

Targeting ataxia telangiectasia-mutated-and Rad3-related kinase (ATR) in PTEN-deficient breast cancers for personalized cancer therapy

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Abstract

Background:

Phosphatase and tensin homolog (PTEN) is a multifunctional gene which acts as a tumour suppressor gene and is involved in DND damage response (DDR) mechanisms. PTEN has been found to be mutated in different types of human cancers including breast cancer. Ataxia-telangiectasia mutated (ATM) and RAD3-related (ATR) are involved in DDR and they have roles in cell cycle regulation and apoptosis. Previously ATM inhibition caused synthetic lethality in prostate and colorectal cancer cells with PTEN deficiency. In this study we hypothesize that PTEN plays key roles in breast carcinogenesis and that inhibition of ATR in the context of PTEN deficiency can provide a novel therapeutic approach through a synthetic lethality mechanism.

Methods:

In this study a large, well-characterised and molecularly annotated series of breast cancer (n=1954) was utilized to evaluate the clinicopathological and biological role of PTEN protein expression assessed using immunohistochemistry (IHC) and tissue microarrays technology. Several breast cancer cell lines (n=4) representing various molecular classes and PTEN status were studied *in vitro* using functional assays. Cellular consequences of ATR inhibitor (VE-821) treatment were investigated in a panel of PTEN-proficient including MCF7 and MDA-MB-231, and PTEN-deficient including BT-549 and MDA-MB-468 breast cancer cell lines. DNA repair expression profiling, MTS cell-proliferation assay, FACS for cell cycle, γH2AX and FITC-annexin V flow cytometry analysis were performed on

PTEN-deficient and PTEN-proficient cells to study the functional consequences of PTEN deficiency on breast cancer cells.

Results:

PTEN was expressed in the nucleus and cytoplasm of malignant cells. The negative nuclear expression was detected in 62.6%, whilst negative/low cytoplasmic expression was found in 40.2% cases of breast cancer. The negative nuclear PTEN IHC expression was associated with features of aggressive behavior including higher grade, nuclear pleomorphism, higher mitotic index, larger tumour size, oestrogen receptor (ER) negativity, high risk Nottingham prognostic index (NPI≥3.4) and shorter breast cancer specific survival (BCSS) (p-value <0.05). Interestingly, in tumours with low nuclear PTEN, high ATR and/or high pChk1 ($pCHK1^{Ser345}$) expression was also linked to poor BCSS (*P-values* <0.05). Preclinical study demonstrated that PTEN-deficient breast cancer cells feature altered transcriptional expression of several genes involved in DNA repair pathways. Compared with ATR inhibitor (VE-821) in PTEN-proficient breast cancer cells, ATR inhibition in PTEN-deficient cells was associated with accumulation of double strand DNA breaks, cell cycle arrest at G2/M phases, and increased apoptosis.

Conclusions:

PTEN deficiency has prognostic significance in breast cancer, and selective targeting of ATR in PTEN-deficient cells with ATR inhibitor (VE-821) can serve as a potential avenue for development of personalized therapy for breast cancer patients.

Publications and Abstracts

Publication arising from this thesis

Targeting ataxia telangiectasia-mutated- and Rad3-related kinase (ATR) in PTEN-deficient breast cancer cells for personalized therapy

Nouf Alsubhi, Reem Ali, Tarek Abdel-Fatah, Paul Moseley, Stephen Y.T. Chan, Andrew Green, Ian O. Ellis, Emad A. Rakha, Srinivasan Madhusudan. Breast cancer research and treatment. 2018 Jun;169(2)277-286. doi:10.1007/s10549-018-4683-4. Epub 2018 Feb 02.

Other related publications

1. **Chk1 phosphorylated at serine^{345} is a predictor of early local recurrences and radio-resistance in breast cancer**

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2. **Clinicopathological significance of ATM-Chk2 expression in sporadic breast cancer: a comprehensive analysis in large cohorts**

Tarek Abdel-Fatah, Arvind Arora, **Nouf Alsubhi,** Devika Agarwal, Paul Moseley, Christina Perry, Rachel Doherty, Stephen Chan, Andrew green, Emad Rakha, Graham Ball, Ian Ellis and Srinivasan Madhusudan. Neoplasia. 2014 Nov; 16(11):982-991. doi: 10.1016/j.neo.2014.09.009. Epub 2014 Sep 22.

3. **Targeting BRCA1-BER deficient breast cancer by ATM or DNA-PKcs blockade either alone or in combination with cisplatin for personalized therapy**

Albarakati N, Abdel-Fatah TM, Doherty R, Russell R, Agarwal D, Moseley P, Perry C, Arora A, **Alsubhi N,** Seedhouse C, Rakha EA, Green A, Ball G, Chan S, Caldas C, Ellis IO, Madhusudan S. Mol Oncol. 2015 Jan;9(1):204-217. doi: 10.1016/j.molonc.2014.08.001. Epub 2014 Aug 27.

4. **Checkpoint kinase1 (CHK1) is an important biomarker in breast cancer having a role in chemotherapy response**

Meethaq Al-kaabi, Alaa Alshareeda, Dena Jerjees, Abir Muftah, Andy Green, **Nouf Alsubhi,** Chris Nolan, Stephen Chan, E Cornford, Srinivasan Madhusudan, Ian Ellis and Emad Rakha. BJC, 2015 Feb;112: 901-911. doi: 10.1038/bjc.2014.576. Epub 2015 Feb 17.

Abstracts presented at conferences

1. **Deciphering the role of PTEN in DNA damage response in breast cancer**

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2. **Synthetic lethal targeting of ATR-Chk1 pathway in PTEN-deficient breast cancers for personalisation of therapy**

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Abbreviations

APE1	Human apurinic/apyrimidinic endonuclease
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
ATRIP	ATR-interacting protein
BCSS	Breast cancer specific survival
BER	Base excision repair
Bp	Base pair
BRCA1	Breast cancer susceptibility 1
BRCA2	Breast cancer susceptibility 2
CHK1	Checkpoint kinase 1
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNA-PK	DNA-protein kinase
DNA-PKcs	DNA dependent protein catalytic subunit
DSB	Double strand break
EGRF	Epidermal growth receptor factor
ER	Estrogen receptor
HER	Human epidermal receptor
HR	Homologous recombination
IHC	Immunohistochemistry
MMR	Mismatch repair
MRN complex	MRE11-RAD50-NBS1
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining recombination
NPI	Nottingham prognostic index
PARP1	Poly (ADP-Ribose) Polymerase 1
PCHK1	Phosphorylated Checkpoint Kinase 1
PCNA	Proliferation cell nuclear antigen
PI	Propidium iodide
PLK1	Polo-Like-Kinase-1
Polβ	DNA Polymerase beta

PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RNAse	Ribonuclease
RPA	Replication Protein A
RT-PCR	Real Time – Polymerase Chain Reaction
SDF-1α	Stromal cell-derived factor-1α
SEM	Standard Error of Mean
SL	Synthetic lethal
SSB	Single strand break
TNBC	Triple negative breast cancer
XRCC1	X-ray repair cross complementing gene 1
γH2AX	Phosphorylated histone H2AX

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1. General Introduction

1.1 Molecular Classification of Breast Cancer

Breast cancer is a leading cause of mortality among women in the developed countries of the world (Spitale et al., 2009). It consists of distinct histopathological features and clinical implications due to involvement of various biologically distinct entities; therefore, it is not a single diseased state. The researchers have shown that breast cancer with different biological and histopathological characteristics displays different symptoms which require different treatments, and consequently is treated with different therapeutic strategies (Tang et al., 2008; Iwamoto and Pusztai., 2010; Desmedt et al., 2009; Reis-Filho et al., 2010; Sotirirou and Pusztai, 2009; Weigelt et al., 2010). Therefore, the decision of choice of therapeutic interventions highly depends on the grouping of the breast cancer into subtypes. In this way, the grouping of breast cancer is required for choosing effective and efficient therapeutic interventions, and is normally based on the distinct biologically and chemically distinct entities affecting the proliferation and progression of the breast carcinoma (Blows et al., 2010; Dai et al., 2015). The different markers such as immunohistochemistry markers – ER, PR and HER2 – along with pathological variables such as tumour size, nodal involvement, and tumour grade are used for the classification of breast carcinoma. These markers and clinicopathological variables are also employed for disease prognosis and prediction of therapeutic outcomes, thereby carrying prognostic and predictive value for patient prognosis and management (Vallejos et al., 2010; Cheang et al., 2009). The discussion on the different classification based on clinicopathological

variables and immunohistochemistry markers has been given in the subsequent subsections.

1.1.1 Luminal-like tumours

Luminal like tumours are known to express the genes based on pattern of gene expression found in the luminal epithelial component of the breast (Perou et al., 2000). The pattern of genetic expression found in luminal-like tumours involves expression of ER and genes associated with ER activation such as *LIV1* and *CCDN1* and luminal cytokeratin 8/18 (Perou et al., 2000; Sotiriou et al., 2003). The luminal-like tumour has two subtypes called luminal A and luminal B. The luminal A subtype is characterised by ER or PR positive and HER2 negative and denoted by ([ER+] [PR+] [HER2-]) using IHC nomenclature. Similarly, luminal B subtype is featured by ER or PR positive and HER2 positive, and according to IHC nomenclature is represented by ([ER+] [PR+] [HER2+]). Compared to luminal B, the luminal A subtype shows a lower expression pattern of genes related to cellular proliferation, and a higher expression of ER and associated genes. Consequently, the luminal B is characterised as higher-grade tumour than the luminal A subtype cancer (Dai et al., 2015).

Luminal B subtype is found to be most common in young female patients compared to luminal A (Hashmi et al., 2018). In the breast cancer study carried out in Carolina, 64.3% of the breast cancer incidents were related to luminal like cancer, among which, luminal A subtype was found in 57% of premenopausal white, 67% of postmenopausal white women, 40% of premenopausal African American women and 55% of the postmenopausal African American women (O'Brien et al.,

2010). Generally speaking, the luminal like cancer has the best prognosis compared to other cancer subtypes. Among luminal-like cancer, the luminal B has significantly worst prognosis compared to luminal A subtype (Sorlie et al., 2003).

Luminal-like cancer subtypes show poor response to the conventional chemotherapy; however, they respond well to the endocrine therapy (Brenton et al., 2005). However, the response to the endocrine therapy differs between luminal subtype A and luminal subtype B. The luminal B showed the higher recurrence score than the luminal subtype A, which indicated that the latter can be treated effectively by the endocrine therapy (Paik et al., 2004). Luminal subtype B, therefore, can be treated using the combined treatment involving endocrine and chemotherapeutic agents.

Some other treatments are underway for approval or approved for treating luminal like cancer. For example, mTOR inhibitors such as everolimus were approved in combination with exemestane to treat luminal A subtype cancer, because they have been shown to reverse AKT-induced resistance to hormonal therapy and transtuzumab (Abbas et al., 2008). This is because of the fact mTOR inhibitors target the mTOR pathway for preventing the uncontrolled proliferation of tumour cells. Similarly, a cyclin-dependent kinase 4/6 inhibitor such as Palbociclib being developed by Pfizer is under approval process to treat such patients following the positive outcomes from a phase II study (Morikawa and Henry, 2015).

1.1.2 HER2 Positive Breast Cancer

This type of breast cancer contains cells overexpressing HER2 protein, and based on results from the fluorescence in situ hybridization and immunostaining, this subtype has been developed, which showed ER negative, PR negative and HER2 positive phenotype ([ER-] [PR-] [HER2+]) (Vallejos et al., 2010). However, the results obtained from these assay do not match with different clinical cases analysed, as HER2 positive or overexpression tumours also overexpress some other genes such as *GRB7* (Perou et al., 2000, Dai et al., 2014) and PGAP3 (Dai et al., 2014). HER2 positive tumours are graded as grade 3 tumours, and most of them carry TP53 mutation. In addition, there was no association discovered for HER2 positive tumours with the age and race, and carry no data of known risk factors (Brenton et al., 2005; Colditz et al., 2004).

HER2 positive tumours show poor prognosis and less response to the chemotherapeutic agents except taxane and anthracycline-based neoadjuvant chemotherapy (Brenton et al., 2005). It is also reported that HER2 positive tumour cells showed good response to anti-HER2 monoclonal antibody such as trastuzumab, nonetheless, it was discovered that all HER2 positive patients do not show the response to trastuzumab. The resistance to trastuzumab treatment has been reported due to loss of PTEN and upregulation of CXCR4 which is a receptor for the natural ligand stromal cell-derived factor-1α (SDF-1α). Nagata et al (2004) reported that trastuzumab rapidly activates the phosphatase function of PTEN prior to downregulation of HER2 receptors, thereby showing a novel mechanism of PTEN activation which contributes to the antitumor activity of trastuzumab

independent of HER2 receptors downregulation. Therefore, the loss of PTEN results in the resistance of trastuzumab therapy in HER2 positive breast cancer patients. Moreover, the function of CXCR4 and SDF-1α are essential for HER2-mediated breast tumour metastasis (Nagata et al., 2004, Brenton et al., 2005, Li et al., 2004).

1.1.3 Triple Negative Breast Cancer (TNBC)

Triple negative breast cancer (TNBC) is characterised by ER negative, PR negative and HER2 negative, and symbolically this is denoted as ([ER-] [PR-] [HER-]). Thus the expression profile of TNBC mimics that of normal myoepithelial breast cells and basal epithelial cells in other body parts (Perou et al., 2000). TNBC contains higher expression of proliferation related genes and basal markers including EGFR and keratins (Perou et al., 2000; Sotiriou et al., 2003). The tumours with higher cytokeratins expression contain low expression of BRCA1 and Tp53 mutation (Sorlie et al., 2001; O'Brien et al., 2010). Like HER2 positive tumours, TNBC is characterised by grade 3 tumours (Sorlie et al., 2001; O'Brien et al., 2010). About 60-90% cases of basal tumours are TNBC cases (Fan et al., 2006; Swenson et al., 2009).

TNBC has the most aggressive phenotypes of BC, occurrence in young patients, prevalence in the premenopausal individuals, poor disease-specific survival of such patients and display of poor response to standard targeted systemic therapies (Carey et al., 2006). In addition, risk factors include earlier menarche and abnormality in breast-feeding such as breast lumps in lactating women. The studies have also

showed the time-dependent survival in TNBC patients is around 5 years with poor early diagnosis and prognosis (Dai et al., 2015).

The metastasis pattern displayed by TNBC also differs from other subtypes of breast cancer, which show more metastatic tendency to the visceral organs and without involving the lymph nodes (Ho-Yen et al., 2012). In addition, the tumour size (approximately 2 cm) contained by TNBC patients is higher than that possessed by patients of other breast cancer subtypes (Rakha et al., 2006). This class of tumours shows rapid and uncontrolled cell proliferation, and not amenable to conventional treatments, whereby leaving the only option of chemotherapeutic treatment for TNBC patients (Ho-Yen et al., 2012).

Two independent studies have demonstrated that TNBC is sensitive to anthracycline and taxane-based chemotherapy. The poor prognosis does not stem from the chemoresistance, but it is mainly due to the lesser chemotherapeutic options available to therapeutic armamentarium for such patients (Brenton et al., 2005). Chang et al (2005) showed that basal like tumours may be driven by EGFR. Many studies have demonstrated that genes associated with angiogenesis and matrix remodelling and 'wound response' signatures are associated with basal like tumour or TNBC, suggesting the potential new targets for treating TNBC patients (Dai et al., 2015; Chang et al., 2005).

The patients' outcome of luminal-like tumours, HER2 overexpression tumours and basal-like tumours/TNBC have been shown in figure 1.1.

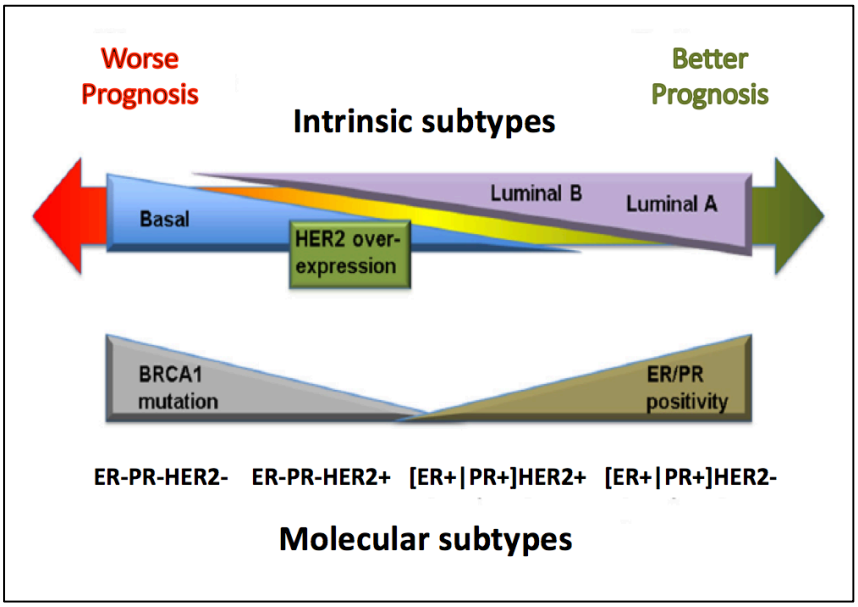


Figure 1-1 The outcomes of intrinsic molecular subtypes of breast cancer and their associated outcomes.
Luminal A, luminal B, HER2 overexpression and basal-like tumours in the breast cancer patients. Adapted from (Dai et al., 2015).

The summary of molecular subtypes of the breast cancer along with their IHC status, grades, outcomes and prevalence patterns have been presented in the table 1.1 .

Table 1-1 Intrinsic molecular subtypes of breast cancer along with IHC status, grades, patients' outcome and prevalence.

Molecular subtypes	IHC status	Grade	Patients outcomes	Prevalence
Luminal A	[ER+|PR+] HER2-KI67-	1|2	Good	23.7% (Spitale et al, 2009; Cheang et al., 2009)
Luminal B	[ER+|PR+] HER2-KI67+	2|3	Intermediate	38.8% (Spitale et al, 2009; Cheang et al., 2009)
	[ER+|PR+] HER2+KI67+		Poor	14% (Spitale et al, 2009; Cheang et al., 2009)
HER2 overexpression tumours	[ER-|PR-] HER2+	2|3	Poor	11.3% (Spitale et al, 2009; Cheang et al., 2009)
Basal-like tumours/TNBC	[ER-|PR-] HER2-, basal marker+	3	Poor	12.3% (Spitale et al, 2009; Cheang et al., 2009)
Normal-like	[ER+|PR+] HER2-KI67-	1|2|3	Intermediate	7.8% (Tang et al, 2008; Smid et al., 2008)

1.2 Formation of DNA breaks and repair mechanisms

The DNA breaks may be formed due to damage caused by various DNA damaging agents such as reactive oxygen species resulting from metabolic sources (endogenous source) or ionizing radiations, drugs or chemicals (exogenous source) (Polo and Jackson, 2011; Friedberg et al., 2006; Jackson and Barret, 2009; Ciccia and Elledge, 2010). In order to cope with both exogenous and endogenous sources of DNA damage, cells are equipped with DNA repair mechanisms which play role

in protecting the DNA integrity and stability (Ciccia and Elledge, 2010; Hoeijmakers, 2001). The main mechanisms responsible for DNA repair are bases excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and Double stranded breaks repair (DSBR). The DNA damaging agents causing damage to individual bases of DNA induce NER and BER mechanisms to repair such damage (Barzilai and Yamamoto, 2004; Ciccia and Elledge, 2010). Similarly, the damage to either DNA single strand (ssDNA) or double strand (DSB) is repaired by single strand annealing, homologous recombination (HR) or non-homologous end joining (NHEJ) mechanism (Polo and Jackson, 2011; Jackson and Bartek, 2009). DNA damage sources and DNA repair pathways are summarised in figure 1.2.

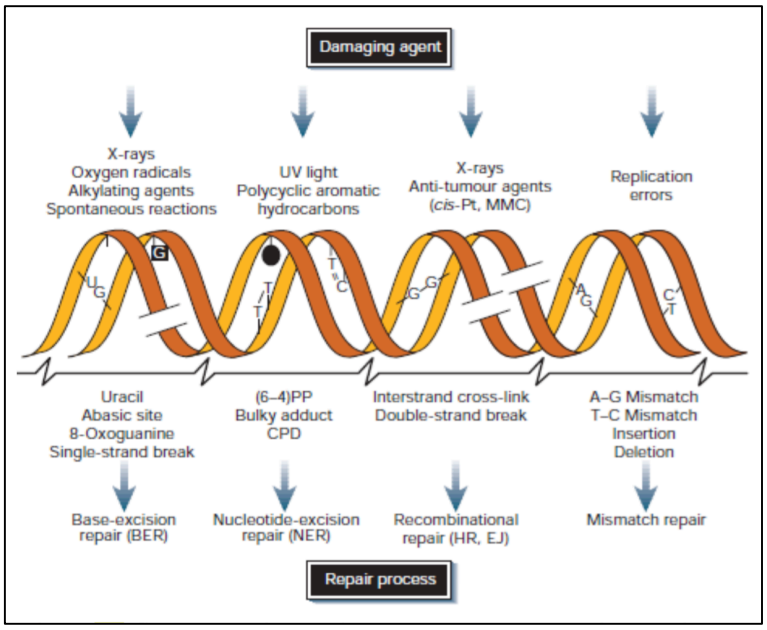


Figure 1-2 Overview of DNA damage and corresponding repair pathway. Specific DNA damage can be derived from endogenous and exogenous agents and the corresponding DNA repair pathway for each type of damage. Adopted from (Hoejmakers, 2001).

DNA DSBs are regarded as the lethal damages, as in the case of DSB, template strand is not available to be copied in order to repair the DSB. If such breaks remained unrepaired, they can cause different developmental and genetic syndromes such as sterility, radio-sensitivity, predisposition to cancer, immunodeficiency and neurodegeneration (Jackson and Bartek, 2009). DSBs are normally generated by drugs mimicking the action of ionizing radiation, such as X-rays and gamma-rays, and topoisomerase II inhibitors which hamper the process of unwinding stimulated by topoisomerase II activity. DSBs are also generated as a result of replication of DNA containing some lesions such ssDNA breaks, retroviral integration, meiotic recombination in germlines, apoptotic cell death and class switch recombination in vertebrates (Friedberg et al., 2006, Jackson and Bartek, 2009). DNA is also prone to DSBs when the chromosomal ends become exposed as a consequence of telomere shortening due to cell aging (d'Adda di Fagagna et al., 2003).

There are two fundamental mechanisms implicated in the DSBs repair, which include HR and NHEJ (Hartlerode and Scully, 2009; Pardo et al., 2009). These mechanisms seem complementary to each other and are activated under different conditions. The important step which allows the choice between HR and NHEJ is the end resection of DSB. HR requires the DSB end-resection step, while it is not required for the NHEJ mediated repair pathway (Polo and Jackson, 2011).

A large fraction of DNA DSBs in S and G2 phases of the cell cycle is repaired by HR repair pathway which begins resectioning 5'-end of DNA flanking DSB. The

reaction is catalysed by MRN complex containing MREII, RAD50 and Xrs/NBS1. MREII possesses both exo-and endo-nuclease activities to process the 'clean ends' of DNA by endonuclease activities. The function of MRN is assisted by the endonuclease Sae2/CTPI/CTIP, RECQ family helicases, nucleases Dna2 and Exo1 which are involved in processing hairpin structures attached to proteins (You and Bailis, 2010; Huertas, 2010; Longhese et al., 2010; Mimitou and Symington, 2009; Bernstein ad Rothstein, 2009).

The end-resection of DSBs creates 3'-ssDNA which is bound by replication protein A (RPA) which is subsequently replaced by RAD51 recombinase with the aid of some other auxiliary proteins such as RAD51 paralogs, RAD52 and proteins comprising Fanconi anemia (FA) pathway involving CD1/breast cnacer-2 (BRCA-2) and FANCN/PALB2 (Partner and Localizer of BRCA2) which are implicated in detecting and repairing cross-links between two strands at the site of stalled replication fork (Moldovan and D'Andrea, 2009). RAD51 together with other factors searches for complementary DNA parts on sister-chromatid, followed by invasion of damaged strand into homologous region of sister-chromatid through action of DNA polymerase. Following this, DNA ends are ligated by the DNA ligase 1 (LIG1), and it is followed by cleavage and resolution of HR intermediates by DNA helicase and resolvase, thereby leaving repaired and intact DNA (Mazon et al., 2010).

Unlike HR repair pathway, NHEJ functions during G0 and G1 phases of the cell cycle (Cannan and Pederson, 2016), which is activated following the binding of Ku70/Ku80 complex to the DNA DSB's ends. Ku70/Ku80 heterodimer complex

exists in a ring form which encircles 3-4bp of DNA (Walker et al., 2001). Once bound to DNA, it recruits X-ray repair cross complementing protein 4 (XRCC4) and Cernunnos X4 like factor (Cer-XLF) which constitute the bridge between two ends of DSB. In the event of complementary ends and without any damage, DSB ends are joined together directly through action of XRCC4 associated DNA ligase IV (Reynolds et al., 2012). Otherwise, further end processing is required before the ends of DSB are joined together.

In order to ligate such ends, DNA protein kinase (DNA-PK) is recruited by Ku heterodimer complex, which is then activated by its catalytic subunit called DNA-PKcs). The activation of DNA-PK leads to phosphorylation and subsequent activation and recruitment of nuclease Artemis to the site of DSB. The broken ends at DSB are juxtaposed to each other by DNA-PK which allows recruitment of several factors such as nuclease Artemis, polynucleotide kinase (PNK), Apratxin, PNK-like factor (APLK) and Aprataxin in the complex. At DSB, ssDNA overhangs are removed by nuclease Artemis, thereby leaving a substrate for DNA ligase IV which ligates ends by acting in conjunction with XRCC4 and Cer-XLF (Lieber and Wilson, 2010).

In addition, in the event of mutation or missing components in classical NHEJ pathway, some alternative end-joining pathways exist, which mostly rely on microhomologies in the ends at DSB for mediating the end-joining reaction, and are dependent on factors involved in HR and single strand break (SSB) repair such as MRN complex (MRE11-RAD50-NBS1), PARP1 (ploy (ADP-ribose) polymerase-1), DNA ligase I/III and XRCC1 (McVery and Lee, 2008).

SSBs are also toxic to the cells, though they are not as lethal and harmful as the DSBs. The deficiency in NER repair pathway has reported to be implicated in the neurodegeneration syndromes such as Xeroderma syndrome is caused by defects in XPA, XPB and XPD genes involved in NER repair pathway (Caldecott, 2008). SSB results from the attack of free radicals such as H2O2, ionizing radiations, radiomimetic drugs, and are initially detected by PAPRP1 in most of the eukaryote. PARP1 has the ability to bind the DNA breaks and subsequently triggers the poly-ADP-ribosylation of itself and several nuclear proteins. These modifications help recruit XRCC1 to the site of DNA break which further acts as a scaffold to recruit numerous SSB repair factors implicated in end-processing, filling of gaps and end-ligation. Various factors implicated in single strand and double strand breaks repair pathways have been presented in table 1.2.

Table 1-2 Protein factors taking part in different DNA break repair pathways in mammals and *S. cerevisiae*

Repair pathways	Steps	Factors in mammals	Factors in *S. cerevisiae**
HR-DSBR	Resection	MRN, CTIP, EXO1, BLM, DNAs	MRX, Sae2, Exo1, sgs1, Dnas
	Homologous pairing and strand exchange	RPA, RAD51, RAD52, RAD54, RAD51 paralogs, BRCA2-PLAMB2 (FANCD1-FANCN),	Rfa, Rad51, Rad52, Rad54, Rad55, Rad57
	DNA synthesis	PCNA, Pol δ	PCNA, Pol δ
	HR resolvases	MUS81-EME1, GEN1, SLX1-SLX4, XPF-ERCC1	Mus81-Eme1, Yen1, Slx1-Slx4, Rad1-Rad10
	Dissolution of HR intermediates	BLM, TOPIII-RMII_RM12, RTEL1	Sgs1-Top3-Rmi1, Srs2
NHEJ-DSBR	End binding	MRE11-RAD50-NBS1 (MRN), Yku70-Yku80, DNA-PKcs	Mre11-Rad50-Xrs2 (MRX), Ku70-Ku80
	End-Processing	Artemis, APLF, PNK, APTX	Not been found yet
	Ligation	LigaseIV-XRCC4-XLF	Lig4-Lif1-Nej1
SSBR	Detection	PARP1	Not been found yet
	End-processing	APE1, XRCC1, PNK, APTX	
	Gap-filling, ligation	LigIII, Pol β	

* genes in *S. cerevisiae* are presented in table along with mammals for comparison purpose, because some factors/genes have same or similar origin in both mammals and *S. cerevisiae*

1.3 DNA damage response signalling Pathways

Cells face different stresses both from extrinsic and intrinsic environments, and which can pose a threat to their survival and integrity. In order for surviving these adverse conditions and challenges and to copy the genetic information intact from one generation to the other generation, the cells have developed a highly organized

and concerted framework called the DNA damage response (DDR). The DDR signalling pathway is conferred upon the cells with ability to sense the damage/stresses on the genome, initiate signalling to arrest the cell-cycle checkpoints and activate the DNA repair pathways or to eliminate the cells with unrepairable damage to the DNA structure. The significance of DDR signalling pathway in preserving the DNA integrity and maintenance can be realised by considering the fact that mutations or aberrations in function of DDR can lead to development of various diseases and cancer disposition syndromes (Ciccia and Elledge, 2010).

The cellular response to DNA damage and stress is regulated by DDR signalling pathway, and like any classical signalling pathway, DDR signalling pathway contains sensors, transducers and effectors as shown in figure 1.3 (Zhou and Elledge, 2000). However, unlike other signal transduction pathways which are activated by the ligands binding to the receptor kinases, DDR signal transduction pathway is activated through the presence of aberrant or abnormal DNA structure. There are specialised proteins which are designed to sense these aberrant DNA structure, and are called sensors in DDR network, which activate the upstream DDR kinases. For example, MRN and RPA proteins for ATM and ATR signalling pathways sense the lesions in DNA during replication or in response to external stimuli such as ionizing radiations as shown in table 1 (Polo and Jackson, 2011).

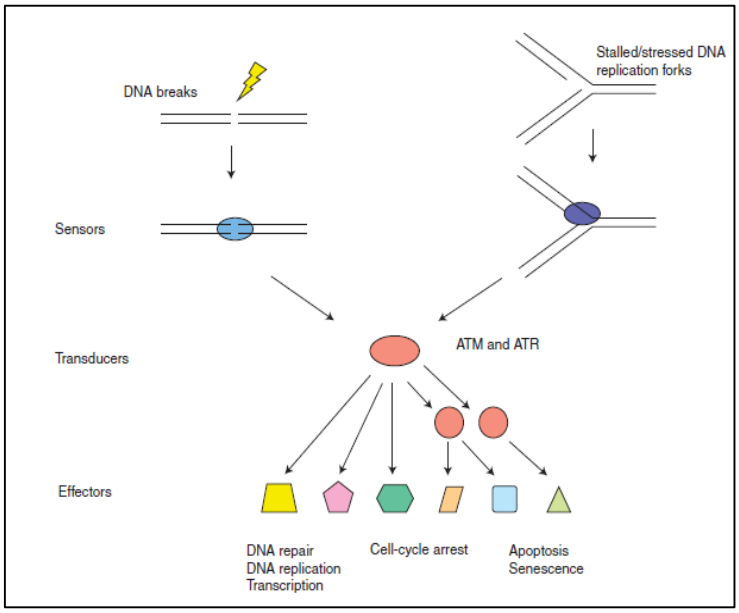


Figure 1-3 DNA damage repair signal transduction pathway
Like any other classical signal transduction pathway, the DNA damage such as DNA breaks or stalled replication forks are sensed by sensor proteins. ATR, ATM and downstream kinases act as transducers, while proteins involved in regulation of DNA repair, DNA replication, transcription, cell-cycle arrest and apoptosis serve as effectors of DDR signalling pathway, and are important for genomic integrity. Adapted from (Marechal and Zhou, 2013).

The DDR signalling network consists of transducers which are kinases and mediators which facilitate the phosphorylation events (Table 1.3). The effectors are the kinases' target proteins which perform the required functions relating to preserving the genomic integrity and stability within the network such as DNA repair, DNA replication and cell cycle checkpoints (Harper and Elledge, 2007; Houtgraaf et al., 2006; Dai and Grant, 2010). Thus it can be argued that DDR signalling pathway is mainly driven by the protein phosphorylation.

The important transducers of DDR signalling pathway include the ataxia-telangiectasia mutated (ATM), ATM-and Rad3-Related (ATR) and DNA-

dependent protein kinases (DNA-PKcs) which constitute the upstream kinases belonging to the phosphatidylinositol-3-kinase-like kinase family (PIKKs) (Lempiainen and Halazonetis 2009; Lovejoy and Cortez 2009). Both ATM and ATR are responsible for phosphorylating hundreds of proteins at their Ser/Thr-Glu motifs in response to DNA damage, while DNA-PKcs are responsible for phosphorylation of proteins involved in non-homologous end joining (NHEJ) repair pathway (Matsuoka et al. 2007; Smolka et al., 2007; Stokes et al., 2007; Bensimon et al., 2010; Beli et al., 2012). ATR and ATM initiate the second wave of phosphorylation by activating some other kinases such as CHK1, CHK2 and MK2 kinases (Liu et al., 2000; Reinhardt et al., 2007).

Though the functional importance of most of these phosphorylation events remains unclear, a variety of ATM/ATR substrates are involved in DDR signalling pathway. ATM and ATR had been found to be the main signal transducers of DDR signalling pathway, which orchestrate different processes and reactions in order to maintain genomic stability (Marechal and Zou, 2013). The *in vivo* and *in vitro* studies have demonstrated that ATM and ATR performs distinct functions in regulating the DDR signalling pathway. ATM is mainly activated by the double stranded DNA breaks (DSB), whereas ATR is responsible to initiate the repair mechanisms in response of a variety of DNA damages such as DSB, signal stranded DNA (ssDNA), and DNA lesions interfering with DNA replication. Given the aim and focus of this study, the preceding sections of this chapter will focus only on ATR and its role in DDR signalling pathway (Zou and Elledge, 2003; Brown and Baltimore, 2003; Wang et al., 2004).

Table 1-3 DNA damage signalling and its components in mammals and yeast

DNA damage signalling	Mammals	*S. cerevisiae*
Sensors	MRNA RPA (+RFC-like, PCNA-like checkpoint-clamp)	RRX Rfa (+RFC-like, PCNA-like checkpoint-clamp)
Transducers	ATM ATR-ATRIP DNA-dependent protein kinases	Tel1 Mec1-Dcd2
Mediators		
ATM signalling	53BP1, MDC1, BRCA1, MCPH1 PTIP	Rad9
ATR signalling	TOPBP1, Claspin	Dpb11, Mrc1
Effectors	CHK1, CHK2	Chk1, Rad53

1.4 Structure and functions of ATR

1.4.1 ATR structure

ATR consists of five important domains, which are HEAT (Huntington-elongation factor 3-protein phosphatase 2A-TOR1) repeats, FAT (FRAP-ATM-TRRAP), Kinase, PIKK-regulatory domain (PRD), FATC. The organization of domain in the structure of ATR can be seen in the figure 1.4.

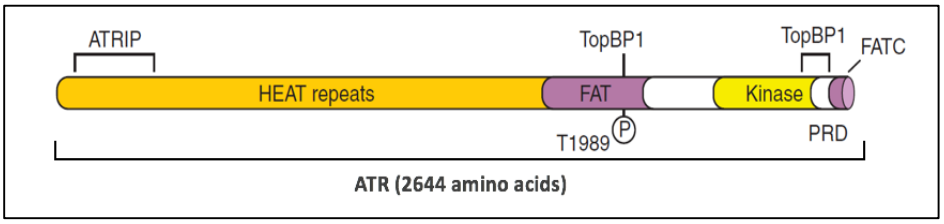


Figure 1-4 Organization of functional domains in ATR structure.
HEAT repeats domain is located on N-terminal, which contains multiple repeats, some of them interacts with other proteins. Kinase domain is flanked by FAT and FATC domain. FAT and FATC domains are regulator domains which are important for ATR interaction with TOPBP1. PRD domain located between kinase and FATC is important for TOPB1 interaction and ATR activation. Adapted from (Marechal and Zhou, 2013).

HEAT repeats domain consist of multiple repeats (40-50 alpha helical repeats) which are involved in interactions with proteins, which play fundamental role in regulation and function of ATR (Perry and Kleckner, 2003). Furthermore, ATR forms the complex with ATR-interacting protein (ATRIP) though its HEAT repeat domain located at N-terminal of ATR, and this interaction does not seem to be controlled by the DNA damage (Ball and Cortez, 2005; Itakura et al., 2005; Ball et al., 2005). The HEAT repeats domain also contain Tel2-Tti1-Tti2 (TTT) complex which is fundamentally important for the folding of HEAT repeats domain and interaction with ATRIP. The depletion of different components of this complex comprised not only the expression level of ATR but also reduced the interaction between ATR and ATRIP (Takai et al., 2010).

FAT domain located downstream of HEAT repeats domain, which is autophosphorylated at Thr1989 to become activated in response to DNA damage. The stimulation of ATR through TOPBP1 is mediated through this domain (Liu et

al., 2011a). The FATC domain is essential for activation of basal kinase activity of ATR. Importantly, both FAT and FATC constitute regulatory domains of ATR The shortest domain located between FATC and kinase domains is called PRD domain which is indispensable for interaction between TOPBP1 and ATR and consequent activation of ATR (Mordes and Cortez, 2008).

1.4.2 Mechanism of ATR activation

The physiological functions of ATR and it significance in regulating the DNA damage response is mainly derived from the signalling leading to its activation. Therefore, in the following section, mechanisms of ATR activation are discussed.

1.4.2.1. Recognition of DNA damage

DNA damage induced by various DNA damaging agents yields different structures such as single stranded DNA, junctions between single stranded and double stranded DNA which are recognized initially in order to activate ATR. Most commonly found structure of DNA originating from either the interference with DNA replication or the action of DNA damaging agents. When the DNA polymerase and helicase activities are compromised during the DNA replication, the single stranded DNA structures are accumulated at the sites of damage (Byun et al., 2005; Walter and Newport, 2000). Furthermore, several repair systems including long-patch base excision repair, nucleotide excision repair and mismatch repair cause the generation of increased amount of single stranded DNA (Friedberg, 2003; Lopes et al., 2006).

The activities of exo- and end-nucleases at the double stranded breaks results in generating single stranded DNA structures. Shiotani and Zou (2009) argue that accumulation of single stranded DNA, junctions between the single stranded DNA and junctions between the single stranded and double stranded DNA are hallmarks of action of DNA damaging agents or the stalled replication forks, and are always present together at the site of DNA damage. In addition, these damaged structures were found to be sufficient to activate the ATR mediated activation of the SSBs repair pathways to preserve the DNA integrity (McDougall et al., 2007).

The ssDNA produced by the DNA damaging agents is detected initially by replication protein A (RPA) in all eukaryotes. RPA binds to the ssDNA (RPA-ssDNA complex), which is followed by the binding of ATR-interacting protein (ATRIP), thereby forming the ATRIP-ssDNA complex which then localized to the DNA damage sites (Ball et al., 2005; Namiki and Zou, 2006; Zou and Elledge, 2003). The TRPIP-ssDNA complex recruits ATR to the DNA damage site. Furthermore, there are additional interactions between ATR-ATRIP complex and other proteins and ATR-ATRIP and RPA which increase the association of ATR-ATRIP complex with the DNA damage site (Ball et al., 2005; Namiki and Zou, 2006; Yoshioka et al., 2006). Even though the RPA-ssDDNA complex might b suffice to localize the ATRIP-ATR complex, but is not capable for ATR activation (Stokes et al., 2002; Michael et al., 2000; Byun et al., 2005; McDougall et al., 2007).

The association of ATR-ATRIP complex to the DNA damage site is not sufficient to execute the DNA damage checkpoint response. However, some additional

proteins are required, such as RAD9, RAD17, HUS1 and RAD1, which regulate the activity of ATR. RAD17 is structurally similar to RFC and forms the RFC-like complex through interaction with its fours small subunits which share resemblance with RFC2, RFC3, RF4 and RFC5. Nevertheless, RAD1, RAD9 and HUS1 interact with each other in order for forming RAD9-RAD1-HUS1 complex, also called 9-1-1-complex, which share resemblance with the processivity factor for DNA polymerase called proliferation cell nuclear antigen (PCNA) [Parilla-Castellar et al., 2004; Ellison and Stillman, 2003).

Colocalization of ATIP-ATR complex with 9-1-1-complex trigger the ATR signalling. The loading of 9-1-1-complex on DNA damage site/primer-template junction is caused by DNA damage specific clamp loader in ATP dependent reaction. This means that 9-1-1-compelx recognizes the DNA damage site loaded with RPA which confers specificity to the loading the additional proteins required to play crucial role in DNA damage response. This highlights the importance of RPA and 9-1-1-complex in recognition of DNA damage site, and subsequent activation of ATR-mediated DNA damage-checkpoint (Ellison and Stillman, 2003; Zou et al., 2003; Bermudez et al., 2003).

ATR kinase is activated by a complex mechanism involving recruitment of ATR to the RPA-bound ssDNA via its binding partner ATRIP and ATR stimulation by TopBP1 (detailed in section 1.4.2.2.) (Zou et al., 2003; Bermudez et al., 2003; Majka et al., 2006). This is supported by the experiment involving *X. laevis* egg extracts containing ssDNA. ATR signalling could not be activated during this experiment, however, the extracts containing ssDNA with free 5' primer end was

found to be activating the ATR signalling (McDougall et al., 2007). The primed ssDNA with a free 5' primer end is a structure normally generated during the stalled replication process, end resection at double stranded DNA, and base excision repair; and it is required for activation of DNA replication checkpoints (Byun et al., 2005; Pacek and Walter, 2004; Nedelcheva et al., 2005).

1.4.2.2. Activation of ATR by TOPBP1

ATR is activated following the 9-1-1-complex and ATR-ATRIP are recruited to the DNA damage site, In *S. cerevisiae*, 9-1-1 complex (Ddc1-RAD17-Mec3 complex) is sufficient to activate the function of ATR-ATRIP complex (Mec1-Ddc2 complex) [Majka et al., 2006b]. However, the role of 9-1-1-complex in activating the ATR in other organism is poorly defined. In humans and *Xenopus*, the DNA topoisomerase binding protein-1(TOPBP1) is reported to play an important role in activating the ATR-ATRIP complex even when the DNA is not present in vitro (Kumagai et al., 2006). TOPBP1 is a breast cancer like C-terminal (BRCT) domain containing protein, which is reported to contribute to ATR activation in vitro. In S. cerevisiae, Dpb11 which is TOPBP1 homologue is known to stimulate Mec1-Ddc2 complex (Mordes et al., 2008b, Navadgi-Patil and Burgers, 2008). This shows that TOPBP1 is regarded as an evolutionary conserved activator of ATR kinase. TOPBP1 binds to binding domain in ATRIP in ATR-ATRIP complex through ATR activation domain located between BRCT domains VI and VII. In order for ATR signalling activation, human TOPBP1 or yeast Dpb11 interacts with RAD9 in humans and Ddc1 in yeast (Makiniemi et al., 2001; Wang and Elledge, 2002). Subsequent studies have demonstrated that TOPBP1 interacts with C-terminus of

RAD9 and phosphorylated Ser387 located in RAD9 (Delacroix et al., 2007; Lee et al., 2007). Thus the interaction between RAD9 and TOPBP1 results in recruiting the TOPB1 to 9-1-1 complex at the site of DNA damage. Furthermore, it is also reported that TOPBP1 induces the phosphorylation of RAD1 rather than RAD9 in *Xenopus* extracts, which indicate that activation of DNA damage checkpoint is also regulated by surrogate mechanism (Lupardus and Cimprich, 2006), which is independent of RAD9 phosphorylation. In addition, this also shows that RAD9 and RAD1 components in 9-1-1-complex are essential for bringing the TOPBP1 to the DNA damage site (Delacroix et al., 2007).

Apart from stimulating ATR kinase activity, TOPBP1 serves as a substrate for ATR or ATM. The ser1131 located in TOPBP1 is phosphorylated by ATR in the event of replication stalling or by ATM in the event of creation of DNA structures containing polyA/polydT (Hashimoto et al., 2006; Yoo et al., 2007). The phosphorylation of TOPBP1 at ser1131 serves as a stimulator of interaction between ATR-ATRIP and TOPBP1. This indicates that TOPBP1 amplifies ATR mediated signals through mechanism of feed-forward signalling loop containing phosphoserine 1131 (Yoo et al., 2007).

1.4.2.3. ATR signalling at sites of DNA damage

Once ATR is activated, and assembled at the site of DNA damage such as stalled replication fork or a DNA lesion, ATR mediated signalling starts at the DNA damage site to regulate repair and replication. Although, ATR substrates are still under discovery and expanding due to advances in proteomics methodologies, but CHK1 is the best studied Ser/Thr kinase working at checkpoint to regulate the

transition of cell cycle from G2 to M phase (Matsuoka et al., 2007; Mu et al., 2007; Stokes et al., 2007; Smolka et al., 2007).

1.4.2.4. ATR mediated activation of CHK1

CHK1 is phosphorylated by ATR on Ser317 and Ser345 to convert it into an active CHK1. The presence of ATR in the cell is the reliable indicator for CHK1 activation (Walworth et al., 1996; Liu et al., 2000; Lopez-Girona et al., 2001). In contrast to other proteins working at the site of DNA damage, CHK1 sends the signals throughout the nucleus so that DNA damage response can be triggered at the nucleus which is the main hub for the DNA replication. Presumably, the location of CHK1 at the DNA damage site is transient, where it is activated by the claspin protein which serves as mediator located near the moving replication fork (Kumagai et al., 2000). In the event of stalled DNA replication fork or DNA lesion, the claspin becomes activates following phosphorylation at Ser864 and Ser895 (in *X. laevis*) which is through mediated through ATR, however the responsible kinase is still unknown. In this way, claspin brings CHK1 and ATR close to each other at the site of DNA damage (Kumagai and Dunphy, 2003). The activates claspin binds to RAD17 in 9-1-1-complex recruited at DNA damage site, and plays a role in sustaining phosphorylation state of CHK1 (Wang et al., 2006). In addition, the phosphorylation of RAD17 is dependent on ATR, which provides an alternative mechanism for amplification of signals (Bao et al., 2001).

Once CHK1 is phosphorylated by ATR and becomes active, it is released from chromatin to cause phosphorylation of many CHK1 substrates (Smits et al., 2007). CHK1 may phosphorylate CDC25 phosphatases, and make it an inactive CDC25.

CDC25 is responsible for removing the inhibitory phosphorylation from Cyclin-dependent kinases (CDKs) and making them active to cause transition from G2 to M stage (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997). CHK1 is also responsible for phosphorylating P53 which enhances the transcription of p21 and its accumulation at the site of DNA damage. The elevated level of p21 protein inhibits the activity of CDK2, thereby blocking the entry of cell into synthesis (S) phase of the cell cycle. Thus ATR mediated phosphorylation of CHK1 prevents or delays the entry of cell cycle into either S phase or M phase until repair of DNA damage site is executed (Yu, 2007).

ATR signalling through CHK1 is also reported to slow down the replication fork elongation (Unsal-Kacmaz et al., 2007; Mickle et al., 2007), inhibit the replication origin firing partly (Maya-Mendoza et al., 2007), and DNA replication even in the absence of DNA damaging agents (Shechter et al., 2004; Maya-Mendoza et al., 2007), whereby playing a critical role in allowing the replication process to occur without errors. This also help to allow the DNA replication to occur at the reduced rate under DNA damaging conditions (Merrick et al., 2004; Mimitrova and Gilbert, 2000; Alvino et al., 2007; Heffernan et al., 2002).

Apart from CHK1, there are several other ATR substrates at the site of DNA replication fork including RPA, RAD9, RAD17, TOPBP1 and minichromosome maintenance protein-2 and -3 (MCM2 and MCM3). The exact roles of most of these ATR-mediated phosphorylation events are still to be discovered. Nonetheless, there is growing evidence suggesting the possible role of these phosphorylation events in fork stabilization and promotion of completion of DNA replication (Brush et al.,

1996; Wang et al., 2001; Cortez et al., 2004; Liu et al., 2006). For instance, the ATR mediated phosphorylation of MCM proteins help regulate binding of Polo-Like-Kinase-1 (PLK1) which is responsible for completion of DNA replication near the replication fork (Trenz et al., 2008).

1.4.2.5. ATR signalling during DNA repair

ATR phosphorylates several proteins involved in the DNA repair. ATR signalling, specifically, can be observed during the DNA recombination at collapsed replication forks or stalled replication forks. Some of the proteins involved in recombination events and are substrates of ATR include Werner syndrome ATRP-dependent helicase (WRN), BRCA1 and Bloom syndrome protein (BLM) [Pichierri et al., 2003; Davies et al., 2004; Li et al., 2004]. It is not clear as to how ATR alters the activities of proteins through phosphorylation during the recombination events. However, it is quite possible that role of ATR mediated phosphorylation of recombination related protein is to limit the frequencies of crossover events. ATR is also responsible for phosphorylation of Fanconi-Anaemia protein (FANCD2) which regulates the interstrand crosslink repair. ATP mediated phosphorylation of FANCD2 results in bringing it to damage foci and its monoubiquitylation (Andreassen et al., 2004). Finally, ATR also regulates the intracellular localization of XPA which is the NER protein (Wu et al., 2007). Thus it can be concluded that ATR mediated phosphorylation facilitates the repair mechanisms for a variety of DNA lesions.

1.4.2.6. Implication of ATR in Double strand break repair

ATR is primarily known as 'replication stress-response kinase' which is activated in response to single strand DNA at the site of DNA damage. However, it is also reported to be activated in response to double-strand break (DBS) along with ATM which usually becomes activated in response to DBS. Nonetheless, ATM remains activates throughout the cell cycle apart from the stages of cell cycle, but active ATR is concentrated slowly and predominantly at S and G phases of the cell cycle (Jazayeri et al., 2006).

The need for the active ATR at the site of DSB can be realized by considering the fact that during the large parts of single stranded region is revealed during the CDK dependent DSB-end resection. Therefore, ssDNA structure arising from the end resection at DSB results in activating ATR. Several researchers have discovered the activation of ATR along with ATM during DSB-end resection (Jazayeri et al., 2006; Myers and Cortez, 2006; Adams et al., 2006). The second tier of crosstalk between ATM and ATM can be imagined by taking the role of TOPBP1 in ATR activation. ATM plays an important role in activating TOPB1 which then is involved in activation of ATR (Yoo et al., 2007). Several publications have showed that in ATM deficient cells exposed to infra-red radiations (IR), the DSB-end resection was delayed but not prevented. These data show that in the absence of ATM (Jazayeri et al., 2006; Myers and Cortez, 2006; Adams et al., 2006), ATR slowly recognizes the DSB damage and execute the ATM-independent end resection. As a result of this interdependency, the ATM-deficient cells were found to have severe defects related to the cell cycle checkpoints. Similar defects were

observed in the ATR-deficient cells which were exposed to IR (Cortez et al., 2001, Brown and Baltimore, 2003). Furthermore, ATM is reported to initiate the cell-cycle checkpoints response, while ATR is known to sustain this response. It was also reported that activation of ATR was observed after 10 minutes of initiation of DBS-end resection in wild type cells (Jazayeri et al., 2006; Myers and Cortez, 2006; Adams et al., 2006). Similarly, the initiation of G2-M checkpoint was found to be impaired in ATR-deficient cells (Brown and Baltimore, 2003). Thus it is more appropriate to think that both ATM and ATR liaise with each other to generate DSB response at the DSB damage site.

1.5 ATR inhibitors

In this section, the ATR inhibitors are described.

1.5.1 VE-821

Vetex pharmaceuticals discovered the two powerful and selective ATR inhibitors through a high throughput screening campaign (Charrier et al., 2011). One of these compound is VE-821 which is competitive ATP inhibitor of ATR, and interestingly it shows the minimum cross-reactivity with other PIKKs such as ATM, MTOR and DNA-PKcs (Reaper et al., 2011). VE-821 inhibits the ATR mediated phosphorylation of CHK1 at Ser345 which is downstream target of ATR. VE-821 also demonstrated synergy with genotoxic agents from different classes in the colorectal cancer cells HCT116. When VE-821 is administered with cisplatin, it shows more pronounced chemosensitization. In addition, the observed chemosensitization was enhanced in the ATM-deficient cell lines with

mutation/knock-down of p53 expression, or in combination with Ku-55933 – ATM inhibitor. The cytotoxicity of VE0821 is recorded to be minimal in the normal cells, the only effect observed in normal cells is the reversible growth arrest without inducing the apoptosis (Reaper et al., 2011). These findings were supported by another study published in the same year (Sangster-Guity et al., 2011). Several other studies showed that treatment of VE-821 to a variety of cancer cells rendered them sensitive to chemotherapy and ionizing radiations (Pires et al., 2012; Prevo et al., 2012; Huntoon et al., 2013). In vitro, it has been shown that effect of VE-821 on hypoxic cells was reasonably better in terms of rendering them to the sensitive to radiotherapy. This is an interesting finding as the hypoxic cells were found resistant to radiotherapy by previous studies (Sprong et al., 2006; Wouters and Brown, 1997).

1.5.2 VE-822

This inhibitor was also discovered by the Vetex pharmaceuticals, and is an analogue of the VE-821 with enhanced selectivity and potency to inhibit the ATR function. These properties of the increased solubility, selectivity, potency and other pharmacokinetic properties were studied in the background do of the pancreatic cancer cell lines, and observed to radio-sensitize these cells potently (Weber and Ryan, 2015). The growth delay was increased by VE-822 in pancreatic cells in combination with the gemcitabine and ionizing radiations. Interestingly, the cytotoxicity caused by VE-822 in normal cells and tissues was found to be minimal (Fokas et al., 2012). This is the first inhibitor which succeeded the clinal trials and was introduced in the market as VX-970. The studies have shown that cytotoxic

effect of VE-822 in the lung cancer cells in combination with gemcitabine and cisplatin (Hall et al., 2014). The safety, tolerability and cytotoxic effects of this inhibitor in combination with chemotherapy are still in the process (Clinical Trials.gov: NCT02157792).

1.5.3 AZD6738

This is another powerful inhibitor of ATR, and was developed by AstraZeneca. This is an analogue of AZ20 which is classed as a selective and potent inhibitor of ATR. This agent demonstrated ATR inhibition in MRE11A-deficient LoVo xenografts model (Jacq et al., 2012; Foote et al., 2013). The bioavailability, solubility and pharmacokinetic properties are much better than that of AZ20, and it is make suitable for the oral application (Jones et al., 2013). On one hand, it inhibits ATR activity by blocking the downstream phosphorylation of CHK1 (serine/threonine protein kinase). This causes disruption in ATR-mediated signaling, resulting in inhibitory effect on the DNA damage checkpoint activation, disruption of DNA damage repairing mechanism, and induction of apoptosis. Furthermore, it also changes of sensitivity of the tumour cells to the radiotherapy and chemotherapy (Min et al., 2017; Kim et al., 2017). On the other hand, it increases the phosphorylation of DNA DSB marker γH2AX. AZD6738 in combination with ionizing radiations or carboplatin showed the significant level of anti-tumour inhibitory activity in vivo. The anti-tumour activity of AZD6738 on its own was demonstrated in ATM-deficient xenograft models (Guichard et al., 2013; Jones et al., 2013).

1.6 Role of ATR in DNA replication stress and apoptosis

The exposure of cells to the radiations, genotoxic agents, reactive oxygen species (intrinsic stress) and incorporation of abnormal nucleotides can cause DNA damage (Berti and Vindigni, 2016; Collins, 1999). The resulting DNA damage can be repaired in the normal cells through the DNA repair mechanisms, and if the DNA is beyond the repair process, the damaged cells are subject to apoptosis. In the case of sustained growth signaling, the cells without effective DNA damage response or with loss of cell cycle checkpoints will continue to replicate the damaged DNA resulting in the unbridled cell proliferation (Gaillard et al., 2015). In such situation, the DNA polymerase halt at the damaged site of DNA during progression of replication fork, and this condition is termed as "fork stalling". As a result of stalled replication forks, the mini-chromosome-maintenance (MCM) is activated, which is responsible for unwinding the DNA downstream of the replication fork. This process exposes the single-stranded DNA patches which in turn activates ATR signaling cascade resulting in phosphorylation of CHK1, RAD17 and H2AX. These events are collectively referred to as "replicative/replication stress" (Berti and Vindigni, 2016; Zeman and Cimprich, 2014).

Importantly, the replication stress is hallmark of the cancer cells, which is rarely observed in the normal cells. Many studies have suggested that replication stress is caused by the premature onset of the G1-S transition either through the activation of oncogene or the inactivation of tumour repressors such as CDKN2A, RB1, and TP53 (Zhang et al., 2016; Burrell et al., 2013). Consequently, the pre-requisites for accurate replication process such level of nucleotides and replicative enzymes

become insufficient, thereby causing the replication stress. During cell unrestrained cell proliferation, mitochondria produce excessive amount of reactive oxygen species which cause the development of 8-oxoguanine which is responsible for causing base pair mismatch. These events contribute to the replication stress (Macheret and Halazonetis, 2015; Zhang et al., 2016; Valko et al., 2006).

The key purpose of generating the replicative stress is to stall the replication forks and accumulate the ssDNA. The latter is coated with ssDNA-binding proteins such as RPA (replication protein A). RPA activates ATR signaling pathway through which CHK1 is phosphorylated (Berti and Vindigni, 2016; Zeman and Cimprich, 2014). This pathway not only stabilizes the stalled replication forks, but it also causes the activation of cell cycle S-M checkpoints to prevent the entry of cell cycle into mitotic phase (M) (Eykelenboom et al., 2013). When the amount of DNA damage exceeds the number of available RPA molecules for binding, causes the collapse of the replication forks resulting in generation of DSBs (Toledo et al., 2013; Zeman and Cimprich, 2014). It leads to mitotic catastrophe, and consequently the apoptosis is triggered due to accumulation of DSBs (Toledo et al., 2013; Camman, 2001).

Nevertheless, if the ATR and CHK1 are absent, the stalled replication forks can continue to exist while simultaneously other replication origins are fired, thereby exhausting the existing pool of deoxynecleotide triphosphate (dNTPS). These conditions aggravate the replication stress and results in accumulation of non-progressive replication forks (Toledo et al., 2003). Thus Zhang et al (2016) argued that replication stress is enhanced by absence of RPA, ATR or CHK1, leading to

genomic instability and ultimately apoptosis. If the replication stress is of mild or low level, it induces the genomic suitability and increases the tumorigenesis (Berti and Vindigni, 2016; Gaillard et al., 2015, Lacona and Fernandez-Captillo, 2014). However, if the replication stress is of high level, it leads to mitotic catastrophe which further induce the cancer cell death. Therefore, the ATR or CHK1 depletion can be used target for chemotherapy and radiotherapy to counteract cell progression.

1.7 Phosphatase and tensin homologue (PTEN)

PTEN gene encodes the plasma membrane lipid phosphatase which is considered an effective tumour suppressor in a variety of tissues (Di Cristofano and Pandolfi, 2000; Keniry and Parsons, 2008). Drawing on genetic and biochemical studies, it was discovered that PTEN negatively regulate the PI3K/AKT signalling pathway; and the deletion of PTEN activated AKT pathway leading to increased cell proliferation, cell survival and cellular spreading and migration which all are considered critical determinants of cancer progression and development (Di Cristofano et al., 1998; Maehama and Dixon, 1998; Carracedo and Pandolfi, 2008). PTEN mutations in germline cells were discovered in hereditary tumour syndromes such as Cowden syndromes characterized by autosomal dominant phacomatosis and cancerous syndromes related to Proteus and Proteus-like syndromes and Lhermitte-Duclos disease - dysplastic cerebellar gangliocytoma. (Zhoy et al., 2001; Zou et al., 200).

These syndromes represent the increased risk of cancer development and different developmental disorders among the carrier population. The deletion of PTEN in mice led to increased risk of cancer development in different organs such as skin, prostrate and mammary glands (Ming et al., 2011; Backman et al., 2004; Yanagi et al., 2007), highlighting the fundamental part played by PTEN in supressing tumour. Besides, PTEN performs a fundamental role in DNA damage and repair responses. The following subsection details the PTEN functions in the context of DNA damage and repair.

1.7.1 Structure and functions of PTEN

The crystal structure of the PTEN protein (a 403 amino acids protein) was determined by Lee et al (1999), revealing five major functional domains: i) phosphatidylinositol-4,5- bisphosphate (PIP2) binding domain (PBD) is a short domain in N-terminal, ii) a phosphatase domain consisting of a 179 amino acid (aa)-residue (residues 7–185), iii) a C2 domain consisting of a 166aa-residue (residues 186–351), which can bind to phospholipid nearly located to plasma membrane membranes, leading to inhibition of cell migration, iv) a Carboxy-terminal tail and v) a Postsynaptic-density protein Disc large Zona occludens Binding Domain (PDZ-BD) interaction motif (Figure 1.5).

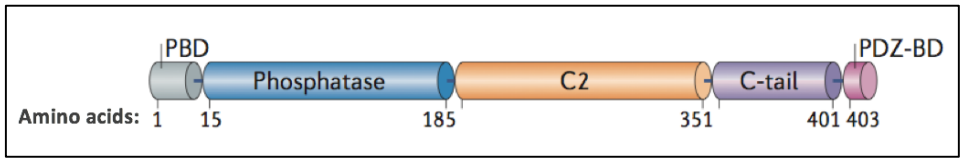


Figure 1-5 Diagrammatic representation of PTEN tumour suppressor protein. PTEN protein comprises of PBD domain phosphatidylinositol-4,5- bisphosphate (PIP2) binding domain; phosphatase domain; C2 domain; C-tail domain carboxyl-terminal tail and PDZ-BD Postsynaptic-density protein Disc large Zona occludens Binding Domain. Adapted from (Brito et al., 2015).

The active sites or signature motif (HCXXGXXR) of protein tyrosine phosphatases (PTPs) and dual specificity protein phosphatases (DSPs, serine/threonine phosphatases) are also found in PTEN lipid phosphatase domain. PTEN phosphatase activity is not restricted to polypeptides (phosphoprotein) such as tyrosine or serine/threonine residues, but it can also act on phosphoinositide substrates (phosphorylated inositol lipids) such as Phosphatidylinositol-triphosphate (PIP3) [(Kakarougkas and Jeggo, 2013; Ratanaphan, 2012; Bozulic and Hemmings, 20009; Brito et al., 2015], which is a phospholipid product of PI3K residues in cell plasma membrane. PTEN also contains a short sequence in the first 190 amino of amino terminal region of acids which includes the signature motif similar to tensin and auxilin (Lee et al., 1999).

1.7.2. PTEN Functions in the context of DNA damage response

The important function of the PTEN is to suppress the spontaneous DNA double breaks in the genome. The study showed that deletion of PTEN from the mouse embryonic fibroblasts (MEF) resulted in higher rate of spontaneous double breaks in the genome than the wild-type MEF (Shen et al., 2007). This showed that genomic stability was compromised in the PTEN-deficient cells. Consistent with

this, Shen et al (2007) showed that double DNA break repair system became defective in the cells lacking in PTEN, which might be due to the absence of PTEN at the centromeres or downregulation of RAD51. In fact, PTEN regulates the RAD51 expression through its action on the chromatin, and is also essential for reducing spontaneous DNA breaks. Several publications have provided evidence relating to association of an increased level of radioresistance and reduced expression of PTEN at cellular level (Rosser et al., 2004). It was revealed that sensitivity of cells to radiation was regained after having sufficient level of PTEN expression which actually repressed the production of gamma-H2AX foci – marker for DNA damage. This suggests that PTEN is responsible for decreasing the levels of DSB (Pappas et al., 2007).

However, another report provided data in contrast to the above findings showing that PTEN was not involved in DSB repair at genomic level in MEF and some other cell lines. They discovered that initial steps in sensing the DNA damage and chromatin modifications were the same in the PTEN-deficient cells as they were in the PTEN-proficient cells (Gupta et al., 2009). This further suggested that the higher level of genomic instability in the PTEN-deficient cells was the result of PTEN deficiency. This discrepancy was likely the outcome of different assays and cell lines used during these experiments. These data indicate that PTEN plays an important role in DSB repair in cell line types and end-points dependent manner. Furthermore, the checkpoint defects in cell cycle within PTEN-deficient cells may result in the DSB formation (Puc and Parsons, 2005). This warrants the further

research to investigate the role of PTEN in different cancer cell types both *in vivo* and *in vitro*.

1.7.2.1. Role of PTEN in NER activity

PTEN is an essential molecule for regulating the NER activity, as the higher expression of PTEN ensured the efficient and effective NER activity (Ming et al., 2007). The mice with downregulated expression of PTEN and exposed to UV radiation were found to predisposed to skin tumour (Ming et al., 2007; Ming et al., 2011). Even in the case of human skin cancer, PTEN was reported to be significantly downregulated in both pre-cancerous and cancerous skin lesions. How does PTEN regulate NER activity? PTEN increases the transcription level of XPC in keratinocytes in order to positively regulate the GG-NER activity. In addition, XPC expression is regulated by the PTEN/AKT/p38 pathway which plays a critical role in GG-NER activity. In the malignant cells with targeted downregulation of PTEN, AKT suppressed the p38 expression level which in turn reduced the XPC expression. Thus the GG-NER activity was impaired in cells with reduced level of XPC in keratinocytes. Furthermore, when the PTEN related deacetylase SIRT1 was inhibited, the consequential acetylation of PTEN inhibited the PTEN activity, AKT activation was increased resulting in suppression of XPC expression through nuclear translocation of p130 – a transcriptional repressor of XPC (Ming et al., 2010). Taken together, these data suggest that PTEN is involved in positive regulation of GG-NER activity by enhancing XPC expression in keratinocytes through AKT, whereby suggesting that PTEN serves as an important genomic

gatekeeper in the epidermis due to its ability to regulate XPC dependent GG-NER as a consequence of DNA damage (Ming et al., 2011).

1.7.2.2. Role of nuclear PTEN in the nucleus

Once PTEN was considered strictly restricted to cytoplasm, however, the research showed that PTEN is also located in nucleus (Lian and Di Cristofano, 2005). In the nucleus, PTEN performs a variety of functions including protecting cells from tumorigenesis and oxidative stress, inducing cell cycle arrest, regulating DNA repair, and cellular stability. PTEN is implicated in regulating the cell cycle by suppressing cyclin D1 expression (Chung et al., 2006). In addition, the apoptotic stimuli activate PTEN which antagonizes pro-survival AKT pathway, thereby preventing the AKT mediated cell proliferation. (Gil et al., 2006). In addition, accumulation of PTEN in the nucleus has been associated with cell cycle arrest at G0/G1 stage of cell cycle (Ginn-Pease and Eng, 2003), showing the potential function of PTEN in cell growth inhibition.

Liu et al (Liu et al., 2005) proposed that translocation of PTEN in the nucleus initiated tumour supressing activities independent of AKT pathway, which was mainly through inhibition of anchorage-independent growth and transition of cells from G1 to S phase of the cell cycle. Independent of ability of PTEN to regulate PI3K/AKT pathway, PTEN is also reported to be implicated in maintaining the genomic integrity through novel function involving its physical interaction with centromere-specific binding protein-C (CENP-C) for sustaining centromere stability and with E2F1 by acting on chromatin in order to regulate transcription of Rad51 for regulating DSB repair and chromosomal destability resulting from the

DSB damage (Shen et al., 2007). The disruption of association between CENP-C and PTEN results in chromosomal aberration at massive scale (Shen et al., 2007). Phosphatase activity is not essential for this association and the resulting protection against chromosomal destability, as the PTEN with mutation in phosphatase domains was still able to interact with CENP-C successfully (Shen et al., 2007).

1.7.2.3. Nuclear localization of PTEN

PTEN does not contain a specific nuclear localization sequences (NLS), however, it is reported that NEDD4-mediated monoubiquitylation of specific sequences in C-terminal domain increases the transport of PTEN from cytoplasm into nucleus (Trotman et al., 2007). Besides, PTEN can be imported to nucleus through several other mechanisms such as passive transport by diffusion (Liu et al., 2005a), active transportation through NLS-like signals (Chung et al., 2005), and cytoplasmic-localization-signal-dependent export. Chung and his colleagues have discovered the Major Vault Protein (MVP) which is a putative nuclear-cytoplasmic protein playing a role in importing PTEN in the nucleus (Chung et al., 2005). This import is dependent on three NLS-like signals including NLS1 - KKDK (amino acids 265-269), NLS2 – RTRDKK (amino acids 260-164), and NLS3 – RREDK (amino acids 233-237). These NLSs direct PTEN movement to nucleus under mediation of MVP, but independent of phosphatase activity of PTEN (Chung et al., 2005). Another research work showed that PTEN is imported to the nucleus in cell cycle-dependent manner and is mediated by PI3K/AKT/mTOR/S6K signalling pathways (Liu et al., 2007). Thus the exact mechanism of PTEN import to nucleus is unclear, and needs further research in this direction. Nonetheless, the partitioning between

cytoplasmic PTEN and nuclear PTEN serves as an important marker for disease severity. For instance, the increased level of cytoplasmic PTEN indicates the aggressive disease state in patients of cutaneous melanoma, esophageal squamous cell carcinoma (Zhou et al., 2000; Whiteman et al., 2002), colorectal and pancreatic tumours (Gu et al., 1998).

1.7.2.4. Interaction of PTEN with CHK1

CHK1, in the cell cycle checkpoint pathway, serves as an important signal transducer, and is found to regulate PTEN activity (Martin and Ouchi, 2008). For example, depletion of CHK1 resulted in reduction in PTEN expression and reduced level of phosphorylation. Importantly, the phosphorylation events mediated by both PTEN and CHK1 are considered essential for recovery of cell cycle from the stalled DNA replication successfully, therefore, CHK1 and PTEN are important components of cell cycle regulatory pathway. ATR phosphorylates CHK1, and phosphorylated CHK1 is essential for phosphorylation of PTEN at Thr383, which is mediated by CKII. The phosphorylated states of both CHK1 and PTEN are essential for cell cycle recovery, which indicates that interaction between CHK1 and PTEN plays a critical role in regulation of cell cycle checkpoints and DNA damage response pathway. The research demonstrated that in PTEN-deficient cells, the CHK1 mediated activation of cell cycle checkpoint was impaired, which was mainly due to sequestration of ubiquinated CHK1 in the cytoplasm (Puc et al., 2005). On exposure to ionizing radiations, PTEN-deficient cells were rendered with partially defective checkpoint. Targeted loss of PTEN was linked to subsequent AKT pathway's activation which impaired CHK1 activation through triggering

phosphorylation, ubuiqtination and decreased localization of CHK1 in nucleus. These events resulted in genomic instability in the carcinogenic cells (Puc et al., 2005). As a result of impaired activity of CHK1 in the PTEN null cells, the DSBs were promoted and accumulated in the cells (Puc and Parsons, 2005). The consequence of PTEN deficiency in cells caused the increased DNA damage along with failure to decrease radiation induced size in human cancer cells, which led to the increased radiosensitivity (Lee et al., 2004).

Another interesting finding reported by (Ming et al., 2011) demonstrated the PTEN loss in UVB induced activation of CHK1 in an AKT independent manner in skill cancer cells. This shows that PTEN influences the DNA checkpoint pathways depending on the wavelength of radiations and associated signalling pathways, types of DNA damage and repair efficiency. (He et al., 2004; Ali, 2000), and response of specific cell types to PTEN loss (Shen et al., 2007; Gupta et al., 2009). Thus it can be concluded that PTEN interaction with CHK1 is critical for regulating DNA damage and cell cycle checkpoints in an AKT independent manner.

1.7.2.5. Cooperative interaction between PTEN and p53

In human cancers, *p53* is found to be the most frequently mutated gene, while *PTEN* is the second most mutated gene (Levine et al., 2004, Yin and Shen, 2008, Simpsons and Parsons, 2001). The p53 is transcriptional factor which regulates several target genes involved in regulation of cell cycle, apoptosis, and genomic stability in response to DNA damage, and is responsible for inducing genes implicated in DNA repair and cell cycle arrest in the event of DNA damage. p53 is also known as tumour suppressor gene which forms an essential link between downstream effector

proteins playing important role in apoptosis or cellular growth arrest (Ali, 2000). There are several publications which have provided evidence showing the interaction between PTEN and p53 (Paez and Sellers, 2003, Ming and He, 2012). PTEN was empirically showed to act as downstream transcriptional target of p53 in order to mediate cellular apoptosis (Backman et al., 2001). The evidence of PTEN as a proapoptotic factor comes from its capacity to antagonize PI3K/AKT signalling pathway (Ali, 2000). Some other studies have suggested that PTEN may also act as an upstream regulator of p53 activity and its expression (Mayo and Donner, 2002; Freeman et al., 2003). PTEN also increases the binding capability of p53 with DNA by having physical interaction with p53 (Freeman et al., 2003). The functional crosstalk between p53 and PTEN was also confirmed by several other studies which showed the direct physical interaction between these two proteins, suggesting the role of PTEN in stability and cooperative action on the function of p53 (Tang and Eng, 2006; Flores-Delgado et al., 2007).

Bilbao et al (2006) demonstrated the stability of acetylated form of p53 in nucleus in response to DNA damage by the physical association of PTEN with p53. These data suggest the cooperative interaction between PTEN and p53 in response to the DNA damage, and which is essential to preserve the integrity of the human genome. In addition, the PTEN and p53 have different functions in different cellular conditions. PTEN is regarded as guardian of genomic integrity in peaceful times, therefore acting like a cellular police force. Nonetheless, p53 role in protecting human genome is performed during emergency situation such as genotoxic stress and DNA damage. Under normal conditions, the expression level of p53 was found

to be extremely low, while it was observed to be increased by many folds under DNA damage and genotoxic stress. Consistent with this, p53 causes the apoptosis and regulates cell cycle checkpoints depending on the nature and severity of the DNA damage. PTEN plays a critical role in p53 mediated growth arrest in G1 phase of the cell cycle, suggesting the PTEN role in genome protection on oxidative stress and damage. p53 conducts its operation during irreparable DNA damage in two phases. In the early phase of the cellular response to DNA damage, p53 induces PTEN and Wip1 (Zhang et al., 2011). The ATM-p53-Wip1 loop coordinates with p53-MDM2 loop in the early phase, whereas the p53-PTEN-AKT-MDM2 loop actively becomes functional in the late phase of cellular response to DNA damage; thus p53 expression is driven to high level in the late phase of DNA damage response through interaction with PTEN (Zhang et al., 2011).

Furthermore, the activities of AKT and MDM2 were inhibited through upregulation of PTEN which also mediated an increase in p53 expression level, whereby inducing apoptosis and G2/M arrest in the case of irreparable DNA damage (Selvendiran et al., 2007). These data showed the role of PTEN in association with p53 to regulate the cell cycle and apoptosis in DNA damage.

1.7.2.6. Interaction of PTEN with PI3K/AKT

PTEN also interacts with PIP3 which is an activator of PI3K/AKT pathway. PTEN is involved in hydrolysing PIP3 and its conversion into PIP2, which means PIP3 is no longer available to activate AKT. The inactivation of AKT triggered by PTEN mediated hydrolysis of PIP3 opposes PI3K by dephosphorylating phosphoinositides at position number 3 (Maehama and Dixon, 1998; Maehama et

al., 2001). Thus PTEN negatively regulates the signalling mediated though AKT-dependent phosphorylation of targets implicated in cellular growth and proliferation, thereby inhibiting the carcinogenesis and oncogenesis (Planchon et al., 2008). Furthermore, PTEN is also responsible for the release of CHK1 into nucleus for DNA repair in response to DNA damage by dephosphorylating PIP3 and consequently preventing AKT activation and CHK1 phosphorylation. Receptor Tyrosine kinases (RTKs) are the high-affinity cell-surface receptors for different peptide/protein hormones such as human epidermal growth factor receptor family (HER) that leads to kinase activation of PI3K in AKT pathway. In PTEN-deficient cells, PIP3 is accumulated at the plasma membrane resulting in activation of AKT, which enhances cell survival, proliferation, growth, metabolism and angiogenesis by regulating AKT dependent phosphorylation of downstream protein targets (Figure 1.6) (Brito et al., 2015). AKT activation can also activate mTORC1 complex (comprised of mTORC, mLST8, DEPTOPC, PRAS40 and PARTORC) by inhibitory phosphorylation of its negative regulators (TSC2 and PRAS40), resulting in the translation of mRNA into proteins which are essential for cell proliferation and growth by phosphorylation of p70 Ribosomal protein S6 kinase (S6K) and eukaryotic translation-initiation factor 4E binding protein 1 (4EBP1) (Zoncu et al., 2011; Vander Haar et al., 2007; Ma et al., 2009). Recent studies had shown that the inactivation of eukaryotic translation-initiation factor 4E (eIF4E), which is a key negative regulator of cell proliferation downstream proteins of mTORC1, plays a prominent role in promoting the growth of sporadic cancers (Dowling et al., 2010; She et al., 2010).

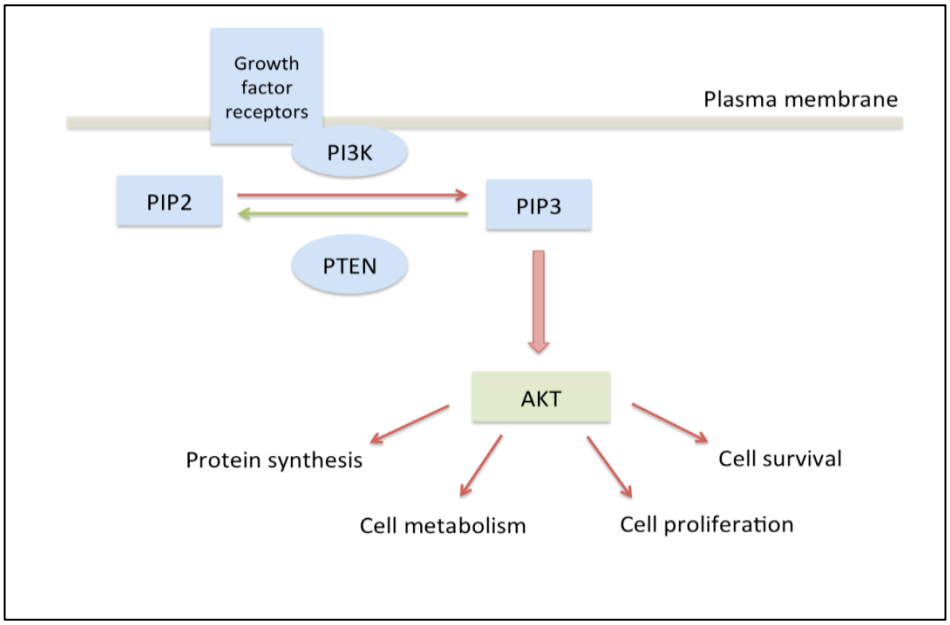


Figure 1-6 Relation between PTEN and PI3/AKT signalling pathway
PTEN phosphatase and tensin homologue; PI3K Phosphatidylinositol-3 kinase; PIP2 Phosphatidylinositol 4,5-bisphosphate; PIP3 Phosphatidylinositol (3,4,5)-triphosphate; AKT Serine/threonine-specific protein kinase B.

Various studies have reported that PTEN-null mice showed high susceptibility to tumour induction in different organs including mammary gland, prostate and skin (Backman et al., 2004; Li et al., 2002). In fact, decreased function of PTEN leads to activation of PI3K/AKT signalling pathway in several tumours types such as prostrate, skin and breast cancer (Khan et al., 2013; Bhaskar and Hay, 2007). To conclude, PTEN performs multiple functions ranging from tumour suppression to the maintenance of genomic integrity by preventing DNA damage and initiating DNA repair by regulating the phosphorylation status of multiple downstream pathways in cytoplasm and nucleus. The summary of the functions performed by PTEN in cytoplasm and nucleus has been presented in figure 1.7. In cytoplasm,

PTEN converts PIP3 into PIP2, and antagonises action of PI3K and AKT, and prevents CHK1 phosphorylation which enables it to enter into nucleus to participate in DNA repair pathways. Cytoplasmic PTEN can enter into nucleus through NEDD4 mediated ubiquitination mechanism. The nuclear PTEN physically interacts with CENP-C which is responsible for centromere stability. PTEN also acts on chromatin to interact with E2F1 in order for regulating RAD51 transcription which is involved in DSB repair pathway.

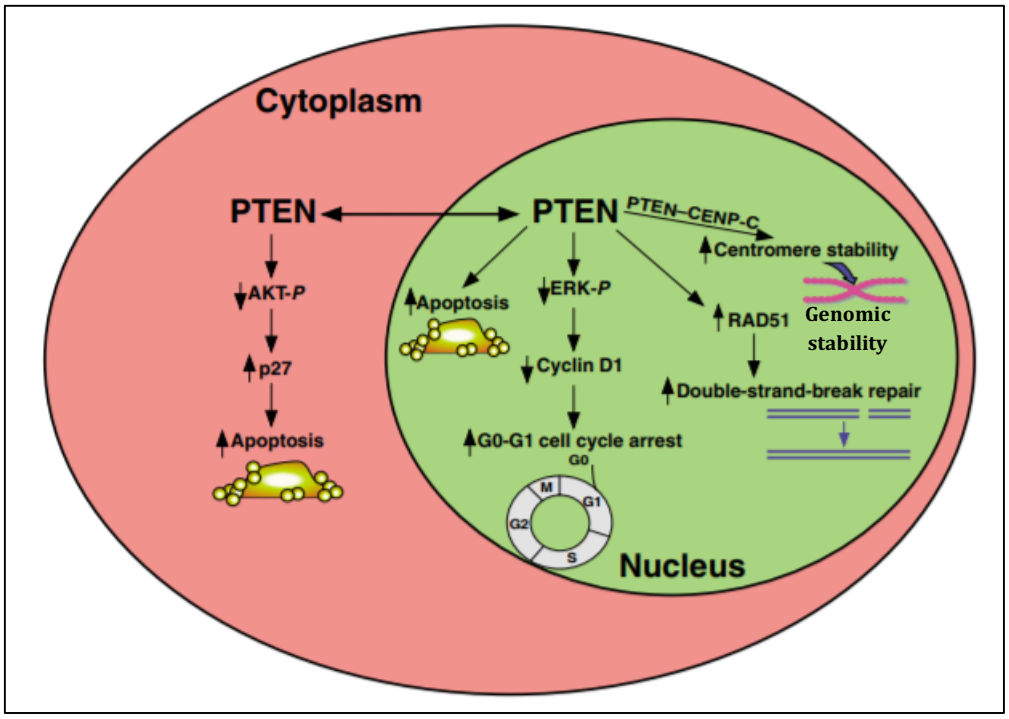


Figure 1-7 Functions mediated by PTEN in cytoplasm and nucleus
In nucleus, PTEN performs a range of functions: downregulation of ERK (MAPK) causing decrease in cyclin D1 and G0-G1 cell cycle arrest; increase in RAD51 levels and DSB repair; enhancement of centromere stability through interaction with CENP-C and overall genomic stability. Adapted from (Planchon et al., 2008).

1.8. Synthetic lethality

According to Rehman et al (2010), synthetic lethality is the phenomenon in which mutation sin two or more than two genes results in cell death, whereas the mutations in any one of them does not cause the cell death or drastic consequences (Figure 1.8). Synthetic word caries its original meaning 'combination of two which gives rise to something new' in Greek language. The synthetic lethality approach is being used to target DNA repair pathways for treating the cancer based on the genetic background of the various cancers.

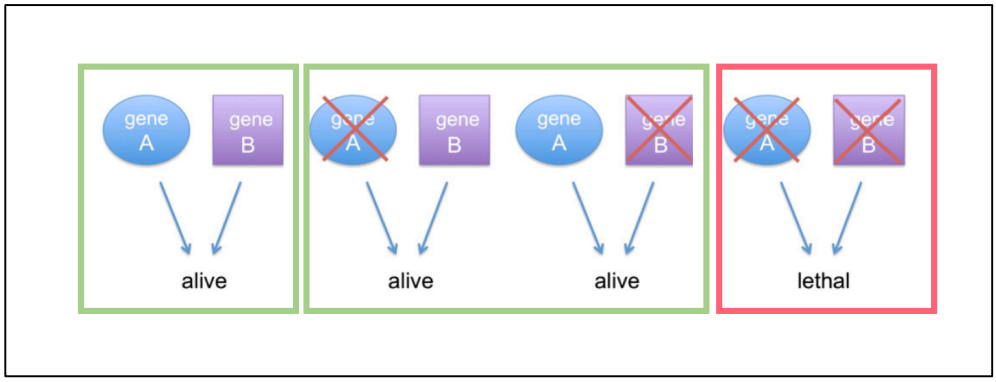


Figure 1-8 Schematic overview of synthetic lethality.
Deletion of either gene A or gene B does not affect viability of cell whereas inactivation of both at the same time is lethal. Adopted from (Nijman, 2011)

The best example of synthetic lethality approach for cancer therapy was shown by the use of PARP1 inhibitors to target BRCA mutations (Bryant et al., 2005; Farmer et al., 2005). PARP1 is an important component of the BER pathway which is implicated in repairing the SSBs. PARP1 deficient cell lines shows aberration in SSB repair, leading to accumulation of DSBs. The cells with defects in DSB repair can not survive. BRCA gene products are involved in the HR pathway. The

BRCA/PARP1 synthetic lethality model suggest that targeting BRCA deficient cell lines (HR deficient cell lines), the PARP1 inhibitors leads to accumulation of SSBs which further leads to development of DSBs during DNA replication. The accumulation of unrepaired DSBs leads to the cell death (Bryant et al., 2005; Farmer et al., 2005). The BRCA cell lines (BRCA1 and BRCA2) treated with PARP1 inhibitors (KU0058684 and KU0058948) showed the reduced survival due to G2/M arrest.

After the experimental evidence of BRCA/PARP1 synthetic lethality models, this model was tested in clinical trials using Olaparib drug (PARP1 inhibitor) to target BRCA deficient tumours in cohort of patients (Fong et al., 2001; Balmana et al., 2011; Tutt et al., 2010). Both Phase I and II trials showed that Olaparib demonstrated antitumor activity in the BRCA deficient tumours, and PARP1 inhibitors were recommended for treating BRCA deficient tumours (Chan and Mok, 2010; Balmana et al., 2011).

1.9. Hypothesis and Aims of the study

In this study, we hypothesized that PTEN plays key roles in breast carcinogenesis and that inhibition of ATR in the context of PTEN deficiency can provide a novel therapeutic approach through a synthetic lethality mechanism. To test this hypothesis, we have used a large and well-characterized series of breast cancer with a large panel of related biomarkers available to investigate the clinicopathological and biological significance of PTEN protein expression in breast cancer. Breast cancer cell lines representing PTEN-proficient and deficient status were investigated for the effect of ATR inhibition to evaluate the potential therapeutic approach and to produce synthetic lethality of PTEN-deficient breast cancer.

Aims

1. To assess expression and subcellular localization of PTEN in a large well-characterised clinically annotated primary invasive BC series using IHC.
2. To investigate the correlation of PTEN with: i) clinicopathological features, ii) other relevant and key biomarkers involved in BC development and progression, iii) Key proteins involved in DDR with focus on ATR-CHK1 axis, iv) patients' outcome in terms of breast cancer specific survival (BCSS).
3. To assess the mRNA and protein expression of DNA repair genes in a panel of PTEN-proficient and PTEN-deficient breast cancer cells and to determine whether PTEN deficiency is associated with DNA repair alterations.
4. To investigate the effect of ATR inhibition in PTEN-deficient and PTEN-proficient human breast cancer cells.

5. To investigate whether synthetic lethality relationship exists between ATR and PTEN using ATR inhibitor in PTEN-deficient and PTEN-proficient human breast cancer cells.

2. Materials and Methods

2.1. Clinical study

2.1.1. Patient characteristics in the study cohort

This study was conducted on a well-characterized consecutive series of 1954 patients of female primary operable invasive breast cancer admitted at Nottingham City Hospital between 1987 and 1998. Patients aged 70 years and under were diagnosed, treated and followed up in a standardized approach. Full clinicopathological data were collected including age, menopausal status, tumour histology such as size, grade (and its components: tubule formation, pleomorphism and mitotic count), lymph node status and Nottingham Prognostic Index (NPI) score. Tumour samples were cored from retrieved formalin fixed- paraffin embedded tissue blocks for Tissue Micro Array (TMA) construction. During TMA construction, several cores were lost or excluded due to insufficient representation of tumour which decreased the total number of informative cases to 1954 cases. The clinicopathological criteria of this series are shown in Table 2.1.

Outcome data were assessed on a prospective basis and these included tumour recurrences (local, regional and distant recurrences) and patients' survival status (alive/lost to follow-up, or dead and death caused by breast cancer, or due to some other cause) and time to event. Breast cancer-specific survival (BCSS) is defined in months as the period between the primary surgery and the death of breast cancer or with evidence of breast cancer (i.e. widely metastatic or locally advanced recurrent disease), with a median survival of 120 months.

Table 2-1 Clinicopathological characteristics of the primary series

Clinicopathological criteria	N (%)
Tumour Size (cm)	
<2.0	1010 (40.4)
>2.0	931 (37.2)
Nottingham Prognostic Index	
Good Prognostic Group (>0.3)	789 (37.2)
Moderate Prognostic Group (3.4-5.4)	1051 (49.6)
Poor Prognostic Group (>5.4)	281 (13.2)
Stage	
1	1502 (66.1)
2	595 (26.2)
3	177 (7.8)
Grade	
1	440 (19.9)
2	757 (34.2)
3	1017 (45.9)
ER	
Negative	549 (26.2)
Positive	1547 (73.8)
PgR	
Negative	761 (40.7)
Positive	1111 (59.3)
HER2	
Negative	1653 (87.8)
Positive	231 (12.3)

2.1.2. Patients' clinical cohort

Adjuvant therapy was given based on a combination of prognostic and predictive factors including NPI, ER status, and menopausal status in 1989. After that year, patients within good prognosis group (NPI score ≤3.4) were not considered to receive adjuvant therapy following surgery. While, tamoxifen [endocrine therapy (anti-ER)] was given to moderate and poor prognosis groups (NPI score >3.4) and ER-positive tumour. Premenopausal patients within the latter group and ER

positive were offered Zoladex [hormonal therapy (reduced ER level)] besides tamoxifen whereas ER negative patients were prescribed chemotherapy if fitted in form of CMF (i.e. a combination of Cyclophosphamide, Methotrexate, and 5-Flourouracil). Adjuvant therapy data of all patients were available including surgical, hormonal, chemotherapy and radiotherapy. None of the patient received either neoadjuvant therapy or any preoperative DNA-damaging therapy.

2.1.3. Molecular biomarkers

Not only clinicopathological and outcome data were available but also a wide range of molecular and biological biomarkers for these patients that carried relevance to breast cancer including hormone receptors (ER and PgR) (done by Prof. Emad Rakha), epidermal growth factor receptor family members (HER1, HER2, HER3 and HER4) (done by Dr. Abd El-Rahim). Moreover, a set of upstream and downstream biomarkers related to PTEN such as PI3K/AKT (done by Dr. Mohammed Aleskandrany) and DNA damage respond [DDR] such as ATR and pCHK1 (done by Paul Moseley and Dr. Tarek Abdel-Fatah) were used for correlation with PTEN (Appendix Table 1).

2.1.4. Tissue arrays and immunohistochemical staining

Construction of the primary series (one core per case) was done by group of pathologist, oncologist and technician (Dr. Dena Akram/PhD Pathology, Dr. Rezvan Abduljabbar/Ph.D Oncology, Dr. Alaa Alshareeda/Ph.D Oncology, Mr. Chris Nolan and Mr. Glynn Donovan/Biobank employer), as previously described (Kononen et al., 1998).

Immunohistochemistry (IHC) approach was used to detect the immunoreactivity of anti-PTEN6H2.1 antibody to the invasive BC tissues and normal breast parenchymal tissues as control with the cores of grid. The key steps involved in immunohistochemical staining have been described as follows.

2.1.4.1 Slide heating

The 4μm thick formalin-fixed paraffin embedded (FFPE) tissue sections were cut from TMAs onto Superforst slides (Surgipath). Following this, the TMA slides were heated on the heat plate (60ºC) for 10 minutes in order for melting paraffin and enhancing the tissue adherence.

2.1.4.2. Deparaffinising and rehydration

Slides were loaded onto Leica Autostainer XL^{TM} (Leica Microsystem) which ran them twice through rehydrating racks and then through xylene racks twice xylene for 5 min each for deparaffinising. After that, slides were soaked 3 times in methanol for 2 minutes each, followed by running them through deionized water 3 times for 5 minutes each for rehydration. The slides were quickly moved to an antigen retrieval step without letting them being dried out.

2.1.4.3. Antigen retrieval

This step was performed on FFPE tissues to retrieve antigen, so that suitable enzymatic and non-enzymatic procedures could be drawn up to analyze such samples. Optimisation of antigen retrieval was done by performing different pH-dependent antigen retrieval methods and the suggested method provided by the manufacture until optimal results were achieved. Antigen retrieval was performed

in three steps. Firstly, the TMA slides were incubated at 98ºC (in water bath) for 25 minutes in target retrieval solution (pH 9.0; Leica Microsystems, Newcastle, UK). Secondly, the slides were incubated for 10 minutes at room temperature in warm Tris buffer Saline (TBS, pH 7.6, 50ºC). In third and final step, slides were cooled by putting them under running tap water (pH 6.0) for 5 minutes.

2.1.4.4. Leica Max Polymer detection system

The manual IHC staining was performed using Novolink Max Polymer Detection System (Leica Microsystems, Newcastle, UK). Slides were loaded onto sequenza plates; and endogenous peroxidase activity was blocked by hydrogen peroxide for 5 minutes to minimize background staining. Peptide activity was blocked by protein blocking for 5 minutes. After each blocking step, the slides were rinsed twice with the wash buffer (TBS) for 5 minutes each. After that, the monoclonal mouse anti-human PTEN antibody (clone 6H2.1, Dako, Denmark) was diluted to 1:25 in commercial diluent called Leica Bond Primary antibody diluent (Leica Microsystems, Newcastle, UK). The diluted primary antibody was applied on to slides, followed by 60 minutes incubation at room temperature. The primary antibody incubation step was followed by washing the slides three times for 5 minutes each. In addition, the negative control was obtained by omitting the antibody addition step, whereas normal ductal breast tissue was taken as an internal positive control based on the Dako data sheet. Following this, Novolink polymer was added on to slides and incubated for 30 minutes, followed by washing slides three times for 5 minutes each. Next, enzyme substrate was added to the tissues on slides and incubated for 5 minutes. Next, the slides were washed once for 5 minutes.

Following this, haematoxylin (counterstaining) was added onto slides and incubated for 6 minutes, followed by rinsing the slides in tap water.

2.1.4.5. Dehydration and mounting

As a final step of the staining procedure, slides were placed 3 times in 100% concentration IMS for 2 minutes each, and cleared twice in xylene for 5 minutes each, and mounted with DPX using the DPX mounter (BDH, Leica Microsystems, Newcastle, UK). Finally, slides were left to dry for 48 hours.

2.1.4.6. Optimization of staining

The optimum staining of tissues is the one which harbors the minimal staining in the background, so that the visualization of characteristics of tumour tissues can easily be made. Therefore, the staining was repeated under different conditions involving various antibody dilutions in order to obtain the optimum staining conditions. Different conditions tried for staining involved serial dilutions and antibody retrieval methods such as anti-PTEN6H2.1 dilution (1:25) and additional dilutions (i.e. 1:175, 1:150, 1:125, 1:100, 1:75, 1:50, **1:25**). Antigen retrieval solution (pH 9.0) as recommended in the supplier (Dako) and from some previous publications.

2.1.4.7. Assessment of immunohistochemical staining (H-score)

Initially, a qualitative assessment of the staining of tumour tissues, negative control and positive control was performed using light microscope. Following this, slides were scanned using a NanoZoomer slide scanner (Hamamtsu Photonics, Welwyn Garden City, UK) to obtain the high-resolution digital images (0.45 μm/pixel)

which were uploaded onto an accessible server using web-based interface (Distiller, SlidePath Ltd, Dublin, Ireland). Immunohistochemical staining was assessed at 20x magnification using a minimum of a 24-inch high resolution screen (1920x1080). In this study, the assessment of the staining and immunoreactivity was performed using a semi-quantitative approach called modified histochemical score (modified H-score) which was obtained by multiplying the percentage of the invasive tumour cells (if malignant cells represent 15% or more of the core) and the intensity of the staining (0, 1, 2, or 3 corresponding to negative, weak, moderate, or high, respectively) as prescribed by Abd-El-Rehim et al (2005). The resultant H-score had been generated in the range of 0-300. To ensure reproducibility of the H-score, at least 25% of the TMA cores were randomly scored by a pathologist colleague Dr. Abir Hadia. Concordance between two H-scorers was determined by using the intraclass correlation coefficient (ICC). An ICC value less than 0.40 represents poor agreement; between 0.41 and 0.60 represents moderate; 0.61 - 0.80 was considered substantial, and greater than 0.81 as a perfect agreement. All cases were scored without knowledge of the patients' pathological or outcome data. In the event of discrepancy between H-scores, two independent scorers revaluated the core and the agreed H-score was used for analysis.

2.1.4.7. Statistical analysis

In this study, statistical analysis was performed using Statistical Package for the Social Sciences SPSS v23 for Mac (Chicago, IL, USA). A p-value less than 0.05 was considered to be significant.

2.1.4.8. Selecting immunohistochemical cut-off points:

In this study, cut-off points regarding the nuclear and cytoplasmic PTEN were determined based on the normality distribution of the H-score data. The data were tested using the Shapiro-Wilk test in which median was selected for the non-normally distributed data while mean for normally distributed data. Moreover, previously generated cut-offs of nuclear and cytoplasmic PTEN from large studies were taken in consideration if they were available. The generated cut-off values classified patients into three categories: PTEN negative group, PTEN low group, PTEN high group. Cutoff values for H-scores were included in the positive/high groups.

2.1.4.9. Statistical procedures used for analysis

Due to multiple comparisons, p-value from the two-tailed t-test was considered significant for all statistical tests if p-value was found to be less than 0.05. Benjamini-Hochberg correction method was applied for multiple testing adjustment, which is a useful approach for reducing the type I error which refers to the chance of the rejecting the true hypothesis (false positive). According to this mothed, p values are arranged in ascending order beginning from higher significant values on top to lower significant values follow the higher values. Following this, the ranks are given to the p values; the highest rank provided to the most significant p values. Benjamin-Hochberg correction used the following formula to calculate the adjusted p value. Adjusted p value = Q(i/m), where Q, I and m are equal to the false discovery rate, individual p values ranks, the total number of tests,

respectively (Benjamini and Hochberg, 1995). If P value's Benjamini-Hochberg is ≤0.05, then it is considered significant.

Univariate analysis using Chi-squared test (x^2) was performed to examine the correlations with: 1) categorical clinicopathological variables that already have cut-off points, 2) range of markers that biologically and clinically relevant to breast cancer. In order to draw prognostic analyses, the Kaplan-Meir survival analysis was performed to plot survival curves using a long-rank test. After finding the significant association of biomarkers with patient's outcomes in Kaplan-Meier analysis, prognostic independency and confounders relating to the investigated biomarkers were determined using cox regression test for multivariate analysis. To improve accuracy and precision, well- known standard prognostic factors: tumour grade and size and tumour stage were used. All non-significant factors were excluded from cox regression test to show the real association between the investigated marker and survival (Willner et al., 1997).

2.2. Pre-clinical study

2.2.1. Compounds and reagents

VE-821[3-Amino-6-[4-(methylsulfonyl)phenyl]-N-phenyl-2-pyrazinecarboxamide] was purchased from selleckchem, UK (10mM/1mL). Further novel ATR inhibitor AZD6738 was obtained from AstraZeneca, UK. All inhibitors were diluted in dimethyl sulphoxide (DMSO, Sigma-Aldrich, UK). Drug-free controls were exposed to less than 1% (v/v) DMSO.

2.2.2. Breast cancer cell lines and culture media

All cell lines were human breast adenocarcinoma adherent cell lines. MCF7 (used between passage 25 and 38) and MDA-MB-231 (used between passage 44 and 56) were purchased from ATCC® then cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) and Minimum Essential Medium Eagle (Sigma-Aldrich) supplemented with 1% L-glutamine (200 mM) and 1% non-essential amino acids (0.1 mM), respectively. BT-549 (used between passage 40 and 55) and MDA-MB-468 (used between passage 48 and 62) were obtained from Cell Line Service (Eppelheim, Germany). In this study, all cell lines were purchased from ATCC® and Cell Line Service which provide a certificate of validation authentication. Experiments were performed in triplicate (i.e. cells lines were passaged at three different times). All cell lines were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, Carlsbad, USA); and all media were supplemented with 10% foetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma). In this study, the selection of cell lines was based on the status of *PTEN*. MCF7 and MDA-MB-231 cells express wild type *PTEN* while BT-549 cells and MDA-MB-468 cells harbor mutations in *PTEN*. The molecular profile of the selected panel is summarized in Table 2.2.

Table 2-2 Selected breast carcinoma cell lines with identified ER/PgR/HER2 immunoprofile and mutations, especially PTEN mutations

Cell line	ER/PgR/HER2 Immunoprofile	*PTEN* status	PTEN mutation type	Mutant genes
MCF7	ER+/PR+/HER2-	Wild type	-	-
MDA-MB-231	ER-/PR-/HER2-	Wild type	-	BRAF,CDKN2A, KRAS, NF2, TP53
BT-549	ER-/PR-/HER2-	Mutant	Deletion 822delG Substitution 821G>T	PTEN, RB1, TP53
MDA-MB-468	ER-/PR-/HER2-	Mutant	Substitution 253+1G>T	PTEN, RB1, SMAD4, TP53

2.2.3. Tissue Culture

All cell lines were cultured in a Microflow Laminar II fume hood (MDH) using sterile tissue culture flasks, multi-well plates and sterile medium and solutions. Cells were cultured as adherent cultures in a humidified 5% CO_2 incubator at 37°C. All media were pre-warmed to 37°C prior to use and were replaced three times per week to maintain the cell lines.

2.2.4. Subculture of cell lines

Cells were grown to 70–80% confluency then washed two times with 1x calcium- and magnesium-free Dulbecco's phosphate buffered saline (DPBS; Sigma). Following this, the cells were incubated with 1x trypsin/ethylene diamine tetra acetic acid (EDTA) solution (PAA/Sigma) for 5 minutes at 37°C to detach the adherent cells. After that, the tissue culture medium was added to neutralize the trypsin solution, followed by centrifugation at 200 x *g* for 5 minutes to remove the residual trypsin. The cell pellet was re-suspended in the culture medium, and re-plated into tissue culture flasks. The tissue culture were routinely checked for the mycoplasmic contamination. Furthermore, all cell-based experiments were performed using cells in the logarithmic phase and within 15 passages after revival.

2.2.5. Cryopreservation of cell lines

Cryopreservation of cells was performed at a low passage number for future use. Prior to freezing cells, they were trypsinised and counted using a haemocytometer. The 1 x 10^6 cells were resuspended in a 1 ml of freezing medium, stored in a cryovial at -80°C overnight, and then transferred to liquid nitrogen for long term

storage. Freezing medium contained complete growth medium for the cell line and 10% DMSO. Cells were recovered from liquid nitrogen by thawing rapidly in a water bath at 37°C, followed by suspending them in the 9 ml fully supplemented culture medium. Next, they were centrifuged at 1000 rpm for 5 minutes in order to remove traces of DMSO. The medium was then removed and the cell pellets were resuspended in medium before transferring them to the tissue culture flasks.

2.2.6. Viable cell counting

All cell counts were carried out using a Neubauer haemocytometer. Cells were diluted in 1:1 ratio with trypan blue solution (i.e. 50 µl cell suspension + 50 µl trypan blue solution), followed by mixing the mixture and incubating it at room temperature for 5 minutes. Next, Cell suspension was loaded onto the Neubauer haemocytometer; and cells were counted using standard methods. Trypan blue is a vital dye which is taken up by cells with damaged membranes; therefore, all the cells without having Tryptan blue dye were counted as viable cells. All counts were carried out in duplicate for each experiment. Average number of cells in one large square of haemocytometer x 10^4 x dilution factor= cells/ml.

2.2.7. Profiling of DNA repair gene expression in breast cancer cell lines using an RT^2 profiler DNA repair PCR array.

The expression of DNA repair genes in PTEN-proficient and PTEN-deficient breast cancer cells was determined using RT2 profiler DNA repair PCR array. The protocol followed to conduct the experiment has been given as follows.

The total RNA extraction was performed using RNeasy Mini Kit (Qiagen) by following manufacturer's instructions. Briefly, RNeasy lysis buffer RLT (350 µl) was added to cell pellet (3 x 10^6 cells), followed by vortexing the mixture to give a homogeneous mixture. The sample was disrupted and homogenized by repeated aspiration through a fine-gauge needle. An equal volume of 70% ethanol was added to the lysate and 700 µl of the sample was then transferred to an RNeasy mini spin column perched in a 2-ml collection tube. The column was centrifuged at 8000 x *g* (≥10,000 rpm) for 15 seconds, and then the flow-through was discarded. Finally, 350 µl kit wash buffer RW1 was added to the column, and the centrifugation step under same conditions was repeated.

DNase digestion was performed using the RNase-free DNase set (Qiagen); 80 µl DNase I incubation mix (pre-prepared as a 1:8 dilution DNase I: buffer RDD) was added to the RNeasy column membrane and incubated at room temperature for 15 minutes. An additional 350 µl wash buffer RW1 was then added to the column, and the centrifugation was performed at 8000 x *g* (≥10,000 rpm) for 15 seconds. Then, buffer RPE (pre- prepared as a 1:5 dilution in absolute ethanol) was added to RNeasy spin column and centrifuging it at 8000 x *g* after each wash for two times. The total RNA was eluted from the spin column by adding the 50 µl RNase-free water to it and centrifuging at 8000 x *g* for 1 minute. The integrity and concentrations of the resulting total RNA were determined by measuring the absorbance of the RNA samples at 260 nm using a nanodrop spectrophotometer (Wilmington, USA). An absorbance of 1.8 or greater indicated a good quality of RNA.

2.2.7.1 cDNA synthesis

cDNA synthesis was performed using the RT^2 First Strand Kit (Cat No; 79254330401, Qiagen) according to manufacturer's instructions. The genomic DNA elimination mixture was prepared by adding the RNA sample (0.5 µg), 2 µl of the GE buffer, and RNase-free water to bring the final volume to 10 µl. The DNA elimination mixture was centrifuged for 10 seconds at 8000 x g. Then the mixture was incubated for 5 minutes at 42°C, followed by placing it immediately on ice for at least 1 minute. Next, 10 µl reverse-transcription mixture [4 µl 5x buffer (BC3), 1 µl of random hexamer Primers & External Control Mix (P2), 2 µl Reverse Transcriptase Enzyme Mix (RE3), and 3 µl nuclease-free water] were added to a sterile 1.5 ml eppendorf. Then, the 10 µl reverse-transcription mixture was added to each Eppendorf tube containing the genomic DNA elimination mixture, and the samples were incubated for 15 minutes at 42°C. The reaction was stopped by incubating it for 5 minutes at 95°C. Finally, 91 µl RNase-free water was added to the cDNA and kept on ice prior to proceeding with qRT-PCR or the cDNA was stored at -80°C.

2.2.7.2 RT^2 Profiler^{TM} DNA Repair PCR Array

The expression of 84 DNA repair genes was evaluated using the RT^2 Profiler DNA repair PCR array, and as a part of procedure, cDNA samples were added to RT^2 SYBR Green Mastermix (Qiagen) by following the manufacturer's instructions. The PCR mixture was prepared as shown (Table 2.3)

Table 2-3 Reaction mix for Quantitative real time PCR (RT-qPCR)

Reagent	Volume
RT2 SYBR Green Mastermix	1350 µl
cDNA synthesis reaction	102 µl
RNase-free water	1248 µl
Total	**2700 µl**

25 µl of the PCR reaction mixture was added to each well of the PCR array plate (PAHS-042ZC); the plate was sealed with the optical adhesive film and centrifuged for 60 minutes to remove the bubbles. Next, RT-PCR was carried out on an Applied Biosystems 7500 FAST cycler under the following PCR reaction conditions: 1:10 minutes at 95 ^{0}C, 2-41:15 seconds at 95 ^{0}C and 1 minute at 60 ^{0}C. Furthermore, the quality control was added to exclude the possibility of DNA contamination.

Qiagen RT2 profiler PCR array was used in this way, which contained 96 wells. Out of 96 wells, 84 wells (A1-G12) contained 84 DNA repair genes, 5 wells (H1-H5) contained 5 house-keeping genes (β–actin, B2M, GAPDH, HPRT1, and RPLP0) which were used for normalization of the array data, and 3 wells (H7-H12) carried the internal controls for assessing PCR reproducibility, RT efficiency, and detection of genomic DNA contamination (Figure 2-1).

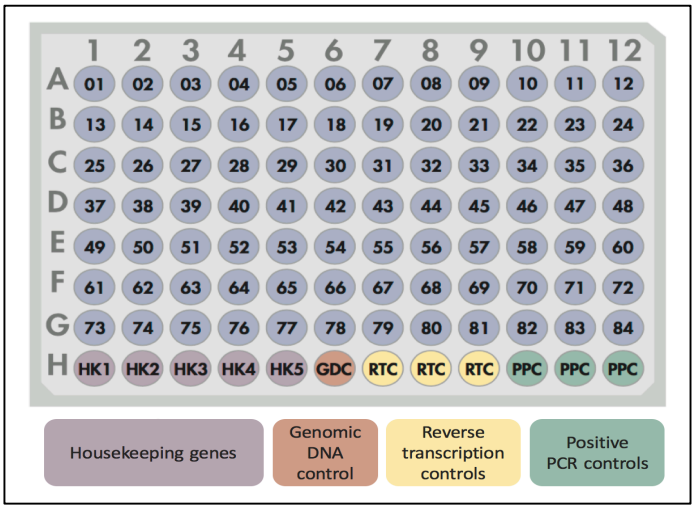


Figure 2-1 Layout of 96-well Qigen RT^2 Profiler^{TM} DNA Repair PCR Array. Adapted from (Qiagen, 2011).

2.2.7.3. Data analysis

Raw data from the RT^2 Profiler was uploaded onto the website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php?target=upload)., and integrated Web-based software package automatically calculated comparative threshold cycle (ΔΔCt) based fold-change calculations. The software automatically used GAPDH to normalize the data. Following normalization, in each cell line, the relative expression of each genes was averaged for two samples. The fold changes in expression of each gene was determined by calculating the difference between the PTEN-deficient cells and PTEN-proficient cells. P-value equal to or less than 0.05 was used as a measure of statistical significance. However, student t-test was performed was performed to obtain statistical significance for RT-qPCR data. GraphPad prism software (6.02 version) was used to analyze the data. This Experiment were performed in duplicate (i.e. cells lines were passaged at two different times) due to the expensive cost of the RT^2 Profiler DNA repair

PCR array

2.2.8. Quantitative real time PCR (RT-qPCR)

RT-qPCR was performed to confirm the upregulation and downregulation of DNA repair genes as demonstrated by RT2 Profiler DNA Repair PCR array. RT-qPCR was conducted using QuantiTect Primer (Qiagen, UK) as per supplier's instructions. Briefly, the reaction mixture was prepared for RT-qPCR as shown in table 2.4.

Table 2-4 cDNA synthesis reaction mix using RT^2 first strand kit.

Reagents	Volume
cDNA from RT^2 first strand synthesis	2µl
10x QuantiTect primer	1µl
Syber Green Mastermix	5µl
RNase-free water	2µl
Total volume	**10µl**

PCR reaction mixture was added to each well in the PCR plate (Applied Biosystems, UK). RT-qPCR reaction was conducted using an Applied Biosystems 7500 FAST cycler to quantify the expression of mRNA using SYBR green detection (Applied Biosystems, UK). The thermal cycler was maintained at the following conditions: initial incubation at 95 ^{0}C for 10 minutes, 40 cycles at 95 ^{0}C for 15 seconds, and 60 ^{0}C for 60 seconds. After 40 cycles, the amplicons were heated from 60 ^{0}C to 95 ^{0}C for 20 minutes in order to perform melting curve analysis. The presence of single melting peak showed the specificity of the products and excluded the possibility of primer-

dimers Non-template control was included to exclude the contamination of PCR reagents. In addition, two RT-negative controls were included to confirm the absence of residual genomic DNA. The plates were sealed with optical adhesive film and centrifuged for 1 minute at 1000 x *g* to remove bubbles. Normalization of samples against GAPDH was performed to determine fold change in expression status of PTEN-deficient breast cancer cells versus PTEN-proficient breast cancer cells. Also, the C_t value of the target gene was normalized to the housekeeping gene GAPDH. Table 2.5 shows genes and primers which are used to conduct RT-qPCR.

Table 2-5 List of primers used in RT-qPCR

Gene symbol	Source	Catalogue number
PTEN	Qiagen	Hs_PTEN_4_SG
ATR	Qiagen	Hs_ATR_1_SG
CHK1	Qiagen	Hs_CHK1_1_SG
GAPDH	Qiagen	Hs_GAPDH_1_SG

2.2.9. Western blot analysis

2.2.9.1 Cell lysate preparation for electrophoresis

70-80% of cells ($2x10^6$) were harvested and washed with PBS. To lyse the cells, the cell pellet was treated with a 100-µl RIPA lysis buffer (20 mM Tris, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS; Sigma) containing 10% protease inhibitor and 1% phosphatase inhibitor 2 & 3 cocktails (Sigma). The cell suspension was incubated on ice for 1 hour, then

was repeatedly aspirated through a fine-gauge needle to maximize lysis before being incubated at -20°C overnight. Following overnight incubation, the suspension was centrifuged at 10,000 x *g* for 30 minutes at 4°C, followed by transferal of the supernatant to a clean Eppendorf tube. The resulting supernatant was stored at -20°C. Equal volumes (10µl) of lysate were mixed with 5 µl lithium dodecyl sulphate (LDS) sample buffer, 1 µl Dithiothreitol (DTT) (Novex Life Technologies) and dH_2O. The mixture was incubated at 100ºC for 10 minutes, and then placed on ice.

2.2.9.2 SDS-PAGE

Polyacrylamide gel electrophoresis and protein transfer samples were loaded onto precast 4-12% SDS gels (Novex Life Technologies) and run in 3-(N-morpholino) propanesulphonic acid-sodium dodecyl sulphate (MOPS-SDS) NuPAGE® MOPS-SDS running buffer (Novex Life Technologies) under reducing condution by adding 1000µl of proprietary antioxidant (Novex Life Technologies), at 150V for 90 minutes,

2.2.9.3 Protein transfer

Using the Xcell II Blot Module (Invitrogen), proteins were transferred from the gel onto a nitrocellulose membrane (GE Healthcare, UK) in transfer buffer [20% (v/v) methanol, 50 mM Tris-HCl, and 380 mM glycine] at 30 V for 60 minutes.

2.2.9.4 Immunoblotting

Membrane was blocked by incubating it with PBS-T (PBS, 0.1% Tween-20) containing 5% non-fat milk for 60 minutes at room temperature. Following this,

membrane was incubated with primary antibody diluted in blocking buffer for 60 minutes at 37°C or overnight at 4°C. After washing the membrane three times for 5 minutes each in PBS-T, the membrane was incubated with infrared dye-labelled secondary antibody in 1:10,000 dilution (Li-Cor, Lincoln). Next, the membrane was washed 3 times (10 minutes each) with PBS-T. Finally, protein expression was detected by a Li-Cor Odyssey Scanner.

2.2.9.4 Primary antibodies

The primary antibodies used in Western blotting experiments are listed with their details in Table 2.6.

Table 2-6 List of primary antibodies used in Western blot

Antigen	Antibody	MW (kDa)	Source	Catalogue#	Dilution
PTEN	Mouse mAb	55	Dako	M362729	1:100
ATR	Rabbit pAb	300-376	Cell signaling	2790	1:1000
CHK1	Rabbit pAb	54	Abcam		1:1000
pCHK1^{Ser345}	Rabbit pAb	54	Abcam	Ab58567	1:1000
β-actin	Mouse mAb	42	Sigma	A2228	1:10000
β-actin	Rabbit mAb	42	Sigma		1:10000

2.2.9.5 Data analysis

β-actin was used for normalization of the data. This assay was performed in duplicate and triplicate; the student t- test was used to calculate the significant difference between control cells and mutant/knockdown cells. A p-value ≤ 0.05 was

defined as significant. Statistical analysis was performed using the GraphPad prism software version 6.02; data were presented in the form of graphs.

2.2.10. Aqueous nonradioactive cell proliferation assay (MTS assay)

The Cell Titer 96 Aqueous One Solution Reagent contains a tetrazolium compound ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The Cell Titer 96 Aqueous One Solution Reagent is bio reduced by cells into a coloured formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) produced by dehydrogenase enzymes in metabolically active cells. The quantity of the formazan product as measured by its absorbance at 490 nm is directly proportional to the number of living cells in the culture.

MTS assay was performed as per the manufacturer's recommendations (Promega, US) in order to assess the cytotoxicity of varying concentrations of VE-821 [ranging from 1μM to 7μM and AZD6738 [ranging from 0.25μM to 3μM]. The selected cell lines were plated in a 96-well plate at a density of 1000 cells per well. The cells were allowed to adhere overnight in an incubator, followed by addition of an inhibitor to the cells at varying concentrations. The solvent-treated control (i.e., cells treated with 0.1% DMSO, which exceeds the concentration in the treated samples) was included in all experiments to test whether the cytotoxicity effects were related to vehicle or inhibitor exposure. The

MTS assay was performed on day 7 by adding 20 µl of CellTiter 96 Aqueous One Solution Reagent per well and incubating the plate for 1–4 hours under normal incubator conditions. An absorbance at 490 nm was recorded with a 96-well plate reader (Magellan, Tecan Group Ltd., Switzerland).

2.2.10.1. Data interpretation and analysis

Results are expressed as the surviving percentages of cells and were calculated using the relative absorbance by comparing the absorbance in the presence of the inhibitor to the absorbance in the absence of the inhibitor (in the solvent-treated control). The MTS assay was performed in triplicate in three independent experiments.

$$\textbf{Surviving percentage} = \frac{\textbf{Absorbance of treated cells (in \%)}}{\textbf{Absorbance of solvent-treated control cells (in \%)}}$$

The percentage of surviving cells was plotted on the Y-axis versus different concentrations of inhibitor on the X-axis. Data analysis was performed using Microsoft Excel.

2.2.11. Gamma-H2AX and cell cycle analysis by flow cytometry

The breast cancer cell lines under investigation were grown to 70-80% confluency, and were harvested. Approximately 100,000 cells were seeded in 6-well plate, and left overnight to allow them adhere to the wells of 6-well plate. Next, the VE-821 compound, an inhibitory compound for ATR, was added to cells, and cells were harvested at 24 hours and 48 hours of exposure to the

inhibitor. Following this, the cells were fixed in 70% ethanol and stored at 4 ^{0}C. Suspensions were centrifuged to discard the ethanol from the fixed cells. Resulting sampls were processed using H2AX Phosphorylation Assay Kit (Merckmillipore, Nottingham, UK) as per instructios from the manfucaturer. The fixed cells were washed twice in 1 x PBS, and after each wash, the cells were centrifuged at 1000rpm for 5 minutes to obtain the cell pellet. The cell pellets were resuspended in 50 µl of 1 x permealization solution (Millipore Company, UK) diluted in Diethylpyrocrabonate water (DEPC H2O). The cells were incubated with 3.5 µl of primary anti-phosphohistone H2AX-FITC (Ser-139) antibody (Millipore Corp) on ice for 15 minutes. In order to wash away excessive FITC labeled primary antibody, the cells were washed with 200 µl of the washing solution, and were centrifuged to remove the supernatant. Following centrifugation, the cell pellets were resuspended in 100µl of 100µg/ml RNAse diluted in PBS, and incubated at room temperature for 15 minutes. Following incubation, the cells were incubated with 400 µl of 2.5µg/ml PI diluted in PBS for 15 minutes before the FACS analysis was performed on cells.

2.2.11.1. Data analysis

The analysis on samples were performed using Cytomics F500 Flow cytometer (Beckman Coulter, USA). Propidium iodide was excited using 488 nm laser beam; and 620 nm bandpass filter (FL3) was used to detect the fluorescence emission. FITC-anti-phospho-histone H2AX was excited with 488 nm laser beam, and 525 nm bandpass filter (FL1) was used to collect the fluorescence

emissions.

At least 10000 cells from each sample were analyzed, and after this, Weasel (Victoria, Australia) flow cytometry analysis software was utilized to analyze the data obtained from the samples. The Statistical analysis and graphical presentation of data were performed using GraphPad Prism 6 (GraphPad, LaJolla, USA).

2.2.12. Apoptosis detection by FITC-Annexin V flow cytometry

Apoptosis detection by FITC-Annexin V flow cytometry was performed using the (Fluorescein isothiocyanate FITC-Annexin V apoptosis Detection Kit (BD Pharmingen, San Jose, USA) as per manufacturer's instructions. The cells were grown to 70-80% confluence and harvested. 100,000 cell were seed in 6-well plate; and allowed to adhere to wells for 24 hours. Following this, the inhibitory compound (VE-821) at 5 µm concentration was added to the cells. The cells were collected at two different time points - 24 hours and 48 hours. Next, the cells were fixed in 70% ethanol, and washed twice with the cold PBS. Following this, the cells were resuspended in 1 x Binding Buffer (BioLegend, UK) at a concentration of 1 x 10^{-5} cells/ml. From the solution, 100µl (1 x 10^{-5} cells) was placed in a 5ml culture tube to which 5 µl of FITC Annexin-V (ImmunoTools, Germany) and 5 µl of propidium iodide (PI) were added. Following this, the cells were vortexed gently, and incubated for 15 minutes at room temperature in the dark. 400 µl of 1 x Binding solution was added to each tube, and samples were analyzed by FACS within an hour.

2.2.12.1. Data analysis

Cytomics F500 flow cytometer (Beckman Coulter, USA) was used to analyze the samples at 488nm excitation wavelength using a 620 nm band pass filter for data collection. Data was analyzed using Weasel (Victoria, Australia). The assay was performed in triplicate, and the student t-test was applied to calculate p-value (statistical significance) for difference of sensitivity between PTEN-deficient and PTEN-proficient cells before and after the treatment with inhibitors. The statistical significance was defined as $p \leq 0.5$ value. Percentage of apoptotic cells in early apoptosis (FITC Annexin V positive, PI negative) and late apoptosis (FITC-Annexin V positive, PI positive) were ascertained, and compared between the untreated and treated samples. The Graph pad prism software (6.02 version) was used to calculate the statistical values and to draw the graphs.

3. PTEN Protein Expression in Breast Cancer

3.1. Introduction

The phosphatase and tensin homolog (PTEN) encodes the lipid-protein phosphatase, and functionally it is well-known tumour suppressor in a variety of tissues in the human body (Saal et al., 2005; Zhou et al., 2007). PTEN is a negative regulator of PI3K/AKT pathway through considerable genetic and biochemical studies. The loss of PTEN function in different cancers led to the activation and increased activity of AKT which is responsible for cellular proliferation, cell growth, spreading, migration, survival and tumour progression (Keniry and Parsons, 2008; Di Cristofano et al., 1998; Maehama and Dixon, 1998; Carracedo and Pandolfi, 2008). The PTEN was found to be mutated in germline in several forms of hereditary tumour syndromes such as Proteus syndrome and Proteus-like syndrome, Bannayan-Zonana syndrome, Lhermitte–Duclos disease and Cowden disease (CD) (Liaw et al., 1997; Marsh et al., 1997; Suzuki et al., 1998; Zhou et al., 2000; Zhou et al., 2001). In addition, the mice with PTEN deletion were found to be susceptible to increased risk of cancer in multiple organs including, skin, mammary glands and prostrate (Ming et al., 2011; Backman et al., 2004; Li et al., 2002; Suzuki et al., 2003; Yanagi et al., 2007; Ming et al., 2011; Ming et al., 2008), thereby showing the increased potential of PTEN in suppressing the induction and progression of tumours.

The loss of PTEN has been associated with progression of breast cancer, which is mediated through several mechanisms involving an epigenetic silencing, degradation of PTEN and post-translational modifications, germline/somatic mutations in PTEN (Leslie et al., 2010; Shetty et al., 2011; Singh et al., 1998,

Garcia et al., 1999). The studies in last ten years showed evidence regarding the PTEN loss with the failure of chemotherapy and radiotherapy to the breast cancer (Kechagioglou et al., 2014). It has also been reported that downregulation of PTEN is associated with poor prognosis and prediction of the breast cancer (Depowski et al., 2001). PTEN also plays an important role in HER2-overexpressing breast tumours and effectiveness of trastuzumab in treating such carcinomas (Azim and Azim, 2008; Pandolfi, 2004; Esteva et al., 2010; Dave et al., 2011).

The overexpression of HER2 is reported in 15-20% of the breast carcinomas (Azim and Azim, 2008); and HER2 positivity resulted in an increased aggressiveness of breast tumours and poor prognosis (Vant-Veer et al., 2002; Wang et al., 2005). It has been reported that loss of PTEN decreased the response of breast cancer cells to chemotherapy (trastuzumab) up to 20%, whereas the restoration of PTEN function increased the effectiveness of treatment up to 70% (Nagata et al., 2004; Lan et al., 2004). These findings were corroborated by study undertaken by Fujita et al (2006) who showed that the PTEN-positive breast cancer cells showed 89% response rate to the treatment compared to the response rate of 12.5% for the PTEN-negative cells.

Dave et al (2011) demonstrated that loss of PTEN increased the levels of p-AKT and p-MAPK, and trastuzumab was unable to lower the levels of p-AKT in PTEN-negative breast cancer cells, thereby showing the role of PTEN loss in resistance to chemotherapy. Several other studies linked the loss of PTEN with poor prognosis of breast carcinomas (Singh et al., 1998; Garcia et al., 1999; Kechagioglou et al., 2014). PTEN loss has also been reported in the triple negative cell lines carrying

BRCA1/BRCA2 mutations in breast cancer patients (Saal et al., 2005; Phuah et al., 2012). These data indicate that PTEN is a potential predictor of the clinical outcomes of the targeted therapy in breast cancer patients. However, there is accumulating evidence regarding the impact of loss of PTEN on the expression of various factors involved in aggressiveness and proliferation of the breast cancer.

PTEN loss is reported to play an important role in regulation of PI3K/AKT pathway which is considered an important factor in the progression of tumorigenesis. PTEN exerts its influence on PI3K/AKT pathway through dephosphorylating PIP3, and consequently inhibits phosphorylation of AKT which is dependent on PIP3 (Maehama and Dixon, 1998). Several other studies supported that PI3K is deregulated in in different form of human cancer including breast cancer through loss of PTEN, or HER2 overexpression or mutations in catalytic subunit PIK3CA (Saal et al., 2005; Tokunaga et al., 2007; Dillon et al., 2007; Stemke-Hale et al., 2008). However, in breast cancer, the regulation of AKT as a result of loss or downregulation of PTEN in different breast cancer cell lines is not supported by sufficient literature, which warrants further investigation in this context.

Ataxia-telangiectasia mutated (ATM) and RAD3-related (ATR), members of the PIKKs (PI3K-like protein kinases) family of serine threonine kinases are key players in the maintenance of genomic integrity (Brown and Baltimore, 2003). ATR is activated in response to single strand breaks (SSBs), which in turn phosphorylates numerous DDR effectors involved in regulating cell cycle progression and DNA repair pathways (Ming et al., 2012). A key substrate of ATR is CHK1 whose phosphorylation at Ser317 and Ser345 activates it, which in turn phosphorylates

other downstream proteins such PTEN to regulate DNA repair and cell cycle (Ming et al., 2012; Paez and Seller, 2003). Proficient ATR-CHK1 signaling network is therefore essential for coordination of DNA repair, cell cycle progression and apoptosis in response to DNA damage (Martin et al., 2004). Depletion of ATR or its pharmacological inhibition through small molecule inhibitors has been shown to increase radio-sensitivity and decreased level of phosphorylated CHK1 and PTEN (Lee et, 2004; Puc et al., 2005). Both active PTEN and CHK1 are essential for recovery of stalled replication, suggesting the implication of these proteins in regulatory pathways for cell cycle. Thus ATR-CHK1-PTEN axis play a critical role in establishing the checkpoints and delaying progression of cell cycle from one phase to another phase due to DNA damage, so that sufficient time is provided for action of repair machinery.

3.2. Aims

1. To explore the expression of PTEN in a large well-characterized clinically annotated primary invasive BC series and comparing its association with clinical, pathological and biological features of the cohort.
2. To examine the association of PTEN with a range of PI3K/AKT pathway related proteins to understand the biological context the markers behave within.
3. To profile expression of PTEN with key proteins involved in DNA damage response (DDR) with particular focus on ATR/CHK1 axis.

3.3. Materials and methods

The IHC staining was performed using Novolink™ Max Polymer Detection System from Leica Biosystems (Newcastle, United Kingdom). Twenty-minute Microwave antigen retrieval was used to unmask PTEN using citrate buffer was used for 20 minutes at 37 °C to unmask PTEN antigen. Slides were incubated with primary antibody diluted to their optimal working concentrations for 1 hour at room temperature (1:25) (see chapter 2). H-score was used for IHC staining assessment; the cutoff point was detected using medians and X-tile software (see Table 2.4). Western blotting was performed using whole cell lysates from MCF7, MDA-MB-231 and BT-549 as detailed in Chapter 2.

3.4. Results

3.4.1. Overview of PTEN expression

Of all the cases analyzed, only 811 cases were scorable and suitable for analysis of *PTEN* expression. PTEN expression was detected in the nuclei and cytoplasm of the malignant cells. Median was used to dichotomies H-score expression of nuclear PTEN (median=0 [IQR 0-30]; negative = 0, positive > 0) and cytoplasmic PTEN (median=90 [IQR 40-180]; negative/low < 90, high ≥ 90) into two categories. PTEN showed nuclear expression in 303 cases (37.4%) while 508 cases (62.6%) were negative for PTEN nuclear expression. 485/811 (59.8%) of the cases were positive for PTEN cytoplasmic expression, and 326/811 (40.2%) of the cases were negative for PTEN cytoplasmic expression. Examples from negative to strong immunoreactivity for nuclear and cytoplasmic expression of PTEN are shown in

Figure 3.1. Moreover, agreement between two scorers showed substantial concordance for PTEN scoring on TMA (ICC=0.75).

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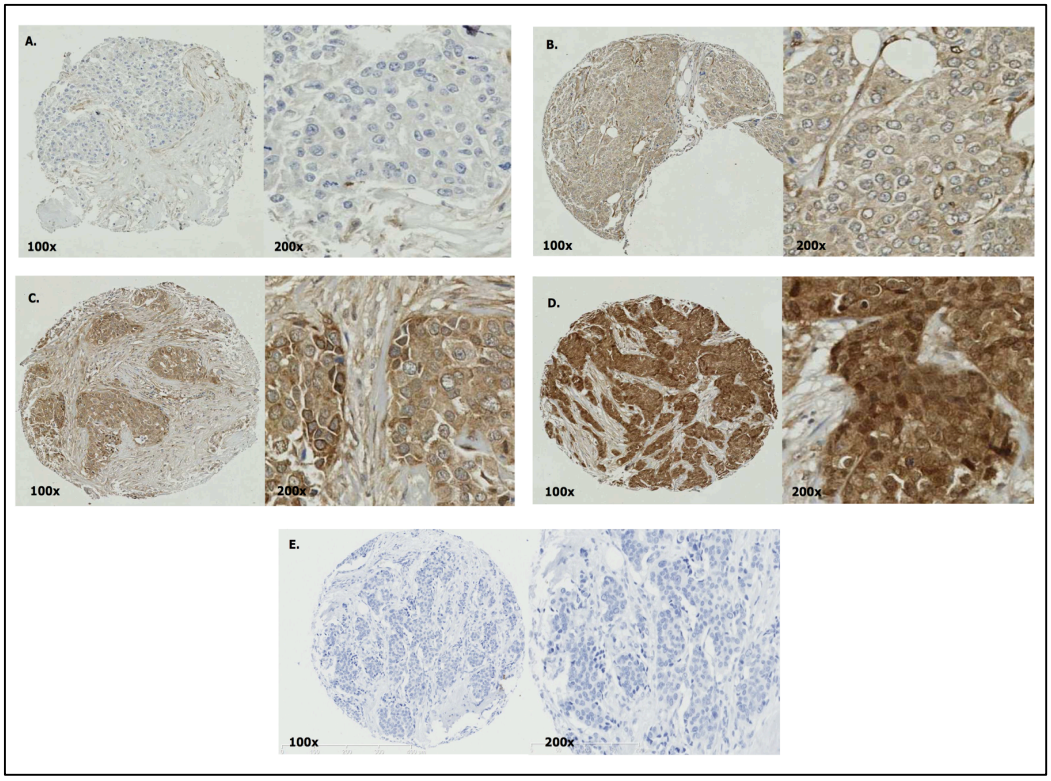


Figure 3-1 Different intensities of nuclear and cytoplasmic staining of PTEN
A: negative staining for both, **B:** negative staining for nuclei and weak for cytoplasm, **C**: some weak staining for nuclei and moderate for cytoplasm **D**. strong staining for both and **E:** negative control TMA cores pictures were taken using digital pathology interference at 100x (left) and 200x (right)

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3.4.2. Antibody specificity

Western blotting analysis of MCF-7 and MDA-MB231 and BT-549 breast cancer cell lines displayed two bands: 55kD and 42kD, corresponding to PTEN and β-actin, respectively (Figure 3.2).

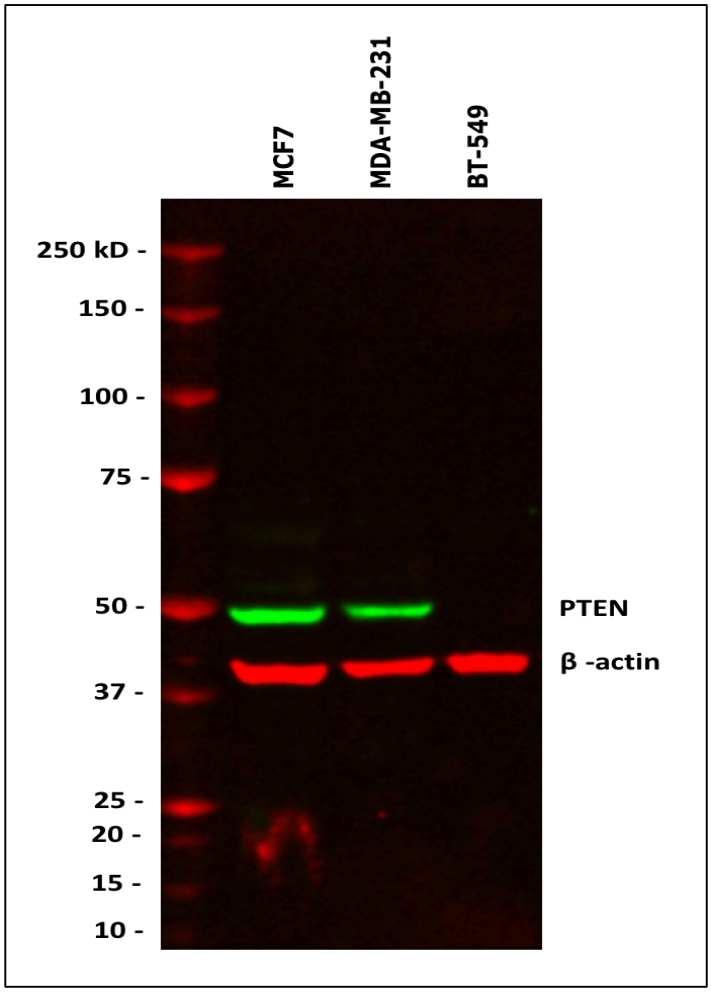


Figure 3-2 Western blot of PTEN protein detected by anti-PTEN6H2.1 in breast cancer cell lines.
Predicted molecular weight PTEN 55kDa (green bands), β-actin 42kDa (red bands).

3.4.3. Association with clinicopathological characteristics

The absence of nuclear PTEN is significantly associated with higher tumour grade, pleomorphism, mitotic frequency, less tubular formation, large tumour size, higher proportion of moderate and poor Nottingham Prognostic Index (NPI). Furthermore, no significant association of cytoplasmic and nuclear PTEN expression was found with HER2 status, lymphovascular invasion, and tumour stage (Table 3.1).

Absence or low expression of the cytoplasmic PTEN was significantly associated with features of aggressive phenotype such as higher tumour grade, more mitotic frequency, larger tumour size, and moderate NPI. In addition, negative or low expression of PTEN within cytoplasm was significantly associated with less tubular formation (Table 3.2). In addition, the negative/low expression of cytoplasmic PTEN was positively associated with the positive HER2. However, low or negative expression of cytoplasmic PTEN had no significant association with pleomorphism, tumour stage, lymphovascular invasion (Table 3.2).

Table 3-1 Association between Nuclear-PTEN expression and clinicopathological variables in whole cohort

Clinicopathological parameters	Negative (= 0) (%)	Positive (> 0) (%)	Adjusted *P*-value
Grade			**1.6×10^{-6}**
1	49 (9.7%)	55 (18.3%)	
2	149 (29.6%)	122 (40.5%)	
3	305 (60.6%)	124 (41.2%)	
Tubules			**0.0021742**
1	20 (4.1%)	17 (5.8%)	
2	144 (29.3%)	118 (40.4%)	
3	328 (66.7%)	157 (53.8%)	
Pleomorphism			**0.00116725**
1	8 (1.6%)	5 (1.7%)	
2	154 (31.4%)	131 (44.9%)	
3	329 (67.0%)	156 (53.4%)	
Mitosis			**0.0000035**
1	123 (25.0%)	121 (41.4%)	
2	94 (19.1%)	59 (20.2%)	
3	275 (55.9%)	112 (38.4%)	
Stage			3.3250
1	294 (58.4%)	189 (62.8%)	
2	167 (33.2%)	89 (29.6%)	
3	42 (8.3%)	23 (7.6%)	
Tumour size			**0.024040333**
< 2.0	218 (43.3%)	156 (51.7%)	
≥ 2.0	286 (56.7%)	146 (48.3%)	
Nottingham Prognostic index			**0.000028**
>0.3	107 (22.3%)	109 (37.8%)	
3.4-5.4	291 (60.6%)	147 (51.0%)	
>5.4	82 (17.1%)	32 (11.1%)	
Lymphovascular invasion			0.754018
Probable /Negative	318 (63.5%)	195 (64.6%)	
Definite	183 (36.5%)	107 (35.4%)	
HER2 status			0.6150924
Negative	412 (84.4)	241 (82.8)	
Positive	75 (15.4)	50 (17.2)	

Table 3-2 Association between Cytoplasmic-PTEN expression and clinicopathological variables in whole cohort

Clinicopathological parameters	Negative/low (< 90) (%)	High (≥90) (%)	Adjusted *P*-value
Grade			**0.006393333**
1	27 (8.3%)	77 (16.0%)	
2	107 (33.0%)	164 (34.2%)	
3	190 (58.6%)	239 (49.8%)	
Tubules			**0.001183**
1	9 (2.9%)	28 (6.0%)	
2	84 (26.7%)	178 (38.0%)	
3	222 (70.5%)	263 (56.1%)	
Pleomorphism			6.937315
1	5 (1.6%)	8 (1.7%)	
2	114 (36.3%)	171 (36.5%)	
3	195 (62.1%)	290 (61.8%)	
Mitosis			**0.0015925**
1	85 (27.0%)	159 (33.9%)	
2	48 (15.2%)	105 (22.4%)	
3	182 (57.8%)	205 (43.7%)	
Stage			0.544063333
1	203 (62.7%)	280 (58.3%)	
2	96 (29.6%)	160 (33.3%)	
3	25 (7.7%)	40 (8.3%)	
Tumour size			**0.017472**
< 2.0	133 (41.0%)	241 (50.0%)	
≥ 2.0	191 (59.0%)	241 (50.0%)	
Nottingham Prognostic index			**0.005444425**
>0.3	69 (22.4%)	147 (32.0%)	
3.4-5.4	198 (64.3%)	240 (52.2%)	
>5.4	41 (13.3%)	73 (15.9%)	
Lymphovascular invasion			0.136490
Probable /Negative	215 (67.0%)	298 (61.8%)	
Definite	106 (33.0%)	184 (38.2%)	
HER2 status			**0.000016**
Negative	286 (91.1%)	367 (78.9%)	
Positive	28 (8.9%)	97 (20.9%)	

3.4.4. Association with key biomarkers in breast cancer and DNA damage response

The associations of nuclear PTEN expression with important biomarkers in the breast cancer have been shown in table 3.3. The absence of nuclear PTEN was significantly associated with the negative expression of ER ($p < 0.001$), PgR ($p < 0.001$), N-pCHK1 ($p < 0.001$), and C-pCHK1 ($p = 0.003$). In addition, the negative expression of the nuclear PTEN was also significantly associated with high expression of PI3K ($p = 0.012$).

Conversely, the negative expression of the nuclear PTEN was significantly associated with positive expression of HER1 ($p = 0.036$), HER4 ($p = 0.031$), Ki 67 ($p = 0.0028$), and p53 ($p = 0.027$). The negative expression of the nuclear PTEN was also associated with high expression of PI3K ($p = 0.012$). The expression of nuclear PTEN, however, did not show significant association with HER2, HER3, P-AKT, and ATR.

The association between expression of cytoplasmic PTEN and other important biomarkers in the breast cancer is presented in table 3.4. The negativity of PTEN expression was found to be highly significantly associated with negative expression of ER ($p < 0.001$), PgR ($p < 0.001$), HER2 ($p < 0.001$), ATR ($p = 0.006$), and C-pCHK1 ($p < 0.001$).

Nonetheless, the expression of cytoplasmic PTEN was not associated with certain biomarkers including HER1, HER3, HER4, Ki 67, p53, P-AKT, PI3K, and N-pCHK1.

Table 3-3 Association between nuclear expression of PTEN and important biomarkers of BC in whole cohort

Biomarkers	Negative (= 0) (%)	Positive (> 0) (%)	Adjusted *P*-value
ER Status			**2.5×10^{-10}**
Negative	175 (34.8%)	42 (13.9%)	
Positive	328 (65.2%)	261 (86.1%)	
PgR Status			**4.8×10^{-10}**
Negative	251 (51.6%)	82 (27.9%)	
Positive	235 (48.4%)	212 (72.1%)	
HER2 Status			0.6150924
Negative	412 (84.6%)	241 (82.8%)	
Positive	75 (15.4%)	50 (17.2%)	
HER1			**0.035597**
Negative	379 (77.3%)	249 (83.6%)	
Positive	111 (22.7%)	49 (16.4%)	
HER3			0.142075
Negative	32 (7.1%)	28 (10.1%)	
Positive	421 (92.9%)	248 (89.9%)	
HER4			**0.031488**
Negative	45 (9.3%)	42 (14.3%)	
Positive	440 (90.7%)	252 (85.7%)	
Ki 67 Status			**0.002874**
Negative	130 (32.5%)	107 (44.2%)	
Positive	270 (67.5%)	135 (55.8%)	
P53 Status			**0.027004**
Negative	327 (68.4%)	220 (75.9%)	
Positive	151 (31.6%)	70 (24.1%)	
P-AKT			0.633023
Negative	75 (22.2%)	51 (23.9%)	
Positive	263 (77.8%)	162 (76.1%)	
PI3K			**0.012390**
Low	75 (18.9%)	60 (26.5%)	
Medium	95 (24.0%)	64 (28.3%)	
High	226 (57.1%)	102 (45.1%)	
ATR			4.417836
Negative	87 (36.6%)	57 (38.3%)	
Positive	151 (63.4%)	92 (61.7%)	
N-PCHK1			**1.1×10^{-7}**
Negative	294 (63.5%)	122 (43.1%)	
Positive	169 (36.5%)	161 (56.9%)	
C-PCHK1			**0.0039405**
Negative	127 (30.2%)	51 (19.8%)	
Positive	293 (69.8%)	207 (80.2%)	

Table 3-4 Association between cytoplasmic expression of PTEN and important biomarkers of BC in whole cohort

Biomarkers	Negative/low (< 90) (%)	High (≥90) (%)	Adjusted *P*-value
ER Status			**0.000004**
Negative	115 (35.7%)	102 (21.1%)	
Positive	207 (64.3%)	382 (78.9%)	
PgR Status			**0.000319**
Negative	158 (50.5%)	175 (37.5%)	
Positive	155 (49.5%)	292 (62.5%)	
HER2 Status			**0.000008**
Negative	286 (91.1%)	367 (79.1%)	
Positive	28 (8.9%)	97 (20.9%)	
HER1			0.135928
Negative	242 (77.1%)	386 (81.4%)	
Positive	72 (22.9%)	88 (18.6%)	
HER3			0.989158
Negative	24 (8.2%)	36 (8.2%)	
Positive	267 (91.8%)	402 (91.8%)	
HER4			0.885193
Negative	34 (11.0%)	53 (11.1%)	
Positive	276 (89.0%)	416 (88.7%)	
Ki 67 Status			0.461768
Negative	89 (35.2%)	148 (38.0%)	
Positive	164 (64.8%)	241 (62.0%)	
P53 Status			0.105414
Negative	208 (68.0%)	339 (73.4%)	
Positive	98 (32.0%)	123 (26.6%)	
P-AKT			0.128762
Negative	56 (26.3%)	70 (20.7%)	
Positive	157 (73.7%)	268 (79.3%)	
PI3K			0.601488
Low	59 (23.2%)	76 (20.7%)	
Medium	67 (26.4%)	92 (25.0%)	
High	128 (50.4%)	200 (54.3%)	
ATR			**0.0063864**
Negative	66 (46.2%)	78 (32.0%)	
Positive	77 (53.8%)	166 (68.9%)	
N-PCHK1			2.381982
Negative	169 (57.7%)	247 (54.5%)	
Positive	124 (42.3%)	206 (45.5%)	
C-PCHK1			**4.9×10^{-6}**
Negative	96 (36.8%)	82 (19.7%)	
Positive	165 (63.2%)	335 (80.3%)	

3.4.5. Outcome analysis

3.4.5.1. PTEN in whole Cohort

In the whole cohort, negative expression of nuclear PTEN was associated with shorter breast cancer specific survival (BCSS), with survival of 120 months (LR = 7.718; p =0.005) (Figure 3.3A). In contrast to nuclear PTEN, the expression of cytoplasmic PTEN was not associated with BCSS, indicating the critical role of nuclear PTEN in BCSS of patients (Figure 3.3).

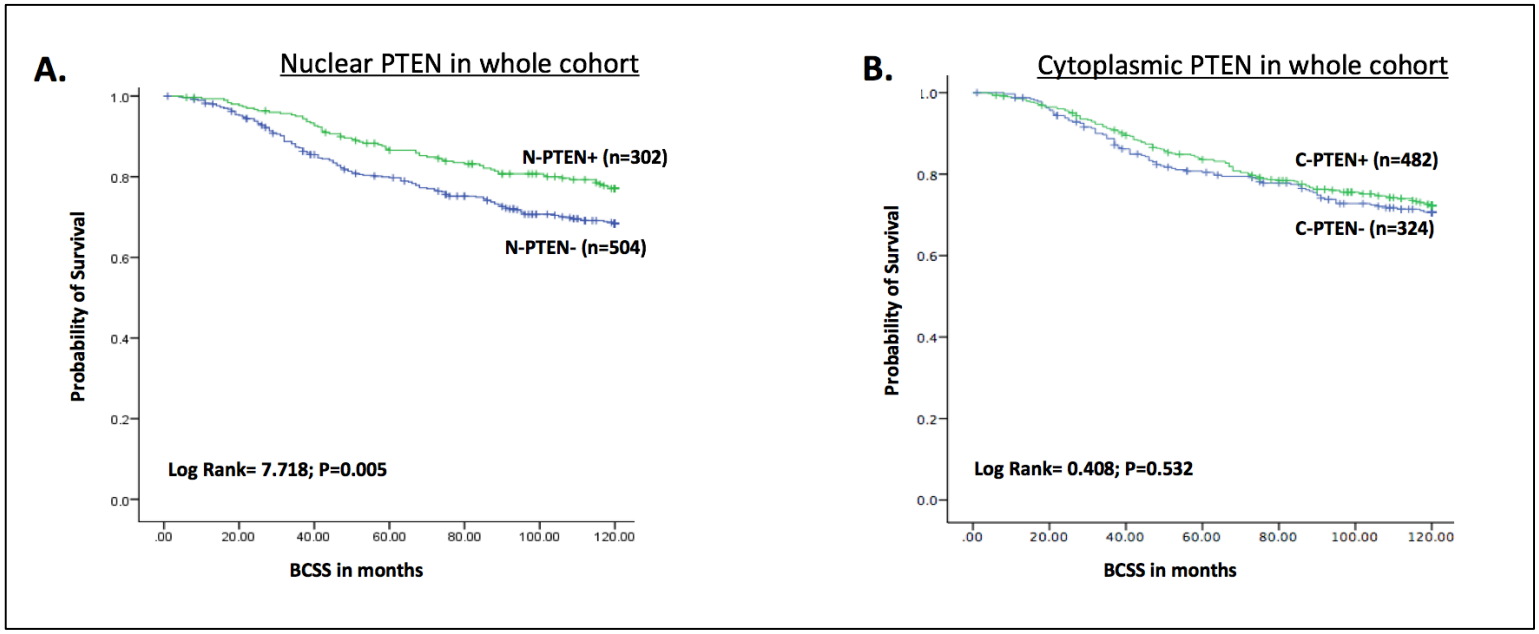


Figure 3-3 Kaplan Meier plots showing 10 years BCSS for N-PTEN and C-PTEN in the whole series
A. represents Kaplan Meier plot in whole series with N-PTEN expression showing 10 years BCSS. **B**. represents Kaplan Meier plot with C-PTEN expression in whole series showing 10 years BCSS; **N**: nuclear, **C**: cytoplasmic

3.4.5.2. Nuclear and cytoplasmic PTEN in combination

At follow-up of 10 years, the following combination of expressions: N-PTEN-negative/C-PTEN-positive and N-PTEN-negative/C-PTEN-negative were associated with the worst survival in the breast cancer patients compared to the tumours with positive nuclear PTEN in combination (LR = 8.374; p = 0.039). In other words, the combination involving N-PTEN-positive showed better survival compared to those combination involving N-PTEN-negative (Figure 3.4). This corroborated the role of N-PTEN in the BCSS of patients.

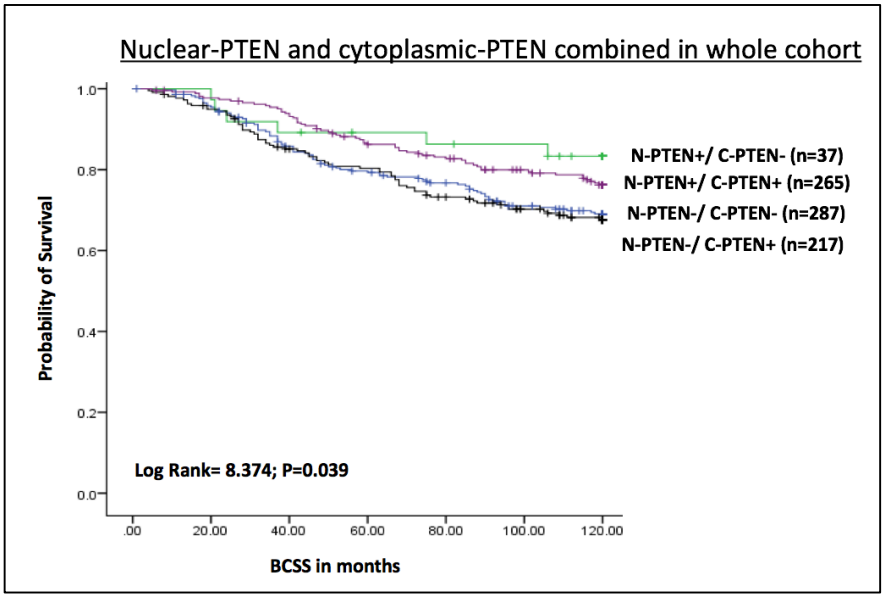


Figure 3-4 Kaplan Meier plot showing 10 years BCSS for combined expression of nuclear-PTEN and cytoplasmic-PTEN in whole series.
N: nuclear, **C:** cytoplasmic

3.4.5.3. PTEN and ATR combined in whole cohort

We investigated the impact of expression of nuclear PTEN and ATR on survival in patients. It was found that negative nuclear PTEN and high ATR expression together was associated with the worst BCSS ($p = 0.029$) (Figure 3.4A).

Opposite results were obtained for the cytoplasmic PTEN and ATR expressions at 10 years follow-up (Figure 3.5B). The positive expression of cytoplasmic PTEN and negative expression of ATR was not significantly associated with BCSS (LR = 5.517; $p = 0.138$).

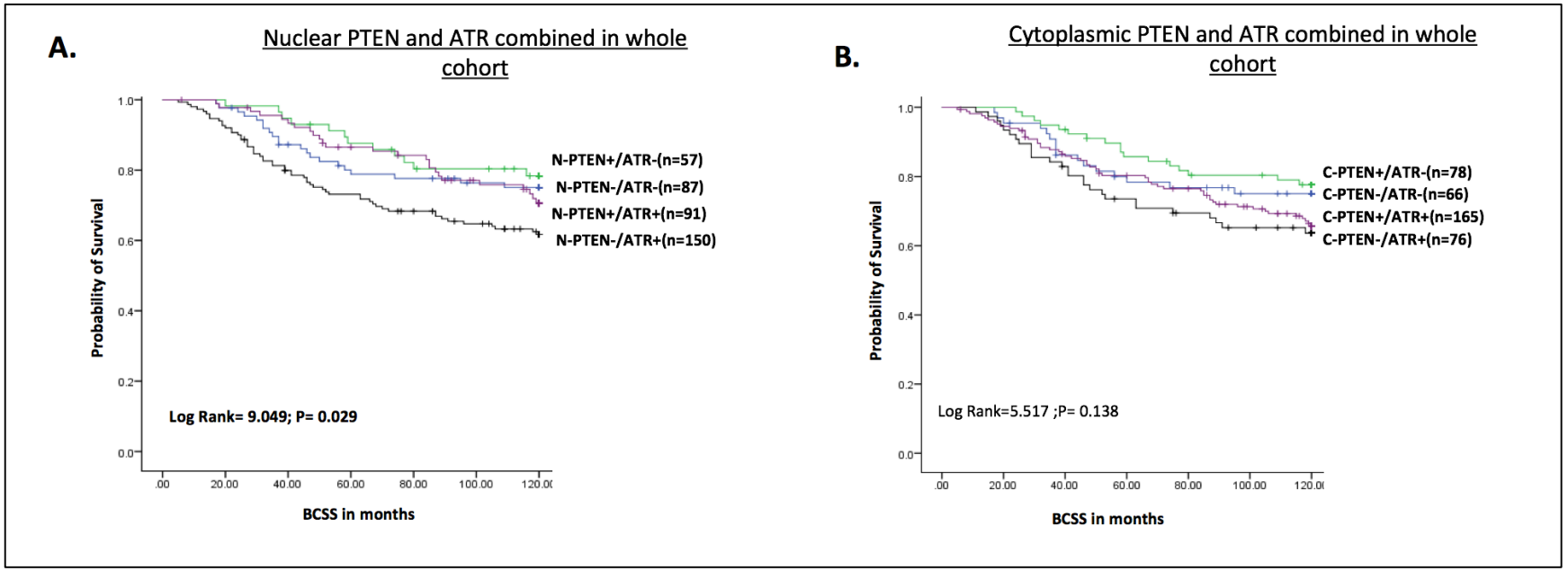


Figure 3-5 Kaplan Meier plots illustrating 10 years BCSS for combined expression of ATR and PTEN in the whole series
A. represents Kaplan Meier plot in whole series with nuclear-PTEN and ATR expression. **B**. represents Kaplan Meier plot with cytoplasmic-PTEN and ATR expression combined in whole series; **N**: nuclear, **C**: cytoplasmic.

3.4.5.4. PTEN and pCHK1 in whole cohort

In the whole cohort, the effect of nuclear PTEN and pCHK1 expressions was investigated on BCSS in patients. We found that patients whose tumours have PTEN and pCHK1 expressions in the following combinations: N-PTEN-negative/N-pCHK1-postive and N-PTEN-negative/N-pCHK1-negative, showed the worst BCSS at 10 years of follow-up compared to the tumours that have the following combinations of expression: N-PTEN-positive and N-pCHK1-negtaive, N-PTEN-positive and N-pCHK1-positive (LR = 8.488; p = 0.037) (Figure 3.6A).

Cytoplasmic PTEN and nuclear phosphorylated CHK1 expression together was not significantly associated with the BCSS in patients (LR =0.712; p = 0.664) (Figure 3.6B). In contrast, in whole cohorts, the patients with tumours having negative nuclear PTEN expression and positive cytoplasmic pCHK1 expression together have the worst survival compared to tumours having positive nuclear PTEN and negative cytoplasmic pCHK1 (LR = 5.517; p = 0.031) (See figure 3.5C). In contrast to N-PTEN-positive/Cyto-pCHK1, the cytoplasmic PTEN and cytoplasmic pCHK1 expression together was not significantly associated with BCSS in patients at 10 years of follow-up (LR – 0.0607; p = 0.895). The results are shown in figure 3.6D.

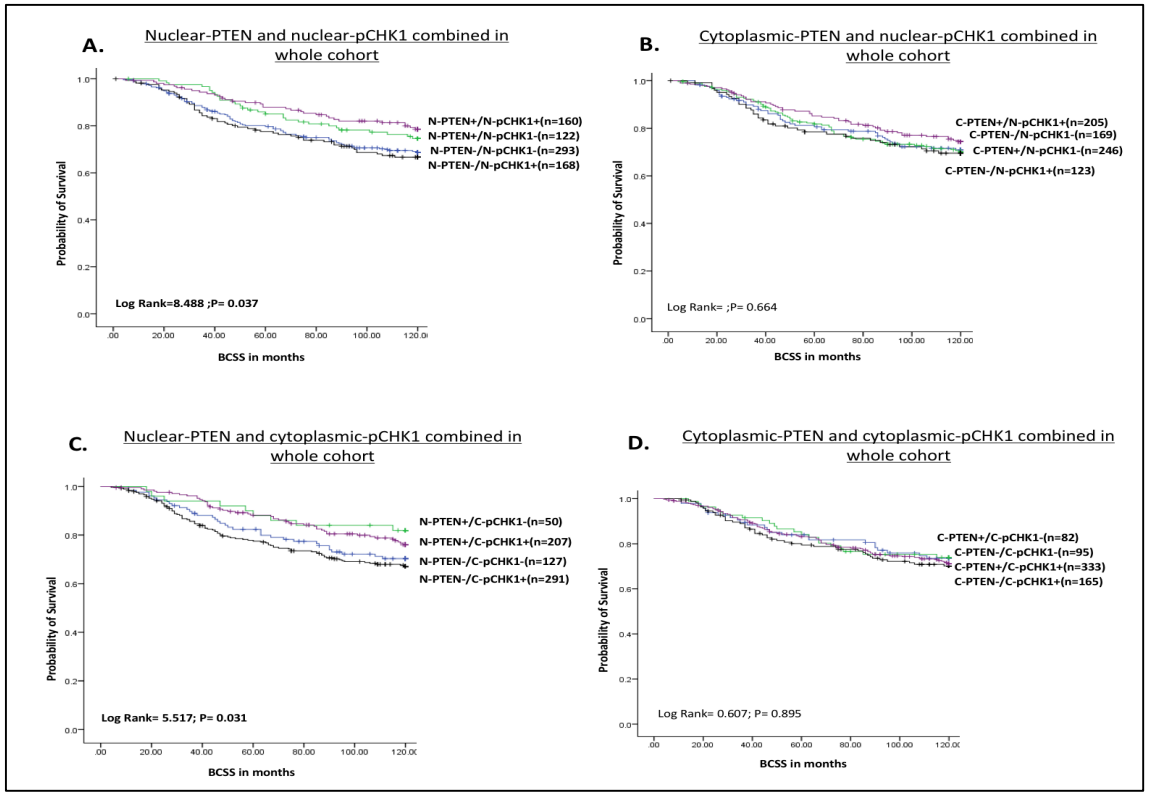


Figure 3-6 Kaplan Meier plots demonstrating 10 years BCSS for combined expression of PTEN and pCHK1 in the whole series. **A**. represents Kaplan Meier plot in whole series with N-PTEN and N-pCHK1. **B**. represents Kaplan Meier plot with C-PTEN and N-pCHK1 expression combined in whole series **C**. represents Kaplan Meier plot with N-PTEN and C-pCHK1 expression combined in whole series. **D.** represents Kaplan Meier plot with C-PTEN and C-pCHK1 expression combined in whole series; **N**: nuclear, **C**: cytoplasmic

3.4.5.5. ATR and pCHK1 in whole cohorts

The effect of expressions of ATR and pCHK1 together BCSS in patients was investigated. The survival analysis using Kaplan-Meier plot showed that expressions of ATR and nuclear pCHK1 had no significant association with the survival of patients in the whole cohort after 10 years of follow-up (LR = 5.663, p= 0.129) (3.7A). In contrast, the tumours with positive expressions of ATR and cytoplasmic pCHK1 showed significantly worst survival in the whole cohort compared to those with negative expressions of ATR and cytoplasmic pCHK1 (LR = 7.691; p = 0.053). (Figure 3.7B).

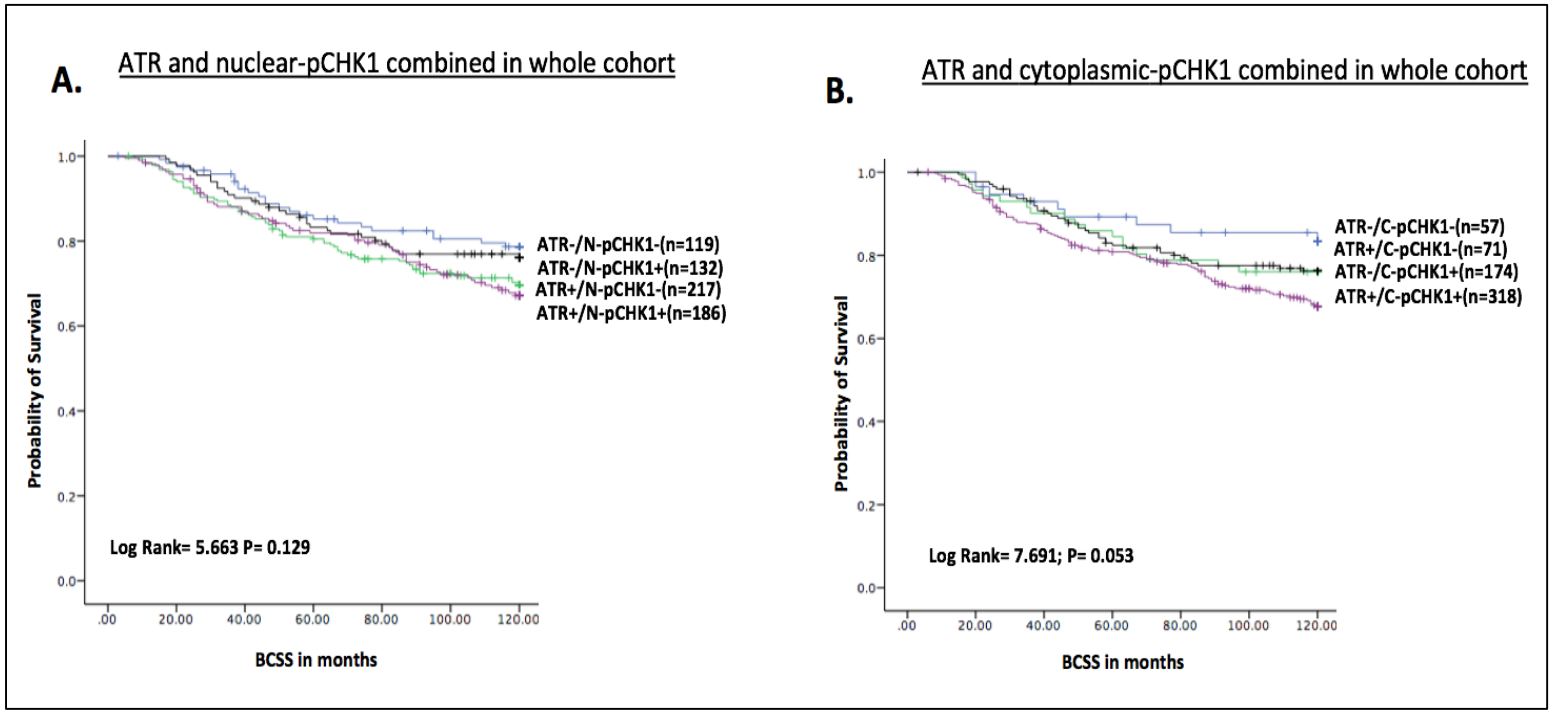


Figure 3-7 Kaplan Meier plot showing BCSS for the combined ATR pCHK1 in the whole series
A. represents Kaplan Meier plot in whole series with ATR and N-pCHK1. **B**. represents Kaplan Meier plot in whole series with ATR and C-pCHK1 expression; **N**: nuclear, **C**: cytoplasmic

3.4.5.6. ATR and cytoplasmic pCHK1 combined in absence and presence of nuclear PTEN expression

The effect of expression of ATR and cytoplasmic pCHK1) together in the absence of nuclear PTEN on the survival of the patients in whole cohort was investigated. The survival analysis demonstrated that ATR-positive/C-pCHK1-positive in the absence of nuclear PTEN was significantly associated with the worst survival in patients after 10 years of follow-up (LR = 10.651; P = 0.014). (3.8A). In contrast, the negative expressions of ATR and nuclear pCHK1 in the absence of the nuclear PTEN was not significantly associated with survival (LR = 5.547; P = 0.136) (Figure 3.8B)

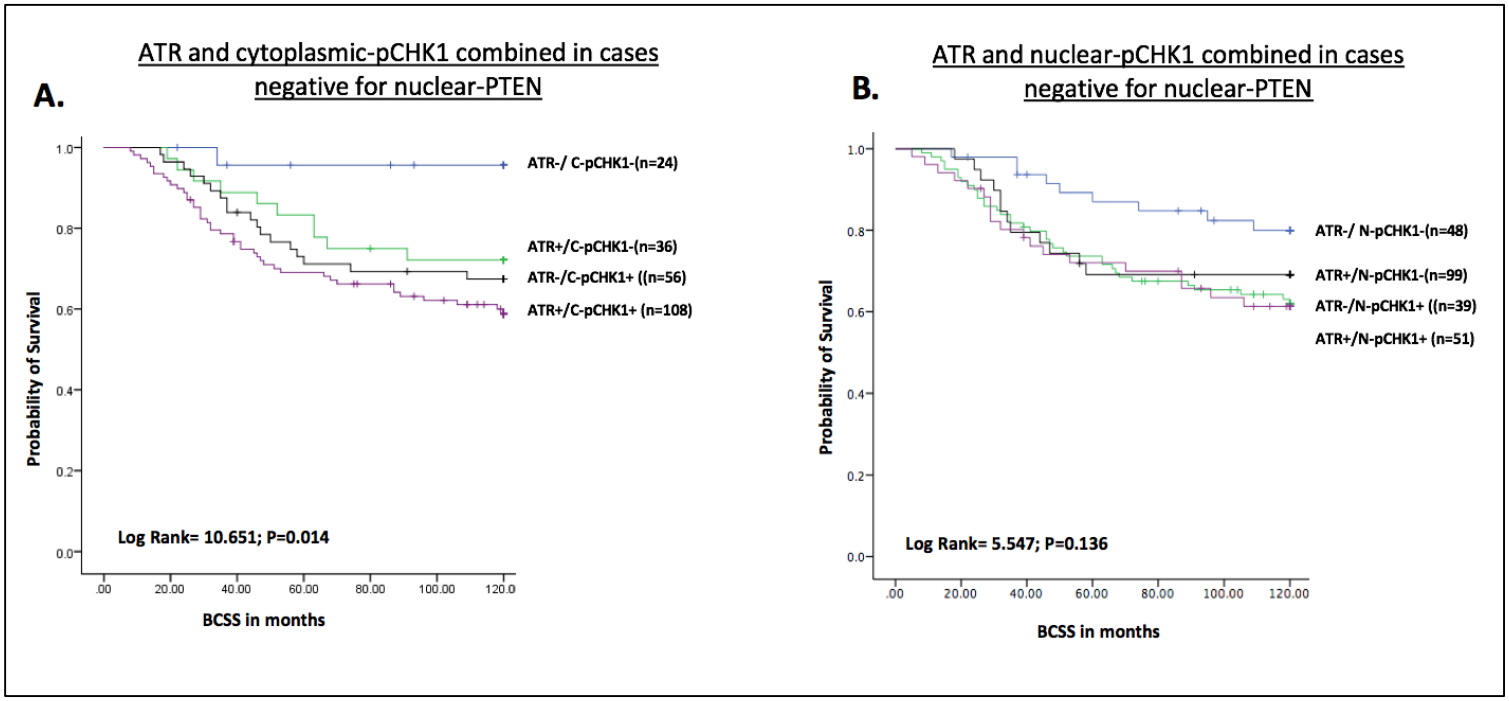


Figure 3-8 Kaplan Meier plot illustrating BCSS for ATR and pCHK1 in absence of nuclear-PTEN
A. represents Kaplan Meier plot in whole series with ATR and C-pCHK1 in absence of N-PTEN. **B**. represents Kaplan Meier plot in whole series with ATR and N-pCHK1 expression in absence of N-PTEN; **N**: nuclear, **C**: cytoplasmic

3.4.5.6. ATR and pCHK1 combined in presence of nuclear PTEN expression

In the whole cohort, expression of ATR and nuclear pCHK1 together in presence of nuclear PTEN expression was not significantly associated with the survival in patients at 10 years of follow-up (LR = 1.463; P = 0.691) [Figure 3.9]. Similarly, expression of ATR and cytoplasmic pCHK1 together in presence of nuclear PTEN was not associated with the BCSS in patients (LR = 0.900; P = 0.825), showing the significance of PTEN expression in affecting the BCSS in the whole cohort (Figure 3.9B).

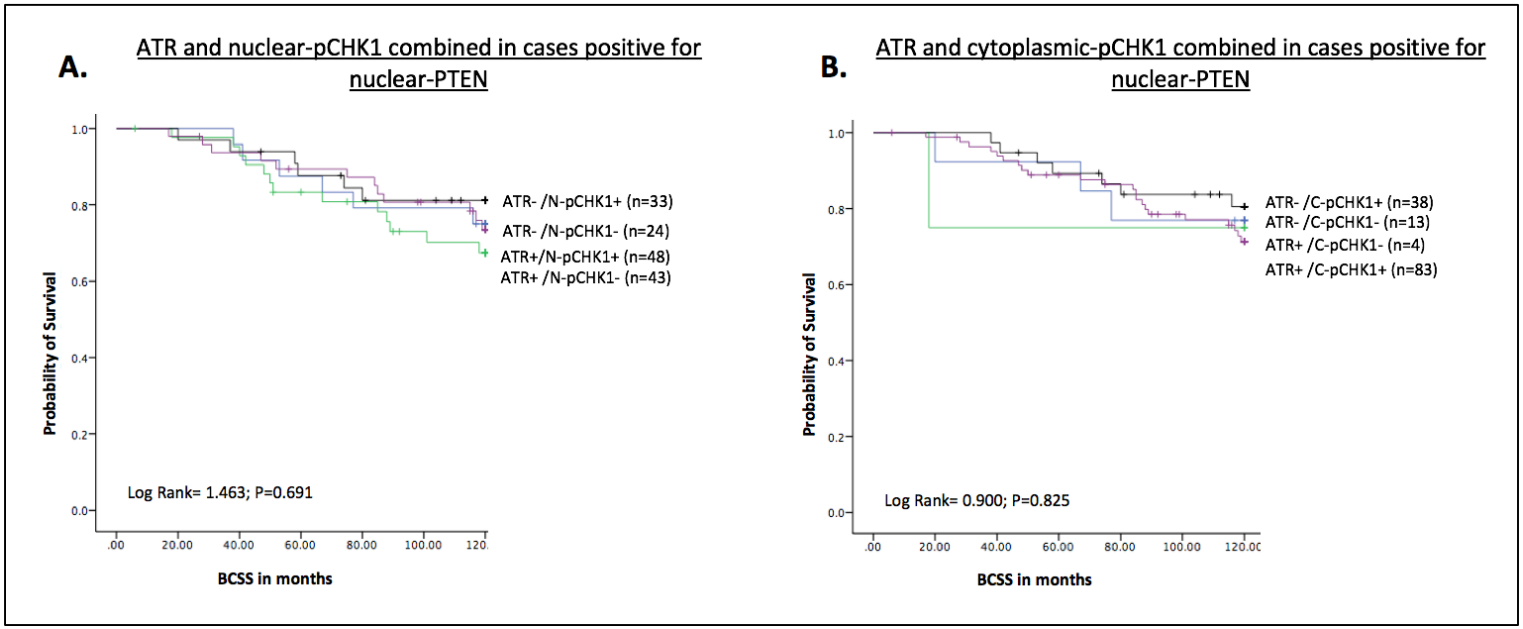


Figure 3-9 Kaplan Meier plot showing 10 years BCSS for ATR and pCHK1 in presence of nuclear PTEN
A. represents Kaplan Meier plot in whole series with ATR and N-pCHK1 in presence of nuclear PTEN. **B**. represents Kaplan Meier plot in whole series with ATR and C-pCHK1 expression in presence of nuclear PTEN. **N**: nuclear, **C**: cytoplasmic

3.5. Discussion

PTEN is an important tumor suppressor, and has been reported by several studies to be implicated in the pathogenesis of a wide range of human cancers. The researchers have found that PTEN was either mutated or deleted in a variety of human cancers (49). This study has reported intriguing findings about the alternations of cytoplasmic and nuclear PTEN and their associations with the different clinicopathological features and key biomarkers for breast cancer.

3.5.1. Cytoplasmic and nuclear PTEN expression and clinicopathological features

This study showed that loss/negativity of both cytoplasmic and nuclear PTEN expressions were positively associated with tumour grade (III), mitosis, tumour size, TN status. Different studies have reported that loss of PTEN was associated with the triple negative or basal like subtypes of breast cancer (Dourdin et al., 2008; Hu et al., 2009; Lopez-Knowles et al., 2001; Saal et al., 2008). Another study conducted by Bose et al (2002) confirmed that the loss of PTEN expression was associated with the high tumour grade and tumour size. Similar results were reported by many other studies (Lin et al., 2014; Depowski et al., 2001). Consistent with this study, Lee et al (2004) reported that loss of PTEN was responsible for increased mitosis and tumour grade. Nevertheless, there was no significant associations found between the loss of PTEN and tumour size and lymphovascular invasion.

In agreement with Lee et al (2004), this study found that PTEN within cytoplasm and nucleus did not affect the lymphovascular invasion. Similarly, the lack of association between PTEN loss and tumour size found by Lee et al (2004) was contrary to what was found by this study. This study also found that loss of cytoplasmic PTEN was significantly associated with HER2-positive tumour status, while nuclear PTEN was not significantly associated with HER2.

However, some studies reported no significant association between the tumour grade, tumour size and lympovascular invasion (Bakarakos et al., 2010; Capodanno et al., 2009; Panighari et al., 2004). In addition, contrary to this study, some studies reported that there was no correlation between the proliferative activity (mitotic index) and loss of PTEN (Lin et al., 2014; Perez-Tenorio et al., 2007) The discrepancy of results of this study with other studies may be related to number of cases examined and methodological factors. Furthermore, the studies showing results opposite to this study has not differentiated between the cytoplasmic and nuclear PTEN, which may be an additional factor causing the discrepancy in results. Similarly, this study demonstrated that loss of nuclear PTEN was associated with higher pleomorphism and less tubular formation. Higher pleomorphism was only associated with nuclear PTEN loss rather than cytoplasmic PTEN, and this may show the variation in function of cytoplasmic and nuclear PTEN. These results are consistent with Li et al (2009) who provided evidence that mitotic index and nuclear pleomorphism were increased as a result of reduced or negative nuclear PTEN expression.

Furthermore, the loss of PTEN is significantly associated with moderate or poor Nottingham Prognostic Index, which is logical as higher mitotic index, nuclear pleomorphism, and less tubular formation are associated to the loss of PTEN.

3.5.2. Cytoplasmic and nuclear PTEN expression and biomarkers

This study found that cytoplasmic PTEN was associated with ER, PgR and HER2 biomarkers which constitute the basis of classification of breast cancer tissues expressing these biomarkers. Low or negative expression of PTEN was found to be significantly associated with the negative ER and PgR status, which means that tissues without expression of ER and PgR receptors in the human breast cancer showed either low or negative expression of the PTEN protein within the cytoplasm. This finding carry the great implication from the predictive prospective, considering almost half of the breast cancer express PgR receptor (Allred et al., 2009). Similarly, 83% of the ER positive breast cancer also express PgR receptor (Dunnwald et al., 2007). Given these data, the negative expression of PTEN. The previous studies Hammond et al (2010) reported that presence or absence of ER and PgR receptor expression have prognostic values for hormone positive breast cancer patients. Because the current study found the significant association with the negative ER and PgR receptor status in the breast cancer tumour, it shows the significance of PTEN in favorable prognosis of breast cancer tumour along with ER and PgR status. Several other studies suggested the expression of ER and PgR associated with the success of the chemotherapeutic agents to suppress the proliferation of breast tumours (Dowsett, 2005; Viale, 2007; Yamashita, 2006). Based on the finding of the current study, the negative or low expression of PTEN

is associated with negative ER and PgR, which may show the predictive benefits for the breast cancer patients. The negative status of ER and PgR is related to loss or absence of PTEN expression. These results are confirmed by several other retrospective studies (Samulak et al., 2013; Patani et al., 2013; Dowsett, 2008).

In addition, the negative ER, PgR and HER2 status is also related to the triple negative cells. This study also showed that negative expression of PTEN was highly significantly associated with the negative expression of HER2. The negative expressions of ER, PgR and HER2 receptors are characterized in the triple negative (TN) breast cancers cells. TN breast cancers are characterized as the disease with higher cell proliferation, aggressive histology, lower survival and poor clinical outcomes among the breast cancer patients (Krishnamurthy et al., 2012; Dent, 2009; Tang and Tse, 2016). Loss of PTEN expression was also reported to be linked with the aggressiveness of the breast cancer among patients (Lee et al., 2004).

Taken together, these data suggest that negative expression of PTEN holds a critical position in sustaining the disease progression, poor prognosis and low survival among breast cancer patients; which is actually in accordance with the role of PTEN in tumour suppression. The study conducted by Brown et al (2008) demonstrated that HER2 expression does not significantly affect the survival rate of patients carrying ER-negative, PgR-negative and HER2- positive phenotype, indicating that negative expression of PTEN may play a fundamental role in decreasing the survival of patients. This hypothesis has been supported by several studies showing the loss or mutations of PTEN decrease the survival of patients with a variety of cancers (Lee et al., 2001; Kwabi-Addo et al. 2001; Mikhail et al., 2005). Thus it

can be argued that TN tumours with negative cytoplasmic PTEN expression may contribute to the higher tumour grade/disease grade, poor clinical outcomes, and aggressive histology.

In contrast to significant association negative expression of cytoplasmic PTEN with negative expression of HER2, the results demonstrated that cytoplasmic PTEN expression was not significantly associated with HER1, HER3 and HER4 expressions. The data about the role of HER1, HER3, and HER4 in the progression of breast cancer are limited and controversial, and do not show the significant association with the progression of breast cancer. In spot of this, the current study could not find an association between the PTEN expression and expressions of these transmembrane receptors. Iqbal and Iqbal (2014) showed that HER1 and HER3 do not act independently, instead of this, they partner with HER2 through heterodimerization process. The HER2/HER1, HER2/HER3 and are reported to be the potent mitogenic combinations to activate the PI3K/AKT and MAP pathways to enhance the cell proliferation.

Therefore, the independent expressions of HER1 and HER3 did not show any significant association with the PTEN expression. The HER2-induced overexpression of HER4 has been reported to cause reduction in cell proliferation and increased apoptosis within the HER2-positive cells, which suggest that HER4 expression lessens the mitogenic impact of HER2 on the signaling pathways leading to cell proliferation (Barnes et al., 2005). Another study showed that HER2 only affects the apoptosis controlling sides of the PI3K/AKT pathway instead of cell proliferation. Thus HER2 overexpression is related to good outcome compared

to HER1, HER2 and HER3 receptors. The current study could not demonstrate the significant association between the cytoplasmic expression of PTEN and some other biomarkers such as P53, Ki67, PI3/AKT and nuclear-pCHK1. This study had not reported the association between the Ki-67 expression (negative/positive) and PTEN expression within cytoplasm.

This might be due to the fact that ki-67 is a nuclear localized protein (Gerdes et al., 1983), and consequently is not affected by PTEN within cytoplasm. However, low or negative expression of PTEN within nucleus was found to be significantly associated with positive expression of Ki-67, which was an expected outcome and supported by the existing literature (Gokaslan et al., 2005; Halvorsen et al., 2003; Uegaki et al., 2005). The effect of PTEN on the expression of Ki-67 is in accordance with differences in the functions of PTEN and ki-67. PTEN is tumour suppressor protein while the Ki-67 is high cellular proliferation activity. Thus the loss or downregulation of PTEN was associated with overexpression of proliferation marker Ki-67.

These findings are in agreement with the association between PTEN and Ki-67 reported by several other studies in several tumours (Ji et al., 2014; Calhoun et al., 2016; Gokaslan et al., 2005). The expression of Ki-67 is predominant during S, G1, and G2 phases of the cell cycle, while it is absent during G0 phase. Contrary to Ki-67, the expression of PTEN is predominant during G0-G1 arrest, indicating that both proteins can not exist together in their highly expressive forms during the cell cycle within the nucleus (Gerdes et al., 1984; Scholzen and Gedes, 2000).

The presence of PTEN at G0-G1 checkpoint stops the cell cycle from entering into growth and cell proliferation stage, while the high concentration of ki-67 during G1-S checkpoint drives the cell into the growth and proliferation stage. It was observed that negative expression of cytoplasmic PTEN did not affect the expression of phosphorylated CHK1 within nucleus (N-pCHK1), however, it was significantly associated with the negative expression of cytoplasmic phosphorylated CHK1 (C-pCHK1). Similarly, it was found that the negative expression of nuclear PTEN was found to be significantly associated with the negative expressions of N-pCHK1 and C-pCHK1. Different explanations can be given for these associations of PTEN with pCHK1. The CHK1 is known to play an important role in the cell cycle initiation at the right time and the controlling the DNA damage response (Schmitt et al., 2006; Liu et al., 2000; Wilsker et al., 2008). Because these events occur only within the nucleus, therefore, the nuclear PTEN rather than cytoplasmic PTEN affects the level of pCHK1 within nucleus (Puc et al., 2005).

In addition, the loss of PTEN status inhibits the phosphorylation of CHK1 at S345 and promotes the AKT mediated phosphorylation at S280, which causes the low response to DNA damage and chromosomal instability. This is in accordance with finding of this study showing that PI3K ($p < 0.05$). Many other studies have suggested that mutations leading to decreased or negative expression of PTEN positively impacted the PI3K levels and subsequently increased the active form of AKT (Tang et al., 2006; Kanamori et al., 2001; Tarakawa et al., 2003). Hence the loss of PTEN activates the PI3K/AKT pathway which plays an essential role in

tumorigenesis and cell cycle through regulating the CHK1 activity (Chang et al.,2003; Vara et al., 2004; Luo et al., 2003; Gao et al., 2004).

The absence of PTEN leads to production of PCHK1-S280, the covalent binding of PCHK1-S280 to 1-2 molecules of ubiquitin and localization to the cytoplasm (Li et al., 2009), which is consistent with the results of this study showing negative status of C-pCHK1. Thus it can be noted that in the absence of PTEN expression, the level of PCHK1-S345 decreases while that of PCHK1-S280 increases due to activation of AKT; and these phosphorylation events control the subcellular localization of CHK1. Supporting the finding of the current study, Puc (2005) found that the absence of PTEN leads to activation of AKT which impairs CHK1 through phosphorylation, ubiquitination and reduced nuclear localization which subsequently promote genomic instability in tumour cells. Several other researchers also provided the evidence in support of the results of this study by showing that negative expression of PTEN negatively impacts the level of activated CHK1 though increasing the activation of PI3K/AKT pathway, lowering the DNA damage response and increasing the chromosomal instability (Mahrhofer et al., 2006; Syljuasen et al., 2005; Cho et al., 2005; Durkin et al., 2006; Gorgoulis et al., 2006).

The loss of nuclear PTEN was significantly associated with positive expression of p53, however, the cytoplasmic PTEN was not associated with p53 expression. This result is in agreement with several other researchers who reported that concomitant loss of both PTEN and p53 is not common in tumour cells (Kurose et al., 2002; Vousden and Lu, 2002; Salmena et al., 2008; Oren, 2003; Freeman et al., 2003). Different studies have highlighted the tight link between p53 and PTEN which are

reported to major tumour suppressors involved in regulating cell death, DNA damage response and cell proliferation (Bargonetti and Manfredi, 2002; Yamada and Araki, 2001). In different types of tumour either PTEN or p53 can be mutated, but concomitant mutations in both is very rare, which is mainly due to the fact that loss of PTEN reduces the selective pressure on tumour cells to lose p53 or vice versa. Therefore, in this study, the loss of PTEN boosted the expression of PTEN (Mayo and Donner, 2002; Chen et al., 2005).

This study demonstrated that the loss of cytoplasmic PTEN was associated with the negative status of ATR, while the nuclear PTEN did not show any association with the ATR. This is the novel finding reported by this study, as previous literature does not show the direct impact of loss of PTEN expression on the ATR expression. This shows that expression of nuclear PTEN does not play an important role in regulating ATR expression. In addition, it also shows that cytoplasmic PTEN expression might be an important player for regulating ATR expression. Given the role of ATR in regulating P53 and CHK1 functions through phosphorylation (Shiloh, 2001; Stiff et al., 2006; Hurley and Bunz, 2007), this study suggests that cytoplasmic PTEN may impact the DNA damage response signaling pathways and tumour suppressing activities through another molecule called ATR. However, the mechanisms by which PTEN controls the ATR expression is not known, which warrants the further research to investigate the ATR expression in cells containing and lacking of PTEN. In the next chapter, the ATR expression in PTEN-deficient and proficient cells will be analyzed. It has been found that positive expression of nuclear PTEN is an independent prognostic and predictive factor which increases

the survival rate of patients with breast tumours. Breast tumours expressing nuclear PTEN showed good survival, while cytoplasmic PTEN was found to be negative prognostic factor for the survival of patients. In addition, the loss of nuclear PTEN expression apart from the expression status of other markers such ATR, cytoplasmic PTEN and pCHK1 (cytoplasmic/nuclear) worsened the survival through increasing the aggressiveness of the disease. This finding is consistent with several other researchers showing the positive impact of the lack of nuclear PTEN expression on the aggressiveness of the disease with a wide variety of cancers (Zhou et al., 2000; Whiteman et al., 2002; Gu et al., 1998). This clearly shows the implication of nuclear PTEN in the pathogenesis of the tumours.

Similarly, breast tumours in whole cohorts expressing nuclear PTEN with negative ATR and pCHK1 (cytoplasmic/nuclear) status showed better survival compared to that with negative nuclear PTEN expression. However, those groups of tumour expressing only cytoplasmic PTEN in the absence of ATR and pCHK1 (cytoplasmic/nuclear) expressions showed the worst survival. Thus nuclear expression was found to be distinguishing feature predicting and prognosticating the survival of patients. The lack of expressions of ATR, pCHK1 (cytoplasmic) and cytoplasmic-PTEN were found to be good prognostic markers showing the better survival in the whole series. These are useful prognostication strategies which can be used to design the personalized treatment for different groups of patients. To conclude expression of nuclear PTEN is positive while the cytoplasmic PTEN, ATR and pCHK1 can be classified as negative predictors of survival among patients with breast cancer. The expression of PTEN, in the light of results of this

study, can serve as important factor for the prognosis, tumorigenesis and development of breast cancer. Some combinatorial indices emerged from the results of this study, which can have useful prognostic value for the practitioners and clinicians, include N-PTEN +/C-pCHK1-, N-PTEN +/N-pCHK1- , and ATR - / C-pCHK1 - / N-PTEN –.

4. Profiling expression DNA repair pathways in PTEN-proficient and PTEN-deficient cells

4.1. Introduction

PTEN has been demonstrated by several other studies to be mutated in several forms of cancer, implicating its role in tumorigenesis (Hunt et al., 2012; Puc et al., 2005; Gupta et al., 2009; Ming et al., 2011a, Ming et al., 2011b). It acts as a major tumour suppressor, which suppresses the cell proliferation mainly through the suppressing activity of PI3K/AKT pathway Ming et al., 2012; Shen et al., 2007). The loss of PTEN activity increases the accumulation of PIP3 level which sustains the activity of AKT signalling cascade in which CHK1 is phosphorylated by AKT (Hunt et al., 2012). Therefore, loss of PTEN is implicated in the DNA damage response as CHK1 is considered an important player which regulates the delays at S/G2 checkpoint and subsequent progression of the cell cycle in order to allow the sufficient time for repair machinery to repair the damaged site of DNA (Toulany et al., 2008, Ming et al., 2012, Shen et al., 2009).

The increased level of active AKT in the cytoplasm leads to the phosphorylation of CHK1 which triggers the cytoplasmic sequestration and ubiquitination process, resulting in reduced level of CHK1 in the nucleus. Consequently, ATM/ATR can not phosphorylate or activate it in the nucleus (Puc et al., 2005). Importantly, the active form of CHK1 is reported to phosphorylate its substrate called CDC25A which is degraded following its phosphorylation. After degradation of CDC25A, the cell cycle is arrested, thus PTEN, CHK1, and ATR/ATM are the important components of axis regulating the cell cycle arrest in the event of DNA damage (Hunt et al., 2012).

In the case of CHK1 inhibition, the DSBs arises, because CHK1 delays the progression of S-phase so that aberrant DNA will not replicate to avoid generating DNA lesions (Syljuasen et al., 2005). Therefore, the loss of PTEN mechanistically abrogates the critical checkpoints of the cell cycle, leading to accumulations of SSBs and DSBs in the daughter chromosome. This proposition was supported by the result showing the increased sensitivity of PTEN-deficient cells to the ionizing radiations through abrogating cell S/G2 checkpoint which leads to 'mitotic arrest bypass'. This results in suppression of apoptosis induced by ionizing radiations during radiotherapy. AKT activated as a result of PTEN loss is reported to cause the radiotherapy and chemotherapy through the activation/phosphorylation of DNA-protein kinase (DNA-PK) which suppresses apoptosis and enhances DNA repair (Toulany et al., 2008). Interestingly, DNA-PKcs is an important component of non-homologous end joining pathway (NHEJ) which is active during the G1-phase of the cell cycle.

The chromosomal aberrations were reported in PTEN-deficient cells, which was contributed by impaired S/G2 checkpoints or due to aberration in homologous recombination functions (Pandita and Richardson, 2009; Gupta et al., 2009). This means that PTEN may be involved in the expression of factors implicated in NHEJ and HR pathways. Shen et al (Shen et al., 2007) confirmed this assumption by showing that PTEN-deficient cells showed the reduced expression of RAD51, nevertheless, these results could not be confirmed when the same cell lines and reagents were used (Gupta et al., 2009). They further demonstrated the increased mitotic index and chromosomal aberrations associated with the PTEN-deficient

cells following their exposure to ionizing radiations rather than PTEN-proficient cells, which suggested that PTEN is involved in checkpoints regulation during progression of cell cycle. Mendes-Pereira et al (2009) showed that PTEN affected the HR pathway and RAD51 expression, but they could not reveal the mechanism by which it exerts its influence on them.

However, Fraser et al (2012) showed that there was no association between PTEN deletion and loss of RAD51 in prostate cancer, while PTEN deficiency made the prostate cancer cells sensitive to PARP inhibitors. PARP proteins are expressed in BER pathway, which means that PTEN deficiency in prostate cancer affects the BER pathway. In another study, McEllin et al (2010) come with findings which were in agreement with Mendes-Pereira et al (2009) in astrocytes with PTEN-deficiency, but they could not show any difference in RAD51 levels between the PTEN-null and PTEN-proficient astrocytes. Another report concluded that PTEN-deficient H1299 cells exhibited the accumulation of DSBs after IR exposure compared to PTEN-proficient H1299 cells (Pappas et al., 2007).

Another report showed that deficiency of PTEN in the cancer cells was associated with an elevated DNA DSBs lesions, accumulation of reactive oxygen species and ATM activation in PC3 and HCT116 isogenic models. However, the subsequent inactivation of ATM in PTEN-deficient cells resulted in catastrophic DNA damage, increased apoptosis, mitotic cell arrest compared with wild type cells (McCabe et al., 2015). In skin cancer, different reports have demonstrated that PTEN was downregulated, and as a result of this, DSBs were accumulated. In addition, the loss of PTEN has been reported to negatively regulate global genome NER pathway

through suppression of XPC (Ming et al., 2011a, Ming et al., 2011b, Ming et al., 2010).

Taken together, these data indicate that PTEN deficiency in different types of cancer cells can regulate HR, NHEJ, and NER pathways, and some other factors regulating DNA damage response. However, profiling DNA repair gene expression in PTEN-deficient breast cancer cells has not been previously investigated. Therefore, this study aims to investigate the loss of PTEN on the expression of different factors involved in DNA repair pathways in the breast cancer. The next section will give the aims in detail.

4.2. Aims

This chapter addresses the following aims of the study**:**

1. To examine the status of PTEN expression in different breast cancer cell lines for selection of PTEN- proficient and PTEN-deficient cells (MCF7, MDA-MB-231, MDA-MB-468 and BT-549)
2. To investigate the DNA repair gene expression in PTEN-proficient and PTEN-deficient cells using RT^2 Profiler^{TM} DNA Repair PCR Array.

4.3. Materials and Methods

The Western blotting analysis was performed to test the expression of PTEN in MCF7, MDA-MB-231, MDA-MB-468, BT-549 cell lines. For quantification of PTEN expression, RT-qPCR was utilized, which help select PTEN-deficient cells and to confirm the Western blotting results. The detailed material and methods

about Western blotting and RT-qPCR can be found in chapter 2. RT^2 Profiler™ DNA Repair PCR Array was used to profile expressions of DNA repair genes, and the detailed material and methods about the PCR array can be found in chapter 2.

4.4. Results

4.4.1. MCF7 and MDA-MB-231 shows PTEN expression

In order to examine the status of the PTEN expression (cytoplasmic and nuclear PTEN) in different cells such MCF7, MDA-MB-231, BT-549 and MDA-MB-468, western blotting analysis was performed. The cell lysate was extracted from 70-80% confluent cells; and run on the 4-12% precast SDS gel. Proteins were transferred onto a nitrocellulose membrane using the PTEN-specific antibody. B-actin was employed as a loading control and for normalization of concentration of PTEN protein in the sample. The Western blot demonstrated that MCF7 and MDA-MB-231 were the PTEN-proficient cells, whereas BT-549 and MDA-MB-468 were deficient of PTEN protein, which enables to classify these cell lines as PTEN-proficient and PTEN-deficient cells for this study regardless of their other distinguishing characteristics. Protein quantification was performed by infrared dye-labelled secondary antibody (Li-Cor, Lincoln, USA; 1:10000 dilution in blocking buffer) detected by Li-Cor Odyssey Scanner, which confirmed that expression of PTEN was found in almost equal proportion in both MCF7 and MDA-MB-231 cells, no expression was detected in BT-549 and MDA-MB-468 (Figure 4.1). Significant lack of expression of PTEN protein was observed in BT-549 ($p < 0.01$) and MDA-MB-468 cell lines ($p < 0.005$) compared to MCF7 and

MDA-MB-231 cells, respectively. This experiment was done in triplicate (i.e. cell lines were passaged three at three different times).

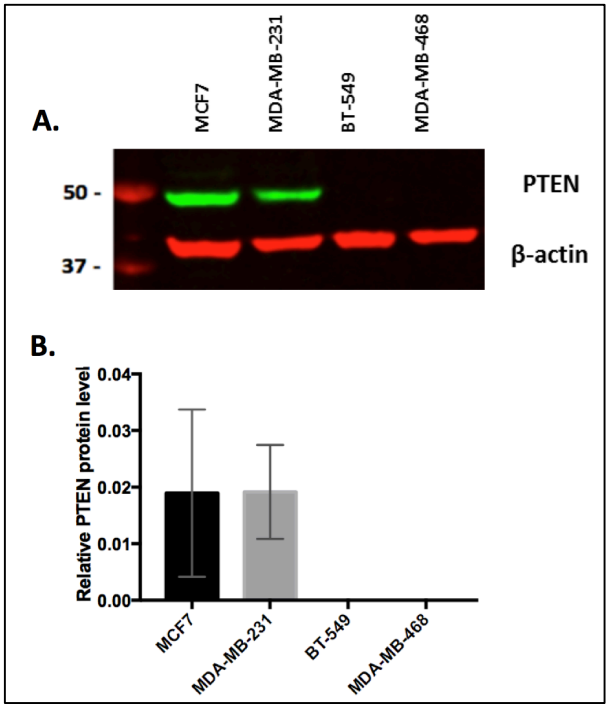


Figure 4-1 PTEN western blotting analysis in breast cancer cell lines
A. PTEN protein expression (cytoplasmic and nuclear) in the selected cell lines. **B**. shows the quantification of PTEN protein. P-value of BT-549 versus MCF7 and MDA-MB-231 were 0.01 and 0.05, respectively. Similarly, P. value of MDA-MB-231 versus MCF7 and MDA-MB-231 were 0.01 and 0.05, respectively. The experiment was done in triplicate.

4.4.2. RT-qPCR confirmed the lack of expression of PTEN in BT549 and MB-468 cells

The expression of mRNA level of PTEN in selected cell lines was examined using RT-qPCR assay as described in chapter 2. For each cell line, the relative PTEN expression was measured and compared with each other. The expression of housekeeping gene HPRT1 was used to normalize the PTEN expression in the

samples. The relative expression level of mRNA of PTEN was calculated by taking ratio between PTEN mRNA and HRPRT1 mRNA. The comparison between the mRNA level in MCF7 and BT-549 showed that PTEN mRNA level was 5 fold higher than BT-549, and 121 fold higher than MDA-MB-468 cells. Similarly, the PTEN mRNA level in MDA-MB-231 demonstrated 4 fold higher than BT-549, and 108 fold higher than MDA-MB-468 cells (Figure 4.2). However, there was no significant difference in expression level of PTEN either between MCF7 and MDA-MB-231 or between BT-549 and MDA-MB-468. These data indicate that PTEN protein was expressed significantly in MCF7 and MDA-MB-231 cells, which is why these cell lines were classed as PTEN-proficient cell lines. There was no expression of mRNA in BT-549 and MDA-MB-468 cell lines, and hence significant difference in PTEN expression was reported by data (Figure 4.2). Therefore, BT-549 and MDA-MB-468 were categorized as PTEN-deficient cell lines for this study.

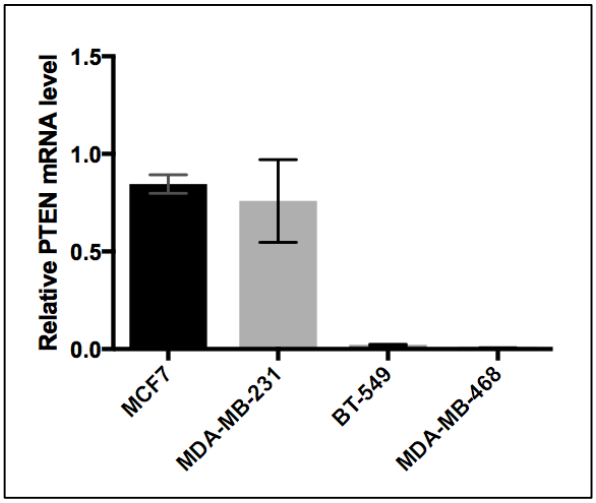


Figure 4-2 Quantification of PTEN expression at mRNA level using RT-qPCR.

4.4.3. Profiling of DNA repair genes Using RT^2 Profiler PCR array

The Qiagen RT^2 Profiler PCR array is a powerful and highly reliable assay which is used to analyze the expression of genes involved in signal transduction/disease related pathways or any biological process. In the current study, it was employed to analyze a panel of DNA repair genes in the selected cell lines using two-step reverse transcription PCR (RT-PCR). Two-step RT-PCR begins with the conversion of isolated RNA to cDNA using reverse transcriptase (RT). Next, the cDNA was synthesized by priming the gene-specific primers using a PCR master mix under specific thermal cycling conditions. This master mix contains deoxyribonucleotides, thermostable polymerase, and SYBR green dye which fluoresces on binding to the minor groove of any double-stranded DNA (dsDNA). The PCR cycle software determines the fluorescent 'noise', also called baseline fluorescence, and automatically calculated. At the beginning of exponential phase of PCR cycle, the dsDNA is amplified, and hence the fluorescence increases with an increase in dsDNA contents, and reaches the pre-set baseline intensity which is called baseline threshold. The PCR cycle at which the fluorescent signal of the reaction crosses the threshold. The C_T is used to calculate the initial DNA copy number because the C_T value is inversely related to the starting amount of the target. A higher level of target gene means that the reaction enters the exponential phase earlier and therefore gives a lower C_T value. Furthermore, normalization of C_T of the gene of interest with the C_T of the house-keeping gene (ΔC_T) allows the

comparison of gene expression between different samples using $\mathbf{\Delta\Delta C_T}$ method.

Amount of target gene = $2^{-\Delta\Delta C_T}$

Where $\Delta\Delta C_T = \Delta C_T$ of sample - ΔC_T of control

ΔC_T is the C_T of the target gene subtracted by the C_T of the housekeeping gene.

A melting curve analysis can identify the presence of a nonspecific product, such as primer dimers, which appear as additional peaks on the melting curve and often form in a no-template control (NTC). The products are heated from 60°C to 95°C for 20 minutes after the 40 cycles because the primer dimers exhibit a lower melting temperature than the amplicon. When the melting point (Tm) is reached, the florescent signals decreases as the dsDNA breaks into ssDNA at this temperature. The melting temperature is affected by various factors such as GC content, template DNA concentration and length and mismatches), therefore, different PCR products may show different melting temperatures. Using the melting curve, it is possible to detect the presence of nonspecific products as additional peaks by plotting $-\Delta F/\Delta T$ (change in fluorescence/change in temperature) against the temperature to give a melting profile. Figures 4.3 shows the melting curve analysis for each cell line.

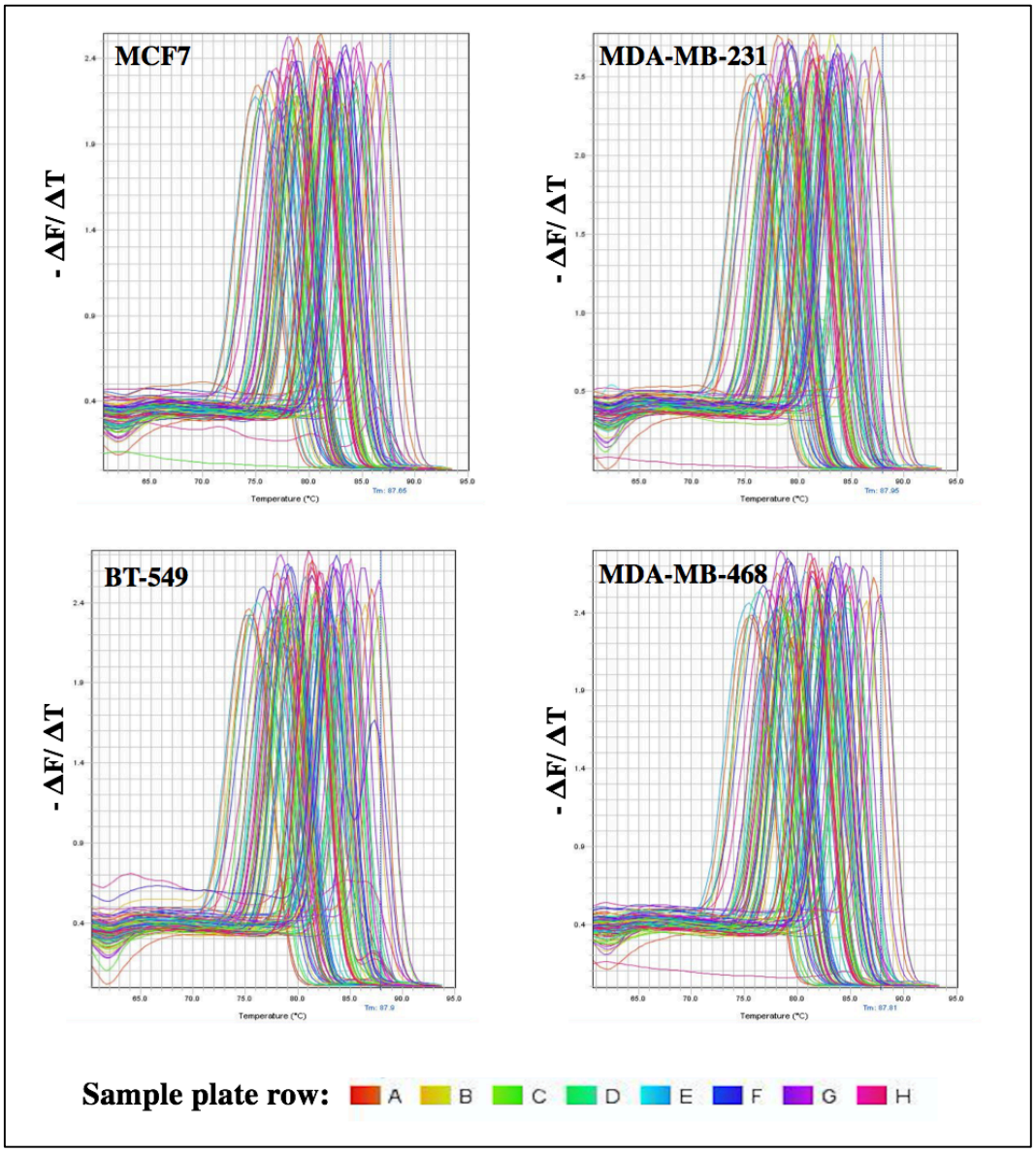


Figure 4-3 Melting curve analysis for selected breast cancer cell lines using RT^2 Profiler PCR Array.
Single melting peaks for all 84 primers used for the selected cell lines, showing the high specificity.

4.4.4. Altered DNA Repair Genes expression in PTEN-deficient Breast cancer cells

In order to assess the impact of PTEN deficiency in breast cancer cells, the PTEN-deficient cell lines including BT-549 and MDA-MB-468 were used as treatment cell lines and PTEN-proficient cell lines including MDA-MB-231 and MCF7 were used as control cells. RT2 Profiler PCR array was employed to investigate the impact of PTEN deficiency on expression of 84 genes with known functions in DNA damage response pathways. The detailed protocol of RT2 PCR DNA repair profile assay can be found in chapter 2. Data analysis was performed as detailed in in chapter 2. Briefly data from the Profiler was imported into Qiagen analysis template which can be used to make comparison of PTEN-deficient cell lines against PTEN-proficient cell lines.

The integrated Web-based software package was used to analyze data, which automatically performed all calculations relating to C_T, ΔC_T, $\Delta\Delta C_T$ and fold changes in the expression of the 84 genes in comparison with the control cell lines (MCF7 and MDA-MB-231). The expression data for MDA-MB-231 and MCF7 were normalized using a housekeeping gene called GAPDH and Beta-actin, respectively. The scatter plots were obtained showing the comparative gene expression in PTEN-deficient BT-549 and MDA-MB-468 cells to the PTEN-proficient MDA-MB-231 and MCF7 cells. It can be observed from the scatter plots that PTEN deficiency in BT-549 cells remarkably downregulated most of genes involved in DNA damage signaling (Figure 4.4.), while that in MDA-MB-468 upregulated the DNA repair genes in comparison with PTEN-

proficient MDA-MB-231 cells (Figure 4.5). Similarly, scatter plots that PTEN deficiency in BT-549 cells remarkably downregulated most of genes involved in DNA damage signaling (Figure 4.6.), while that in MDA-MB-468 upregulated the DNA repair genes in comparison with PTEN-proficient MCF7 cells (Figure 4.7). In addition, this shows that PTEN serves as transcription enhancer in BT-549 cells, whereas it does as a transcription suppressor in MDA-MB-468 cells.

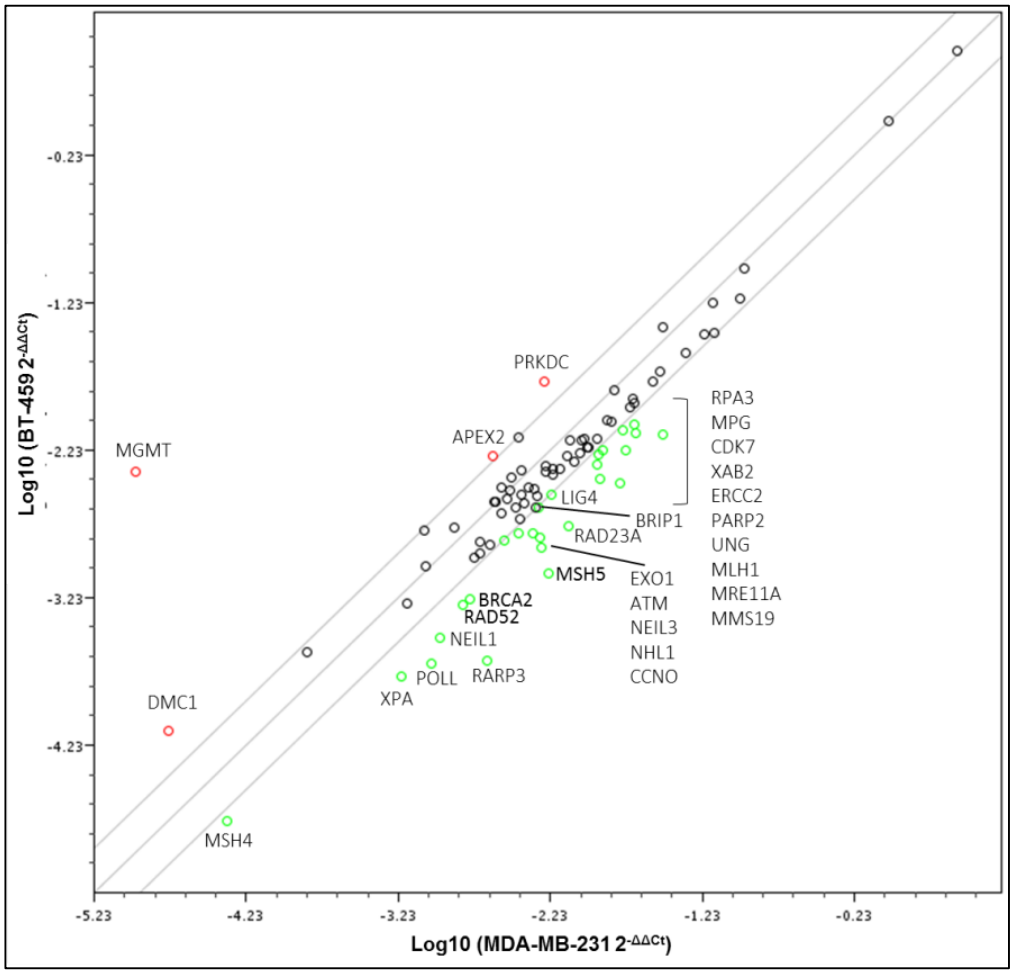


Figure 4-4 : Scatter plot showing comparison of gene expression in PTEN-deficient BT-549 and PTEN-proficient MDA-MB-231 cells.
The genes above and below the grey lines showed 2-fold changes in expression with respect to control MDA-MB-231 cells. Green circles indicate genes under-expressed, the red circles indicate genes overexpressed compared to PTEN-proficient cells. The grey circles indicate genes whose expression are not altered.

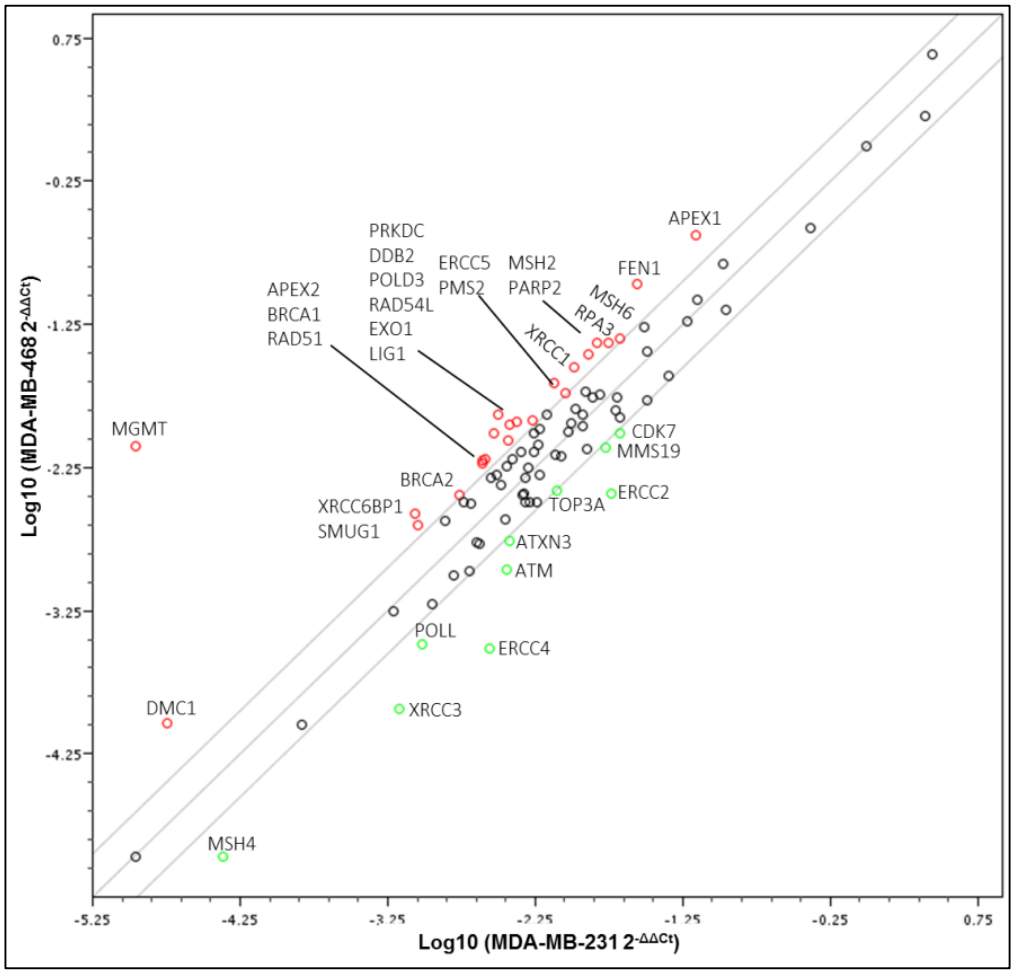


Figure 4-5 Scatter plot showing comparison of gene expression in PTEN-deficient MDA-MB-468 and PTEN-proficient MDA-MB-231 cells.
The genes above and below the grey lines showed 2-fold changes in expression with respect to control MDA-MB-231 cells. Green circles indicate genes under-expressed, the red circles indicate genes overexpressed compared to PTEN-proficient cells. The grey circles indicated genes whose expression was not altered.

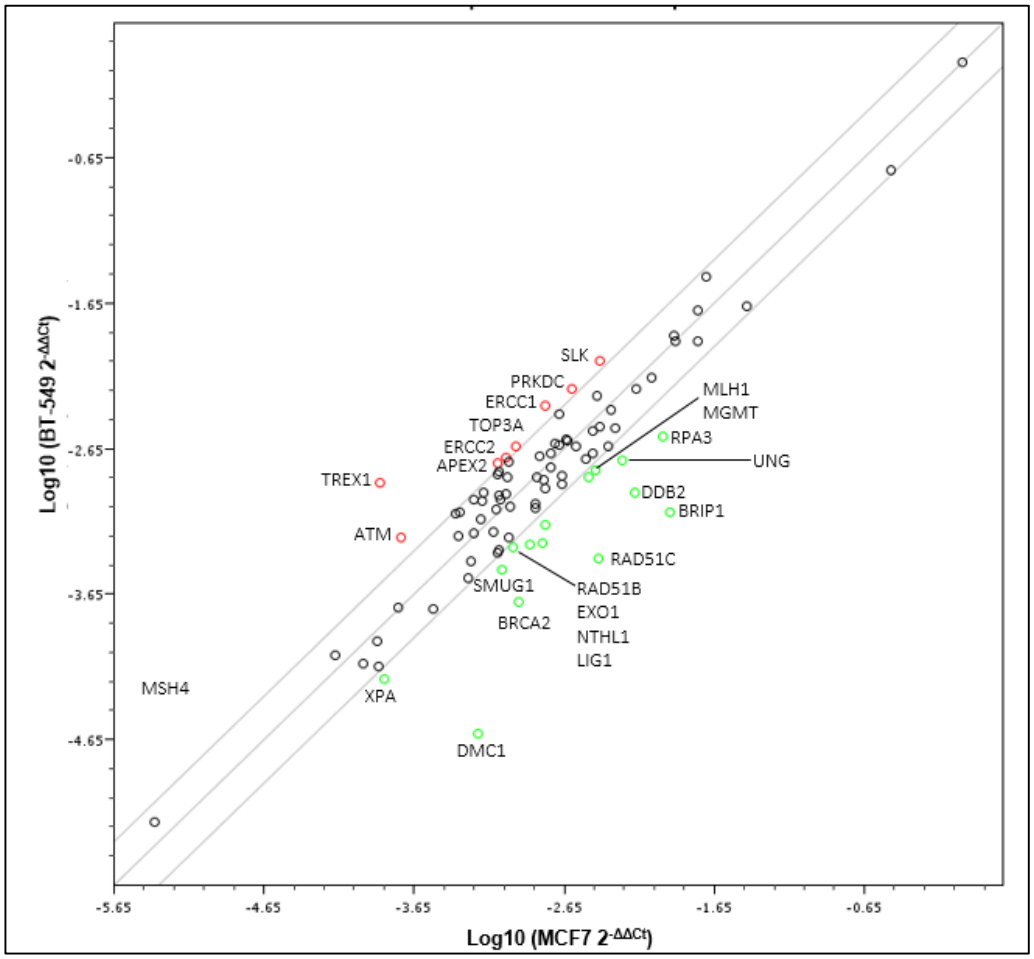


Figure 4-6 Scatter plot showing comparison of gene expression in PTEN-deficient BT-549 and PTEN-proficient MCF7 cells.
The genes above and below the grey lines showed 2-fold changes in expression with respect to control MCF7 cells. Green circles indicate genes under-expressed, the red circles indicate genes overexpressed compared to PTEN-proficient cells. The grey circles indicated genes whose expression was not altered.

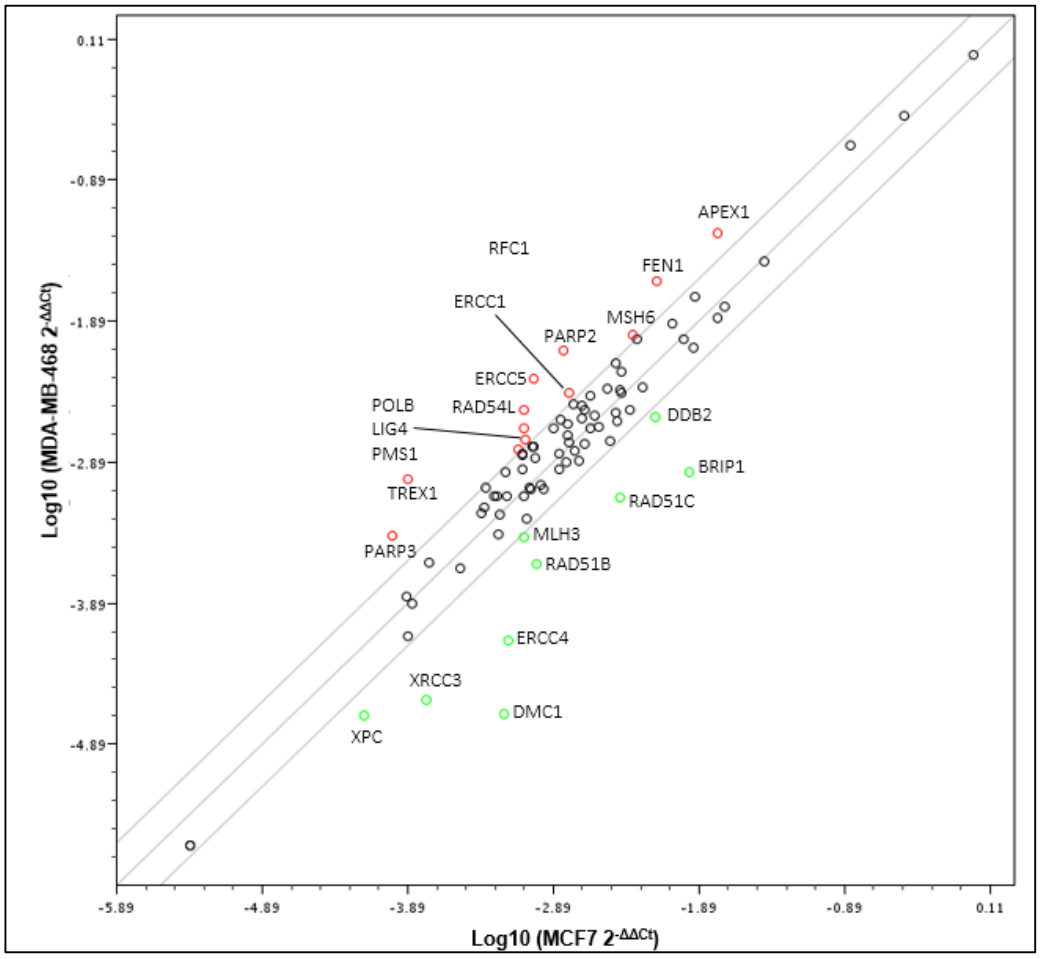


Figure 4-7 Scatter plot showing comparison of gene expression in PTEN-deficient MDA-MB-468 and PTEN-proficient MCF7 cells.
The genes above and below the grey lines showed 2-fold changes in expression with respect to control MCF7 cells. Green circles indicate genes under-expressed, the red circles indicate genes overexpressed compared to PTEN-proficient cells. The grey circles indicated genes whose expression was not altered.

The genes showing the 2-fold upregulation or downregulation in their expression compared to the control cells and had p-value less than 0.05 were considered to be genes with significantly altered expression. Student t-test was used to compare the gene expression from the PTEN-deficient cells to that of PTEN-proficient cells. The data showed that several genes from BER (Table 4.1), HR pathway (Table 4.2), NHEJ pathway (Table 4.3), NER pathway (Table 4.4), MMR pathway (Table 4.5) and Other DNA repair related genes (Table 4.6) were comparatively downregulated and upregulated which might be due to PTEN deficiency in BT-549 and MDA-MB-468. However, a majority of these DNA repair genes in both BT-549 and MDA-MB-468 were not found with significantly altered expression compared to the PTEN-proficient MDA-MB-231 cells. Similarly, most of genes did not meet the criteria of 2-fold change in their expression levels and p-value lesser than 0.05.

Six genes in PTEN-deficient BT-549 cells met the criteria of 2-fold change expression, showing that their expression was significantly altered compared to PTEN-proficient cells MDA-MB-231 cells. Out of six genes, two genes belonged to BER (APE2 and OGG1), 1 gene from HR (DMC1), two genes from NHEJ (XRCCBP1 and PRDKC1 and one from epigenetic DNA repair (MGMT). Seven genes including NTHL1 (BER pathway) and DMC1 (HR pathway), XRCCBP1 (NHEJ pathway), MGMT (epigenetic DNA repair) and TDG (MMR pathway) showed the significantly altered expression in PTEN-deficient BT-549 compared to PTEN-proficient. Interestingly none of NER genes showed significant changes in their expression levels. Similarly,

comparison of profiler data from PTEN-deficient MDA-MB-468 cells with the PTEN-proficient MDA-MB-231 cells showed that 22 genes met the criteria of 2-fold change in expression levels and six genes met the criteria of p-value lesser than 0.05. From 21 genes with 2-fold altered expression, there were six BER pathway genes (APE1, APE2, PARP2, SMUG1, FEN1 and XRCC1), eight HR pathway genes (BRCA1, BRCA2, RAD50, RAD51, RAD51D, RAD54L, XRCC2 and DMC1), three NHEJ pathway genes (PRKDC, XRCC6, XRCC6BP1), 4 NER pathway genes (ERCC5, RPA3, DDB2 and LIG1) and MMR pathway gene (EXO1). Likewise, the genes significant expression level with *P-values* less than 0.05 included PARP3 (BER pathway), XRCC6BP1 (NHEJ pathway), MGMT (epigenetic DNA repair) and LIG1, CDK7, RPA3 and SLK (NER pathway).

Table 4-1 Base excision repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MDA-MB-231.

Base Excision Repair	BT-549 cells compared to MDA-MB-231cells		MDA-MB-468 cells compared to MDA-MB-231 cells	
	Fold Change	P-value	Fold change	P-value
APE1	**0.8409**	0.644	**3.41**	0.177
APE2	**2.1409**	0.136	**2.56**	0.315
PARP1	**0.6646**	0.306	**1.231**	0.645
PARP2	**0.4513**	0.069	**2.691**	0.057
PARP3	**0.0974**	0.070	**0.753**	0.460
Pol β	**0.8289**	0.188	**1.765**	0.073
SMUG1	**1.0783**	0.945	**2.50**	0.300
NTHL1	**0.2969**	0.037	**1.122**	0.631
UNG	**0.4508**	0.338	**1.544**	0.544
FEN1	**0.6177**	0.468	**3.95**	0.152
OGG1	**1.9623**	0.148	**1.592**	0.496
NEIL1	**0.2803**	0.213	**0.555**	0.379
NEIL2	**0.6761**	0.108	**0.704**	0.081
NEIL3	**0.3544**	0.103	**1.594**	0.300
XRCC1	**0.5896**	0.142	**2.694**	0.067
CCNO	**0.2496**	0.120	**0.628**	0.353
LIG3	**0.5788**	0.082	**0.793**	0.851
MUTYH	**0.7783**	0.592	**1.70**	0.578

P-values were determined using two-tailed unpaired student t-test.

Table 4-2 Homologous recombination repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MDA-MB-231.

Homologous Recombination Repair Genes	BT-549 cells compared to MDA-MB-231cells		MDA-MB-468 cells compared to MDA-MB-231 cells	
	Fold Change	P-value	Fold change	P-value
BRCA1	**1.0125**	0.973063	**2.532**	0.156862
BRCA2	**0.332**	0.20239	**2.058**	0.230322
RAD21	**0.9083**	0.658759	**0.652**	0.267711
RAD50	**0.654**	0.283137	**1.735**	0.120646
RAD51	**1.0523**	0.924436	**2.4267**	0.177566
RAD51B	**0.6843**	0.337469	**0.523**	0.273475
RAD51C	**0.5739**	0.160754	**1.536**	0.202792
RAD51D	**1.2799**	0.939635	**1.729**	0.587591
RAD54L	**0.9831**	0.770987	**4.103**	0.213757
RAD52	**0.3349**	0.273047	**0.636**	0.482307
XRCC2	**0.5976**	0.495719	**1.744**	0.70808
XRCC3	**0.8049**	0.706057	**0.173**	0.087876
ATM	**0.4435**	0.228304	**0.297**	0.190541
DMC1	**4.0699**	0.056979	**5.178**	0.283165

P-values were determined using two-tailed unpaired student t-test.

Table 4-3 Non-Homologous end joining repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MDA-MB-231.

Non-homologous end joining Repair Genes	BT-549 cells compared to MDA-MB-231cells		MDA-MB-468 cells compared to MDA-MB-231 cells	
	Fold Change	P-value	Fold change	P-value
DNA-PKcs	**3.1767**	0.151938	**2.2424**	0.196427
XRCC4	**0.6943**	0.235329	**0.7773**	0.294372
XRCC5	**0.6016**	0.190123	**1.4348**	0.474652
XRCC6	**0.6525**	0.21293	**1.7663**	0.151416
XRCC6BP1	**1.9332**	**0.038247**	**3.1041**	**0.009402**
LIG4	**0.4924**	0.064275	**1.3758**	0.500142

P-values were determined using two-tailed unpaired student t-test.

Table 4-4 Nucleotide excision repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MDA-MB-231.

Nucleotide Excision Repair Pathway	BT-549 cells compared to MDA-MB-231cells		MDA-MB-468 cells compared to MDA-MB-231 cells	
	Fold Change	P-value	Fold change	P-value
BRIP1	**0.4956**	0.380722	**0.9789**	0.94284
LIG1	**0.5405**	0.202439	**2.3439**	0.038526
ATXN3	**0.7828**	0.517317	**0.455**	0.341401
RAD23A	**0.2282**	**0.040881**	**0.8829**	0.863456
RAD23B	**0.596**	0.17283	**0.9852**	0.88487
RPA1	**0.7202**	0.594418	**1.1208**	0.766113
CCNH	**0.5783**	0.200337	**0.7189**	0.364546
CDK7	**0.3626**	0.033358	**0.4628**	**0.0368**
DDB1	**0.5131**	0.118977	**1.1882**	0.576579
DDB2	**0.7791**	0.480425	**2.758**	0.15099
PNKP	**0.7202**	0.475932	**1.1627**	0.818808
POLL	**0.2203**	0.111151	**0.3397**	0.105248
RPA3	**1.2153**	**0.013077**	**2.4142**	**0.011999**
SLK	**0.5859**	0.105294	**0.5387**	**0.011999**
ERCC1	**0.6378**	0.449785	**0.8553**	0.726503
ERCC2	**0.3126**	0.214585	**0.2004**	0.182421
ERCC3	**0.6408**	0.441911	**1.9336**	0.353102
ERCC4	**1.1797**	0.749765	**0.1096**	0.12279
ERCC5	**0.7156**	0.155827	**2.8671**	0.114258
ERCC6	**0.5076**	0.307206	**0.7834**	0.680832
ERCC8	**0.5965**	0.237951	**0.6749**	0.370102
XAB2	**0.2312**	0.077427	**0.5188**	0.169362
XPA	**0.2823**	**0.053668**	**0.9**	0.925235
XPC	**1.7057**	0.506012	**0.6168**	0.279155

P-values were determined using two-tailed unpaired student t-test.

Table 4-5 Mismatch repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MDA-MB-231.

Mismatch Repair pathway Genes	BT-549 cells compared to MDA-MB-231cells		MDA-MB-468 cells compared to MDA-MB-231 cells	
	Fold Change	P-value	Fold change	P-value
POLD3	**1.1125**	0.969898	**2.928**	0.322472
PMS1	**0.7638**	0.247354	**1.3166**	0.484463
PMS2	**0.6174**	0.378696	**2.0421**	0.354854
MLH1	**0.3949**	0.327065	**0.9371**	0.833309
MLH3	**0.564**	0.352031	**0.6934**	0.595247
MSH2	**0.6145**	0.396381	**2.8051**	0.142376
MSH3	**0.717**	0.418346	**0.8239**	0.625614
MSH4	**0.413**	0.398092	**0.2465**	0.254666
MSH5	**0.1484**	**0.030613**	**0.5616**	0.29892
MSH6	**0.5834**	0.303527	**2.1445**	0.304173
MPG	**0.4148**	0.277013	**0.6059**	0.406125
TREX1	**1.177**	0.627562	**1.304**	0.575987

P-values were determined using two-tailed unpaired student t-test.

Table 4-6 Other related DNA repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MDA-MB-231.

Other related Genes	BT-549 cells compared to MDA-MB-231cells		MDA-MB-468 cells compared to MDA-MB-231 cells	
	Fold Change	P-value	Fold change	P-value
ATR	**0.898**	0.70747	**1.6469**	0.192378
EXO1	**0.4901**	0.328215	**3.3103**	0.24269
MGMT	**380.5219**	**3.87E-05**	**730.693**	**0.009658**
RAD18	**0.7234**	0.427313	**1.0555**	0.925664
RFC1	**0.588**	0.358980	**1.3744**	0.540982
TOP3A	**0.8697**	0.550096	**0.4878**	0.214917
TOP3B	**0.638**	0.367829	**1.6348**	0.378102
MRE11A	**0.3016**	0.178277	**0.5998**	0.3634
MMS19	**0.2073**	0.181555	**0.4552**	0.287729
TDG	**0.5845**	**0.016382**	**1.1143**	0.491562

P-values were determined using two-tailed unpaired student t-test.

The profiler data were also generated for the comparison of PTEN-deficient cell lines – MDA-MB-468 and BT-549 – with another PTEN-proficient control – MCF7. It was hypothesized that PTEN-deficient cells generate different expression against different PTEN-proficient cell line. The data demonstrated that several genes from BER (Table 4.7), HR pathway (Table 4.8), NHEJ pathway (Table 4.9), NER pathway (Table 4.10) and MMR pathway (Table 4.11) and other DNA repair related genes (Table 4.12) were comparatively downregulated and upregulated in PTEN-deficient cells (BT-549 and MDA-MB-468).

When the expression of genes in PTEN-deficient cells (BT-549 and MDA-MB-468) were compared with PTEN-proficient control (MCF7), it was observed that six genes in BT-549 come up to the criteria of 2-fold change for Signiant change in expression. 1 gens (APE2) belonged to BER pathway, 1 gene (ATM) came from HR pathway, 1 gene (DNA-PKcs) from the NHEJ pathway, two genes (SLK and ERCC2) from NER pathway, and a gene topoisomerase function (TOP3A).

Likewise, when the profiler data from PTEN-deficient cells (MDA-MB-468) were compared with that from PTEN-proficient cells (MCF7), total of 11 genes demonstrated the 2-fold change in expression level. Out of 11 genes, five genes (APE1, PARP2, PARP3, Polβ, FEN) were associated with BER pathway, 1 gene with HR pathway, one gene with NHEJ pathway, and 4 genes with NER pathway. These data indicated that total number of upregulated genes in MDA-MB-468 was approximately 2-fold higher than that in BT-549. In addition, out

of 11 genes, only five genes showed the P-value equal to or lesser than 0.5, suggesting that their expression was significantly upregulated in response to PTEN deficiency in MDA-MB-468.

Most genes in PTEN-deficient cells BT-549 and MDA-MB-468 were downregulated with expression level lesser than 1 in comparison with PTEN-proficient cell line-MCF7. For instance, 34 genes were upregulated, out of which 14 genes were significantly downregulated with P-value less than 0.5. Similarly, 37 genes in MDA-MB-468 were downregulated, out of which 7 genes were significantly downregulated with P-value lesser than 0.5. This suggest that most of genes in BT-549 and MDA-MB-468 are not significantly downregulated.

Table 4-7 Base excision repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MCF7.

Base Excision Repair	BT-549 cells compared to MCF7 cells		MDA-MB-468 cells compared to MCF7 cells	
	Fold Change	P-value	Fold change	P-value
APE1	**1.134**	0.534346728	**3.165**	0.077389959
APE2	**2.2144**	**0.003765604**	**1.8198**	0.346699963
PARP1	**0.8126**	0.151551814	**1.0367**	0.82466032
PARP2	**1.2917**	**0.039254537**	**5.3036**	**0.010790538**
PARP3	**0.7302**	0.707669141	**3.8858**	**0.033022423**
Pol β	**1.8875**	**0.001009209**	**2.7647**	**0.008665958**
SMUG1	**0.3815**	**0.024267498**	**0.6089**	0.321570652
NTHL1	**0.3138**	**0.003953073**	**0.8168**	0.144247533
UNG	**0.3431**	**0.008889204**	**0.809**	0.209691279
FEN1	**0.857**	0.454091194	**3.7696**	**0.075152988**
OGG1	**1.2335**	0.228864956	**0.6892**	0.530643411
NEIL1	**0.8325**	0.645695668	**1.134**	0.832819459
NEIL2	**1.9038**	**0.021892712**	**1.3645**	0.075385073
NEIL3	**0.5696**	0.058959544	**1.7638**	0.253142661
XRCC1	**0.5932**	0.035480913	**1.867**	**0.047039938**
CCNO	**0.5325**	0.218388409	**0.9217**	0.818505099
LIG3	**0.9066**	0.496378868	**0.8553**	0.913240011
MUTYH	**1.1928**	0.418573929	**1.7891**	0.225289064

P-values were determined using two-tailed unpaired student t-test.

Table 4-8 Homologous recombination repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MCF7.

Homologous Recombination Repair Genes	BT-549 cells compared to MCF7 cells		MDA-MB-468 cells compared to MCF7 cells	
	Fold Change	P-value	Fold change	P-value
BRCA1	**0.6113**	0.360956274	**1.0526**	0.732767697
BRCA2	**0.175**	0.108677344	**0.7472**	0.477312193
RAD21	**1.732**	0.084017589	**0.8558**	0.575710633
RAD50	**0.8262**	0.162375573	**1.5089**	**0.016079013**
RAD51	**0.9102**	0.891470189	**1.4453**	0.127974768
RAD51B	**0.4656**	0.090315111	**0.2454**	**0.052796642**
RAD51C	**0.1054**	**0.05221297**	**0.1942**	0.065248505
RAD51D	**1.0606**	0.758104931	**0.9867**	0.891601794
RAD54L	**1.2787**	0.182790629	**3.675**	0.146083305
RAD52	**0.5936**	0.074492571	**0.7761**	0.358994604
XRCC2	**0.6969**	**0.009798675**	**1.4006**	0.151760648
XRCC3	**1.0498**	0.980894035	**0.1549**	0.159222718
ATM	**3.0004**	**0.036485412**	**1.3834**	0.443235337
DMC1	**0.0415**	**0.006709991**	**0.0364**	**0.007284999**

P-values were determined using two-tailed unpaired student t-test.

Table 4-9 Non-Homologous end joining repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MCF7.

Non-homologous end joining Repair Genes	BT-549 cells compared to MCF7 cells		MDA-MB-468 cells compared to MCF7 cells	
	Fold Change	P-value	Fold change	P-value
DNA-PKcs	**2.2861**	0.098401401	**1.1111**	0.665246492
XRCC4	**1.1855**	0.336803311	**0.9139**	0.549002971
XRCC5	**0.581**	0.056618621	**0.954**	0.997342457
XRCC6	**0.7945**	0.416431556	**1.4808**	0.344701882
XRCC6BP1	**1.2949**	0.359515051	**1.4316**	0.172480656
LIG4	**1.1927**	0.363745459	**2.2946**	0.218849738

P-values were determined using two-tailed unpaired student t-test.

Table 4-10 Nucleotide excision repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MCF7.

Nucleotide Excision Repair Pathway	BT-549 cells compared to MCF7 cells		MDA-MB-468 cells compared to MCF7 cells	
	Fold Change	P-value	Fold change	P-value
BRIP1	**0.0724**	**0.009449532**	**0.0984**	**0.011917912**
LIG1	**0.4079**	**0.022142076**	**1.2178**	0.198047403
ATXN3	**1.7967**	0.07712499	**0.7191**	0.62705637
RAD23A	**0.7942**	0.2269973	**2.1156**	0.208085694
RAD23B	**0.6925**	0.077431268	**0.7883**	**0.023259957**
RPA1	**1.1014**	0.687888918	**0.6994**	0.115448517
CCNH	**1.8786**	**0.035806046**	**1.608**	0.070958135
CDK7	**1.1143**	0.622503182	**0.9794**	0.860479619
DDB1	**1.0064**	0.908280137	**1.6048**	0.084634975
DDB2	**0.1703**	**0.003464647**	**0.4151**	**0.026849343**
PNKP	**1.1564**	0.178683972	**1.2855**	0.465415389
POLL	**1.8875**	**0.001009209**	**2.7647**	**0.008665958**
RPA3	**0.2649**	**0.024316833**	**0.9631**	0.743807589
SLK	**2.3143**	0.111571338	**1.465**	0.245951594
ERCC1	**2.6196**	**0.036780145**	**2.4188**	0.14746883
ERCC2	**2.1557**	**0.003751439**	**0.9517**	0.962465521
ERCC3	**0.6791**	**0.027953864**	**1.411**	0.339955707
ERCC4	**1.7228**	0.10536229	**0.1102**	**0.016134846**
ERCC5	**1.9184**	**0.047407516**	**5.2924**	**0.046770439**
ERCC6	**1.7938**	**0.031522068**	**1.9062**	0.250756319
ERCC8	**1.5269**	0.105853817	**1.1897**	0.499983283
XAB2	**1.0562**	0.489950787	**1.632**	**0.011249645**
XPA	**0.4135**	0.092282152	**0.9079**	0.988339307
XPC	**1.2768**	0.597351367	**0.3179**	**0.003453731**

P-values were determined using two-tailed unpaired student t-test.

Table 4-11 Mismatch repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MCF7.

Mismatch Repair pathway Genes	BT-549 cells compared to MCF7 cells		MDA-MB-468 cells compared to MCF7 cells	
	Fold Change	P-value	Fold change	P-value
POLD3	**0.966**	0.761292507	**1.7509**	0.375013495
PMS1	**1.5236**	0.0573716	**1.8083**	0.189582098
PMS2	**0.6173**	0.17138005	**1.4058**	0.4772086
MLH1	**0.436**	**0.044039978**	**0.7123**	0.38207997
MLH3	**0.5447**	0.062720605	**0.4611**	0.127050561
MSH2	**0.6345**	**0.032991509**	**1.9945**	0.091309389
MSH3	**1.8306**	0.076910479	**1.4484**	0.155524255
MSH4	**1.4621**	0.170030601	**0.6009**	0.655323754
MSH5	**0.5638**	0.15735373	**1.4688**	0.426495729
MSH6	**0.9063**	0.51920916	**2.2939**	0.199751812
MPG	**0.8424**	0.271317633	**0.8472**	0.326168235
TREX1	**9.9652**	**0.002984869**	**7.6019**	0.093206862

P-values were determined using two-tailed unpaired student t-test.

Table 4-12 Other related DNA repair genes with altered expression in PTEN-deficient BT-549 and MDA-MB-468 cells compared to PTEN-proficient cells MCF7.

Other related Genes	BT-549 cells compared to MCF7 cells		MDA-MB-468 cells compared to MCF7 cells	
	Fold Change	P-value	Fold change	P-value
ATR	**0.6412**	0.316144328	**0.8097**	0.543198272
EXO1	**0.3663**	**0.038631548**	**1.7035**	0.331053882
MGMT	**0.4347**	0.055601808	**0.5747**	0.086389658
RAD18	**0.8584**	0.638124439	**0.8624**	0.539641954
RFC1	**1.1473**	0.37757121	**1.8465**	**0.049541664**
TOP3A	**2.1606**	**0.025075982**	**0.8344**	0.669457359
TOP3B	**1.0807**	0.575003427	**1.9067**	0.170436478
MRE11A	**0.5921**	**0.024795657**	**0.8107**	0.270367735
MMS19	**0.7137**	0.192009396	**1.0791**	0.622481763
TDG	**0.5347**	0.146441052	**0.7019**	0.253802915

P-values were determined using two-tailed unpaired student t-test.

4.5. Discussion

4.5.1 Loss of PTEN expression associated with reduction in HR recombination genes expression

The role of PTEN in the DNA damage response has been confirmed by several other studies, which suggests the interaction of PTEN with BRCA1, BRCA2 and RAD51 which are involved in the cellular processes such as mitotic and meiotic cell cycles (Ming et al., 2010, Ming et al., 2011a, Ming et al., 2011b, Fraser et al., 2012). The impact of the PTEN expression on the expression of several genes involved in HR recombination also suggest the role of PTEN in mediating the genomic stability through HR recombination (Mendes-Pereira et al , 2009). Nuclear PTEN seems to play a variety of roles in conjunction with BRCA1 to repair DSBs, such as causing cell cycle arrest and maintaining genomic stability. On the other hand, PTEN recruits RAD51 on the chromatin at the damages site, thereby reducing the spontaneous DSBs (Shen et al., 2007; Shen et al., 2013).

The findings of this study showed that RAD51B and RAD51C are significantly downregulated in BT-549 and MDA-MB-468, respectively (Table 4.8). The previous reports showed that RAD51 and BRCA1 regulate the repair of damaged DNA in meiotic cells (Chen et al, 1998). This indicated that PTEN deficiency associated with downregulation of RAD51 and BRCA1, and subsequently indirectly impairs the DNA damage response.

Another study showed the direct interaction between PTEN and RAD51 during the stalled replication fork at mitotic cell cycle (He et al., 2015). Song et al showed that

PTEN is required to regulate expression of RAD51 to mediate HR based double strand break repair (Song et al., 2012). McEllin et al (2010) demonstrated that DNA damage caused by the DNA damaging agent in lethal brain tumour – Glioblastoma multiforme - was not repaired effectively in PTEN-null astrocytes which triggered the apoptosis, which was mainly because of the compromised HR pathway. This suggested that the lack of PTEN expression at cellular level can change the expression landscape of the genes involved in the HR repair pathway for repairing DSBs. Many other studies showed the involvement of PTEN in regulating the expression of RAD51 which is essential for the HR mediated repair of DSBs (Salmena et al., 2008; Shen et al., 2007; Yin and Shen, 2008).

Previous studies have empirically proved that BRCA1 deficiency causes the impairment in the DNA damage repair mechanism, decreased genomic stability and increased level risk of ovarian and breast tumorigenesis (Paul and Paul, 2014; O'Donovan and Livingston, 2010; Wang and Di, 2014). Accumulating evidence associated with loss of PTEN expression and BRCA1 associated tumorigenesis are reported, which may be the potential mechanism of causing DSBs and homozygous deletions (Saal et al., 2008). The downregulation of ATM along with RAD-related proteins in PTEN-deficient may be the prime cause of aberration in the repair pathways, as these proteins are interlinked with each other and their functions are interdependent at the site of DNA damage. ATM is a major kinase which senses the DNA damage and activates CHK2 (Clark et al., 2012; Bowen et al., 2005); CHK2 phosphorylates BRCA1 to contribute to DNA repair, and BRCA1 in concert with Rad50 and Rad51 is involved in maintaining the genomic stability (Khanna et

al., 2004). Given strong interactions with PTEN, ATM, RAD-related proteins, and BRCA1 for repairing DSBs, PTEN seems to play a pivotal role in regulating the expression of other proteins prerequisite for the DNA damage response in breast cancer.

It is also an increasing possibility that loss of PTEN serves as precursor of the BRCA1 related cancer and inefficiency of BRCA1 to mediate DSBs repair (Martins et al., 2012). This conclusion is supported by findings of this study revealing the loss of PTEN expression triggered the downregulation of BRCA mRNA level in BT-549 compared to the MCF7. However, the upregulation of BRCA1 and BRCA2 were observed in the case of MDA-MB-468 but not in MCF7, which suggest the complicated regulation involving multiple factors in BRCA expression in MDA-MB-468 cells rather than MCF7 tumor cells. This further urges the research to personalize the therapy based on the individual cell lines in breast cancer.

This study showed that the loss of PTEN expression also led to downregulation of XRCC2, XRCC5, XRCC3 in PTEN-deficient cells, and which are the important players in the HR induced repairing of DSBs. Many other studies corroborated this finding showing that XRCC2 and XRCC3 have mutations or down regulation in the breast cancer cells (Johnson et al., 1999; Pierce et al., 1999; Liu et al., 1998). The studies also suggested that mutated or low level of XRCC2 expression is associated with increased risk of development of breast tumours (Johnson et al., 1999). The downregulation of XRCC2, BRCA1 and RAD-related proteins due to PTEN deficiency highlights the complexity of the genetic architecture which not only increase the susceptibility to cancer and posing some challenges for

developing personalized risk models and therapies for breast cancer patients (Park et al., 2012).

Similarly, expression of Dmc1, a homolog of RAD51, was also significantly upregulated by average of 4 – fold in the PTEN-deficient cells compared to the PTEN-proficient cells, showing the important impact of PTEN on Dmc1-induced HR during meiosis. This indicates that PTEN not only alters the expression of many other key proteins involved in DSBs during the mitotic cell cycle, but it also regulates expression of proteins involved in HR during meiosis. The downregulation of Dmc1 and compromised HR mediated DNA repair has been previously reported by many other studies in melanoma, cervical, breast, lymphoma and colon tumors (Kalejs et al., 2006; Ianzini et al., 2009; Lindsey et al., 2013). Dmc1 was also shown to be upregulated in response in different cancer cell types such as human lymphoma cell lines (Ianzini et al., 2009). Consequently, upregulation of Dmc1 could give an indication of the increased DNA replication stress and DNA damage in the absence of PTEN. Rivera et al (2015) showed that reduced expression of Dcm1 activated CHK1, decreased the cell proliferation and enhanced RPA foci presence indicating the increased replication stress, and increased radiosensitivity by inhibiting DNA damage response. These data indicate that PTEN expression may suppress the Dcm1 expression in order to exert its functions as tumor suppressor and induce DNA damaging response, which also support the findings of this study.

4.5.2. Loss of PTEN expression upregulates proteins involved in BER pathway

This study has reported that PTEN deficiency in BT-549 downregulated most of the BER genes except APE2, SMUG1 and OGG1 which were upregulated compared to the PTEN-proficient cells. However, the reverse was observed in the case of PTEN-deficient cell line MDA-MB-468 where most of the BER genes were upregulated except PARP3, LIG3, NEIL2, CCNO which were downregulated. This suggests that PTEN deficiency is cell specific and does not affect the expression of proteins involved in BER evenly in all tumour cells. The explanation of the uneven effect on the regulation of BER proteins may be associated with the variations in the protein expression portfolio of BT-549 and MDA-MB-468. In addition, this effect might also be caused by mutation in the PICK3CA pathway. This also indicates the complexity of the DNA damage response which involves different factors for repairing the DNA damage sites in different cells. In addition, based on the portfolio of upregulated and downregulated BER proteins in PTEN-deficient cells, the optimized and customized radio- and chemo-therapeutic options can be explored.

Considering PTEN deficiency in BT-549 cells, the downregulation of APE1, PARP1/2/3, pol-β, NTHL1, UNG, FEN1, NEIL1/2, XRCC1, CCNO, LIG3, MUTYH were observed. Considering AP1/2 regulation in cells, PTEN deficiency may impair the ability of cells to perform AP site incision mediated by APE1 (Stevnezer et al., 2014(. In addition, the repair complex initiated in the BER pathway incudes pol-β, XRCC1, LIG3 (Ahel et al., 2006; Demple and Harrison,

1994; Dianov and Hubscher, 2013), and these components are downregulated in PTEN-deficient cells of BT549, showing the unstable repair complex resulting in creation of the unfilled AP gaps, SSBs, and DSBs during the G1 phase of the cell cycle in which the BER is supposed to operate predominantly (Dianov and Hubscher, 2013). However, the upregulation of OGG1 in BT-548 PTEN-deficient cells shows that cells are able to repair guanine bases damaged by oxidative agents and suppresses carcinogenesis. However, this protein was downregulated in MDA-MB-468, which means that these cells may not be able to repair such damages. OGG1 is also reported to be associated with tumorigenesis, and exists in polymorphic variants with lower activities than the wild type (Shinumur and Yokota, 2001). The upregulation of OGG1 in BT-549 and downregulation of it in MDA-MB-468 may be related to existence of its different polymorphic variants in these cells. The polymorphism generates different variants with carrying 'ON' and 'OFF' states through changes in sequence of promoter, thereby affecting the gene expression in cells.

PARP1/2 are downregulated in BT-549 PTEN-deficient while, they were upregulated in MDA-MB-468 PTEN-deficient cells. PARP1/2 proteins are involved in protecting the SSDB from further deleterious damage such as DSBs, and help recruiting BER components to SSBs (Ahel et al., 2008; Woodhouse and Dianov et al., 2008; Woodhouse et al., 2008; Parsons et al., 2012). Based on the findings, it can be inferred that PTEN deficiency in BT-549 renders the cells potentially at risk of developing DSBs compared to that in MDA-MB-468 cells.

Taken together, these results indicate that impairment in BER genes in PTEN deficient cells cast a negative influence on the repairing of SSBs compared to that in MDA-MB-468 cells, indicating that PTEN deficiency may lead to existence of unrepaired SSBs in BT-549 cells.

4.5.3. PTEN deficiency associated with reduction of protein expression in NER and MMR pathways

The previous studies have exhibited the expression of PTEN is essential for the effective functioning of NER pathway (Ming et al., 2011). The downregulation of PTEN in mice's skin revealed their increased sensitivity to the skin tumorigenesis (Ming et al., 2011a; Ming et al., 2011b). The current study demonstrated that in response to PTEN deficiency, several of NER factors were downregulated compared to the PTEN-proficient cells, which showed agreement to the previous studies that PTEN expression is prerequisite for NER activity. The factors which were downregulated included LIG1, BRIP1, ATXN3, RAD23A, RAD23B, RPA1/3, ERCC1-8, XPA, XPE (DDB1-DDB2), and XPC (Table 4.4). It was demonstrated that PTEN promotes the global genome NER activity by enhancing XPC expression.

In line with the findings of the previous studies, this study showed that deficiency of PTEN resulted in downregulation XPC expression. Several studies showed that XPC along with RAD23B (XPC/RAD23B complex) is essential for recognition of DNA damage site, and recruiting other NER components such as ERCC1-XPF for

the incision of the damaged part of DNA (McNeil and Melton, 2012; Naegeli and Sugasawa, 2011, Hanawalt and Spivak, 2008).

Another study showed that PTEN suppression led to activation of AKT and suppression of XPC through increased localization of repressor p130 into the nucleus (Ming et al., 2010). Downregulation of RPA indicates that an area closer to DNA damage site might be left degenerated in PTEN-deficient cells, as RPA protects the undamaged DNA while repairing the damaged DNA site (McNeil and Melton, 2012). Thus deficiency of PTEN can destabilize the recognition complex and DNA damage incision complex by regulating transcription of a number of proteins involved in forming these complexes in NER pathway for repairing of DNA lesions.

The role of PTEN in context of MMR pathways is not well-understood. In the current study, the loss of PTEN upregulated PolD3 and TREX1 at mRNA level. TREX1 is a ‘three prime exonuclease 1’ which is involved in excision of nucleoside phosphates from 3’-end of DNA (157). It is still not clear as to which type of role TREX1 plays in context of repairing of the damaged DNA site and in combination of which factors it mediates its role. The upregulation of TREX1 in response to PTEN loss may serve as an alternative mechanism to remove the damage site, and may be defensive strategy used by PTEN-deficient cells against genotoxins. Transcription of several other genes involved in the mismatch repair pathway include MSH1-5, PMS1-2 and MPG was downregulated by PTEN loss, suggesting the unrepaired DSBs in DNA structure which can lead to tumorigenesis.

Thus the findings of this study suggest that deficiency of PTEN results in impaired activities of NER and MMR pathways leading to an increase in mismatches within cells in breast cancer, thereby making the cells susceptible to accumulate SSBs and DSBs. Nonetheless, further research is required to investigate the efficiency of these pathways.

4.5.4. Loss of PTEN induces reduction in expression of proteins of NHEJ pathway

NHEJ is the main pathway responsible for repairing DSBs from the exogenous and endogenous sources (Yan et al., 2007). The reduced expression of its components such as LIG4, DNA-PKcs, and XRCC4 caused severe issues of radiosensitivity, impaired survival proliferation, and genomic instability (Gao et al., 2000). In the current study, PTEN loss reduced the expression of many genes involved in NHEJ pathway, XRCC4, XRCC5, XRCC6, and LIG4. Previously, PTEN loss has been linked to the reduction of XRCC4 through deregulation in p53 and CK2 (Kang et al., 2017). Therefore, it can be suggested that PTEN loss in breast cancer cells might exert its influence on expression of XRCC4 through mechanism described by Kang et al (2017). In addition, it is possible that PTEN loss might be involved in impairing the DNA damage recognition through ATM /CHK2 axis, which has been supported by this study that ATM was downregulated in response to PTEN loss (Table4.3). This may provide an explanation of deregulated CHK2 which in concert with P53 would have reduced XRCC4 expression at mRNA level.

In addition, this study reported that PTEN loss upregulated expression of DNA-PKcs which is a catalytic subunit of DNA-protein kinase playing critical role in repairing DSBs via NHEJ pathway. The explanation of this phenomenon can be sought from the activation of PI3/AKT axis in response to PTEN loss. The activated AKT phosphorylates DNA-PKcs, which enhances the DNA repair of DSBs, suppresses the apoptosis, and offering resistance to chemotherapy and radiotherapy (Liang et al., 2012; Kang et al., 2017). Therefore, DNA-PKcs might be required by proliferating cells to avoid excessive accumulation of DSBs which might ultimately trigger the apoptotic process. Similarly, PTEN loss significantly upregulated XRCC6BP1 which is a DNA dependent protein kinase, and is involved in increased cell proliferation and improves DSB repair (Erasimus et al., 2016).

Barr et al (2016) also showed the increased expression of XRCC6BP1 during progression of lung tumour. Furthermore, the conclusion drawn by Easimus support the findings of this study that upregulation of DNA-PKcs and BRCC6BP1 actually support the cell proliferation and improves DSB repair in brain tumours. Similar mechanisms may be operational in the breast cancer, which means that both upregulated proteins in PTEN–null cells may be involved in progression of breast cancer. Some other NHEJ related genes were downregulated in response to PTEN loss, such as XRCC5, XRCC6 and XRCC6BP1 and LIG4 which impair NHEJ function in cancer cells leading to development of tumours. Wang et al (2013) showed that reduced expression of XRRC5/Ku80 and XRCC6/Ku-70 factors resulted in chromosomal instability, transformation of pre-cancerous hepatocytes, development of hepatocellular carcinoma, and failure in DNA repair.

Thus PTEN loss leads to the genomic instability and cell proliferation through impairing NHEJ pathway in the breast cancer cells. The ability of cells lacking PTEN to repair DNA damage and suppress proliferation is mainly contributed to the impaired expression of several factors involved in NHEJ, HR, NER and BER pathways. This conclusion is in line with one drawn by Kang et al (2017) that in absence of PTEN, cells are unable to activate DNA repair pathways to their full extent which is essential for mounting an adequate DNA damage response. In addition, the loss of PTEN promoted the downregulation in mRNA levels of ATM and ATR, DNA damage sensing factors, and which may increase the chromosomal aberrations, cell cycle arrest and apoptosis processes, as it has been shown in the case of prostate cancer (McCabe et al., 2015). Therefore, it will be interesting to explore the impact of combined loss of PTEN and ATR on the cell cycle, apoptosis and DNA damage in the breast cancer cells. Thus, this study warrants the further investigation to explore the response of breast cancer cells to the combined loss of PTEN and ATR. The results relating to this will be presented in the next chapter.

5. Targeting PTEN deficiency in breast cancer for personalized therapy using ATR inhibitors

5.1. Introduction

PTEN has been reported to be an important factor playing a vital role in tumour suppression, and is involved in controlling a variety of cellular processes and signals (Song et al., 2012). The role of PTEN in antagonizing the PI3/AKT signaling pathway is well established, through which it influences the cell proliferation and tumorigenesis (Ming and He, 2012). Apart from this canonical function, the accumulating evidence have been provided in the support of the role of PTEN in maintaining the genomic stability (Shen et al., 2007). Several researchers have supported the notion that PTEN is implicated in regulating the DNA damage repair and preserving the structural integrity of the genome (Shen et al., 20027; McEllin et al., 2010; Ming et al., 2011). The interaction of PTEN with histones has been suggested to be involved in chromatin remodeling (Chen et al., 2014). Another study reported that PTEN is a main player in checkpoint control and regulation of cell cycle, for instance, deficiency of PTEN within cells leads to the progression of cell cycle from the G0 phase to the G1 phase (Groszer et al., 2006). However, the overexpression of PTEN resulted in cell cycle arrest at G1 phase (Zhu et al., 2001; Wneg et al., 1999). Puc et al (2005) also showed that deficiency of PTEN caused some G2-arrest related defects in response to exposure of cells to the ionization radiations, which was mainly attributed to the phosphorylation of CHK1 and its subsequent localization from the nucleus to the cytoplasm. Therefore, these empirical data support the role of PTEN in the regulation of progression of very important phases of the cycle cell: G1 and G2. Thus stalling or slowing down progression of the replication fork induces the

replication stress in the PTEN-deficient cells, which in turn induces the genomic instability (Zeman and Cimprich, 2014; He et al., 2015).

PTEN has been demonstrated to be critical for DNA damage repair. The deletion of PTEN from the mouse embryonic fibroblasts had induced the double strand breaks spontaneously (Hue et al., 2008). Shen et al (2007) exhibited that PTEN-deficient cells were prone to the double stand DNA breaks and defective repair of such breaks, which demonstrated the implication of PTEN in the genomic instability. PTEN was also found to restore the radio-sensitivity of the cells by suppressing the γ-H2AX foci formation, which alternatively indicates the role of PTEN in decreasing the double strand breaks in DNA (Gwak et al., 2012). Ming et al (2011) exhibited that PTEN downregulation inhibited the expression of xeroderma pigmentosum C (XPC), a key protein involved in the global genome (GG)-nucleotide excision repair pathway, via AKT/p38 signaling pathway. Taken together, inhibition of PTEN expression decreases the ability of the XPC-dependent NER pathway, thereby decreasing the ability of the cells to perform repair following DNA damage. Supporting the finding of the Ming and his colleagues, Bassi et al (2013) reported that deficiency of PTEN made the cells hypersensitive to exogenous DNA damaging agents, and stressed on the proposition that PTEN can be used for personalized therapy. Recently, the evidence has been provided about the phosphorylation of PTEN by ATM and its entry into nucleus. The PTEN translocation into nucleus following the DNA induces the autophagy following exposure to the DNA damaging agents. This shows that deficiency of the PTEN

causes the cells to persist following the exposure to DNA damaging agents (Chen et al., 2015).

On activation, both ATR and ATM have the ability to phosphorylate several proteins involved in DNA damage response and damage repair; the phosphorylation is normally mediated at single or multiple sites (Ser/Thr). ATR phosphorylates CHK1 while ATM does CHK2. ATR and ATM phosphorylate H2AX following the DNA double strand, and are helpful in recruiting proteins implicated in checkpoint activation and DNA damage repair (Savic et al., 2009). ATR and ATM on activation phosphorylate BRCA1, and are required for S and G2/M checkpoints and repair of DNA damage mediated by HR pathway (Xie et al., 2010; Chen, 2000). The signals initiated by ATR complex cause the checkpoint arrest during cell cycle, initiate DNA damage repair, suppress replication of DNA and stabilize the stalled replication forms. The research also exhibited that ATR mediated signaling in response to UV irradiations phosphorylate H2AX at both S and G1 phases, which indicates the predominant role of ATR in DNA damage response (Hanasoge and Ljugman, 2007; Marti et al., 2006; Branzei and Foiani, 2010; Cimprich and Cortez., 2008). The study has also demonstrated that XPC and DDB2 – NER initiation factors - induce the recruitment of ATR to the damage site and activate it through the phosphorylation event (Ray et al., 2013).

The consequences of ATR inhibition are drastic for the cells' survival, because it causes the accumulation of toxicity to the cells in addition to altering sensitivity of cells to the DNA damaging agents. However, its effect is not studied in many of the cancer cell types and mutational profiles (Gavande et al., 2016). For instance,

the impact of inhibition of ATR on the PTEN- deficient cells has not been studied until now. Previous publications have showed that sensitivity of the p53-deficient pancreatic cells to the ionizing radiations in vitro was increased as a result of VE-821 and VE-822 – two important ATR inhibitors (Fokas et al., 2014; Prevo et al., 2012). The radiosensitization of p53 deficient cells affects adversely on the homology directed repair, which seems to occur due to two mechanisms. In the first instance, ATR inhibition caused by ATR inhibitor results in cell cycle arrest and increased replication stress which in turn induces the double strand breaks at sites of the DNA damage. Secondly, ATR inhibition impairs the homology directed repair which is further complicated by exposure of p53/ATM deficient sensitive cells to the ionizing radiation. Both cell types are commonly found mutated in the tumour cells (Fokas et al., 2014; Wang et al., 2004; Prevo et al., 2012). The discussion about the ATR inhibitors can be found in Chapter 1 general introduction (section 1.5).

5.2. Rationale for study

Although studies discussed in section 5.1 reflect the role of ATR in repairing the UV-induced DNA damage, however, this is not clear as to how ATR implements its function of DNA damage response and repair in conjunction with PTEN. In chapter 3, the negative ATR expression was significantly associated with the negative expression of nuclear PTEN. In this chapter, it is explored if PTEN-deficient cells treated with the ATR inhibitor can be targeted for the personalized therapy.

5.3. Aims

This study tends to achieve the following aims:

1. To investigate the impact of ATR inhibitors on the cell viability using MTS assay
2. To test the proposition that ATR inhibition in the PTEN-deficient BC cells induces the accumulation of DNA double strand breaks following the treatment with ATR inhibitor.
3. To investigate the impact of ATR inhibitor on the cell cycle in the PTEN-deficient BC cells.
4. To determine the impact of ATR inhibitor on the induction of apoptosis in PTEN-deficient BC cells following treatment with the PTEN inhibitor.

5.4. Results

5.4.1. ATR is expressed by selected breast cancer cell lines

In order to investigate the ATR expression in different cells such MCF7, MDA-MB-231, BT-549 and MDA-MB-468, western blotting analysis was performed. The cell lysate was extracted from 70-80% confluent cells; and electrophoresed on the 4-12% precast SDS gel. Proteins on the SDS gel were transferred onto a nitrocellulose membrane using the ATR-specific antibody. β-actin was used as a loading control and to normalize ATR concentration in the sample.

The Western blot showed that ATR was fairly expressed by all of the selected lines including MCF7, MDA-MB-231, BT-549 and MDA-MB-468 (Figure 5.1). ATR

protein was quantified using infrared dye-labelled secondary antibody (Li-Cor, Lincoln, USA; 1:10000 dilution in blocking buffer), and detected by Li-Cor Odyssey Scanner. The results confirmed that ATR was expressed in almost equal proportion in all of four cell lines used in this study (Figure 5.1). No significant difference in expression of ATR between PTEN-deficient cells (BT-549 and MDA-MB-231) and PTEN-proficient cells (MCF7 and MDA-MB-231) was found ($p > 0.05$). *P-values* were calculated from means of three independent experiments using unpaired t-test.

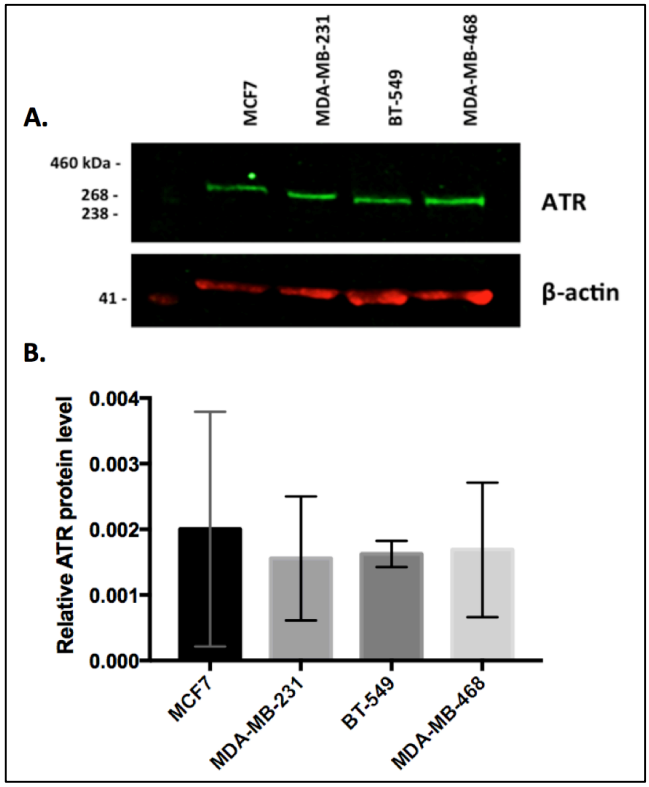


Figure 5-1 Western blotting analysis of ATR in the selected breast cancer cell lines.
A. shows the status of ATR expression in the selected cell lines and **B**. shows quantification of ATR protein levels in different cell lines. *P-values* calculated for MCF7 versus BT-549, MCF7 versus MDA-MB-468, MDA-MB-231versus BT-549 and MDA-MB-231 versus MDA-MB-468 were 0.78, 0.86, 0.95, 0.90, respectively.

5.4.2. RT-qPCR confirmed ATR expression in all four cell lines

The expression of ATR mRNA level in different cell lines including MCF7, MDA-MB-231, BT-549 and MDA-MB-468 cell lines was checked using RT-qPCR assay as described in chapter 2. The relative ATR expression level for each cell line was calculated and compared with each other. The expression of housekeeping gene HPRT1 was used to normalize the expression of ATR in the given cell lines. The relative expression level of ATR mRNA was measured by taking ratio between mRNA levels of ATR and mRNA levels of HRPRT1 in the given cell lines. The data indicated that ATR was expressed significantly in all of four cell lines, however, there was a significant difference in the mRNA expression levels of ATR between MCF7/MDA-MB-468 and MDA-MB-231/BT-54. The results can be viewed in figure 5.2.

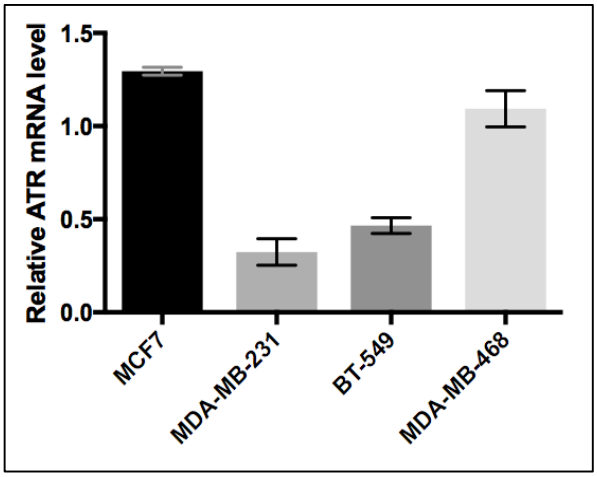


Figure 5-2 Quantification of ART expression at mRNA level in different cell lines using RT-qPCR.

5.4.3. CHK1 and phosphorylated CHK1 are expressed by the selected cell lines

In order to examine the expressions of CHK1 and phosphorylated CHK1 (pCHK1) in the MCF7, MDA-MB-231, BT-549 and MDA-MB-468, the western blotting analysis was performed. No significant difference in the expression of CHK1 and pCHK1 was found between PTEN-deficient cells (BT-549 and MDA-MB-468) and PTEN-proficient cells (MDA-MB-231 and MCF7) ($p > 0.5$). In addition, there was no difference in the ratio of total CHK1 and pCHK1 in PTEN-deficient cell lines and PTEN-proficient cell lines. The results obtained from the Western blotting analysis has been summarized below (Figure 5.3).

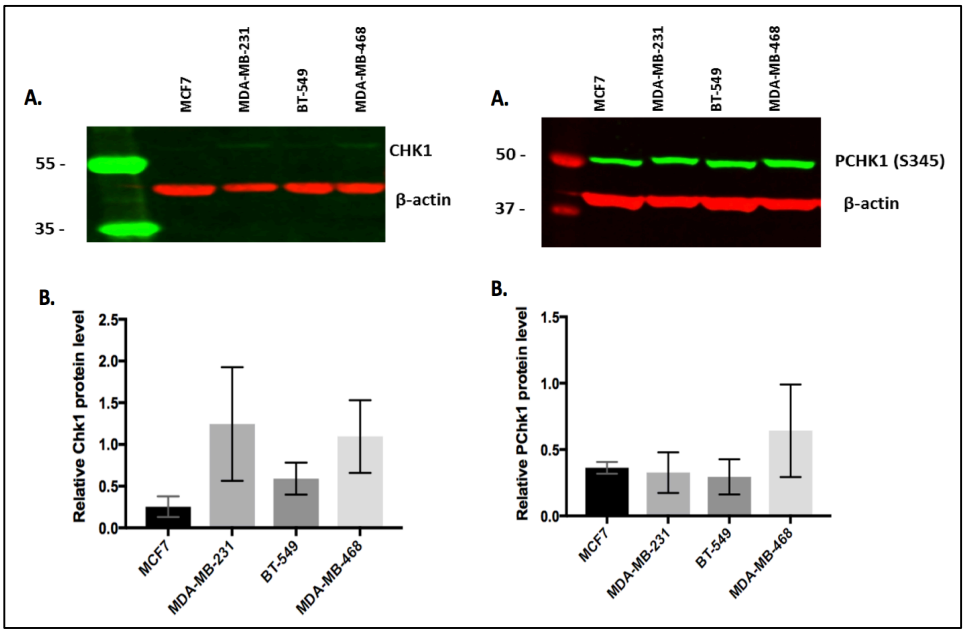


Figure 5-3 Western blotting analysis of CHK1 and pCHK1^{Ser345} in breast cancer cells.
A. shows the status of CHK1 and pCHK1^{Ser345} in the selected cell lines and **B.** shows quantification of CHK1 and pCHK1^{Ser345} protein levels in different cell lines. *P-values* calculated for MCF7 versus BT-549, MCF7 versus MDA-MB-468, MDA-MB-231versus BT-549 and MDA-MB-231 versus MDA-MB-468 were

0.57, 0.37, 0.85, 0.36, respectively. *P-values* were calculated from means of three independent experiments using unpaired t-test.

Following normalization of CHK1 and pCHK1(S345) against β-actin, the expression of pCHK1 was found to be significantly higher than that of non-phosphorylated CHK1 in all cell lines under investigation. This indicated that due to presence of ATR expression, the most of the CHK1 is being phosphorylated by ATR within these cell lines, which is why the level of non-phosphorylated CHK1 was discovered to be lower compared to the pCHK1. The highest concentration of pCHK1 was observed to be in MDA-MB-468 cells, whereas the MCF7 demonstrated the lowest concentration of pCHK1 compared to the others. The level of pCHK1 in MDA-MB-231 and BT-549 was found to be in higher than that in MFC7 and lower than that in MDA-MB-468. This shows that ATR mediated phosphorylation on Ser345 in CHK1 may vary in different cell lines.

5.4.4. Sensitivity of PTEN-deficient breast cancer cells in response to ATR inhibitor (VE-821)

Previously, PTEN-deficient cells showed the sensitivity to various chemotherapeutic drugs targeting the mTOR pathways in triple negative cell lines (Neshat et al., 2001; Steelman et al., 2008). However, the fate of the PTEN deficiency and its impact on the ability of cells to respond to ATR inhibitor was not known. In order to investigate the impact of PTEN deficiency on the growth of triple negative cells in response to ATR inhibitor such VE-821, the MTS assay was performed to examine the growth inhibition of the PTEN-deficient cells – BT-549 and MDA-MB-468 - and PTEN-proficient cells – MDA-MB-231 in response to the VE-821 ATR inhibitor. The cells were treated with varying concentrations of VE-821 ranging from 1μM to 7μM for 7 days. The data demonstrated PTEN-

deficient cells were sensitive to VE-821 compared to MDA-MB-231 PTEN-proficient cells. However, comparatively, between two PTEN-deficient cell lines, the BT-549 cells showed higher sensitivity to VE-821 at concentrations greater than 3uM. In contrast to BT-549, the cell viability of MDA-MB-468 cells was found to be stable in response to VE-821 concentrations greater than 3uM (Figure 5.4). The error bars are shown by calculating SD from each concentration of drug from three independent experiments.

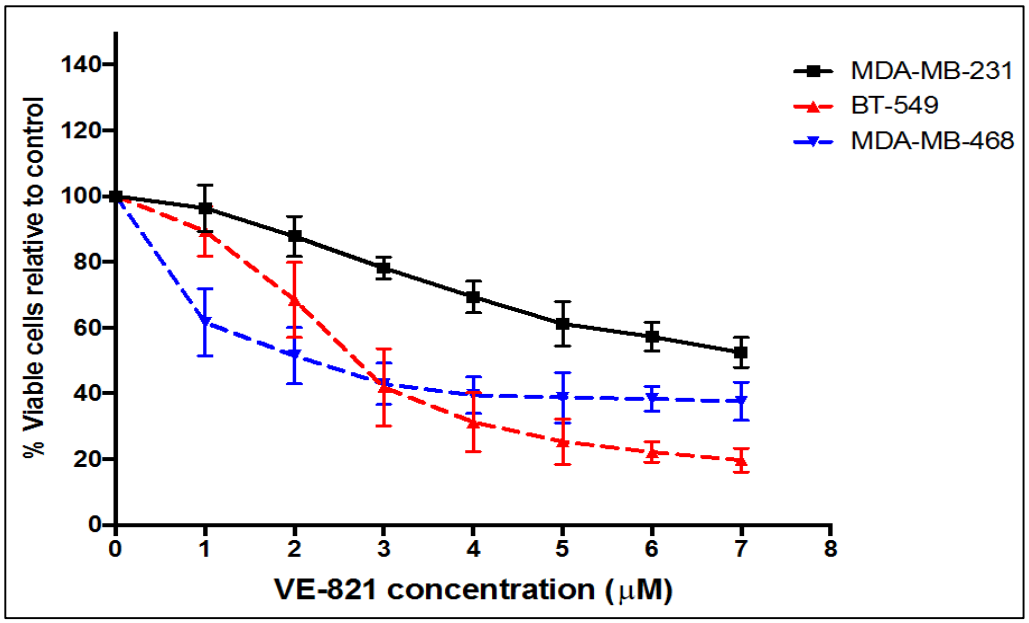


Figure 5-4 . MTS growth inhibition assay in a panel of PTEN-proficient and PTEN-deficient human breast cancer cell lines treated with ATR inhibitor VE-821.
PTEN-deficient cells showed increased sensitivity after exposure of cells to the VE-821 (ATR inhibitor). MTS data are shown as the mean and SD values for each concentration from three independent experiments.

5.4.5. Sensitivity of PTEN-deficient breast cancer cells in response to ATR inhibitor (AZD6738)

The cytotoxic impact of AZD6738 was determined against PTEN-deficient cell lines – MDA-MB-468 and BT-549 – and PTEN-proficient cell line – MDA-MB-231. The results demonstrated that AZD6738 reduced the cells viability of PTEN-deficient cell lines in a concentration dependent manner in comparison with the PTEN-proficient cells. Higher level of toxicity of AZD6738 was observed in the case of BT-549 cells compared to MDA-MB-468 cells at the same concentrations, showing that PTEN deficiency made the former more sensitive to AZD6738 than PTEN-deficient MDA-MB-468 (Figure 5.5). This showed the degree of specificity of the AZD6738 for the BT-549. Nevertheless, PTEN proficiency significantly increased the cell viability of MDA-MB-231 against 0.2uM and 0.5uM concentrations of AZD6738, and for the higher concentrations, the cell viability followed the decreasing trend, which was an expected outcome for increasing lethal concentrations of AZD6738. Thus the results indicated that PTEN proficiency in MDA-MB-231 decreased the cytotoxic impact of AZD-6738 ATR inhibitor compared to PTEN-deficient cell by increasing the phosphorylation of DNA DSB marker gamma-H2AX.

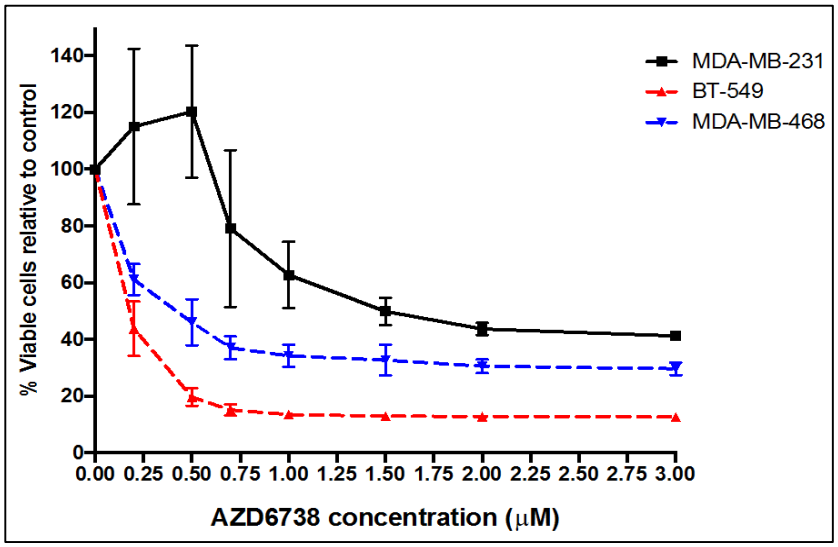


Figure 5-5 . MTS growth inhibition assay in apenl of PTEN-proficient and PTEN-deficient breast cancer cell lines treated with ATR inhibitor AZD6738. PTEN-deficient cells (BT-549 and MDA-MB-468) were found to be more sensitive to the ATR inhibitor than PTEN-proficient cells (MDA-MB-231). MTS data are shown as the mean and SD values for each concentration from two independent experiments.

5.4.6. γH2AX accumulation in PTEN-deficient breast cancer cells treated with ATR inhibitor (VE-821)

Phosphorylated H2AX (γH2AX) is produced as a result of double strand breaks, showing the accumulation of γH2AX foci as a marker of DNA damage. PTEN-proficient and PTEN-deficient BC cell lines were treated with the 5μM of ATR inhibitor (VE-821) for 24 hours and 48 hours as shown in the figure 5.6. The data demonstrated the significant increase in the accumulation of γH2AX in treated MDA-MB-231 compared to untreated cell lines ($p = 0.033$). Similarly, in BT-549 cell lines, the treated cells showed significant accumulation of γH2AX compared to the untreated cells. A significant increase in the accumulation of the γH2AX was

observed in the case of treated MDA-MB-468 compared to untreated MDA-MB-468 cell lines. PTEN-deficient cells – BT-549 ($p < 0.001$) and MDA-MB-468 ($p < 0.001$) – showed to be more statistically significant accumulation of the γH2AX foci compared to the PTEN-proficient cells MDA-MB-231 ($p=0.004$). This shows that VE-821 inhibitor was toxic to both PTEN-proficient and PTEN-deficient cell lines, but its impact was significantly higher in PTEN-deficient cells than in PTEN-proficient cell lines.

Similar results were obtained for 48 hours exposure of PTEN-proficient and PTEN-deficient cell lines to the VE-821 ATR inhibitor. Treated MDA-MD-231 cell line showed significantly higher accumulation of the γH2AX than untreated MDA-MB-231 cell line ($p = 0.005$). The significant increase in accumulation of γH2AX foci was observed for treated BT-549 cells compared to untreated BT-549 cells ($p = 0.001$). Similar results were also observed in treated MDA-MB-468 cells, in which a significant increase in γH2AX accumulation as compared to untreated MDA-MB-468 cells ($p = 0.005$).

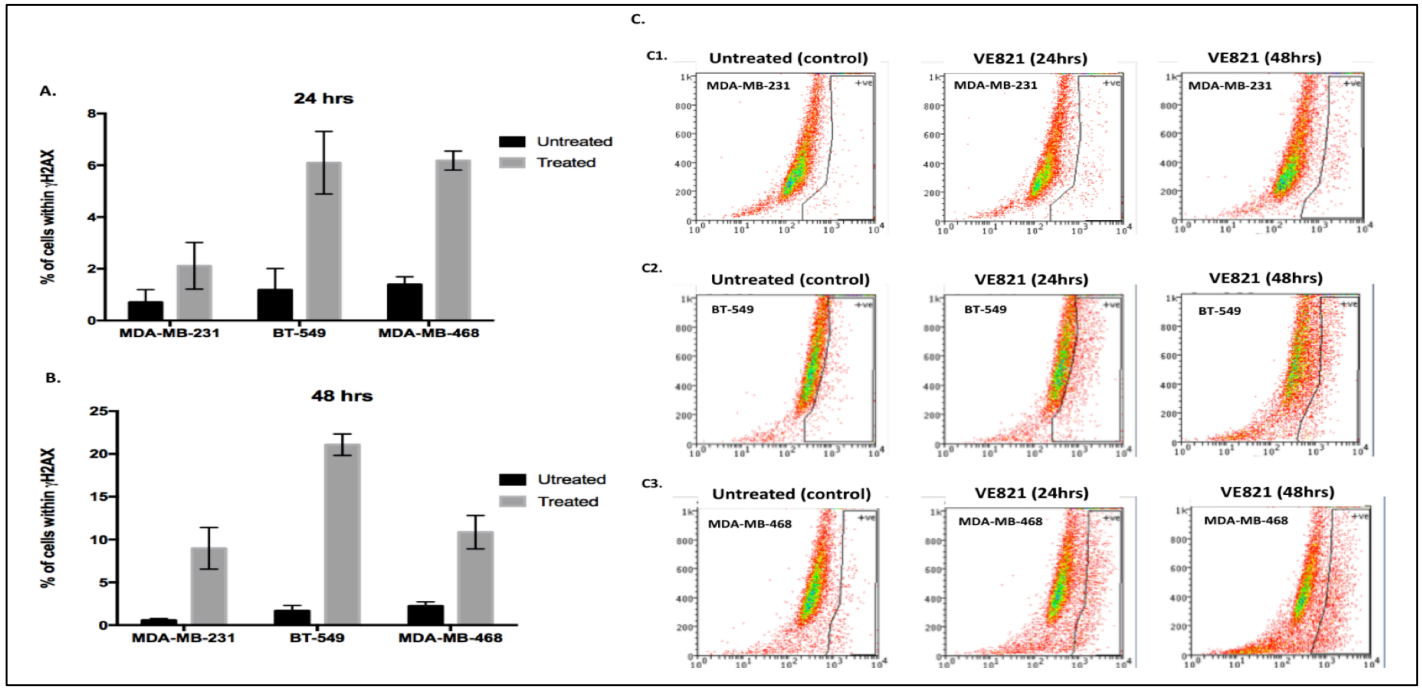


Figure 5-6 γH2AX accumulation by flow cytometry in breast cancer cell lines treated with ATR inhibitor (VE-821) for 24 and 48 hours. **A.** PTEN-proficient cells MDA-MB-231 and PTEN-deficient cells BT-549 and MDA-MB-468 following treatment with 5μM (VE-821) for 24 hours compared to untreated cells **B.** PTEN-proficient cells MDA-MB-231 and PTEN-deficient cells BT-549 and MDA-MB-468 after 48 hours of exposure to 5μM of VE-821 in comparison to untreated cells. **C.** Representative graphs of FACS images of γH2AX analysis following VE-821 treatment for 24 and 48 hours in **C1**. MDA-MB-231, **C2.** BT-549 and **C3.** MDA-MB-468. Data shown in figures were drawn from means and SD values calculated from three independent experiments each was done in duplicate. T-test was performed to obtain *P-values* and GraphPad prism was used to draw graphs and perform statistical analysis.

5.4.7. Cell cycle arrest in PTEN-deficient cells treated with ATR inhibitor (VE-821)

The formation of double strand breaks as demonstrated through the development of γH2AX foci activates the cell cycle checkpoints, so that the cell cycle arrest can be triggered in order for allowing the DNA damage repair mechanism to operate and repair the DNA damage. However, in the event of irreparable or extensive damages to the DNA integrity, apoptosis is initiated (Ming et al., 2012). In order to examine the cell cycle arrest and progression, FACS analysis was performed as described in chapter 2.

The PTEN-deficient and PTEN-proficient cells were treated with 5μM of ATR inhibitor VE-821 for 24 hours and 48 hours (Figure 5.7). After 24 hours of treatment, no significant arrest was observed between treated and untreated cells for G1, S and G2/M phases of the cell cycle (Figure 5.7A). However, after 48 hours of treatment (Figure 5.7B), treated MDA-MB-468 cell line showed the significant cell cycle arrest at G2/M phases ($p = 0.0013$) compared to the untreated MDA-MB-468 cell line.

However, no significant arrest was observed between the treated MDA-MB-231 cell lines and untreated cell lines at G1 ($p = 0.954$), G2 ($p = 0.261$) and G2/M ($p = 0.173$) phases. Similarly, the treated BT-549 had not demonstrated any significant arrest at G1 phase ($p = 0.0774$), G2 phase ($p = 0.075$) and G2/M phase ($p = 0.0771$). Even though the cell cycle arrest at G1, G2, and G2/M phases was not significant, but it was observed comparatively higher in treated cells compared to non-treated

cells and was very close to the threshold significant level ($p = 0.05$). This indicates that cell cycle is arrested in both PTEN-deficient cell lines – MDA-MB-468 and BT-549 compared to PTEN-proficient cell line – MDA-MB-231 which did not show any significant difference in terms of cell cycle arrest at G1, S and G2/M phases.

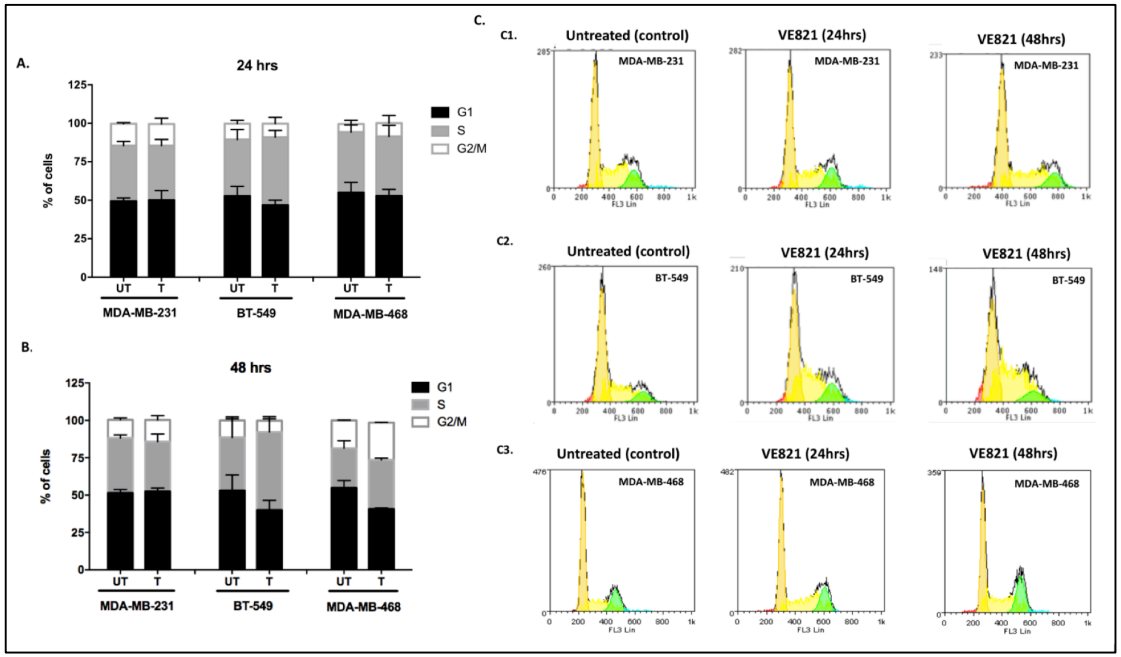


Figure 5-7 Cell cycle by Flow cytometric cell cycle analysis in breast cancer cells treated with ATR inhibitor (VE-821) for 24 and 48 hours. **A**. PTEN-deficient cells (MDA-MB-468, BT-549) were compared with PTEN-proficient cells (MDA-MB-231) following exposure of cells to 5µM of ATR inhibitor for 24 hours. **B**. PTEN-deficient cells (MDA-MB-468, BT-549) were compared with PTEN-proficient cells (MDA-MB-231) following exposure of cells to 5µM of ATR inhibitor for 48 hours. **C**. Representative graphs of FACS images of cell cycle analysis following VE-821 treatment in **C1**. MDA-MB-231, **C2**. BT-549 and **C3**. MDA-MB-468. Data represents means and SD values calculated from three independent experiments each was performed in duplicate. T-test was performed to calculate *P-values* and $p<0.05$ was considered significant. Graphs were produced and statistical analysis and graphs were done using GraphPad prism.

5.4.8. Apoptosis detection by Annexin-V after treatment with ATR inhibitor

Annexin-V has the ability to bind to phosphotidylserine (PS) – a phospholipid). If it is labeled with the fluorescent molecule such as propidium iodide (PI), it can be used to detect the early and late apoptosis in the tumour cells. The PI only permeates through the damaged membrane when the cells are dead, which means that dead cells or cells at their late apoptotic phase are found to be Annexin-V positive and PI positive. Before the viable cells start losing their membrane integrity at the early apoptosis phase, the PS molecules translocate from inner membrane to the outer surface of plasma membrane. This demonstrates that cells at the early apoptotic phase are Annexin-V positive and PI negative while late apoptosis cells are Annexin-V positive and PI negative. However, viable cells with intact membrane have PS inside the plasma membrane, so they are Annexin-V and PI negative. The cells undergoing necrosis are PI positive but Annexin-V negative (Riccardi and Nicoletti, 2006; Hingorani et al., 2011).

PTEN-proficient and PTEN-deficient BC cell lines were treated with 5μM of VE-821 ATR inhibitor for 24 hours and 48 hours (Figure 5.8). Generated data was normalized against apoptotic fraction of untreated cells to detect the percentage increase in apoptosis. After 24 hours of VE-821 treatment, none of cell lines - MDA-MB-231 (PTEN-proficient) and (PTEN-deficient) BT-549 and MDA-MB-468 show any significant difference in the early and late apoptosis compared to untreated cells (Figure 5.8). After 48 hours of VE-821 treatment, the early apoptosis

demonstrates a statistically significant increase in percentage with treated MDA-MB-231 cells compared to the untreated MDA-MB-231 cells ($p = 0.0011$). Similar results were also observed in treated MDA-MB-231 cells, in which a significant increase in late apoptosis as compared to untreated MDA-MB-231 cells ($p = 0.0014$). Similarly, treated BT-549 cells showed significant increase in early apoptotic phase compared to the untreated BT-549 cells ($p = 0.0001$). Similar results were obtained for late apoptotic phase in BT-549 treated for 48 hours ($p = 0.0036$). It can be noticed that treated BT-549 cells showed statistically higher cells in early apoptotic phase ($p = 0001$) compared to treated MDA-MB-231 cells ($p = 0.0011$). Showing that ATR inhibitor induced early apoptosis in the PTEN-deficient cells. However, the treated MDA-MB-468 cells did not show significant difference between the treated cells in early apoptotic phase compared to the untreated MDA-MB-468 cells after a 48 hours treatment of VE-821 ($p = 0.131$). Significant increase in late apoptosis was observed in treated MDA-MB-468 compared to untreated MDA-MB-468 cells after 48 hours treatment with 5µM of VE-821.

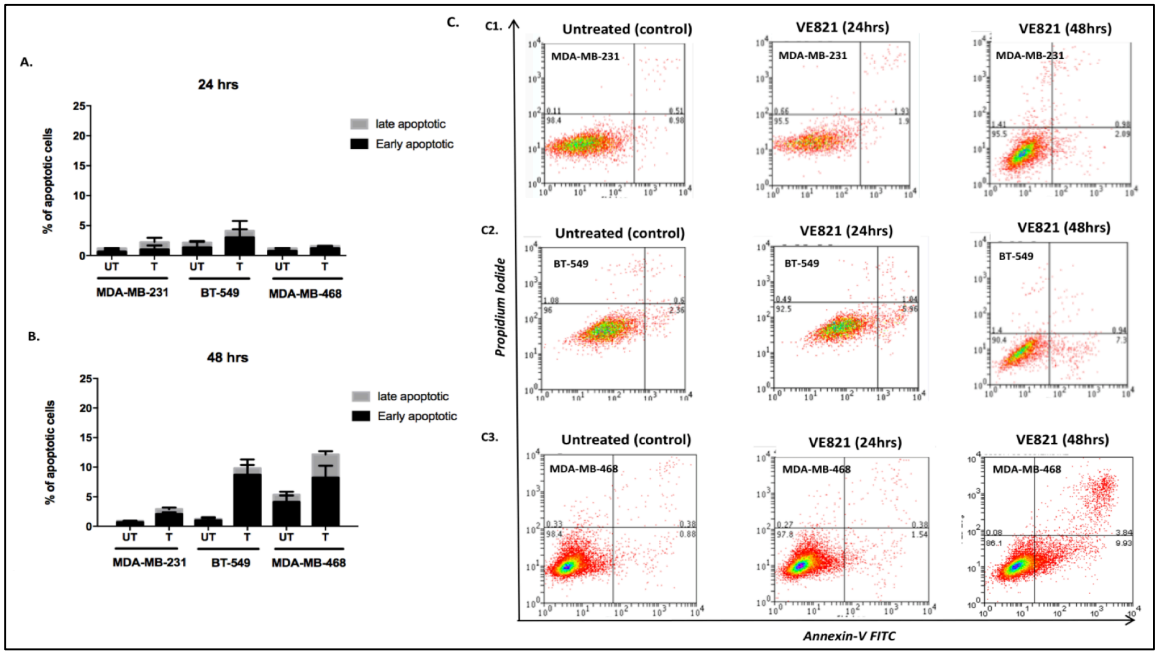


Figure 5-8 Apoptosis detection by Annexin V-FITC FACS in breast cancer cells treated with ATR inhibitor (VE-821) for 24 and 48 hours.
Treatment of cells with 5µM of VE-821 ATR inhibitor for 24 hours and 48 hours was related to significant increase in early and late apoptosis. Generated data was normalized against apoptotic fraction of untreated cells to detect the percentage increase in apoptosis **A**. after 24 hours of treatment with 5µM of VE-821, early and late apoptotic phases in treated cells were compared to the untreated cells. **B**. After 48 hours of 5µM of VE-821, early and late apoptotic phases in treated cells were compared to the untreated cells. Bottom left quadrant represents viable cells (Annexin V and PI negative), bottom right quadrant represents cells in early apoptosis (Annexin V positive and PI negative), top right quadrant represents cells in late apoptosis (Annexin V and PI positive), top left quadrant represents necrotic cells (Annexin V negative and PI positive). **C**. Representative graphs of FACS images for apoptosis assay following VE-821 treatment in **C1.** MDA-MB-231, **C2.** BT-549 and **C3.** MDA-MB-468. *P-values* were calculated using t-test comparing the treated with the untreated cells for each cell line. Graphs were drawn and statistical analysis calculated using the GraphPad Prism.

5.5. Discussion

In the previous chapter, Chapter 4, the expression of different proteins involved in HR, NER and BER pathways were downregulated/upregulated in the PTEN-deficient cells compared to the PTEN-proficient cells. This suggested the variations in the expression of different factors associated with DNA damage response pathways under the regulatory influence of PTEN expression in the selected breast cancer cell lines.

5.5.1. ATR inhibition in PTEN-deficient cells increases DNA damage

The ATR inhibition in the PTEN-deficient cells significantly increased the accumulation of γH2AX foci in the order of 10 times higher than the PTEN-proficient treated cells. The accumulation of γH2AX foci means that the single strand or double stranded DNA damage increases, showing that accumulation of the DNA damage was increased in the event of ATR inhibition within PTEN-deficient cells compared to the ATR inhibition in the PTEN-proficient cells. However, both PTEN-proficient and PTEN-deficient cells were observed to be sensitive to the ATR inhibitor. The γH2AX foci accumulated due to the phosphorylation of γH2AX mediated by ATM in the cells lacking in PTEN. Therefore, PTEN-proficient and PTEN-deficient cells showed the accumulation of γH2AX foci due to inhibition of ATR. In addition, the greater magnitude of accumulation of γH2AX foci in the PTEN-deficient cells compared to the PTEN-proficient cells indicated that PTEN plays a critical role in mediating the DNA

damage repair along with ATR. Ming and He (2012) reviewed that PTEN is a major player in mediating the DNA repair and DNA damage response, especially in nucleotide excision repair and DNA double strand break.

ATR is known as an important transducer to trigger the phosphorylation of mediators such as CHK1. Therefore, ATR inhibition within PTEN-proficient cells also resulted in accumulation of DNA damage. CHK1 is phosphorylated by ATR in order to trigger the signaling cascade ranging from arresting the cell cycle checkpoints and initiating the apoptosis in the cells in the case of irreparable DNA damage. However, in the event of ATR inhibition, the downstream signaling pathways phosphorylated by ATR such as CHK1 are not initiated, thereby causing the accumulation of DNA lesions during DNA replication. Partial loss of ATR was previously reported to cause the replication stress and DNA damage resulting from the genomic instability and replication errors at the repetitive sequences (Mirkin and Mirkin, 2007; Murga et al., 2010). Similarly, Casper et al (2002) demonstrated that ATR inhibition resulted in the accumulation of DNA double strand breaks and chromosomal instability, which caused the accumulation of γH2AX foci. The findings of Capser et al (2002) are in line with this study. Supporting the findings of this study, several other studies have reported that ATR inhibition mediated by ATR inhibitors caused the accumulation of the γH2AX foci (Leszczynska et al., 2016; Matsuoka, et al., Huntoon et al., 2013; Fokas et al., 2012; Syljuåsen et al., 2005; Prevo et al., 2012).

5.5.2. Cell cycle arrest increases after treatment with ATR inhibitor in PTEN-deficient cells

It can be inferred from the accumulation of γH2AX foci that double strand breaks might be resulted from stalled replication fork. The presence of the DNA lesions triggers the cell cycle arrest through the activation of the cell cycle checkpoints, which allow the enough time to repair the DNA damage, or to initiate the process if the damage is extensive and beyond repair (Kurtz and Lees-Miller, 2004; Ming et al., 2012). It was found that in PTEN-deficient cell line MDA-MB-468, G2/M phase were arrested significantly compared to untreated MDA-MB-468 cells after 48 hours of treatment with ATR inhibitor VE-821. The treated cells of the BT-549 (PTEN-deficient cells) showed the modest increase in G1, S and G2/M phases of the cell cycle, nevertheless, these arrests could not reach the statistically significant level ($p = 0.077$) (Figure 5.7A). The difference in the cell cycle arrest at G1, S and G2/M phases between two different PTEN cell deficient cell lines in response to ATR inhibition may be related to the fact that role of PTEN in the DNA damage repair in different cell lines is dependent on the cell type and endpoints as noted by Gupta et al (2009). The increase in arrest at the S/G1 and G2/M phases was in agreement with the previous published literature (Gamper et al., 2013; Shaltiel et al., 2014), showing that prolonged arrest at G2/M allowed the DNA damage repair to happen.

Shen et al (2007) showed that PTEN deficiency within the tumour cells reduced the ability of cells to repair the double strand breaks, which may be associated with the downregulation of Rad51 and absence of PTEN at the centromere. They further

stated that the PTEN acts on the chromatin for regulation of Rad51 expression which subsequently decreases the events of double strand breaks. The expression of PTEN also reduces the formation of γH2AX foci (Pappas et al., 2007), which is in accordance with finding reported by this study.

It is interesting to note that arrest at the cell cycle checkpoint (G2/M) in the event of inhibition of ATR in PTEN-deficient cells, which can be explained by considering the overlapping roles of ATR/CHK1 and ATM/CHK2 pathways which phosphorylate the same substrates in their downstream pathways (Smith et al., 2010). For example, the p53 is involved in activation of DNA damage response pathways at the level of checkpoint arrest activation. In the case of deficiency of p53, both ATR and ATM mediate the cell cycle checkpoint arrest following the DNA damage in order to allow the repair (Reinhardt et al., 2007).

5.5.3. Early and late apoptosis depends on the amount of DNA-damage and cell cycle arrest in PTEN-deficient cells

A flow-cytometry was performed to detect the early and late apoptosis within the PTEN-deficient cells in response to ATR inhibition. BT-549 showed the significant increase in proportions of the cells in the late phase of apoptosis compared to the early phase after 24 hours and 48 hours of the treatment with ATR inhibition. However, after 48 hours of treatment with ATR inhibitor, both BT-549 and MDA-MB-231 showed significant proportion of cells in late apoptosis phase compared to that in early apoptosis phase. However, in response to ATR inhibition, the MDA-MB-468 (PTEN-deficient cells) did not show statistical significance in early and

late apoptotic phases. These results can be explained by taking into account the amount of DNA damage and cell cycle arrest. The greater the cell cycle arrest, the greater the time for the DNA damage repair machinery to repair the DNA damage. This means that cells would not be submitted to apoptosis. The cells only meet the apoptotic fate when DNA damage is extensive or beyond the repair (Ming et al., 2012). In the case of MDA-MB-468, in this study, the cell cycle arrest at G1, S an G2/M phases was highly significant in response to ATR inhibition compared to MDA-MB-231 and BT-549. Therefore, the ATM mediated phosphorylated substrates such as CHK2 and p53 might have played a role in repairing the DNA damage in PTEN-deficient cells MDA-MB-231 (Shiloh, 2006). Therefore, the cells were not significantly increased in late apoptotic phase compared to the early apoptotic phase.

However, the BT-549 cells showed an increase in the cell cycle arrest at G1, S and G2/M phases compared to the MDA-MB-231 cells in response to ATR inhibition. This demonstrates that both PTEN-deficient strains had showed sensitivity to ATR inhibitor, and this had increased the repair time for the repair pathway to repair the DNA lesions. Therefore, the proportion of cells in late apoptotic phase was significantly higher than in those in the early apoptotic phase. In addition, the replication stress caused by the ATR inhibition could not be repaired due to absence of PTEN, which can also be a contributory factor in mediating the late apoptotic phase (Ming et al., 2012). Furthermore, the accumulation of unrepaired DNA at the mitotic stage might have results in mitotic catastrophe and finally triggered apoptosis of the PTEN-deficient cells (Kwok et al., 2016)

5.5.5. Toxicity of AZD6738 (ATR inhibitor) to the cell growth of PTEN-deficient cells

The results showed that PTEN-deficient cells were significantly sensitive to the AZD6738 ATR inhibitor compared to the PTEN-proficient cells. Even though PTEN proficient cells showed sensitivity to the drug, but it was statistically non-significant. The growth of the cells was inhibited to a greater extent in BT-549 cells compared to the MDA-MB-468 cells. This finding is in line with the previously published results showing that AZD6738 (ATR inhibitor) caused the cell death and senescence in lung cancer cells (Vendetti et al., 2015) and leukemia cells (Kwok et a., 2016). However, the action of AZD6738 in the PTEN-deficient of breast tumours was a novel finding reported by this study, and it also shows that impact of AZD6738 is potentiated in the PTEN cells compared to the PTEN-proficient cells. Therefore, the consideration of PTEN deficiency along with AZD6738 administration can impact greatly the clinical outcome of the breast cancer treatment.

6. General discussion

6.1 General Discussion

The genomic stability is the primary concern among all living organisms, as DNA is liable to be attacked by the exogenous and endogenous agents that are threatening DNA integrity. When DNA is damaged permanently and these changes are accumulated, it causes aggressive tumorigenesis. The role of DNA in the carcinogenesis was observed when it was demonstrated that all endogenous or exogenous DNA damaging agents cause the structural changes in the sequence of DNA. Therefore, such agents (carcinogens) were termed as mutagens. The living organisms have developed the specialized mechanism for sensing the DNA damage and repairing it in order to maintain the genomic stability, which is called DNA damage response. Interestingly, it has been revealed that cancer cells are mutated in factors taking part in the DNA damage response and become more dependent on compensatory or back-up DNA repair systems for repairing life-threatening mutations in DNA structure. Recently, it has become evident that targeting these back-up DNA repair systems using specific inhibitors has emerged as a promising anticancer therapy. The data imply that PTEN-deficient cells with impaired DNA repair may be reliant upon ATR signalling pathway for survival. Therefore, ATR blockade in this context could lead to synthetic lethality. This strategy in which DNA repair factors are inhibited using the inhibitors to prevent cell proliferation is termed as synthetic lethality (Lord and Ashworth, 2008; Rasool and Tomkinson, 2010; Abbots, 2014b). In this strategy, two or more factors are selected as a part of inhibition strategy via using the mutation or inhibitor approach which ultimately induces apoptosis among the proliferating cancerous cells, however, mutation in

one of these factor does not lead to selective death of cells. This is a promising approach which profiles the genetic make-up of cancer cells in context of DNA repair pathways active in cancer cells and targets one or more pathways as an anticancer therapy of selecting the factors involved in DNA repair pathways.

The alterations in DNA repair factors have been shown to have predictive and prognostic value in cancer patients. Loss of PTEN activity has been demonstrated in several cancer forms such as ovarian, colon, prostate, glioblastoma and breast cancers (Saal et al., 2008; Perez-Terorio et al., 2007). Perturbations in PTEN genetic landscape is critical for the cell proliferation and increases the susceptibility to tumorigenesis. The tumour formation was found to be increased after decreasing PTEN dosage in mice (Hollander et al., 2008). In breast cancer, PTEN loss was responsible for increasing the risk of breast carcinomas (Saal et al., 2008). PTEN was reported to lose immunoreactivity in 40% of breast cancer cases, through only 5% of cases carry mutations in PTEN protein (Saal et al., 2008). PTEN loss has also been reported in cancer stem cells, for example, PTEN deletion was detected in neural cells which was the cause of cell proliferation and growth (Groszer et al., 2001). Unlike PTEN loss in neural cells, PTEN loss was found in radial glia-like precursors in mouse dentate gyrus, which resulted in reduction of cell pool rather than expansion. This suggests that PTEN loss in stem cells show different outcomes depending on cell lineage (Bonaguidi et al., 2011). In addition, in the late stage of cancer, functional losses of PTEN and p53 have been found to be prevalent (Alimonte et al., 2010), which suggested the indispensable role of PTEN in tumour suppression. Furthermore, PTEN loss also caused the increased accumulation of

double strand breaks, suggesting the role of PTEN in cell cycle arrest (Ming et al., 2012). Therefore, this study aims to investigate the correlation of PTEN with: i) clinicopathological features, ii) other relevant and key biomarkers involved in BC development and progression, iii) Key proteins involved in DDR particularly those involved in ATR/CHK1 axis, iv) patients' outcome in terms of breast cancer specific survival (BCSS).

The IHC data showed that negative nuclear PTEN was significantly associated with increased tumour size, mitotic activity and NPI values and reduced tubules formation, showing the aggressiveness of breast tumour in the absence of PTEN. Furthermore, cytoplasmic and nuclear PTEN was significantly correlate with negative ER, HER2 and PgR, suggesting PTEN loss is associated with aggressive triple negative phenotype. These findings are supported by several other studies (Bose et al., 1998; Perren et al., 1999; Depowski et al., 2001). Additionally, cytoplasmic PTEN loss was negatively associated with ATR and cytoplasmic pCHK1, while nuclear PTEN loss was negatively associated with nuclear pCHK1. These findings were reported by none of previous studies.

In addition, this study examined the impact of PTEN expression on survival of the whole cohort; and it was revealed that nuclear PTEN loss was significantly associated with reduced survival rate compared to the cytoplasmic PTEN loss. Concomitant loss of PTEN, ATR and cytoplasmic pCHK1 further reduced the survival rate in the whole cohort. Several other studies reported the loss of PTEN and progressive loss of ATR, and was found to be absent in late stage cancer (Waha et al., 1998; Ding et al., 2004; Ye et al., 2007; Tommiska et al., 2008; Bartek et al.,

2007). The association between ATR and PTEN indicated that these components may be used to establish synthetic approach.

Synthetic lethality approach has been used by various research groups as an effective strategy to develop the therapeutic strategies for cancer. For instance, the data provides that VE-821 induces synthetic lethality in PTEN-deficient cells through accumulation of DSBs, cell cycle arrest and induction of apoptosis. Nada et al (2015) employed synthetic lethality strategy to inhibit the function of ATM in BRCA1-deficient cells to increase apoptosis, cell-replication and to reduce the survival of tumour cells. Similarly, inhibition of BER pathway in HR deficient cell background was shown to be synthetically lethal (Mohammed et al., 2011a). Similarly, inhibition of ATM, ATR, or DNA-PKcs using their cognate inhibitors in XRCC1 deficient cells were found to be an effective synthetic lethal strategy (Sultana et al., 2013a; Sultana et al., 2013b). It was hypothesized that impairing ATR expression in PTEN-deficient cells may serve as a novel synthetic lethality approach for breast cancer patients. To test this hypothesis, the DNA repair genes were profiled in PTEN-deficient and PTEN-proficient cells (Chapter 3). Following this, the ATR was targeted by ATR inhibitor (VE-821) in PTEN-deficient cells to compare whether targeting ATR in PTEN-deficient was synthetically lethal to that in PTEN-proficient breast cancer cells (Chapter 4).

Total of 84 genes involved in DNA repair were profiled using RT^2 Profiler^{TM} DNA Repair PCR array in PTEN-deficient and PTEN-proficient cells. The profiler data revealed that several genes were downregulated in consequence of deficiency of PTEN in PTEN-deficient cells compared to the PTEN-proficient. It was found that

PTEN-deficient breast cancer cells have impaired HR, NHEJ, NER and MMR pathway due to global reduction in expression level of key DNA repair genes involved in these pathways. Interestingly ATR and ATM were downregulated as well in PTEN-deficient breast cancer cells compared to PTEN-proficient cells. Nevertheless, further investigation can be conducted to determine the impact of these pathways in development and progression of breast carcinomas.

Previously, ATM inhibition in BRCA1-deficient cells and PTEN-deficient cells were reported to be an example of effective synthetic lethality. Thus it was assumed that ATR deficiency in PTEN-deficient background may potentially cause synthetic lethality in breast cancer cells. MTS assay was performed to determine cytotoxicity to the cell growth in response to ATR inhibitor (VE-821). Data obtained from the MTS assay showed that PTEN-deficient cell lines were more sensitive to cytotoxicity caused by ATR inhibitor than the PTEN-proficient cell lines used in this study. To further determine the mechanism causing selective cytotoxicity, several other functional studies were performed. For example, the accumulation of DSBs as a result of ATR inhibition in PTEN-deficient cells were performed using γH2AX immunofluorescence microscopy assay, and it was revealed that γH2AX foci were significantly increased after 24 h and 48 h in response to ATR inhibitor treatment in PTEN-deficient cells (BT549 and MDA-MB-468) compared to the PTEN-proficient cells (MDA-MB-231). In addition, ATR inhibition in PTEN-deficient cells (MDA-MB-468), cell cycles were arrested at G2/M phase after 48 hours of treatment of ATR inhibitor in PTEN-deficient background compared to the PTEN-proficient one. Cell cycle arrest at G2/M phase

after ATR inhibition was consistent with many other studies (Lapenna and Giodano, 2009; Jazayeri et al., 2006). Apoptosis detection was performed by using FITC-Annexin-V flow cytometry, and was found that both early and late apoptosis rates were significantly increased in PTEN-deficient cells compared to the PTEN-proficient cells. Increase in apoptosis in response to ATR inhibitor in different cancer cell lines was previously published by several researchers (Leszczynska et al., 2016; Brunner, 2012). Taken together, these data underscored the importance of ATR in the cells survival and DNA repair, and support the hypothesis of PTEN/ATR synthetic lethality, and highlight the significance and vitality of ATR in maintaining the genomic stability in PTEN-deficient cells. Thus this study proposes that PTEN/ATR synthetic relationship can be tuned for the personalised breast cancer therapy.

Interestingly, in response to PTEN deficiency, both ATR and ATM were already downregulated. Even in low dosage ATR was able to arrest the cell cycle and induce the apoptosis. Despite ATR, ATM or PTEN impairment, the DSBs may be repaired by DNA back-up repair pathways and consequently cells may continue to grow and survive. However, the function of back-up repair pathways also depends on or overlap with the NER, HR and NHEJ pathways (Schipler and Iliakis, 2013, Chapman et al., 2012). Considering the downregulation of several genes implicated in these repair pathways, the function of DNA back-up repair pathways may be interrupted, which may result in increasing accumulation of DSBs beyond a threshold level which led to cell cycle arrest and apoptosis.

6.2. Future research directions

In this study, only few breast cancer cell lines were used, which is one of limitations of this study. Moreover, MCF7 was not included in the functional studies due to the shortage of time. The future studies should consider including more cell lines so that data representative of different molecular classes of breast cancer cell lines can be obtained. In addition, some drugs such as PARP inhibitors can be used as positive control to further validate the findings of this study, because they have been used widely in other studies conducted to show the synthetic lethality relationship between two genes in breast cancer cells. This study has raised many questions where it answered several questions. The future studies can be planned to validate the findings of current study. In addition, the pharmaceutical grade ATR under development can also be used to test and validated these findings. Moreover, to test the overexpression of PTEN on the regulation of proteins involved in DNA repair pathways, future studies can clone PTEN into plasmid allowing enhanced expression of PTEN. Targeting PTEN knock-down breast cancer cells by ATR inhibitors as an additional validation to explore the synthetic lethality relationship. As this study demonstrated impaired DNA repair pathways due to downregulation of key proteins involved in HR, NER, NHEJ and BER. Future studies may target these repair pathways separately in PTEN-deficient breast cancer cells.

6.3. Summary of key findings of this research

1. Negative expression of nuclear and cytoplasmic PTEN was associated with aggressive tumour phenotype.

2. Negative/low expression of nuclear and cytoplasmic PTEN was negatively associated with ET, HER2 and PgR.
3. Loss of nuclear PTEN, ATR and pCHK1 was associated with poor BCSS
4. Loss of PTEN was associated with an altered regulation of several genes involved in DNA repair pathways such as BER, NER, HR, NHEJ and MMR.
5. PTEN-deficient BC cells were more sensitive to ATR inhibitors (VE-821 and AZD6738) than PTEN-proficient BC cells.
6. DSBs accumulation was increased significantly in PTEN-deficient BC cells in response to ATR inhibitor treatment compared to the PTEN-proficient BC cells.
7. Cell cycle was arrested at G2/M phases in PTEN-deficient BC cells after 48 hours treatment of ATR inhibitor in comparison with PTEN-proficient BC cells.
8. ATR inhibitor treatment increased significantly the early and late apoptotic rate in PTEN-deficient BC cells compared to PTEN-proficient BC cells.

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8. Appendices

Appendix Table 1. List of antibodies used in this study for different correlation with PTEN

Antibody	Clone	Supplier	Dilution/ Incubation	Pre-treatment	Categorical Cut-off points
ER	1D5	DAKO	1:150/ 1 hour	Antigen retrieval for 20 minutes microwave	≥ 1%
PgR	636	DAKO	1:100/ 1 hour		≥ 1%
HER1	EGFR.113	Novacastra	1:10		≥ 10%
HER2	cerbB-2	DAKO	1:250/ 1 hour		≥ 10%
HER3	RTJ1	Novacastra	1:20		≥ 10%
HER4		Neomarkers	1:20		≥ 10%
Ki67	MIB1	DAKO	1:25/ 1 hour		> 34%
P53	DO7	Novocastra	1:50/ 1 hour		≥ 5%
AKT (Phospho S474)		Neomarkers	1:150/1 hour		≥ 65 H-score
PI3KCA		Sigma	1:50/1 hour		Negative (<30), Low (30-100), High (101-300)
ATR	IE9	Nevous Biologicals	1:20 overnight		≥60 H-score
CHK1 (Phospho S345)	Ab 58567	Abcam	1:150 1h		≥50 H-score nuclear, ≥50 H-score cytoplasmic, x-tile.

Appendix Table 2. DNA repair genes assayed in Qiagen RT^2 profiler DNA repair pathways

Symbols	Descriptions
APE1	APEX nuclease (multifunctional DNA repair enzyme) 1
APE2	APEX nuclease (apurinic/apyrimidinic endonuclease) 2
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ATXN3	Ataxin 3
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BRIP1	BRCA1 interacting protein C-terminal helicase 1
CCNH	Cyclin H
CCNO	Cyclin O
CDK7	Cyclin-dependent kinase 7
DDB1	Damage-specific DNA binding protein 1, 127kDa
DDB2	Damage-specific DNA binding protein 2, 48kDa
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2
ERCC3	Excision repair cross-complementing rodent repair deficiency, complementation group 3
ERCC4	Excision repair cross-complementing rodent repair deficiency, complementation group 4
ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5
ERCC6	Excision repair cross-complementing rodent repair deficiency, complementation group 6
ERCC8	Excision repair cross-complementing rodent repair deficiency, complementation group 8
EXO1	Exonuclease 1
FEN1	Flap structure-specific endonuclease 1
LIG1	Ligase I, DNA, ATP-dependent
LIG3	Ligase III, DNA, ATP-dependent
LIG4	Ligase IV, DNA, ATP-dependent
MGMT	O-6-methylguanine-DNA methyltransferase
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MLH3	MutL homolog 3 (E. coli)
MMS19	MMS19 nucleotide excision repair homolog (S. cerevisiae)
MPG	N-methylpurine-DNA glycosylase

MRE11A	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
MSH3	MutS homolog 3 (E. coli)
MSH4	MutS homolog 4 (E. coli)
MSH5	MutS homolog 5 (E. coli)
MSH6	MutS homolog 6 (E. coli)
MUTYH	MutY homolog (E. coli)
NEIL1	Nei endonuclease VIII-like 1 (E. coli)
NEIL2	Nei endonuclease VIII-like 2 (E. coli)
NEIL3	Nei endonuclease VIII-like 3 (E. coli)
NTHL1	Nth endonuclease III-like 1 (E. coli)
OGG1	8-oxoguanine DNA glycosylase
PARP1	Poly (ADP-ribose) polymerase 1
PARP2	Poly (ADP-ribose) polymerase 2
PARP3	Poly (ADP-ribose) polymerase family, member 3
PMS1	PMS1 postmeiotic segregation increased 1 (S. cerevisiae)
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)
PNKP	Polynucleotide kinase 3'-phosphatase
POLB	Polymerase (DNA directed), beta
POLD3	Polymerase (DNA-directed), delta 3, accessory subunit
POLL	Polymerase (DNA directed), lambda
DNA-PKcs	Protein kinase, DNA-activated, catalytic polypeptide
RAD18	RAD18 homolog (S. cerevisiae)
RAD21	RAD21 homolog (S. pombe)
RAD23A	RAD23 homolog A (S. cerevisiae)
RAD23B	RAD23 homolog B (S. cerevisiae)
RAD50	RAD50 homolog (S. cerevisiae)
RAD51	RAD51 homolog (S. cerevisiae)
RAD51B	RAD51 homolog B (S. cerevisiae)
RAD51C	RAD51 homolog C (S. cerevisiae)
RAD51D	RAD51 homolog D (S. cerevisiae)
RAD52	RAD52 homolog (S. cerevisiae)
RAD54L	RAD54-like (S. cerevisiae)
RFC1	Replication factor C (activator 1) 1, 145kDa
RPA1	Replication protein A1, 70kDa
RPA3	Replication protein A3, 14kDa
SLK	STE20-like kinase
SMUG1	Single-strand-selective monofunctional uracil-DNA glycosylase 1
TDG	Thymine-DNA glycosylase
TOP3A	Topoisomerase (DNA) III alpha
TOP3B	Topoisomerase (DNA) III beta

TREX1	Three prime repair exonuclease 1
UNG	Uracil-DNA glycosylase
XAB2	XPA binding protein 2
XPA	Xeroderma pigmentosum, complementation group A
XPC	Xeroderma pigmentosum, complementation group C
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6
XRCC6BP1	XRCC6 binding protein 1