using novel pharmacological approaches.

We hypothesized that exploiting a synthetic lethal strategy based on inhibition of PARP-mediated single-stranded break repair in BRCA1-immunodeficient cells [2,4] would lead to catastrophic DNA damage and therefore render the mitochondrial apoptosis pathway dispensible. In modelling 'BRCAness' by silencing BRCA1 expression, we confirmed the expected sensitization to PARP inhibition, as has been reported in the context of *BRCA1* mutation or loss of *BRCA2* in breast cancer [2–4]. Silencing of BAX and BAK, although capable of completely blocking the mitochondrial apoptosis pathway, was unable to attenuate cell killing induced by PARP inhibition in *BRCA1*-silenced cells. This implies that the catastrophic levels of DNA damage that result from a combined defect in ssDNA repair and HR-mediated DSB repair is sufficient to kill without recourse to mitochondrial apoptosis signalling.

Data from the Spanish Lung Cancer Group strongly suggests that reduced *BRCA1* mRNA expression levels in NSCLC patients correlate with increased sensitivity to cisplatin [10], as was originally hypothesized from preclinical studies [11,15]. Accordingly, we were able to demonstrate that silencing of *BRCA1*, using siRNA, sensitizes cells to cisplatin. Additionally, NSCLC cells selected for resistance to cisplatin have previously been demonstrated to harbour defects in mitochondrial apoptotic signalling [24]. Given that PARPi-induced synthetic lethality in *BRCA1*-depleted NSCLC cells appears to be independent of this pathway, we hypothesized that *BRCA1* depletion would still sensitize cisplatin-resistant cells to PARP inhibitor. Accordingly, selection for resistance to cisplatin did not prevent PARP inhibitor-mediated synthetic lethality. BRCA1 levels in cisplatin-resistant cells were equal to those in parental cells. Silencing of *BRCA1* alone did not restore sensitivity to these cells. Together with their sensitivity to PARP inhibition following *BRCA1* silencing, this strongly suggests that DNA repair, associated with H460-resistant cells, also utilizes the single-strand break-repair pathway. This conserved reliance, as in parental cells, can be exploited by PARP inhibition to induce cell death. *BRCA1* may therefore be a predictive marker of response to PARP inhibition in platinum-resistant NSCLC. This provided a rationale for investigating whether sporadic NSCLC with absent or low levels of BRCA1 protein expression may represent an important subgroup with a high likelihood of responding to PARP inhibition.

Using a cohort of primary NSCLC specimens, we have identified a molecular subset of non-small cell lung cancers (NSCLCs) that are *BRCA1*-immunodeficient. The exact mechanisms underlying *BRCA1*-immunodeficiency in sporadic breast, ovarian and prostate cancer and NSCLC remain unknown; however,

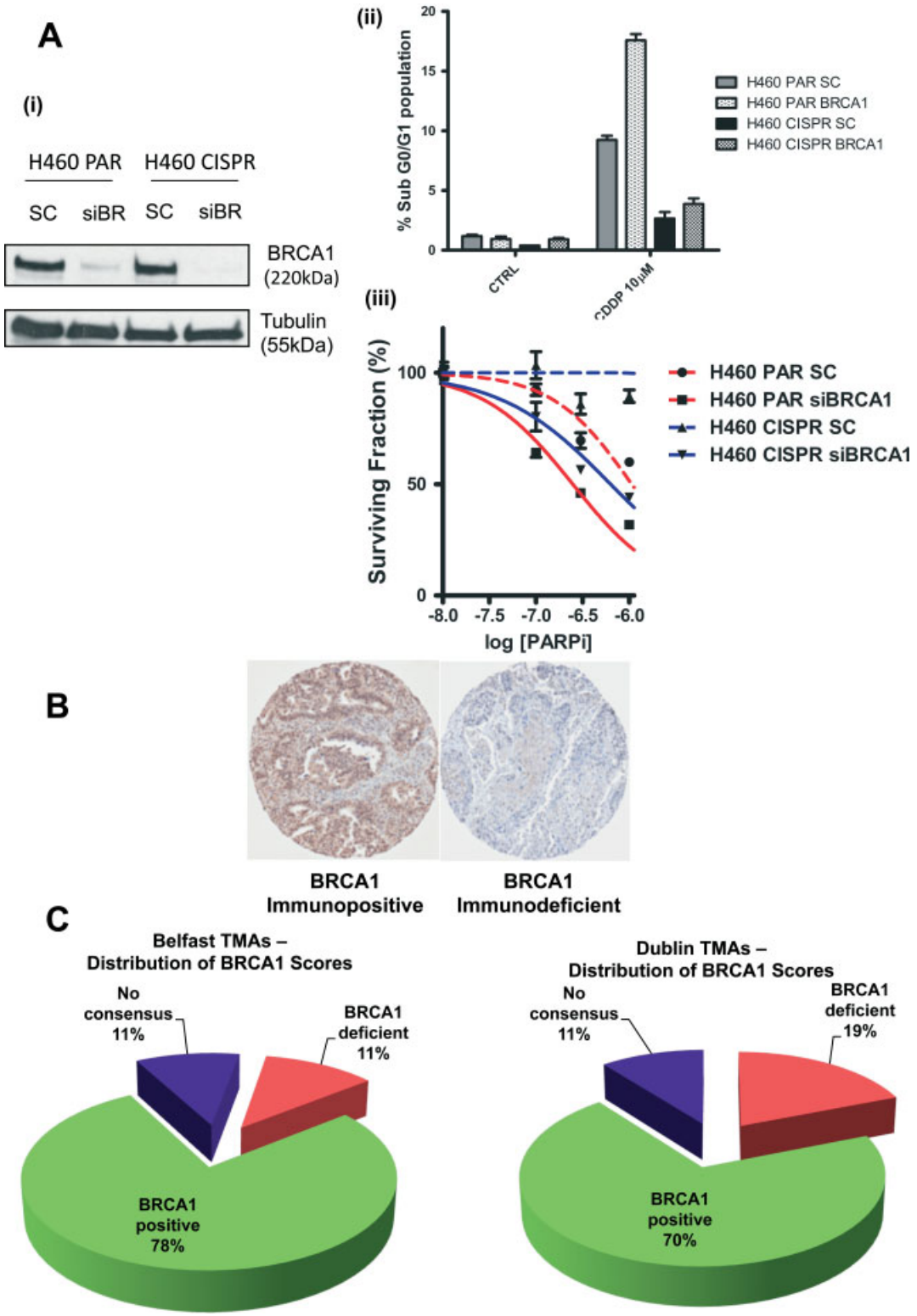

Figure 5. PARP inhibitor-induced synthetic lethality in *BRCA1*-silenced, platinum-resistant NSCLC cells. (A) (i) Western blot showing siRNA-mediated silencing of BRCA1 in H460 parental (H460 PAR) and cisplatin-resistant (H460 CISPR) cells transfected with scrambled (SC) or *BRCA1* (siBRCA1)-targeted siRNA; (ii) apoptotic cells, indicated by sub-G_0/G_1 fraction as assessed by flow cytometry of PI-stained H460 PAR and H460 CISPR cells, transfected as above, following 48 h treatment with 10 μM cisplatin. *BRCA1* silencing confers increased sensitivity to cisplatin-induced apoptosis in parental but not cisplatin-resistant cells; (iii) clonogenic dose–response assays after siRNA-mediated silencing of *BRCA1* in H460 PAR and CISPR cells. PARP inhibition induces an equivalent degree of synthetic lethality in BRCA1-silenced H460 PAR (red) and H460 CISPR (blue) cells. (B) Representative images of BRCA1 immunohistochemistry, demonstrating positive (left panel) versus negative cores (right panel) in a tissue microarray. (C) Pie-charts summarizing the comparative BRCA1-immunodeficiency frequency in primary NSCLC specimens from the Belfast cohort (left) versus the Dublin cohort (right).

Table 1. Distribution of BRCA1 immunostaining by NSCLC histological subtype in both cohorts combined

	Distribution by histology Number (% of histological subtype)	
	Squamous	Non-squamous
BRCA1-immunodeficient	15 (8.7)	34 (26.2)
No consensus	16 (9.3)	16 (12.3)
BRCA1-immunopositive	141 (82)	80 (61.5)
Total	172 (100)	130 (100)

epigenetic alterations, such as promoter methylation, have been reported to be a potential mechanism [8].

In addition, interrogation of the tumorscape database reveals loss of *BRCA1* copy number as a significant somatic event in NSCLC [39]. The *BRCA1* mutation frequency, on the other hand, as revealed by the Cosmic database, is low at 0.3% [5]. Nonetheless, BRCA1 IHC presents a robust, relatively simple and cost-effective approach for identifying a subgroup of *BRCA1*-immunodeficient NSCLC patients who may derive benefit from treatment with PARP inhibitors. This biomarker assay now merits testing in the clinical trial setting.

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Author contributions

PH, JQ and DF initiated and designed the project; IP, KS, EL and JQ carried out experiments; JB carried out data analysis; KG, KOB, JJ, DF developed TMAs; PH developed digital pathology for subsequent scoring; KK and MS carried out BRCA1 scoring on TMAs; KA carried out IHC staining of TMAs; DR contributed to experimental design and critical review of the manuscript; and IP, KS, DF and JQ wrote the manuscript. All authors critically reviewed and approved the manuscript prior to submission.

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