ANTI-CANCER EFFECTS OF THYMOQUINONE IN BREAST CANCER CELLS: INVOLVEMENT OF NON-HOMOLOGOUS END-JOINING AND TELOMERE-TELOMERASE HOMEOSTASIS

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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10th July 2012

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SUMMARY

Recent trends in cancer management have sparked a growing interest in discovering novel natural compounds that aim to effectively and specifically target cancer cells with minimal toxicity in normal cells. The anti-neoplastic effects of thymoquinone (TQ), a main active constituent of *Nigella Sativa* seeds, had been demonstrated in various *in vitro* and *in vivo* cancer models with minimal toxicity in normal cells. However, studies till date have only examined the proliferative ability of breast cancer cells upon TQ treatment and the possible underlying mechanisms of action of TQ are not well understood.

Recently, our laboratory had shown that TQ induced telomere shortening, DNA damage and apoptosis in glioblastoma cells. Based on the foregoing accounts, this study investigated the anti-cancer potential of TQ in breast cancer cells, MDA-MB-231 and MCF-7. Reduced proliferative capacity was observed only in breast cancer cells, which showed inefficient or delayed repair of TQ-induced deoxyribonucleic acid (DNA) damage in comparison to normal epithelial cells. Specifically, TQ-induced DNA double strand breaks (DSBs) in the breast cancer cells could possibly involve the non-homologous end-joining (NHEJ) pathway as the main DNA DSB repair mechanism in this study.

However, the regulation of telomere-telomerase homeostasis by TQ in MDA-MB-231 and MCF-7 cells appeared to be dissimilar. In MDA-MB-231 cells, the observations were likely associated with telomerase inhibition via c-myc regulatory pathway of telomerase reverse transcriptase (hTERT) expression with concomitant telomeric repeat-binding factor-2 (TRF2) down-regulation and subsequent telomere shortening. The acute effects of such de-regulation have been shown to induce DSBs

at telomeric sites and also ataxia telangiectasia mutated (ATM)-independent activation of DNA-protein kinase catalytic subunit (DNA-PKcs) via mediation of NHEJ repair pathway. On the other hand, in MCF-7 cells, telomerase inhibitory effects were evident only at high TQ doses and upon chronic low dose exposure for up to 8 weeks. The inhibitory effects could possibly involve indirect modulation of the c-myc regulatory pathway of hTERT expression with subsequent progressive telomere shortening. Likewise in MDA-MB-231 cells, there was subsequent activation of DNA-PKcs via mediation of NHEJ repair pathway.

Taken together, our findings suggest that the common activation of DNA-PKcs in TQ-treated breast cancer cells could serve as an important observation for future possible combinatory treatment with TQ and the potential of translating this nature endowed compound for cancer treatment in humans.

LIST OF FIGURES

ABBREVIATIONS

(6-4)PP	6-4 photoproduct
ADP	adenosine diphosphate
ALT	alternative lengthening of telomeres
A-T	ataxia telangiectasia
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BARD1	BRCA1-associated RING domain-1
BER	base excision repair
BFB	breakage fusion bridge
BRCA1	breast cancer type 1 susceptibility protein
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
Cis-Pt	cisplatin
CPD	cyclobutane pyrimidine dimer
DIG	digoxgenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-protein kinase subunit
DNA-PKcs	DNA-protein kinase catalytic subunit
EDTA	ethylenediaminetetraacetic acid

ER	estrogen receptor
ER(+)	estrogen receptor-positive
ERα	estrogen receptor alpha
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
FL	fluorescence emission
HCl	hydrochloric acid
HEK	human embryonic kidney
HER2 or Neu	human epidermal growth factor receptor 2
HR	homologous recombination
IRAK4	interlukin-1 receptor-associated kinase-4
MDC1	mediator of DNA damage checkpoint protein-1
MEFs	mouse embryonic fibroblasts
MMC	mitomycin
MPF	Mitotic promoting factor
MRN	Mre11/ Rad50/ Nbs1
NER	nucleotide excision repair
NF-κB	nuclear factor-kappa B
NHEJ	non-homologous end-joining
PAGE	polyacrylamide gel electrophoresis
PARP-1	poly (ADP-ribose) polymerase-1
p-ATM	phosphorylated ataxia telangiectasia mutated

PBS	phosphate buffered saline
PCA	principle component analysis
PCR	Polymerase chain reaction
PD	population doubling
PDN	population doubling number
p-DNA-PKcs	phosphorylated DNA-protein kinase catalytic subunit
PI-3K	phophatidylinositol 3-kinase
PNA	peptide nucleic acid
POT1	protection of telomeres-1
PPAR-γ	peroxisome proliferator activated receptor-gamma
PR(+)	progesterone receptor-positive
pRB	retinoblastoma
Q-FISH	quantitative-fluorescence in situ hybridisation
R	sulforhodamine emission
RING	really interesting new gene
RNA	ribonucleic acid
RNF8	ring finger protein-8
SDS	sodium dodecyl sulphate
SEM	standard error mean
TEP1	telomerase-associated protein-1
TEP3	telomerase-associated protein-3
TERC	telomerase RNA component

TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor-beta
TIF	telomere dysfunction induced foci
TIN2	TRF1-interacting protein-2
TQ	thymoquinone
TRAP	telomeric repeat amplification protocol
TRF	terminal restriction fragment
TRF1	telomeric repeat-binding factor-1
TRF2	telomeric repeat-binding factor-2
WINMDI	windows multiple document interface

LIST OF PUBLICATIONS

1. Gurung RL, **Lim SN**, Khaw AK, Soon JF, Shenoy K, Mohamed Ali S, Jayapal M, Sethu S, Baskar R, Hande MP. Thymoquinone induces telomere shortening, DNA damage and apoptosis in human glioblastoma cells (2010). PLoS One. Aug 12; 5(8)

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1. **Lim SN, Gurung RL, Hande MP. Effects and mechanisms of action of Thymoquinone in breast cancer cells. 70th Annual meeting of the Japanese Cancer Association. October 3-5, 2011, Nagoya, Japan.**

CHAPTER 1

1. Introduction

1.1 DNA damage and repair

Deoxyribonucleic acid (DNA) is a stable macromolecule, which carries the genetic material essential for all processes of life and maintenance of cellular functions. Nevertheless, DNA can be damaged when exposed to environmental agents, oxidative stress and spontaneous degradation (Friedberg E. C., 2005a; Hoeijmakers, 2001). This may manifest as single or double strand breaks (DSBs), base deletions, insertions or point mutations, instability of hydrogen bonds between complementary strands and even formation of base adducts (Fig. 1A) (Hoeijmakers, 2001). However, the most common DNA lesions are the formation of strand breaks, especially DSBs which is considered the most deleterious form of DNA damage (Jackson, 2002). When a DNA lesion is detected in cells, cell regulatory mechanisms are activated to allow correction of any possible DNA or chromosomal defects (Hartwell and Kastan, 1994; Hartwell and Weinert, 1989). Inadequate or unsuccessful repair may lead to the accumulation of DNA lesions culminating to apoptosis or in rare instances progressing to cancer (Fig. 1B) (Hoeijmakers, 2001). Due to the essential roles of DNA as aforementioned, it is the only biological macromolecule that undergoes repair when damaged so as to preserve genomic integrity and hence stability.

Cells with resistance to DNA damaging agents are likely associated with increased cellular repair activities. On the other hand, defective DNA repair pathways contribute to hypersensitivity to these agents. Interestingly, somatic or inherited

mutations in DNA repair proteins in tumour cells have been reported to rely much more than normal cells on the remaining functional DNA repair mechanisms for damage repair (Damia and D'Incalci, 2007).

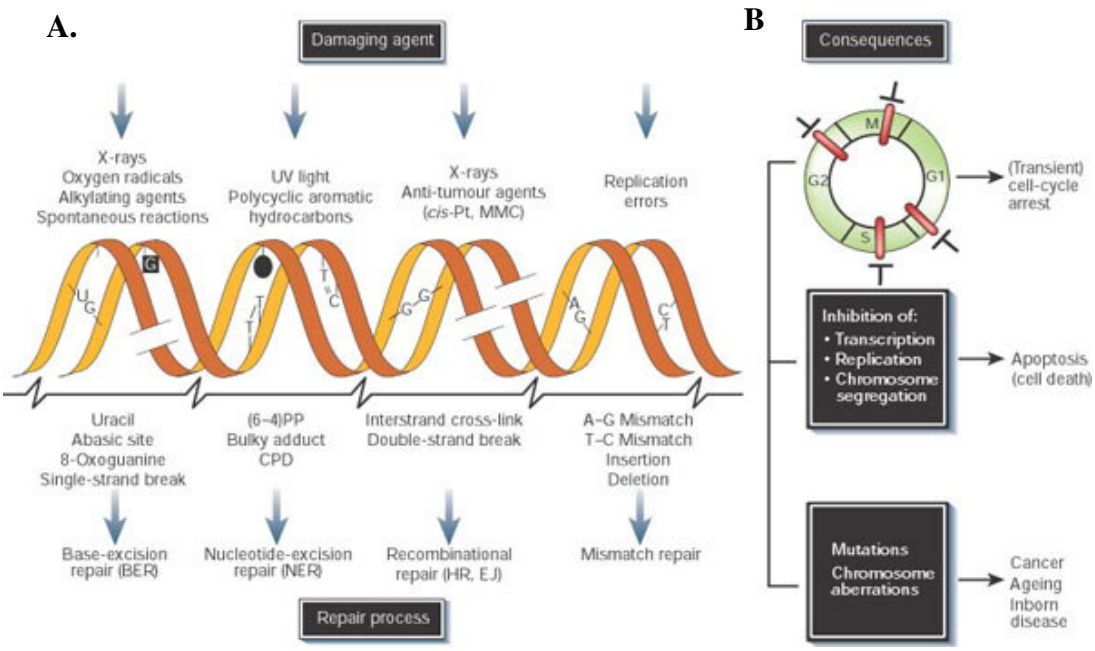


Figure 1. Possible sources of DNA damage, DNA repair mechanisms and subsequent consequences of immediate and sustained DNA damage. (A) Common DNA damaging agents (top), which are capable of inducing the various types of DNA lesions (middle) and can be repaired by specific repair pathways (bottom). (B) Immediate effects of unrepaired DNA damage causes cell cycle arrest (top) or apoptosis (middle), while continued accumulation of DNA damage is likely to lead to permanent changes in DNA sequences and hence, cancer. Abbreviations: cis-Pt and MMC, cisplatin and mitomycin C, respectively; (6-4) PP and CPD, 6-4 photoproduct and cyclobutane pyrimidine dimer, respectively; BER and NER, base- and nucleotide-excision repair, respectively; HR, homologous recombination; EJ, end joining. Reproduced from Nature: Genome maintenance mechanisms for preventing cancer. (Hoeijmakers, 2001)

1.2 DNA repair pathway – Non-homologous end-joining (NHEJ)

Non-homologous end-joining (NHEJ) pathway is one of the major DNA DSB repair pathways in mammalian cells (Jackson, 2002; Lieber et al., 2004). NHEJ mediates the repair of DSBs by directly re-joining the broken ends, which will ultimately cause deletions of small DNA sequences at the sites of DNA breakages (Jackson, 2002). Although NHEJ is active in all cell cycle phases, it has been shown to be particularly important for recombination repair in G_0 and G1 cell cycle phases as such cells do not possess a homologous chromosome, which is required for repair of DNA damage (Hendrickson, 1997; Rothkamm et al., 2003). However, the human genome is complex and only a small percentage encodes for proteins. Therefore the risks associated with such an error-prone repair pathway are not as detrimental as cells entering S phase with unrepaired DSBs.

1.2.1 Major players in the NHEJ pathway

The NHEJ pathway is governed by a highly regulated protein complex comprising of a large DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and its regulatory Ku 70 and 80 subunits (Burma and Chen, 2004; Smith et al., 1999; Smith and Jackson, 1999). The importance of the protein complex in NHEJ has been shown by several studies, which reported greater occurrences of chromosomal aberrations and genomic instability in mouse embryonic fibroblasts (MEFs) and mammalian cells lacking in either DNA-PKcs, Ku70 or Ku80 proteins (Barnes et al., 1998; Ferguson et al., 2000; Gao et al., 1998a; Gao et al., 1998b; Gu et al., 2000; Taccioli et al., 1998).

In a cellular response to DSBs (Fig. 2), DNA end-binding protein Ku70/80 complex recognizes and binds to each of the DSB sites (Mahaney et al., 2009; Yuan et al., 2010). This consequentially signals the recruitment of DNA-PKcs, which stimulates its catalytic activity via phosphorylation of ser-2056 or Thr-2609 clusters (Chan et al., 2002; Chen et al., 2005; Ding et al., 2003). Compromised DSB repair function of DNA-PKcs has been shown to occur when these two identified cluster sites were mutated (Chan et al., 2002; Ding et al., 2003). The interaction of DNA-PKcs and Ku70/80 forms the DNA-PK complex, which aids in synapsis of the DSB sites (Mahaney et al., 2009). The XRCC4-DNA ligase IV is recruited for ligation of the double strand ends for completion of the repair process. The kinase activity of DNA-PKcs can be regulated by auto-phosphorylation of Ser-2056 cluster, which leads to inactivation of its kinase activity and subsequent dissociation of the DNA-PK complex after damage repair (Chan et al., 2002; Ding et al., 2003). Serine/threonine phosphorylation sites are commonly present in DNA repair proteins and are cognate substrates of phophatidylinositol 3-kinase (PI-3K) members (Mahaney et al., 2009; Poltoratsky et al., 1995).

Ataxia telangiectasia-mutated (ATM) protein kinase, which also belongs to the PI-3K super family, is a general DNA damage sensor (Shiloh, 2006). In the event of DNA damage, ATM will be activated to phosphorylate downstream targets involved in cell cycle arrest, DNA repair and stress response (Riballo et al., 2004; Shiloh, 2006). Although the involvement of ATM in homologous recombination (HR) of DSBs repair has been well-established, recent evidence has revealed a possible complementary involvement of ATM in mediating DNA-PKcs phosphorylation at the

Thr-2609 cluster upon detection of DSBs contributing to the NHEJ pathway (Chen et al., 2007).

ATM and DNA-PKcs have been shown to be separately involved in regulating the phosphorylation of H2AX (An et al., 2010; Burma et al., 2001; Park et al., 2003; Stiff et al., 2004). H2AX is a component of chromatin and comprises of a central globular domain, an N-terminal tail and a unique C-terminal tail with a conserved motif connected by a linker of variable sequence and length (Bonner et al., 2008). The conserved motif contains the omega-4 serine 139 that becomes phosphorylated to generate gamma-H2AX (γ-H2AX) (Rogakou et al., 1998). γ-H2AX is a specific and efficient coordinator in the early response for DNA DSB repair (Kinner et al., 2008). It is a well-established biomarker employed in immunofluorescence experiments for detection of DSBs (Kinner et al., 2008).

Once the initial DNA damage sensor proteins as described previously becomes activated, a nucleation reaction is initiated with the recruitment of MDC1 and continuing with that of the MRN (Mre11/Rad50/NBS1) complex to further activate DNA-PK and ATM (Yuan and Chen, 2010). This generates a feedback loop that leads to further phosphorylation of H2AX and chromatin modifications required for the recruitment of 53BP1 (Lee et al., 2010; Yuan and Chen, 2010). The activation cascade culminates with the recruitment of RNF8 to phosphorylated MDC1 and the polyubiquitinylation of H2AX to recruit BRCA1/BARD1 (Wei et al., 2008).

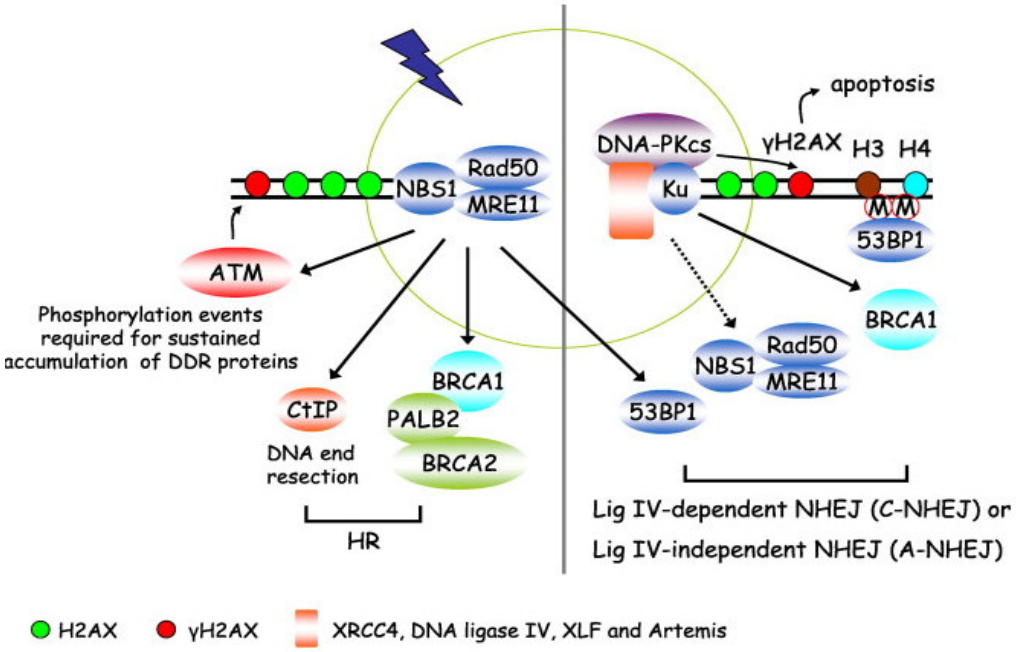


Figure 2. Double strand break recognition and repair pathways. Ku70/80 heterodimer recognizes and binds directly to broken DNA double strand ends. Recruitment of DNA-PKcs initiates a series of phosphorylation events, including generation of γ-H2AX. General DNA damage sensor, ATM, can also activate DNA-PKcs. Subsequent nucleation reaction completes the repair process. Reproduced from FEBS Letters: Focus on histone variant H2AX: To be or not to be. (Yuan et al., 2010)

1.3 Telomeres and its structure

Telomeres are chromosomal end-capping structures first discovered by Hermann Muller in 1938 (Rodier et al., 2005). These specialized nucleoprotein complexes function to protect from end-to-end chromosomal fusions, prevent the recognition of chromosomal ends as DSBs and also from nuclease degradation (Greider and Blackburn, 1985; Shay and Wright, 2006)

Mammalian telomeres consist of repetitive non-coding sequences of TTAGGG with a single-stranded 3' G-rich overhang, which invades into the duplex telomeric region forming a secondary structure (Rodier et al., 2005). The secondary structure consists of a telomere-loop (T-loop) and a displacement-loop (D-loop), which aid to stabilize and cap telomeric DNA (Fig. 3). Specific protein complexes such as protection of telomeres-1 (POT1), telomeric repeat-binding factor-1 (TRF1), TRF2, TRF1-interacting protein-1 (TIN1), TIN2, TINF2-interacting protein (TPP1) and transcriptional repressor/activator protein (RAP1) form the shelterin complex, which play additional roles in protecting telomeres and hence maintaining its function (de Lange, 2004). Telomere-specific proteins are able to interact and bind directly or indirectly to either the single- or double-stranded telomeric DNA (Shay and Wright, 2006).

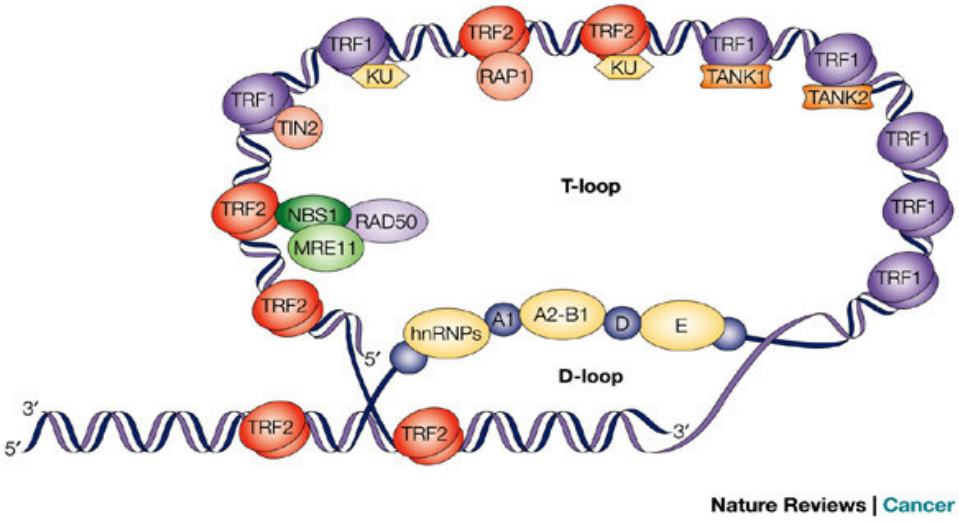


Figure 3. The proposed structure of telomeres and their associated proteins. TRF1 and TRF2 interact with the double-stranded duplex telomeric DNA. Indirectly-binding proteins bind to telomeric DNA through interaction with directly-binding proteins, especially through TRF1 and TRF2. Abbreviations: POT1, protection of telomeres-1; RAP1, transcriptional repressor/activator protein; TRF1 and TRF2, telomeric repeat-binding factor-1 and telomeric repeat-binding factor-2, respectively; TIN2, TRF1-interacting protein-2, respectively; NBS1, nijmegen breakage syndrome 1; TANK1 and TANK2, tankyrase 1 and tankyrase 2, respectively; hnRNPs, heterogeneous nuclear ribonucleoproteins. Reproduced from Nature Reviews: Molecular Cell Biology 5. (de Lange, 2004)

1.3.1 Telomeric end-replication problem

Considering the important roles that telomeres are involved in as aforementioned, it is thus necessary to maintain functional telomeres for continued cell proliferation.

Each round of DNA replication in normal somatic cells leads to progressive loss of terminal telomeric sequences of approximately 50 to 200 base pairs with each cell division (Lansdorp, 2000). The loss of telomeric repeats is primarily attributed to the end replication problem of the lagging strand or in some cases due to the presence of exonucleases (Xin and Broccoli, 2004). The end replication problem arises due to the intrinsic inability of DNA repair mechanisms to fill in the 5’ gap contributed by

the short RNA primers, which are required for initiating replication by DNA polymerase (Rodier et al., 2005). As a result, progressive telomere attrition occurs with each cell division and thus, telomeres are known to serve as mitotic clocks recording proliferative history.

However, continued telomere shortening will eventually lead to the triggering of multiple safeguard mechanisms in cells under normal circumstances. Cells stop dividing and undergo permanent G_0 ,a process also known as replicative senescence or mortality stage 1 (M1) (Fig. 4) (Hayflick, 1965). This prevents deregulation of proliferation pathway that may otherwise predispose cells to the development of cancer.

However, when a mutation imparts a selective survival advantage, further mutations such as those involving the dominant gain-of-function of proto-oncogenes or recessive loss-of-function of tumour suppressor genes will tend to accumulate (Lengauer et al., 1998; Loeb et al., 2003a). Therefore, cells may bypass M1 when somatic mutations that inactivate retinoblastoma (pRB) or p53 tumour suppressor genes occur. Consequently, cells continue to proliferate until telomeres are critically shortened and are unable to form the secondary telomere structure. This telomere dysfunction will then lead to a continuous break-fusion-bridge (BFB) cycle resulting in massive gene dosage changes and genetic instability. Eventually, cells will enter cellular crisis or mortality stage 2 (M2) (DePinho, 2000), which serves as a potential barrier for the road to immortalization.

As rare event prior to M2, telomerase reactivation or up-regulation allows cells to escape crisis and proliferate indefinitely with subsequent progression to invasion and metastasis of cancerous cells (Fig. 4) (Greider and Blackburn, 1985). Majority of

advanced human malignant tumour cells undergo telomerase reactivation enabling cancer cells the capacity for unlimited proliferation. Hence, up-regulation of telomerase can be considered as an almost universal marker for most (approximately 85 to 90 %) tumour cells in diagnostics. However, an alternative pathway exists for cancer cells to attain immortality, which is through recombination via alternate lengthening of telomeres (ALT) (Shay and Bacchetti, 1997).

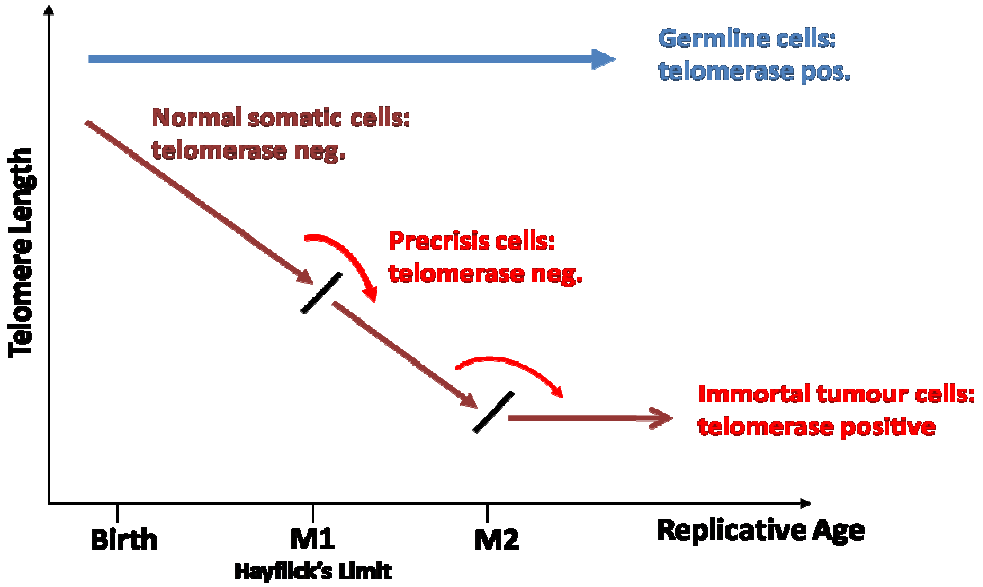


Figure 4. The telomere-telomerase hypothesis of cell aging and immortalization. Telomerase activity persists in germ line cells but not in somatic cells; hence telomeres shorten with age and time in normal somatic cells but not in germ line cells. In normal somatic cells, when a critical telomere length is reached, a rare event causes the cell to reactivate or up-regulate telomerase. This allows the cell to maintain the length of telomeres and attain immortalization. Modified and reproduced from Cold Spring Harbour Symposium: Quantitative Biology. (Harley et al., 1994)

1.4 Telomerase – a regulator of telomere length

The holoenzyme, telomerase, was first purified by Greider and Blackburn (1985). It is a cellular ribonucleic acid (RNA)-dependent DNA polymerase consisting of three major components: (1) the telomerase RNA (TERC) subunit, (2) the catalytic telomerase reverse transcriptase (TERT) subunit and the (3) protein dyskerin, with

other associated proteins; telomerase-associated proteins (TEP1, TEP3) (Fig. 5) (Chen et al., 2009).

The TERC subunit comprises of an integral 11 base pairs RNA template complementary to the TTAGGG repeats. The TERT subunit reverse transcribes and elongates the 3' telomeric end with hexameric repeats through employment of the TERC subunit (Feng et al., 1995; Nakamura et al., 1997). Hence, telomerase plays a pivotal role in the stabilization of telomere length by compensating for the loss of telomeric DNA with each round of replication. This is further validated by Hahn et al. (1999) reportedly showing that the retroviral introduction and expression of hTERT into large T-antigen expressing human embryonic kidney (HEK) cells and normal human BJ fibroblast cells not only stabilizes telomere length but plays a central role in cellular resistance to apoptosis (de Lange and DePinho, 1999). Furthermore, deletions in genes encoding for the TERC and dyskerin proteins in stem cells have also shown to alter their renewal capacity due to the failure of a functional telomerase to maintain telomere length (Marciniak and Guarente, 2001; Mitchell et al., 1999; Vulliamy et al., 2001; Wong et al., 2004).

All cells and tissues express telomerase at birth, which is subsequently suppressed when differentiation occurs (Mattson MP, 2000-). Thus, telomerase expression can be considered as a hallmark for human cancers since it is detectable in 85 to 90 % of tumours and not in normal somatic cells, with the exception of germline cells and the proliferating cells of renewal tissues (e.g. bone marrow cells, intestinal epithelial cells) (Wright et al., 1996). However, it is important to note that telomerase expression alone does not induce a transformed phenotype in cancer but rather the association of other internal and external factors too (Hanahan and Weinberg, 2011).

Telomerase-mediated telomere length compensation is favoured in cells with the shortest telomere length (Gellert G.C., 2005; Shay and Wright, 2005). Such scenarios may occur even when most telomeres in the cell population are long, since it is the shortest telomere length that determines the fate of the cell (Gellert G.C., 2005).

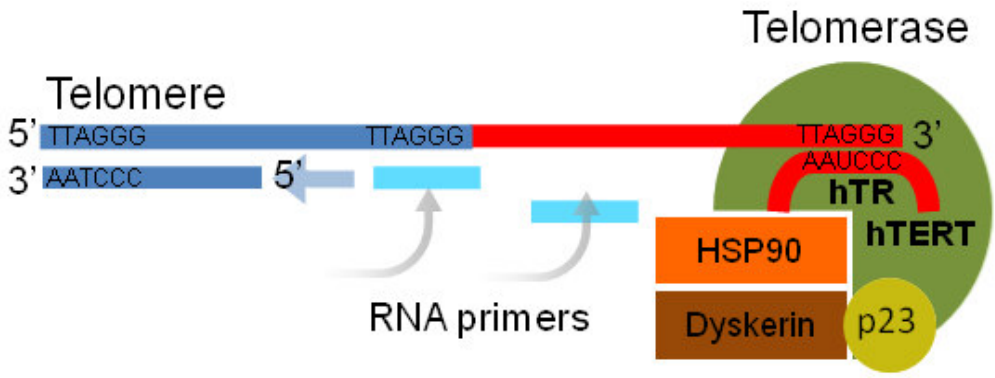


Figure 5. Simplified structure of telomerase and telomere maintenance mechanism. Telomerase complex comprises the TERC, hTERT, dyskerin, HSP90 and p23 subunits. It extends the 3' telomere end by adding TTAGGG repeats using the complementary RNA template in the TERC subunit. This enables the RNA primers to be located further away from 5' end and hence prevents loss of telomere ends associated with the end-replication problem. Modified and reproduced from Frontiers in Bioscience: Telomere protein complexes and interactions with telomerase in telomere maintenance. (Pinto et al., 2011)

1.4.1 Regulation of telomerase

The regulatory pathway for telomerase is not fully elucidated and has always been an area of interest for most researchers. Nevertheless, reports have shown that since hTERC and hTEP-1 are constitutively expressed in mammalian cells, the transcription and alternative splicing of TERT will then be the rate-limiting step for telomerase expression (Meyerson et al., 1997; Nakayama et al., 1998; Takakura et al., 1998). A further study by Seimiya et al (2000) also postulated a possible post-translational involvement of the TERT subunit in telomerase regulation (Seimiya et al., 2000).

Some upstream positive transcriptional regulators of the TERT gene include c-myc proto-oncogene, AKT and estrogen receptor α (ERα), while negative regulators include pRB tumour suppressor gene, E2F transcription factors and transforming growth factor beta (TGF-β) (Fig. 6) (Grandori and Eisenman, 1997; Horikawa and Barrett, 2003; Wang et al., 1998).

The c-myc proto-oncogene is commonly known to be involved in cell proliferation and immortalization when constitutively expressed in primary fibroblasts (Askew et al., 1991; Kohl and Ruley, 1987) . Consequently, c-myc has been touted as a key molecular switch positively regulating telomerase activity and expression of TERT, where c-myc binding sites can be found at the TERT promoter region (Greenberg et al., 1999; Schneider-Stock et al., 2003; Wu et al., 1999).

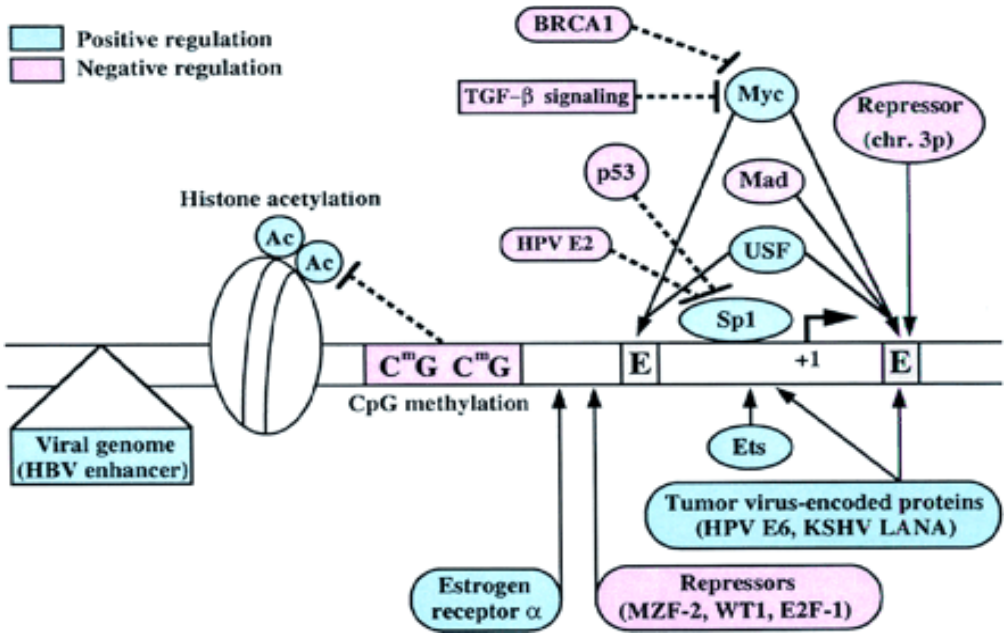


Figure 6. Multiple mechanisms for the transcriptional regulation of hTERT gene. Various mechanisms act on the hTERT promoter to regulate hTERT transcription. Some positive regulators include c-myc and estrogen receptor α, negative regulators include p53, pRB and BRCA-1. E: two canonical E-box (CACGTG) elements upstream and downstream of the transcription initiation site (+1). Reproduced from Carcinogenesis: Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanisms. (Pinto et al., 2011)

1.5 Regulation of telomere function

Both telomere length and stability of the secondary telomeric structure play vital roles in regulating the function of telomeres. Telomere length is stabilized by achieving a balance between telomere shortening due to the intrinsic end-replication problem and the accessibility to telomerase, which aids in adding hexamer repeats to the shortened telomeres. In addition, the expression and activity of shelterin proteins in cells affect telomere length, stability of the secondary telomeric structures and the access to telomerase.

1.5.1 Telomere binding proteins – regulators of telomere function

Telomere binding proteins, e.g. TRF1 and TRF2, are also important in controlling telomere length in cells. TRF1 and TRF2 bind to double-stranded telomeric sequences and maintain the t-loop secondary structure *in vitro*. However, their modes of telomere regulation are vastly dissimilar (Smogorzewska et al., 2000).

Studies have shown that TRF1 is a negative regulator of telomere length as TRF1 over-expression leads to telomere shortening, while a mutant DNA-binding domain TRF1 variant results in progressive telomere lengthening (Smith and de Lange, 1997; Smogorzewska et al., 2000; van Steensel and de Lange, 1997). In addition, the regulatory effects of TRF1 on telomeres are independent of telomerase activity, suggesting that TRF1 controls telomerase access to telomeres (Smogorzewska et al., 2000).

On the other hand, TRF2 plays an important role in telomere end capping (Smogorzewska et al., 2000). This prevents telomeres from chromosomal end-to-end fusions through interaction with DNA-damage signalling and repair factors (Chen et

al., 2009). Recently, TRF2 has been shown to migrate and localise to sites of DNA DSBs suggesting the protective role of TRF2 at telomeric regions (Bradshaw et al., 2005; Stauropoulos, 2005). The localization of TRF2 to such sites has been observed to be faster than the recruitment of ATM (Bradshaw et al., 2005).

1.5.2 DNA repair proteins involvement in telomere maintenance

There have been various studies reporting the close knit relationship between DNA repair proteins and telomere function (d'Adda di Fagagna et al., 1999; d'Adda di Fagagna et al., 2001; Gilley et al., 2001; Hande, 2004; Slijepcevic et al., 1997). It is probably the tendency of telomeric sites to be highly prone and sensitive to DNA damage with subsequent recruitment and localization of DNA repair factors to damaged sites that fuels such reports (Hewitt et al., 2012). In certain circumstances, the shelterin complex serves as the connection for interaction between DNA repair factors and telomere associated proteins.

1.5.2.1 ATM and telomere maintenance

ATM is the first reported DNA repair protein to alter telomere dynamics (Metcalfe et al., 1996). Defective ATM in mouse cells causes accelerated telomere attrition, fusions and extrachromosomal telomere fragments (Hande et al., 2001). In addition, cells derived from ataxia telangiectasia (A-T) patients had increased chromosomal aberrations and telomere loss (Hande et al., 2001). Although ectopic expression of hTERT in A-T cells was able to extend the lifespan of these cells, manifestation of telomere instability still occurred.

Interestingly, ATM has also been shown to interact with telomere-associated proteins, specifically TRF1 and TRF2. ATM phosphorylation of TRF1 results in the

release of TRF1 from telomeres (Wu et al., 2007). This is likely to promote telomerase access to telomeres and hence telomere lengthening. On the other hand, TRF2 binding represses ATM kinase activity and protects telomeres from the activation of ATM-dependent DNA damage response pathway (Karlseder et al., 2004).

1.5.2.2 DNA-PKcs and telomere maintenance

DNA repair proteins involved in NHEJ, specifically DNA-PKcs and Ku70/80, play a role in telomere capping and hence prevent chromosomal fusions. Mammalian cells defective in either Ku70/80 or DNA-PKcs exhibited higher occurrence of end-to-end telomeric fusions (d'Adda di Fagagna et al., 2001). The role of telomere capping by DNA-PKcs arises from observations of DNA-PKcs deficient mouse cells displaying higher levels of telomere fusions with no significant changes in telomere length (Goytisolo et al., 2001; Hande et al., 1999). Furthermore, the pharmacological inhibitor of DNA-PK phosphorylation, IC86621, disrupted telomere end capping (Bailey et al., 2004). This further signifies the crucial role of DNA-PK kinase activity in performing its telomere end-protection role.

1.5.2.3 PARP-1 and telomere maintenance

Poly(ADP)-ribose polymerase 1 (PARP-1) is an abundant nuclear DNA damage sensor mediating repair of DNA single strand breaks implicated in the base excision repair (BER) pathway (Huber et al., 2004). Studies have shown the localization of sporadic PARP-1 at normal telomeres and accumulation when telomere erosion occurs (Gomez et al., 2006). Although PARP-1 is dispensable for telomere end protection, PARP-1-deficient MEFs displayed hypersensitivity to genotoxic

agents and heightened genomic instability due to a greater occurrence of telomere attrition (d'Adda di Fagagna et al., 1999; Gurung et al., 2010a).

No differences in telomerase activity between PARP-1-deficient and wild type cells suggest that PARP-1 does not regulate telomerase activity (Samper et al., 2001). Further studies also showed no direct interaction between PARP-1 and TERT proteins via the yeast-two hybrid assay. Therefore, PARP-1 is most likely to associate with telomere-associated proteins, TRF2, in regulating telomere length (Gomez et al., 2006). In particular, PARP-1 poly(ADP-ribosyl)ates TRF2 and this alters the DNA-binding domain of TRF2 (Dantzer et al., 2004). Subsequently, TRF2 dissociates from telomeres causing relaxation of the t-loop structure, which then provides access to DNA repair factors.

1.6 Dysfunctional telomere-induced genomic instability in cancer

Continuous epithelial turnover over time leads to telomere shortening. When coupled with inactivation of tumour suppressor genes, replicative senescence can be bypassed and subsequent proliferation results in further progressive telomere attrition. Hence, the function of telomeres is compromised. The ‘naked’ telomeres are then recognized as DSBs by DNA repair machineries resulting in futile end-to-end joining. The resultant dicentric chromosome leads to anaphase bridging during segregation in mitosis, which breaks apart when pulled across opposite spindle poles. The broken chromosome will be repaired once again through fusion with another chromosome generating another dicentric chromosome. This eventually perpetuates a breakage-fusion-bridge (BFB) cycle that facilitates the accumulation of genetic changes and hence instability enabling precancerous cells to emerge from crisis to malignancy (Maser and DePinho, 2002). In addition, studies by various groups have demonstrated

that telomere shortening leads to chromosomal and genomic instability with subsequent tumour development in telomerase-deficient mouse models. (Hahn et al., 1999; Hande, 2004; Pinto et al., 2011; van Steensel and de Lange, 1997; Xin and Broccoli, 2004)

Recently, it has been proposed that the ten hallmarks of cancer (Fig. 7) are acquired by most cancer cells (Hanahan and Weinberg, 2011). Interestingly, two of the hallmarks are an unstable genome with mutations and limitless proliferative capacity. As mentioned previously, telomere dysfunction culminates in massive gene dosage changes leading to chromosomal instability, which then drives multiple genetic changes (e.g. reactivation of telomerase) paving the road to immortalization (Maser and DePinho, 2002). Hence, we are interested to understand the association of telomere dysfunction and possible implications for cancer therapy in this study.

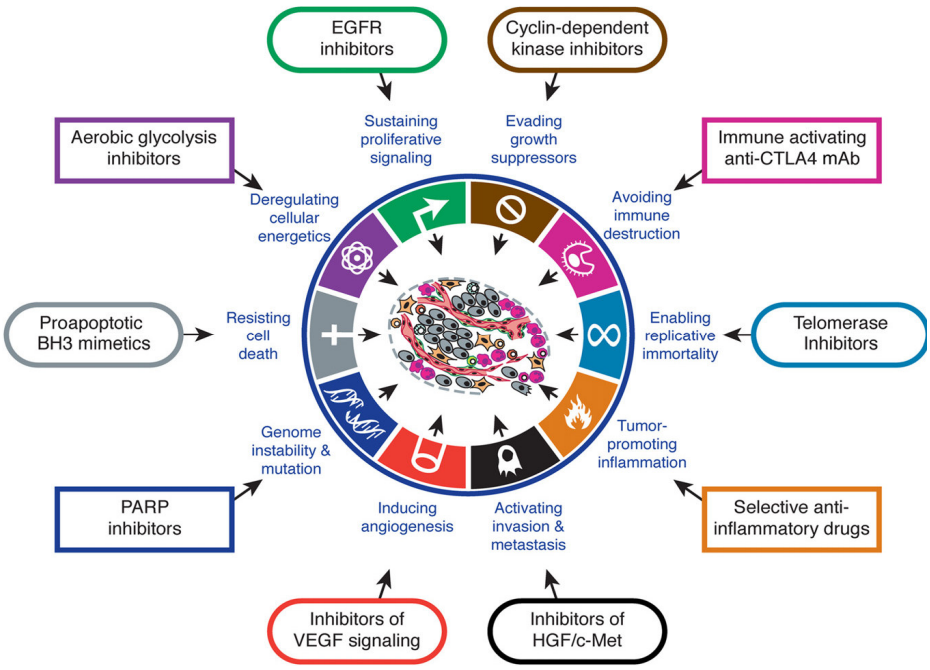


Figure 7. The ten hallmarks of cancer and specific therapeutic targets for each of the cancer hallmarks. Ten acquired capabilities necessary for cancer development and progression. Drugs (boxed) are being developed to target each of the enabling characteristics and emerging hallmarks. Reproduced from Cell: Hallmarks of cancer: the next generation. (Hanahan and Weinberg, 2011)

1.7 Trends in breast cancer

The prevalence of cancer has been increasing in recent years and is one of the leading causes of death in Singapore, accounting for one in every four deaths (Poh, 2008; Lim G.H., 2012). Of concern is the rapid rise of breast cancer, which is approximately an increment of three percent per year since 1968 (Fig. 8) (Sim et al., 2006).

Breast cancer is chosen as a model for this study due to the much received attention in Singapore over the years. It is the most common female cancer in Singapore with incidence rate doubling over the years and is expected to reach the much higher incidence rate in western countries (Sim et al., 2006). Not only is breast cancer the most common female cancer, it has the highest mortality rate in females. Survival from breast cancer is related to tumour size at time of diagnosis and treatment at an early stage will lead to a more effective and improved outcome (Rickard and Donnellan, 1998). In addition, the natural history of the disease involves a pre-invasive phase, for which treatment at this phase allows complete cure for breast cancer patients before metastasis sets in (Wee, 2002).

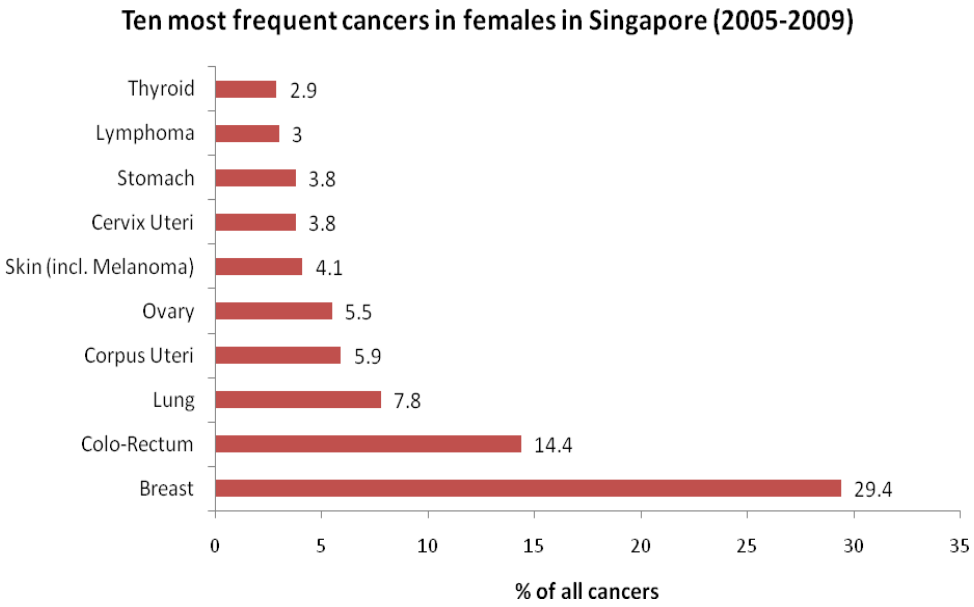


Figure 8. Ten most frequent cancers in Singapore females (2005-2009). Breast cancer tops the chart with being the most frequently occurring cancer in females in Singapore. Reproduced from Annual Registry Report: Trends in Cancer Incidence in Singapore. (National Registry of Diseases Office, 2011; Lim G.H., 2012)

1.7.1 Current treatment for breast cancer

The standard treatments available for breast cancer patients include surgery, chemotherapy (e.g. tamoxifen, paclitaxel), radiotherapy (e.g. x-rays, radioactive substances), hormonal therapy (e.g. estrogen blockers) and targeted therapy (e.g. monoclonal antibodies, tyrosine kinase inhibitors, PARP inhibitors) (National Cancer Institute, 2011). No treatments are fool-proof and relapses do occur depending on the stage and type of breast cancer first detected and treated. Thus, it is necessary to search for novel agents to contribute towards a more successful treatment of breast cancer in future.

The foremost concern of current chemotherapeutic cancer agents is the side effects caused by non-specific targeting of both normal and cancerous cells. It has been reported that hormonal therapy with adjuvant tamoxifen therapy in breast cancer

patients has a tendency to act on other body cells and hence develop endometrial cancer (Bernstein et al., 1999; van Leeuwen et al., 1994). Therefore, much emphasis has been placed on discovering novel natural or synthetic compounds that target tumour cells more efficiently and selectively with minimal toxicity to normal cells.

1.8 Possible development of telomerase inhibition in cancer therapeutics

Telomerase inhibition has been viewed as an attractive target for cancer therapeutics in view that a therapeutic window exists in which cancer cells can be effectively targeted without affecting normal somatic cells. Furthermore, the length of telomeres in cancer cells is shorter in comparison to normal somatic cells (Chen et al., 2009), thus significantly affecting the survival of cancer cells to a greater extent. Yet, one may contest that telomerase inhibition will ultimately affect telomerase positive germline cells, stem cells and the proliferating cells of renewal tissues. It is true that telomerase inhibition do affect such telomerase positive cells, but all these cells generally have longer telomeres than cancer cells and the initial length of telomeres is an important factor for telomerase inhibition leading to telomere shortening, coupled with growth arrest, senescence or apoptosis (Hahn et al., 1999; Zhang et al., 1999).

1.9 Natural plant products in cancer therapy

Over the recent years, natural plant products have sparked a growing interest in the area of research for prevention or treatment of cancer (Vuorelaa et al., 2004). The identification of pharmacologically active constituents in potential plant products as chemo-preventive agents has always been the focus since treatment results have been promising (HemaIswarya and Doble, 2006). During the period of 1983 to 1994,

approximately 41 % newly approved drugs originated from natural products, of which 60 % are anti-cancer agents (Cragg et al., 1997).

Although natural plant products mediate their effects through multiple targets, they are known to produce relatively lesser side effects with minimal toxicity (Vuorelaa et al., 2004). In comparison to synthetic compounds, natural plant products are inexpensive and easily available in ingestive forms with reported years of intake by humans. There is also growing evidence linking cancer risk and dietary factors. Hence, dietary phytochemicals can be said to be a promising class of compounds with health benefits (Sa and Das, 2008). Epidemiological data has shown that a lower cancer risk occurs in populations with a greater reliance on spices, fruits and vegetables in their diets (Wargovich, 1999). Some reportedly well-known active constituents in natural plant products to prevent or treat illnesses or diseases include genistein (in soy), epigallocatechin gallate (in green tea) and curcumin (in spice) (Gerhauser et al., 2003; Moiseeva et al., 2007).

However, there are also other natural plant products that have not been extensively researched on and might have the potential for new discoveries. An example would be thymoquinone (TQ) as evidenced by the limited number of published studies and reports over the years (Gali-Muhtasib et al., 2004a; Shoieb et al., 2003). Hence, the therapeutic potential of TQ should not be undermined and further in-depth investigations should be warranted.

1.9.1 Thymoquinone

TQ is the most abundant component in black seed oil (Gali-Muhtasib et al., 2006; Padhye et al., 2008). Black seeds can be harvested from the *Nigella sativa* plant

(Gali-Muhtasib et al., 2006; Padhye et al., 2008). Not only are the seeds used as a food spice, but also a natural remedy for over two thousand years to treat a plethora of illnesses and diseases, especially in Mediterranean and West Asian countries (Gali-Muhtasib et al., 2006). In addition, the seeds have been characterised by a low degree of toxicity *in vivo* (Ali and Blunden, 2003). Studies have shown that TQ is the main bioactive constituent in the black seeds contributing to its effects (Ali and Blunden, 2003).

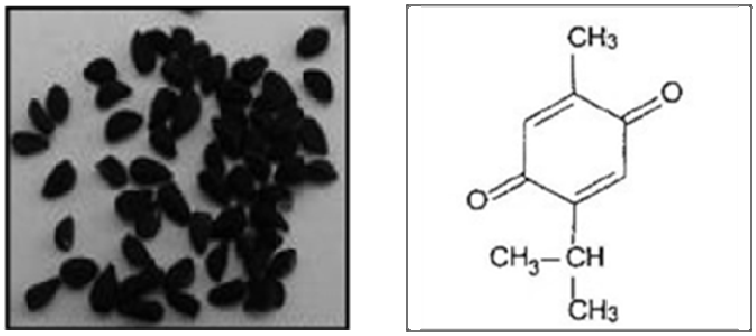


Figure 9. Black cumin seeds (left) and chemical structure of thymoquinone (right). The seeds are obtained from the *Nigella Sativa Linn* plant. Thymoquinone is the bioactive constituent extracted from the seeds. Reproduced from Cancer therapy: From here to eternity-the secret of Pharaohs: Therapeutic potential of black cumin seeds and beyond. (Padhye et al., 2008)

1.9.1.1 Reported biological effects of TQ

Being a pleiotropic agent, TQ mediates its effects through multiple signalling pathways in many patho-physiological conditions. Some of the reported biological effects include anti-neoplastic, anti-oxidant and anti-inflammatory (Gali-Muhtasib et al., 2006). Literature have well-documented the anti-neoplastic effects of TQ in various *in vitro* and *in vivo* cancer models. The cytotoxicity of this compound has been reported in various cancer cell lines including prostate cancer, colorectal cancer, human glioblastoma and ovarian cancers. However, the extent of cytotoxicity has been shown to be dependent on the tumour cell type with minimal toxicity in normal

cells (Shoieb et al., 2003). Several possible targets, which have been identified to contribute to the cancer cell specific effects of TQ, include the regulation of cell cycle and apoptotic proteins.

1.10 Motivation and significance

Recently, we have shown the novel effects of TQ on DNA damage and telomerase activity in brain cancer cells (Gurung et al., 2010b). An interesting finding from this study is that telomerase positive hTERT-BJ1 fibroblasts and human glioblastoma cells demonstrated increased sensitivity to TQ induced anti-proliferative effect as compared to normal cells. Cells with higher basal telomerase activity were more affected by TQ's telomerase inhibition, which is also evidenced by a down regulation in hTERT protein expression. In addition, long term TQ treatment significantly shortened telomeres suggesting that TQ disrupts telomere length maintenance by inhibiting the activity of telomerase over time in cancer cells. Dysfunctional telomeres have been shown to activate DNA damage response pathways leading to either senescence or apoptosis, which in this study predominately led to apoptosis (Herbert et al., 1999). Therefore, it is of particular interest to investigate if TQ also affects telomeres and DNA integrity in breast cancer cells.

Most of the earlier reports investigated the effects of TQ in relation to proliferative ability, cell cycle regulation and apoptotic effects in cancer models (Gali-Muhtasib et al., 2004b; Hsieh et al., 2006; Roepke et al., 2007; Shoieb et al., 2003). In breast cancer models, current studies have only examined the anti-proliferative effects after TQ treatment. The mechanism of action of TQ in breast cancer cells has yet to be fully understood, although studies have postulated the involvement of the nuclear factor kappa-B (NF-κB) and peroxisome prolierator-activated receptors-gamma

(PPAR-γ) pathways (Chaturvedi et al., 2011; Sayed and Morcos, 2007; Woo et al., 2012).

Therefore, this study aims to focus on the DNA damaging and telomere-telomerase effects and hence the mechanism of action of TQ in breast cancer cells. In addition, it is of interest to investigate the effects of TQ in normal breast epithelial cells and thereby establish if the effects of TQ are indeed selective towards the cancer phenotype.

Based on the foregoing account, efforts should be placed on gaining further understanding of the molecular mechanisms of action of TQ and subsequently on its bioavailability in clinical studies. This might then lead to the ultimate goal of translating this nature endowed compound for cancer treatment in humans.

1.11 Breast cancer cells as the model of study

Two breast cancer cell types chosen for this study are MCF-7 and MDA-MB-231. Both are well characterized *in vitro* model systems for invasive cancer. Being the first hormone-responsive breast cancer cell line discovered, estrogen and progesterone receptor-positive (ERα(+), PR(+)) MCF-7 cells have been adopted by many laboratories as an investigative tool in the mechanisms of cancer therapeutics (Simstein et al., 2003). On the other hand, triple-negative (ERα(-), PR(-), HER2/neu(-) MDA-MB-231 cells expressing lysine-280 mutant p53 (Kravchenko et al., 2008) are much more aggressive in nature (Balduyck et al., 2000) and have been correlated with cancer progression, metastasis and apoptosis resistance (Greenblatt et al., 1994). Hence, it will be of interest to identify anti-proliferating agents that are able to inhibit such invasive cancer cell growth.

In addition, the immortalized but non-transformed human mammary epithelial cell line, MCF-10A, will also be used in this study to investigate the selective cytotoxic effects of TQ, if any, on normal and cancer cells.

1.12 Objectives

1. To evaluate the growth inhibitory effects of TQ in breast cancer cells.
2. To investigate the DNA-damaging effects of TQ in breast cancer cells.
3. To investigate the effects of TQ on telomerase and telomere dysfunction.
4. To investigate a possible close knit relationship of DNA repair factors and telomeres in TQ-pre-treated breast cancer cells.

It is important to elucidate the anti-proliferative effects of TQ in the breast cancer cells, followed by evaluating the effectiveness of TQ in impairing DNA damage repair as well as in inhibiting telomerase in breast cancer cells, which could ultimately help to develop novel therapeutic agents for human malignancies.

CHAPTER 2

2. Materials and Methods

2.1 Cell lines and drug treatment

Two human breast carcinoma cell lines, MCF-7 and MDA-MB-231 (ATCC, Manassas, VA, USA), were grown as monolayer in 75 cm^2 flasks in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 U/mL penicillin/streptomycin (Gibco, Grand Island, NY, USA). An immortalised but non-transformed human mammary epithelial cell line, MCF-10A (ATCC, Manassas, VA, USA), was cultured in MEGM (Lonza Corporation, Walkersville, MD, USA) supplemented with 52 µg/mL bovine pituitary extract (Lonza Corporation, Walkersville, MD, USA), 10 % fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 U/mL penicillin/streptomycin (Gibco, Grand Island, NY, USA). All three cell lines were maintained in a humidified 5 % CO_2 incubator at 37 °C.

Stock solution of Thymoquinone (TQ) (Sigma-Aldrich, St. Louis, MO, USA) was prepared in dimethylsulfoxide (DMSO) and suitable working concentrations were made from the stock in complete medium. Exponentially growing cells were exposed to 0 to 20 µM TQ for 48 h. However for telomere length analysis, the cells were treated with TQ for up to 8 weeks. Under these conditions, most subcultures before crisis exhibited growth rates that were comparable with the control and required cell passage weekly. However, cells with reduced growth rates at later passages were given fresh medium and test compounds every two to three days. For all experiments, vehicle control cells were treated with DMSO (0.1 %).

2.2 Cell viability

The short-term anti-proliferative effect of TQ on the three cell lines was assessed by the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, WI, USA) according to the manufacturer's protocol. Cells were seeded in 12-well plates at an initial concentration of 2 x 10^4 cells per well. Following overnight incubation, these cells were exposed to 0 to 20 µM TQ for 48 h. The number of viable cells in culture was determined by measuring the luminescent signal generated, which is proportional to the amount of ATP present. The quantification of ATP can then be correlated to the presence of metabolically active cells after TQ treatment.

2.3 Wound healing assay

To investigate the migration ability of cells after TQ exposure, cells were seeded in six-well plates until 80 % confluence. A 'wound' was created using a pipette tip and rinsed with 1 x PBS to remove detached cells. Following, cells were incubated with indicated concentrations of TQ for 48 h. Microscopic images of the 'wound' were captured before and after TQ exposure.

2.3 Cell cycle analysis

The changes in cell cycle phase distributions after TQ exposure were determined by flow cytometry. Cells were seeded in 100 x 20 mm culture dishes at an initial concentration of 5 x 10^5 cells per culture dish and incubated overnight. Following 48 h TQ exposure, harvested cells were washed with 0.1 % bovine serum albumin (BSA): 1 x PBS, fixed in ice-cold 70 % ethanol: 1 x PBS and stained with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA): RNase A (Roche, CA, USA) (2 mg PI and 2 mg RNase A/100 mL 0.1 % BSA in 1 x PBS). Samples were analysed

using a fluorescence-activated cell sorter (FACS) calibur (Becton Dickinson, NJ, USA) at 488 nm excitation λ and 610 nm emission λ. A total of 10,000 events per sample were evaluated using the WINMDI software (Windows Multiple Document Interface).

2.5 Alkaline single cell gel electrophoresis (comet) assay

The extent of DNA damage induced by TQ was investigated by the comet assay. Cells were seeded in 100 x 20 mm culture dishes at an initial concentration of 5 x 10^5 cells per culture dish. Following 48 h TQ exposure, harvested cells were resuspended in Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO, USA) with 10 % DMSO and 0.5 M EDTA, mixed with 0.7 % low melting point agarose (Conda, Madrid, Spain) at 37 °C and applied onto comet slides (Trevigen, Gaithersburg, MD, USA). Subsequently, cells were subjected to lysis in lysis solution (2.5 M NaCl, 0.1 M pH 8.0 EDTA, 10 mM Tris-HCl, 1 % Triton-X-100) at 4 °C for an hour. The slides were then loaded into a gel electrophoresis tank filled with chilled alkaline electrophoresis buffer (0.3 M NaOH, pH 13 with EDTA) for denaturation for 40 minutes in the dark. Electrophoresis was then carried out at constant 25 V/300 mA for 20 minutes. Subsequently, slides were immersed in neutralization buffer (0.5 M Tris-HCl, pH 7.4), dehydrated in 70 % ethanol and dried at 37℃. DNA was stained with SYBR Green (Trevigen, Gaithersburg, MD, USA). The images were captured using Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with triple band filter. A total of 50 randomly selected cells per sample were analysed using the Comet Imager Software (Metasystems, Altlussheim, Germany) to determine the tail moment, which represents the fraction of DNA in the comet tail.

2.6 Telomeric Repeat Amplification Protocol (TRAP) assay

Detection of telomerase activity in cell extracts was performed using the TRAPeze® XL Telomerase Detection Kit (Chemicon International, Billerica, MA, USA) according to manufacturer's protocol (Piotrowska et al., 2005). Cells were seeded in 100 x 20 mm culture dishes at an initial concentration of 5 x 10^5 cells per culture dish. Following 48 h TQ exposure, harvested cell pellets were lysed with CHAPS lysis buffer provided on ice for an hour. The supernatant (1.5 µg protein) obtained was added to 48 µl of reaction mixture containing TRAP buffer, dNTP mix, TS primer, RP Amplifluor® primer, K2 Amplifluor® primer and 2 units of Taq polymerase. Subsequently, polymerase chain reaction (PCR) was initiated by the telomerase mediated elongation products (i.e. the telomeric DNA repeats) as template. Following, normal PCR cycle was performed using primer pairs with quenched fluorescein to amplify the telomeric DNA repeats. Fluorescence signals of the PCR products were generated by unquenching the fluorescein on PCR primers and measured using the fluorescence plate reader TECAN SpectraFluor Plus. Controls used for this experiment include CHAPS-only telomerase negative control and Taq polymerase-negative control. The ratio between the net increase of fluorescence emission (ΔFL), which is derived from the telomerase-dependent synthesis of the PCR product, and the net increase of sulforhodamine emission (ΔR), which is on the other hand derived from the product synthesized on the internal control representing PCR efficiency, for each cell line is calculated. The final ratio is then expressed as a percentage of the control.

2.7 Population doubling (PD) study

The long-term anti-proliferative effect of TQ on MCF-7 and MDA-MB-231 cells was assessed by trypan blue exclusion assay. Trypan blue will not be extruded by non-viable cells and thus only unstained viable cells were counted with a haemocytometer. Cells were harvested and measurements were performed weekly for up to 8 weeks of TQ exposure for each cancer cell type. A fresh tissue culture flask was then reseeded with 5 x 10^4 cells, or all cells if the cell number were less than 5 x 10^4. The population doubling number (PDN) was calculated as follows:

$$PDN = \log_2(N_0/N_x),$$

where N_0 = number of cells at harvest and N_x = number of cells seeded (ref)

The remaining cells were kept for DNA extraction for Telomere Restriction Fragment (TRF) length analysis.

2.8 Telomere Restriction Fragment (TRF) length analysis

To analyze the average telomere length in a population of cells, pure genomic DNA from the harvested cell pellets was firstly extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. Following, the TRF length analysis assay was performed using the Telo-TAGGG Length Assay Kit (Roche Applied Science, CA, USA). DNA (1.5 µg/sample) was digested with restriction enzymes, *Hin*f1 and *Rsa*1, for 10 minutes at 37 ^{o}C, where the whole genomic DNA except the sub-telomeric and telomeric regions was digested. Digested DNA fragments were fractionated by gel electrophoresis in a 0.8 % agarose at 80 V for 3 hours. The gel was then washed in hydrochloric solution (0.25 M HCl), denaturation solution (0.4 M NaOH) and neutralisation solution (1 M Tris 7.4, 5 M

NaCl). The DNA fragments in the gel were transferred to the Nytran® positively-charged nylon membrane (Sigma-Aldrich, St. Louis, MO, USA) via capillary action overnight. Subsequently, cross-linkage of DNA with the membrane was processed by ultraviolet light (Stratagene, Santa Clara, CA, USA), hybridized with Digoxgenin (DIG)-labelled telomere probe at 42 ^{o}C for 3 hours and washed with a series of anti-DIG alkaline phosphatase washing solutions. The membrane was incubated with avidin-conjugated horseradish peroxidase for 5 minutes, followed by horseradish peroxidase substrate solution, tetramthylbenzidine, for 5 minutes. Visualization of the DNA fragments were detected on X-ray films (Kodak, Rochester, NY, USA). The chemiluminescent signals were scanned by the Kodak Gel imaging system and analyzed by the Kodak imaging software for quantitative measurements of the mean TRF length.

2.9 Immunofluorescence staining for γH2AX

Briefly, 5×10^4 cells were seeded on cover slips in six-well plates overnight and then subjected to 48 h TQ exposure. Cells were fixed in 4% paraformaldehyde, permeabilize in 0.1% Triton-X-100 and incubated with anti-phospho-γH2AX (Ser139) (Upstate Biotechnology, Waltham, MA, USA) diluted in 1 x PBS with 4% FCS and 0.1% Triton X-100. Cells were washed and incubated with FITC-conjugated anti-mouse secondary antibody (1:500) at room temperature for an hour in the dark. Subsequent washes with 1 x PBS were also conducted in the dark. The cover slips were sufficiently dried prior to mounting onto slides containing DAPI (Vectashield®, Vector Laboratories, Burlingame, CA, USA). Fluorescent images were captured through confocal microscopy (Olympus Fluoview FV1000, Center Valley, PA, USA).

2.10 Immunofluorescence staining for telomere dysfunction

Detection of telomere dysfunction induced foci (TIF) analysis was performed as described previously. Cells were incubated with anti-phospho-γH2AX (Ser139) (Upstate Biotechnology, Waltham, MA, USA) and then with FITC-conjugated anti-mouse secondary antibody (1:500) as described previously with the exception that TBS-T was used for washing instead of 1 x PBS. Following, slides were placed in 4% formaldehyde for 20 minutes for cross-fixing to preserve antibodies. Subsequently, Cy3-labelled telomere sequence-specific peptide nucleic acid (PNA) probes were applied onto post-fixed slides and thermal co-denaturation was carried out at 80 ^{o}C for 6 minutes, following which slides were hybridised in a moist chamber at 37 ^{o}C for 2 hours. After hybridisation, the slides were washed in formamide and 10 % Tween-20 and counterstained with DAPI (Vectashield®, Vector Laboratories, Burlingame, CA, USA). All images were captured on the Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with filters for observation of DAPI (blue), FITC (green), Cy3 (red) and a triple filter for simultaneous observation of DAPI, FITC and Cy3.

2.11 Western blot analysis

Protein expression was analysed by immunoblotting. Briefly, cells were seeded in 100 x 20 mm culture dishes at an initial concentration of 5 x 10^5 cells per culture dish. Following 48 h TQ exposure, harvested cells were lysed with RIPA (radio-immunoprecipitation assay) buffer (1 % nonidet P-40, 1 % sodium deoxycholate, 0.1 % SDS, 0.15 M NaCl, 0.01 M sodium phosphate, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate and 100 U/mL aprotinin, pH 7.2) to obtain whole cell lysates containing total cellular proteins. Protein concentration was

determined by bicinchoninic acid assay with bovine serum albumin as the standard (Pierce Biotechnology Inc., Rockford, IL, USA). Equivalent amounts of protein (60 µg/assay) were resolved on 10 % SDS-polyacrylamide gel (SDS-PAGE) and transferred to Protran® nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO, USA). Membranes were incubated with 1 x PBS containing 0.05 % Tween 20 and 5 % non-fat milk to block non-specific binding for an hour, followed by incubation with primary antibodies to target proteins and then with the appropriate secondary antibodies conjugated to horseradish peroxidise. Primary antibodies used to probe for the different proteins include p53 (Santa Cruz, USA), p21 (Santa Cruz, USA), cyclin D1 (Santa Cruz, USA), cyclin B (Santa Cruz, USA), survivin (Santa Cruz, USA), c-myc (Santa Cruz, USA), hTERT (Santa Cruz, USA), TRF2 (Santa Cruz, USA), phosphor-ATM (Santa Cruz, USA), ATM (Santa Cruz, USA), phosphor-DNA-PKcs (Santa Cruz, USA), DNA-PKcs (Santa Cruz, USA), PARP-1 (Cell Signalling, USA) and actin (Santa Cruz, USA). Immunoreactive bands were visualised by using either SuperSignal West Pico or SuperSignal West Femto chemiluminescent reagents (Pierce Biotechnology Inc., Rockford, IL, USA) and subsequently quantified using the Kodak imaging software.

2.12 Gene expression analysis

Total RNA was extracted from QIAmp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA) and quantified using NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). RNA integrity was checked using Bio-Analyzer (Agilent Technologies, Santa Clara, CA, USA). Five hundred nanograms of extracted RNA from each sample were used for the gene expression study. TotalPrep RNA Amplification Kit (Ambion®, TX, USA) was used for cRNA amplification process.

The biotinylated amplified RNA thus generated was used for hybridization with HumanRef8 V3.0, Human Whole-Genome Expression BeadChips (Illumina Inc., USA) for 16 hours at 58 °C. After the incubation period, the arrays were washed and stained with Streptavidin-Cy3 (GE Healthcare, Bio-Sciences, UK). Illumina Bead Array Reader was used to scan the arrays. The array data thus obtained after scanning was imported and analysed using Partek® Genomics Suite™ (Partek GS) (Partek Incorporated, MO, USA).

2.13 Statistical analysis

All data were expressed as mean ± SEM and statistically subjected to Student's paired *t*-test using Microsoft Excel 2007 (Microsoft Corp., USA). The difference was considered to be statistically significant when $p<0.05$.

CHAPTER 3

3. Results

3.1 Effects of TQ on proliferative ability of normal and breast cancer cells

3.1.1 Breast cancer cells are sensitive to the anti-proliferative effects of TQ

Most cancer cells possess the potential of limitless replicative capacity, which is one of the hallmarks of cancers, contributing to the ability to proliferate indefinitely and invade into other tissues (Hanahan and Weinberg, 2011). Hence, it is of interest to investigate possible anti-proliferative effects of thymoquinone (TQ) in breast cancer cells. Moreover, normal mammary epithelial cells were also examined to determine whether the anti-proliferative effects of TQ were only specific to cancer cells.

TQ treatment resulted in growth inhibitory effects in breast cancer cells, MDA-MB-231 and MCF-7 (Fig. 10). In particular, the cell viability and migratory ability were considerably affected after 48 h incubation within the indicated concentration range of TQ tested (0 – 20 μM).

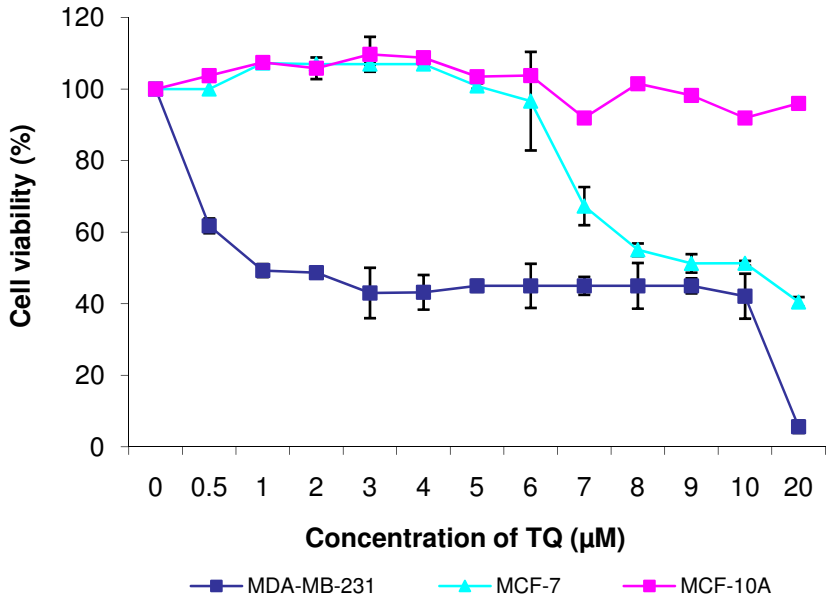
Both breast cancer cells exhibited a decline in viability of cells after TQ treatment for 48 h within the indicated concentration range tested (0 – 20 μM) (Fig. 10A). MDA-MB-231 cells displayed a drastic reduction in viability from 1 μM TQ, while MCF-7 cells showed a similar drastic reduction in viability from a higher TQ concentration of 7 μM. On the other hand, spontaneously immortalised but non-transformed mammary epithelial cells, MCF-10A, appeared to be the least sensitive with no apparent changes in cell viability within the indicated concentration range of TQ tested (0 – 20 μM).

To further evaluate the growth inhibitory effects of TQ on breast cancer cells, the migration ability of MDA-MB-231 and MCF-7 cells were investigated using the scratch wound assay. MDA-MB-231 and MCF-7 cells had reduced migration in a dose-dependent manner, especially at higher TQ concentrations (Fig, 10B). In contrast, the migratory potential of MCF-10A cells remained unaffected relative to untreated after exposure to 48 h TQ at the concentrations tested (1 µM, 10 µM and 20 µM).

Interestingly, MDA-MB-231 cells appeared to be more sensitive than MCF-7 cells to TQ treatment with substantial reduction in cell viability from concentrations of 0.5 µM and above as compared to from concentrations of 6 µM and above for MCF-7 cells (Fig. 10A). In addition, there was a greater suppression in migratory ability in MDA-MB-231 cells than MCF-7 cells (Fig. 10B) at all three concentrations (1 µM, 10 µM and 20 µM) of TQ tested.

The IC50 is defined as the concentration of drug resulting in 50 % growth inhibition *in vitro* (Yung-Chi and Prusoff, 1973). The IC50 values for TQ for the breast cancer cells were derived from the dose-response curve in Figure 10A. MDA-MB-231 and MCF-7 cells were found to have an IC50 value of 1 µM and 10 µM respectively, which were used for subsequent functional studies.

A.


120
100
80
60
40
20
0
Cell viability (%)
0 0.5 1 2 3 4 5 6 7 8 9 10 20
Concentration of TQ (µM)
MDA-MB-231
MCF-7
MCF-10A

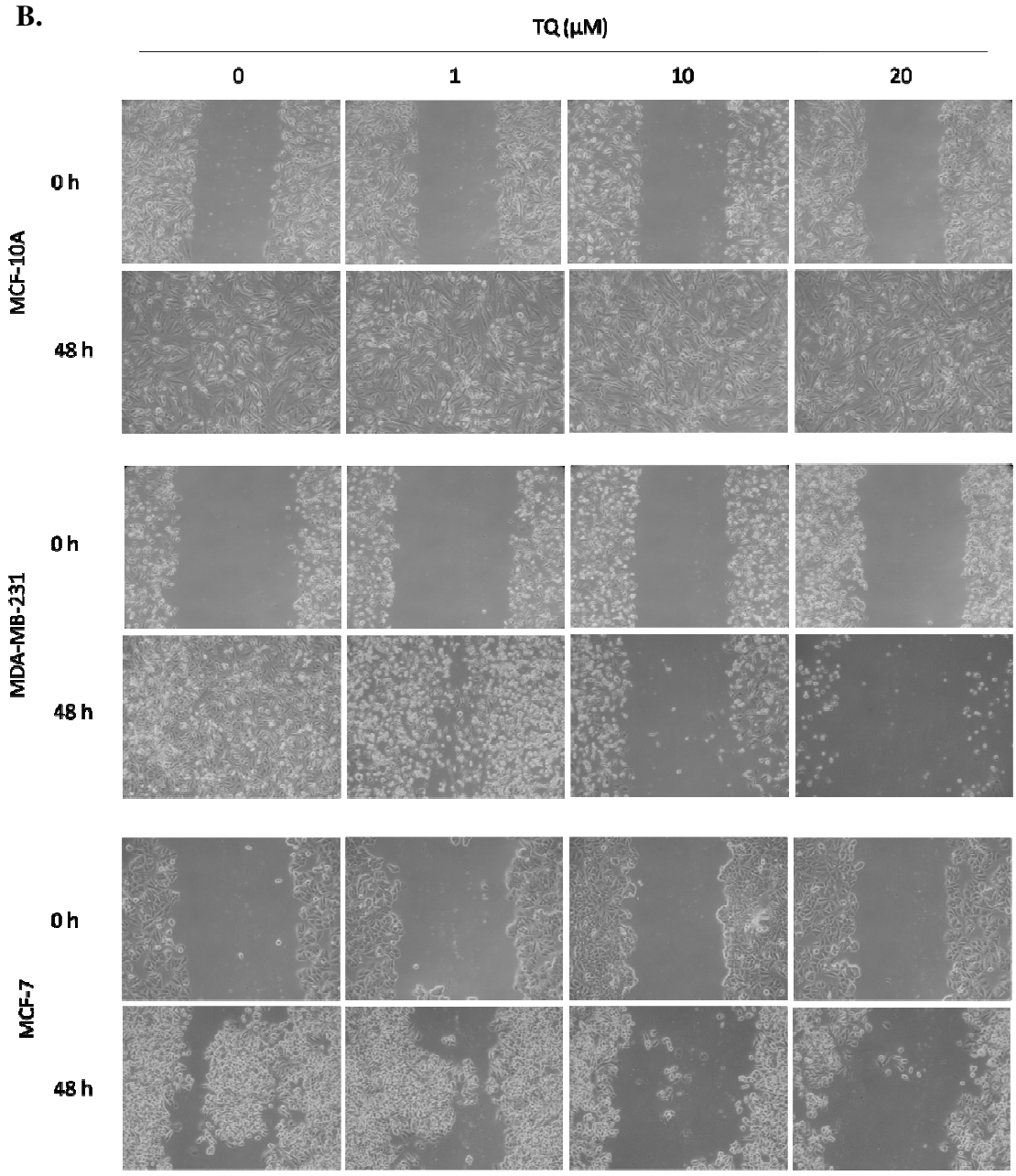


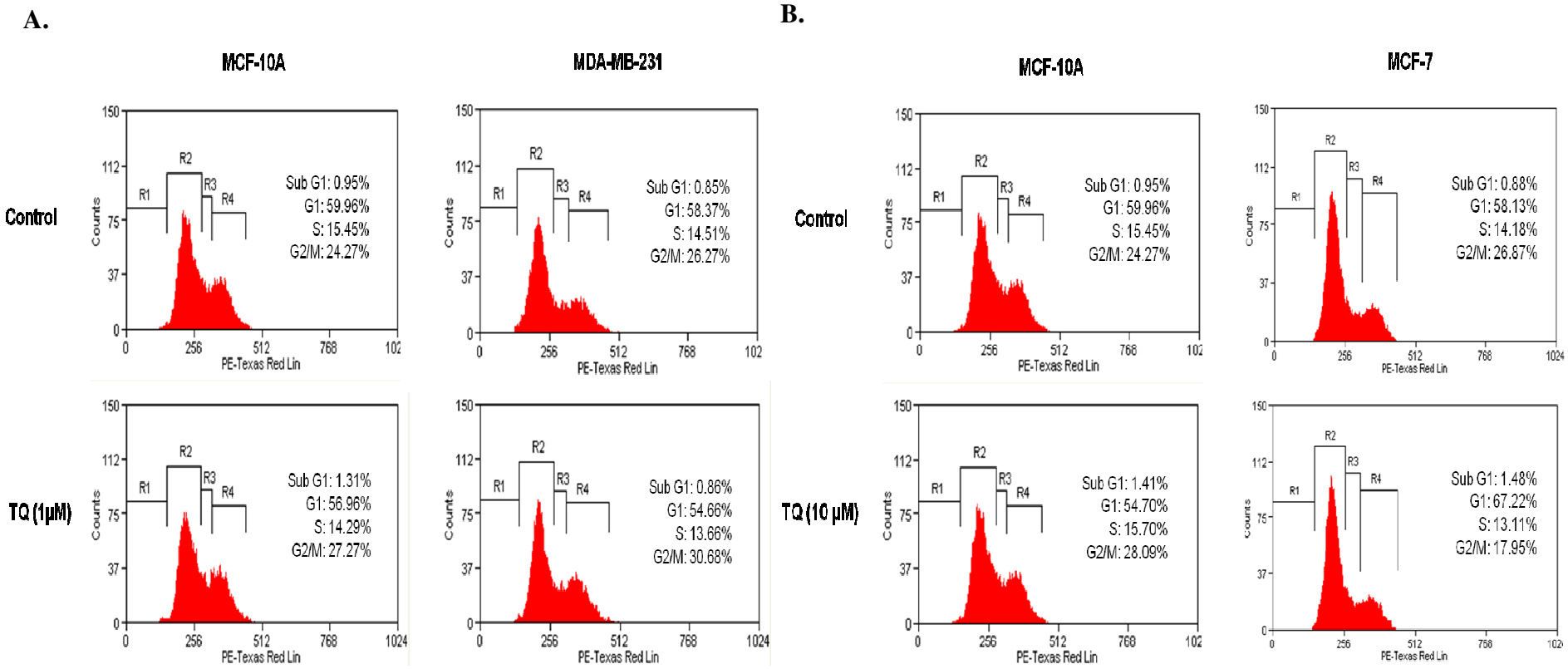
Figure 10. Growth inhibitory effects of TQ on breast cancer cells. (A) TQ reduced viability of breast cancer cells, but not immortalized human mammary epithelial cells. Dose-response curve following 48 h TQ exposure at the indicated concentrations in MCF-10A, MDA-MB-231 and MCF-7 cells. Cell viability was assessed by CellTiter-Glo® luminescent cell viability assay and the percentage cell viability was normalised against control (0.1 % DMSO) for each cell line. Data represent the mean ± SEM of three independent experiments carried out in triplicates. (B) TQ suppressed migratory ability of breast cancer cells as determined by the scratch wound assay. Representative microscopic images of the growth of MCF-10A, MDA-MB-231 and MCF-7 cells following 48 h TQ exposure at the indicated concentrations. Data is representative of three independent experiments.

3.1.2 TQ causes deficiencies in cell cycle checkpoint function in breast cancer cells

The observed differences in the cell viability and migratory trends prompted our investigation of cell cycle changes in the cells following 48 h exposure to TQ.

Figures 11A and 11B illustrate the cell cycle histograms of MCF-10A, MDA-MB-231 and MCF-7 cells after 48 h exposure to TQ. No significant shifts in cell cycle profiles were observed in MCF-10A cells after 48 h TQ (1 µM and 10 µM) (Fig. 11C). However, exposure to TQ led to delays in cell cycle progression in breast cancer cells. MDA-MB-231 cells presented an increase ($p<0.05$) of 3.24 % G2/M phase of cells accompanied by a decrease in G1 phase after 48 h TQ (1 µM) (Fig. 11D). Concomitant increases ($p<0.05$) of 0.60 % and 9.09 % in sub-G1 (apoptotic) and G1 phase of cells respectively were exhibited in MCF-7 cells upon 48 h TQ treatment (10 µM) (Fig. 11E).

Hence, the growth inhibitory effects in breast cancer cells as observed previously (Fig. 10) could likely be attributed to cell cycle arrest in G2/M and G1 phases for MDA-MB-231 and MCF-7 cells respectively.


A.
MCF-10A
MDA-MB-231
Control
TQ (1μM)
Sub G1: 0.95%
G1: 59.96%
S: 15.45%
G2/M: 24.27%
Sub G1: 0.85%
G1: 58.37%
S: 14.51%
G2/M: 26.27%
Sub G1: 1.31%
G1: 56.96%
S: 14.29%
G2/M: 27.27%
Sub G1: 0.86%
G1: 54.66%
S: 13.66%
G2/M: 30.68%
B.
MCF-10A
MCF-7
Control
TQ (10 μM)
Sub G1: 0.95%
G1: 59.96%
S: 15.45%
G2/M: 24.27%
Sub G1: 0.88%
G1: 58.13%
S: 14.18%
G2/M: 26.87%
Sub G1: 1.41%
G1: 54.70%
S: 15.70%
G2/M: 28.09%
Sub G1: 1.48%
G1: 67.22%
S: 13.11%
G2/M: 17.95%
Counts
PE-Texas Red Lin
R1
R2
R3
R4

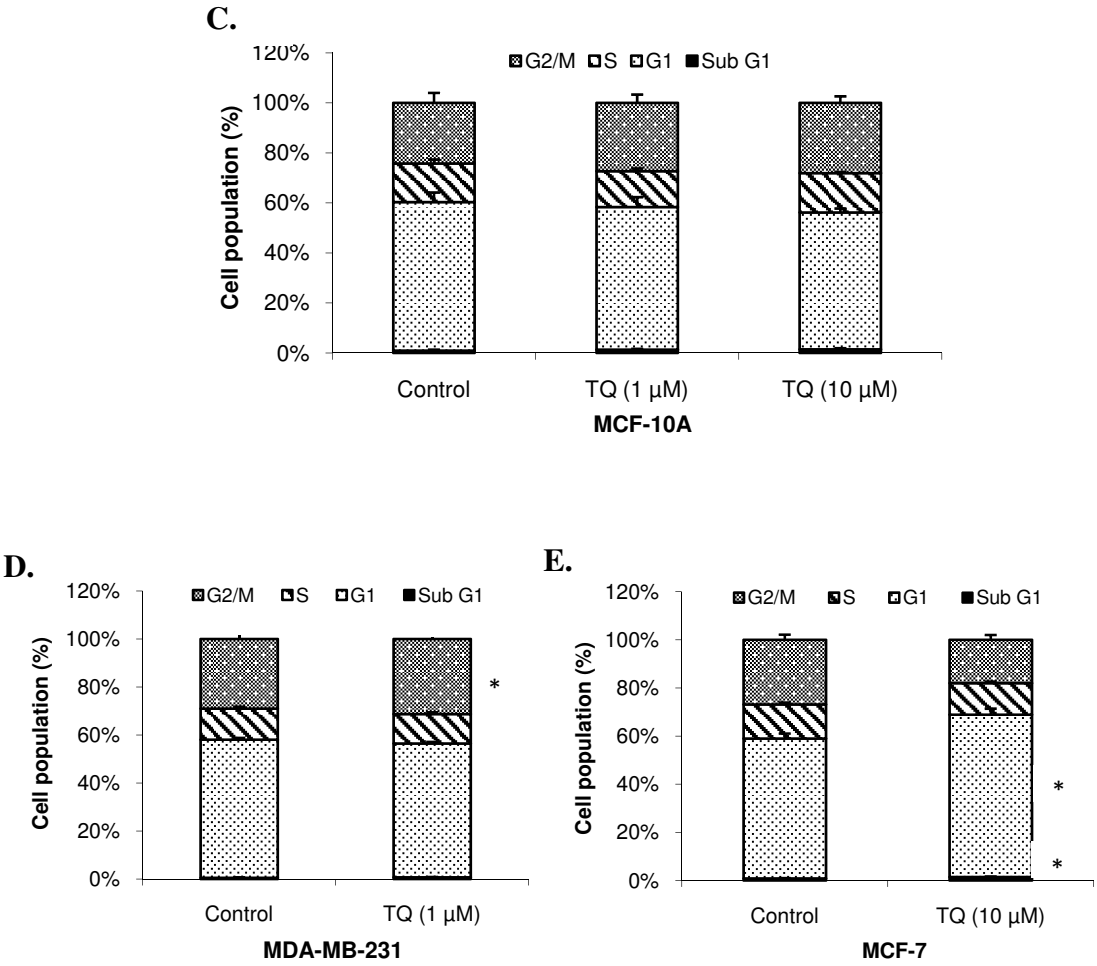


Figure 11. Growth inhibition of breast cancer cells following 48 h TQ exposure is largely attributed to changes in cell cycle profiles. (A-B) Cell cycle profiles for (A) MCF-10A and MDA-MB-231 without (0 µM) or with (1 µM) TQ treatment and (B) MCF-10A and MCF-7 without (0 µM) or with (10 µM) TQ treatment as measured by propidium iodide staining. Data is representative of three independent experiments. (C-E) Percentages of cells in each phase of the cell cycle for (C) MCF-10A, (D) MDA-MB-231 and (E) MCF-7 cells without (0 µM) or with (1 µM, 10 µM) TQ treatment. Data represent the mean ± SEM of three independent experiments carried out in duplicates.

3.1.3 Changes in cell cycle protein expressions in TQ-treated breast cancer cells

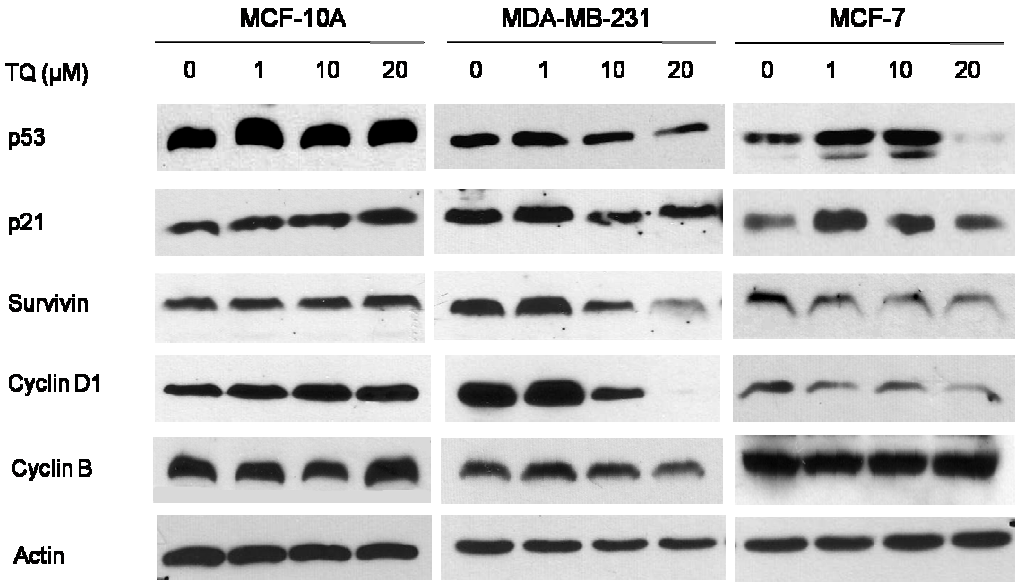
Alterations in cell cycle profiles were observed in TQ-treated breast cancer cells (Fig. 11) and to further elucidate the possible mechanisms behind such changes, expression of cell cycle related proteins (e.g. p53, p21, cyclin D1, cyclin B and survivin) were investigated.

In immortalised but non-transformed mammary epithelial cells, MCF-10A, cell cycle protein expression levels of p53, p21, survivin, cyclin D1 and cyclin B persisted and remained fairly unchanged upon 48 h TQ exposure at the indicated concentrations tested (1 μM, 10 μM and 20 μM) (Fig. 12).

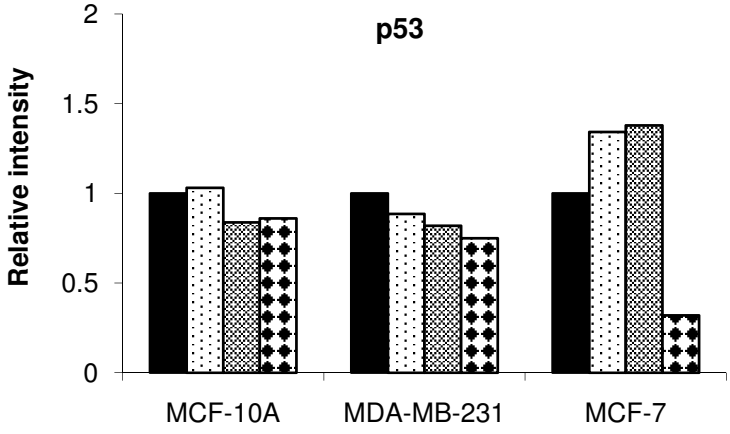
In contrast, MDA-MB-231 cells displayed dose-dependent down-regulation in p53, p21, survivin and cyclin D1 expression levels after 48 h TQ treatment (Fig. 12A-E). However, cyclin B expression levels showed slight down-regulation upon 48 h TQ treatment (Fig. 12F). This observation corroborates well with cell cycle profiles indicative of a G2/M arrest in TQ-treated MDA-MB-231 cells.

Similarly, in MCF-7 cells, down-regulation of survivin and cyclin D1 expression levels were evident upon 48 h TQ exposure (Fig. 12A, 12D, 12E). Expression levels of p53 and p21 were up-regulated at low doses of TQ (1 μM and 10 μM) but were down-regulated at a high TQ dose of 20 μM (Fig.12B, 12C). Only cyclin B protein expression levels persisted and remained fairly unchanged at the indicated TQ doses tested (Fig. 12F). Cell cycle profiles showed TQ-induced G1 arrest in MCF-7 cells, hence when treated at IC50 TQ doses concomitant down-regulation of cyclin D1 and up-regulation of p53 and p21 expressions were observed.

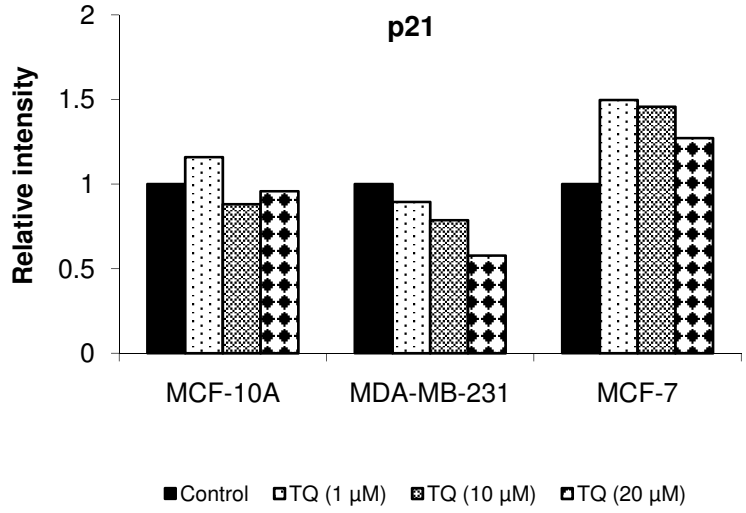
A.


MCF-10A
MDA-MB-231
MCF-7
TQ (μM)
0 1 10 20 0 1 10 20 0 1 10 20
p53
p21
Survivin
Cyclin D1
Cyclin B
Actin

B.


p53
Relative intensity
2
1.5
1
0.5
0
MCF-10A
MDA-MB-231
MCF-7

C.


p21
Relative intensity
2
1.5
1
0.5
0
MCF-10A
MDA-MB-231
MCF-7
Control
TQ (1 μM)
TQ (10 μM)
TQ (20 μM)

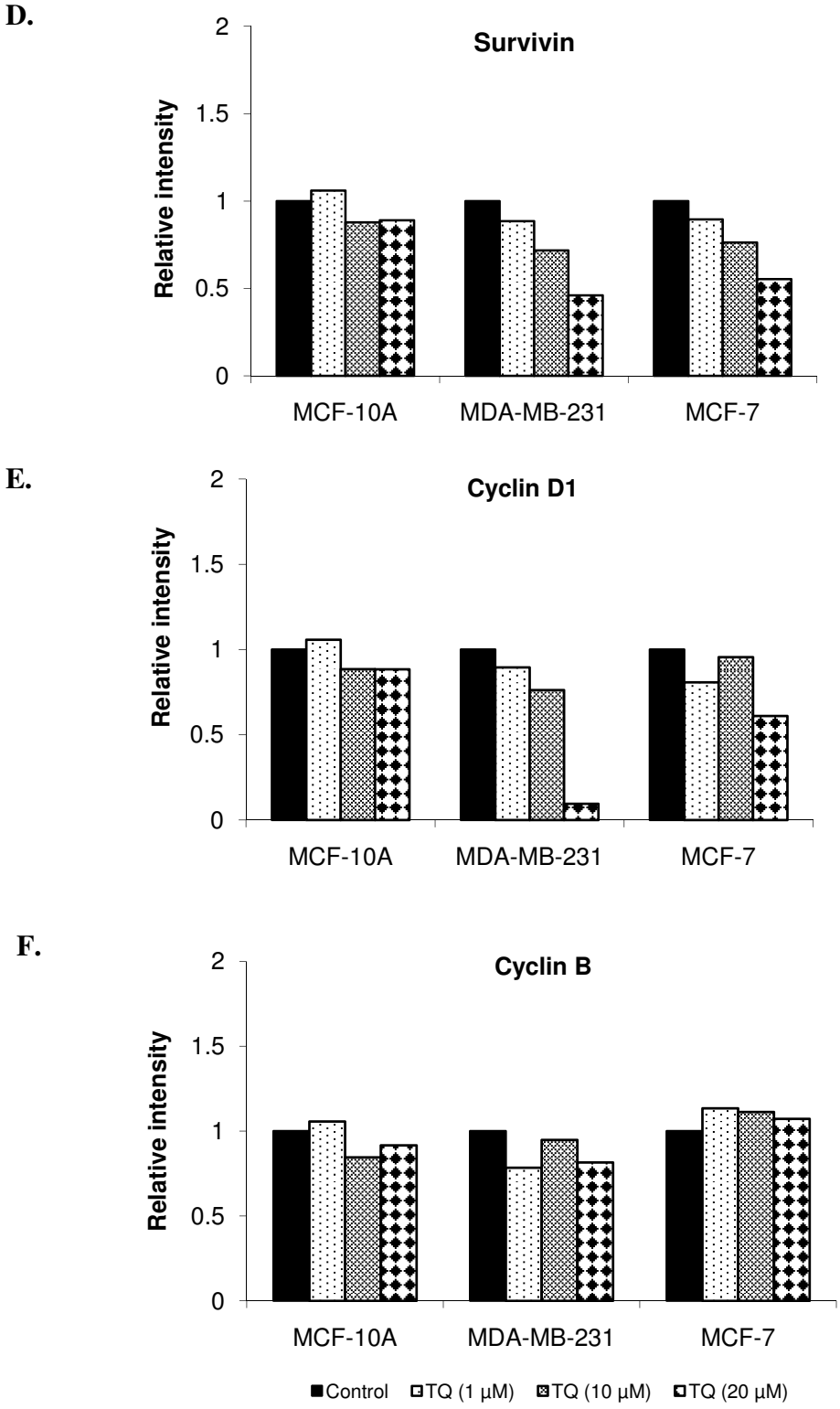


Figure 12. Changes in expression levels of cell cycle proteins in TQ-treated breast cancer cells. (A) Protein expression levels were assessed by western blot following 48 h TQ exposure at the indicated concentrations for MCF-10A, MDA-MB-231 and MCF-7 cells. (B-F) Relative intensity of western blots for (B) p53, (C) p21, (D) survivin, (E) cyclin D1 and (F) cyclin B in MCF-10A, MDA-MB-231 and MCF-7 cells following 48 h TQ exposure at the indicated concentrations for displayed blot. β-Actin serving as the internal control.

3.2 DNA damaging effects of TQ in normal and breast cancer cells

3.2.1 TQ induces significantly greater DNA damage in breast cancer cells

Growth arrest serves as a safeguard mechanism to ensure that cells have sufficient time for repairing the DNA damage incurred (Beckerman, 2009). In addition, our laboratory had previously shown TQ induced DNA damage in glioblastomas (Gurung et al., 2010b). Hence, to determine if the observed cell cycle profile changes in the cells or lack thereof were associated with DNA damage, the alkaline single cell gel electrophoresis (comet) assay was performed to evaluate DNA damage at 48 h following TQ exposure. In addition, to determine the repair capacity of cells following induction of DNA damage, if any, was evaluated after a 48 h recovery period. Nuclei with undamaged DNA appear round (Fig. 13A); nuclei with damaged DNA in the form of strand breaks resulted in DNA fragments which migrated faster during gel electrophoresis giving rise to a 'tail' (Fig. 13B). Mean comet tail moment, a function of tail length and fraction of DNA in 'tail', was used as a measure of DNA damage.

In line with the cancer cell specific anti-proliferative effects as mentioned earlier, TQ induced elevated levels of DNA damage in breast cancer cells as compared to normal cells. Upon 48 h TQ exposure, MDA-MB-231 and MCF-7 cells displayed significant mean tail moment increases ($p<0.001$) of 4.93 µm and 3.13 µm respectively in comparison to controls (Fig. 13C, 13D). Also, it was noted that MCF-10A cells displayed significantly lower ($p<0.01$) tail moments at 48 h TQ exposure compared to the breast cancer cells. Although the damage was significant at a lower TQ dose of 1 µM, no significant changes in cell cycle profiles were observed.

The decrease in tail moment back towards the baseline is indicative of damage repair and also if the DNA damage induced was mainly due to the effects of the drug. Following 48 h recovery period, reductions in tail moments for all cells, in particular the breast cancer cells, were observed (Fig 13C, 13D). However, the reduction in tail moments for recovered MDA-MB-231 and MCF-7 cells did not achieve levels prior to TQ treatment. MCF-10A cells displayed no differences in post-recovery tail moments between treated (1 μM and 10 μM) and untreated cells.

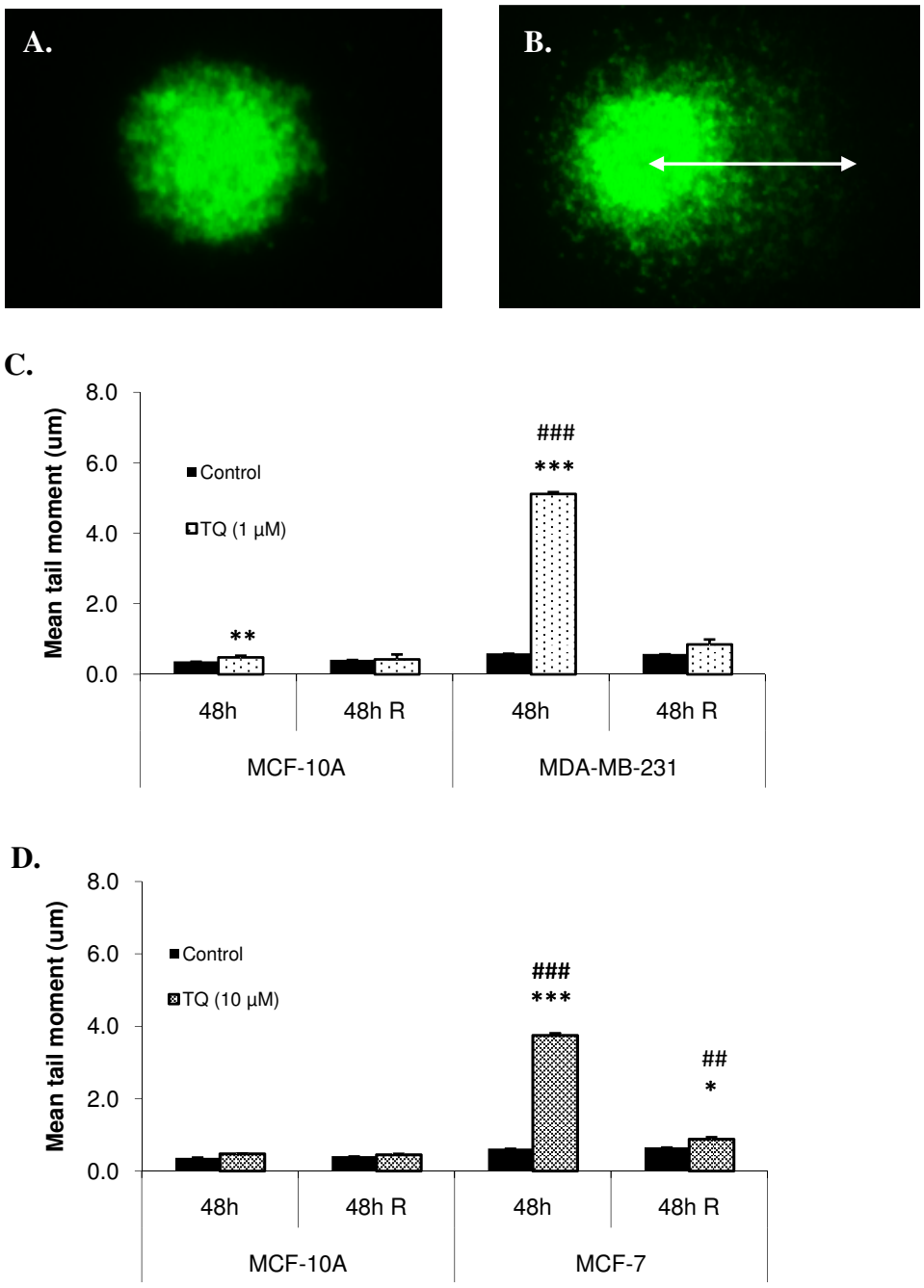


Figure 13. TQ induced greater amount of DNA damage in breast cancer cells. Representative images of (A) normal and (B) damaged (presence of tail as depicted by an arrow) nucleus following alkaline single gel electrophoresis (comet) analysis in breast cancer cell, MDA-MB-231. (C-D) Extent of DNA damage measured in terms of mean tail moment (product of tail length and fraction of DNA in tail) after 48 h exposure to TQ (1 µM or 10 µM), which was followed by a 48 h recovery period for (C) MCF-10A and MDA-MB-231 cells and (D) MCF-10A and MCF-7 cells. Data represent the mean tail moment (µm) ± SEM of three independent experiments. * indicates the change in DNA damage with respect to control is statistically significant, i.e. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. # indicates the change in DNA damage with respect to control between cell lines for same treatment is statistically significant, i.e. $p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$.

3.2.2 TQ induces DNA double strand breaks with subsequent inefficient/delayed repair in breast cancer cells

Various forms of DNA damage exist and may manifest as single or DSBs, simple base deletions, insertions or point mutations, instability of hydrogen bonds between complementary strands and adducts between adjacent bases or with other molecules (Hoeijmakers, 2001). For the focus of this study, it is of interest to examine if TQ specifically induces DNA DSBs by performing the γ-H2AX immunofluorescence staining assay (Kinner et al., 2008). Representative images show the nuclei (stained blue with DAPI) of an untreated (Fig. 14A) and TQ-treated (Fig. 14B) breast cancer cell, MCF-7. The amount of DNA DSBs was quantified by enumerating the number of positive γ-H2AX foci (stained green) formed.

Significantly higher average number of foci positive for γ-H2AX were observed in breast cancer cells, MDA-MB-231 ($p<0.05$) and MCF-7 ($p<0.01$), upon 24 h and 48 h TQ exposure relative to their respective controls (Fig. 14C, 14D). Interestingly, the average number of positive γ-H2AX foci formed from 24 h to 48 h TQ exposure remained at fairly similar levels for both cells. No significant differences in the average number of positive γ-H2AX foci formed were observed in MCF-10A cells after 24 h and 48 h TQ exposure.

In the breast cancer cells, with subsequent 24 h and 48 h recovery periods, the average number of positive γ-H2AX foci formed was significantly sustained at levels after TQ treatment (Fig. 14C, 14D). This observation could be an indication of a delayed or inefficient repair capacity of the TQ-treated breast cancer cells. However, MCF-10A cells displayed no differences in post-recovery average number of positive γ-H2AX foci formed between treated (1 μM and 10 μM) and untreated cells.

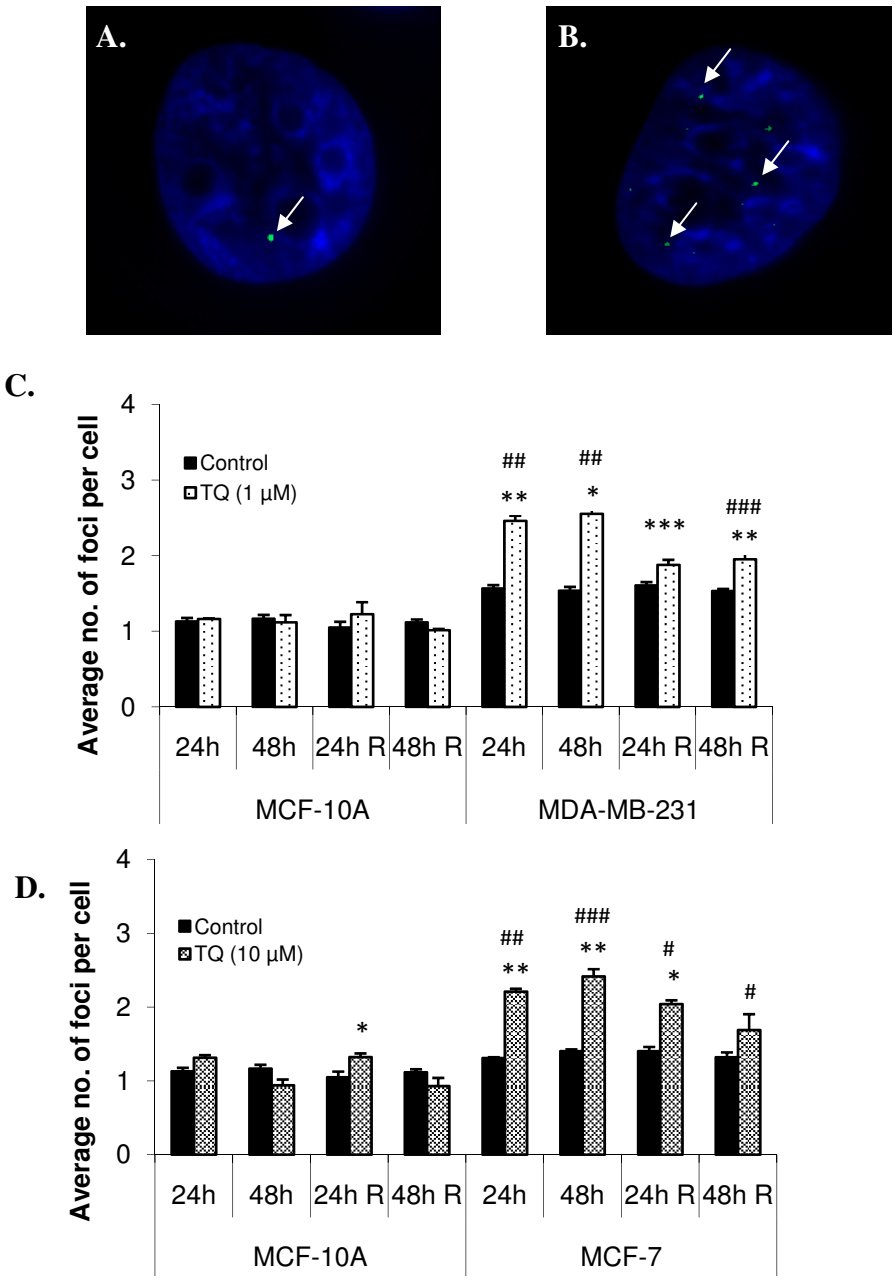


Figure 14. TQ induced significant DNA double strand breaks with subsequent inefficient/delayed repair in breast cancer cells. Representative images of γ-H2AX foci (green) and cell nuclei (blue) in (A) untreated and (B) TQ-treated breast cancer cell, MCF-7, assessed by the γ-H2AX immunofluorescence assay. Average number of γ-H2AX foci per cell following exposure with TQ (1 µm or 10 µM) for 24 h and 48 h, which was followed by 24 h and 48 h recovery periods in (C) MCF-10A and MDA-MB-231 cells and (D) MCF-10A and MCF-7 cells. Data represent mean ± SEM of three independent experiments. * indicates the increase in the average number of foci per cell with respect to control is statistically significant, i.e. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. # indicates the change in DNA damage with respect to control between cell lines for same treatment is statistically significant, i.e. $p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$

3.2.3 Increased expression levels of p-DNA-PKcs and PARP-1 in TQ-treated breast cancer cells

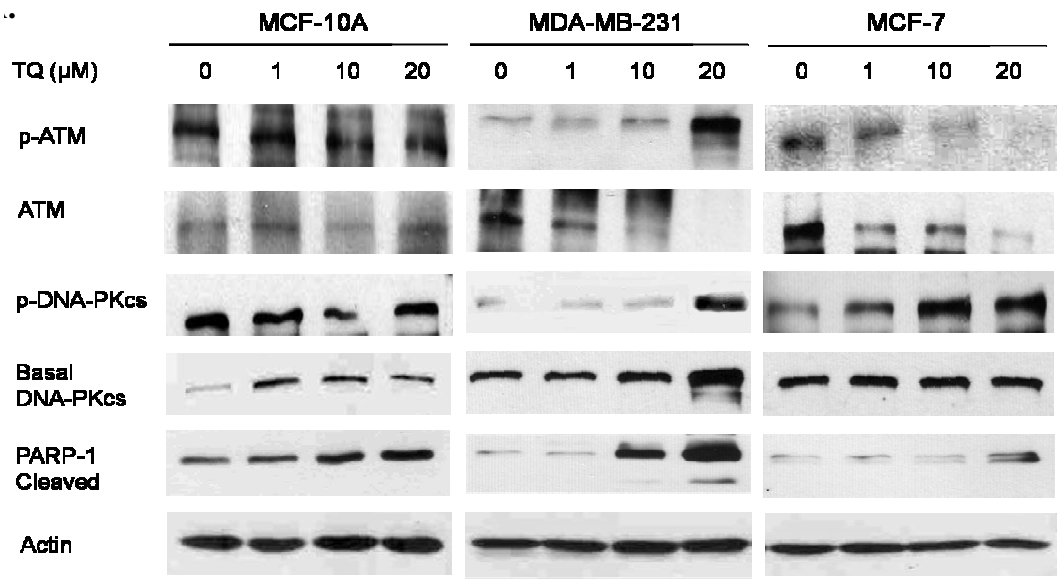
It is clearly evident that TQ induced DSBs specifically in breast cancer cells as compared to normal cells (Fig 14C, 14D) and the immediate response to DNA insult involves activation of DNA repair pathways. Consistent with these findings, activation of DNA-repair pathway was observed only in breast cancer cells.

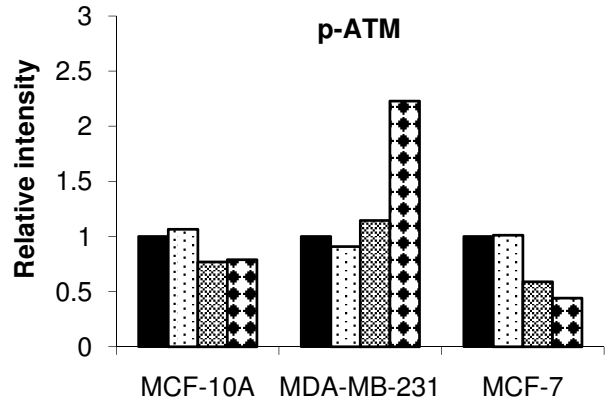
After 48 h TQ exposure, ATM phosphorylation and basal ATM expression levels remained fairly unchanged in MCF-10A cells (Fig. 15A, 15B, 15C). DNA-PKcs phosphorlyation expression levels persisted unchanged at the indicated TQ doses (Fig. 15D), while a slight up-regulation in basal DNA-PKcs expression could be observed at all doses of TQ (Fig. 15E). No differences in PARP-1 expression levels could be observed at the indicated concentration range of TQ tested (Fig. 15F)

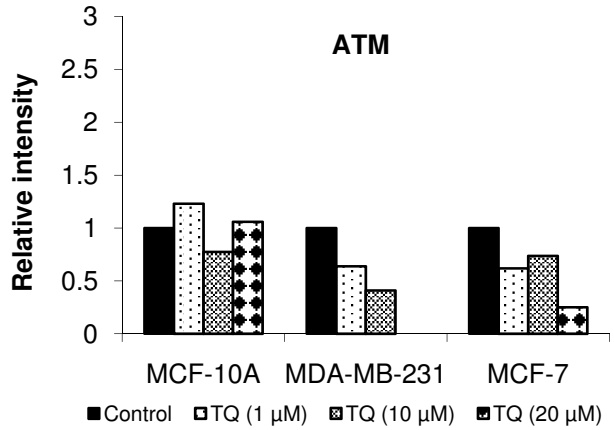
In contrast, activation of ATM was observed in MDA-MB-231 cells at a high TQ dose of 20 μM (Fig. 15B). This was accompanied by the concomitant dose-dependent decrease in basal ATM expression (Fig. 15C). Slight up-regulation of both phosphorylated (Fig. 15D) and basal DNA-PKcs expression levels (Fig. 15E) could be observed at a low TQ doses (1 μM and 10 μM), with significant up-regulation at a high TQ dose (20 μM). Dose-dependent increase in PARP-1 expression levels was evident with the concomitant up-regulation of the cleaved PARP-1 expression level (Fig. 15A, 15F).

On the other hand, MCF-7 cells displayed concomitant dose-dependent decrease in phosphorylated and basal ATM expression levels at 48 h TQ exposure at the indicated concentration range (Fig.15B, 15C). DNA-PKcs phosphorylation increased in a dose-dependent manner (Fig. 15D), while basal DNA-PKcs expression

levels persisted and remained fairly unchanged at the indicated concentration range of TQ tested (Fig. 15E). Increased in PARP-1 expression level could be observed only at a high TQ dose (20 µM) (Fig. 15F).







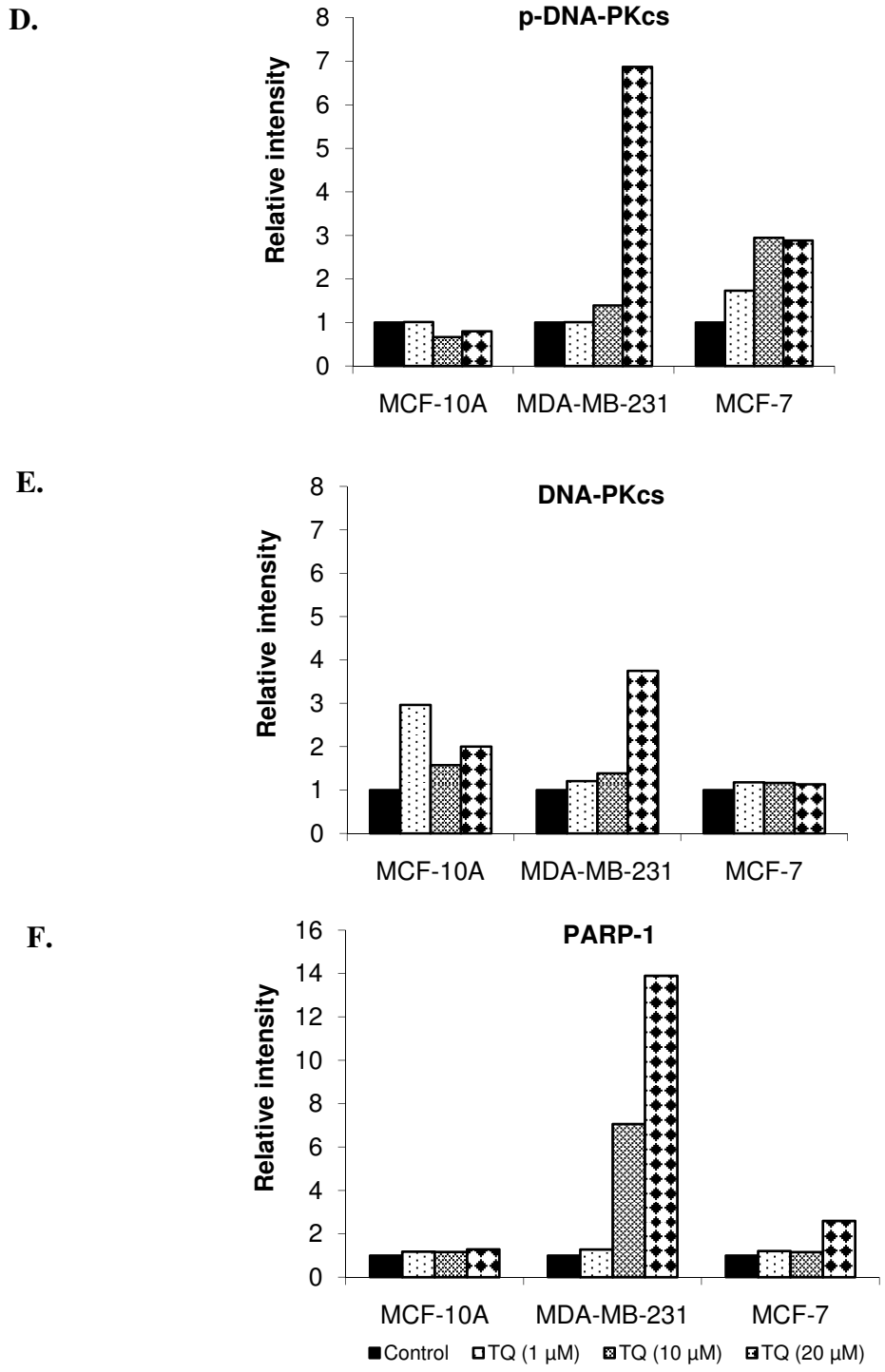


Figure 15. Activation of DNA-PKcs and PARP-1 in TQ-treated breast cancer cells. (A) DNA damage response protein expression levels were assessed by western blot following 48 h TQ exposure at the indicated concentrations for MCF-10A, MDA-MB-231 and MCF-7 cells. (B-F) Relative intensity of western blots for (B) p-ATM, (C) ATM, (D) p-DNA-PKcs, (E) DNA-PKcs and (F) PARP-1 in MCF-10A, MDA-MB-231 and MCF-7 cells following 48 h TQ exposure at the indicated concentrations for displayed blot. β-Actin serving as the internal control.

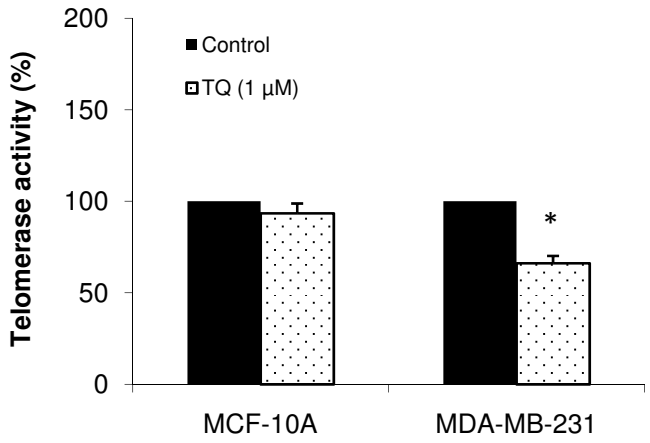
3.3 Immediate effects of TQ on telomerase expression and activity

3.3.1 TQ reduces telomerase activity only in MDA-MB-231 cells

Our previous study showed the reduction in telomerase activity and hTERT expression levels in hTERT-BJ fibroblasts upon exposure to TQ (Gurung et al., 2010b). Hence, it would be of interest to investigate if similar effects were also observed in TQ-treated breast cancer cells. Telomerase activity in the cells after 48 h TQ exposure was assessed by TRAP assay. Quantification of PCR products is deduced by calculating the ratio between the net increase of fluorescence emission (ΔFL), which is derived from the telomerase-dependent synthesis of the PCR product, and the net increase of sulforhodamine emission (ΔR), which is derived from the product synthesized on the internal control. The ratio $\frac{\Delta FL}{\Delta R}$ is then calculated as a percentage of the respective control.

MDA-MB-231 cells displayed a significant ($p<0.05$) reduction (34 %) in telomerase activity at 48 h TQ (1 µM) (Fig. 16A), while MCF-7 cells exhibited insignificant increased percentage in telomerase activity at 48 h TQ (10 µM) (Fig. 15B). No differences in telomerase activity were recorded in MCF-10A cells after 48 h TQ (1 µM and 10 µM) exposure (Fig. 16A, 16B).

A.



B.

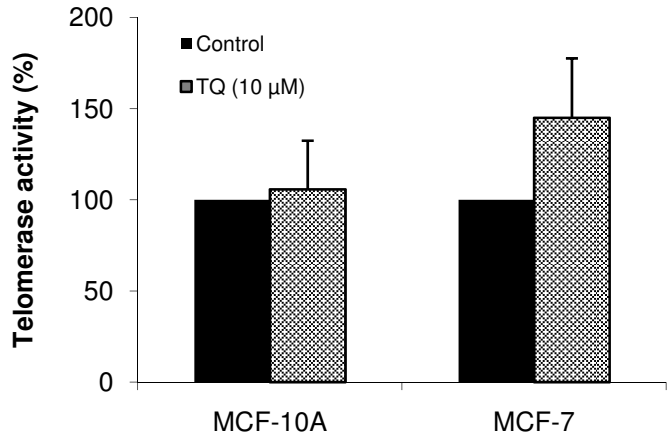


Figure 16. Effects of TQ on telomerase activity. Graphs shows telomerase activity (measured in terms of $\frac{\Delta FL}{\Delta R}$ as a % of control) as assessed by TRAP assay. Telomerase activity in (A) MCF-10A and MDA-MB-231 cells and (B) MCF-10A and MCF-7 cells subjected to TQ (1 µm or 10 µM) for 48 h. Data represent the mean ± SEM (% of control) of three independent experiments. * indicates change in telomerase activity with respect to control is statistically significant, i.e. p-value < 0.05.

3.3.2 TQ alters c-myc regulatory pathway of hTERT expression in breast cancer cells and affects TRF2 expression levels

The c-myc proto-oncogene is commonly known to be involved in cell proliferation and immortalisation when expressed constitutively in primary fibroblasts (Askew et al., 1991; Kohl and Ruley, 1987). Consequently c-myc has been touted as a key molecular switch positively regulating telomerase activity and expression of TERT, where c-myc binding sites can be found at the TERT promoter region (Greenberg et al., 1999; Schneider-Stock et al., 2003; Wu et al., 1999). TERT is the catalytic component of telomerase, which regulates telomere length in cells (Feng et al., 1995; Nakamura et al., 1997). Hence, western blot was employed to determine if any changes could be detected in the c-myc regulatory pathway affecting hTERT protein expression levels. To further examine if the observed effect of TQ on telomerase activity was accompanied by additional changes in telomere structure, telomere-associated protein TRF2, which have been reported to play a role in telomere length maintenance was also investigated (Smogorzewska et al., 2000).

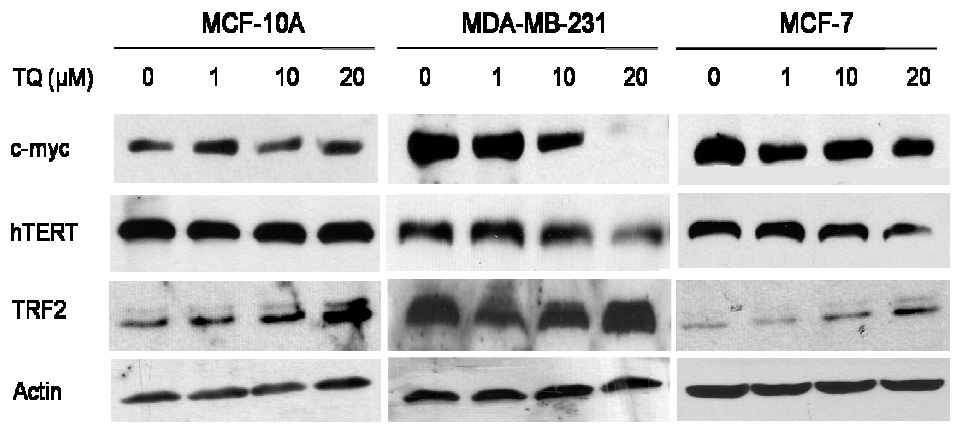
A dose-dependent down-regulation in c-myc and hTERT expression was observed in 48 h TQ-treated MDA-MB-231 cells (Fig. 17A, 17B, 17C). On the other hand, TRF2 expression decreased upon exposure to a low TQ dose (1 μM) and gradually returned back to basal levels at higher TQ doses (10 and 20 μM) (Fig. 17A, 17D).

MCF-7 cells exhibited a down-regulation in c-myc upon TQ exposure (1 – 20 μM) (Fig. 17B), while hTERT expression persisted at lower dose of TQ (1 μM) with a prominent down-regulation occurring at a higher dose of TQ (20 μM) (Fig. 17C). A similar trend in TRF2 expression was observed in TQ-treated MCF-7 cells as in

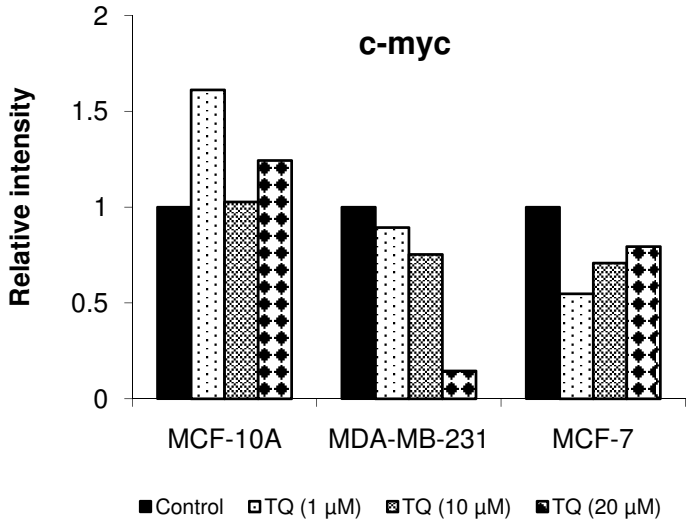
MDA-MB-231 cells, where TRF2 expression was reduced at a low TQ dose (1 µM), which was subsequently increased at higher TQ doses (10 and 20 µM) (Fig. 17D).

Within the indicated range of TQ concentrations tested (0 – 20 µM), c-myc and hTERT expression levels remained fairly unaffected in MCF-10A cells (Fig. 17B, 7C). However, a dose-dependent increase in TRF2 expression was recorded in MCF-10A cells (Fig. 17D).

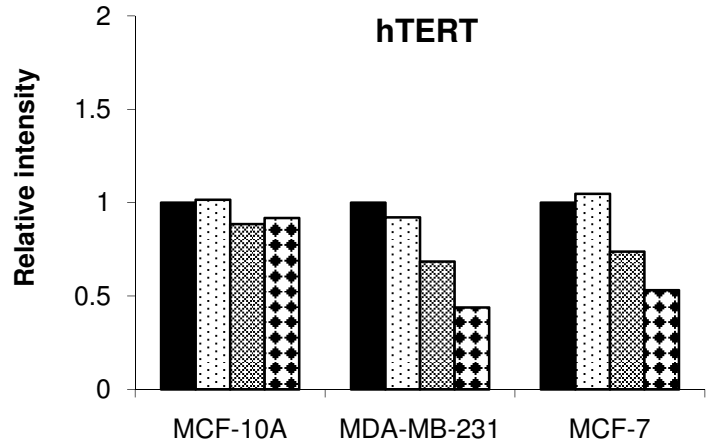
A.



B.



C.



D.

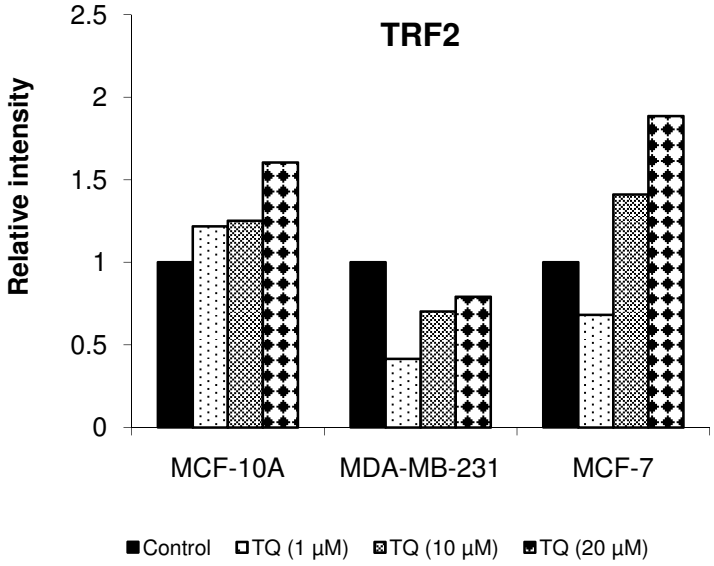


Figure 17. Alteration of c-myc. hTERT and TRF2 expression levels upon TQ treatment. (A) Expression levels of c-myc, hTERT and TRF2 assessed by western blot in MCF-10A, MDA-MB-231 and MCF-7 cells following 48 h TQ exposure at the indicated concentrations. (B-D) Relative intensity of western blots for (B) c-myc, (C) hTERT and (D) TRF2 in MCF-10A, MDA-MB-231 and MCF-7 cells following 48 h TQ exposure at the indicated concentrations for displayed blot. β-Actin serving as the internal control.

3.4 Long-term effects of TQ on cell proliferation and telomere-telomerase homeostasis

3.4.1 Prolonged TQ exposure reduces proliferative capacity of breast cancer cells

Chemotherapy usually involves prolonged low-dose treatment for cancer patients (Peres, 2000). Two *in vivo* studies have illustrated that continuous low-dose chemotherapeutic drugs (e.g. cyclophosphamide and methotrexate) eradicated tumours in mice (Bocci et al., 2005; Hanahan et al., 2000). Moreover, the extrapolation to clinical setting with a continuous low-dose regimen in breast cancer patients exhibited shrunken or stabilized tumour size with reduced side effects. Therefore, in order to understand the effects of long term exposure to TQ in breast cancer cells, population doubling (PD) study using the trypan blue-exclusion assay was performed in these cells.

With prolonged exposure for up to 8 weeks to a non-toxic TQ dose based on cell viability assay (Fig. 10A), a gradual decline in proliferative capacity of the breast cancer cells could be observed (Fig. 18). The decline in cumulative PD for MDA-MB-231 cells was greater than for MCF-7 cells. This was expected due to a greater sensitivity of MDA-MB-231 cells to TQ as established earlier. The gradual decline in PD confirmed that the prolonged effects of TQ were less likely attributed due to cytotoxicity of TQ. Consequently, this observation further prompted our investigation into a possible telomere length maintenance and telomerase-inhibitory mechanism upon chronic low dose TQ exposure in MDA-MB-231 and MCF-7 cells.

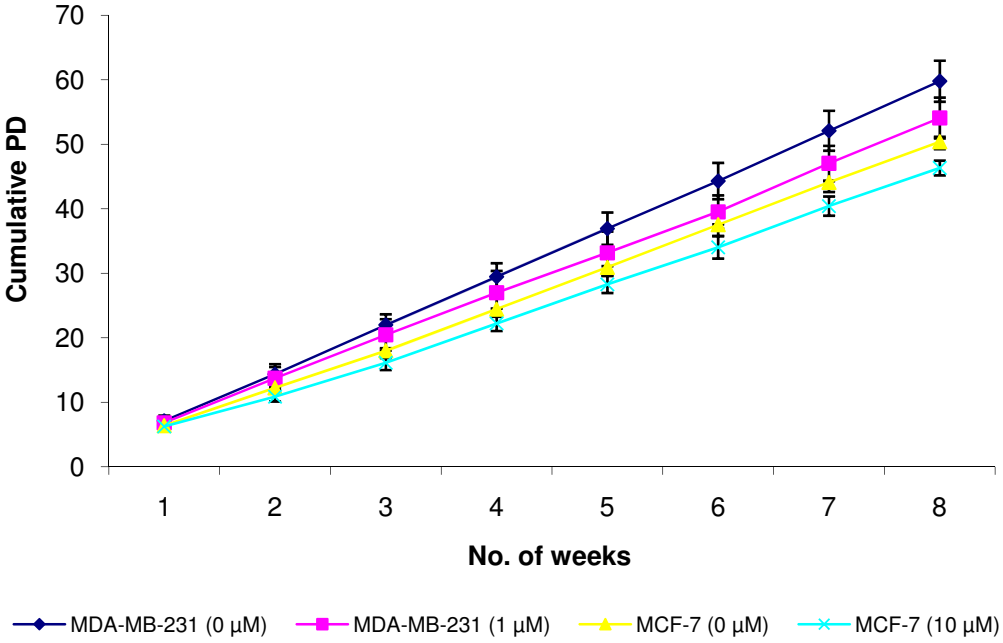


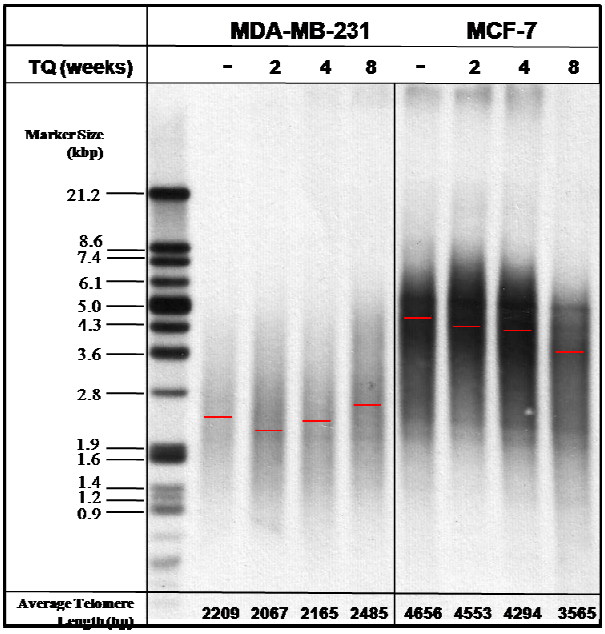
Figure 18. Prolonged exposure to TQ reduced proliferative capacity of breast cancer cells. Cumulative population doubling (PD) curve assessed by trypan blue-exclusion PD study. MDA-MB-231 and MCF-7 cells were treated with 1 µM and 10 µM TQ respectively for the indicated weeks shown. Data represent the mean ± SEM of two independent experiments.

3.4.2 Telomere shortening in breast cancer cells at 2 weeks of TQ treatment

The effects of TQ, if any, on telomere length were monitored by performing the TRF assay. At 2 weeks of TQ treatment, MDA-MB-231 cells displayed an average telomere shortening of 142 bp relative to untreated cells (Fig. 19B). However upon continued TQ treatment for up to 8 weeks, average telomere length increased by 276 bp relative to untreated cells. On the other hand, progressive telomere shortening was displayed in MCF-7 cells during the course of cell proliferation for up to 8 weeks (Fig. 19A). The greatest reduction in average telomere length was recorded at 8 weeks of TQ exposure in MCF-7 cells and is equivalent to approximately 1.09 kbp (Fig. 19B).

Telomere shortening is a factor of cell division and hence, population doublings derived from the PD study was taken into account. MDA-MB-231 and MCF-7 cells showed comparable telomere attrition rates at 2 weeks of TQ treatment (Fig. 19C). Prolonged TQ treatment for 8 weeks gradually decreased attrition rates in MDA-MB-231 cells, while exponentially increased attrition rates were recorded in MCF-7 cells.

A.



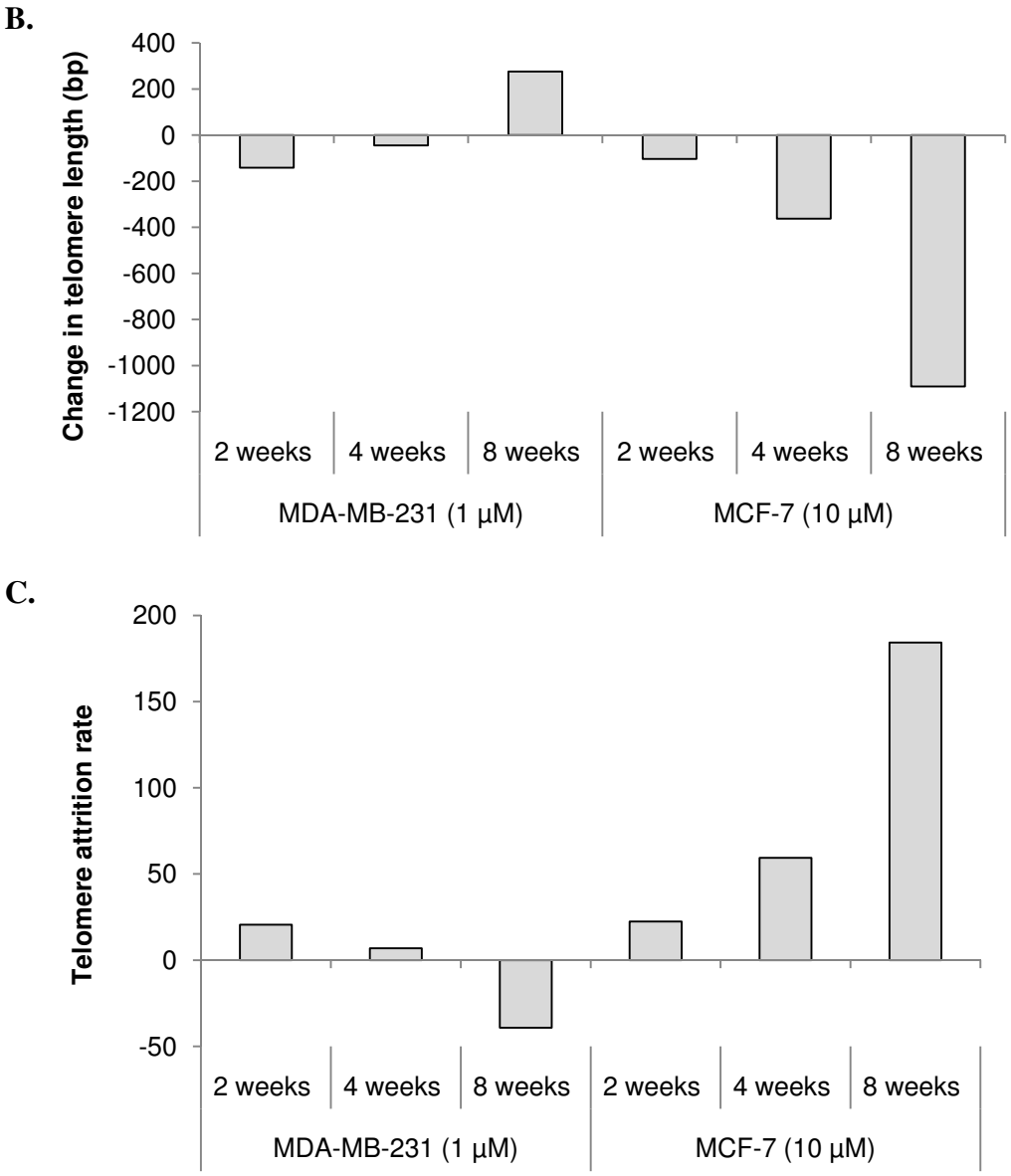


Figure 19. TQ induced telomere attrition in breast cancer cells upon prolonged exposure for up to 8 weeks. (A) TRF analysis for average telomeric length in a cell population. Telomeric restriction fragments detected in Southern blot for breast cancer cells, MDA-MB-231 and MCF-7, treated without (0 µM) or with TQ (1 µm or 10 µM) for the indicated number of weeks. Data represent the mean change (% of control). (B) Corresponding histograms displaying the total change in telomere length for MDA-MB-231 and MCF-7 cells for displayed blot. (C) Corresponding histograms displaying the telomere attrition rate for MDA-MB-231 and MCF-7 cells for displayed blot. Telomere attrition rate was derived by dividing the TRF length decrease or increase by the number of PD.

3.4.3 Prolonged exposure to TQ alters hTERT and TRF2 expression levels in breast cancer cells

To investigate the possible mechanism (e.g. telomerase-dependent mechanism or telomerase-independent mechanism via regulation of proteins involved in shelterin complex) involved in inducing telomere shortening following TQ treatment, we checked protein expression levels of hTERT and TRF2. As aforementioned, telomere length maintenance may be altered by telomerase-dependent (e.g. hTERT expression) or telomerase-independent (e.g. disruption in shelterin complex) mechanisms.

In MDA-MB-231 cells, down-regulation of hTERT expression relative to control was evident at 2 weeks after TQ treatment and levels subsequently returned to basal control levels at 4 and 8 weeks after TQ treatment (Fig.20A, 20B). However, MCF-7 cells displayed a down-regulation of hTERT expression level only at 4 and 8 weeks TQ treatment.

For both breast cancer cells, up-regulation of TRF2 expression was observed at 2 weeks after TQ treatment with levels subsequently reduced to basal control levels at 4 and 8 weeks after TQ treatment (Fig. 20C).

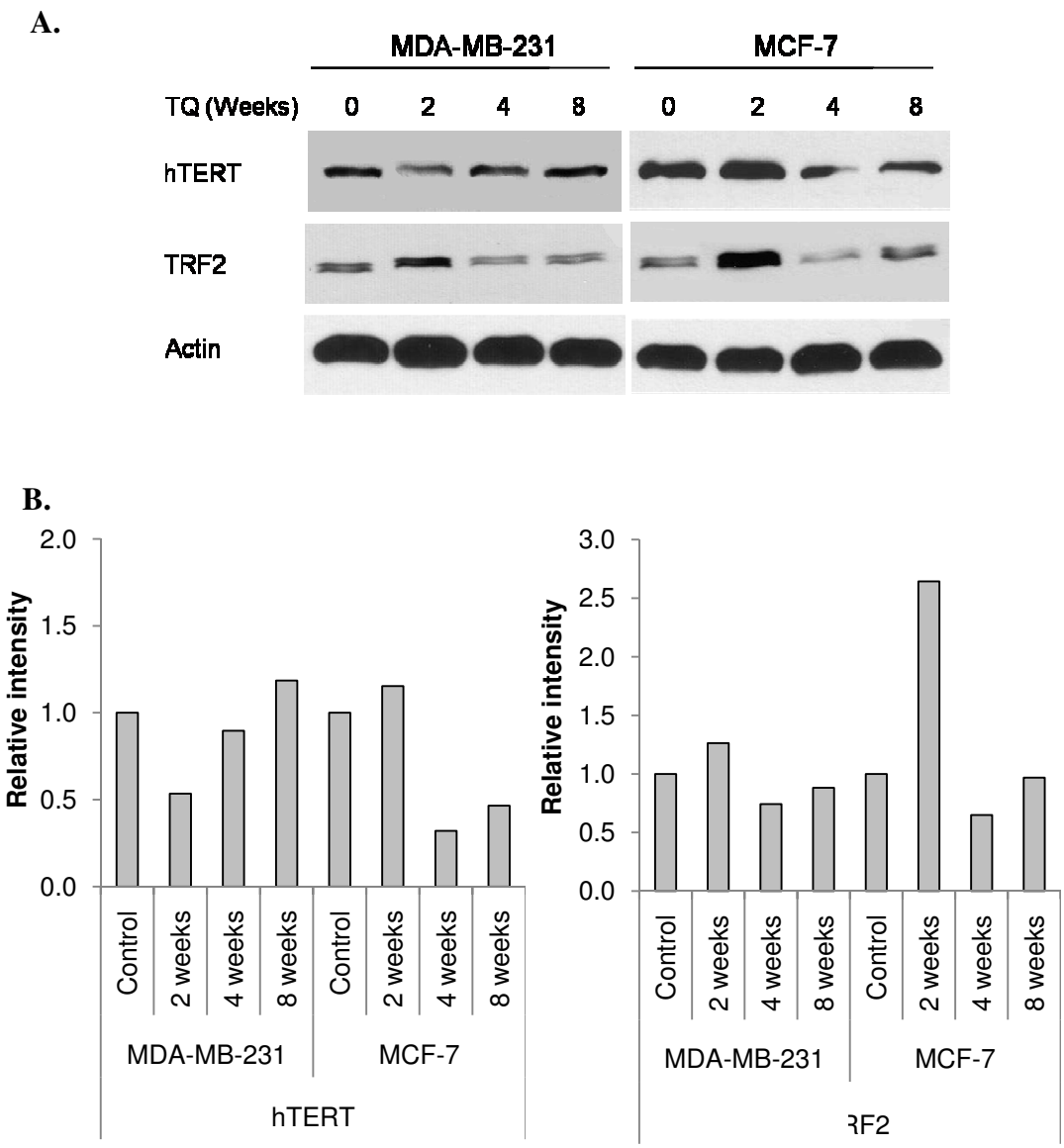


Figure 20. Continued exposure to TQ for 8 weeks altered hTERT and TRF2 expression levels in breast cancer cells. (A) Expression levels of hTERT and TRF2 assessed by western blot in MDA-MB-231 and MCF-7 cells following TQ treatment (1 µm or 10 µM) for the indicated number of weeks. (B-C) Relative intensity of western blots for (B) hTERT and (C) TRF2 in MDA-MB-231 and MCF-7 cells following TQ treatment (1 µm or 10 µM) for the indicated number of weeks for the displayed blot. β-Actin serving as the internal control.

3.5 Possible relationship between DNA damage and telomeres

3.5.1 TQ induces DNA double strand breaks at telomeric regions in breast cancer cells

Results obtained so far have shown the induction of DNA DSBs and the perturbation of telomere maintenance in TQ-treated breast cancer cells. Hence, the TIF assay was performed to investigate if initial DNA damage activation occurred at telomeric sites where double immunostaining co-localisation of γ-H2AX in conjunction with telomere specific probes represents cells with positive TIFs (Fig. 21A) (Brugat et al., 2010; Takai et al., 2003).

Significantly higher proportion of cells positive for TIFs (≥2) were observed in breast cancer cells, MDA-MB-231 ($p<0.05$) and MCF-7 ($p<0.05$), upon 48 h TQ exposure relative to their respective controls (Fig. 21B). Interestingly, the highest proportion of cells positive for TIFs (≥2) formed for both MDA-MB-231 and MCF-7 cells were treatment at their IC50 doses of 1 µM and 10 µM, respectively. Moreover, breast cancer cells which were treated at doses above their IC50 values showed a subsequent reduction in proportion of cells positive for TIFs (≥2). However, MCF-10A cells displayed minimal differences in proportion of cells positive for TIFs (≥2) between the treated and untreated cells.

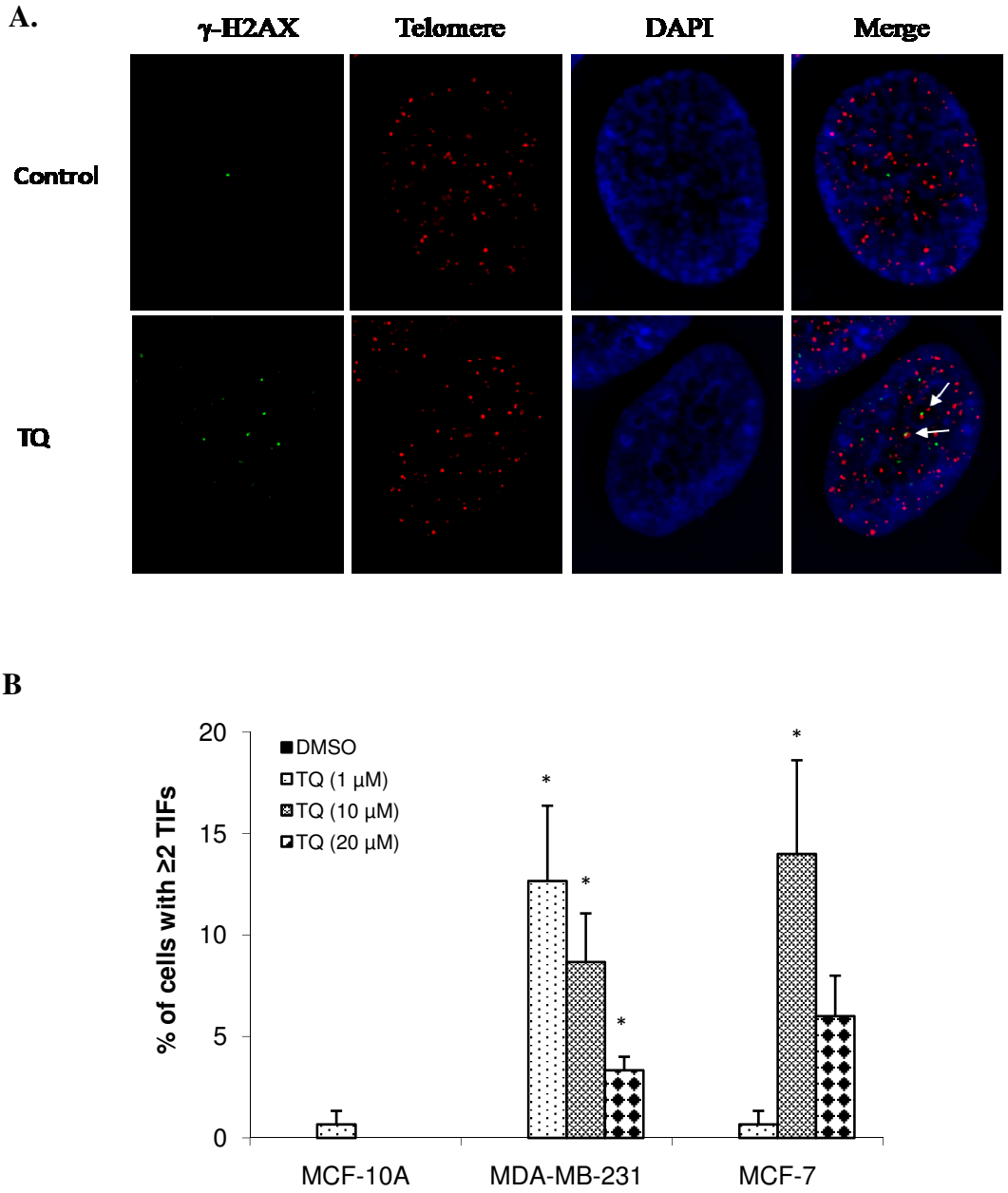


Figure 21. TQ induced co-localisation of γ-H2AX with telomeres in breast cancer cells. TIF analysis following 48 h TQ exposure in MCF-10A, MDA-MB-231 and MCF-7 cells. (A) Representative images of γ-H2AX foci (green), telomeres (red) and cell nuclei (blue) in untreated and TQ-treated breast cancer cell, MCF-7. (B) % of cells positive for TIFs (≥2) following exposure with 48 h TQ at the indicated concentrations in MCF-10A, MDA-MB-231 and MCF-7 cells. Data represent mean ± SEM of three independent experiments. * indicates the increase in the average number of foci per cell with respect to control is statistically significant, i.e. $p < 0.05$, $^{**}p < 0.01$.

3.6 Gene expression profiles of normal and breast cancer cells

3.6.1 Differential gene expression profiles in breast cancer cells

In order to further understand the differential responses of the two breast cancer cell lines, MDA-MB-231 and MCF-7, towards the effects of TQ, gene expression profiling of the cell lines for inherent similarities and differences was performed. Following microarray analysis, a total of 2,518 genes (approximately 7 % of genes analysed) were differentially expressed in MCF-7 as compared to MDA-MB-231 cells based on the set of criteria assigned ($p<0.05$; fold difference of 2) (Fig. 22A). This suggests that many genetic differences exist amongst the two different cell types at basal levels and could likely contribute to the differential responses and sensitivities towards the effects of TQ.

In addition, we investigated prominent gene expression patterns and correlations between cell types and treatment through the principle component analysis (PCA) graph (Fig. 22B). Data shows mutually exclusivity between cell types, which are carried out in duplicates, and is indicative of minimal experimental errors during the microarray process.

To elucidate transcriptomic differences between control and TQ-treated cells, we determined the changes in gene expression levels and selected genes involved in cell cycle, DNA damage response, telomere-telomerase regulation and other signalling pathways. Different genes were observed to be deregulated for the different cell types (Fig. 22C-F).

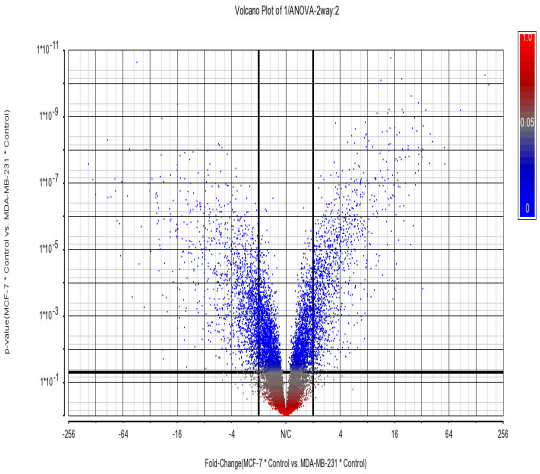
Exposure to 48 h TQ in MDA-MB-231 cells resulted in the down-regulation of cell cycle gene *ZFHX3*, which encodes for a transcription factor involved in transactivating p21 (Fig. 22C). This observation corroborated with the down-regulated

p21 protein expression obtained from western blot (Fig. 20C). Gene expression of DNA damage response gene *BRAP*, which encodes for a BRCA1-associated (breast cancer type 1 susceptibility) protein involved in the repair of DNA DSBs, also displayed a down-regulation. Interestingly, only two differentially regulated genes involved in telomere and telomerase regulation, *SIRT2* and *SERF1A*, were detected. Both were down-regulated in TQ-treated MDA-MB-231 cells. *SIRT2* gene encodes for Sirtuin protein involved in chromatin silencing at telomeres, while *SERF1A* encodes for a protein located at telomeric regions. In addition, the expression of gene encoding for interlukin-1 receptor-associated kinase 4 (IRAK4) proteins, which activates nuclear factor kappa-B (NF-κB) in mammals, was down-regulated.

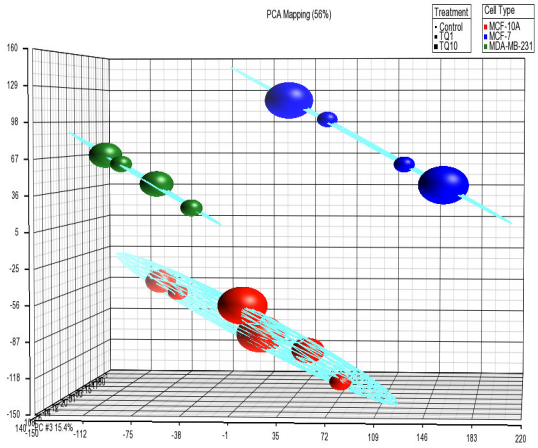
In TQ-treated MCF-7 cells, a down-regulation in a number of cell cycle genes, such as *E2F3* and *CDC6*, involved in G1 to S transition, were observed (Fig. 22D). Hence accounting for the G1 phase cell cycle arrest observed previously (Fig. 11B, 11E). In addition, a greater number of DNA damage response genes, such as *GADD45G*, and *FANCC*, were shown to be down-regulated upon TQ exposure for 48 h. The *FANCC* gene encodes for the fanconi anaemia group C protein that delays apoptosis and promotes homologous recombination of DNA DSBs. Moreover, there was a down-regulation in *MYCBP* and *PKCZ* genes, which are indirect positive regulators of hTERT transcription.

Only approximately 0.01% of genes for the different functional groupings were deregulated in MCF-10A cells exposed to either TQ doses (1 μM and 10 μM) (Fig. 22E, 22F). Some of which included the up-regulation of *BIRC2* (anti-apoptotic) and *PRKCB* (positive regulator of hTERT transcription) genes.

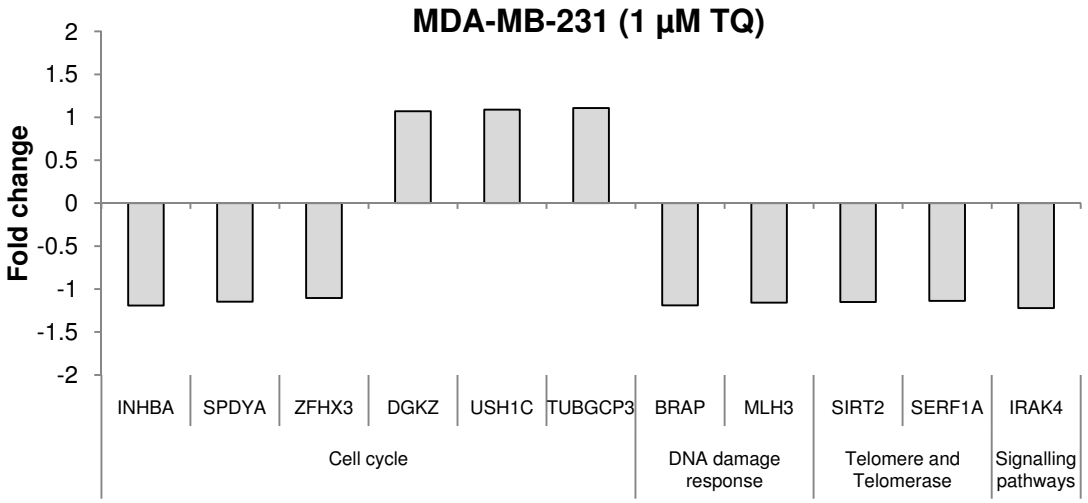
A.


Volcano Plot of 1/ANOVA-2way 2
p-value(MCF-7 * Control vs. MDA-MB-231 * Control)
Fold-Change(MCF-7 * Control vs. MDA-MB-231 * Control)

B.


PCA Mapping (56%)
Treatment
Control
TQ1
TQ10
Cell Type
MCF-10A
MCF-7
MDA-MB-231

C.


MDA-MB-231 (1 µM TQ)
Fold change
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
INHBA
SPDYA
ZFHX3
DGKZ
USH1C
TUBGCP3
BRAP
MLH3
SIRT2
SERF1A
IRAK4
Cell cycle
DNA damage response
Telomere and Telomerase
Signalling pathways

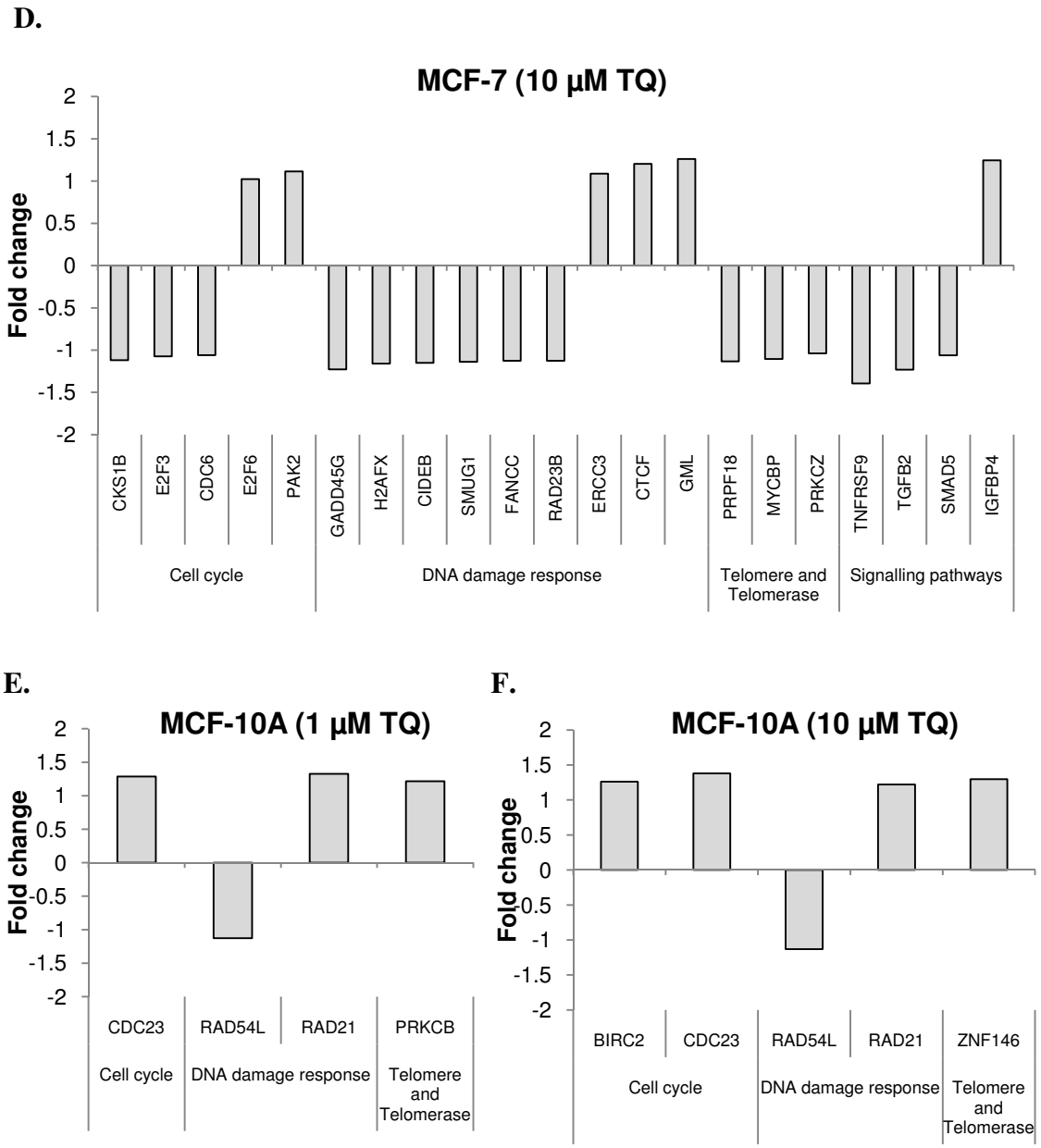


Figure 22. Differential gene expression profiles in MDA-MB-231 and MCF-7 cells. (A) Volcano plot indicates differentially regulated genes. Clusters in blue indicate genes differentially regulated ($p<0.05$; 1 way ANOVA). Black bold lines (X axis) indicate fold change cut off of 2. (B) PCA analysis of breast cancer cell lines, MDA-MB-231 and MCF-7, and immortalised mammary epithelial cell line, MCF-10A, following 48 h TQ treatment. 56 % of the data are represented in the scatterplot. PCA separates data sets according to distance from a base vector (PC #1-3). (C-F) Differential gene expression in breast cancer cells following 48 h TQ treatment. Functional groupings of selected differentially expressed genes in (C) MDA-MB-231, (D) MCF-7 and (E-F) MCF-10A cells treated with either 1 µM or 10 µM TQ. Data expressed as fold change ($p<0.05$) in TQ-treated cells as compared to their respective controls.

CHAPTER 4

4. Discussion

A major concern of cancer chemotherapy is the non-specific targeting of both normal and cancerous cells by radiotherapy as well as cytotoxic therapeutic drugs. This highlights the importance of discovering potential bioactive constituents in natural plant compounds, which have been reported to target tumour cells more efficiently and selectively with minimal toxic effects on normal cells (Vuorelaa et al., 2004).

In this study, investigations into how TQ affects cell viability, migration and cell cycle checkpoints demonstrated the anti-proliferative effects of TQ, which were selective towards breast cancer cells. More importantly, minimal effects on the growth of normal epithelial cells, MCF-10A, were observed following exposure to TQ. This is consistent with other reports indicating TQ's anti-neoplastic effects without affecting corresponding normal cells (Gali-Muhtasib et al., 2004a; Shoieb et al., 2003). MDA-MB-231 cells showed significant reduction in cell viability and migration than MCF-7 cells upon 48 h TQ exposure. This illustrates that MDA-MB-231 cells, which are highly metastatic, are more sensitive towards the anti-proliferative effects of TQ.

The growth inhibition observed is likely attributed to cell cycle arrest in G2/M and G1 phases for MDA-MB-231 and MCF-7 cells, respectively. Findings from cell cycle profiles corroborated with cyclins B and D1 protein expression levels, which were down-regulated in TQ-treated MDA-MB-231 and MCF-7 cells, respectively. Cyclin B forms a mitotic promoting factor (MPF) through binding to cyclin dependent

kinase (cdk) and regulates entry into the mitotic (M) phase of the cell cycle (LeBrasseur, 2003). On the other hand, cyclin D1 is a proto-oncogene and an important cell cycle regulator in G1 to S phase transition. Cyclin D1 binds cdk4/6 and hyperphosphorylates pRB, which releases RB-sequestered E2F transcription factors allowing transcription of genes required for entry into G1 phase (Alao et al., 2006).

Apart from cyclin proteins, expression levels of p53, its downstream target p21 and survivin (an inhibitor of apoptosis) were also investigated (Levesque et al., 2008; Wang et al., 2004). In normal cells, p53 becomes activated upon DNA damage leading to p21-triggered growth inhibitory effects for damage repair to set in (Gasco et al., 2002; Weiss et al., 2003). This scenario was observed in low dose TQ-treated MCF-7 cells, where an up-regulation of p53 and p21 primarily led to p53-mediated p21-dependent G1 arrest allowing for DNA damage repair.

However, attenuation of p21 in malignant cells has been shown to possibly prevent normal repair process and promote apoptosis (Weiss et al., 2003). This could be a likely explanation of the sudden reduction in p53 and p21 expression upon exposure to high TQ dose in MCF-7 cells. Being a more sensitive cell line towards TQ's effects, MDA-MB-231 cells also exhibited dose-dependent down-regulation of survivin, p53 and p21 proteins. At the gene expression level, there was also a significant down-regulation in *ZFHX3* gene, which encodes a transcription factor transactivating p21. The down-regulation of *ZFHX3* gene and survivin, p53, p21 protein expressions might contribute to the G2/M arrest-induced growth inhibitory effects of low TQ dose, while also possibly inducing apoptosis at high TQ doses as evidenced by the presence of cleaved PARP protein expression levels in MDA-MB-231 cells.

The modus operandi for majority chemotherapeutic drugs is through induction of DNA damage with subsequent obligatory cell cycle arrest for repair and execution of cell death for irreparable damage (Chu and Vincent T. DeVita, 2007; Luo and Leverson, 2005; Madhusudan and Hickson, 2005; Madhusudan and Middleton, 2005). As DNA damage and repair play a central role in cellular response to therapy, it is of significant interest to understand the correlation between the DNA damage and repair and the sensitivity or resistance phenotype of the breast cancer cells towards TQ in this study. To investigate this possibility, the alkaline single cell gel electrophoresis (comet) assay and immunofluorescence staining for γ-H2AX were performed to measure general DNA damage and DNA DSBs, respectively. γ-H2AX is involved in the early response to DNA DSBs and hence employed as an established biomarker for DSBs (Kinner et al., 2008). In conjunction, a recovery period was employed to make inferences in subsequent damage repair. Collectively, TQ induced significant DNA DSBs in both breast cancer cell types with minimal damage in MCF-10A cells. Furthermore, the breast cancer cells exhibited sustained DNA damage after recovery periods possibly indicating an inefficient or delayed repair capacity of damaged DNA. Hence, the induction of DNA DSBs by TQ in MDA-MB-231 and MCF-7 cells is likely to lead to G2/M and G1 cell cycle phase arrest, respectively, with consequent reduced cancer cell growth.

Human tumour cells harbour numerous mutations that allow irregular and unregulated growth (Hanahan and Weinberg, 2011; Loeb et al., 2003b; Vogelstein and Kinzler, 1993). Acquired resistance in tumour cells could be due to either up-regulation of DNA repair genes or defective signalling pathways which alter the apoptotic response (Damia and D'Incalci, 2007). In order to further elucidate the

mechanism involved in TQ-induced DNA damage, expression levels of related DNA repair proteins known to be involved in DNA DSB repair were investigated. ATM serves as a general DNA damage sensor but the main player in the repair of DSBs via NHEJ pathway is DNA-PKcs (Burma and Chen, 2004; Shiloh, 2006; Smith et al., 1999; Smith and Jackson, 1999).

In MDA-MB-231 cells, a dose-dependent up-regulation of p-ATM and p-DNA-PKcs expression is likely to be a response to TQ-induced DNA damage and DSBs, respectively. Apart from being a primary sensor protein, ATM is one of the upstream proteins involved in the HR pathway for the repair of DSBs via recruitment of the MRN complex (Chen et al., 2007). In addition, recent evidences have also shown that ATM serves as an upstream regulator of DNA-PKcs and hence the phosphorylation and activation of DNA-PKcs at Thr-2609 cluster for mediating double strand repair (Chen et al., 2007). In this study, the phosphorylation of DNA-PKcs in TQ-treated MDA-MB-231 cells was evaluated only at the ser-2056 clusters; therefore the activation of DNA-PKcs is likely to be ATM-independent. However, we are not able to conclude if activation of DNA-PKcs also occurs via ATM dependent phosphorylation at the Thr-2609 clusters. Further experiments such as using an antibody that is specific for detecting phosphorylated DNA-PKcs at Thr-2609 clusters can help validate ATM-dependent phosphorylation of DNA-PKcs. Nevertheless, the increase in p-DNA-PKcs expression is greater than for p-ATM expression demonstrating that NHEJ pathway, rather than HR pathway, as the predominant mediation of DSB repairs. Interestingly, DNA-PKcs is preferentially activated in MDA-MB-231 cells upon exposure to low TQ dose, while ATM activation occurs at high TQ dose. This could possibly be due to the complementary roles that ATM and

DNA-PKcs have in double strand repair breaks (Martin et al., 2012). The initial end-joining of the broken sites is carried out by recruiting DNA-PKcs with the completion of the repair process by ATM.

Numerous reports have demonstrated that DNA DSBs not only arise from ionising radiation or reactive oxygen species, but also from single strand breaks (Kuzminov, 2001). If single strand breaks are not repaired by base excision repair (BER) before replication in S phase, duplication of these 'lesions' will lead to the formation of DSBs. PARP-1 is a modulator of BER, which binds to single strand breaks with subsequent synthesizing and transferring of poly(ADP-ribose) polymers to various nuclear proteins for initiation of repair (Pachkowski et al., 2009). Interestingly, a dose-dependent up-regulation of PARP-1 in TQ-treated MDA-MB-231 cells was observed and could likely be a response to increased formation of TQ-induced single strand breaks (Zaremba et al., 2011). We have since shown that TQ induced DNA DSBs but further investigations would be required to determine if greater single or DSBs were specifically induced in MDA-MB-231 cells after 48 h TQ exposure.

NHEJ pathway occurs in all phases of cell cycle but is predominantly involved in the repair of DSBs in G1 phase of cell cycle (Hendrickson, 1997; Rothkamm et al., 2003). Hence, the up-regulation of phosphorylated DNA-PKcs in TQ-induced G1 cell cycle arrest in MCF-7 cells is expected. Similar to MDA-MB-231 cells, the increased PARP-1 expression in TQ-treated MCF-7 could also be a possible response to formation of TQ-induced single strand breaks. However, this was observed only at the highest TQ dose tested in MCF-7 cells and could possibly be due to their reduced sensitivity at low TQ doses. The down-regulation in basal ATM and p-ATM

expressions in TQ-treated MCF-7 cells indicate that NHEJ pathway could be the main mediator in TQ-induced DSB repair. In addition, gene expression study in TQ-treated MCF-7 cells revealed the down-regulation of FANCC gene, which encodes a protein delaying apoptosis while allowing HR of DSBs. Hence, the mechanism for TQ-induced DNA DSBs involves cell cycle arrest followed by the activation of DNA repair protein, DNA-PKcs, for NHEJ repair pathway in both breast cancer cells.

Recently, our laboratory discovered a novel effect of TQ on telomerase inhibition in human glioblastomas, which led to telomere shortening upon prolonged exposure to TQ (Gurung et al., 2010b). Telomerase activity is directly correlated with protein expression of hTERT, which is known to be the rate-limiting subunit of telomerase (Feng et al., 1995; Nakamura et al., 1997). hTERT is a downstream target of c-myc factor and c-myc binds to E-box (CACGTG) hTERT promoter region with subsequent expression of hTERT gene (Greenberg et al., 1999; Schneider-Stock et al., 2003; Wu et al., 1999). Hence, c-myc is a positive regulator of hTERT expression. In this study, telomerase activity was significantly reduced in 48 h TQ-treated MDA-MB-231 cells but not in MCF-7 or MCF-10A cells. The telomerase inhibitory effects in MDA-MB-231 cells were associated with a down-regulation in c-myc and hTERT expression. Although telomerase inhibition was not observed in MCF-7 cells treated with 48 h TQ at its IC50 value, a higher TQ dose displayed slight reduction in c-myc and hTERT protein levels. The reduction of telomerase activity at IC50 observed only in MDA-MB-231 cells could possibly contribute to the greater sensitivity of TQ in MDA-MB-231 cells as compared to MCF-7 cells. A reduction in telomerase activity has shown to increase sensitivity of the cells to numerous chemotherapeutic agents and radiation (Nakamura et al., 2005). Furthermore, the different observations suggest

that TQ might exert its anti-proliferative effects in MDA-MB-231 and MCF-7 cells via different biological pathways. Studies have shown that estradiol primarily regulates c-myc transcription in estrogen receptor (ER) positive cells, while regulates c-myc post-transcriptional level in ER negative cells (Duangmano et al., 2010). This could be a possible explanation for the observed differential telomerase inhibition of TQ in the breast cancer cells. In ER(-) MDA-MB-231 cells, TQ could directly affect the ribonucleic acid (RNA) stability of c-myc by reducing its half-life and leading to hTERT down-regulation. On the other hand, in ER(+) MCF-7 cells, TQ is required to modulate either ER or its cellular signal transduction pathways (e.g. ERK/MAPK) first before downstream regulation of transcription factor, c-myc. Hence only a sufficiently higher TQ dose would cause the reduction in c-myc and hTERT protein levels in MCF-7 cells.

Telomeres are not only regulated by telomerase but also by telomere-associated proteins (de Lange, 2004). In particular, TRF2 plays a key role in protecting telomeres by maintaining the correct TTAGGG structure at telomere termini. TRF2 expression levels were down-regulated upon TQ exposure in MDA-MB-231 cells. A possible implication would be the loss of the telomeric protection cap. The 'naked' telomeres would be more vulnerable to the external environment and be recognized as DSBs activating the DNA repair pathway (Peuscher and Jacobs, 2011).

However, in TQ-treated MCF-7 cells, elevated levels of TRF2 were expressed at high TQ doses. The elevation of TRF2 at higher TQ doses might be a response to eroded telomeres, where TRF2 could be involved in suppressing ATM kinase activity and hence protect telomeres from ATM-dependent HR repair (Karlseder et al., 2004).

However, it is important to note that TRF2 may be present in other cellular components other than on telomeres (Baker et al., 2011; Hiyama, 2009). Isolation of nuclear proteins is highly recommended rather than the isolation of total cellular protein for investigating TRF2 protein expression levels at telomeres.

Results have in so far shown the inhibition of telomerase, especially so for hTERT expression, in both cancer cells at different TQ doses. Telomerase activity has implications on telomere length, which reduces by approximately 50-200 bp with each cell division (Lansdorp, 2000). Low dose chronic exposure to TQ allows for more pronounced changes in telomere length since TRF assay measures the average telomere length in the cell population rather than individual telomeres of a chromosome (Kimura et al., 2010). Telomere shortening was observed within 2 weeks of TQ exposure in both breast cancer cells with comparable rates of telomere attrition. Continued TQ treatment for 4 and 8 weeks in MCF-7 cells further reduced telomere length, while MDA-MB-231 cells showed gradual increase in telomere length. These observations were consistent with hTERT protein expression where significant hTERT down-regulation occurred at 4 and 8 weeks of TQ exposure in MCF-7 cells, while hTERT down-regulation occurred at 2 weeks of TQ exposure with subsequent hTERT up-regulation at 4 and 8 weeks of TQ exposure in MDA-MB-231 cells. Surprisingly, TRF2 up-regulation in both breast cancer cells were observed at 2 weeks TQ treatment with levels subsequently down-regulated at 4 and 8 weeks. A recent study found elevated levels of TRF2 in breast cancer cells with short telomeres (Diehl et al., 2011). The higher levels of TRF2 expression after 2 weeks TQ exposure could be a feedback response for increasing binding to telomeres for protecting critically shortened telomeres. Another contentious issue is the down-regulation of TRF2 in

MDA-MB-231 cells which exhibited lengthened telomeres and there could be two possible speculations. The original function of TRF2 had been proposed to protect telomeres, so the loss of TRF2 could contribute to the loss of telomere function (Smogorzewska et al., 2000). Many studies have shown that the telomere lengths in cells with absence or loss of function of TRF2 were longer and due to a higher occurrence of telomere fusions (Smogorzewska et al., 2000; van Steensel et al., 1998). In addition, MDA-MB-231 cells have shorter initial telomere length than MCF-7 cells and shorter telomeres would require lesser cell divisions to reach the critical length threshold due to the end replication problem coupled with the inhibition of telomerase (Scheinberg et al., 2010). Therefore the subsequent gradual increases in telomere length could be a result of telomere fusions arising from loss of TRF2 end-capping function. The second possibility arises from MDA-MB-231 cells acquiring alternative lengthening of telomere (ALT) mechanism due to acquired resistance to TQ. Since MDA-MB-231 cells are more sensitive towards TQ's effects, there could be a strong selection pressure for the emergence of resistant cells that employ or acquire the ALT mechanism over 4 and 8 weeks of TQ exposure. Moreover the ALT mechanism has been shown to be independent of telomerase (Bryan et al., 1995). By performing a more sensitive assay, the quantitative fluorescence in-situ hybridisation (Q-FISH), measurements of telomere length will have an improved resolution of 200 bp as compared to TRF assay (Slijepcevic, 2001). Cancer cells are heterogeneous in nature and variability in telomere length exists in cell populations (Savre-Train et al., 2000). Hence Q-FISH also allows for measurement of telomere length of individual chromosome arms in a cell and would allow us to safely conclude if the occurrence of telomere lengthening is due to telomere fusions or the ALT mechanism.

From the population doubling study that was carried out in concordance with TRF assay, there was a gradual decline in proliferative capacity of the breast cancer cells upon chronic non-toxic TQ dose over a period of 8 weeks. Hence, the perturbations in telomere length maintenance were likely attributed to the direct or indirect effects of TQ on telomerase and/or telomere binding protein, TRF2, rather than the direct cytotoxicity of TQ.

As telomeres are fragile sites that function to protect DNA, inhibition of telomerase in tumour cells could render telomeres more susceptible to the induction of greater DNA damage. Evidences exist that preferential DNA damage can result from disruption in telomerase activity or telomere-associated regions when exposed to certain chemotherapeutic agents (e.g. doxorubicin) and this leads to loss of telomere cap protection (Gurung et al., 2010b; Tamakawa et al., 2010). Such a phenomenon may result in the uncapped telomeres being recognized as DNA DSBs. In this study, we have in so far shown that TQ not only induced acute DNA damage, but also telomere dysfunction via regulation of telomerase activity and/or telomere binding protein, TRF2. Further investigation of the co-localization of γ-H2AX with telomere specific probes revealed the possibility of activation of DNA damage response at telomeres upon TQ exposure. Specifically significant percentage of cells with positive TIFs could be observed in the breast cancer cells when treated at their IC50 TQ values. However, higher TQ doses had lesser percentage of cells with positive TIFs. This is expected as higher TQ doses had greater reduction in viability, migration and increased induction of DNA damage of the breast cancer cells. When cells sustain DNA damage, cell cycle arrest allows for damage repair and irreparable damage in

cells is likely to lead to apoptosis as evidenced by increased PARP-1 cleavage, especially so for MDA-MB-231 cells.

Taken together, breast cancer cells are indeed more sensitive to the anti-proliferative effects of TQ than normal cells and different mechanisms seem to be responsible for the effects of TQ in MDA-MB-231 and MCF-7 cells (Fig. 23). In MDA-MB-231 cells, TQ not only altered telomerase activity via c-myc regulation of hTERT expression but also reduced TRF2 expression. The long term effects of such regulation led to telomere shortening within 2 weeks of TQ treatment with reduced proliferative capacity of MDA-MB-231 cells. The acute effects of such regulation have shown to possibly induce DNA DSBs at telomeres (increased positive TIFs formation) and ATM-independent activation of DNA-PKcs at these sites for mediating repair via the NHEJ pathway. The increased DNA damage induced possibly led to a G2/M arrest for damage repair and also led to apoptosis at higher TQ dose exposure. These events consequently reduced cell viability and migratory ability of MDA-MB-231 cells.

However, in MCF-7 cells, TQ could likely modulate c-myc down-regulation directly via ER or indirectly via ER signalling pathways (e.g. ERK/MAPK) leading to hTERT down-regulation and telomerase inhibition, especially at high doses. The long term effects of such regulation led to progressive telomere shortening throughout 8 weeks of TQ treatment with reduced proliferative capacity of MCF-7 cells. Evidence has also shown a possible dysregulation of TRF2 in telomerase-independent pathway leading to progressive telomere shortening. The acute effects of such regulation could induce DNA DSBs at telomeres (increased positive TIFs formation) or at random DNA sites with activation of DNA-PKcs at these sites for mediating repair also via the

NHEJ pathway. The increased DNA damage induced possibly led to a p53-mediated p21-induced G1 arrest for damage repair. These events consequently reduced cell viability and migratory ability of MCF-7 cells.

The direct or indirect involvement of telomerase in mediating the effects of TQ in MCF-7 cells is still unclear and could also possibly involve telomerase-independent mechanisms (e.g. dysregulation of telomere binding proteins). Further insights into the differential responses of the two breast cancer cells via comparison of gene expression profiling at basal levels revealed a total of 2,518 differentially regulated genes and is equivalent to approximately 7 % of total genes analysed. This implies that genetic differences exist amongst the two different cell types at basal levels and could likely to contribute to the differential responses and sensitivities towards the effects of TQ. For example, MCF-7 cells possess wild-type p53 and functional ER, unlike MDA-MB-231 cells, which possess mutant p53 and lack of functional ER (Gasco et al., 2002). The property of estrogen receptors has been illustrated to regulate c-myc expression at transcriptional and post-transcriptional levels in ER(-) and ER(+) cells, respectively.

Since estrogen receptors have been shown to be implicated in the differential sensitivity of the two cell lines, the status of p53 might also contribute to such an observation. A study carried out by Avila et al. (1994) demonstrated that quercetin, which is a natural bioflavanoid, showed different extent of growth inhibitory effects in wild-type p53 MCF-7 cells and mutant p53 MDA-MB-468 cells. Higher concentrations of quercetin were required for MCF-7 cells to achieve inhibition levels in MDA-MB-468 cells (Avila et al., 1994). Therefore, the p53 status may be implicated in the sensitivity of the two breast cancer cells towards TQ, where MDA-

MB-231 cells possess a mutant p53 status, specifically an arginine to lysine mutation at codon position 280 in the DNA-binding domain (Gasco et al., 2002) while MCF-7 cells possess wild-type p53 status (Takahashi and Suzuki, 1993).

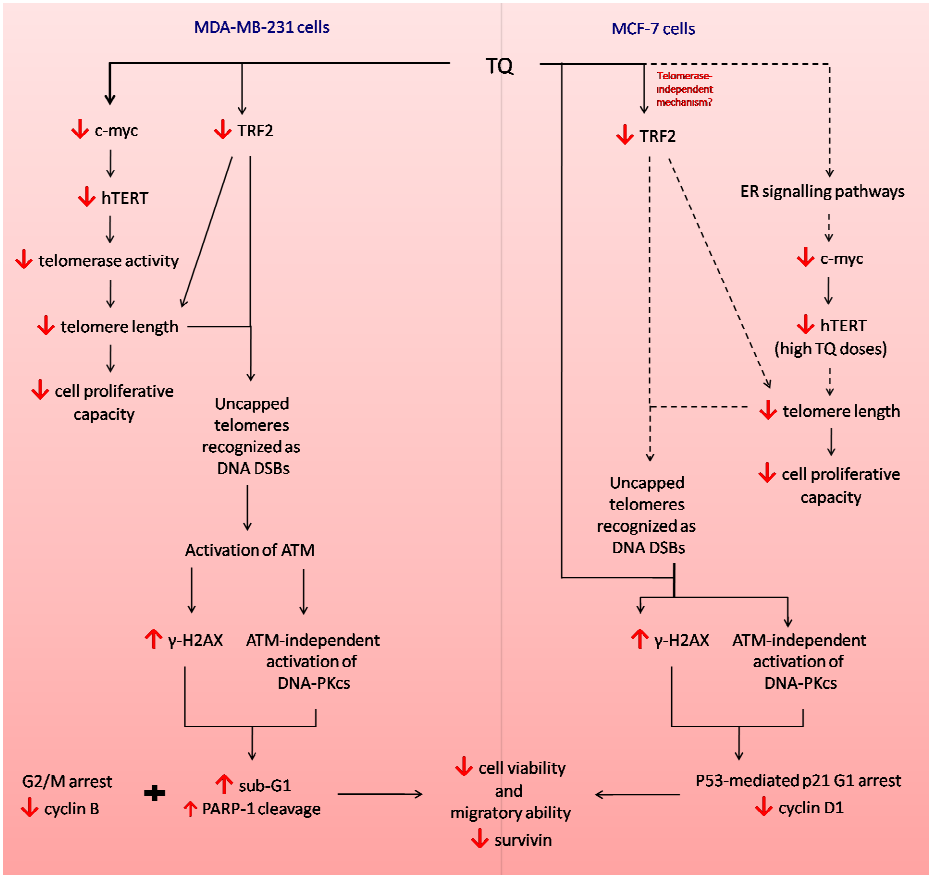


Figure 23. Systematic summary for the functional interaction of the different signaling pathways studied. The signaling pathways studied include changes, if any, in cell cycle profiles, DNA damage repair pathways and telomere-telomerase homeostasis. The effects of TQ appeared to be dissimilar in both breast cancer cell lines studied, especially so for the effects of TQ on telomere-telomerase homeostasis.

CHAPTER 5

5. Limitations and Future Directions

Taken together, the *in vitro* results have in so far demonstrated the selectivity of TQ towards the cancer phenotype with minimal toxic effects in the immortalised normal cells tested. However, further assessment is required when extrapolated to *in vivo* settings to determine if the effects are mirrored by *in vitro* TQ effects.

Results have shown that TQ induced significant DNA DSBs and caused telomere dysfunction in the breast cancer cells. More importantly, an up-regulation of activated DNA-PKcs following TQ exposure was observed. Therefore, it would be worthwhile to determine if DNA-PKcs are recruited specifically due to DNA DSBs at telomeric sites or random DNA damage at non-telomeric sites by performing dual immunofluorescence staining studies involving the co-localisation of DNA-PKcs with telomere specific probes.

Studies have validated the promising results of combinatorial therapy by inhibition of DNA repair pathway and other signalling pathways to facilitate efficient anti-cancer therapy with improved outcomes. For example an inhibitor of DNA-PKcs, NU7441, which is currently in early phase of clinical trials, has been shown to increase radiosensitivity of cells and also sensitise cells to topoisomerase II poisons (Cowell et al., 2005; Helleday et al., 2008; Madhusudan and Hickson, 2005). In a previous study, we have shown that DNA-PKcs is important in mediating the cytotoxic effects of TQ in human glioblastoma cells (Gurung et al., 2010b). It would be of interest to investigate if DNA-PKcs plays a similar role in breast cancer cells, given that DNA-PKcs has been shown to be activated following TQ exposure and also

NHEJ pathway seemingly activated for TQ-induced DSB repair. These cancer cells could be pre-treated with NU7026, a pharmacological inhibitor of DNA-PKcs (Willmore et al., 2004), before incorporation of TQ to assess if impairment of DNA-PKcs and the NHEJ pathway could render cancer cells greater sensitivity towards TQ with ultimate cell death.

In this study, both breast cancer cells displayed differential sensitivities towards TQ exposure. As described previously, the different estrogen receptor status in MDA-MB-231 (ER+) and MCF-7 cells (ER-) could influence and contribute to the altered c-myc regulation of hTERT expression. Particularly, the loss of estrogen receptor function could possibly increase sensitivity of the cells towards TQ as observed in TQ-treated MDA-MB-231 cells. Hence, it would be interesting to observe if siRNA mediated silencing of estrogen receptor in MCF-7 cells could render greater sensitivity towards to TQ, especially so for effects on telomerase.

Some immortalised human cell lines and tumours maintain telomeres in the absence of any detectable telomerase activity by alternative lengthening of telomeres (ALT) mechanism, where a possible role of recombination has been suggested (Bryan et al., 1995). Since ALT and telomerase-dependent maintenance co-exist in some immortalised cells, it is possible that recombination and other repair mechanisms may render some tumours to be resistant to conventional therapy. Hence, the action of TQ on telomere homeostasis in ALT cells (e.g. U2OS cells) could also be elucidated.

CHAPTER 6

6. Conclusion

The compound, thymoquinone, which is derived from black seed oil, is an ingredient used in Asian food and has shown to have a potential use in anti-cancer therapy. Being a natural plant product, TQ is a pleiotropic agent that is likely to affect multiple signalling pathways in many patho-physiological conditions. Our study shows promising anti-proliferative effects of TQ in breast cancer cells and the involvements of DNA repair pathways and telomere-telomerase homeostasis have shown to be implicated in contributing to the observed effects. However, we cannot discount the possible roles of other signalling pathways in mediating the effects of TQ. Nevertheless, the outcome of the proposed study would provide a better understanding and contribution to the molecular mechanism of the anti-cancer properties of TQ and subsequently in combinatorial approach to facilitate efficient cancer cell killing.

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