POST-TRANSLATIONAL MODULATION OF PTEN AND ITS ROLE IN GENOMIC INSTABILITY

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Thesis submitted to Jadavpur University for the degree of Doctorate of Philosophy in Science under the supervision of

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Post-Translational Modulation of PTEN and its role in genomic stability"** submitted by Smt. **Ginia Ghosh** who got her name registered on **27/04/2016** for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Prof. Parimal Karmakar** and that neither this thesis nor any part of it has been submitted for either any degree or any other academic award anywhere before.

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DECLARATION

I do hereby, declare that the work embodied in the thesis entitled **"POST-TRANSLATIONAL MODULATION OF PTEN AND ITS ROLE IN GENOMIC INSTABILITY"** submitted for the award of Doctorate of Philosophy (Ph.D.) in Science, is the completion of the work carried out under the supervision of Prof. Parimal Karmakar at the Department of Life science and Biotechnology, Jadavpur University. Neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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DEDICATION

I Dedicate this thesis to Almighty God, my Parents and my Daughter

PREAMBLE

The thesis consists of four chapters. The first chapter includes a general introduction on PTEN, the function of PTEN, and the post-translational modification of PTEN. In the second chapter, we mostly focused on different post-translational modifications of PTEN in the context with genomic stability. In the third chapter, we focused on the role of SUMOylated PTEN in maintaining genomic stability. During this study, we also found that C-terminal phosphorylation-dependent phosphatase activity as well as SUMOylation of PTEN is necessary for maintaining the genomic integrity of the cell along with its catalytic activity. Collectively in the last chapter, we discussed the biological significance of post-translational modification of PTEN in preventing cytological damage.

Methods and techniques used in this thesis are described at the end of the thesis as some common methods are used in both chapters.

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constructive criticism and scientific suggestions. Lastly, I would like to thank almighty god for creating nature and allowing us to unveil it. There might be a few more names I may have missed out, but my sincere thanks and regards remain for all.

CONTENTS

List of Abbreviations

APC/C	Anaphase Promoting Complex/Cyclosome
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
AURKA	Aurora Kinase A
BSA	Bovine serum albumin
CBMN	Cytokinesis block micronucleus
CDK	Cyclin Dependent Kinase
CENP-C	Centromeric Protein C
Chk	Checkpoint kinase
CK2	Casein Kinase 2
CLS	Cytosolic Localization Signal
CS	Cowden Syndrome
DAPI	4',6-Diamidino-2-phenylindole
DAXX	Death domain-associated protein
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
DSB	Double strand break
dsDNA	Double-stranded DNA
ECL	Enhanced chemiluminescence
EDTA	Ethylene di-amino tetra- acetate
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
EtOH	Absolute ethanol
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FOXO	Forkhead Box O
GFP	Green Fluorescence Protein

GSK3	Glycogen Synthase Kinase 3 Beta
GTPase	Guanosine Tri Phosphatase
H3k9me3	Histone 3 Lysine 9 Trimethylation
HAT	Histone Acetyltransferase Protein
HAUSP	Herpes Virus-Associated Ubiquitin-Specific Protease
HDAC	Histone deacetylase
HP1	Heterochromatin Protein 1
HR	Homologous Recombination
HRP	Horse Radish Peroxidase
HU	Hydroxyurea
IR	Ionizing radiation
KCl	Potassium Chloride
MAD1	Mitotic Arrest Deficiency 1
MAGI-2	Membrane-Associated Guanylate Kinase Inverted 2
MAMs	Mitochondria-associated Membranes
MAPK	Mitogen-Activated Protein Kinase
MAST2	Microtubule-Associated Serine and Threonine Kinase 2
MCC	Mitotic Checkpoint Complex
MCM2	Minichromosomal Maintenance Protein
MEF	Mouse Embryonic Fibroblast
MEM	Minimal Essential Medium
mi-RNA	Micro-Ribonucleic Acid
MMAC1	Mutated in Multiple Advanced Cancers
MNi	Micronucleus
MVP	Major Vault Protein
NaOH	Sodium hydroxide
NBUD(s)	Nuclear bud(s)
NEDD4-1	Neural precursor cell Expressed Developmentally Down-regulated protein
NEM	N-ethylmaleimide
NHEJ	Non-homologous end joining

NLS	Nuclear Localization Signal
NP-40	Nonidet P-40
NPB(s)	Nucleoplasmic bridge(s)
NSCLC	Non-Small Cell Lung Carcinoma
OTUB1	Ubiquitin thioesterase
PAGE	Polyacrylamide gel electrophoresis
PAR3	Protease-Activated Receptor 3
PBM	PIP2 Binding Module
PBS	Phosphate buffered saline
PCA	Principal Component Analysis
PDZ	PSD-95, Discs-large, ZO-1
PHTS	PTEN Hamartoma Tumor Syndrome
PI3K	Phosphatidylinositol 3-kinase
PICT-1	Protein Interacting with Carboxyl Terminus 1
PIP2	Phosphatidylinositol-4,5-Bisphosphate
PIP3	Phosphatidylinositol-3,4,5-Triphosphate
PLK1	Polo Like Kinase 1
PML	Promyelocytic Leukaemia Protein
PPAR	Peroxisome Proliferator-Activated Receptor
PTEN	Phosphatase and Tensin homolog deleted on chromosome Ten
PTMs	Post-translational modifications
PVDF	Polyvinylidene-difluoride
RFP	Red Fluorescent Protein
RIPA	Radioimmune precipitation assay Buffer
RNA	Ribonucleic acid
ROCK	Rho-A associated kinase
ROS	Reactive Oxygen Species
RPA1	Replication Protein A
RPMI-1640	Roswell park memorial institute-1640
RT	Room Temperature
SAC	Spindle Assembly Checkpoint
SD	Standard deviation

SDS	Sodium Dodecyl Sulphate
ssDNA	Single-Stranded DNA
SUMO-1	Small ubiquitin-like modifier 1
TBST	Tris Buffer Saline –Tween 20
TEMED	Tetramethyl ethylenediamine
TEP1	TGF-regulated and Epithelial cell-enriched Phosphatase
TOP2A	DNA Topoisomerase II
WWP2	WW domain-containing E3 ubiquitin protein ligase 1
XIAP	X-linked inhibitor of apoptosis protein
XPC	Xeroderma Pigmentosum C

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List of Symbols

%	Percentage
:	Ratio
~	Approximate
+Ve	Positive
<	Less than
=	Equal
>	Greater than
±	Plus-minus
×	Times
°C	Degree centigrade
µg	Microgram
µl	Microliter
µM	Micromolar
µmol	Micromole
g	Acceleration due to gravity
gm	Gram
h	Hour
M	Molar
mA	Milliampere
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
rpm	Rotation per minute
U	Unit
V	Volt
-Ve	Negative

w/v	Weight/volume
α	Alpha
γ	Gamma
λ	Lamda

Abstract

Abstract

Multiple malignancies have been identified to frequently harbor mutations in the dual phosphatase tumor suppressor protein PTEN. The function of PTEN is not limited to its canonical function ie regulation of PI3K pathways. The genome of PTEN null cells is highly unstable and shows a greater number of aneuploidy. PTEN now emerges as a protein that seems to act as a caretaker of different cellular events like autophagy, senescence, DNA repair, etc. These multiple functions of PTEN are regulated by its post-translational modification or its specific association with other proteins. PTEN's subcellular localization has been reported to link with its PTM. Subcellular localization of PTEN is also associated with its catalytic activities like DNA repair, Activation of PI3K, maintenance of cell cycle, etc. However, how these post-translational alterations impact aneuploidy and cytological damage has not been thoroughly investigated. We concentrate on the relationship between phosphatase activity and PTEN's C terminal phosphorylation in light of cytological damage such as the creation of nuclear bridges, nuclear buds, and micronuclei. Our findings imply that in PTEN-deficient PC3 cells, wild-type PTEN greatly lessens cytological damage but not phospho-mutant PTEN. When PTEN is phosphatase dead, cytological damage markers rise throughout the first 24 hours following DNA damage. Utilization of phosphorylation and phosphatase-dead dual mutant PTEN showed the degree of cytological DNA damage parameters similar to phosphatase-dead PTEN. A failure in the mitotic phase of the cell cycle, persistent DNA damage, or an accumulation of aberrant replication intermediates can result in multinucleated cells and MNi development. Restoration of Wt-PTEN function reduces the generation of multinucleated cells caused by DNA damage, suggesting that PTEN is crucial for preserving genomic stability. We also discovered that both phosphatase activity and PTEN phosphorylation are necessary for preserving chromosome numbers. Significantly

abnormal γ-tubulin pole development is seen in PTEN null cells during metaphase. It's interesting to note that PLK1, Aurora kinase A, and p-PTEN are all localized to spindle poles. The expression of phosphorylated Aurora kinase A (T288) and phosphorylated PLK1 (T210) increases in PTEN's phosphorylation and phosphatase mutant in comparison to cells expressing wild-type PTEN. Again, the Wild-type-PTEN can interact physically with Aurora Kinase A and PLK1 but not the phosphorylation dead mutant. As a result, our research reveals that PTEN physically interacts with and dephosphorylates Aurora kinase A and PLK1 in a manner that is dependent on C-terminal phosphorylation. PTEN prevents abnormal chromosomal segregation in metaphase by reducing the hyperphosphorylation state of these proteins.

Again, PTEN/PI3K/AKT pathway creates a vital signaling pathway regulating several biological processes like cell metabolism, cell growth, apoptosis, and cell proliferation. Phosphatidyl inositol-3 kinase, a class I PI3Ks family of lipid membrane constituents that are negatively charged formed by hydroxyl groups phosphorylation by specific kinases on 3, 4, 5 positions of the inositol ring of phosphatidyl inositol (PI). The phosphatase activity of PTEN dephosphorylates PIP3 to PIP2 at the position of D3 of the inositol ring by removing one phosphate which is attributed to its canonical function. Thus, inhibiting the PI3K/AKT pathway. leads to tumor suppression. This function needs PTEN to localize in the membrane. Therefore, membrane localization of PTEN requires sumoylation, a post-translational modification that occurs at the K254 site of the PTEN protein. Cells containing PTEN mutation at K254R are reported not to respond to DNA damage. So, we investigated the significance of PTEN sumoylation with DNA damage-induced cytological damage, such as the generation of nuclear buds, nuclear bridges, and micronuclei. According to our data, sumo-dead PTEN suffers more cytological damage compared with Wt-type PTEN. The cytological damage indicators are elevated during the 24-hour recovery period following

DNA damage in the case of sumo-dead PTEN. Next, we evaluated how well the sumo-dead mutant (K254R-PTEN) prevented aneuploidy and discovered that sumoylation is crucial for preserving chromosomal numbers. We analyzed the γ-tubulin pole generation in cells transfected with the K254R-PTEN clone qualitatively and quantitatively because chromosomal number fluctuation in daughter cells is caused by multiple spindle pole formation. In cells transfected with the K254R-PTEN clone, we discovered a considerably higher rate of aberrant pole formation as compared to the cells transfected with wild-type PTEN. Compared to cells expressing wild-type PTEN, the expression of the phosphorylated form of Aurora kinase A (T288) and phosphorylated form of PLK1 (T210) proteins increases when the sumoylation activity of PTEN is diminished in the presence or in the of absence an agent depolymerizing microtubule, nocodazole.

Thus, different post-translational modifications such as C-terminal phosphorylation along with phosphatase activity and SUMOylation of PTEN are involved in the catalytic activity as well as maintaining genomic stability of cells.

Chapter 1

General Introduction & Literature Review

1.1 Cancer and PTEN

Cancer is described as more than 100 associated malignant diseases. Cancer is one of the foremost cause of death in the world (Jemal et al, 2011, Hanahan and Weinberg, 2000). PTEN has emerged as a gene playing a role in tumor suppression which is the 2nd most frequently mutated in nearly 10% of cases across all types of cancer (Shah et al, 2012, Lawrence et al, 2014). Genetic alterations of PTEN are more in endometrial cancer (35%), prostate cancer (17%), glioblastoma (32%), non-small cell lung carcinoma (NSCLC) (12%), melanoma (13%), and breast cancer (9%). Apart from genetic mutation epigenetic silencing of promoters can also affect PTEN expression resulting in down-regulation of the expression of PTEN in endometrial, breast, thyroid, lung, brain tumors, ovarian, and gastric (Hollander et al., 2011). Several micro-RNAs overexpression including miRNA22, miRNA-21, miRNA 25a, or the miRNA 106b-25 cluster adds extra complexity in prostate, glioma, and lung cancer when PTEN expression is reduced (Hollander et al., 2011).

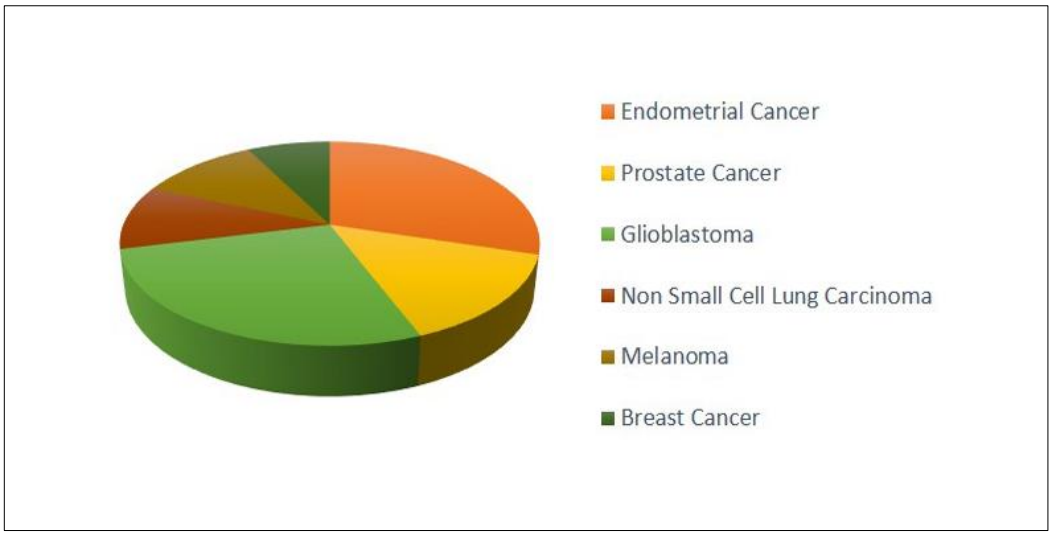
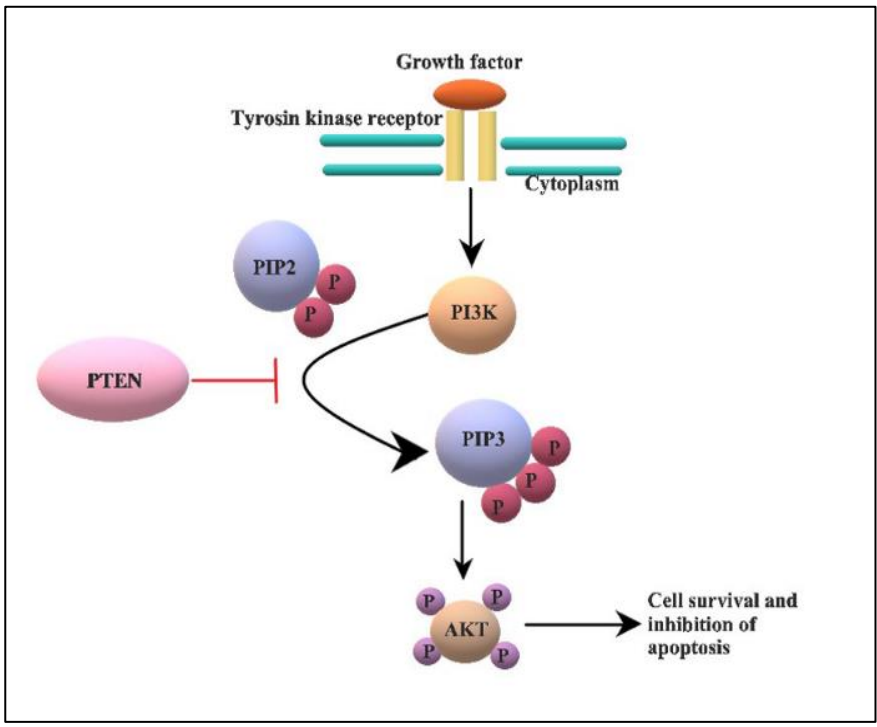


Figure 1.1: *Percentage of genetic alteration of PTEN in different cancers*

1.2 Introduction to PTEN

PTEN (phosphatase and tensin homolog) deleted on chromosome 10q23.3 identified in 1997 by three separate groups (Li et al) is a gene that plays a role in tumor suppression, like p53, which is implicated in the widely held malignancies. PTEN is also known as TEP1 and MMAC1. It is a phosphatase that phosphorylates both proteins as well as a lipids. PTEN dephosphorylates proteins such as tyrosine, serine, and threonine, as well as PIP3 (phosphatidyl 3, 4, 5 triphosphate) to PIP2 (phosphatidyl 4, 5 bisphosphate) by removing one phosphate from the inositol ring's D3 position. Because it suppresses the PI3K/AKT signaling pathway, its lipid phosphatase function is crucial for tumor suppressive action. (Myers et al., 1998, Stambolic et al., 1998) (Fig 1.1).



Adopted from Molinari F. Frattini M. et al. Front. Oncol., 2014

Figure 1.2: *Classical tumor-suppressing activity of PTEN antagonizing the PI3K pathway by dephosphorylating PIP3 to PIP2.*

PTEN deficiency accompanies a variety of neoplastic disorders, including, glioblastoma, endometrial cancer, colon, and melanoma (Cantley et al, Bonneau et al). Germline gene mutations of PTEN cause a variety of syndromes, including Bannayan-Riley-Ruvalcaba syndrome, Cowden syndrome, Lhermitte-Duclos syndrome, Bannayan-Zonana syndrome, Proteus-like syndrome, and Proteus syndrome, all are characterized by benign hamartomas and developmental abnormalities (Mester et al., Arch et al., 1997; Marsh et al., 1998; Zhou et al., 2001; Eng et al, Liaw et al., 1997, Zhou et al., 2003) collectively called PHTS (PTEN

Hamartoma Tumor Syndrome). It is a genetic condition that causes hamartomas, non-cancerous growths termed in various parts of the body. Patients may have other physical abnormalities such as larger-than-average head size, intellectual disabilities, and abnormal skin growths in addition to hamartomas. People who have PTEN hamartoma tumor syndrome are more likely to acquire thyroid, breast, kidney, uterine, skin, and colorectal cancers. PHTS is hereditary, which means it can be handed down from one generation to the next. PTEN also has critical functions in stem-cell biology (Hill et al., 2009), melanoma (Mirmohammadsadegh et al., 2006), pancreatic cancer (Ying et al., 2011), colorectal cancer (Frattini et al., 2005), lung cancer (Zhang et al., 2010), breast cancer (Zhang et al., 2013), and leukemia (Cheong et al., 2003). Research has linked PTEN mutation to Autism Spectrum Disorder and macrocephaly (Butler et al., 2005)

PTEN has a variety of cellular roles, including a contentious involvement in the cellular metabolism of insulin-mediated glucose absorption. It also includes the inhibition of PPAR, PPAR-coactivator 1a, and forkhead box O (FOXO) 1 (Ortega-Molina et al., 2012). PTEN also has a function in cell motility, namely in modulating chemotaxis directionality, as demonstrated in Dictyostelium by (Iijima et al., 2002, Huang et al., 2003, Funamoto et al., 2001) through the PI3K pathway. Both in vivo and in vitro experiments showed

reconstitution of PTEN expression in PTEN-/- glioma cells proved controlled angiogenesis (Wen et al., 2001).

1.3 Structure of PTEN

PTEN's crystal structure revealed that it is made up of 403 amino acids and has numerous domains. It is made up of the following domains: (a) PBM domain, (b) N-terminal phosphatase domain, (c) C2 domain, and (d) C-terminal fragment. (a) The adherence of PTEN to its position of action via PIP2 molecules in the membrane is enabled by PIP2 binding module, PBM. (b) The N-terminal domain has a specificity for both protein and lipid phosphatase, the domain is called (PD) with the catalytic motif HCKAGKGRS/T. There is a sequence homology of this region with actin-binding protein tensin 1 and ATPase heat shock cognate 70 which has a non-catalytic cofactor, auxillin. (Li et al., 1997). (c) In the plasma membrane, there are phosphatidyl serine residues which are associated by positively charged residues in the C2 domain of PTEN (d) C-tail (C-terminal region), contains PSD-95, Discs-large, ZO-1 (PDZ-binding domain) that regulates the stability and membrane association via multiple phosphorylation events (Vazquez et al., 2000). (Fig 1.3A).

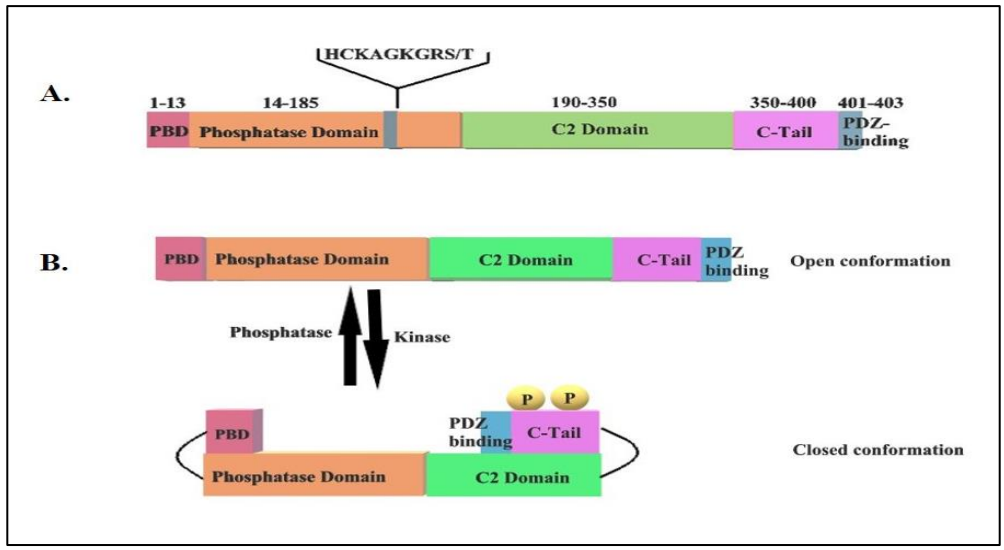
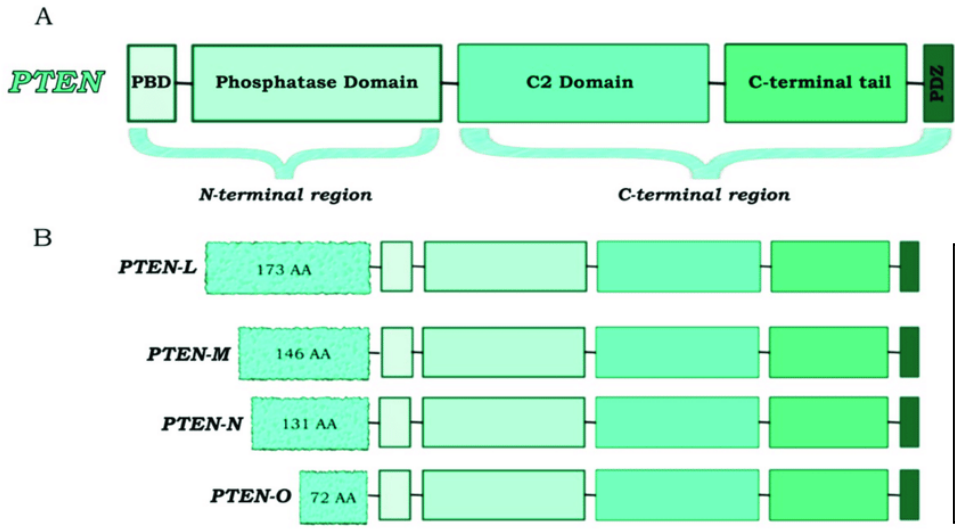


Figure 1.3: A. *PTEN protein structure is composed of 403 amino acids containing PIP2 Binding Domain (PBD), Phosphatase Domain, C2 Domain, C-Tail containing two PEST (Proline, glutamic acid, Serine, Threonine) domains, and a PDZ binding Domain.* ***B.*** *PTEN exists in closed conformation when phosphorylated favoring accumulation in the cytoplasm and in open conformation when dephosphorylated favoring its accumulation in the nucleus.*

Phosphorylation is required for intramolecular contact between the catalytic domain of PTEN and the C-tail, which results in PTEN in a 'closed' conformation, limiting recruitment of PTEN for PIP3 access to the plasma membrane (Tibarewal et al., 2012, Rahadar et al., 2009). (Fig 1.3B). The primary sites for phosphorylation are Ser380, Thr382, Thr383, and Ser385 cluster and Thr366, Ser370 (Vazquez et al., 2000, Miller et al., 2002). This phosphorylation mechanism is carried out by two kinases. PTEN is phosphorylated by CK2 (casein kinase 2) at Ser370 and the S380-Ser385 cluster. Ser362 and Thr366, respectively are phosphorylated by GSK3 (glycogen synthase kinase 3 beta) (Torres and Pulido, 2001, Al-Khouri et al., 2005). Furthermore, phosphorylation at the C-tail suppresses phosphatase activity and increases PTEN half-life (Vazquez et al., 2000). Src family kinases and ROCK (Rho-A associated kinase) phosphorylates phosphorylation sites at the C2 domain which regulates protein-protein interactions and PTEN phosphatase activity (Hopkins et al., 2014).

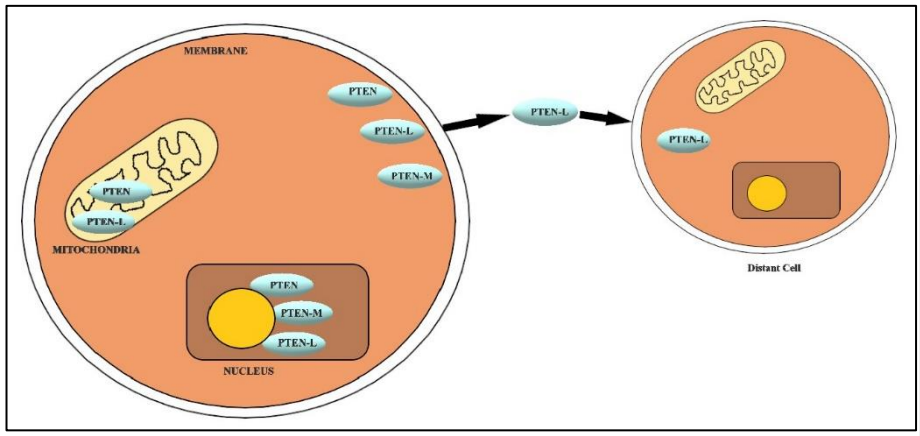
1.4 Proteoforms of PTEN

Various translational isoforms of the PTEN, a 403 amino acid protein have recently been found (Fig 1.4.1A). These isoforms are translated from non-canonical upstream start codons but from the same mRNA, resulting in distinct N-terminal extensions for each isoform (Tzani et al., 2016). PTEN-M (146 amino acid additions), PTEN-L (173 amino acid additions), PTEN-O (72 amino acid additions), and PTEN-N (131 amino acid additions) are the isoforms (Tzani et al., 2016). The isoform PTEN-M is the most common alternative isoform of PTEN that preserves phosphatase activity (Tzani et al., 2016). The longest PTEN proteoform with constitutively active phosphatase is PTEN-L (Fig 1.4.1B). It has been demonstrated that it exhibits cell-penetrating and secretory capabilities, making it an appealing option for protein delivery where PTEN function is restored in tumor cells (Wang et al., 2015, Hopkins et al., 2013, Johnston et al., 2015, Malaney et al., 2013, Masson et al., 2016). PTEN-L levels are reduced in renal and breast cell carcinoma (Hopkins et al., 2013, Wang et al., 2015). PTEN-L is found in mitochondria and affects energy metabolism (Liang et al., 2014). The therapeutic viability of the PTEN-L isoform and the functional importance of the other isoforms of PTEN in various disorders must be investigated (Fig 1.4.2).



Adopted from Bazzichetto. C et al., Cancers., 2019

***Figure 1.4.1**: **A**. The canonical PTEN isoform consists of 403 amino acids structured in five functional domains. **B**. PTEN has four translational variants (PTEN-L, -M, -N, and -O) which contains an additional N-terminus and differ in functions and subcellular localization. Translation begins from a translation site upstream of the canonical initiation sequence and results in N-terminal extensions for each of the variants.*



Adopted from Malaney P. et al. Cell Mol Life Sci., 2017

***Figure 1.4.2:** Proteoforms of PTEN and its functional relevance. Translational variants of PTEN give rise to different proteoforms that localize to various sub-cellular compartments regulating cell signaling.*

1.5 Sub-cellular localization of PTEN

The lipid phosphatase activity of PTEN at the plasma membrane is the focus of the majority of the research. Recent research, however, indicates that PTEN has a function in subcellular localizations other than the membrane. PTEN has emerged not only as a key regulator of chromosomal integrity and the cell cycle in the nucleus, but it is also located in the mitochondria, MAMs, and ER (Bononi et al., 2013, Zu et al., 2011, Shen et al., 2015, Zhu et al., 2006). Mitochondria-dependent apoptosis is controlled by PTEN in the mitochondria, in the occurrence of ROS (Zhu et al., 2006, Zu et al., 2011). The PTEN-L isoform regulates the metabolism of energy in a way dependent on protein-phosphatase-in mitochondria (Liang et al., 2014). PTEN is found in the MAM and ER, where it controls the transport of Ca2+ ions to the mitochondria from the ER, where cells become susceptible to Ca2+-mediated apoptosis (Bononi et al., 2013). According to Naquib et al., 2015, PIP3 dephosphorylation of PIP3 happens at the endosomes when PTEN attaches to endosomes along microtubules in the cell. This occurs in contrast to the localization of PTEN to the plasma membrane inhibiting PI3K/AKT signaling which is the traditional paradigm of PTEN action (Naquib et al., 2015). PTEN-L can re-enter neighboring cells after it is secreted (Hopkins et al., 2013). Functionally active PTEN can also be transported outside of cells through exosomes (Gabriel et al., 2013, Putz et al., 2012, Chowdhury et al., 2022).

1.6 Cellular Functions of PTEN

1.6.1 Cellular Polarity

Cell polarity is regulated by PTEN via both PIP3-dependent and independent pathways. PIP2 spatial arrangement in epithelial cells aids in the definition of the basolateral and apical surfaces (Gassama-Diagne et al., 2006). PTEN binds to the apical surface and increases resident PIP2 levels. This promotes the recruitment of Par/aPKC complex, CDC42, and annexin2, which results in the creation of a lumen and polarity formation(Von Stein et al., 2005, Martin-Belmonte et al., 2007). As a result, PTEN deficiency may affect apical surface and lumen development, resulting in the loss of epithelial features. This changes the cells' phenotypic to mesenchymal, resulting in Epithelial-Mesenchymal Transition (EMT) (Worby et al., 2014, Song et al., 2012). PTEN interacts with proteins containing numerous PDZ domains, including PAR3 and MAGI1b, to regulate the development of junctional complexes (Kotelevets et al., 2005, Yan et al., 2015). PTEN also regulates ciliogenesis in the ependyma and trachea, which helps to regulate planar cell polarity. Disheveled phosphorylation promotes cilia disintegration (Shnitsar et al., 2015). PTEN maintains Dishevelled in a hypophosphorylated state via its protein phosphatase activity regulating ciliary homeostasis (Shnitsar et al., 2015). As a result, (PTEN Hamartoma Tumor Syndrome) PHTS and autism patients who have PTEN mutations have an abnormal ciliary function (Louvi et al., 2011, Ohtoshi et al., 2008, Shnitsar et al., 2015).

1.6.2 Cellular Metabolism

PTEN controls metabolism through both PI3K/AKT independent and dependent mechanisms (Garcia-Cao et al., 2012). PTEN also regulates lipid metabolism via the

transcription factor MAF1 (Palian et al., 2014). Increased energy expenditure might be attributable to increased oxidative phosphorylation in mitochondria and a concurrent anti-Warburg effect which is the reduction in anaerobic glycolysis (Ortega-Molina et al., 2012, Garcia-Cao et al., 2012). Mice overexpressing PTEN have been demonstrated to increase insulin sensitivity when they were fed with a high-fat or normal diet compared to wild-type mice (Ortega-Molina et al., 2012). Mice also have higher energy expenditure, lower body weight, and lower fat accumulation (Ortega-Molina et al., 2012, Garcia-Cao et al., 2012). Furthermore, mice showed lower glutamine metabolism and absorption (Garcia-Cao et al., 2012). As a result of PTEN-mediated glycolysis suppression and glutaminolysis, a tumor-suppressive metabolic state is promoted. Furthermore, because it influences metabolism and energy expenditure, PTEN is an appealing medicinal molecule for decreasing obesity. Thus, PTEN mutation may be associated with insulin sensitivity and obesity (F. Mitchell., 2012).

1.7 Nuclear Functions of PTEN

1.7.1 Nuclear localization

Since PTEN lacks both a traditional NLS (nuclear localization signal) and a CLS (cytosolic localization signal), there are several hypotheses regarding the processes of PTEN nuclear import and export. Post-translational modification, protein-protein interaction, active shuttling, and simple diffusion are all examples of this. Because the processes are very dynamic and complicated depending on the cell cycle phase, they may change for various tissues and cell types.

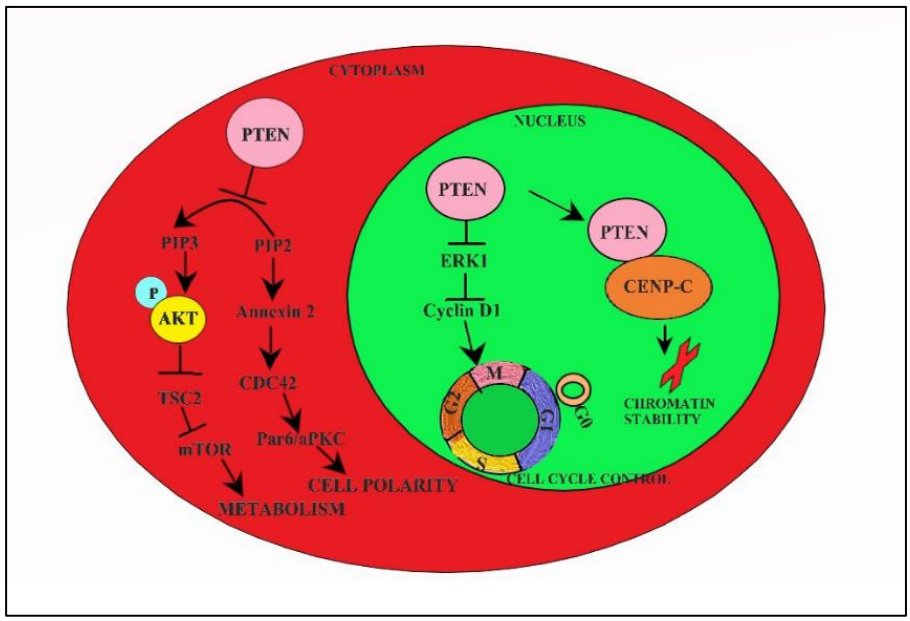


Figure 1.7.1: *PTEN localizes to the cytoplasm as well as in the nucleus. Cytoplasmic functions lead to cell polarity and metabolism whereas nuclear functions lead to G0-G1 arrest and also interact with CENP-C enhancing chromatin stability.*

Liu et al. proposed simple diffusion as the route of nuclear import of PTEN (Liu et al., 2005). Proteins having a molecular weight of 20 kDa might diffuse through nuclear pores, with a top limit of 60 kDa molecular weight. Despite this restriction, other bigger fusion proteins could not enter the nucleus, whereas GFP-PTEN fusion proteins (approximately 75 kDa) do. Even though having a 75 kDa molecular weight of GFP-PTEN, Liu et al. contended that this cut-off was determined by the structure of the protein rather than its molecular weight. However, without GFP fusion, the 50 kDa molecular weight of PTEN, makes it appropriate for easy diffusion.

Terrien et al. identified another route of PTEN nuclear import, demonstrating that PTEN nuclear localization is dependent on interactions of MAST2 with the PTEN PDZ domain (Terrien et al., 2012). A viral glycoprotein disrupts this connection and enhances PTEN nuclear exclusion in human neuroblastoma cells.

Gil et al. discovered active shuttling of PTEN to the nucleus while investigating a potential PTEN transporter, Ran-GTPase (Gil et al., 2006). Immunofluorescence experiments demonstrated that (amino acid residues 8-32) at the N-terminus PTEN have a nuclear localization domain when wild-type and mutant PTEN constructs were overexpressed in the PTEN-negative U87MG cell line. Wild-type Ran GTPase mediates active nuclear import requires these N-terminal residues, but not by GTPase-deficient dominant-negative mutant RANQ69L, which was unable for PTEN nuclear import. Furthermore, (aa 370-385) in the C-terminal region point mutations at phosphorylation sites and C-terminus truncations appear to prefer PTEN nuclear localization. Chung et al., however, were unable to confirm the significance of C-terminal phosphorylation sites in the nuclear localization of PTEN. (Chung et al., 2005). They found no difference in PTEN nucleo-cytoplasmic location in PTEN WT MCF7 cells when they overexpressed some of these PTEN phospho-mutants. These disparities might be attributed to various model systems (breast cancer and brain cancer) or differing PTEN status of the cell lines. Chung et al employed wild-type PTEN-expressed breast cancer MCF7 cells, whereas Gil et al used glioblastoma cells U87MG that lack endogenous PTEN protein. Endogenous PTEN protein may influence the subcellular distribution of phospho-mutants via protein-protein interactions. C-tail phosphorylation of PTEN regulates its dimerization. PTEN C-tail phosphorylation forms a monomer whereas the absence of phosphorylation causes dimeric conformation (Papa et al., 2014).

Yu et al. proposed a second mechanism for active PTEN shuttling to the nucleus. (Yu et al., 2002), An innovative interacting partner of PTEN MVP (major vault protein) was identified by using a yeast-two hybrid by him. MVP hypothesized to have involvement in nuclear-cytoplasmic trafficking, is a 13 MDa ribonucleoprotein complex and a vault component (Mossink et al., 2003). The binding of MVP and PTEN is mediated via the EF hand-like motif of MVP and the C2 domain of PTEN. Experiments using co-immunoprecipitation in

HEK293T and HeLa cells verified this connection. Further research indicated that phosphorylation of PTEN and phosphatase activity of PTEN is not necessary (Chung et al., 2005) for this interaction which is Ca2+-dependent (Yu et al., 2002; Minaguchi et al., 2006). Interestingly, mutations in NLS-like regions discovered in PTEN might impair this connection. Immunofluorescence and site-directed mutagenesis investigations in MCF7 cells revealed four distinct NLS-like sequences (NLS1 to NLS4). Only mutations of two or more of these NLS-like sequence locations, namely NLS3 and NLS4 or NLS2 and NLS3, exclude nuclear PTEN, preventing its binding to MVP, as opposed to mutations of any individual sequence, which do not affect PTEN localization (Chung et al., 2005).

PTEN nuclear import needs monoubiquitination, especially at lysine 289 is another possible method. (Trotman et al., 2007). PTEN nuclear exclusion was a result of a mutation at lysine 289 residue in a patient with Cowden syndrome (CS) from lysine to glutamate (K289E). Immunofluorescence studies with GFP-PTEN K289E and GFP-PTEN revealed that the GFP-PTEN was present in both the nucleus and the cytoplasm, whereas the GFP-PTEN K289 mutant was mostly detected in the cytosol. K289E mutation retains its PTEN membrane localization or phosphatase activity as the import of PTEN K289 to the nucleus is reduced which is independent of the phosphatase activity of PTEN (Georgescu et al., 2000). Interestingly, a member of the NEDD4 family of E3 ubiquitin ligases, NEDD4-1, may ubiquitinate lysines 289 and 13. It operates as an E3 ligase as well as a PTEN binding partner. as involved PTEN polyubiquitination involves a proto-oncogenic ubiquitin ligase NEDD4-1 (Wang et al., 2007). Mutations in either K13 or K289 cause a shuttling deficiency that can be repaired by monoubiquitination. Furthermore, RNAi suppression of NEDD4-1 enhances PTEN levels and protein stability, resulting in cytosolic localization of PTEN, whereas overexpression increases nuclear localization of PTEN, resulting in proteasomal destruction of endogenous PTEN by polyubiquitination. PTEN has two

classical PEST motifs, these sequences are degraded by the ubiquitin pathway and linked with short intracellular half-life proteins. NEDD4-1 is not required for the control of PTEN stability and localization discovered by Fouladkou et al. (Fouladkou et al., 2008). The interaction of Nedd4-1 with PTEN using cells and tissues from two separate Nedd4-1 deficient mouse strains did not confirm by them. Furthermore, the addition of Nedd4-1 did not affect PTEN subcellular distribution, suggesting that it is not a universal E3 ligase for PTEN. Indeed, it was later postulated that the WWP2 and XIAP, a RING domain E3 ligase, and a NEDD4-like E3 ligase, aid in the ubiquitination of PTEN (Van Themsche et al., 2009, Maddika et al., 2011). However, it is unknown if all three ubiquitin ligases can produce PTEN mono- and polyubiquitination.

HAUSP or USP7 is the deubiquitinating enzyme that has been identified as the enzyme that signals PTEN for nuclear export removing ubiquitin from it. Song and colleagues (2008) Experiments with co-immunoprecipitation revealed a direct connection between HAUSP and PTEN. Furthermore, siRNA-mediated HAUSP knockdown enhances PTEN monoubiquitination and promotes PTEN mutant at lysine residues 289 or 13 and WT PTEN localization to the nucleus. This implies that to alter PTEN compartmentalization, it is enough that either of the lysines is monoubiquitinated/de-ubiquitinated. Furthermore, PTEN cannot form functional nuclear bodies because PTEN is abnormally located in acute promyelocytic leukemia, where promyelocytic leukemia protein (PML) activity is disturbed. PML inhibits HAUSP's action in these nuclear bodies towards PTEN via a mechanism that involves the adaptor protein DAXX, implying that a PML-DAXX-HAUSP molecular network regulates PTEN de-ubiquitination and trafficking, which can be disrupted in human cancer.

Another route for PTEN nuclear localization has been proposed: SUMOylation (small ubiquitin-like modifier, SUMO). Gonzalez-Santamaria et al. (2012) utilized the SUMOplot

prediction method to identify (IKVE, aa 253-256) a conserved SUMOylation region in the PTEN protein sequence that comprised SUMO-conjugation sites with three lysines (K266, K102, and K289). as SUMO acceptor sites point mutations in C2 domain of PTEN at lysines 266 and 289 were identified. Interestingly, a lysine 266-point mutation in PTEN shows a lowered conjugation of SUMO to PTEN than a lysine 289-point mutation which was previously discovered (Trotman et al., 2007). Overall, these PTEN mutants defective in SUMOylation are mostly found in the cytoplasm and are not found in the nucleus. Because the monoubiquitination site was identified at lysine 266 and it was shown that PTEN SUMOylation inhibits its conjugation with monoubiquitin, indicating the interaction of ubiquitination and PTEN SUMOylation (Gonzalez-Santamaria et al., 2012).

A 75 kDa PTEN species was found in 2013 found to be sensitive when treated with an inhibitor of cysteine-based enzymes, N-ethylmaleimide (NEM) such as deSUMOylases and deubiquitinases that was missing from PTEN-/- cells (Bassi et al., 2013). They inferred that this species was a SUMOylated version of PTEN since mono-SUMOylation can presumably increase the molecular mass of PTEN by 20 kDa. SUMOylation prediction program found a lysine at 254 as the SUMO acceptor site in the PTEN C2 domain. Lysine 254 was verified as the primary site of PTEN SUMOylation (Bassi et al., 2013).

Overall, the pathways hypothesized for PTEN nuclear import imply that the localization of PTEN to the nucleus is controlled by a range of cell types, tissue, and situation-specific variables. Several years following its discovery, the majority of research efforts focused on PTEN's role in the plasma membrane. However, throughout the last decade, researchers have discovered several unique nuclear functions for PTEN. The majority of these actions are unrelated to its lipid phosphatase activity.

1.7.2 Chromosomal Stability

An important role is played by PTEN in the control of the repair of damaged DNA and chromosome integrity. The stability of the centromere is regulated by the C-tail of PTEN associated with CENP-C, a centromere protein. Consequently, PTEN loss results in centromere instability and breakage (Shen et al., 2007). Further, the lack of PTEN C-tail region in transgenic mice develops multiple spontaneous benign and malignant tumors (Sun et al., 2014). A crucial role in regulating DNA damage repair is also played by PTEN. Spontaneous double-stranded breaks (DSBs) are a result of loss of PTEN. Rad51 and Rad52 expression is regulated by PTEN interaction with chromatin facilitating DNA damage repair upon exposure to DNA double-strand damaging agents (Choi et al., 2013, Shen et al., 2007). The PTEN function in efficient DNA replication has been highlighted in several recent studies. ATM-mediated phosphorylation of PTEN also plays a role in response to the damage of DNA (Chen et al., 2015). Due to exposure to UVB PTEN is involved in nucleotide excision repair upon DNA damage. Increasing the transcription of XPC (Xeroderma pigmentosum C), a nucleotide excision repair protein by PTEN positively regulates DNA repair (Ming et al., 2011). DNA forms catenated duplexes in the G2 phases of the cell cycle at the end of replication. Chromosomal instability is a symbol of cancer cells that is the consequence of inappropriate decatenation that causes the delay of cells to enter the M phase of the cell cycle. Associating physically with DNA topoisomerase II (TOP2A) and stabilizing it, DNA decatenation is efficiently regulated by PTEN. In contrast, during anaphase, ultra-fine bridges forms as a result of several decatenation defects in PTEN-deficient cells (Kang et al., 2015).To regulate the DNA replication process PTEN associates with the replication fork components. During the cell cycle in the S phase, several factors such as insufficiency of replication enzymes and nucleotide deficiency cause replication stress as DNA is susceptible to breakage. PTEN associates and MCM2 (mini-

chromosome maintenance 2) is dephosphorylated, a protein involved in replication under these replication stress conditions, progression of the replication fork, and avoiding chromosomal instability and DNA replication defects (Feng et al., 2015).

1.7.3 Cell Cycle Control

Chung et al. presented the first evidence of nuclear PTEN essential for cell cycle arrest (Chung et al., 2006, Chung and Eng, 2005). PTEN regulates the cell cycle is regulated by PTEN via AKT-independent as well as dependent mechanisms (Liu et al., 2005, Sun et al., 1999). PTEN specifically prevents G1/S and G2/M transitions during the cell cycle. Likewise, cell cycle inhibitors p16, p21, and p27 in the G1 phase are also increased by PTEN. Although the G2/M phase of the cell cycle is affected by the phosphorylation of PTEN, the Cyclin B1/CDK1 complex retention to the nucleus is enhanced by dephosphorylation of PTEN which induces arrest at prometaphase (Kim et al., 2016). Further, through the mitotic phase progression of cells occurs when PLK1 phosphorylates PTEN at Ser380 and associates with chromatin. Consequently, mitotic exit is caused by phosphorylation-deficient PTEN (Choi et al., 2014). Song et al proposed another piece of evidence that PTEN controls the cell cycle in the nucleus. He showed that APC/C is regulated by PTEN in a manner independent of phosphatase function in the nucleus (Song et al., 2011). Progression of the cell cycle regulation by proteolysis mediated by ubiquitination of several proteins which are assumed to be active to the late G1 phase from mitosis by APC/C, one of the major E3 ubiquitin-ligases. The degradation of ETS proto-oncogene 2 (Ets2), a transcription factor is promoted by restraining the cyclin D1 expression in the G1 phase. CDC20 and CDH1 control at least 11 different subunits that make the APC/C complex fully functional. The collaboration between APC activator CDH1

and APC is promoted by the activity of E3 ligase of the APC/C complex which in turn is enhanced when PTEN binds to APC/C constituents APC3, APC4, APC5, and APC7 in the nucleus. This leads to a reduction of the oncoproteins PLK1 and Aurora kinase which are the targets of proteasome-dependent APC/C. In non-proliferating cells, cellular senescence is induced when there is a reduction of APC/C-CDH1 activity due to the loss of nuclear PTEN (Chen et al., 2005). In MCF7 cells localization of PTEN occurs in the nucleus where it coincides with the G0-G1 phases of the cell cycle. Cyclin D1 is down-regulated, which is the regulatory subunit of cyclin-dependent kinases 6 and 4 (CDK6 and CDK4) results in cell cycle arrest and regulates the G1/S phase transition and S-phase entry. Previously, it had been shown that the MAPK dephosphorylation as well as cyclin D1 down-regulation requires protein phosphatase activity of PTEN (Weng et al., 2001, Weng et al., 2002). Thus, the cascade which is responsible for the cell cycle arrest mediated by PTEN is the down-regulation of transcription of cyclin D1 which is the consequence of MAPK downregulation in a protein phosphatase-dependent manner by nuclear PTEN. The transition of the G1 phase of the cell cycle is regulated by RB, an important tumor suppressor. By targeting Cyclin D1 which inactivates RB, the G1 phase of the cell cycle is inhibited by PTEN. PTEN is known to inhibit, and Cyclin E/CDK2 and Cyclin D3 activity is inhibited by PTEN thereby activation occurs by hypophosphorylation of RB (Radu et al., 2003, Weng et al., 2001, Paramio et al., 1999, Hlobikova et al., 2006, Zeng et al., 2013, Chung et al., 2006, Zhu et al., 2001, Moon et al., 2004). However, it is not known about the effects of phosphorylation of PTEN on the G1 phase of the cell cycle. Thus, PTEN effects the cell cycle by enhancing p27/p21 and Cyclin D1 down-regulation.

1.7.4 DNA Replication Control

When cells are incapable of repairing their damaged DNA and multiply with unrepaired DNA owing to checkpoint inaccuracies, mutations arise in the genome. As a result, daughter cells pick up mutations from their parents. It poses a major danger to genomic integrity, leading to cancer if Achilles' heel which is the replication stress is not properly managed to inhibit the progression of DNA replication. DNA damage and genomic instability result when cells are unable to adequately overcome stress DNA replication is de-regulated. PTEN protects genome integrity during replication because PTEN-negative or PTEN mutant cells have unstable genomes and high growth potential. A number of publications imply that PTEN post-translational regulation has a role in DNA replication. Initially, two distinct study groups discovered a replication problem indicating that wild-type cells have a lower frequency of lagging chromosomes and anaphase bridges than, PTEN-negative cells (Kang et al., 2015, He et al., 2015). Further investigation revealed that PTEN-deficient cells have impaired replication progression, indicating endogenous replication stress. Following aphidicolin/Hydroxyurea treatment replication fork restart is slower in PTEN-negative cells than in cells transfected with wild-type form. According to the Molecular iPOND study, PTEN itself localizes to halted forks made by HU and binds Rad 51 on chromatin to support effective recovery by PTEN (He et al., 2015). RPA1 interacts with the ssDNA close to freshly generated dsDNA during replication stress. Loading of RPA1 serves to shield single bare strands, stop fork movement, and prepare cells to withstand stress (Haring et al., 2008). PTEN has been shown to enhance RPA1 buildup on the replication fork by binding to the C-terminal region of RPA1. PTEN stabilizes RPA1 by inhibiting its ubiquitination activating the deubiquitinase OTUB1 (Wang et al., 2015). PTEN phosphatase activity is not necessary for RPA1 stabilization at the replication fork. Simultaneously, another study showed that PTEN, via its phosphatase

activity, is implicated in the limiting of replication fork migration rate in response to external replication stress. They discovered that during HU-induced replication stress, PTEN dephosphorylates by physically interacting at the Ser 41 residue of the MCM2 protein, preventing replication fork advancement (Feng et al., 2015). So, under replication stress, for effective fork recovery RPA1 is recruited ahead of replication fork on ssDNA which is created by polymerase uncoupling on helicase by PTEN on one hand, trailed by recruitment of RAD 51 and PTEN dephosphorylates MCM2 helicase on the other. ssDNA coated with RPA functions as a shared intermediate structure required for intra-S checkpoint activation (Zou et al., 2006). As a result, PTEN preserves intra-S-phase checkpoint integrity. PTEN also supports delayed fork recovery when replication stress is eliminated. Furthermore, replicative DNA damage accumulates when loading of RPA1 is hindered because dephosphorylation of MCM 2 does not occur in PTEN null cells, causing genomic instability.

1.7.5 Maintain Spindle Architecture

In the cell cycle of eukaryotic organisms, mitosis is an integral stage where daughter nuclei are formed. Mitosis must be constant and proportional for genetic transmission to be successful and faithful in an organism. Mitosis necessitates a regulatory system, with mitotic spindles playing the most important function (McIntosh et al., 2016). The mitotic spindle is a component of the cytoskeleton structure, which is made up of non-motor microtubule-binding proteins, microtubule-dependent motor proteins, and microtubules (Fraschini et al., 2017). PTEN-negative cells exhibit chromosomal damage such as aneuploidy, chromosomal bud, anaphase bridge, lagging chromosome, micronucleus, and centrosome amplification (Vuono et al., 2016, Di Cristofano et al., 2001, Leonard et al., 2013, Shen et al., 2007, He et al., 2015, Sulkowski et al., 2018). During

mitosis, centrosome integrity and composition are regulated by the PTEN-AKT pathway. To preserve centrosome integrity PTEN interacts with γ-tubulin and PLK1 in the centrosome (Choi et al., 2014, Zhang et al., 2016, Li et al., 2014). In an Akt-independent way, PTEN knockdown decreases the pericentrin level in the centrosome. There is an increased error in metaphase separation and centrosome number that results from hampered recruitment of PTEN at the centrosome and lowered levels of PLK1 and γ-tubulin in the centrosome when AKT is suppressed (Leonard et al., 2014). during metaphase, PTEN detects faulty kinetochore attachment which is also a component of the mitotic checkpoint complex. When there is impairment in the microtubule-kinetochore attachment, the MCC / SAC steps in and stops anaphase, so that attachment can take place correctly. PTEN helps in dimerization, nuclear envelop localization, and SAC timing maintenance by physically interacting with the MAD1 protein, (Liu et al., 2017). PTEN null cells spend much lower time M phase than WT cells, indicating SAC arrest (Zhang et al., 2016, Choi et al., 2014). Another study found that two key components of MCC Mad2 and Cdc20 physically interact with PTEN (Choi et al., 2017). As PTEN knockdown decreases Dlg 1 recruitment at the centrosome, it was found that the scaffold protein, Dlg1 recruitment is PTEN-dependent. For the recruitment of PTEN to the centrosome PTEN is phosphorylated by PLK1 at the C-terminus. This provides a docking site for phospho-EG5 (Ser1033, Thr926) and dlg1 (induced by NEK6, Cdk1), a kinesin required for centrosome movement and correct bipolar spindle formation (Van Ree et al., 2016). By rearranging microtubules a member of the kinesin motor protein superfamily EG5 is important for the assembly and maintenance of mitotic spindles (Valentine et al., 2006). However, another study found that spindle disorder results from inhibition of EG5 recruitment to the mitotic spindle apparatus and abnormal phosphorylation of EG5 caused by loss of PTEN. PTEN dephosphorylates EG5 by physically interacting with EG5 at its Thr 926 residue at the centrosome/spindle pole and

aids in its positioning at the spindle pole (He et al., 2016). These contradictory studies showed that maintaining the phosphorylation state of EG 5 is critical for motor protein efficiency. EG5 phosphorylation must be accurately managed as either hyper- or hypo-phosphorylation of EG5 results in erroneous mitosis. It appears that PTEN-mediated mitotic spindle length maintenance is inhibited by hyperphosphorylation of EG5 but for the PTEN-Dlg1-EG5 complex formation on centrosomes phosphorylation of EG5 is essential. In PTEN-deficient cells, for example, hyperphosphorylation of EG5 at Thr 926 leads to a decreased affinity for spindle microtubules and compromises spindle assembly.

1.7.6 DNA Repair

PTEN deficiency is frequently linked to chromosomal instability (Cantley et al., 1999, Simpson et al., 2001). Furthermore, in Mouse Embryonic Fibroblasts (MEFs) genetic loss of PTEN produces an increase in double-strand breaks in unrepaired DNA (Shen et al., 2007). PTEN deficiency is expected to affect genomic integrity via at least two molecular pathways (Trotman et al., 2007, Puc et al., 2005). PTEN promotes metaphase-to-anaphase transition and kinetochore assembly by connecting with the CENP-C in the nucleus, a centromeric binding protein (Shen et al., 2007). Furthermore, PTEN in the nucleus seems to control the expression of a crucial component of the DNA repair process, Rad51, by functioning as a cofactor for the transcription factor E2F1 (Shen et al., 2007). However, further research (Gupta et al., 2009) has shown inconclusive findings, indicating that control of RAD51 by PTEN at the transcriptional level may be confined to certain cell settings. Loss of PTEN disrupts many checkpoints of the cell cycle, perhaps allowing less time for chromosomal segregation and/or DNA repair (Gupta et al., 2009). To ensure competent and error-free cell division, the progression of the cell cycle necessitates the faultless execution

of various molecular processes executed in a timely way. The cyclin-dependent kinases (CDKs) phosphorylate critical substrates to facilitate mitotic progression and DNA synthesis, determining the rate at which these processes occur. Checkpoints of the cell cycle control the proper execution of the cell cycle's key events and regulate the catalytic activity of CDKs. Checkpoints are fail-safe systems that guarantee that cell division occurs only when ideal conditions are fulfilled (Barnum et al., 2014). All organisms require the maintenance of proper genomes via cell division to enable optimal reproduction, development, and disease prevention, including cancer., induced by abortive topoisomerase I and topoisomerase II activity induces endogenous mechanisms such as DNA strand breaks, DNA mismatches generated during DNA replication or ROS created from normal metabolic wastes that may attack DNA can all cause DNA damage. Mutagenic substances, UV, and ionizing radiations are the most common exogenous sources (IR). Cell cycle checkpoints can detect DNA damages, indicate their existence, and trigger pathways that repair DNA lesions, delay cell cycle progression, or remove genetically unstable cells by cell death (Hoeijmakers., 2001, Lukas et al., 2004, Bartek et al., 2007, Bartek et al., 2004, Shiloh., 2003, Kastan et al., 2004). The eukaryotic cell repairs its own damaged DNA through either NHEJ or HR. NHEJ is an error-prone mechanism that occurs throughout the cell cycle, whereas HR is a more specific and error-free mechanism that happens primarily in the S phase. Cancer-related mutation accumulation and instability result from compromising one or both of these networks (Furgason et al., 2013, Kelley et al., 2014, Helleday et al., 2008). several groups showed PTEN depletion reduces the frequency of homologous recombination repair, using an in vivo plasmid-based assay. When they transiently transfected PTEN into PTEN-negative cells HR frequency was substantially increased (Mansour et al., 2018, Bassi et al., 2013, McEllin et al., 2010). According to recent research, DNA ligation and end bridging are involved through the production of an

XRCC4-like factor (NHEJ1/XLF) which is directly induced by PTEN enhancing the NHEJ repair pathway. PTEN induces XLF expression by interacting and binding the PCAF and CBP, histone acetyltransferases to the XLF promoter when acetylated at Lys128 (Sulkowski et al., 2018). Again, for non-homologous end joining (NHEJ) mediated by 53BP1 protein, H3K9me3 is an epigenetic marker is required (Zhang et al., 2018, Svobodova' Kovarikova' et al., 2018). Because PTEN deficiency results in the disorganization of chromatin and impairs expression and foci formation of H3K9me3 protein, PTEN via the 53BP1-mediated pathway might indirectly favorably regulate NHEJ.

1.7.7 Maintain chromatin Architecture

PTEN, in addition to chromatin interaction, inhibits centromere breaking and aneuploidy by stabilizing centromeric areas. The interaction of PTEN with centromeres promotes chromosomal integrity, potentially by regulating homologous recombination (Di Cristifano et al., 2001, Shen et al., 2007, Leonard et al., 2013, Vuono et al., 2016). PTEN requires its C-terminal tail to localize and physically interact with the centromeric protein CENP-C in the absence of phosphatase activity (Shen et al., 2007). By interacting with and stabilizing HP1, PTEN has been discovered to maintain the heterochromatin state of chromatin. Furthermore, in Mouse Embryonic Fibroblast cells, deletion of PTEN resulted in a considerable drop in the intensity of H3K9me3 foci, indicating heterochromatin de-condensation (Gong et al., 2015). Again, it is discovered that PTEN loss causes histone H1 separation from chromatin and, as a result, higher levels of acetylation histone H4 at lysine 16 occur which is an epigenetic marker for activation of chromatin (Chen et al., 2014) and PTEN physically interacts via its C-terminal domain with histone H1, but shortened PTEN does not bind with histone H1. Compromised heterochromatin might result in widespread epigenetic instability, potentially affecting gene expression patterns and eventually leading

to aberrant growth characteristics (Carone et al., 2013). Epigenetic signature H3K9me3 disruption, which is linked with late-replicating heterochromatin, enhances accessibility to chromatin, which advances the time of replication and leads to re-replication (Du et al., 2019). As a result, the maintenance of heterochromatin is critical for the stability of cancer cell genome, which is linked to replication starts. Aside from replication, lack of heterochromatin affects transcription of the universal gene, which is another feature of PTEN-negative cells. Therefore, PTEN prevents uncontrolled transcription and replication by keeping chromatin in an inactive condensed state.

1.8 Post-Translational Modifications (PTMs*)* of PTEN

PTEN activity is modulated by a variety of PTMs, which include phosphorylation, acetylation, oxidation, and ubiquitination. Some of these PTMs are described below.

1.8.1 Phosphorylation

The major PTM is phosphorylation which controls PTEN stability and its activity. Phosphorylation of PTEN in the C-terminal tail and C2-domain include threonine, tyrosine, and serine residues. The c-tail region consisted of almost 50% of the phosphorylation modifications. C-tail folds upon phosphorylation, producing a “closed” conformation (Bolduc et al., 2013, Rahdar et al., 2009, Odriozola et al., 2007,). Due to the membrane-binding C2 domain blocking, there is a reduction in membrane binding of the closed-form, and hence phosphatase activity is reduced. But, “closed” conformation characterizes a more stable form of the protein (Rahdar et al., 2009, Odriozola et al., 2007, Vazquez et al., 2000). The “open” conformation represents serine-threonine cluster

dephosphorylation, which has a PIP3 phosphatase activity and greater membrane binding affinity, even if stability is low (Rahdar et al., 2009, Odriozola et al., 2007, Vazquez et al., 2000). Therefore, an inactivation mechanism is represented by the phosphorylation of PTEN at the C-tail cluster. Oxidative stress phosphorylates Ser380 by S6K, which results in the nuclear exclusion of PTEN (Wu et al., 2013). Again, PTEN is phosphorylated at Ser362, Thr366, and Ser370 by glycogen synthase kinase 3-beta (GSK3-β) (Maccario et al., 2007, Singh et al., 2011, Al-Khouri et al., 2005). In addition, PTEN is phosphorylated at Thr366 and Ser370 by Plk3 (Xu et al., 2010). PTEN is phosphorylated at Ser113 and Thr398 by ATM which regulates the transport of PTEN to the nucleus (Bassi et al., 2013, Chen et al., 2015). PTEN is phosphorylated by Plk1 at Ser380, Thr382, and Thr383 where the Ser380 phosphorylation is crucial for the localization of PTEN to chromatin during the mitosis of the cell cycle (Choi et al., 2014). PTEN is phosphorylated at Ser385 by PLK1, which reduces the monoubiquitination of PTEN reducing its accumulation in the nucleus, and inhibits its polyubiquitination also decreases the catalytic activity (Li et al., 2014). PTEN phosphorylation enhanced by PICT-1 at Ser380 protects it from degradation (Singh et al., 2011, Okhara et al., 2004). RhoA-associated protein kinase (ROCK) modulates intracellular localization of PTEN during cell migration and chemotaxis by phosphorylating PTEN at Thr223, Ser229, T319, and T321 (Song et al., 2012, Li et al., 2005). PTEN is phosphorylated at Tyr336 a tyrosine kinase, Rak which downregulates its ubiquitination by NEDD4-1, the E3 ligase (Song et al., 2012, Yim et al., 2009). PTEN is phosphorylated at Tyr240, Tyr336, and Tyr315 by Src kinase occurs destabilization of PTEN by upregulating the PI3K pathway (Lu et al., 2003, Singh et al., 2011,). PTEN is phosphorylated at Tyr27 and Tyr174 by IRβ which physically binds with PTEN (Liu et al., 2014). Therefore, the level of various kinases in the cytoplasm involved in PTEN phosphorylation differentially

controls PTEN stability. PTEN C-tail regions are phosphorylated at Ser380, Thr382, Thr383, and Ser385 regulating its stability and activity.

1.8.2 Acetylation

PTEN is acetylated at Lys128 and Lys125 by a histone acetyltransferase protein (HAT), PCAF (P300/CBP-Associated Factor). The phosphatase activity of PTEN interferes with acetylation as the residues which are modified are situated within the catalytic cleft increasing PI3K activity (Singh et al., 2011, Okumura et al., 2006). translocation of PTEN to cell junctions is increased when PTEN is acetylated at Lys402 by CBP (CREB Binding Protein) acetyltransferase that increases the PDZ-domain interaction containing protein MAGI-2 (Singh et al., 2011, Ikenoue et al., 2008). PTEN tail and body interaction causing its membrane translocation is reduced when the acetylation of PTEN occurs at Lys163 in presence of HDAC inhibitors (Meng et al., 2016).

1.8.3 Ubiquitination

Polyubiquitination and monoubiquitination of PTEN cause proteasomal degradation and nuclear translocation respectively. A recently known E3 ligase, RFP controls poly-ubiquitination of PTEN at multiple lysine residues which reside in its C2 domain (Lee et al., 2013), inhibiting the phosphatase activity of PTEN and thereby potentiating PI3K activity (Lee et al., 2013). The stability of PTEN protein does not affect by K27-linked PTEN polyubiquitination at Lys342 and Lys344 but suppresses its dimerization (Papa et al., 2014, Pandolfi et al., 2016).

1.8.4 Oxidation and S-nitrosylation

between Cys71 and Cys124 a disulfide bridge is formed within the catalytic pocket of PTEN which inactivates it (Lee et al., 2002) due to oxidative stress. Peroxiredoxin, a peroxidase enzyme shields PTEN itself from oxidative inactivation (Singh et al., 2011, Cao et al., 2009), whereas catalytically active PTEN is regenerated by thioredoxin once PTEN is oxidized (Hui et al., 2008). At the residues Cys83, Cys71, and Cys124 s-nitrosylation enhance the degradation of PTEN via ubiquitination (Singh et al., 2011, Kwak et al., 2010). In animal models of cerebral ischemia and early Alzheimer's disease PTEN nitrosylation is observed (Singh et al., 2011).

1.8.5 SUMOylation

Post-translational modification SUMOylation is involved in many physiological processes, including apoptosis, transcriptional control, nuclear-cytoplasmic localization, stress response, cell cycle progression, and protein stability (Melchior and Geiss-Friedlander, 2007). Ubiquitin-like SUMO proteins are a kind of molecule that operates through an enzyme cascade similar to ubiquitin. It can covalently adhere to and detach from proteins in order to change their functionality.

1.9 Other Post-Translational Modifications

Other post-translational modifications of PTEN include lysine methylation and ribosylation. Methylation and ribosylation inhibit PTEN function (Mund et al., 2010, Nakakido et al., 2015). PTEN is methylated at Lys349 and regulates DNA damage repair (Zhang et al., 2016).

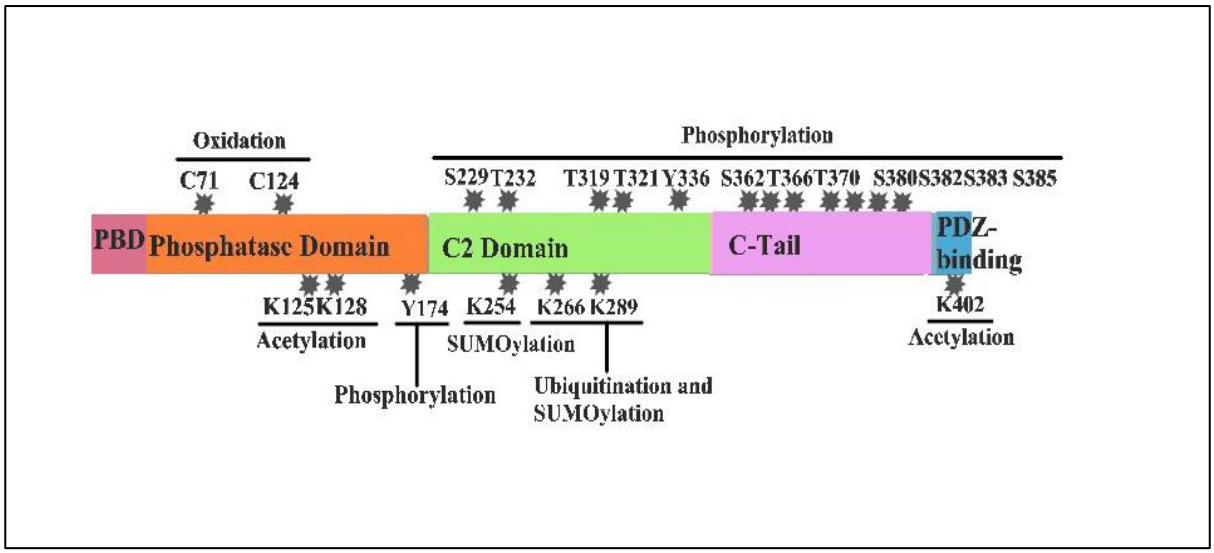


Figure 1.9: *Post Translational Modification of PTEN occurs by oxidation, acetylation, and phosphorylation in the phosphatase domain, phosphorylation, ubiquitination, and sumoylation in the C2 domain, phosphorylation in C-tail and acetylation in PDZ binding domain.*

Rationale Behind the Study

The Rationale Behind the Study

The function of PTEN which has been described till now, suggests that its tumor-suppressive properties extend beyond its ability to inhibit AKT and include DNA damage repair. In order to develop a strategy to combat cancer and other diseases associated with PTEN mutations, it is imperative to have a detailed understanding of the role of the protein PTEN which acts in suppressing tumors, particularly the role of nuclear PTEN in genomic stability maintenance. This is because the majority of chemotherapeutic drugs currently in use target proteins involved in the DNA repair pathway. Additionally, the catalytic function of PTEN is maintained by numerous post-translational modulations, which are also accountable for its cellular and nuclear trafficking. Additionally, it has been revealed that nuclear PTEN is resilient to the destruction caused by ubiquitination. It would appear that PTEN function in the nucleus is delicately controlled by posttranslational regulation. It is crucial to investigate how PTEN's post-translational alterations appear to relate to its nuclear activity. As a result, efforts have been made to address PTEN's additional role in genomic instability which is controlled by its post-translational modification. As mentioned earlier major modifications of PTEN which may contribute substantially to maintain genomic instability are SUMOylation and phosphorylation, along with its phosphatase activity, the canonical function of PTEN. So, we aimed to create SUMO-dead and phosphatase and phosphorylation dead dual mutant PTEN and look over the effect of these mutants in terms of aneuploidy and genomic stability.

Therefore, an approach for treating cancer when PTEN is altered or deleted can be created if we understand how PTEN modulation influences the genomic stability of cells.

Chapter 2

Dephosphorylation of the mitotic kinases Aurora Kinase A and PLK1by phospho PTEN to maintain genomic stability and avoid aneuploidy

Dephosphorylation of the mitotic kinases Aurora Kinase A and PLK1 by phospho PTEN to maintain genomic stability and avoid aneuploidy

2.1 Introduction

PTEN, a well-recognized protein playing the role of a tumor suppressor is the 2nd most commonly mutated protein after p53 (Yin et al., 2008). Inhibition of the cancerous PI3K/Akt pathway is the canonical function of PTEN which has phosphatase activity for dephosphorylating both protein and lipid (Stambolic et al., 1998, Myers et al., 1998). Bestowing numerous studies shows abnormal chromosomal characteristic like the nucleoplasmic bridge, telomere fusion, chromosomal breakage, etc, and genomic instability are the features of PTEN null cells (Shen et al., 2007). As a matter of fact, the preservation of chromatin architecture and genomic stability is a consequence of the localization of PTEN to the nucleus/chromatin (Shen et al., 2007). Numerous research provided shreds of evidence that genomic instability is inhibited by PTEN through a variety of functions, including controlling cell cycle checkpoint (Brandmaier et al 2017), homologous recombination (HR) repair (Mansour et al., 2018), regulation of SAC (Bassi et al., 2013, Hou et al., 2017), and replication fork stability (He et al., 2015). During cell division in metaphase, PTEN also prevents unregulated aneuploidy and segregation of chromatin (Puc et al., 2005, Sun et al., 2014, Yuen et al., 2005).

Some reports also proposed that the post-translational modulations, including ubiquitination, acetylation (Meng et al., 2016), phosphorylation (Vazquez et al., 2001), sumoylation (Lang et al., 2015), and oxidation (Zhang et al., 2020) attributed to the catalytic functions of PTEN. PLK (Choi et al., 2014), GSK-3 (Vazquez et al., 2000), and CK2 (Al-Khouri et al., 2005) are some protein kinases that significantly phosphorylate PTEN at the C-tail region. In the C-terminal tail, several sites of phosphorylation at serine 370, serine 380, threonine 382,

threonine 383, and serine 385 residues have been found. It's interesting to note that STT motif (Ser 380, Thr 382, and Thr 383 residues) phosphorylation increases PTEN stability while concurrently reducing its phosphatase activity (Misra et al., 2014). It has been proposed that this STT cluster's mutations or dephosphorylation render it accessible for ubiquitination (Torres et al., 2001) while also increasing its phosphatase activity (Ginn-Pease et al., 2003, Sun et al., 1999).

Among several mitotic kinases, PLK1 controls various events of the cell cycle, including DNA repair, mitotic entry, and bipolar spindle formation (Barr et al., 2004, Strebhardt., 2010, Bassermann et al., 2008, Liu et al., 2003, Smits et al., 2000, Liu et al., 2010, Van Vugt et al., 2004). Aurora kinases (AURKs), another mitotic kinase, on the other hand, are involved in mitotic entry, spindle assembly, and centrosome activity (Fu et al., 2007). Evidence suggests that AURKA is autophosphorylated as a result of the accumulation of the CEP192 complex in the pericentriolar material. AURKA's kinase activity activation by this autophosphorylation causes PLK1 to be phosphorylated. The CEP192-AURKA-PLK1 complex produces attachment sites for the γ-tubulin ring complex during microtubule nucleation, whereas hyperactivity causes aneuploidy and hinders normal chromosomal segregation (Joukov et al., 2018). Numerous malignancies, including esophageal squamous cell carcinoma, bladder cancer, and breast cancer have been linked to the overexpression of AURKA (Yang et al., 2007, Staff et al., 2010, Sen et al., 2002). AURKA hyperactivation encourages centrosome amplification, which leads to the failure of cytokinesis and the subsequent creation of multinucleation (Meraldi et al., 2002). Additionally, despite the formation of an abnormal spindle anaphase bridge, breakage or fragmentation of chromosomes, and lagging chromosomes which result from overexpression of AURKA in cells skipping the spindle checkpoint during mitosis and causing cells to enter anaphase

(Anand et al., 2003). Multinucleated cell formation has previously been linked to PTEN deficiency (Mukherjee et al., 2013).

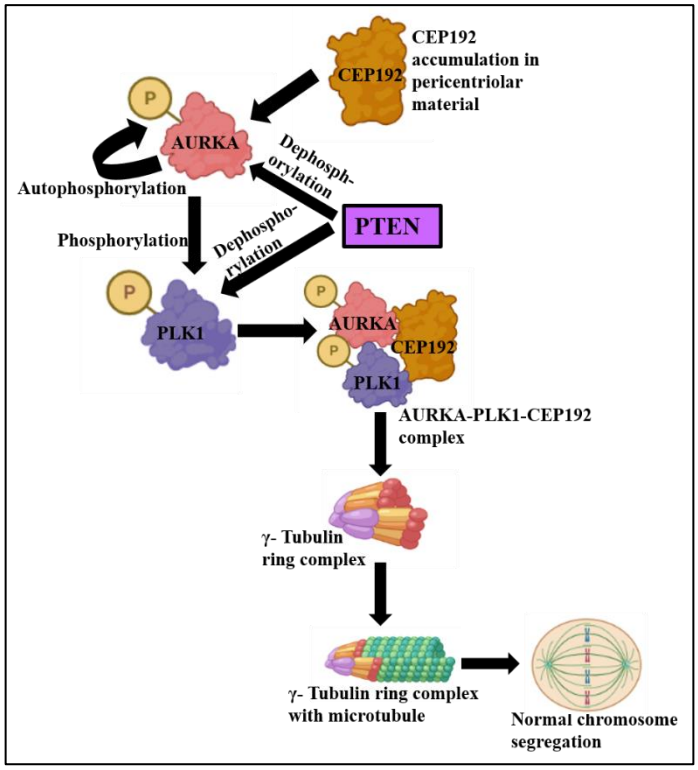


***Figure 2.1**: Illustration of PTEN regulating PLK1 and AURKA for normal chromosomal segregation.*

There are separate studies on PTEN phosphatase and phosphorylation activity in the context of genomic stability. However, no reports are available on the interplay of the PTEN phosphatase and phosphorylation activity together with genomic stability and cytological damage to the best of our knowledge.

2.2 Aims and Objective of the Study

- To generate different PTEN post-translational modulations by creating various point mutations in Wt-PTEN through Site-Directed Mutagenesis eg. Phosphatase mutant and phospho-phosphatase double mutant.

- To show the effect of different PTEN modulations in regulating cytogenetic damage and mitotic abnormality.

- To study the co-relation between cytological damage, aneuploidy, spindle pole, and different PTEN modulation.

2.3 Results

2.3.1 Generation of different PTEN clones by site-directed mutagenesis and their expression

Different PTEN mutant clones were constructed from Wildtype-PTEN by a site-directed mutagenesis kit purchased from Agilent according to the instructions given by the manufacturer. A single colony was isolated after transformation, sequenced, and confirmed with the help of sequencing primer.

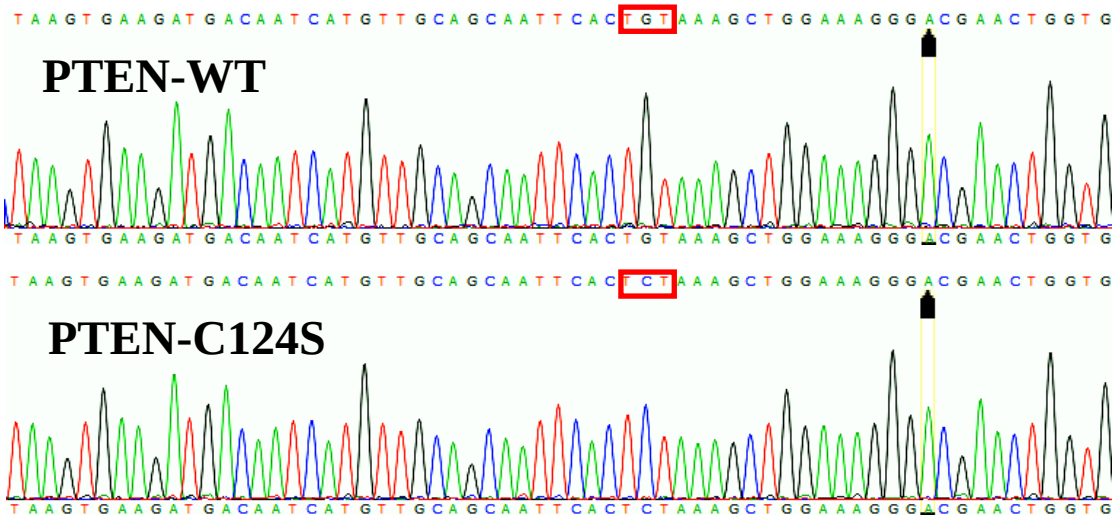


***Figure 2.3.1**: DNA sequencing result of Phosphatase dead PTEN (C124S).*

2.3.2 Depletion of PTEN phosphatase and phosphorylation activity causes an elevation in the level of cytological damage.

Micronuclei, nuclear buds, and nucleoplasmic bridges are chromosomal instability biomarkers that may be detected with the Cytokinesis Block Micronucleus test. Micronuclei are probably the fate of genotoxic events because they arise from chromosomal breaks.

[Hatch et al., 2013]. In the micronuclei of cancer cells, catastrophic nuclear envelope collapses. (Hatch et al., 2013). Missegregated chromosomes in the nucleus create cytoplasmic DNA during successive cell divisions, resulting in the buildup of micronuclei. To quantify global chromosomal damage, we employed the CBMN test, which is extensively used to measure both unreplicated DNA breaks and chromosome loss (El-Zein et al., 2008).

Table I: *The results of cytokinesis-block micronucleus assay in different clones of PTEN after 4h exposure to 100µM etoposide.*

MUTANTS	MNi per 1000 BN cells		NBud per 1000 BN cells		NPB per 1000 BN cells	
	0h	24h	0h	24h	0h	24h
UT	77±2.98	65±3.79	28±1.82	37±1.5	17±2.21	20±2.1
PTEN-WT	49±1.89[a]	25±2.06[b]	14±1.73[a]	9±1.26[b]	6±0.58[a]	3±0.5[b]
PTEN-A4	48±1.15	36±2.16	36±0.82	20±1.82	10±1.29	7±0.96
PTEN-C124S	23±2.36[c]	49±3.20[d]	15±3.09[c]	27±3.30[d]	5±0.5[c]	14±2.38[d]
PTEN-A4-C124S	56±3.16[c]	66±2.88[d]	32±2.58[c]	47±2.5[d]	15±2.75[c]	20±2.94[d]

Note: MNi: Micronuclei; NBud: Nuclear Buds; NPBs: Nucleoplasmic Bridges

Values are shown as for means ± SEM of three independent experiments.

a $p<0.05$ compared with UT 0h (Student's t-test).

b $p<0.05$ compared with UT 24h (Student's t-test).

c $p<0.05$ compared with PTEN 0h (Student's t-test).

d $p<0.05$ compared with PTEN 24h (Student's t-test).

Transfection with A4-PTEN, C124S-PTEN, Wildtype-PTEN, and A4-C124S PTEN (Fig 2.3.2A) in PTEN deficient PC3 cells, etoposide treatment was done for 4 h, and CBMN assay was used to score NBUDs, NPBs, and MNi at 0 h and recovery time point of 24 h. (Table 1).

A considerable surge in MNi (Fig 2.3.2BIV) production induced by etoposide after 24-hour recovery was found in cells transfected with both (phosphatase and phosphorylation dead) A4-C124S-PTEN and (phosphatase dead) C124S- PTEN mutants (66±2.88 and 49±3.20, respectively) compared to Wildtype-PTEN transfected cells (25±2.06). Formation of Nuclear bud (Fig 2.3.2BIII) was also increased in double mutant and phosphatase dead mutant transfected cells compared to Wildtype-PTEN (A4-C124S-PTEN 47±2.5 and C124S-PTEN-27±3.30 vs.9±1.26 at 24h) and NPBs (Fig 2.3.2BII), which are markers of DNA mis-repair or telomere end fusion and amplified DNA elimination, was higher in both C124S-PTEN and A4-C124S-PTEN.

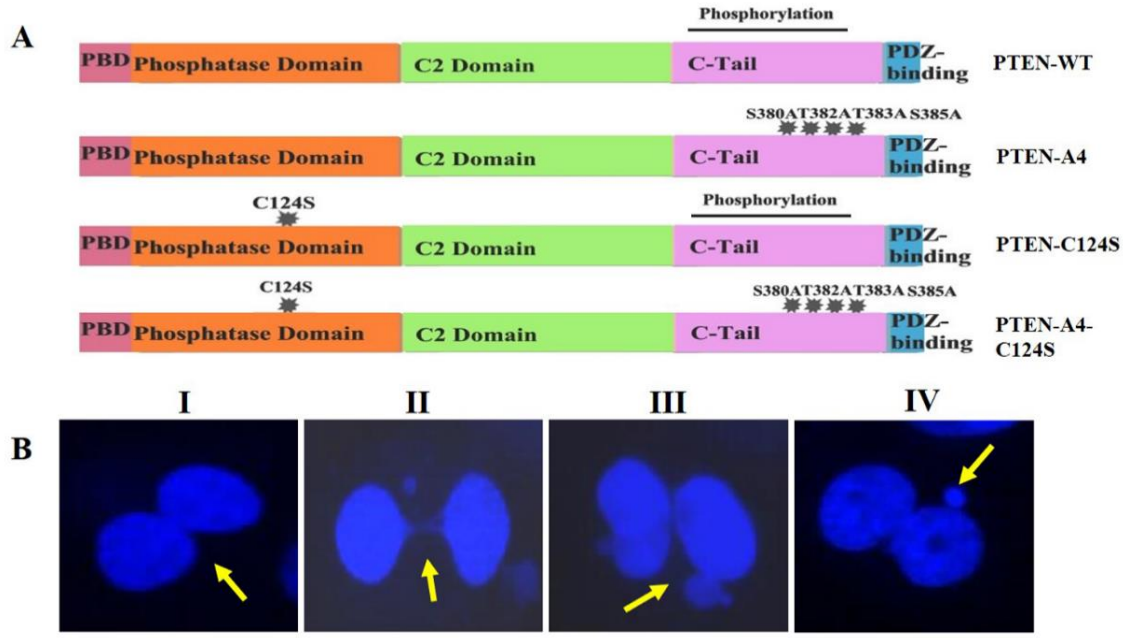


Figure 2.3.2*:Occurrence of cytological damage in different mutants of PTEN. A. Map of different mutants of PTEN (Wt-PTEN, A4-PTEN, C124S-PTEN, A4-C124S-PTEN). B. Microscopic images of cells incubated in presence of etoposide (100µM for 4h) and allowed recovering for 24h. Cells were stained with DAPI. Arrowheads indicate I. Binucleated cells II. Binucleated cells with MNi and NPB III. Binucleated cells with MNi and NBud IV. Binucleated cells with MNI.*

Interestingly, (A4-PTEN) phosphorylation-dead PTEN efficiently eliminates MNi but less than the other PTEN mutant clone transfected cells but not as efficiently as cells transfected with Wildtype-PTEN (Table 1). Thus, our finding suggests that conventional phosphatase activity is crucial for preventing the buildup of cytological damage. Further C-terminal phosphorylation of PTEN, which acts as a molecular switch to negatively limit phosphatase activity (Vazquez et al., 2001), is also required, although it appears to be less significant than phosphatase activity since the PTEN mutant cells exhibit more genomic instability. A4 - PTEN has abnormal phosphatase activity, however, it is unstable in vitro and in vivo. DNA damage repair has previously been linked to PTEN phosphorylation (Misra et al., 2014). Higher levels of phospho-Akt in cells transfected with C124S-PTEN may operate as a driving factor for cell division with damaged unrepaired DNA, circumventing the checkpoint block that leads to the buildup of NBud, NPB, and MNi at the recovery time point of24-hour. Unstable PTEN is unable to repair DNA damage, which may lead to the buildup of MNi. Thus, when both phosphorylation-dead PTEN and phosphatase-dead were utilized, damage buildup is magnified due to increased levels of phospho-AKT and impaired DNA repair capabilities.

2.3.3. PTEN phosphatase and phosphorylation activity trigger the formation of multiple poles during metaphase and suppress spontaneous aneuploidy.

Development of the γ-tubulin pole at the centrosome is the foremost important step for proper chromosomal segregation in metaphase. Multiple pole formation during metaphase is detrimental to cell fate as this causes chromosome missegregation and aneuploidy. It has been described that PTEN-deficient cells impulsively display aneuploidy reflecting whole chromosome instability.

Microscopic images represent the formation of multiple poles after transfection with different mutants of PTEN together with control in $PTEN^{-/-}$ cell line as detected with γ-tubulin and α-tubulin followed by double thymidine block (Fig 2.3.3.1A). Evaluation of the γ-tubulin pole formation devoid of both phosphatase and phosphorylation activity of PTEN detected 1.3 fold drop in bipolar cell percentage in the PTEN clones together with untransfected cells compared to Wildtype-PTEN (Fig 2.3.3.1B) transfected cells. There is a 10-12 fold rise in multipolar cells in untransfected, C124S-PTEN and A4-C124S-PTEN transfected as well as an 8-10 fold escalation in cells transfected with A4-PTEN in contrast to Wildtype-PTEN (Fig 2.3.3.1C) transfected U87MG cells. A 1.5-fold decline in bipolar cells percentage was detected in untransfected, A4-C124S–PTEN, C124S- PTEN, and 1.4 fold decrease was detected in A4-PTEN compared to Wildtype-PTEN transfected PC3 cells (Fig 2.3.3.1E). On the other hand, there is a 6-11 fold surge in multipolar cell percentage in all the phosphorylation and phosphatase dead clones of PTEN that includes untransfected cells than Wildtype-PTEN transfected PC3 cells (Fig 2.3.3.1F).

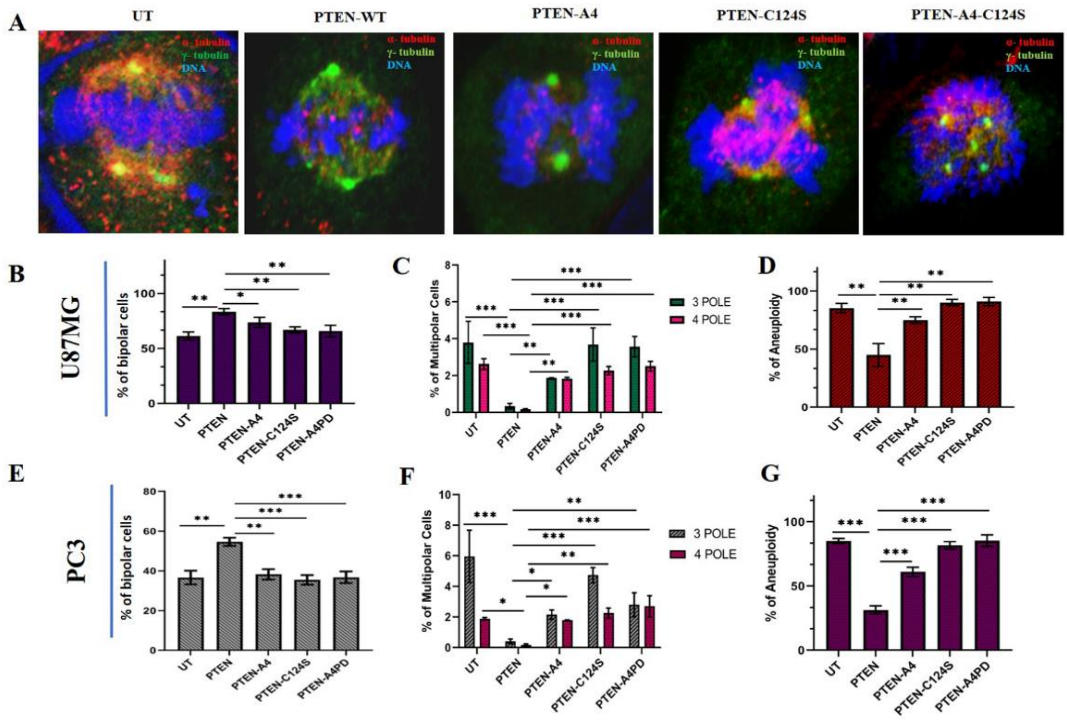


Figure 2.3.3.1.*Multiple pole formation causing chromosome missegregation. A. Microscopic images of the formation of multiple poles during metaphase in different PTEN clone transfected cells. Images are labeled with α-tubulin (red), γ-tubulin (green), and DNA (blue). Graphical representation of decrease in the percentage of bipolar and increase in the percentage of multipolar cells in U87MG (B, C) and in PC3 cells (E, F) transfected with different PTEN clones compared to Wt-PTEN. Quantitative estimation of increase of aneuploidy in various mutant PTEN and decrease of aneuploidy in Wt-PTEN transfected in U87MG (D) and PC3 cells (G). Values are mean ± SEM from three independent experiments *p<0.05;**p<0.01;***p<0.001.*

As our findings suggest that both phosphorylation and phosphatase function of PTEN is vital for genomic stability, the ploidy status was examined after transient transfection of different mutated PTEN clones in both $PTEN^{-/-}$ PC3 and U87MG cell line.

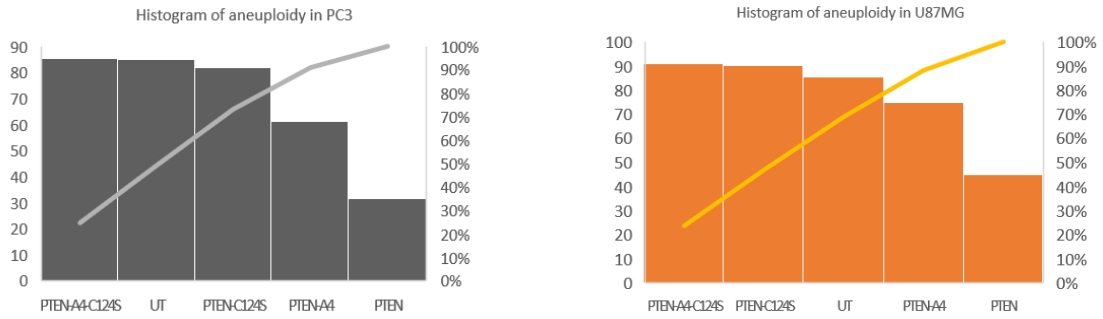


Figure 2.3.3.2. *Histogram of aneuploidy in U87MG cell line (H) and PC3 cell line (I) showing around 90% aneuploidy in UT, PTEN-C124S, PTEN-A4-C124S which decreases to 70% in PTEN-A4 and 40% in Wt-PTEN.*

Aneuploidy status for each mutant was studied after the treatment of cells with a microtubule de-polymerizing reagent, nocodazole for 16 h. Here, we found that 1.6 fold increase in the percentage of aneuploidy in A4-PTEN transfected cells, and an approximately 2-fold increase in cells transfected with both A4-C124S-PTEN and C124S-PTEN which is shown as a histogram (Fig 2.3.3.2H). Likewise, 1.9 fold rise in the aneuploidy percentage was recorded in untransfected cells compared to cells transfected with Wildtype-PTEN (Fig 2.3.3.1D). This trend is persistent with the other PTEN-deficient PC3 cell line. A 2.6, 1.9, and 2.7 fold increase in the aneuploidy percentage in C124S-PTEN, A4-PTEN, and A4-C124S-PTEN respectively to Wildtype-PTEN and 2.7 fold less in Wildtype-PTEN than untransfected (Fig 2.3.3.1G) cells were observed. This is also shown in a histogram (Fig 2H) which presents that almost 90% percent cells are aneuploid C124S-PTEN, UT, and A4-C124S-PTEN which decreases to 70% in A4-PTEN, and 40% in Wildtype-PTEN in U87MG cell line. Similarly, the histogram of the PC3 (Fig 2.3.3.2I) cell line also showed around 90% aneuploidy in C124S-PTEN, UT, and A4-C124S-PTEN which decreases to 65% in the case of A4-PTEN and 30% upon introduction of Wildtype-PTEN. This observation shows that phosphatase and phosphorylation of PTEN are required for the proper segregation of chromosomes.

2.3.4. γ- tubulin co-localizes with phospho-PTEN at the spindle pole during metaphase.

A prior study has shown that chromosomal segregation is regulated by PTEN (Sun et al., 2014). Dysregulation of spindle pole growth and loss of mitotic pole integrity induces aneuploidy (Cosenza et al., 2016, Maiato et al., 2014). We investigated to know if PTEN is restricted to the spindle pole since PTEN loss results in aneuploidy. The phosphorylated form of PTEN (Ser 380, Thr 382,383), binds to γ-tubulin after localizing to the spindle pole which was revealed by immunofluorescence findings (Fig 2.3.4A). γ-tubulin on the other hand, co-localizes with AURKA and PLK1, in the centrosomes of the PTEN-deficient PC3 cell line, where PTEN is unable to dephosphorylate the PLK1 and AURKA proteins, resulting in chromosomes that are incorrectly segregated (Fig 2.3.4B).

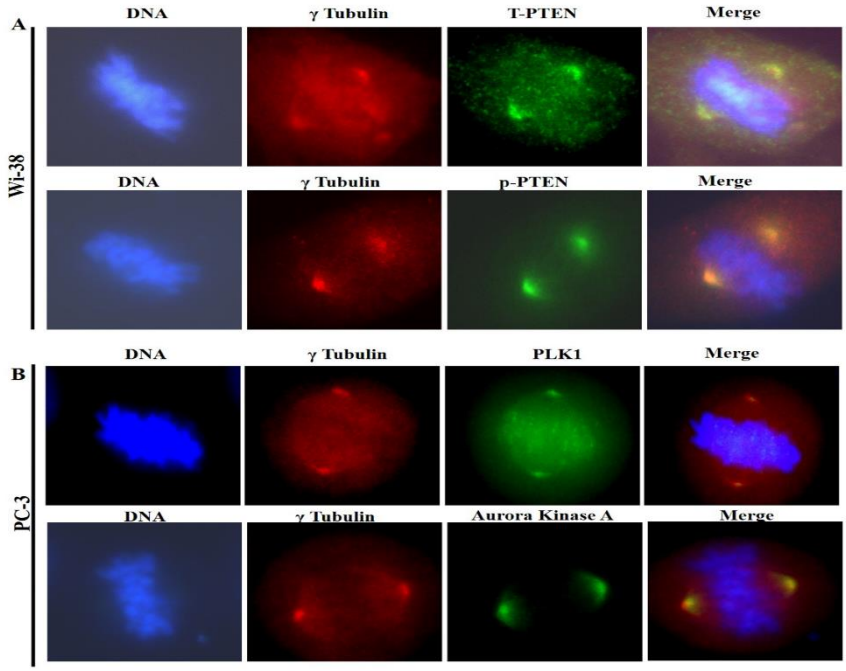


Figure 2.3.4. *PTEN localizes at the spindle pole. A. Wi-38 cells are synchronized at metaphase and immunolabelled with γ-tubulin antibody (red) with either total PTEN antibody (green) or phospho Ser 380, Thr 382,383 PTEN antibody (green). B. PTEN null PC3 cells are synchronized at metaphase and immunolabelled with γ tubulin antibody (red) with either PLK1 antibody or Aurora Kinase A antibody (green).*

2.3.5. Depletion of PTEN phosphorylation and phosphatase activity leads to differential expression of PLK1 and Aurora Kinase A

Investigations have revealed that AURKA autophosphorylation is the primary regulating mechanism in centrosomes during the early phases of mitosis (Zorba et al., 2014). Overactivation of mitotic kinases results in mitotic pole disintegration and unregulated chromosomal segregation (Lens et al., 2010). We transiently transfected several clones of PTEN in PTEN-null cells to test this idea. After treatment with nocodazole for 16 hours, western blots were done to determine the levels of Aurora kinase A and PLK1 expression.

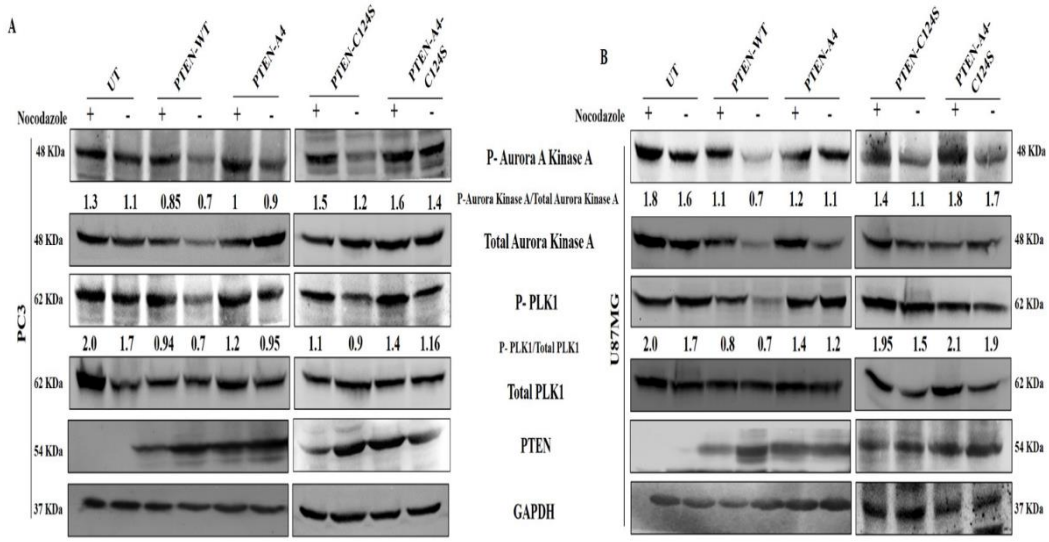


Figure 2.3.5.1.*Differential expression of mitotic kinases in different PTEN mutants. A and B. Wt-PTEN exhibited a reduction in phosphorylation of AURKA and PLK1 whereas a rise in phosphorylation in UT, C124S-PTEN, and A4-C124S-PTEN, PTEN clones after transfection in PTEN$^{-/-}$ cell lines (PC3 and U87MG) before and after nocodazole treatment for 16 h. GAPDH acts as a loading control.*

The expression of both phosphorylated-PLK1 and phosphorylated-Aurora kinase A were significantly reduced in Wildtype-PTEN as compared to untransfected U87MG and PC3 cell lines. These mitotic kinases were likewise found to be overexpressed in cells transfected with

A4-C124S-PTEN and C124S-PTEN as compared to Wildtype-PTEN in PC3 (Fig 2.3.5.1A) and U87MG cells (Fig 2.3.5.1B).

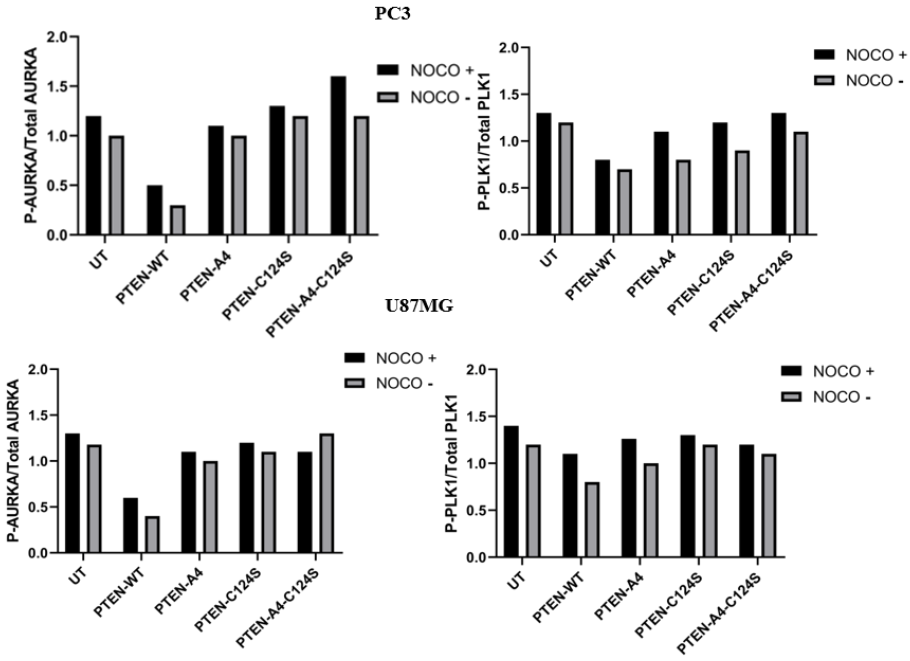


Figure 2.3.5.2. *Graphical representation of the phosphorylation status of phospho-Aurora kinase A/Aurora Kinase A and phospho-PLK1/PLK1 in PC3 and U87MG cell lines. Values were calculated by image J software, indicating the ratio of p-PLK1/ total PLK1 and p-AURKA/ total AURKA antibody. Untransfected control is normalized to 1.*

Interestingly, cells transfected with phospho-dead PTEN had greater levels of phospho-Aurora Kinase A (T288) and phospho-PLK1 (T210) due to increased phosphatase activity. P-PLK1/Total PLK1 and P-AURKA/Total AURKA bar plots for PC3 and U87MG cell lines in the absence and presence of nocodazole (Fig 2.3.5.2C). A4-PTEN is unable to dephosphorylate certain proteins in vivo which seems that there might exist an alternative mechanism for this function.

2.3.6. Wt-PTEN physically interacts with PLK1/ Aurora Kinase A whereas A4-C124S PTEN or A4-PTEN cannot

We carried out an immuno-precipitation experiment to analyze the interaction of PTEN and its mutant with Aurora Kinase A and PLK1. Transfection was done with different PTEN clones in PTEN-negative PC3 cells, treated with nocodazole, and immuno-precipitated with anti-HA antibody after preparation of whole cell lysate and blotted with Aurora kinase A and PLK1. We found that (A4-C124S-PTEN) double mutant PTEN (lane 5) and (A4-PTEN) phospho mutant PTEN (lane 3) do not efficiently physically interact with PLK1 compared to Wildtype-PTEN (lane 2). But (C124S) phosphatase dead PTEN (lane 4) efficiently interacts with PLK1. Interestingly from our immuno-precipitation experiment, we also found that double mutant PTEN (lane 5) & phospho-dead PTEN (lane 3) cannot interact with Aurora Kinase A whereas phosphatase-dead PTEN (lane 4), as well as, Wildtype-PTEN (lane 2) efficiently interact with Aurora kinase A. Therefore, it is possible that for PTEN to physically interact with Aurora kinase A & PLK1, the phosphorylation at the C-terminal may be important. Even if phosphatase activity is higher in A4-PTEN it cannot interact efficiently with Aurora Kinase A and PLK1, therefore unable to dephosphorylate both of them, which we found in our western blot experiment.

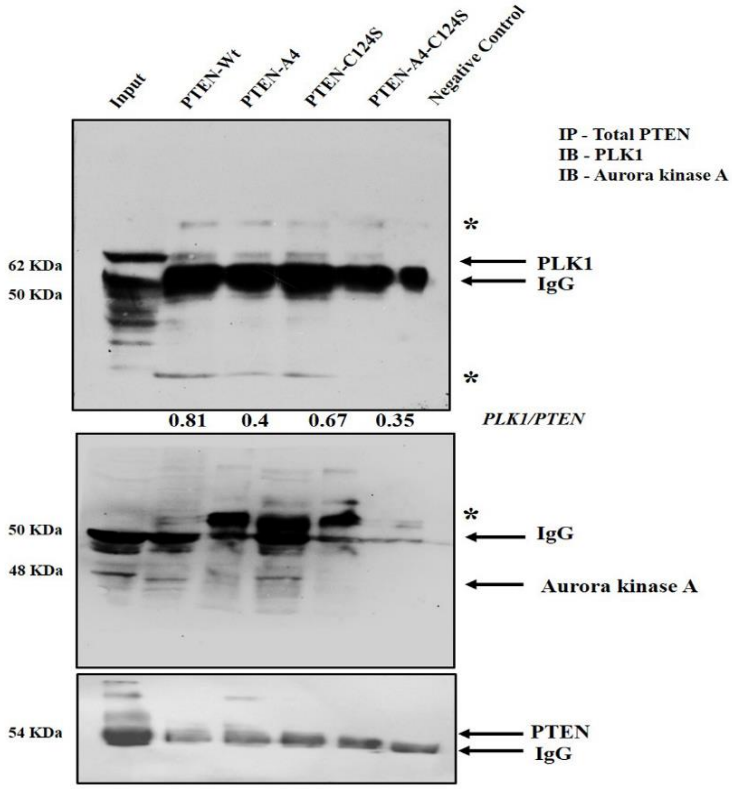


Figure 2.3.6. *PTEN null PC3 cells are transfected with indicated PTEN clone and after nocodazole treatment whole cell lysate was prepared and immune precipitated with HA antibody and subsequently immunoblotted with PLK1 and Aurora Kinase A antibody showed that PTEN-C124S and PTEN-A4-C124S were unable to efficiently interact with PLK1 and Aurora Kinase A and dephosphorylate them.*

2.3.7. A4-C124S PTEN has a positive correlation with different abnormalities

Principal Component Analysis is the interpretation of variables that are most closely connected with each component and which of the values are high in magnitude and the furthest from zero in either direction. The basic principle is to first determine which direction corresponds to the greatest variation between the data points. Our principal component analysis (PCA) explained a total variance of 87.0%. A4-C124S-PTEN revealed higher cytological damage, multiple pole formation, and aneuploidy. The aberrations like 3 poles, 4

poles, aneuploidy, and NBud 24 h, MNi 24 h, NPB 24 h are positively correlated with A4-C124S-PTEN similar to untransfected and negatively correlated with Wildtype-PTEN. A4-C124S-PTEN, on the other hand, is negatively correlated with 2 poles.

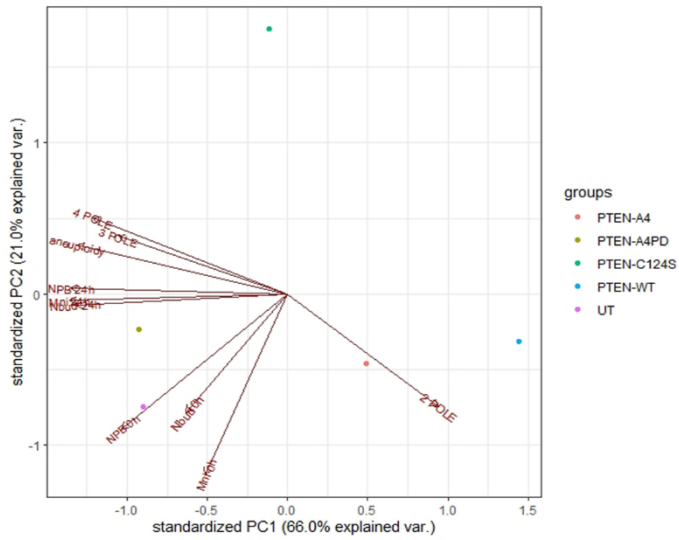


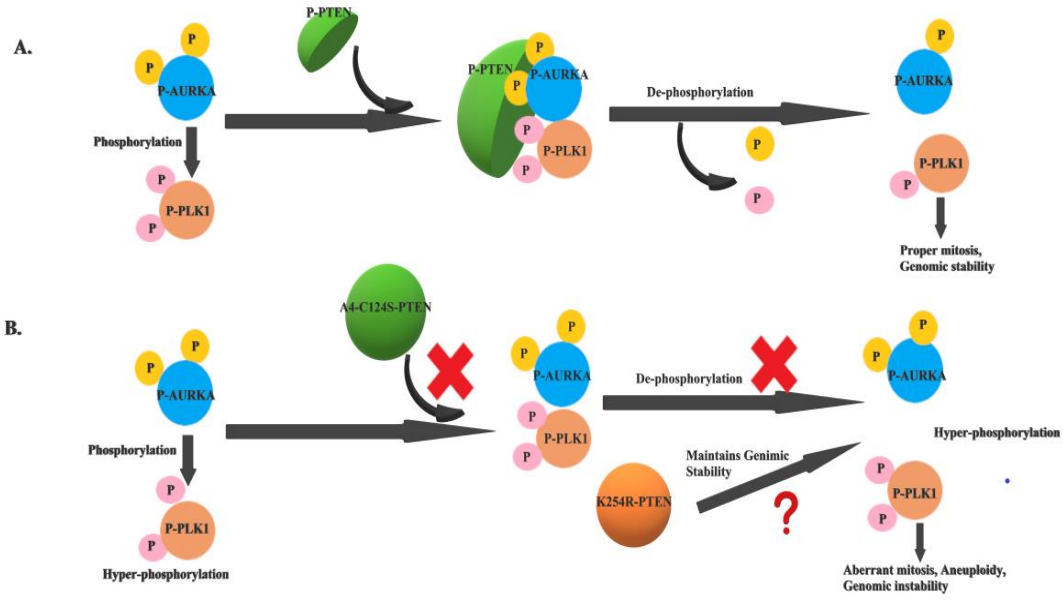
Figure 2.3.7. *Principal Component Analysis (PCA) shows a total variance of 87%. PTEN-A4-C124S showed a positive correlation and PTEN-WT showed a negative correlation with different aberrations.*

Discussion

Numerous studies have addressed the significance of PTEN's phosphatase activity and phosphorylation, but none of them have yet focused on how these two processes interact to maintain chromosomal integrity (Misra et al., 2014, Steelman et al., 2008). In this investigation, we discovered that PTEN phosphatase and phosphorylation activity is both necessary to decrease the damage-induced accumulation of MNi, indicating impaired DNA repair with ineffective cell cycle checkpoint functions. NBud and NPBs have also lessened when Wildtype-PTEN were compared to phosphatase-dead (C124S-PTEN) and phosphatase- and phosphorylation-impaired double mutant (A4-C124S-PTEN). In C124S-PTEN

transfected cells, overexpressed p-Akt appears to induce cell division with damaged unrepaired DNA, bypassing the checkpoint blockade, and causing the accumulation of NBud, NPB, and MNi throughout the recovery phase of 24 hours. When a DNA repair system is absent, the accumulation of MNi is contributed by sporadic phospho-deficient PTEN. Due to the twin effects of increased phospho-AKT levels and diminished DNA repair capacity, a significant accumulation of damage was found when we used PTEN which was both phosphorylation-dead and phosphatase-dead (A4-C124S-PTEN). We also observed according to a previous study, that PTEN-deficient cells exhibit aberrant segregation of chromosomes that results in aneuploidy (Sun et al., 2019). We discovered the creation of multiple γ-tubulin poles in PTEN-deficient cells as we moved upstream along this pathway. It's interesting to note that, in contrast to Wildtype-PTEN transfected cells, enhanced multiple pole formation also occurs in cells transfected with phosphorylation-dead and phosphatase-dead PTEN. Our findings strongly imply that γ-tubulin localized together with phospho-PTEN to the spindle pole. The excessive activity of mitotic kinases including Aurora Kinase A and PLK1 is another factor in centrosome amplification. We discovered that PTEN-deficient cells express these mitotic kinases and their phosphorylated version more frequently. In contrast to Wildtype-PTEN, PTEN phosphorylation and phosphatase dead mutant transfected cells exhibit hyperphosphorylated mitotic kinase. The production of Wildtype-PTEN reduces phospho-Aurora Kinase A (T288) levels and phospho-PLK1 (T210) is independent of the treatment with nocodazole. In the case of both A4-C124S-PTEN and C124S-PTEN expression of phospho-Aurora Kinase A and phospho-PLK1 proteins are identical in the absence and in presence of nocodazole treatment. Even while there is increased phosphatase activity in A4-PTEN, it barely decreased the expression of phospho-Aurora Kinase A and phospho-PLK1. To answer this question, we used an immunoprecipitation test, it was discovered that phospho-dead PTEN had a reduced interaction with PLK1 compared to

Wildtype-PTEN and was not able to physically engage with Aurora Kinase A. Therefore, PTEN's impaired ability to associate with these proteins results in hyperphosphorylation, which increases centrosome amplification and ultimately leads to aneuploidy. PCA accounted for 87.0% of the overall variation, which supports this. A4-C124S-PTEN is favorably correlated with the aberrations like 3-poles and 4-poles, aneuploidy NBud-24 h, MNi-24 h, and NPB-24 h, while Wildtype-PTEN is negatively correlated with both. While Wildtype-PTEN is positively connected to 2 poles, A4-C124S-PTEN is adversely related to 2 poles. The data taken together support a hypothesis in which phosphorylation mediates the interaction of PTEN with Aurora Kinase A and PLK1, leading to the dephosphorylation of those proteins leading to the proper segregation of chromosomes. So, phosphorylated PTEN maintains genomic stability. Our next work of focus is to investigate whether sumoylation also is important in preserving the genetic integrity of cells.



Chapter 3

Sumoylation at PTEN-K254R is crucial for PTEN's action in preserving genomic stability in the nucleus

Sumoylation at PTEN-K254R is crucial for PTEN's action in preserving genomic stability in the nucleus

3.1 Introduction

PTEN is a dual phosphatase tumor suppressor protein whose main function is to inhibit PI3K/Akt pathway regulating cell growth (Myers et al., 1998). PTEN also has a function in subcellular localization other than its activity in the membrane. PTEN has a role in maintaining genomic stability and nuclear PTEN serves several roles in DNA repair and chromosomal structural integrity in exposure to genotoxic stress (Bassi et al., 2013). Furthermore, through regulating both DNA replication and transcription, PTEN is directly engaged in the elementary machinery of genetic transmission as well as chromosomal segregation. In cells lacking PTEN, inaccuracy in chromosomal transmission causes chromosomal instability. PTEN-deficient cells have centromere breaking as a marker of chromosomal instability, indicating that PTEN is required in preserving chromosomal structural integrity (Shen et al., 2007). PTEN has been revealed to co-localize with heterochromatin and to sustain chromatin compaction, which is consistent with its physical interaction with centromeres (Gong et al., 2015). In absence of PTEN C-terminus, chromatin compaction is disturbed, especially in heterochromatin areas including centromeres and telomeres. PTEN was first found in mitotic centrosomes and its localization to the centrosomes was demonstrated to be dependent on AKT-mediated employment of polo-like kinase 1 and γ-tubulin. Knockdown of PTEN causes increased whole-cell levels of PLK1 and γ-tubulin, resulting in the amplification of centrosome. Furthermore, inhibiting AKT prevents the growth of PLK1 and γ-tubulin in cells with PTEN knockdown and decreases the percentage of cells with extra centrosomes. PTEN inhibits an AKT-

mediated cellular repository of PLK1 and γ-tubulin to prevent amplification of centrosome, while recruitment of PTEN to the mitotic spindle poles requires centrosome deposit of PLK1 and γ-tubulin (Leonard et al., 2013).

PTEN is largely post-translationally modulated by acetylation, sumoylation, phosphorylation, and mono- and poly-ubiquitination where sumoylation has a dual role in regulating membrane localization as well as nuclear translocation (González-Santamarĭa et al., 2012). SUMOylation is a significant post-translational regulation where the reversible attachment of SUMO (small ubiquitin-related modifier) has emerging roles in the functional regulation of proteins (González-Garcĭa et al., 2020). SUMO2 and SUMO3 are closely related proteins to SUMO1 found in mammalian cells. These proteins have a similar structure to ubiquitin. They are covalently connected by a SUMO-conjugation system to target proteins that include numerous E3 ligases with different specificities to target-protein along with an E2 ligase (UBC9, also known as UBE2I), and an E1 activating enzyme (SAE1/SAE2). Conjugation of SUMO regulates a variety of cellular functions sometimes by opposing or contributing to ubiquitin conjugation (Hay., 2005, Ulrich., 2005, Schimmel et al., 2008, Meulmeestal et al., 2008, Bergink et al., 2009). Subsequent research identified SUMO roles in signal transduction, DNA repair, nuclear transport, and cell cycle regulation. PTEN has different SUMOylation sites of which K254 is the major site. SUMO1 has the ability to modify PTEN at the K254 site in the C2 domain. Mutation of this site leads to enhanced tumor proliferation (Bassi et al., 2013). Bassi et al also reported that cells expressing PTEN-K254R mutant cannot localize to the nucleus and resolve 53BP1 (p53 binding protein 1) foci, an indicator of double-strand break, after 24 hours of ionizing radiation exposure. PTEN-K254R also cannot resolve γ-H2AX foci in the nucleus which is a biomarker of DNA damage.

As both catalytic activity and localization of PTEN are regulated by sumoylation, we investigated the role of PTEN sumoylation in context with genomic stability.

3.2 Aims and Objective

- The objective of this work is to generate a PTEN-K254R clone and investigate that in maintaining the genomic stability of the cell there is a role of the sumoylation of PTEN.

3.3 Results

3.3.1 Generation of PTEN-K254R clone by site-directed mutagenesis

We generated a K254R-PTEN clone from Wt-PTEN using a site-directed mutagenesis kit from Agilent and followed the instructions provided by the manufacturer. Following transformation, a single colony was extracted, sequenced, and validated using sequencing primers.

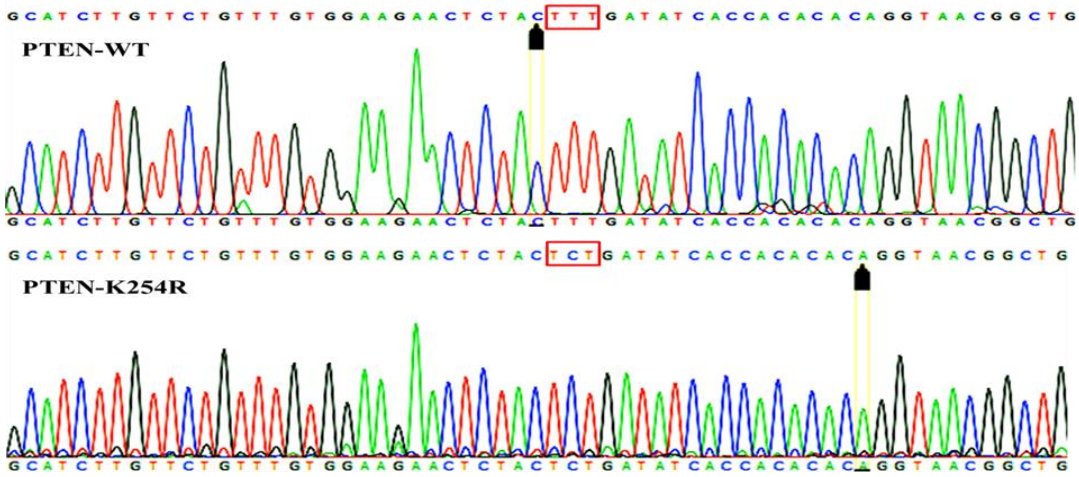


Fig 3.3.1*.DNA sequencing result of sumo dead PTEN (K254R).*

3.3.2 PTEN-K254R mutation leads to elevated cytological damage in cells

CBMN assay was used to measure both unreplicated DNA breakage and chromosome loss to assess global chromosomal damage. PTEN-negative PC3 cells were transfected with Wildtype-PTEN and K254R-PTEN (Fig. 3.3.2 (A)), treated with etoposide for 4 h, and MNi, NPBs, and NBUDs were scored by CBMN assay at 0 h and recovery time point 24 h (Table 2). There is a significant rise in MNi (Fig. 3.3.2(B.II)) formation induced by etoposide in cells transfected with (sumoylation dead) after a 24 h recovery period (34±2.64) compare to cells transfected with Wildtype-PTEN (25±2.1). Formation of NBud has also amplified in cells transfected with K254R-PTEN as compared to Wildtype-PTEN transfected cells (K254R-PTEN 15±2.36 versus 9±1.3 at 24 h) (Fig. 3.3.2(B.III)). Also, NPBs (Fig. 3.3.2(B.IV)), the markers of telomere end fusion or DNA mis-repair and elimination of amplified DNA were higher in the case of K254R-PTEN than cells transfected with Wildtype-PTEN. This observation directs that for the inhibition of accumulated cytological damage sumoylation activity is required.

MUTANTS	MNi per 1000 BN cells		NBud per 1000 BN cells		NPB per 1000 BN cells	
	0h	24h	0h	24h	0h	24h
UT	77±2.94	65±3.8	28±1.83	37±1.51	17±2.22	20±2.0
PTEN-WT	49±1.9[a]	25±2.1[b]	14±1.7[a]	9±1.3[b]	6±0.6[a]	3±0.5[b]
PTEN-K254R	65±2.63[c]	34±2.64[d]	20±2.71[c]	15±2.36[d]	8±0.9[c]	5±0.58[d]

Note: MNi: Micronuclei; NBud: Nuclear Buds; NPBs: Nucleoplasmic Bridges

Values shown as for means ± SEM of three independent experiments.

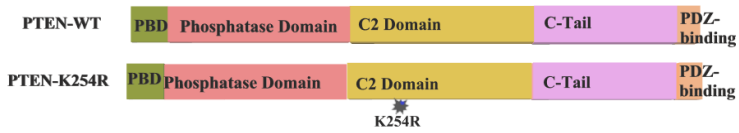
a $p<0.05$ compared with UT 0h (Student's t-test).

b $p<0.05$ compared with UT 24h (Student's t-test).

c $p<0.05$ compared with PTEN 0h (Student's t-test).

d $p<0.05$ compared with PTEN 24h (Student's t-test).

A.



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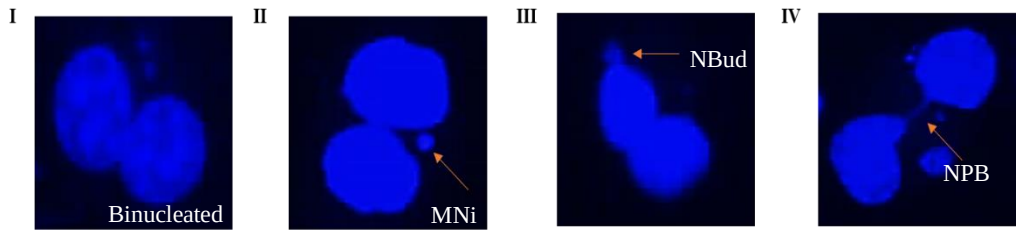


Fig 3.3.2*. A. Map of K254R mutant of PTEN. B. Microscopic images of cells incubated in presence of etoposide (100µM for 4h) and allowed recovering for 24h. Cells were stained with DAPI. Arrowheads indicate I. Binucleated cells II. Binucleated cell with MNi III. Binucleated cell with NBud IV. Binucleated cell with NPB. Micronucleus, bud, and bridge formation are more in the case of K254R mutant than WT PTEN in 24-hour recovery.*

3.3.3 PTEN-K254R mutation leads to multiple pole formation and chromosomal missegregation causing aneuploidy

The development of γ-tubulin poles at the centrosome is the first and most important step in chromosomal segregation during metaphase. Furthermore, aneuploidy causes the production of micronucleated cells. Whole-chromosome instability is the indicator of aneuploidy in PTEN-deficient cells. Microscopic pictures of multiple pole development in untransfected, wild-type PTEN and K254R-PTEN transfected PC3 cell line (PTEN null) and probed with α-tubulin and γ-tubulin followed by double thymidine block were obtained (Fig. 3.3.3(A)). In this study, we looked at γ-tubulin pole development in the absence of PTEN's sumoylation activity and discovered a 1.3fold drop in the bipolar cells percentage in untransfected and K254R-PTEN cells

compared to Wildtype-PTEN (Fig. 3.3.3(B)). In terms of the proportion of multipolar cells, K254R-PTEN transfected U87MG cells have an 8-10fold increase over Wt PTEN (Fig. 3.3.3(C)). In PC3, the bipolar cell percentage in K254R-PTEN and untransfected cells is 1.4 times lower than in cells transfected with Wildtype-PTEN (Fig. 3.3.3(E)). In contrast, the multipolar cell percentage in untransfected including K254R-PTEN cells is 5-10 times higher than in PC3 cells transfected with Wildtype-PTEN (Fig. 3.3.3(F)). We investigated the ploidy status by transfecting K254R-PTEN in both PTEN-negative PC3 and U87MG cell lines after discovering that PTEN sumoylation is critical for genomic integrity. After 24 hours of transfection, treatment was done with a microtubule depolymerizing reagent, nocodazole, and aneuploidy status was determined. We discovered in cells transfected with K254R-PTEN aneuploidy increased 1.5-fold. Similarly, the aneuploidy percentage in untransfected cells is 1.7 times higher than in Wildtype-PTEN transfected cells (Fig. 3.3.3(D)). This pattern is also seen in PC3, another PTEN-deficient cell line. We found that aneuploidy increased 1.8-fold in K254R-PTEN compared to cells transfected with Wildtype-PTEN and a 2.6-fold rise in untransfected than Wildtype-PTEN (Fig. 3.3.3(G)). Thus, our finding suggests that the PTEN sumoylation is required for normal chromosomal segregation.

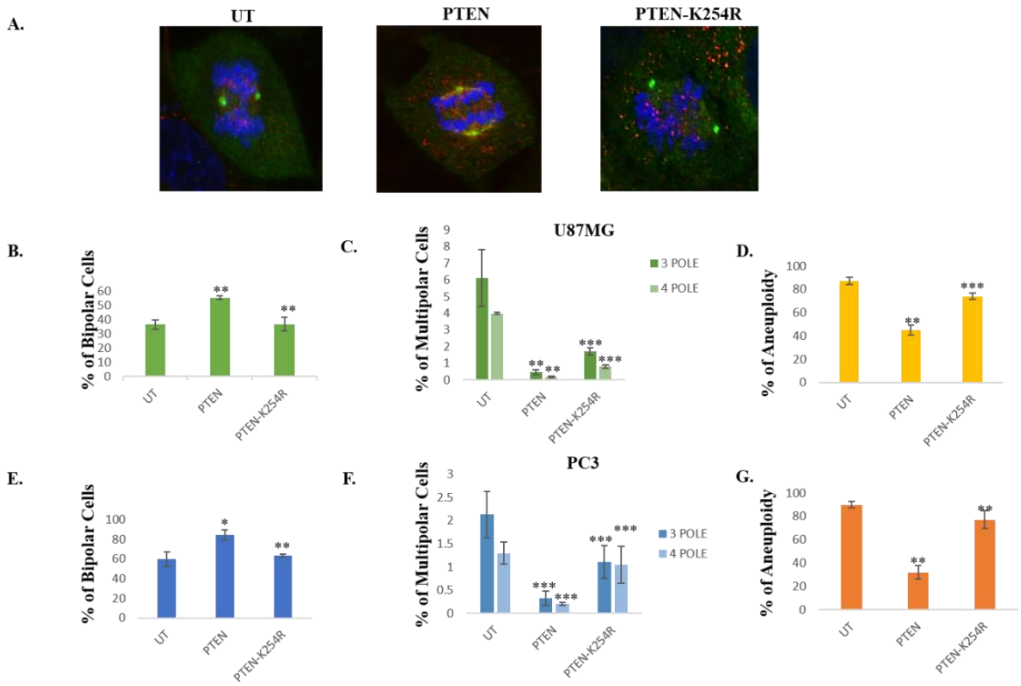


Fig 3.3.3. *A. Microscopic image of multi-pole formation during metaphase in various PTEN clone transfected cells. Graphical representation of the percentage of bipolar and multipolar cells in the K254R PTEN clone transfected U87MG (B, C) and PC3 cells (E, F). Quantitative estimation of aneuploidy in Wt-PTEN and various mutant PTEN transfected in U87MG (D) and PC3 cells (G).*

3.3.4 PTEN-K254R mutation leads to increased expression of phosphorylated PLK1 and AURKA

Overactivation of PLK1 and Aurora kinase A mitotic kinases consequences in mitotic pole disintegration and abnormal chromosomal segregation. We expressed transiently K254R-PTEN clone and wild-type PTEN in PTEN-deficient U87MG and PC3 cell lines to verify our assumption. Nocodazole was treated to cells after 24 hours and a western blot was performed to determine the PLK1 and AURKA expression levels. We discovered that phospho-PLK1 and phospho-AURKA levels were significantly lower in Wildtype PTEN-transfected cells compared to untransfected in U87MG and PC3 cells. Cells transfected with K254R-PTEN also expressed more mitotic kinases

than cells transfected with Wildtype-PTEN in both PC3 (Fig. 3.3.4(A)) and U87MG cells (Fig. 3.3.4(B)).

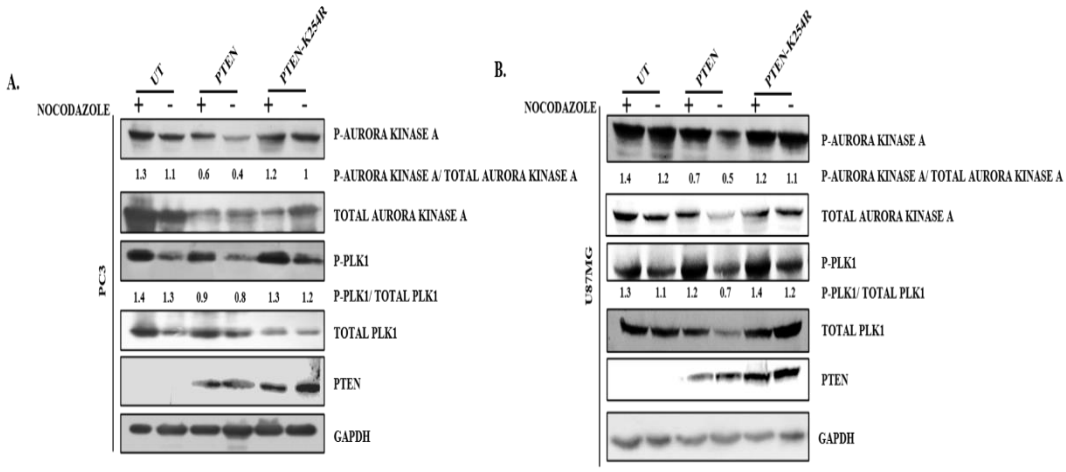


Fig 3.3.4. *A and B. Phosphorylation status of Aurora kinase A and PLK1 of K254R clone of PTEN in pten$^{-/-}$ cell lines (PC3 and U87MG) with and without nocodazole treatment for 16 h.*

3.4 Discussion

Aside from its participation in the classical PI3K/AKT pathway, PTEN also acts in the repair of DNA and response to DNA damage (Ming et al., 2012). In the context of the stability of chromosomes, the role of PTEN sumoylation has been addressed. PTEN sumoylation is needed for cellular genomic stability has been revealed in this study. According to our previous study, inactive PTEN in hepatocellular carcinoma causes cell survival via DNA damage-induced multinucleation formation (Mukherjee et al., 2013). We discovered that the accumulation of NBud, NPBs, and MNi is less in wild-type PTEN than in K254R-PTEN (sumo-dead) throughout the recovery time in 24 hours. There is an alteration in the ploidy status as a result of this genetic error.

Wildtype-PTEN overexpression reduces aneuploidy in both U87MG and PC3 cell lines, as expected. During metaphase, multiple spindle pole development is another justification for aneuploidy. PTEN is directly engaged in the proper alignment and segregation of chromosomes. During mitosis, PTEN deficiency impairs spindle assembly leading to incorrect chromosomal segregation (Hou et al., 2017). In PTEN-deficient cells γ-tubulin levels are high, resulting in centrosome amplification. Interestingly, we discovered that the frequency of multipole generation in K254R-PTEN transfected cells is higher than in cells transfected with Wildtype-PTEN. As a result, it appears likely that PTEN's defective sumoylation function causes aberrant chromosome segregation in daughter cells. There are reports of PTEN localization at mitotic centrosomes (Leonard et al., 2013). PLK1 and Aurora kinase A overexpression has been testified in several cancers, and it is thought to be a potential target of cancer treatment (Ming et al., 2012). The overactivation of mitotic kinases results in centrosome amplification and the development of numerous spindle poles (Agircan et al., 2014, Fry et al., 2017). We found that cells transfected with K254R-PTEN have higher levels of γ-tubulin pole formation than untransfected cells which is consistent with the report. For PTEN recruitment to spindle poles, γ-tubulin is required which results in a feedback loop of signaling at centrosomes to maintain spindle pole stability (Hou et al., 2017). PTEN localization at centrosomes is similarly dependent on PLK1 recruitment. Furthermore, for PTEN recruitment to spindle poles, PLK1 is also required (Leonard et al., 2013). PTEN loss causes abnormal PLK1 phosphorylation, which affects spindle bipolarity, according to research (Hou et al., 2017). PTEN also inhibits the production of Aurora kinase A, which is required for mitosis (Li et al., 2018). According to the findings of our investigation, expression of phospho-PLK1 (T210) is reduced in the presence and in absence of nocodazole

treatment when wild-type PTEN is expressed, relative to untransfected cells, rendering consistency with the previous research. In comparison to untransfected cells, expression of phospho-Aurora kinase A (T288) was reduced in wild-type PTEN in the absence and in presence of nocodazole treatment. In the case of K254R-PTEN, both phosphorylated-PLK1 and phosphorylated-Aurora kinase A protein expression is analogous to untransfected in the presence and in absence of treatment with nocodazole. PTEN mutation causes a variety of cellular abnormalities, which eventually contribute to cancer formation. As a result, our finding suggests that for preserving the genomic stability of the cell, the sumoylation of PTEN is also important.

Chapter 4

Conclusion

Conclusion

The capacity to retain genomic integrity following external DNA damage or replication is critical for reducing neoplastic features. The faithful and exact transfer of DNA in daughter cells is a basic need for life. PTEN, a renowned protein that suppresses tumor formation, is increasingly being recognized as a protein required in DNA repair in addition to its involvement in suppressing the oncogenic PI3K/AKT pathway. PTEN is required for normal DNA damage repair, including DSB repair and NER. PTEN functions as a tumor suppressor due to its activities in DNA repair and or replication. PTEN deficiency in human malignancies may increase radiosensitivity. PTEN deficiency, on the other hand, enhances cell survival while decreasing DNA repair, which might lead to genomic instability and jeopardize treatment effectiveness. Repairing DNA is crucial for cells to maintain proper function and genetic stability (Ming et al., 2012). Rather than a dynamic signaling protein PTEN was formerly thought to be a universally expressed phosphatase acting as a negative regulator of the kinase pathway in the PI3K field. Subsequent research contradicted this initial view, finding that PTEN activity is heavily regulated, mostly by post-translational modification and this can explain the diverse functions of PTEN in cellular metabolism. Different mutations of PTEN lead to different kinds of genomic stability. So, there might be a post-translational modification or protein-protein interaction that regulates the function of PTEN. Several mutations are involved in different catalytic and regulatory functions of PTEN but the particular mutation that involves in maintaining genomic integrity is still unknown. In this study, both PTEN phosphatase and phosphorylation functions were found to decrease the damage-induced MNi accumulation indicating the compromisation of DNA

repair that includes cell cycle checkpoint function that is non-functional. MNi primarily initiates from the acentric chromosome fragments during anaphase as a result of replication error where the entire chromosomes fail to include in the daughter nuclei because of inappropriate attachment of chromosomes with the spindle fibers and segregation. These fragmented chromosomes are then encircled by nuclear membrane generating morphologically similar nuclei (Fenech et al., 2011). NBuds are alike MNi having similar morphology with the exemption that they are connected through a wide or narrow branch of nucleoplasmic material to the nucleus (Fenech et al., 2011). Formation of NBuds signifies a process of elimination of DNA repair complex, amplified DNA, and also additional chromosomes of aneuploid cells. Again, the nonappearance of breakage of anaphase bridge causes the formation of NPB or Nuclear bridge. It initiates from dicentric chromosomes as a result of telomere end fusion and chromosome breaks and occurs when there is a defect in the sister chromatids separation during anaphase due to decatenation failure (Fenech et al., 2011). Our earlier study anticipated that hepatocellular carcinoma harboring inactive PTEN causing the cells to survive with DNA damage-induced multinucleation (Staff et al., 2010).

The generation of MNi, NBud, and NPBs was enhanced when A4-C124S-PTEN, phosphatase and phosphorylation inhibited double mutant, and C124S-PTEN, phosphatase dead were compared to wild-type PTEN. PTEN C-terminal phosphorylation, which functions as a molecular switch to negatively restrict phosphatase activity (Choi et al., 2014, Puc et al., 2005), is also required but appears to be less important than phosphatase activity because dual PTEN mutant cells have higher genomic instability. Although A4-PTEN has aberrant phosphatase activity, it is unstable both in vitro and in vivo (Yang et al., 2015). As previously proven (Misra et al., 2014), PTEN phosphorylation is essential for DNA repair. The elevated phospho-Akt level in cells transfected with C124S-PTEN appears to operate as a driving force for cell division with damaged unrepaired DNA, bypassing the checkpoint blockade

that leads to the accumulation of NBud, NPB, and MNi throughout the 24-hour recovery phase. MNi accumulation is possible in the absenteeism of DNA repair by sporadic phospho-deficient PTEN. Consequently, due to elevated phospho-AKT levels and decreased DNA repair capacity, using phosphatase dead and phosphorylation dead PTEN together an accumulation of excessive damage was found. As a result, phosphatase function appears to be more crucial than phosphorylation.

All of these genetic disorders have an impact on cell ploidy. Wt-PTEN overexpression reduces aneuploidy in both the PC3 and U87MG cell lines, where both C terminal phosphorylation and phosphatase activity are required. A4-PTEN is incapable to localize at metaphase chromosomes, according to previous research (Van Ree et al., 2016), but localization alone is inadequate to prevent aneuploidy, as our findings demonstrated. This study demonstrates PTEN's importance in the MCC/SAC complex, where phosphorylation and phosphatase activity is required and hence require further exploration. Furthermore, aneuploidy, which is one of the symbols of neoplastic development, results in the creation of micronucleated cells (Ehlers et al., 2008). The development of many spindle poles during anaphase is another source of aneuploidy. PTEN is directly involved in chromosomal segregation and alignment (Hou et al., 2017). Again, chromosomal slippage induced by faulty spindle formation has the potential to increase these spontaneous defects (Puc et al., 2005, Shen et al., 2007). PTEN deficiency disrupts mitotic spindle formation, resulting in chromosomal segregation problems (He et al., 2016, Zhang et al., 2016). PTEN-deficient cells contain higher amounts of γ-tubulin, which causes centrosome amplification (Leonard et al., 2013). PTEN catalytic activity, as well as its phosphorylation state, appears to be needed for normal mitotic spindle assembly and chromosomal segregation, according to our results.

PTEN physically interacts with the Mad2, Cdc20, and Mad1 proteins, which are all essential for MCC (Liu et al., 2017, Choi et al., 2017). PTEN also creates attachment sites for Eg-5

and dlg1, which are also essential for appropriate bipolar spindle development (Van Ree et al., 2016). In addition, we determined that phosphorylated PTEN was restricted to the spindle pole. Interestingly, we discovered that C124S-PTEN, A4-PTEN, and A4-C124S-PTEN transfected cells have a greater frequency of multiple pole production than Wt-PTEN transfected cells. As a result, defective mitosis appears to be caused by a lack of phosphatase and PTEN phosphorylation activity in daughter cells, resulting in abnormal chromosomal segregation.

Several investigations (He et al., 2016) have found PTEN localization in mitotic centrosomes. Aurora Kinase A and PLK1 overexpression has been detected in a range of cancers, making them intriguing targets for cancer therapy. (Lens et al., 2010). Mitotic kinase hyperactivity leads to centrosome amplification and the creation of numerous spindle poles (Agircan et al., 2014, Fry et al., 2017). Autophosphorylation increases the catalytic activity of AURKA (Walter et al., 2000). However, since PLK1 is directly dephosphorylated by PTEN, PLK1 dephosphorylation may result in a reduction in total PLK1 expression (Zhang et al., 2016), allowing for proper segregation. We discovered that cells transfected with A4-C124S-PTEN and C124S-PTEN exhibit larger amounts of γ-tubulin pole formation than untransfected cells, which is consistent with earlier findings. PTEN recruitment, on the other hand, requires γ-tubulin, which establishes a feedback loop signaling at centrosomes and so preserves spindle pole stability (Hou et al., 2017).

For centrosomal PTEN localization, PLK1 is also required for PTEN recruitment to spindle poles. (He et al., 2016). PTEN regulates spindle bipolarity, hence its absence causes abnormal PLK1 phosphorylation (Hou et al., 2017). Although PTEN, which is required for mitosis, lowers Aurora Kinase A expression (Li et al., 2018), our immunofluorescence investigation showed that Aurora Kinase A and PLK1 localization to the spindle pole is independent of

PTEN status. Our findings reveal that Wildtype-PTEN expression suppresses phospho-Aurora Kinase A (T288) and phospho-PLK1 (T210) expression in the absence of nocodazole administration. This is persistent in previous research (Giubettini et al., 2011, Sana et al., 2018). Both phosphorylated-Aurora Kinase A and phosphorylated-PLK1 protein expression are equal before and after treatment with nocodazole in C124S-PTEN and A4-C124S-PTEN. In spite of having increased phosphatase activity, A4-PTEN had a modest reduction in p-PLK1 and p-Aurora Kinase A expression.

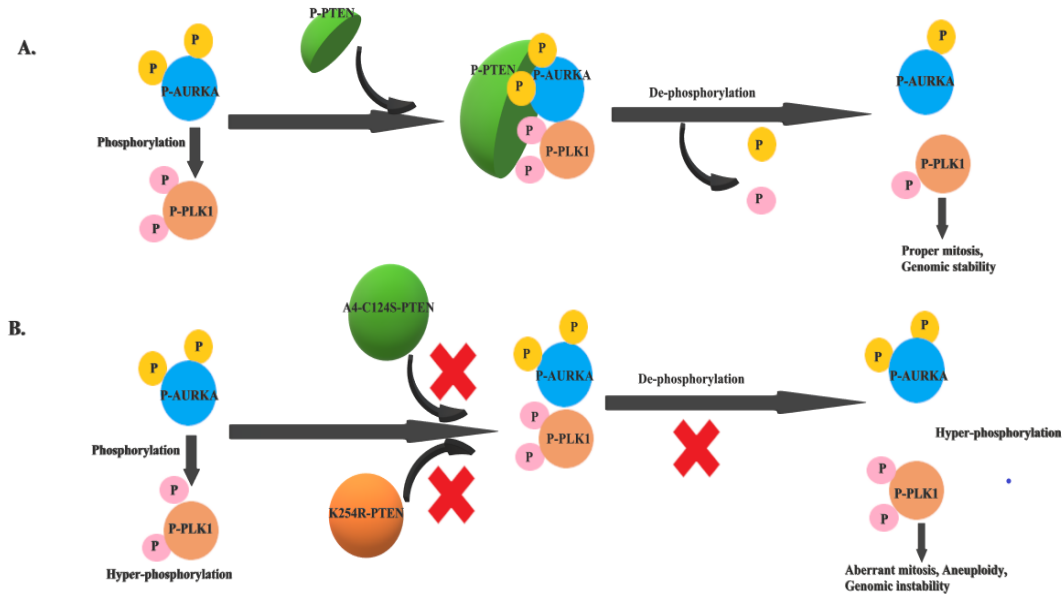
An immunoprecipitation experiment was used to investigate this variation and observed that phosphorylation dead PTEN and double mutant PTEN cannot physically interact with Aurora kinase A and PLK1. Therefore both phosphorylated mitotic kinases are physically and functionally controlled by PTEN. According to this study, PTEN phosphatase and phosphorylation collaborate to dephosphorylate PLK1 and Aurora kinase A, hence reducing genomic instability.

Our PCA, which elucidated 87.0% of the overall variation, confirms this. A4-C124S-PTEN is positively associated with the aberrations aneuploidy, 3 poles, 4 poles, MNi 24 h, NPB 24 h, and NBud 24 h, while negatively associated with wild type-PTEN. On the other hand, A4-C124S-PTEN has a negative relationship with two poles.

The canonical function of PTEN is the dephosphorylation to PIP2 from phosphorylated PIP3 by removing one phosphate from the inositol ring's D3 position which is mainly attributed to its membrane localization as PIP3 is located in the membrane. This localization of PTEN to the membrane is regulated by sumoylation, a post-translational modification. PTEN SUMOylation at various locations may regulate the diverse functions of PTEN by regulating its localization to various subcellular compartments. PTEN is SUMOylated at lysine 254, and the mutation of this amino acid is crucial for PTEN's nuclear localization. DNA repair by

PTEN is directly impacted by SUMOylation and therefore, PTEN SUMOylation mutation at the K254 site (K254R) is important in DNA damage response and maintaining genomic stability of the cell. We discovered that the sumo-dead K254R-PTEN accumulates more MNi, NBud, and NPBs over the course of a 24-hour recovery period than the wild-type PTEN. MNi buildup may be brought on by a lack of DNA repair. This genetic anomaly alters the ploidy condition of the cells, where we showed that overexpressing Wildtype-PTEN decreased aneuploidy in both U87MG and PC3cell lines. The production of multiple spindle poles during metaphase is another cause of aneuploidy. Chromosome alignment and segregation accuracy are directly impacted by PTEN (Hou et al., 2017). PTEN depletion affects spindle assembly during mitosis, which leads to incorrect chromosomal segregation (Van Ree et al., 2016, Zhang et al., 2016, He et al., 2016). We revealed that K254R-PTEN transfected cells have a higher frequency of multiple pole generation than cells transfected with Wildtype-PTEN. Therefore, it appears likely that poor mitosis caused by PTEN's deficient sumoylation function leads to aberrant chromosome segregation in daughter cells. Furthermore, PLK1 and Aurora Kinase A overexpression have been observed in a variety of cancers (Ming et al., 2012) and are thought to be promising targets for cancer treatment. Centrosome amplification and the formation of multiple spindle poles are caused by the over-function of these mitotic kinases (Fry et al., 2017, Agircan et al., 2014). We showed that K254R-PTEN transfected cells exhibit increased tubulin pole production similar to that of untransfected cells. PTEN regulates spindle bipolarity, hence studies have shown that its loss causes abnormal PLK1 phosphorylation (Hou et al., 2017). The expression of Aurora Kinase A, which is crucial for mitosis, is also downregulated by PTEN (Li et al., 2018). The findings of our investigation revealed that wild-type PTEN decreased the expression while PTEN-K254R increased the expression of phospho-Aurora Kinase A (T288) and phospho-PLK1 (T210) both with and without nocodazole treatment.

Apart from these, other post-translational modulations of PTEN may be responsible for various cellular events. Few have been explored already but a majority of such modulation relating to the cellular activity of PTEN remains unexplored. Dysfunction of PTEN leads to different cellular abnormalities. Apart from large deletions in the PTEN gene, fine tunings like post-translational modifications of PTEN protein are not only involved in the catalytic activity of PTEN but also participate in regulating the genomic stability of cells.



Materials & Methods

A. Materials

I. Antibodies

Cell Signaling Technology has provided anti-PLK1 (4513S), anti-Aurora Kinase A (9980S), anti-Phospho-Aurora Kinase A (8025S), and anti-Phospho-PLK1 (3738S) antibodies. Abcam supplied the total PTEN (32199). Alexa Fluor 568 conjugated anti-rabbit IgG (1:150), Alexa Fluor 488 conjugated anti-mouse IgG (1:150), purchased from Life Technologies, USA, and HRP conjugated anti-rabbit IgG (1:25000), purchased from Abcam were used as secondary antibodies.

II. Cell lines and culture conditions

Human glioblastoma cell U87MG was obtained from a female patient with malignant glioma, while human prostate cancer cell PC3 was obtained from a Caucasian male of a 62-year-old diagnosed with grade 4 prostatic adenocarcinoma and Wi38, derived from a female fetus of a 3-month-old (Pune, India). PC3 was cultivated in RPMI 1640 media, whereas Wi38 and U87MG were cultured in MEM. 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml) were added to the cell culture medium. All cells were kept at 37°C, 5% CO2, and 95% relative humidity (RH).

III. Plasmids

For transient transfection, mammalian expression vectors were employed. The wild-type plasmids pSG5L-HA-Wt-PTEN (HA-WT-PTEN) containing hemagglutinin tagged wild-type PTEN protein, and the phospho-deficient pSG5L-HA-A4-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala) were generously provided by Prof. Dr. W.R. Sellers, Harvard Medical School. Phospho-deficient phosphatase dead pSG5L-HA-A4PD-PTEN (Ser380Ala, Thr383Ala, Ser385Ala, Cys124Ser), SUMOylation dead pSG5L-HA-K254R-PTEN

(Lys254Arg) and phosphatase-dead pSG5L-HA-PD-PTEN (Cys124Ser) encoded hemagglutinin tagged mutant proteins.

IV. Reagents

In DMSO (Dimethyl sulphoxide, Sigma), Cytochalasin B (Sigma), Etoposide (Sigma), and Nocodazole (Sigma) were dissolved. The reagents were dissolved to stock concentrations of 5 mg/ml, 25 mM, and 1 mg/ml respectively. The working dilutions were made fresh in the medium. As a mounting material, DAPI was employed.

B. Methods

I. Aneuploidy analyses

A 35 mm culture plate was used to seed cells. Nocodazole (final concentration 50ng/ml) was used for 16h for the treatment of transfected cells. PBS was used to scrape the cells and the cells were centrifuged for 3mins at 2500 rpm. 75mM KCl was used to incubate the pellet after the discard of the supernatant for 15 min and centrifuged for 5 min at 2000 rpm. Methanol: acetic acid (ice-cold) in the ratio of 3:1 was used as fixative for 15 min at 4˚C to fix the pellet and then centrifuged for 5 min at 2000 rpm. The fixative solution was then used to resuspend the pellet after the discard of the supernatant. The resuspended cells were dropped on a cold grease-free glass slide, immediately flame dried, and mounted with DAPI containing mounting medium, and then a fluorescence microscope was used to examine the slides (Leica, Germany).

II. Cellular Transfection

Transfection reagent Lipofectamine 3000 (Invitrogen) was used as per the instruction given by the manufacturer. The antibiotic-free medium was used to seed cells 18 h before transfection. For transfection in 35mm, and 60mm plate, 2.5µg of DNA, 5 µg of DNA, and 5µl, 10µl of lipofectamine 3000, and p3000 reagents respectively mixed and incubated for 15

minutes. Then this mixture was added to 35mm, and 60mm plates respectively, and incubated overnight.

III. Cytokinesis-block micronucleus (CBMN) assay

18 mm sterile glass coverslips were used to seed cells at a density of 2.5×10^4 cells/ml. Transfection was done after 18h. Then 100 µM of etoposide was added to the medium for 4h. The 1× PBS was used twice to wash the cells and cytochalasin B was added (final concentration 5µg/ml) after the treatment. After 36h of incubation at 37˚C, 1× PBS was used to wash the cells after the discard of the cytochalasin B-containing medium. Then 75 mM KCl (hypotonic solution) was used to incubate coverslips at 37˚C for 5 minutes and paraformaldehyde (4%) was used to fix with the cells at RT for 15 min. fluorescence microscope (Leica, Germany) was used to examine the fixed cells after mounting with DAPI on microscopic slides. for each experimental time point in 1000 binucleated cells (BNC) the numbers of NBUDs, NPBs, and MNi were counted and scored according to criteria published by Fenech.

IV. Double thymidine Block

Sterile glass coverslips in a 35mm plate were used to seed cells in MEM and RPMI 1640 medium and after overnight incubation at 37 °C, a thymidine-containing medium (2 mM) was added. The thymidine-containing medium was discarded after 18 h of incubation. PBS was used to wash the cells, and the cells were incubated in a fresh medium for 9 h. Then again for another 18 h, a 2 mM thymidine-containing medium was added to the cells. Then the cells were subjected to immunofluorescence assay after incubation in a fresh medium for 6 h.

V. Indirect immunofluorescence

18 mm sterile coverslips were used to seed cells. 4% paraformaldehyde solution (freshly prepared) at RT for 15 min was added to fix the cells after a double thymidine block.

0.2% Triton X-100 was used for 15 min on ice to permeabilize cells. (5% BSA in 1× PBS) was used as a blocking solution and then total PTEN antibody or phospho-PTEN antibody (1:100), γ-tubulin antibody (1:100), PLK1 antibody (1:100), Aurora Kinase A antibody (1:100), purchased from Cell Signaling, USA diluted in wash buffer (0.1% BSA and 0.05%Tween 20 in 1× PBS) was used as primary antibody for incubating overnight at 4 °C. Wash buffer was used to wash cells several times before probing with Texas Red conjugated anti-mouse antibody (1:200) and fluorescein isothiocyanate (FITC) conjugated anti-rabbit antibody (1:200) for 1 h at RT. A fluorescence (Leica, Germany) was used to examine the cells after washing and mounting with a DAPI-containing mounting medium.

VI. In vivo immunoprecipitation

Cells were cultured 18 h before transient transfection with different clones of PTEN. 1X RIPA lysis buffer (ab156034) was used to prepare cell lysates after 24 h, with phosphatase and protease inhibitors (G Bioscience) on ice for 40 min., the anti-HA antibody (1:150) was added to the pre-cleared lysates and were incubated overnight at 4ºC. Then the lysates were incubated for 2h at 4ºC after the addition of 30µl of protein A/G agarose. Then the lysates were centrifuged at 5000 rpm and ice-cold RIPA buffer was used to wash the beads 3 times for 5 minutes at 5000 rpm. 50µl of 2X Lamellae dye was added to the beads, boiled for 10 minutes, and subjected to western blotting with anti-Aurora Kinase A and anti-PLK1 antibody as described above.

VII. Site-Directed Mutagenesis

Phospho-deficient phosphatase dead pSG5L-HA-A4-pd-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala, Cys124Ser), SUMOylation dead pSG5L-HA-K254R-PTEN (Lys254Arg) and phosphatase dead pSG5L-HA-pd-PTEN (Cys124Ser) encode hemagglutinin tagged mutant proteins was created with site-directed mutagenesis purchased from Agilent according to the instruction provided by the manufacturer and sequencing was

used to confirm. Mutagenic primer for phosphatase dead mutant PTEN was created by primer X software.

For phosphatase dead mutant C124S-PTEN the following primer was used:

```
      Mutation site at center:      Yes

------------------------------------------------------------------------

3 primer pair(s) generated.

Primer pair 1
                                  *
    Forward: 5' GTTGCAGCAATTCACTCTAAAGCTGGAAAGGG 3'
    Reverse: 5' CCCTTTCCAGCTTTAGAGTGAATTGCTGCAAC 3'
                                 *
     GC content: 46.88%             Location: 355-386
     Melting temp: 76.7°C           Mismatched bases: 1
     Length: 32 bp                  Mutation: Substitution
     5' flanking region: 16 bp      Forward primer MW: 9897.55 Da
     3' flanking region: 15 bp      Reverse primer MW: 9750.46 Da
```

For sequencing of the clone following sequencing primer was used:

Sence- 5`GCTAGATCTCGAGCTCTTCAAGAGGATGGATT 3`,

Antisence- 5`CGGGCCCGGGATCCACCGGATCTAG 3`

For Sumo dead mutant K254R-PTEN following primer was used:

```
------------------------------------------------------
2 primer pair(s) generated.
Primer pair 1
                                  *
   Forward: 5' GTGTGTGGTGATATCAGAGTAGAGTTCTTCCAC 3'
   Reverse: 5' GTGGAAGAACTCTACTCTGATATCACCACACAC 3'
                                 *
    GC content: 45.45%              Location: 745-777
    Melting temp: 76.5°C            Mismatched bases: 1
    Length: 33 bp                   Mutation: Substitution
    5' flanking region: 16 bp       Forward primer MW: 10214.74 Da
    3' flanking region: 16 bp       Reverse primer MW: 10050.68 Da
```

For sequencing of the clone following sequencing primer was used:

Sence- 5` ATTACGCTAGATCTCGAGTTTATAGACCAGTG 3`

Antisence- 5` ACAAAACAGTAGAGGATCCACCGGATCTAGA 3`

VIII. Spindle pole assay and confocal microscopy

18 mm sterile glass coverslips were used to seed cells. 4% paraformaldehyde solution (freshly prepared) for 15 min was used to fix the cells and cells and 0.2% Triton X-100 on ice

for 10 min was used to permeabilize the cells after transient transfection. (5% FBS in 1× PBS) was used as a blocking solution for 1h at RT, then anti-γ tubulin and anti-α tubulin antibody (1:100), purchased from Cell Signaling Technology, diluted in wash buffer (0.5% BSA and 0.05% Tween 20 in 1× PBS) was used as primary antibody for incubating overnight at 4◦C. Wash buffer was used to wash cells three times before probing with Alexa Fluor 568 conjugated anti-rabbit antibody and Alexa Fluor 488 conjugated anti-mouse antibody for 1h at RT. A confocal microscope (Leica, Germany) was used to examine the cells after washing and mounting with a DAPI-containing mounting medium.

IX. Statistical analysis

All data in the graphs are presented as means and standard deviations (SD). To generate graphs, Microsoft Excel software or GraphPad Prism 8.0 was used. Microsoft Excel analysis tools were used to perform statistical calculations. For the calculation of statistical differences between groups student's t-test was used. $P<0.05$ was considered statistically significant.

X. Western blotting

60-mm culture dish was used to seed cells 18 h before transfection and different clones of PTEN were transiently transfected. Nocodazole (final concentration 50ng/ml) was used to treat the transfected cells for 16h. 1X RIPA lysis buffer (ab156034), phosphatase, and protease inhibitors (G Bioscience) were used to prepare whole-cell lysates from the cells. Bradford's reagent was used to estimate the protein concentration. After the addition of Lamellae buffer, lysates were boiled for 5 min, and an equal amount of protein (100 μg) containing lysates was electrophoresed on a 10% SDS polyacrylamide gel in Tris–glycine buffer (pH 8.8). Polyvinylidene difluoride (PVDF) membrane (Merck) was used to transfer the proteins. 5% non-fat dry milk and 0.05% Tween-20 in 20 mM Tris–Cl, pH 7.6 (TBS-T) was used to block nonspecific binding. TBS-T was used to wash the membrane after

incubation with the appropriate primary antibody and then HRP-conjugated secondary antibodies were used to reincubate the membrane. ECL detection reagent purchased from Abcam was used to detect the bound antibodies. Cells were overexpressed with different clones of PTEN to understand the modulation of different PTEN clones, so, anti-PTEN, anti-Aurora Kinase A, anti-PLK1, anti-Phospho-Aurora Kinase A, anti-Phospho-PLK1 antibodies were used to immunoblot the cell lysates. Anti-GAPDH antibody was used as a loading control.

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List of Publication

1. **Ginia Ghosh, Sandip Misra, Rachayeeta Ray, Sougata Ghosh Chowdhury, Parimal Karmakar, Phospho PTEN mediated dephosphorylation of mitotic kinase PLK1 and Aurora Kinase A prevent aneuploidy and preserve genomic stability. *Accepted***

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3. **Sandip Misra, Sougata Ghosh Chowdhury, Ginia Ghosh, Ananda Mukherjee, Parimal Karmakar, Both phosphorylation and phosphatase activity of PTEN are required to prevent replication fork progression during stress by inducing heterochromatin, *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, Volume 825, 2022, 111800, ISSN 0027-5107,**

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Poster/Oral Presentation

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ORIGINAL PAPER

Phospho PTEN mediated dephosphorylation of mitotic kinase PLK1 and Aurora Kinase A prevents aneuploidy and preserves genomic stability

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Abstract

PTEN, dual phosphatase tumor suppressor protein, is found to be frequently mutated in various cancers. Post-translational modification of PTEN is important for its sub-cellular localization and catalytic functions. But how these modifications affect cytological damage and aneuploidy is not studied in detail. We focus on the role of phosphatase activity along with C-terminal phosphorylation of PTEN in perspective of cytological damage like micronucleus, nuclear bud, and nuclear bridge formation. Our data suggest that wild-type PTEN, but not phospho-mutant PTEN significantly reduces cytological damage in PTEN null PC3 cells. In case of phosphatase-dead PTEN, cytological damage markers are increased during 24 h recovery after DNA damage. When we use phosphorylation and phosphatase-dead dual mutant PTEN, the extent of different cytological DNA damage parameters are similar to phosphatase-dead PTEN. We also find that both of those activities are essential for maintaining chromosome numbers. PTEN null cells exhibit significantly aberrant γ-tubulin pole formation during metaphase. Interestingly, we observed that p-PTEN localized to spindle poles along with PLK1 and Aurora Kinase A. Further depletion of phosphorylation and phosphatase activity of PTEN increases the expression of p-Aurora Kinase A (T288) and p-PLK1 (T210), compared to cells expressing wild-type PTEN. Again, wild-type PTEN but not phosphorylation-dead mutant is able to physically interact with PLK1 and Aurora Kinase A. Thus, our study suggests that the phosphorylation-dependent interaction of PTEN with PLK1 and Aurora Kinase A causes dephosphorylation of those mitotic kinases and by lowering their hyperphosphorylation status, PTEN prevents aberrant chromosome segregation in metaphase.

Keywords Aneuploidy · Spindle pole · Micronucleus · PTEN · Aurora Kinase · Polo-like kinase

Introduction

PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) is frequently mutated in a variety of malignancies, including prostate cancer, breast cancer, endometrial cancer, and glioblastoma [1–3]. It is a dual protein and lipid phosphatase, whose classical function is to negatively regulate the oncogenic PI3K/Akt pathway [4, 5]. PTEN has recently been identified as a DNA repair protein. Bestowing several studies PTEN null cells shows genomic instability and abnormal chromosomal characteristic like chromosomal breakage, telomere fusion, nucleoplasmic bridge formation, etc. [6]. Significant pool of PTEN localizes to the nucleus/chromatin and plays vital functions in the maintenance of genomic stability and chromatin architecture [6]. PTEN inhibits genomic instability by a variety of functions, including regulation of spindle assembly checkpoint (SAC) [7, 8], controlling cell cycle checkpoints [9], homologous recombination (HR) repair [10], and replication fork stability [11]. According to the findings, PTEN stabilizes the MAD2 protein, a component of the MCC complex, and protects it from ubiquitin-mediated degradation [12]. This results in the prevention of unregulated chromatin segregation and aneuploidy during the metaphase of cell division [13–15].

Number of reports suggested that the catalytic functions of PTEN may be attributed to its post-translational modulations, including phosphorylation [16], ubiquitination, sumoylation [17], acetylation [18], and oxidation

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[19]. Several protein kinases, including PLK1 [20], CK2 [21], and GSK-3 [22] phosphorylate PTEN extensively in the C-terminal region (Ser 370, Ser 380, Thr 382, Thr 383, and Ser 385). Whereas PTEN stability is enhanced upon its phosphorylation, its phosphatase activity is diminished simultaneously [22]. It has been proposed that dephosphorylation or mutations at the STT cluster of PTEN advance its ubiquitination [23] while increasing its phosphatase activity [24, 25]. Thus, the PTEN C –terminal motif regulates its stability and catalytic activity, which may be related to its tumor suppressive functions through maintaining genomic stability. Recently several reports recommended that PTEN C-terminal phosphorylation has been linked with chromatin association and DNA repair [26, 27].

Polo-like kinase1 (PLK1), a mitotic kinase, regulates a variety of cell cycle events, including mitotic entry, bipolar spindle formation, and DNA repair [28–34]. PTEN is directly phosphorylated by PLK1 at Ser 380 and regulating its chromatin interaction [20]. On the other hand, Aurora kinases (AURKs), another mitotic kinase, are a group of preserved serine/threonine protein kinases that play a critical role in carcinogenesis when constitutively activated [35]. AURKA is involved in spindle assembly, mitotic entry, and centrosome activity [36]. Evidence suggests that the build-up of the CEP192 complex in pericentriolar material causes autophosphorylation of AURKA, which activates its kinase activity and leads to PLK1 phosphorylation. During microtubule nucleation, the CEP192-AURKA-PLK1 complex generates γ-tubulin ring complex attachment sites, whereas hyperactivity disrupts normal chromosomal segregation, resulting in aneuploidy [37]. Overexpression of AURKA has been reported in numerous cancers, including bladder cancer, esophageal squamous cell carcinoma, and breast cancer [38–40]. AURKA hyperactivation promotes centrosome amplification, which causes cytokinesis failure and subsequent multinucleation formation [35], which is one of the phenotypes of PTEN null cells [41]. Furthermore, AURKA overexpression in cells skips the spindle checkpoint in mitosis and causes cells to reach anaphase despite the creation of an aberrant spindle, resulting in chromosomal fragmentation or breakage, anaphase bridge, and lagging chromosomes [42].

PTEN phosphorylation and phosphatase activity have been studied individually in the context of genomic stability. However, to the best of our knowledge, there are no reports on the interplay of phosphorylation and phosphatase activity of PTEN together. Hence, in this study, we want to look at the relevance of PTEN's phosphorylation, as well as phosphatase activity, in preserving genomic integrity and subsequent consequence on cytological damage. In this study, in light of ectopic expression of (1) wild-type PTEN, (2) phospho-deficient PTEN mutant, (3) phosphatase-dead PTEN-mutant and (4) phospho-phosphatase dual dead PTEN mutant constructs in the PTEN-null glioma cell line U87MG and the prostate cancer cell line PC3, chromosomal instability was evaluated. Our findings imply that both PTEN phosphorylation and phosphatase activity are important in maintaining genomic stability.

Materials and methods

Cell culture, reagents, and antibodies

PC3 (Human Prostate Cancer Cell); U87MG (Human glioblastoma cell) and Wi-38 (normal lung fibroblast) were purchased from NCCS Pune India. PC3, derived from a prostatic adenocarcinoma grade 4, of a 62-year-old Caucasian male was cultivated in RPMI 1640 medium, U87MG, established from a malignant glioma of a female patient, and Wi-38, derived from a 3 month old female fetus were cultured in MEM, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). Cells were kept at 37 °C, 5% CO_2, and 95% relative humidity (RH). Dimethyl Sulphoxide (Sigma) was used to dissolve Nocodazole (Sigma), Etoposide (Sigma), and Cytochalasin B (Sigma) at concentrations of 1 mg/ml, 25 mM, and 5 mg/ml, respectively. Subsequent dilutions were made fresh in the medium. Anti-Aurora Kinase A (9980S), anti-PLK1 (4513S), anti-P-Aurora Kinase A (T288) (8025S), anti-P-PLK1 (T210) (3738S), and anti-P-PTEN antibodies were purchased from Cell Signalling Technology. Total PTEN antibody (32,199) was purchased from Abcam. Secondary antibodies like anti-rabbit IgG conjugated with Alexa Fluor 568 (1:150, Life Technologies, USA), anti-mouse IgG conjugated with Alexa Fluor 488 (1:150, Life Technologies, USA), and anti-rabbit IgG conjugated with horseradish peroxidase (HRP, 1:25,000, Abcam), were used.

Plasmids, site-directed mutagenesis, and transfection

Mammalian expression vectors for phospho-deficient pSG5L-HA-A4-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala), phosphatase-dead pSG5L-HA-C124S-PTEN (Cys124Ser) and phospho-deficient phosphatase-dead pSG5L-HA-A4PD-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala, Cys124Ser) encode hemagglutinin-tagged mutant proteins were created by site-directed mutagenesis kit (Stratagene) as per manufacturer's instruction and were confirmed by sequencing. The following primer was used for phosphatase-dead mutant PTEN:

Forward: 5′ GTTGCAGCAATTCACTCTAAAGCTGGAAAGGG 3′, Reverse: 5′CCCTTTCCAGCTTTAGAGTGAATTGCTGCAAC 3′

The following sequencing primer was used for sequencing the clone:

Sence- 5′GCTAGATCTCGAGCTCTTCAAGAGGATGGATT 3′, Antisence- 5′CGGGCCCGGGATCCACCGGATCTAG 3′

(HA-WT-PTEN) wild-type pSG5L-HA-Wt-PTEN encodes hemagglutinin-tagged Wt-PTEN protein. Lipofectamine 3000 (Invitrogen) was used for transient transfection. For transient transfection PTEN null PC3 and U87MG cells were cultured in an antibiotic-depleted medium. Transfections were done according to manufacturer instructions.

Cytokinesis-block micronucleus (CBMN) assay

PTEN null PC3 cells were plated on coverslips, transiently transfected, and then subjected to 100 µM etoposide for 4 h, followed by cytochalasin B (final concentration of 5 µg/ml). After 36 h, the coverslips were then incubated for 5 min at 37 °C in a hypotonic solution (75 mM KCl) before being fixed for 15 min at room temperature with paraformaldehyde (4%). The coverslips were put on microscopic slides with mounting medium containing 4 -6-diamidino-2-phenylindole (DAPI, Vecta Shield Laboratories, USA) and observed under a fluorescence microscope (Leica, Germany). The numbers of Micronuclei (MNi), Nucleoplasmic Bridge (NPBs), and Nuclear Buds (NBUDs) were computed in 1000 BNC (binucleated cells) for the respective experimental time points and documented using Fenech's [43] criteria.

Immuno-fluorescence

Cells cultured on glass coverslips were transiently transfected with PTEN clones and synchronized using a double thymidine block where cells were incubated for 18 h with thymidine (2 mM), then 9 h with fresh medium and again 18 h with thymidine (2 mM). After 8 h cells were fixed in a 4% paraformaldehyde solution and permeabilized on ice with 0.2% Triton X-100. The cells were then re-incubated in blocking solution (0.5% FBS in PBS), followed by overnight incubation with anti-α-tubulin and anti-γ-tubulin antibody or anti-γ-tubulin with either anti-PLK1 or anti-Aurora Kinase A antibody in wash buffer. The same technique was used to synchronize WI-38 cells, which were then treated with total PTEN or phospho-PTEN using γ-tubulin antibodies. The cells were then washed with 0.5% FBS and 0.05% Tween 20 in PBS before being probed with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 antibodies at room temperature for 1 h. Cells were mounted with DAPI before being observed under a fluorescence or confocal microscope (Leica, Germany).

Aneuploidy analyses

After transient transfection of different PTEN clones in both PC3 and U87MG cells, nocodazole (50 ng/ml) was added, and treated cells for 16 h. The cell pellet was then collected and incubated for 15 min in 75 mM KCl before being centrifuged. The pellet was then fixed for 15 min at 4 °C in an ice-cold fixative (methanol:acetic acid in a 3:1 ratio) before being centrifuged. The pellet was then resuspended in ice-cold fixative and dropped on a grease-free, cold glass slide, flame dried instantaneously, mounted with DAPI, and studied under a fluorescence microscope (Leica, Germany).

Western blotting

Different PTEN clones were transiently transfected into both PC3 and U87MG cells. Following transfection, the cells were treated for 16 h with nocodazole (50 ng/ml). Using RIPA lysis buffer(ab156034) and protease and phosphatase inhibitors (G Bioscience), whole cell lysates were produced. Protein concentrations in the supernatant were determined by Bradford assay, and an identical amount (90 µg) of protein-containing cell lysates were run on SDS-PAGE. The proteins were then transferred to PVDF (polyvinylidine difluoride) membrane (Merck). The membrane was incubated with appropriate primary antibodies, followed by appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). The membrane was immunoblotted with anti-Aurora Kinase A, anti-PLK1, anti-p-Aurora Kinase A, anti-p-PLK 1, and anti-PTEN. As a loading control, an anti-GAPDH antibody was utilized.

Immunoprecipitation

After transient transfection with various PTEN clones, cell lysates were processed with 1X RIPA lysis buffer containing protease and phosphatase inhibitors. After clearing the lysate with protein A/G conjugated agarose bead, the lysates were incubated with anti-HA antibody overnight at 4 °C. (1:150). Then a protein A/G agarose bead was added and incubated at 4 °C for 2 h. The lysates were then centrifuged, and the beads were washed with RIPA buffer. Proteins were then eluted with 50 µl of 2X Lamellae dye, boiled for 10 min, and blotted with anti-PLK1, anti-AURKA, and anti-PTEN antibodies as previously described.

Statistical analysis

The data in the graphs were all represented as means and standard deviations (SD). GraphPad Prism 8.0 or Microsoft

Excel software was used to make the graphs. Microsoft Excel analytical tools were used to do statistical calculations. The statistical difference between groups was calculated using a student's *t*-test. $p < 0.05$ was determined to be statistically significant. R Studio was used to do the PCA analysis.

Results

PTEN phosphatase activity and phosphorylation results in suppressing cytological damage.

The Cytokinesis Block Micronucleus Assay is the basic technique to detect biomarkers of chromosomal instability such as micronuclei, nucleoplasmic bridges, and nuclear buds. Mis-segregated chromosomes or their pieces generate cytoplasmic DNA in the nucleus, resulting in micronuclei build-up during subsequent cell divisions. At first $PTEN^{-/-}$ PC3 cells were transfected with Wt-PTEN, A4-PTEN, C124S-PTEN, and A4-C124S-PTEN (Fig. 1A) separately, and subsequently, they were treated for 4 h with etoposide followed by CBMN assay. Micronuclei (MNi), Nucleoplasmic Bridges (NPBs), and Nuclear Buds (NBUDs) were counted at 0 h and 24 h recovery time points (Table 1). After 24 h of recovery from etoposide treatment, we found MNi formation is significantly increased (Fig. 1BIV) in both C124S-PTEN (phosphatase-dead) and A4-C124S-PTEN (phosphatase and phosphorylation-dead) transfected cells (49 ± 3.20 and 66 ± 2.88) respectively compared to Wt-PTEN (25 ± 2.06) transfected cells. Nuclear bud development is similarly increased in phosphatase-dead and double mutant transfected cells versus Wt-PTEN (C124S-PTEN-27 ± 3.30 and A4-C124S-PTEN 47 ± 2.5 vs 9 ± 1.26 at 24 h) (Fig. 1BIII). NPBs (Fig. 1BII), which are indications of amplified DNA exclusion and telomere end fusion or DNA mis-repair, were also higher in C124S-PTEN (14 ± 2.38) and A4-C124S-PTEN (20 ± 2.94) transfected cells than in Wt-PTEN (3 ± 0.5) transfected cells. Interestingly, phosphorylation-dead PTEN (A4-PTEN), which has increased phosphatase activity, eliminates MNi more efficiently than other PTEN mutant transfected cells but less efficiently than Wt-PTEN transfected cells (Table 1). This study implies that classical phosphatase activity is critical for excluding accumulated cytological damage.

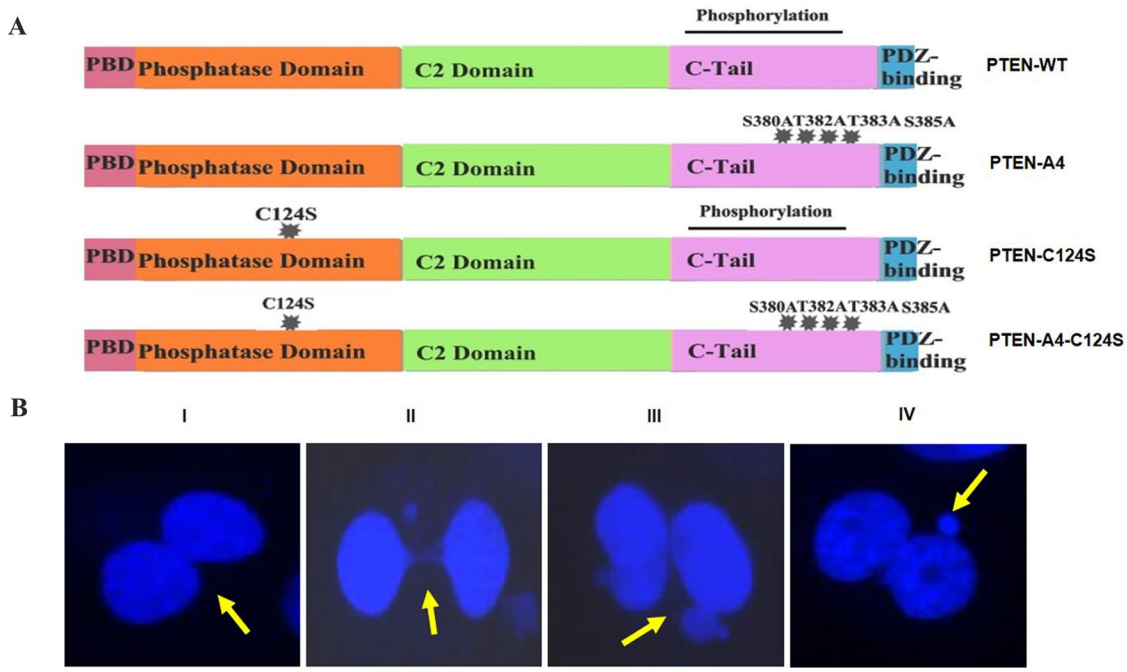

Fig. 1 Occurrence of cytological damage in different mutants of PTEN A. Map of different mutants of PTEN (Wt-PTEN, A4-PTEN, C124S-PTEN, and A4-C124S-PTEN) B. Microscopic images of cells incubated in the presence of etoposide (100 µM for 4 h) and allowed to recover for 24 h. Cells were stained with DAPI. Arrowheads indicate I. binucleated cell II. Binucleated cell with Mni and NPB III. Binucleated cell with NBud IV. Binucleated cell with Mni

Table 1 The results of cytokinesis-block micronucleus assay in different clones of PTEN after 4 h exposure of 100μ M etoposide

Mutants	MNi per 1000 BN cells		NBud per 1000 BN cells		NPB per 1000 BN cells	
	0 h	24 h	0 h	24 h	0 h	24 h
UT	77 ± 2.98	65 ± 3.79	28 ± 1.82	37 ± 1.5	17 ± 2.21	20 ± 2.1
PTEN-WT	49 ± 1.89[a]	25 ± 2.06[b]	14 ± 1.73[a]	9 ± 1.26[b]	6 ± 0.58[a]	3 ± 0.5[b]
PTEN-A4	48 ± 1.15	36 ± 2.16	36 ± 0.82	20 ± 1.82	10 ± 1.29	7 ± 0.96
PTEN-C124S	23 ± 2.36[c]	49 ± 3.20[d]	15 ± 3.09[c]	27 ± 3.30[d]	5 ± 0.5[c]	14 ± 2.38[d]
PTEN-A4PD	56 ± 3.16[c]	66 ± 2.88[d]	32 ± 2.58[c]	47 ± 2.5[d]	15 ± 2.75[c]	20 ± 2.94[d]

Values shown as for means ± SEM of three independent experiments

MNi Micronuclei; *NBud* Nuclear Buds; *NPBs* Nucleoplasmic Bridges

[a] $p < 0.05$ compared with UT 0 h (Student's *t*-test)

[b] $p < 0.05$ compared with UT 24 h (Student's *t*-test)

[c] $p < 0.05$ compared with PTEN 0 h (Student's *t*-test)

[d] $p < 0.05$ compared with PTEN 24 h (Student's *t*-test)

Phosphatase and phosphorylation activities of PTEN prevent multiple γ-tubulin pole formation in metaphase and suppress spontaneous aneuploidy

The most important stage in chromosomal segregation during metaphase is the formation of a γ-tubulin pole at the centrosome. Multiple pole development during metaphase is harmful to cell destiny because they result in chromosome missegregation and aneuploidy. Aneuploidy occurs spontaneously in PTEN-deficient cells, indicating whole-chromosome instability. Microscopic pictures exhibit the variance in the number of pole formations following various PTEN mutant transfection in PTEN$^{-/-}$ U87MG and PC3 cell lines (Fig. 2A). The absence of PTEN phosphatase and phosphorylation activity resulted in a 1.3-fold reduction in normal bipolar cell percentage in PTEN clones against Wt-PTEN (Fig. 2B). Percentage of multiple poles in untransfected, C124S-PTEN, and A4-C124S-PTEN cells is 10–12-fold higher, and 8–10-fold rise in A4-PTEN transfected cells in comparison to Wt-PTEN (Fig. 2C) transfected U87MG cells. Compared to Wt-PTEN transfected PC3 cells, PTEN phosphorylation- and phosphatase-dead clone, phosphorylation-dead clone, and phosphatase-dead clone including untransfected cells, had a 6–11-fold intensification in the percentage of multipolar cells formation (Fig. 2F). Next, we investigated the ploidy status of several mutant PTEN clones after transient transfection in PTEN$^{-/-}$ PC3 as well as U87MG cell lines. After 16 h of treatment with a microtubule depolymerizing reagent, nocodazole, the aneuploidy status of each mutant was investigated. In this study, we detected a 1.6-fold rise in aneuploidy percentage in A4-PTEN, and an approximately twofold increase in both C124S-PTEN and A4-C124S-PTEN transfected U87MG cells and untransfected had a 1.9-times increase in aneuploidy percentage compared with Wt-PTEN-transfected cells (Fig. 2D). This pattern holds true for the other PTEN-negative PC3 cell line. We found a 1.9, 2.6, and 2.7-fold surge in aneuploidy in A4-PTEN, C124S-PTEN, and A4-C124S-PTEN transfected PC3 cells, when compared to Wt-PTEN transfected cells, respectively, and a 2.7-fold increase in untransfected cells when compared with Wt-PTEN transfected cells (Fig. 2G). This finding indicates that the phosphatase activity of PTEN and its phosphorylation is required for proper chromosomal segregation.

Phospho-PTEN co-localized with γ- tubulin at the spindle pole during metaphase.

Previous research has suggested that PTEN controls chromosomal segregation [15]. Owing to the fact that PTEN deletion causes aneuploidy, we want to investigate whether PTEN is localized to the spindle pole. According to our immunofluorescence study, PTEN, or in particular the phosphorylated version of PTEN (Ser 380, Thr 382,383), is localized to the spindle pole and associated with γ-tubulin, as demonstrated in PTEN-positive lung fibroblast Wi-38 cell (Fig. 3A). PLK1 and AURKA, on the other hand, co-localize with γ-tubulin to the centrosomes of the PTEN-negative cell line PC3. (Fig. 3B).

Loss of PTEN phosphatase and phosphorylation activities lead to differential expression of Aurora Kinase A and PLK1

Several investigations have shown that during the early stages of mitosis, AURKA autophosphorylation is the key regulatory mechanism in centrosome formation [44]. Overactivation of these mitotic kinases results in unregulated chromosomal segregation and mitotic pole disintegration [45]. To test this idea, we ectopically expressed various

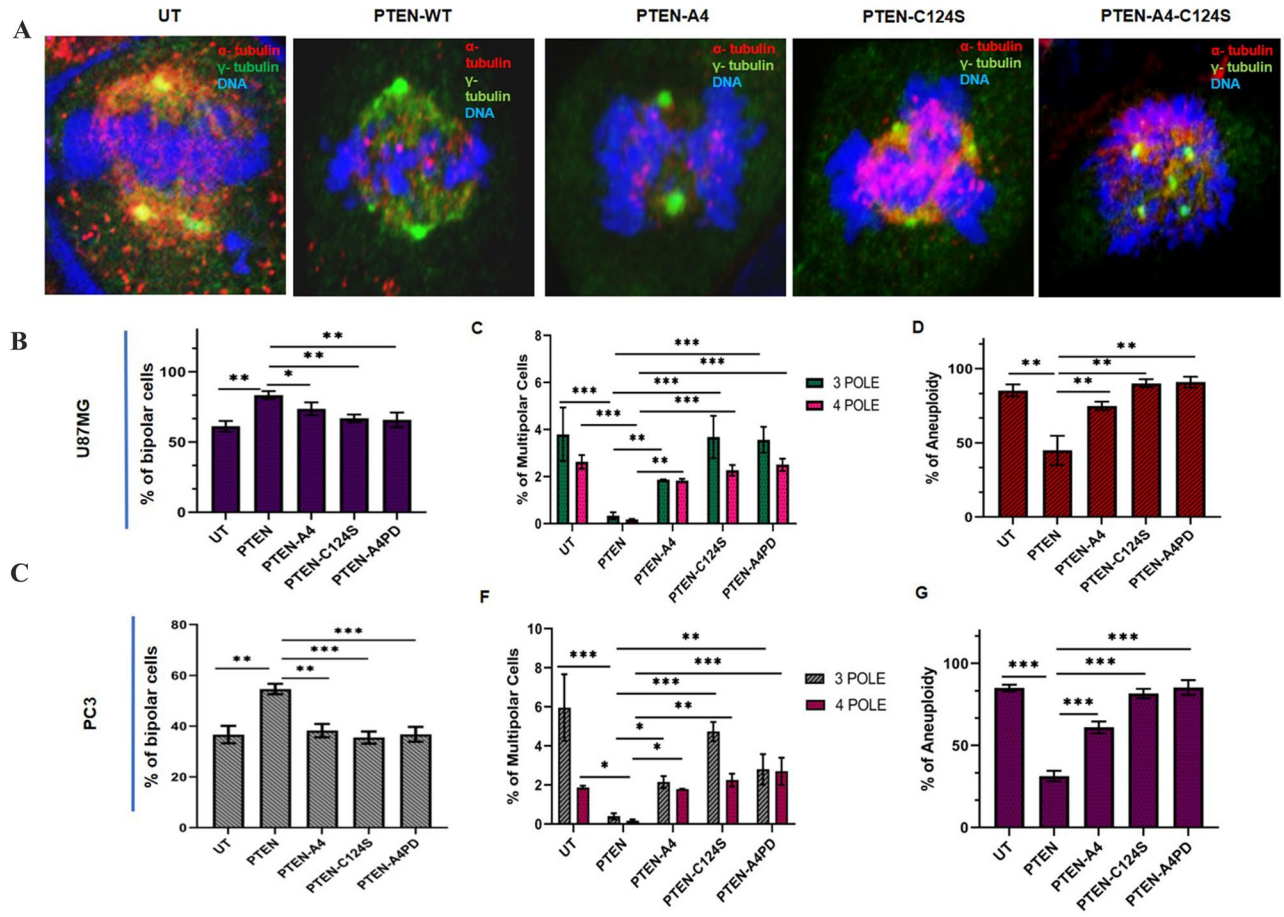

Fig. 2 Multiple pole formation causes chromosome missegregation. **A** Microscopic images of the formation of multiple poles during metaphase in different PTEN-transfected cells Images are labeled with α-tubulin (red), γ-tubulin (green), and DNA (blue). Graphical representation of decrease in the percentage of bipolar and increase in the percentage of multipolar cells in U87MG (**B, C**) and in PC3 cells (**E, F**) transfected with different PTEN clones compared to Wt-PTEN. The increase in aneuploidy in various mutant PTEN and the decrease in aneuploidy in Wt-PTEN transfected U87MG (**D**) and PC3 cells (**G**) were quantified. The values represent the mean SEM of three independent experiments. $*p<0.05$; $**p<0.01$; $***p<0.001$

clones of PTEN in PTEN-deficient PC3 and U87MG cells separately. After 24 h, the cells were treated for 16 h with nocodazole, and western blots were used to evaluate the levels of PLK1 and Aurora Kinase A expression. Both phospho-Aurora Kinase A (T210) and phospho-PLK1 (T288) expression were significantly amplified in untransfected PC3 and U87MG cell lines as compared to Wt-PTEN transfected cells. These mitotic kinases were also observed to be overexpressed in C124S-PTEN and A4-C124S-PTEN transfected cells in PC3 (Fig. 4A) and U87MG cells when compared to Wt-PTEN transfected cells (Fig. 4B). Interestingly, phosphorylation-dead PTEN-transfected cells have higher levels of phospho-PLK1 (T210) and phospho-Aurora Kinase A (T288) though it possesses increased phosphatase activity. Thus, it appears that alternative mechanisms exist in cells that play a role in the inability of A4-PTEN to dephosphorylate certain proteins in vivo.

Wt-PTEN but not A4-PTEN or A4-C124S PTEN physically interact with PLK1/Aurora Kinase A

We next used immunoprecipitation study to see if PTEN and its mutants physically interact with PLK1 and Aurora Kinase A. PTEN clones were transfected into PTEN-null PC3 cells for 24 h before being treated with nocodazole. Anti-HA antibody was used to immunoprecipitate whole cell lysates, which were then blotted with PLK1 and Aurora Kinase A. We observed that phosphorylation-dead mutant PTEN (A4-PTEN) (lane 3) and double mutant PTEN (A4-C124S-PTEN) (lane 5) did not physically interact with PLK1 as efficiently as Wt-PTEN (lane 2). However, phosphatase-dead PTEN (lane 4) interacted

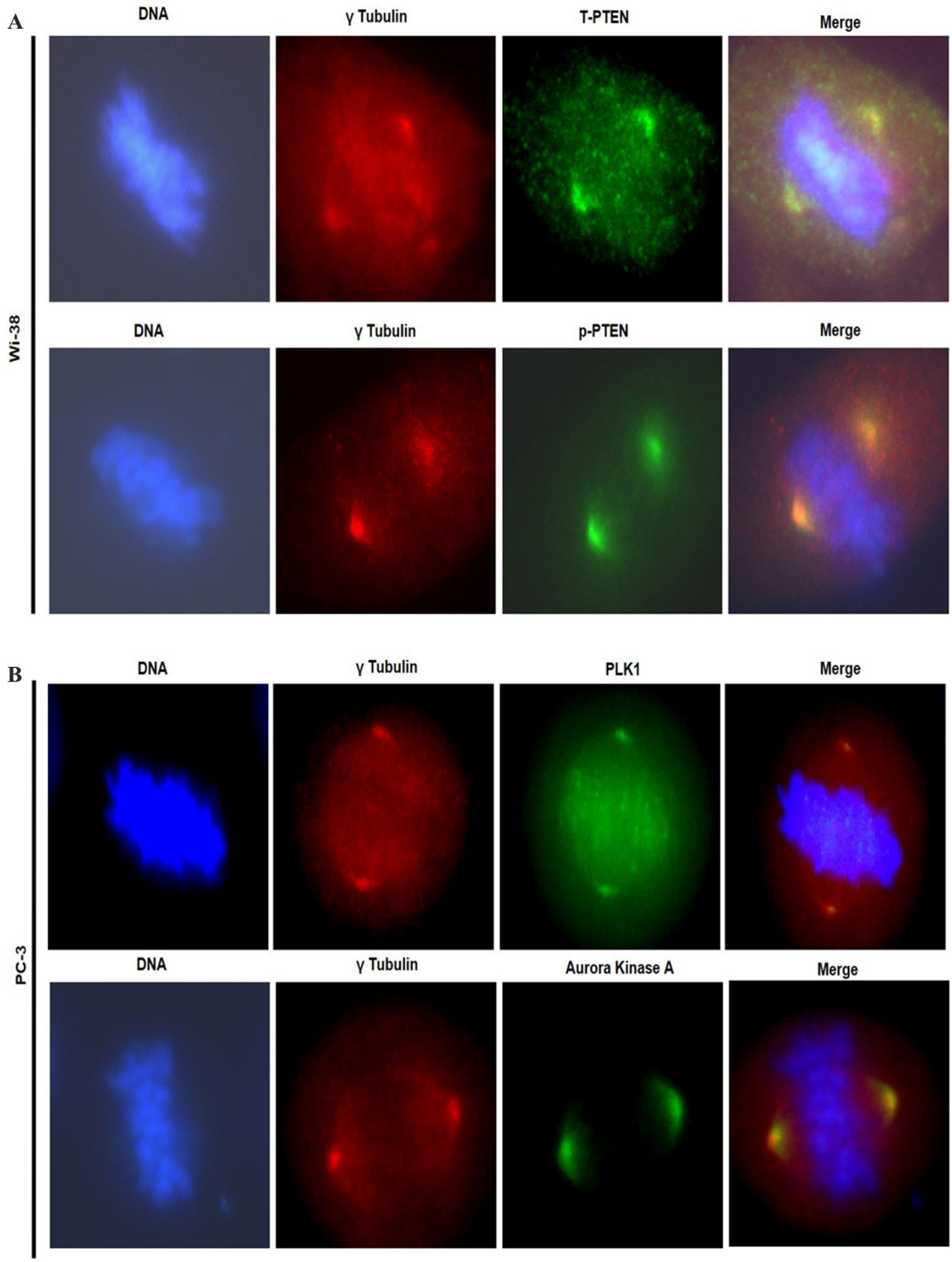

Fig. 3 PTEN localizes at the spindle pole. **A** Metaphase Wi-38 cells are immunolabeled with γ-tubulin antibody (red) and either total PTEN antibody (green) or phospho-Ser 380, Thr 382, and 383 PTEN antibody (green). **B** Synchronized PTEN-null PC3 cells are immunolabeled with γ-tubulin (red) and either PLK1 or Aurora Kinase A (green) antibodies

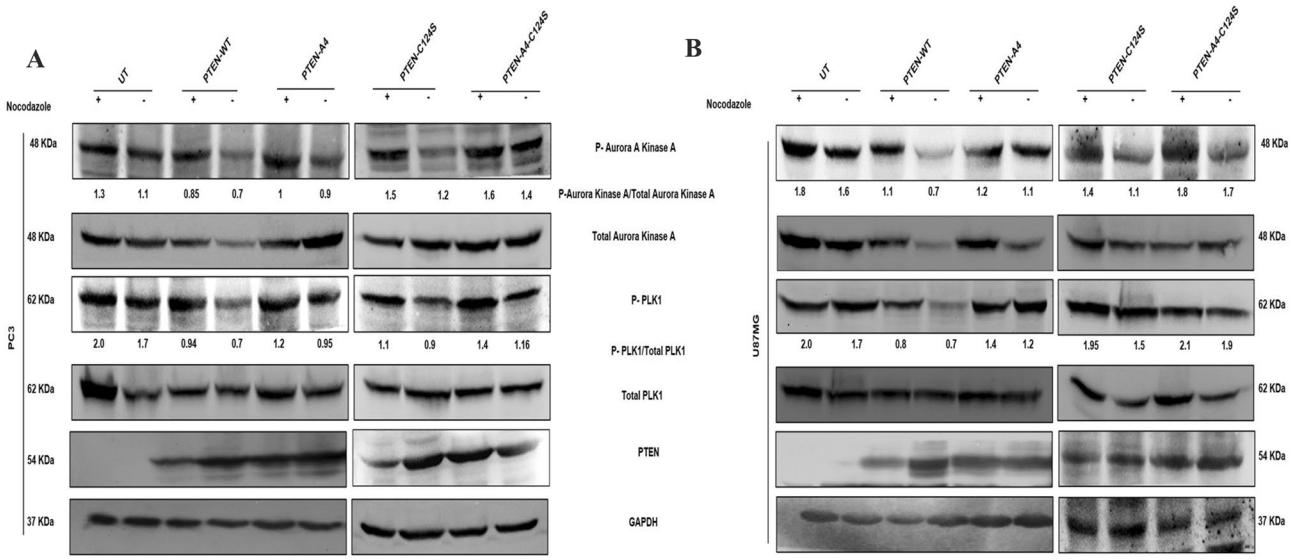

Fig. 4 Differential expression of mitotic kinases in different PTEN mutants A and B Phosphorylation of AURKA and PLK1 was reduced in Wt-PTEN but increased in UT, C124S-PTEN, and A4-C124S-PTEN, PTEN clones after transfection in PTEN-/- cell lines (PC3 and U87MG) before and after nocodazole treatment for 16 h. GAPDH acts as a loading control. Values were calculated by image J software, which indicates the ratio of p-PLK1/ total PLK1 and p-AURKA/ total AURKA antibody. Untransfected control is normalized to 1

with PLK1 efficiently. Next, we found that hosphor-dead PTEN (lane 3) and double mutant PTEN (lane 5) were also unable to associate with Aurora Kinase A, although wild-type PTEN (lane 2) and phosphatase-dead PTEN (lane 4) did. Thus, the C-terminal phosphorylation of PTEN may be critical for its physical interaction with PLK1 and Aurora Kinase A. Even though A4-PTEN has increased phosphatase activity, it is unable to engage efficiently with PLK1 and Aurora Kinase A and so cannot dephosphorylate both, as demonstrated by our western blot assay. (Fig. 5).

Discussion

Maintenance of genomic stability is crucial to prevent neoplastic characteristic of cells. PTEN is one of the important tumor suppressor proteins that not only regulates traditional PI3K/AKT pathway but also plays a critical role in the maintenance of genomic stability by controlling cell cycle regulation, heterochromatin structure maintenance [46, 47], HR repair [46], accurate chromosomal segregation [12, 48]and progression of DNA replication [11, 49]. The importance of phosphatase activity and phosphorylation of PTEN have been investigated in various literature, however, addressing the integrated activity of PTEN phosphatase and phosphorylation activity have not yet been examined in connection to chromosomal integrity [50, 51]. In this study, we used PTEN null PC3 and U87MG cells as a model system as no functional PTEN expressed in these cell lines [52, 53]. The PTEN status of both of these cells is negative. When different mutants of PTEN were transfected, we found PTEN phosphorylation and phosphatase function are both required to reduce damage induced Mni accumulation indicating compromised DNA repair with non-functional cell cycle checkpoint functionary. When phosphorylation- and phosphatase-impaired double mutant (A4-C124S-PTEN) and phosphatase-dead (C124S-PTEN) were compared to Wt-PTEN, Nbud, NPBs also increased. It appears that overexpressed p-Akt in C124S-PTEN transfected cells drives cell division with unrepaired damaged DNA, overcoming the checkpoint barrier, and inducing the build-up of Mni, Nbud, and NPB during the 24 h recovery period. Sporadic phospho-deficient PTEN may also contribute to the Mni build-up in the absence of DNA repair system. As a result, when we utilized both phosphatase-dead and phosphorylation-dead PTEN (A4-C124S-PTEN), excessive damage build-up was detected due to the dual influence of elevated p-AKT levels and reduced DNA repair capabilities. As previously reported, we also observed that PTEN null cells show abnormal chromosomal segregation leading to aneuploidy [12]. This is the consequence of compromised MCC/ SAC functions. PTEN has been shown to physically interact with the Mad1, Mad2, and Cdc20 proteins, all of which are required for MCC [54, 55], and establishes docking sites for dlg1 and Eg-5, both of which are required for normal bipolar spindle formation. Going upstream to this pathway we found that multiple γ-tubulin pole formation occurs in PTEN null

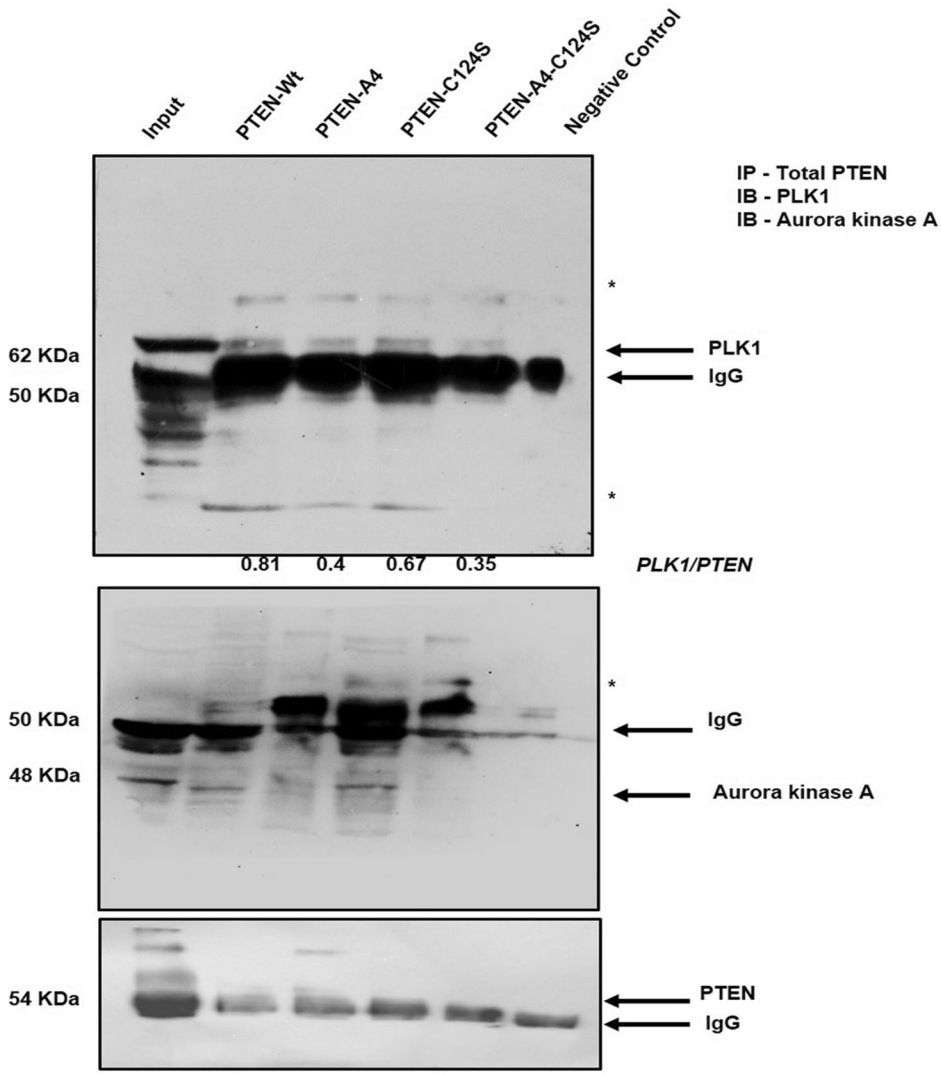

Fig. 5 PTEN null PC3 cells were transfected with the indicated PTEN clone, and after nocodazole treatment, whole cell lysate was prepared and immune precipitated with anti-HA antibody followed by immunoblotting with PLK1 and Aurora Kinase A antibody showed that PTEN-C124S and PTEN-A4-C124S were unable to efficiently interact with PLK1 and Aurora Kinase A and dephosphorylate them

cells. Interestingly we observed that increased multiple pole formation also occurs in PTEN phosphatase-dead and phosphorylation-dead transfected cells when compared to Wt-PTEN transfected cells. A recently published study revealed that PTEN lacking phosphorylation domain (A4-PTEN) is unable to localize at metaphase chromosomes [56]. However, the results of our study strongly suggest that p-PTEN localized to the spindle pole with γ-tubulin. Studies suggest that PTEN-deficient cells have increased γ-tubulin levels, which may lead to centrosome amplification [57]. Another cause of centrosome amplification is the hyperactivity of mitotic kinases like PLK1 and Aurora Kinase A. Nuclear PTEN interacts with APC/C, promotes its interaction with CDH1, and so enhances the tumor suppressive activity of the APC-CDH1 complex by degrading Cyclin A2, Aurora A, PLK1 [58] and regulates proper mitotic exit [9]. Thus, PTEN regulates Aurora A, PLK1 expression indirectly. Accordingly, we found that PTEN null cells show increased expression of those mitotic kinases and their phosphorylated form. PTEN phosphatase and phosphorylation-dead mutant transfected cells show the expression of hyperphosphorylated mitotic kinases compared to Wt-PTEN. Expression of Wt-PTEN suppresses hosphor-PLK1 (T210) and hosphor-Aurora Kinase A (T288) expression, and this is independent of nocodazole treatment. In the case of C124S-PTEN and A4-C124S-PTEN, the expression of both phospho-PLK1 and phospho-Aurora Kinase A proteins is equal before and after nocodazole treatment. A4-PTEN, despite having higher phosphatase activity, showed a negligible decrease in phospho-PLK1 and phospho-Aurora Kinase A expression. To address this question, we carried out an immunoprecipitation assay, where we found that phospho-dead PTEN is unable to physically interact with Aurora Kinase A and diminished association with PLK1 compared to WT-PTEN. Thus, compromised association with these proteins with phospho-mutant PTEN renders it in hyperphosphorylated

form, thus promoting centrosome amplification hence causing aneuploidy. This is supported by principal component analysis (PCA), which explained 87.0% of the total variation. The aberrations (3- and 4-pole, Mni-24 h, Nbud-24 h, and NPB-24 h) and aneuploidy are positively related to A4-C124S-PTEN and negatively related to wild-type-PTEN. A4-C124S-PTEN, on the other hand, is negatively related to two poles whereas wild-type PTEN is positively related to two poles (S1). Taken together our data are in a model where we suggest that phosphorylation-mediated interaction of PTEN with PLK1 and Aurora Kinase A causes dephosphorylation of those proteins and hence maintained those mitotic kinases in hyperphosphorylated form. Thus, phospho PTEN preserves genomic stability. We are the first ones to indicate that PTEN phosphatase activity in conjunction with phosphorylation is crucial in preserving cell genomic integrity.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest All the authors declare there is no conflict of interest.

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PTEN: Sumoylation Function is the Key to the Maintenance of Genomic Stability of Cell

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Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor protein with dual phosphatase activity, is found to be frequently mutated in various cancers. PTEN is post-translationally modulated at various amino acid residues which are crucial for sub-cellular localization as well as its catalytic functions rendering genomic stability. Recent reports suggest that PTEN also acts as a DNA repair protein. But how post-translational modulation of PTEN affects cytological damage and aneuploidy is not studied in detail. Here, we focus on the role of sumoylation of PTEN in context with DNA damage induced cytological damage like micronucleus (MNi), nuclear bud (NB), and nuclear bridge formation. Our data suggest that wild type PTEN but not sumo-dead PTEN significantly reduces cytological damage in PTEN mutant PC3 cells. In case of sumo-dead PTEN, the cytological parameters are increased during 24 h recovery time point after DNA damage. Next, we measured the effectiveness of the sumo-dead (PTEN-K254R) mutant on aneuploidy, where we found that sumoylation is essential for maintaining chromosome number. As chromosome number variation in daughter cell is due to multiple spindle pole formation, we qualitatively and quantitatively evaluate the γ tubulin pole formation in PTEN-K254R clone transfected cells. We found aberrant pole formation is significantly increased in PTEN-K254R transfected cells compared to wild-type PTEN. Further depletion of sumoylation activity of PTEN increases the expression of phosphorylated form of Aurora kinase A (AURKA) (T288) and PLK1 (T210) proteins with or without nocodazole, a microtubule depolymerizing agent compared to cells expressing wild-type PTEN. Thus, sumoylation of PTEN is essential for maintaining genomic stability.

Keywords: PTEN; aneuploidy; micronucleus; Aurora kinase A; polo-like kinase.

1. Introduction

Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN) is frequently mutated in various cancers like endometrial cancer, prostate cancer, glioblastoma and breast cancer.[1–3] It is a lipid and protein dual phosphatase that negatively regulates

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PI3K/Akt pathway.[4,5] Recently, PTEN emerges as a DNA repair protein that prevents genomic instability by multiple modes of mechanisms including cell cycle checkpoint control,[6] replication fork stability,[7] homologous recombination (HR) repair[8] and spindle assembly checkpoint (SAC) activation.[9,10] A significant pool of PTEN is localized to nucleus/chromatin and maintains chromatin architecture and genomic stability.[11] PTEN also plays a critical role in maintaining the structural integrity of individual chromosomes which supports the recognition of PTEN as one of the genome guardians.[11,15] It is also established that deregulated PTEN functions are simultaneous events with aneuploidy[12–14] PTEN is post-translationally modulated by various modes of mechanisms including phosphorylation,[15] oxidation,[16] sumoylation, ubiquitination,[17] and acetylation[18] among which small ubiquitin-related modifier (SUMO) is an important post-transcriptional modifier that involves many important biological functions.[19] Studies showed that PTEN can be modified by sumoylation on K254 and plays an essential role in DNA damage repair. We have previously reported that PTEN depletion is also associated with multinucleated cells' formation.[24] PLK1 directly phosphorylates PTEN at Ser 380 and regulates its chromatin association[20] and Aurora kinases (AURKs); a family of conserved serine/threonine protein kinases is another mitotic kinase that plays an important role in tumorigenesis in active condition.[21] AURKA is mostly involved in centrosome function, mitotic entry, and spindle assembly.[22] Evidences indicate that AURKA is autophosphorylated resulting in activation of its kinase activity which in turn phosphorylates PLK1. Phosphorylated PLK1 then generates attachment sites for γ-tubulin ring complexes in the process of microtubule nucleation affecting the normal segregation of chromosomes that results in aneuploidy.[23] AURKA overexpression causes amplification of centrosome which appears as a result of cytokinesis failure and consequent multinucleation formation.[21] We have previously reported that PTEN depletion is also associated with multinucleated cells' formation.[24] Moreover, hyperactivity of AURKA in cells overrides the mitotic spindle checkpoint and enters anaphase despite abnormal spindle formation leading to the formation of lagging chromosome, anaphase bridge, chromosomal fragmentation or breakage.[25]

As PTEN localization and catalytic activity are regulated by sumoylation, we investigate the role of sumoylation of PTEN in context with genomic stability. In this study, using PTEN deleted prostate cancer cell line PC3 and glioblastoma cell line U87MG as a model, chromosomal instability was compared after ectopic expression of sumo-dead mutant and wild type PTEN constructs. Our study suggests that the sumoylation of PTEN is essential in maintaining genomic stability of the cell.

2. Materials and Methods

2.1. *Cell lines and culture conditions*

Human prostate cancer cell PC3 established from a grade 4 prostatic adenocarcinoma from a 62-year-old male Caucasian was cultured in Roswell Park Memorial Institute (RPMI 1640) medium and human glioblastoma cell U87MG derived from a malignant glioma of a female patient, purchased from National Center for Cell Science (Pune, India) was cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml). All the cells were maintained at 37°C, 5% CO_2 and 95% relative humidity (RH). Both PC3 and U87MG are PTEN negative cell lines.

2.2. *Antibodies, reagents*

Etoposide (Sigma), cytochalasin B (Sigma) and nocodazole (Sigma) were dissolved in DMSO (dimethyl sulphoxide, Sigma) to a concentration of 25 mM, 5 mg/ml and 1 mg/ml respectively. All further dilutions were freshly made in the medium. Anti-PLK1 (4513S), anti-Aurora kinase A (9980S), anti-*p*-PLK1 (T210) (3738S), anti-*p*-Aurora kinase A (T288) (8025S), and anti phospho PTEN antibodies were purchased from Cell Signaling Technology. Anti-PTEN (32199) was purchased from Abcam. Anti-rabbit IgG conjugated with horse radish peroxidase (HRP, 1:25000, Abcam) was used as secondary antibody.

2.3. *Site-directed mutagenesis*

Sumoylation dead pSG5L-HA-K254R-PTEN (Lys254Arg) encodes hemagglutinin tagged mutant proteins was created with site-directed mutagenesis (stratagene) kit according to manufacturer instruction and confirmed by sequencing. For sumoylation dead PTEN following primer was used:

Forward: 5′ GTGTGTGGTGATATCAGAGTAGAGTTCTTCCAC 3′, Reverse: 5′ GTGGAAGAACTCTACTCTGATATCACCACACAC 3′

For sequencing of the clone following sequencing primer was used:

Sence - 5′ ATTACGCTAGATCTCGAGTTT ATAGACCAGTG 3′
Antisence - 5′ ACAAAACAGTAGAGGATCC ACCGGATCTAGA 3′

2.4. *Transfection, plasmids*

Cells were seeded in antibiotic free medium 18 h prior to transfection. For transient transfection, Lipofectamine 3000 (Invitrogen) was used as per manufacturer's instruction. Mammalian expression vectors for wild type pSG5L-HA-Wt-PTEN (HA-WT-PTEN) encodes hemagglutinin tagged wild type PTEN protein and sumoylation dead Lys254Arg encodes hemagglutinin tagged mutant protein. Untransfected cells were also used.

2.5. *Cytokinesis-block micronucleus assay*

Cells seeded on 18 mm sterile glass coverslips at a density of 2.5×10^4 cells/ml were transiently transfected and exposed to 100 μM of etoposide for 4 h. After treatment, cells were washed with 1× phosphate-buffered saline (PBS) and cytochalasin B (final concentration 5 μg/ml) was added to the cells. After 36 h of incubation at 37°C, the medium containing cytochalasin B was removed and cells were washed with 1 × PBS. Coverslips were then incubated in hypotonic solution (75 mM KCl) for 5 min at 37°C and fixed with paraformaldehyde (4%) for 15 min at RT. The coverslips were then mounted with DAPI (4′-6-Diamidino-2-phenylindole, Vecta Shield Laboratories, USA) on microscopic slides and examined under the fluorescence microscope (Leica, Germany). The numbers of MNi micronucleus (MNi), nuclear buds (NBUDs), and nucleoplasmic bridges (NPBs) were counted in 1000 binucleated cells (BNCs) for each experimental time point and were scored according to criteria published by Fenech.[26]

2.6. *Immuno-fluorescence*

PC3 cells were seeded on 18 mm sterile glass coverslips 18 h prior to transfection. Cells were transiently transfected with PTEN-K254R clone of PTEN and then synchronized by double thymidine block. After 7 h cells were fixed in freshly prepared 4% paraformaldehyde solution for 15 min and permeabilized with 0.2% Triton X-100 on ice for 10 min. Fixed cells were pre-incubated in blocking solution (5% FBS in 1 × PBS), followed by overnight incubation with anti-γ tubulin and anti-α tubulin antibody in wash buffer (0.5% BSA and 0.05% Tween 20 in 1 × PBS) at 4°C. Cells were then washed three times with wash buffer and probed with Alexa Fluor 568 anti-rabbit antibody and Alexa Fluor 488 anti-mouse antibody for 1 h at RT. After washing, cells were mounted in the mounting solution containing DAPI (4′-6-Diamidino-2-phenylindole, Vector Shield, USA) and examined under a confocal (Leica, Germany).

2.7. *Aneuploidy analyses*

Cells were seeded on 35 mm culture plate at 1.1×10^6 density. After transient transfection with PTEN-K254R cells were treated with nocodazole (final concentration 50 μg/ml) for 16 h. Cells were scraped in PBS and centrifuged at 2500 rpm for 3 mins. Supernatant was discarded and the pellet was incubated in 75 mM KCl for 15 min and centrifuged at 2000 rpm for 5 min. The pellet was then fixed in ice-cold fixative (methanol: acetic acid in 3:1 ratio) at 4°C for 15 min and centrifuged at 2000 rpm for 5 min. Supernatant was discarded and pellet was resuspended in fixative solution. The resuspended cells were dropped on cold grease-free glass slide, immediately flame dried, mounted with mounting solution containing DAPI (4′-6-Diamidino-2-phenylindole, Vecta-Shield, USA) and examined under a fluorescence microscope (Leica, Germany).

2.8. *Western blotting*

Cells were plated at a density of 3.2×10^6 on 60-mm culture dish 18 h prior to transfection and were transiently transfected with K254R and wild-type clone of PTEN. After transfection the cells were treated with nocodazole (final concentration 50 μg/ml) for 16 h. Whole cell lysates were prepared from the cells with 1X RIPA lysis buffer (ab156034), phosphatase and protease inhibitors (G Bioscience). The supernatant was collected and protein concentration was estimated using Bradford's reagent. Cell lysates containing an equal amount of protein (100 μg) were solubilized in Lamellae buffer, boiled for 5 min, and electrophoresed on a 10% SDS polyacrylamide gel in Tris-glycine buffer (pH 8.8). Proteins were then transferred to polyvinylidene

difluoride (PVDF) membrane (Merck). Nonspecific binding was blocked with 5% nonfat dry milk and 0.05% Tween-20 in 20 mM Tris-Cl, pH 7.6 (TBS-T). After incubation with the appropriate primary antibody, membrane was washed with TBS-T and blot was re-incubated with secondary antibodies conjugated with HRP. Bound antibodies were detected by the ECL detection reagent (Abcam). To understand the modulation of different PTEN clones, cells were overexpressed with the clones, transfected cell lysates were immunoblotted for anti PLK1, anti-Aurora kinase A, anti-*p*-PLK1, anti-*p*-Aurora kinase A, and anti-PTEN antibodies. Anti-GAPDH antibody was used as a loading control.

2.9. *Statistical analysis*

All data in the graphs are presented as means and standard deviations (SDs). Graphs were generated using Microsoft Excel software. Statistical calculations were performed with Microsoft Excel analysis tools. A student's t-test was used to calculate the statistical differences between groups. $P < 0.05$ was considered statistically significant.

3. Result

3.1. *Loss of PTEN sumoylation activity leads to elevated level of cytological damage*

Cytokinesis block micronucleus (CBMN) assay is the simplest method to address micronuclei (MNi), nuclear buds (NBs), and NPBs which are the biomarkers of chromosomal instabilities. To assess the global chromosomal damage we used (CBMN) assay, to measure both chromosome loss and breakage of unreplicated DNA.[27] After transfection with Wt-PTEN and K254R-PTEN (Fig. 1(b)) in PTEN null PC3 cells, they were treated with etoposide for 4 h and MNi, NBUDs and NPBs were scored at 0 and 24 h recovery time point by CBMN assay (Table 1). We observed a significant increase in etoposide induced MNi (Fig. 1(a.II)) formation in K254R-PTEN (sumoylation dead) transfected cells after 24 h recovery period (34 ± 2.64) compare to Wt-PTEN (25 ± 2.1) transfected cells. NB formation was also increased in sumoylation dead transfected cells as compared to Wt-PTEN (K254R-PTEN 15 ± 2.36 versus 9 ± 1.3 at 24 h) (Fig. 1(a.III)). Also, NPBs (Fig. 1(a.IV)) which are the markers of elimination of amplified DNA and DNA misrepair or telomere end fusion were higher in case of both K254R-PTEN than Wt-PTEN transfected cells. This observation indicates that sumoylation activity is essential for prevention of accumulated cytological damage.

3.2. *PTEN sumoylation function leads to multipolar cell formation during metaphase and suppresses spontaneous aneuploidy*

γ-tubulin pole formation at centrosome is primary essential step for proper chromosome segregation in

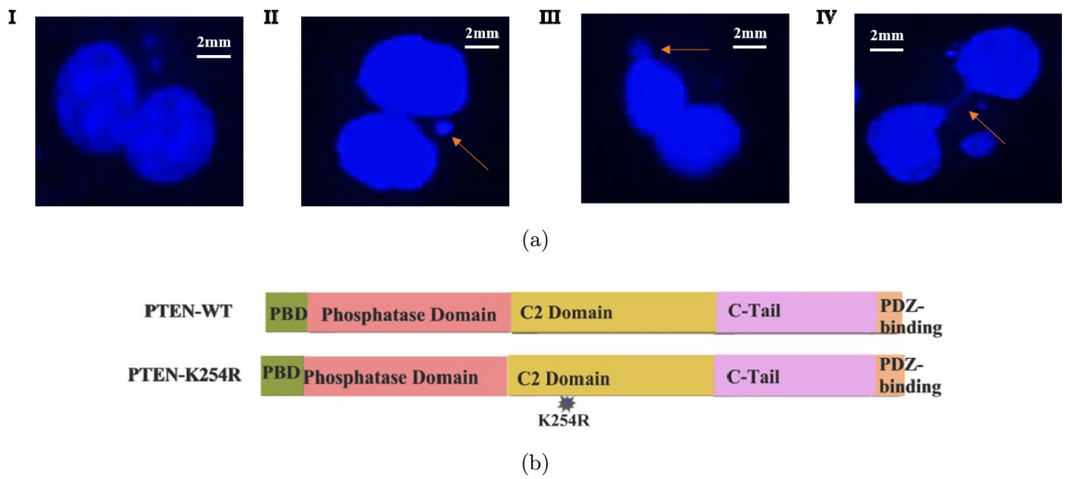


Fig. 1. Occurrence of cytological damage in different mutant of PTEN. (a) Microscopic images of cells incubated in presence of etoposide (100 μM for 4 h) and allowed to recover for 24 h. Cells were stained with DAPI. Arrowheads indicate I. BNC II. BNC with MNi III. BNC with NBud IV. BNC with NPB. (b) Map of sumo-dead mutant of PTEN.

Table 1. The results of CBMN assay in WT-PTEN and PTEN-K254R after 4 h exposure of 100 μM etoposide.

MUTANTS	MNi per 1000 BN cells 0 h	MNi per 1000 BN cells 24 h	NBud per 1000 BN cells 0 h	NBud per 1000 BN cells 24 h	NPB per 1000 BN cells 0 h	NPB per 1000 BN cells 24 h
UT	77 ± 2.94	65 ± 3.8	28 ± 1.83	37 ± 1.51	17 ± 2.22	20 ± 2.0
PTEN-WT	49 ± 1.9^{a}	25 ± 2.1^{b}	14 ± 1.7^{a}	9 ± 1.3^{b}	6 ± 0.6^{a}	3 ± 0.5^{b}
PTEN-K254R	65 ± 2.63^{c}	34 ± 2.64^{d}	20 ± 2.71^{c}	15 ± 2.36^{d}	8 ± 0.9^{c}	5 ± 0.58^{d}

Note: MNi: Micronuclei; NBs: nuclear buds; NPBs: nucleoplasmic bridges.
Values shown as for means $\pm$ SEM of three independent experiments.
[a]$p < 0.05$ compared with UT 0 h (Student's t-test).
[b]$p < 0.05$ compared with UT 24 h (Student's t-test).
[c]$p < 0.05$ compared with PTEN 0 h (Student'st-test).
[d]$p < 0.05$ compared with PTEN 24 h (Student's t-test).

metaphase. Moreover, micronucleated cell formation is a consequence of aneuploidy.[28] It has been reported that PTEN deficient cells spontaneously exhibit aneuploidy reflecting whole chromosome instability. Microscopic images of multiple pole formation were done in untransfected, K254R-PTEN along with wild-type PTEN transfected in PTEN negative PC3 cell line and probed with γ tubulin and α tubulin after double thymidine block (Fig. 2(a)). Here, we analyzed the γ-tubulin pole formation in absence of sumoylation function of PTEN where we observed 1.3 fold decrease in the percentage of bipolar cell in PTEN-K254R and untransfected compared to Wt-PTEN (Fig. 2(b)). In case of percentage of multipolar cells there is 8–10 fold increase in K254R-PTEN with respect to Wt-PTEN (Fig. 2(c)) transfected –U87MG cells. In PC3 there is 1.4 fold decrease in the percentage of bipolar cells in untransfected, K254R-PTEN when compared with Wt-PTEN transfected cells (Fig. 2(e)). In contrast there is a 5–10 fold increase in the percentage of multipolar cells in K254R-PTEN

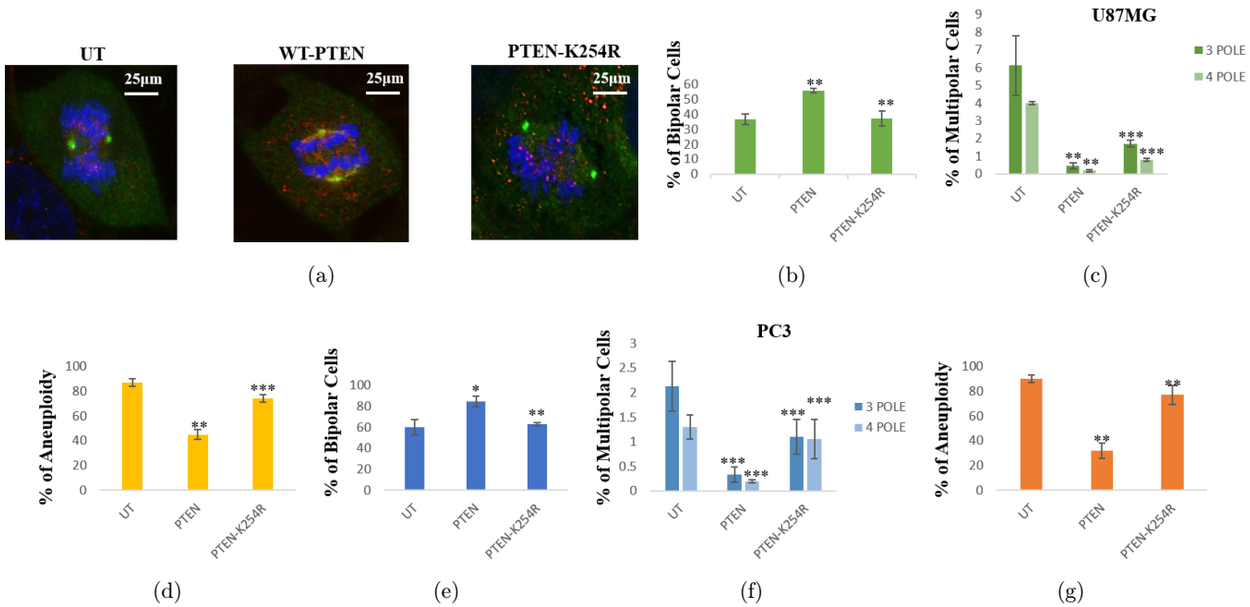


Fig. 2. Multiple pole formation causes chromosome missegregation. (a) Microscopic image of multiple pole formation during metaphase in various PTEN clone transfected cells. Graphical representation of the percentage of bipolar and multipolar cells in various PTEN clone transfected U87MG (b), (c) and PC3 cells (e), (f). Quantitative estimation of aneuploidy in Wt-PTEN and various mutant PTEN transfected in U87MG (d) and PC3 cells (g). Values are mean $\pm$ SEM from three independent experiments $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

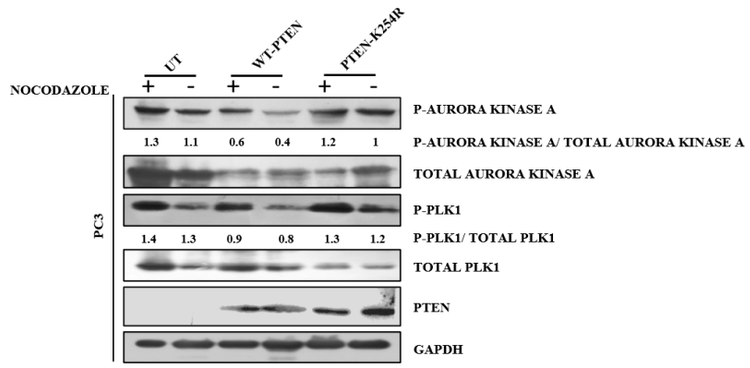
including untransfected cells than Wt-PTEN transfected PC3 cells (Fig. 2(f)). As we found that sumoylation activity of PTEN is important for genomic stability, we studied the ploidy status upon transiently transfecting mutant PTEN in both PTEN$^{-/-}$ U87MG and PC3 cell lines. Cells were treated with nocodazole, a microtubule depolymerizing reagent after 24 h of transfection and aneuploidy status was studied. Here, we found a 1.5 fold increase in the percentage of aneuploidy in K254R-PTEN transfected cells. Similarly, there is 1.7 fold increase in the percentage of aneuploidy in untransfected cells compared to Wt-PTEN transfected cells (Fig. 2(d)). This trend is also consistent in another PTEN null PC3 cell line. We observed 1.8 fold increase in the percentage of aneuploidy in K254R-PTEN compared to Wt- PTEN transfected and 2.6 fold increase in untransfected than Wt-PTEN (Fig. 2(g)). This observation indicates that sumoylation of PTEN is necessary for proper segregation of chromosomes.

3.3. *Loss of PTEN sumoylation activity leads to differential expression of AURKA and PLK1*

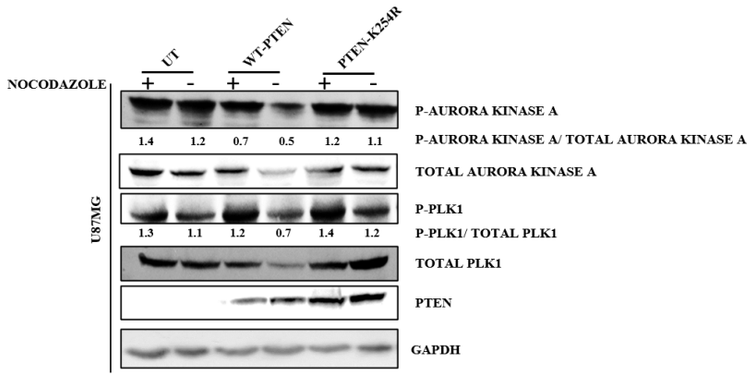
Overactivation of Aurora kinase A and PLK1mitotic kinases causes deregulated chromosome segregation and disintegration of mitotic pole.[29] In order to explain our hypothesis, we ectopically expressed wild-type PTEN and K254R-PTEN clones of PTEN in PTEN deficient PC3 and U87MG cell lines. After 24 h, we treated the cells with nocodazole and performed western blot to assess the expression level of AURKA and PLK1. We found that *p*-AURKA and *p*-PLK1 were markedly increased in untransfected cells compared with Wt-PTEN transfected PC3 and U87MG cells. PTEN-K254R transfected cells also showed higher expression of those mitotic kinases compared to Wt-PTEN transfected PC3 (Fig. 3(a)) and U87MG cells (Fig. 3(b)).

4. Discussion

DNA repair is extremely crucial for genetic stability of cells. PTEN has a potential role in DNA repair and DNA damage response apart from its involvement in the canonical PI3K/AKT pathway.[30] The tumor-suppressing role of sumoylated PTEN has been explored but in context with chromosomal stability has not been studied yet. In this study, we demonstrated that the sumoylation activity of PTEN is required for maintaining genomic stability of cells. MNi mainly originates from fragments of acentric chromosomes due to replication error, entire chromosomes that fail to translocate in the daughter nuclei, improper chromosome attachment with the spindle and segregation of chromosomes during anaphase. These chromosome fragments are enclosed by nuclear membrane forming morphologically similar nuclei.[31] NBuds have similar morphology like MNi but with the exception that they are attached with the nucleus through a narrow or wide stalk of nucleoplasmic material.[31] It represents a process that causes exclusion of DNA repair complex, amplified DNA and also excess chromosomes of aneuploid cells. Nuclear bridge or NPB is formed due to the absence of breakage of anaphase bridge. It originates from dicentric chromosomes which in turn is a result of chromosome breaks and telomere end fusion and occurs when there is a defective separation of sister chromatids during anaphase due to failure of decatenation.[31] Our previous study proposed that hepatocellular carcinoma harbors inactive PTEN that causes the survival of cells with DNA damage-induced multinucleation formation.[24] Here, we found that the accumulation of MNi, NBud and NPBs are higher in the sumo-dead PTEN-K254R compared to wild type PTEN during 24 h recovery. Lack of DNA repair by unstable phospho-deficient PTEN may cause MNi accumulation. This genetic aberration leads to a change in the ploidy status of cells. As expected, we found that overexpression of Wt-PTEN reduced aneuploidy in both PC3 and U87MG cell lines. Another reason for aneuploidy is multiple spindle pole formation during metaphase. PTEN is directly involved in accurate alignment of chromosomes and its segregation.[32] Depletion of PTEN causes impairment in spindle assembly during mitosis resulting in improper chromosomal segregation.[33–35] PTEN null cells show elevated levels of γ tubulin leading to centrosome amplification.[36] Interestingly, here, we found that frequency of multipole formation of PTEN-K254R is higher than Wt-PTEN transfected cells. Therefore, it seems likely that faulty sumoylation function of PTEN results in abnormal segregation of chromosomes in daughter cells as a consequence of defective mitosis. Reports are available for the localization of PTEN at mitotic centrosomes.[36] Overexpression of both PLK1 and Aurora kinase A



(a)



(b)

Fig. 3. Differential expression of mitotic kinase in different mutant of PTEN. (a) and (b). Phosphorylation status of AURKA and PLK1 of different clones of PTEN in pten$^{-/-}$ cell lines (PC3 and U87MG) with and without nocodazole treatment for 16 h showed an increase in phosphorylation of the AURKA and PLK1 in UT and PTEN-K254R whereas a decrease in phosphorylation in wild type PTEN. GAPDH act as a loading control. Values are calculated by image J software, which indicates the ratio of *p*-AURKA with total AURKA and *p*-PLK1 with total PLK1 antibody. Untransfected control is normalized to 1.

has been reported in various cancer[30] and it is regarded as a potential target of cancer chemotherapy. Over-function of these mitotic kinases causes centrosome amplification and multiple spindle pole formation.[37,38] Consistent with the report we demonstrated that PTEN-K254R transfected cells show an elevated level of γ tubulin pole formation similar to the untransfected cell. On the other hand, γ tubulin is necessary for the recruitment of PTEN to spindle poles causing a feedback loop of signaling at centrosomes in maintaining the stability of spindle poles.[32] Localization of PTEN at centrosomes also relies on the recruitment of PLK1. Furthermore, PLK1 is essential for the recruitment of PTEN to spindle poles.[36] Studies revealed that PTEN deficiency results in aberrant PLK1 phosphorylation as it controls spindle bipolarity.[32] PTEN also downregulates the expression of Aurora kinase A which plays an important role in mitosis.[39] From the results of our study it is evident that when wild type PTEN is expressed there is decreased *p*-PLK1 (T210) expression with and without nocodazole treatment compared to untransfected which is consistent with the earlier study. There is also a decrease in *p*-Aurora kinase A (T288) expression in wild-type PTEN before and after nocodazole treatment inp-A comparison with untransfected cells. In case of PTEN-K254R, expression of both *p*-PLK1 and *p*-Aurora kinase A proteins is similar with respect to untransfected with and without nocodazole treatment.

5. Conclusions

Loss of PTEN function leads to different cellular abnormalities which ultimately results in cancer

development. This study will pave the way to find the specific role of PTEN protein in regulating this intricate signaling pathway. So, our results for the first time established that PTEN sumoylation activity is crucial in maintaining genomic stability of cell.

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Review Article

Non-canonical function of nuclear PTEN and its implication on tumorigenesis

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ABSTRACT

Suppression of genomic instability is the key to prevent tumor development. PTEN is a unique tumor suppressor protein having both lipid and protein phosphatase activities. Interestingly though it is a cytoplasmic protein, but a significant pool of PTEN can also be localized in nucleus. The function of cytoplasmic PTEN is well defined and extensively studied in various literatures focusing mainly on the negative regulation of oncogenic PI-3Kinase-AKT pathway but functional regulation of nuclear PTEN is less defined and therefore it is a fascinating subject of research in cancer biology. Post-translation modulation of PTEN such as phosphorylation, sumorylation, acetylation and methylation also regulates its cellular localization, protein-protein association and catalytic function. Loss or mutation in PTEN is associated with the development of tumors in various tissues from the brain to prostate. Here we have summarized the role of nuclear PTEN and its epigenetic modulation in various DNA metabolic pathways, for example, DNA damage response, DNA repair, DNA replication, DNA segregation etc. Further, pathways involved in nuclear PTEN degradation are also discussed. Additionally, we also emphasize probable potential targets associated with PTEN pathway for chemotherapeutic purpose.

1. Introduction

Phosphatase and Tensin homolog deleted on chromosome ten (PTEN) is a unique and bona fide tumor suppressor protein that possesses both lipid and protein phosphatase activity. This protein has been identified simultaneously by two research groups in the year 1997 as tumor suppressor genes located at chromosome 10q23 in glioblastoma and prostate cancer cell lines [1,2]. Soon after its discovery, it has been found that the frequency of monoallelic mutations at this locus has been estimated at 50 %–80 % in sporadic endometrial carcinoma, and at 30 %–50 % in breast, colon, and lung carcinoma. Accumulation of PTEN germline mutations develops in a group of autosomal dominant syndromes characterized by various developmental disorder, neurological deficits, multiple hamartomas, and an increased risk of breast, thyroid, and endometrial cancers which are collectively referred to as the PTEN hamartoma tumor syndromes (PHTS), that includes Cowden syndrome, Lhermitte-Duclos disease, Bannayan-Riley-Ruvalcaba syndrome and Proteus and Proteus-like syndromes [3]. The latest list of COSMIC cancer database includes more than 2700 mutations in PTEN in 28 different tumor types, and the cBio portal of The Cancer Genome Atlas (TCGA) lists 1120 mutations in 27 tumor types. Considering the vast mutational diversity of PTEN in different tumor specimens, it is essential to understand the functional regulation of PTEN in cancer.

The crystal structure of PTEN (403 amino acid) revealed that it is a multi domain protein: N terminal phosphatase domain, the C2 domain and the C-terminal tail. C2 domain as well as 6 to 15 amino acid residues of N-terminus are involved in membrane localization through interaction with phospholipid PIP3 [3–5]. The C-terminal tail contains a number of phosphorylation sites at Serine 370, Serine 380, Threonine 382, Threonine 383, and Serine 385 residues. Interestingly phosphorylation of Ser 380, Thr 382, Thr 383 residues (collectively named STT motif) but not Ser 370 or Ser 385, increases the stability of PTEN and at the same time decreases its phosphatase activity [6]. It has been suggested that mutations or dephosphorylation of this STT cluster unwraps the protein conformation, making it less stable, but increases its phosphatase activity [6,7]. Thus STT motif of PTEN regulates its catalytic activity as well as its stability. Apart from C terminal phosphorylation sites, there are also multiple phosphorylation, sumorylation, acetylation and methylation sites which are important for PTEN nuclear functions (See Fig. 1 and Table 1). PTEN specifically dephosphorylates the D3

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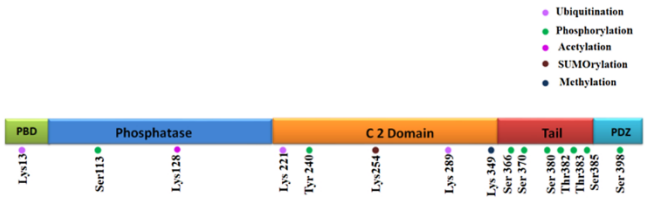

Fig. 1. Domain structure of PTEN with different post translational modulation sites which was reported to be involved in nuclear function.

Table 1
Table indicates different post translational modulation of PTEN which regulate its nuclear localization, chromatin association and DNA repair.

Modification of PTEN	Target site of PTEN	Enzyme responsible for modification	Cellular impact
Phosphorylation	Ser 380, 382, 383	PLK1, CK2, GSK3β	Chromatin associations, DNA repair
	Ser 366 and Thr 370	CK2, GSK3β	Chromatin associations
	Ser 385	PLK 1	Inhibits its nuclear entry
	Tyr 240	FGFR2	Associated with chromatin via Ki67 and recruits RAD51 to promote DNA repair
	Ser113	ATM Ser/Thr kinase	Nuclear accumulation of PTEN and induces autophagy
	Ser 398	ATM Ser/Thr kinase	DNA repair.
SUMOrylation	Lys254	SUMO1	DNA repair via HR Pathway
Methylation	Lys 349	NSD2	Interact with 53bp1 and involve in NHEJ pathway
Ubiquitination	Lys 289, Lys13 (Mono)	NEDD 4−1	Nuclear localization, DNA repair
	Lysine 221(Poly)	FBXO22	PTEN nuclear degradation
Acetylation	Lys128	PCAF	Recruit PCAF and CBP to the promoter of XLF and induces its expression.

position of phosphoinositide-3,4,5-trisphosphate, thus negatively regulates proto-oncogenic PI3K/Akt signaling pathway, which is key for cell survival, proliferation and growth [8]. PTEN significantly contributes in the regulation of cell cycle progression, apoptosis and cell migration. Mechanistically PTEN induces $p27^{kip}$ expression in vivo, which inhibits CDK2 activity and thus inhibits G1-S phase transition [9,10]. In the absence of PTEN, $P27^{kip}$ protein is ubiquitinated by SKP2 of Skp, Cullin, F-box containing complex (SCF^{SKP2}) [11]. Phosphatase activity of PTEN is also necessary for down regulation of cyclin D1 expression, thus preventing G1 transition [12]. Thus PTEN simultaneously down regulates cyclins, which are necessary for cell cycle progression and up-regulates CKI, which are essential for cell cycle arrest, implicating its immense influence on cell cycle control. It has been reported that PTEN interacts with P300 and maintains p53 in acetylated form, which increases PTEN-p53 interaction upon DNA damage. Acetylated p53 is important for G1 arrest [13]. Further PTEN depletion is associated with over expression of cdc6 and cyclin E2 in metastatic human prostate cancer. Decreased expression of cdc6 and cyclin E2 protein levels as a consequence of PTEN expression is associated with G1 arrest [14]. Upon genotoxic stress, PTEN null cell shows premature G2/M exit [15,16]. Mechanistically PTEN null causes hyper phosphorylation of Chk1 at Ser 280 by PI3K/AKT pathway which in turn abrogates G2 cell cycle arrest. Recent studies suggest that a significant pool of PTEN is also localized to nucleus though PTEN lacks classical nuclear localization signal. Partial or complete inactivation of PTEN is associated with radio resistance /chemo resistance attribute of tumor cells [17]. This phenotype is characterized by the ability of PTEN to suppress the formation or accumulation of the γH2AX foci, a well known marker of DNA damage, which may alternatively suggests that PTEN decreases DNA double strand break levels [17]. PTEN deficient cells exhibit an elevated level of chromosomal breaks, gap and aneuploidy either spontaneously or in response to DNA damage induced by various genotoxic agents [18]. Reduced level of ionizing radiation induced DNA repair in PTEN null cells has also been reported [19]. PTEN deficient cells exhibit multinucleated cell formation upon DNA damage which indicates aberrant mitotic entry with damaged DNA or defect in chromosomal segregation, or compromised spindle assembly checkpoint machinery [8]. In the last decade role of PTEN in DNA repair has been extensively studied in various literatures. Here we summarize recent advanced studies emphasizing on the role of PTEN in DNA metabolism.

2. Nuclear translocation of PTEN

As PTEN plays a significant role in DNA repair, a significant pool of PTEN resides in nucleus and loss of nuclear PTEN is closely associated with more aggressive cancers with high rate of genomic instability, making nuclear PTEN a useful prognostic indicator in some cancer types [20]. But there are no specific mechanisms for nuclear translocation of PTEN as it has no classical Nuclear Localization Signal (NLS). So multiple hypotheses exist about the nuclear localization including simple diffusion, protein-protein interaction and post translational modifications [21–24]. It has been reported that oxidative stress induced by H_2O_2 causes nuclear accumulation of phospho PTEN at Ser 380, which in turn interacts with p53 to augment cell cycle arrest. Xenograft studies further suggests that nuclear PTEN, specially p-PTEN at Ser 380, independent of its phosphatase activity, is sufficient to regulate tumorigenesis in vivo [21]. Takeo Minaguchi et al. suggested that PTEN nuclear transport is mediated by tyrosine phosphorylation-independent interaction with Major Vault Protein (MVP) where Ca(2+) concentration is important for PTEN –MVP interaction [22]. The nuclear transport receptor protein importin-11 physically interacts with PTEN, which prevents its degradation by ubiquitin-conjugating enzyme UBE2E1 and helps in nuclear translocation [23]. Reports suggested that monoubiquitination of PTEN at Lys 289 by NEDD 4−1 plays an important role in PTEN nuclear localization as PTEN K289R mutant is unable to enter nucleus and promotes tumorigenesis [24]. Growth factor receptor bound protein 2 (Grb2) binds to PTEN and assists its translocation into nucleus in response to oxidative stress [25]. In endometrial adenocarcinoma, nuclear PTEN accumulation is greatly diminished and aggravated DNA damage marker was observed. Thus it seems that deficient nuclear PTEN induces defective DNA damage response which is associated with tumor development [26]. Bassi et al. reported that the SUMOylation of PTEN at Lys254 is very important for nuclear retention and reduced within hours upon DNA damage. Also Sumo deficient PTEN is unable to repair DNA break as measured by γH2AX foci recovery assay [27]. But arguing with this observation another report shows that PTEN SUMOylation promotes its efficient chromatin removal after DNA damage by MMC, when compared with wild type PTEN [28]. Thus probable explanation may be that upon DNA damage, chromatin PTEN is removed by SUMOylation and accumulates in nucleoplasm. Then it may act as intermediated component of DNA repair pathway. Further studies are required to resolve, how Sumo-PTEN functions in DNA repair pathway. We previously reported that DNA damage induces phosphorylation of PTEN at

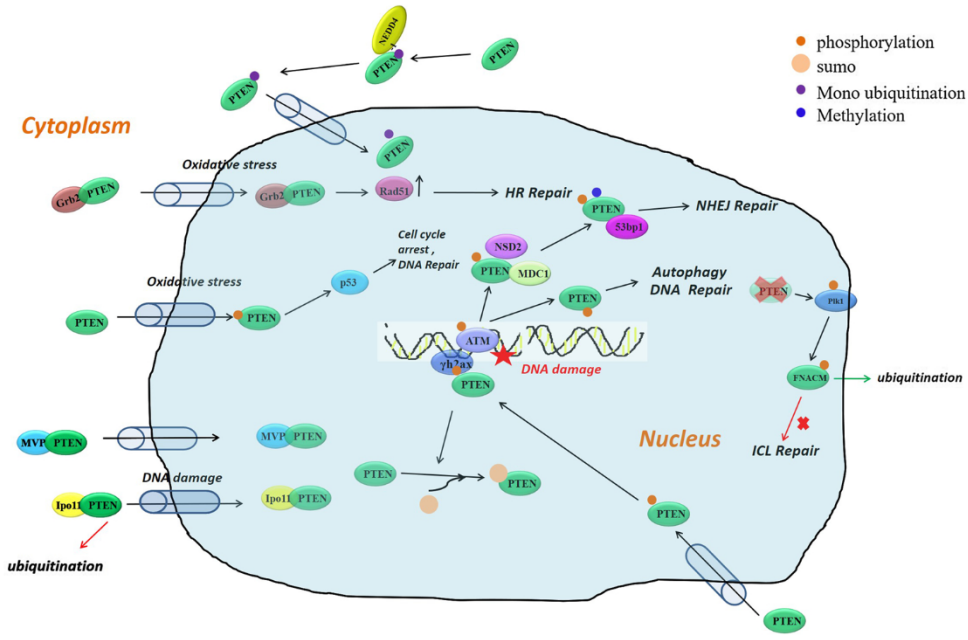

Fig. 2. Proposed different mechanisms of PTEN trafficking in the nucleus. In Oxidative stress PTEN interact with grb 2 or it get phosphorylated at Ser 380 and translocate to nucleus. DNA damage also causes PTEN translocation in nucleus which is governed by Ser 380 phosphorylation. Ipo11 interacts with PTEN and prevents its ubiquitination in cytoplasm and helps in translocation in nucleus. PTEN also get monoubiquitinated at Lys 289 by NEDD4 in cytoplasm and monoubiquitinated PTEN efficiently translocates in nucleus. PTEN also enter in to nucleus by major vault protein mediated interaction. Upon entering into nucleus, PTEN induces the expression of RAD 51, p53 and facilitated cell cycle arrest and HR mediated DNA repair. Phosphorylated PTEN also attached to DNA damage sites alongside with ATM and gamma H2AX, also it get methylated by NSD2 and facilitate 53bp1 mediated NHEJ repair. Sumolrylation also causes PTEN to enter in nucleus and take part in HR repair pathway. Nuclear PTEN also prevent polyubiquitination of FANCM by dephosphorylation and thus involves ICL repair pathway. Thus PTEN nuclear translocation and function depends on its different post translational modifications.

STT motif and which in turn accumulates in nucleus. Further phosphorylation dependent nuclear foci formation of PTEN was also observed after ionizing radiation or etoposide treatment which colocalized with γH2AX [29]. Accordingly, it has been found that PTEN C-terminal (189–403) deletion causes genomic instability and common fragile site re-arrangement in mouse model. Mice heterozygous for PTEN C-terminal deletion also developed multiple spontaneous tumors, and B cell lymphoma [30]. Another report suggested that topotecan (TPT), a topoisomerase I inhibitor, and cisplatin treatment activates ATM Ser/Thr kinase, which induces PTEN phosphorylation at Ser113 residue. This phosphorylation causes nuclear accumulation of PTEN and induces autophagy [31]. ATM mediated PTEN phosphorylation at Ser 398 has also been reported after etoposide induced DNA damage [27]. Going further depth in the molecular mechanism, Jinfang Zhang et al. found that upon DNA damage phospho PTEN forms a ternary complex with MDC1 and NSD2 and promotes NSD2-mediated di-methylation of PTEN at Lys 349. Then methylated PTEN interacts with 53BP1 and recruited to DNA damage sites, initiate DNA repair process partly through de phosphorylating γH2AX via NHEJ pathway [32]. Additionally, it has been reported that PTEN plays an important role in ICL repair. Here PTEN dephosphorylates FANCM and thus prevents it from polyubiquitination. PTEN deficient cells show elevated PLK1 kinase mediated phosphorylation of FANCM, which causes constitutive FANCM polyubiquitination. Thus the assembly of the FA core complex, FANCD2, and FANCI into DNA repair foci is compromised [28]. Therefore PTEN translocation in nucleus is a complex physiologically stimulated phenomenon and largely controlled by post translational modulation. Proposed mechanism PTEN nuclear translocation is summarized in Fig. 2. As in various stress conditions, PTEN accumulates in nucleus, thus it seems that PTEN acts as stress responder which coordinates between external stimulus and in vivo response, aiming to preserve genomic stability.

3. Chromatin association of PTEN depends on its phosphorylation at various sites

As various reports illustrate that PTEN is an important member of DNA repair proteins, so PTEN may be associated with chromatin. Several mechanistic studies have been carried out to focus on the association of PTEN with chromatin. BH Choi and his colleague reported that PTEN became associated with chromatin upon DNA damage and phosphorylation of Ser 366 and Thr 370 of PTEN is essential for this attachment [33]. Further they have found that C2 domain is also responsible for the nuclear translocation of PTEN. After attachment PTEN regulates sumorylation of Rad 52 and thus participates in DNA damage response pathway. Additionally, it has been reported that PLK1 phosphorylates PTEN at Ser 380 in vivo, which is also essential for association of PTEN with chromatin [34–36]. p-PTEN at Ser 380 is important for normal mitotic progression as replacing Ser 380 by Ala resulted in a significant reduction of mitotic cells [36]. As previously reported that PTEN interacts with centromere as well as PLK1 [36,37], thus it may be pertinent to state that phosphorylation of PTEN by PLK1 is an upstream event as C-terminal truncated PTEN is unable to interact with centromere [38]. PTEN can also dephosphorylates PLK1 and suppress polyploid cell populations [34]. Thus phosphorylated PTEN in chromatin causes mitotic arrest whereas soluble PTEN dephosphorylates PLK1 and causes resistance to uncontrolled genome segregation (Fig. 3A). As phosphorylated PTEN has reduced phosphatase activity so in chromatin, it can't dephosphorylates PLK1. So it is tempting to speculate the existence of some mechanisms in cells to dissociate PTEN from chromatin which we will discuss later. But another study suggested that PLK 1 phosphorylates PTEN at Ser 385 and inhibits its nuclear entry [35] rendering promotion of tumor development. Recently Ma et al. also reported that PTEN phosphorylation is associated with chromatin and DNA repair [39,40]. They have identified Tyrosine 240 residue which gets phosphorylated upon IR induced DNA damage by Fibroblast Growth Factor Receptor 2 (FGFR2). Upon phosphorylation, it becomes

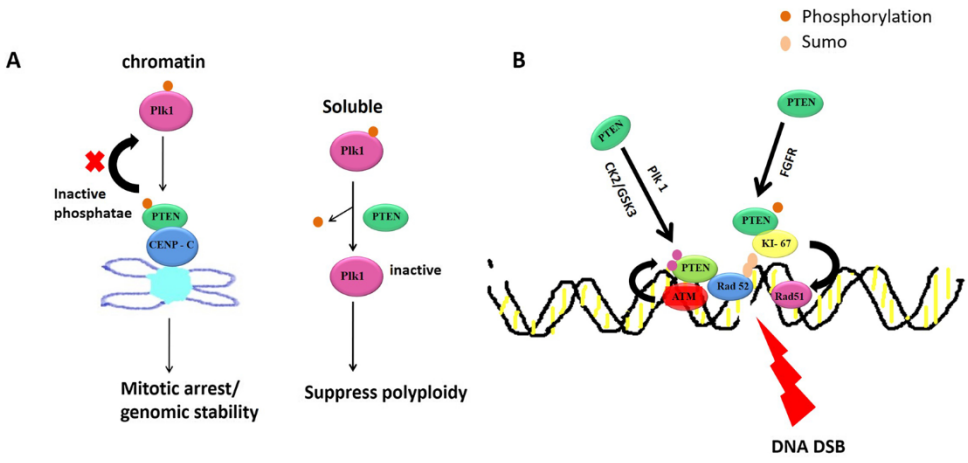

Fig. 3. A. Proposed model of PTEN and PLK 1 functional crosstalk by phosphorylation in maintaining genomic stability. Chromatin PTEN is phosphorylated by phospho-PLK1, associated with CENP-C centromeric protein and maintained genomic stability. As phospho-PTEN is partially inactive phosphatase so its dephosphorylation activity is reduced. In soluble form PTEN dephosphorylates PLK1 and thus inactivate it. Hyper phosphorylation of PLK 1 causes anuploidy. B. PTEN chromatin association is controlled by phosphorylation. Upon DNA damage PTEN gets phosphorylated either by PLK1, CK2, GSK3β or ATM at different phosphorylation site. Then it promotes RAD 52 sumorylation. FGFR also phosphorylated PTEN and in turn helps in RAD51 recruitment at DNA damage site and promotes DNA repair.

associated with chromatin via Ki67 and recruits RAD51 to promote DNA repair in glioblastoma cells and promotes survival (Fig. 3B). Thus inhibition of FGFR 2 may be useful for the radiosensitization of glioblastoma [39,40].

4. PTEN maintained chromatin architecture

Apart from chromatin association, PTEN prevents centromere breakage and anuploidy by stabilizing the centromeric regions. PTEN-centromere interaction maintains chromosomal stability, possibly by modulating homologous recombination [9,28,37,38]. PTEN localizes and physically interacts with CENP-C centromeric protein independent of its phosphatase activity but depends on its C-terminal tail [38]. It has been found that PTEN maintains heterochromatin status of chromatin by interacting and stabilizing HP1α. Further in Mouse Embryonic Fibroblast cells, PTEN knockout led to a significant reduction in H3K9me3 foci intensity which is indicative of de-condensation of the heterochromatin [41]. Again Zhu Hong Chen et al. reported that loss of PTEN leads to dissociation of histone H1 from chromatin and consequently elevated level of histone H4 acetylation at lysine 16, an epigenetic marker for chromatin activation was observed [42]. They have also been found that PTEN physically interacts with histone H1 through their C-terminal domains as truncated PTEN is unable to interact with histone H1. Compromised heterochromatin could lead to broad epigenetic instability, which with a potential impact on gene expression profiles may ultimately lead to abnormal growth characteristics [43]. Disruption of the epigenetic signature such as H3K9me3, which is associated with late replicating heterochromatin, promotes chromatin accessibility, advancing replication timing and leading to re-replication [44]. So heterochromatin maintenance is important for genomic stability of cancer cells which is closely associated with replication initiation. Apart from replication, loss of heterochromatin also positively influences global gene transcription which is another characteristic of PTEN null cells. Thus PTEN maintains chromatin in an inactive condensed form and thus prevents unregulated transcription as well as replication.

5. PTEN involve in both HR and NHEJ

The foremost mechanism that eukaryotic cell carries out to repair its own damaged DNA by either Homologous Recombination (HR) or Non Homologous End Joining (NHEJ). HR is more specific and error less mechanism that occurs predominantly in S phase where NHEJ is error prone and occurs throughout cell cycle. Compromising either or both of these pathways lead to genomic instability and accumulation of cancer driven mutations [45–47]. In vivo plasmid based assay exploit by multiple research groups suggests that PTEN depletion diminished homologous recombination repair frequency. When they ectopically transfect

Table 2
Role of PTEN in DNA double strand break repair.

Homologous recombination	Non Homologous End Joining
1. PTEN physically interact with Rad 51 on chromatin. PTEN also regulates Rad 51 transcription by encompassing Rad 51promoter or by regulating E2F1mediated Rad 51 transcription	1. Acetylated PTEN at Lys 128 localize in NHEJ1 promoter and recruits the histone acetyltransferases, PCAF,CBP. It also induces the expression of XRCC4-like factor (XLF) which bridges and ligates DNA ends by NHEJ. 2.PTEN reduces error prone NHEJ pathway by poly(ADP-ribosyl)ation of Ku70 and prevents binding of Ku 70 to DNA DSB. 3.NSD2-mediated di-methylation of phospho PTEN at Lys 349 causes interaction with 53BP1 thus regulate DNA repair via NHEJ pathway. 4.PTEN increases H3K9me3 expression, thus positively regulate NHEJ by 53BP1 mediated pathway.

PTEN in PTEN null cells, HR frequency significantly up regulated [27,48,49]. Mechanistically PTEN up regulates Rad 51 transcription by encompassing Rad 51 promoter or by modulating chromatin so that E2F1 direct Rad 51 transcription [38]. But another report suggests that PTEN C–terminal phospho mutant physically interacts with E2F1 and represses transcription at the promoter site of various gene [50]. Another report shows that after ionizing radiation induced DNA damage, Rad51 protein expression was significantly up-regulated in Wt-PTEN and phosphatase dead PTEN but not in sumo deficient PTEN transfected cells [51]. Recent studies reveales that PTEN augments NHEJ repair pathway through direct induction of expression of XRCC4-like factor (NHEJ1/XLF) which functions in DNA end bridging and ligation. Again acetylated PTEN at Lys128 interacts and recruits the histone acetyltransferases, PCAF and CBP to the promoter of XLF and induces its expression [52]. But another report suggested that nuclear PTEN reduces error prone NHEJ pathway by poly(ADP-ribosyl)ation of Ku70 and prevents binding of Ku 70 to DNA DSB. Thus DNA PKcs unable to bind DNA broken end to execute repair process [51]. They further shows that phosphatase activity is not required rather sumorylation of PTEN is important for this activity. At Table 2 role of PTEN in HR and NHEJ is summarized. Again H3K9me3 represents epigenetic marker that are important for the function of the 53BP1 protein mediated non-homologous end joining (NHEJ) [53,54]. As PTEN loss causes chromatin disorganization and compromised H3K9me3 protein expression and foci formation, so PTEN may indirectly positively regulate NHEJ by 53BP1 mediated pathway. Thus this PTEN-H3K9-me3–53BP1 axis should be studied elaborately to get insight about the

mechanism. Thus mechanistic role of PTEN and its post translational modulation in regulating NHEJ repair pathway is needed to be explored further which will help in understanding the therapeutic approach for targeting NHEJ pathway in cancer cells.

6. Replicative function of PTEN

Mutation appears in the genome if cells are unable to repair its DNA and replicate with unrepaired DNA due to checkpoint inaccuracy. Thus daughter cells acquire mutations from parental cells. Controlling and sensing DNA damage during replication or replication stress is an Achilles' heel and must be tightly regulated to prevent DNA replication progression, otherwise it causes a serious threat to genomic stability leading to cancer development. De-regulation of DNA replication occurs when cells are unable to overcome stress successfully and may initiate uncontrolled initiation, promoting DNA damage and genomic instability. As PTEN null or PTEN mutant cells shows high growth potential with unstable genome, so PTEN may function in replication machinery to maintain the integrity of genome during replication. There are number of reports suggest that PTEN indeed functions in DNA replication and which may be dependent on its post translational modulation. At first, it has been observed in 2015 by two different research groups that PTEN null cells show higher frequency of anaphase bridges and lagging chromosomes as compared to wild type cells indicating replication defect [55,56]. Further study reveals that PTEN null cells show compromised replication progression which indicates endogenous replication stress. Replication fork restart after aphidicolin/Hydroxyurea treatment is largely impeded in PTEN null cells compared to wild type cells. Molecular iPOND study showed that PTEN itself localized at stalled fork induced by HU and recruits Rad 51 on chromatin to promote its successful recovery [56]. Following replication stress, replication protein A (RPA1) binds to the single-strand DNA adjacent to newly synthesized double-stranded DNA. The purpose of RPA1 loading is to protect single naked strand, halt fork movement and preparing cells to overcome stress [57]. It has been reported that PTEN directly binds to C-terminal domain of RPA1 and promotes RPA1 accumulation on the replication fork. PTEN also recruits the deubiquitinase OTUB1 and prevents RPA1 ubiquitination and thus stabilizes RPA1 [58]. Further phosphatase activity of PTEN is not required to stabilize RPA1 at replication fork. Almost at the same time another report suggested that PTEN is involved in restriction of the replication fork rate movement in response to exogenous replication stress through its phosphatase activity. They have found that PTEN physically associated with and dephosphorylates mini-chromosome maintenance 2 (MCM2) protein at Ser 41 residue and prevents replication fork progression upon HU induced replication stress [59]. Our unpublished Brdu pulse experimental data shows that significantly higher percentage of Brdu positive cells appeared in PTEN null cells compared to Wt–PTEN transfected cells upon aphidicolin induced replication stress, indicating unperturbed DNA replication during Aph treatment in PTEN null cells. Thus PTEN null cells unable to sense replication stress and continue to grow with damaged DNA as we found gamma H2AX foci persists along Brdu foci. PTEN transfection greatly diminished this phenotype. So all these reports suggest that during replication stress PTEN dephosphorylates MCM2 helicase on one hand and recruits RPA1 on ssDNA generated due to uncoupling of polymerase on helicase ahead of replication fork on the other and this is followed by recruitment of RAD 51 for successful fork recovery. RPA-coated ssDNA serves as a common intermediate structure necessary for activation of the intra-S checkpoint [60]. Thus PTEN maintains intra S phase checkpoint integrity. Further when replication stress is removed PTEN promotes stalled fork recovery. Additionallly, PTEN null cell unable to dephosphorylates MCM 2 and RPA1 loading is compromised as a result, replicative DNA damage accumulates that induce genomic instability. We diagrammatically explain the current understanding of functional involvement of PTEN in DNA replication in Fig. 4.

7. PTEN regulate mitotic spindle integrity and Architecture

In eukaryotic organisms, mitosis is an essential part of the cell cycle where replicated chromosomes are segregated into daughter nuclei. Mitosis should be carried out in a continuous and proportionate manner for the successful transmission of genetic informations to the progeny. Mitosis requires a regulatory mechanism in which mitotic spindles play the most essential role [61]. Mitotic spindle is the cytoskeleton structure, comprises of microtubules, microtubule dependent motor proteins and non-motor microtubule binding proteins [62]. As previously mentioned, PTEN null cells show various chromosomal damage including centrosome amplification, aneuploidy, lagging chromosome, anaphase bridge, micronucleus, chromosomal bud [9,28,37,38,52,56]. PTEN–AKT pathway controls the centrosome composition and integrity during mitosis. PTEN is associated with PLK1 [34–36] and γ tubulin at centrosome and maintain centrosome integrity [37]. Knockdown of PTEN reduces centrosomal level of pericentrin in an Akt independent manner. But when Akt is inhibited, centrosomal level of PLK1 and γ tubulin is reduced and recruitment of PTEN at centrosome is compromised, thus increased defect in centrosome number and metaphase separation is observed [37]. PTEN also act as a component of mitotic checkpoint complex which is essential to sense defective kinetochor attachment during metaphase. If microtubule–kinetochore attachment

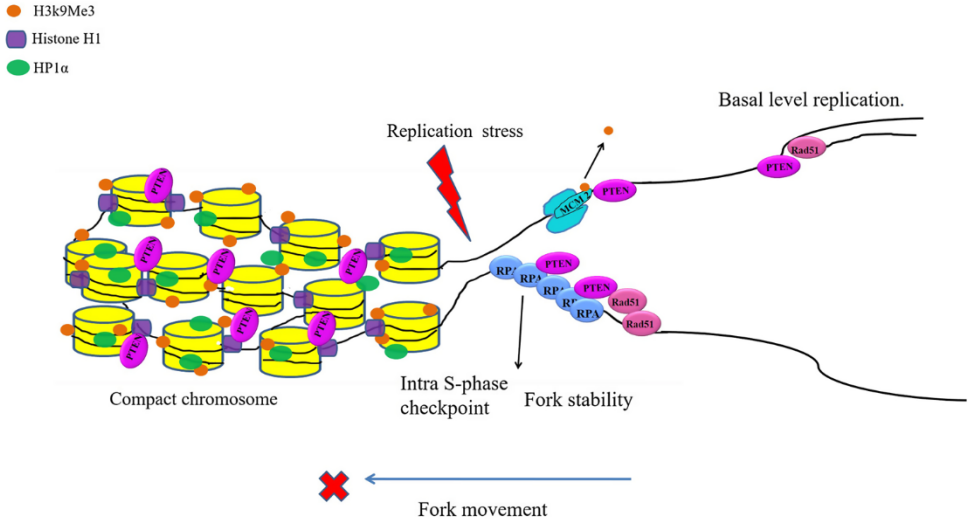

Fig. 4. PTEN localized to replication fork and stabilized it in stress condition. In response to replication stress, PTEN interacts with MCM2 and dephosphorylates it. Thus prevent replication fork progression in stress condition. PTEN also interacts with single-strand DNA (ssDNA) binding protein RPA1 to stabilize DNA replication forks and recruits Rad51 to promote reactivation of stalled forks when stress was relieved. Further PTEN maintain heterochromatin signature of chromatin and it may further prevent fork progression.

is compromised, Spindle Assemble Checkpoint(SAC)/Mitotic Checkpoint Complex(MCC) comes to its function and halts anaphase so that attachment occurs properly. PTEN physically interacts with Mad1 protein and promotes its dimerization, localization in the nuclear envelope and maintains SAC timing [63]. PTEN null cells show significant reduction in M phase compared to WT cells indicating SAC arrest [34, 36]. Another report suggested that PTEN physically interacts with Cdc20 and Mad2, two important components of MCC [64]. PTEN dependent recruitment of a scaffold protein Dlg1 was observed at centrosome as PTEN knockdown impaired Dlg1 recruitment. Mechanistically PLK1 phosphorylates PTEN at C- terminal tail and recruits it to centrosome. This creates docking site for attachment of dlg1 and phospho-EG5 Ser1033, Thr926 (induced by NEK6, Cdk1) [65]. EG5 belongs to the super family of kinesin motor proteins that are responsible for centrosome movement, proper maintenance and assembly of mitotic spindles by rearranging the microtubules [66]. But another report suggests that PTEN deficiency induces aberrant EG5 phosphorylation and abrogates EG5 recruitment to the mitotic spindle apparatus, leading to spindle disorganization. PTEN physically interacts with EG5 at centrosome/spindle pole and dephosphorylates it at Thr 926 residue and helps to station them at spindle pole [67]. These conflicting reports suggests that delicate maintenance of the phosphorylation status of EG 5 is important for the efficient functioning of motor proteins. Thus EG5 phosphorylation must be precisely regulated as erroneous mitosis arises from either hypo or hyper-phosphorylation of EG5. It seems that EG5 phosphorylation is required for the formation of the PTEN-Dlg1-EG5 complex on centrosomes where hyper-phosphorylation of EG5 impairs its PTEN-mediated maintenance of the mitotic spindle length. For example, hyper-phosphorylation of EG5 at Thr 926 in PTEN-deficient cell results in a reduced affinity to spindle microtubules and impairs the spindle architecture [67].

8. Mechanism of the loss of nuclear PTEN

Form the above discussion it is clear that PTEN translocates in to the nucleus to preserve genomic stability by various mechanisms including DNA repair by HR or NHEJ, DNA replication and chromosome segregation. Further various post translational modulation including phosphorylation and sumorylation of PTEN is essential for maintaining the integrity of the genome. It has been reported that in human endometrial tumors, significant nuclear PTEN was lost when compared with normal endometrial tissues [26]. Nuclear PTEN localization is also positively correlated to tumor suppression in primary cutaneous melanoma [68] and colorectal cancer [69]. It is essentially clear that loss of nuclear PTEN causes indigenous DNA damage as well as unsuccessful DNA repair and compromised S phase checkpoint after genotoxic insult which is the initial step of cancer predisposition. Thus the mechanism of degradation of nuclear PTEN and prevention of nuclear entry of PTEN is important to study. Though cytoplasmic PTEN degradation is extensively studied in several literatures but very few studies have been carried out so far regarding nuclear PTEN degradation. One of the four subunits of the ubiquitin protein ligase complex, F-box only protein 22 (FBXO22) is over expressed in various cancer types including colorectal cancer and this induces ubiquitylation of nuclear PTEN at lysine 221 followed by proteasome-mediated degradation of nuclear PTEN [70]. PTEN C–terminal phospho mutant more efficiently interacts with FBXO22 than Wt-PTEN, indicating that though phospho mutant PTEN accumulates in nucleus, but due to degradation it is unable to take its journey towards chromatin. Another report supports this hypothesis where they found that phospho mutant PTEN efficiently interacts with APC–Cdh1 and promotes its degradation. Phosphorylation of PTEN at Ser-380 impaired its interaction with Cdh1, thus positively regulating PTEN stability on chromatin [71]. It has been also reported that phospho-PTEN status is significantly reduced in ATM null cells and promotes its degradation. ATM deficiency resulted in the accumulation of XIAP/p-XIAP levels which ubiquitinated PTEN and Casein kinase 2 [72]. Interestingly we found that p-PTEN co-localized with pATM upon DNA damage indicates close proximity between them. Importin-11 a nuclear import receptor, interacts with PTEN and facilitates its nuclear import. Knockdown of Importin-11 drastically reduces nuclear PTEN. Functionally Importin-11 prevents PTEN degradation by two mechanisms- (1) by sequestering ubiquitinated PTEN that has been primed for cytoplasmic polyubiquitination and degradation and (2) by importing the activated Ub-loaded UBE2E1, a E2 ubiquitin- conjugating enzyme, thus restricting its ability to prime PTEN for degradation in the cytoplasm [23].

9. Conclusion and prospect

Latest understanding of the functional aspect of PTEN reveals that it is not confined in cytoplasm to down regulate PI3-Kinase pathway but maintains genomic stability in nucleus by multiple mode of mechanism as discussed above. Nuclear function of PTEN is also governed by epigenetic regulation e.g phosphorylation, sumorylation, acetylation and ubiquitination at the various sites from N terminal to C-terminal end. Both phosphatase dependent and independent function of PTEN was reported to maintain genomic stability. Phosphorylation of C terminal Ser/Thr residue of PTEN reduces its phosphatase function but simultaneously phosphorylation at this site and other residue increases the affinity of PTEN to chromatin. Probable explanation may be given as phospho PTEN is a stable form and prerequisite stability is required for functioning of PTEN on phosphatase dependent manner [34]. Phosphatase dead PTEN unable to control cell proliferation as it causes up regulation of MCM2 and AKT phosphorylation but able to initiate DNA damage response (Rad 51 mediated) and survive with genomic instability after DNA damage. Thus PTEN null cells show extensive proliferation with unrepaired DNA. Again PTEN phosphorylation mutant unable to attached with chromatin, which may contributes for genomic instability. Thus it may be interesting to observe different epigenetic modification of PTEN and their chromatin association in response to DNA damage. This may additionally reveal how different epigenetic modulation of PTEN is associated with DNA repair-sub pathways. It has recently been reported that the phosphorylated PTEN inhibits its ubiquitination in vivo and in vitro by NEDD4−1 and WWP ubiquitin ligase. But poly ubiquitination of PTEN greatly reduces PTEN phosphatase activity [73]. This conflicting data indicates that PTEN phosphorylation tightly and delicately regulates PTEN phosphatase activity and maintains homeostasis in vivo perhaps through ubiquitination. So there may be fine regulation between PTEN phosphatase activity and PTEN phosphorylation for proper functioning. Further phosphatase activity of PTEN is required for its own de-phosphorylation at C-terminal tail [74, 75] which may transiently activate phosphates activity. PTEN involves in both HR and NHEJ repair pathways but exact mechanism is not clearly understood. In NHEJ pathway, binding to DNA end cause auto-phosphorylation of DNA PKcs at number of sites which causes dissociation of DNA PK complex from DNA end. Phosphorylation of Ku by DNA-PKcs results in the dissociation of the Ku heterodimer from the forked dsDNA and the subsequent free dsDNA ends are available for Exo1-mediated DNA end resection. So instant phosphorylation of this protein must be prevented by some mechanism otherwise NHEJ pathway must be compromised which causes genetic instability. Obviously, by the end of the repair process, either the phosphorylated protein has to be degraded or the phosphate group must be released. Although the (auto) phosphorylation activity of DNA-PK has been shown to be essential for repair of both random double-strand breaks, the corresponding phosphatase has been elusive. As PTEN is phosphatase and involved in DNA repair pathway so it is quite possible that PTEN may be involved in this process. Factors and specific mechanisms that are required for the dissolution of NHEJ complex once repair is completed remain to be studied. Thus the mechanistic role of PTEN and its post translational modulation in regulation NHEJ repair pathway is needed to be explored, which will help in understanding the therapeutic

approach for targeting NHEJ pathway in cancer cells. Further how chromatin modulation in PTEN null cells influence NHEJ repair pathway will also be an interesting aspect of research.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

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Phosphorylation of PTEN at STT motif is associated with DNA damage response

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ABSTRACT

Phosphatase and tensin homolog deleted on chromosome Ten (PTEN), a tumor suppressor protein participates in multiple cellular activities including DNA repair. In this work we found a relationship between phosphorylation of carboxy (C)-terminal STT motif of PTEN and DNA damage response. Ectopic expression of C-terminal phospho-mutants of PTEN, in PTEN deficient human glioblastoma cells, U87MG, resulted in reduced viability and DNA repair after etoposide induced DNA damage compared to cells expressing wild type PTEN. Also, after etoposide treatment phosphorylation of PTEN increased at C-terminal serine 380 and threonine 382/383 residues in PTEN positive HEK293T cells and wild type PTEN transfected U87MG cells. One-step further, DNA damage induced phosphorylation of PTEN was confirmed by immunoprecipitation of total PTEN from cellular extract followed by immunobloting with phospho-specific PTEN antibodies. Additionally, phospho-PTEN translocated to nucleus after etoposide treatment as revealed by indirect immunolabeling. Further, phosphorylation dependent nuclear foci formation of PTEN was observed after ionizing radiation or etoposide treatment which colocalized with γH2AX. Additionally, etoposide induced γH2AX, Mre11 and Ku70 foci persisted for a longer period of times in U87MG cells after ectopic expression of PTEN C-terminal phospho-mutant constructs compared to wild type PTEN expressing cells. Thus, our findings strongly suggest that DNA damage induced phosphorylation of C-terminal STT motif of PTEN is necessary for DNA repair.

1. Introduction

Phosphatase and tensin homolog deleted on chromosome Ten (PTEN), a tumor suppressor gene, located in chromosome 10q23 region is frequently mutated or loss in various tumor cells. Soon after its discovery in 1997, the Phosphoinositide 3 kinase dependent roles of PTEN have been extensively studied in several literatures [1–3]. It is a dual lipid and protein phosphatase, that negatively regulates PI3K/Akt pathway by dephosphorylating D3 position of phosphoinositide 3,4,5-triphosphate. Apart from this, PTEN plays a conserved role in cellular polarity, maintenance of tumor microenvironment, cellular senescence [4] and cell cycle regulation [5,6]. PTEN/Akt signaling axis also regulates DNA damage induced multinucleation and maintains genomic integrity [7,8]. PTEN (403 amino acid) composed of catalytic N-terminal phosphatase domain, lipid binding C2 domain, PEST motif and C-terminal tail [1,2]. The C-terminal tail contains number of phosphorylation sites at serine 370, serine 380, threonine 382, threonine 383, and serine 385 residues. Interestingly phosphorylation of Ser 380, Thr 382, Thr 383 residues (collectively named STT motif) but not Ser 370 or Ser 385 increases stability of PTEN and at the same time decreases it's phosphatase activity [9]. It has been suggested that mutations or dephosphorylation of this STT cluster open the protein conformation, making it less stable, but increases its phosphatase activity [9,10]. Thus STT motif of PTEN regulates its catalytic activity and stability, which perhaps associated with its anti tumor activity. Phosphorylation deficient mutant form of PTEN has strong affinity for PDZ domain of MAGI-2, a multidomain scaffolding protein, and cooperates to inhibit cell migration and Akt activation compare to wild type PTEN [11] despite the fact that PTEN independent Akt regulation after DNA damage has also been reported [12]. Thus an inverse correlation with phosphorylated Akt and PTEN stability indicates the additional role of PTEN in cellular metabolisms in addition to its phosphatase activity. Partial or complete inactivation of PTEN is associated with radioresistance/chemoresistance character of cancer cells and ectopic expression of wild type PTEN overcome this phenotype [7]. This radio sensitization is characterized by the ability of PTEN to suppress the formation of the γH2AX foci, a marker of DNA damage, which alternatively suggests that PTEN decreases DNA double strand break levels [13]. Further

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post-translational modulations of PTEN regulate its intracellular trafficking [14,15]. Recent studies revealed that PTEN also localized to the nucleus and have a functional role to maintain genomic integrity and stability [16,17]. Further, distinct signaling pathways are supposed to be associated with the activation of cytoplasmic and nuclear PTEN [18]. Various molecular mechanisms about the nuclear localization of PTEN have been proposed [19,20]. PTEN null mouse embryonic fibroblast cell show increase centromere breakage reduced RAD51 expression [17]. In skin papilloma and squamous cells carcinoma (SCC), PTEN expression is reduced compared to skin lacking these lesions. In this case, loss of PTEN suppresses the expression of Xeroderma Pigmentosum C (XPC) through the Akt/p38 signaling axis [21]. It is also reported that phosphorylated PTEN mostly present at nucleus [22]. Accordingly it has been demonstrated that in response to oxidative stress PTEN phosphorylated at Ser 380 and localizes to nucleus [23]. These, along with the observation that PTEN participates in DNA DSBs repair [16] indicate that phosphorylation of PTEN is associated with DNA damage response. Thus, we prompted to investigate the role of phosphorylation and subsequent nuclear localization of PTEN in context with DNA repair. Using the PTEN deficient U87MG cells as a model, DNA damage response was compared after ectopic expression of phospho-mutant and wild type PTEN constructs. Our studies suggest that DNA damage augments phosphorylation of C-terminal tail of PTEN. Consequently, nuclear accumulation of PTEN increases which positively regulates the DNA repair process.

2. Materials and methods

2.1. Cell culture and treatment

U87MG and HEK293T cells were purchased from National Center for Cell Science, (Pune, India) and maintained at 37 °C, 5% CO_2 and 95% relative humidity (RH) in MEM and DMEM respectively, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1× non essential amino acid, penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were seeded for overnight and subsequently treated with etoposide (25 μM for 3 h) after withdrawal of etoposide, incubated in fresh medium for indicated time period.

2.2. Antibodies, reagents, plasmids

Etoposide (Sigma) were dissolved in dimethyl sulphoxide (Sigma) to a concentration of 25 mM. All further dilutions were freshly made in the medium. Akt, phospho-Akt (Ser 473), total PTEN, phospho-PTEN (Ser 380), Mre11, were purchased from Cell Signaling Technology (USA). Phospho-PTEN (Ser 380, Thr 382/383), β-actin, HA antibody, Ku70, γH2AX were purchased from Santa Cruze Biotechnology, USA. Anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (HRP, 1:1000, Bangalore Genei, India) and anti-rabbit IgG conjugated with Texas Red and FITC (1:200, Santa Cruze Biotechnology) were used as secondary antibody. Gene constructs pSG5L-HA-Wt-PTEN (HA-wt-PTEN) encodes hemagglutinin tagged wild type PTEN protein, phospho-deficient pSG5L-HA-A4-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala) and phospho-mimicking pSG5L-HA-E4-PTEN (Ser380Glu, Thr382Glu, Thr383Glu, Ser 385Glu) encode hemagglutinin tagged mutant PTEN proteins. pSG5L-HA was used as control empty vectors.

2.3. Cellular transfection

U87MG cells were seeded in antibiotic free medium and incubated overnight prior to transfection. For transient transfection, Fu Gene 6 transfection reagent (Roche, USA) was used as per manufacturer's instruction (reagent: DNA = 3:1).

2.4. MTT assay

After etoposide treatment cells were washed with 1 × PBS and incubated in medium with 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (0.5 mg/ml) for 3 h at 37 °C. The resulting formazan crystals were dissolved in MTT solubilization buffer and the absorbance were taken with a UV–vis spectrophotometer (Hitachi) at a wavelength of 570 nm.

2.5. Fluorescence microscopy

Cells were seeded on 18 mm sterile glass cover slips. After treatment cells were fixed in freshly prepared 4% paraformaldehyde solution for 15 min and permeabilized with 0.2% Triton X-100 on ice for 10 min. Fixed cells were pre-incubated in blocking solution (1% BSA in 1× PBS), followed by overnight incubation with either anti-HA antibody, MRE11 antibody, Ku70, γH2AX antibody or total PTEN antibody (Santa Cruz Biotechnology, USA; 1:100), and phospho PTEN antibody (Cell Signaling, USA, 1:100) in wash buffer (0.1% BSA and 0.05%Tween 20 in 1× PBS) at 4 °C. Cells were then washed six times with wash buffer and probed with Texas Red conjugated anti mouse antibody (1:200) and fluorescein isothiocyanate (FITC) conjugated anti rabbit antibody (1:200) for 1 h at RT. After washing, cells were mounted with the mounting solution containing DAPI (4-6-diamidino-2-phenylindole,Vector Laboratories, USA) and examined under a fluorescence microscope (Leica, Germany).

2.6. Western blotting and immunoprecipitation

Cell pellets were collected after treatment with etoposide and cell lysates were prepared by cell lysis buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 2 μg/ml aprotinin, 2 μg/ml leupeptin and phosphatase inhibitor) and resolved by 10% SDS–PAGE and then were transferred to methanol activated PVDF membrane. After blocking for 1 h at room temperature (RT) with TBS containing 0.1% (w/v) Tween-20 and 5% (w/v) non-fat milk, membranes were incubated with primary antibodies at 4 °C of overnight, followed by washing with TBS containing 0.1% Tween-20 and incubated with HRP-conjugated anti rabbit or anti mouse antibody for 1 h at RT. Detection was carried out using ECL reagents (Amersham Biosciences, Buckinghamshire, UK). For immunoprecipitation, cell lysate was prepared by using RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 10 mM Tris–Cl (pH 6.5),0.5% sodium deoxycholate, 0.2 mM PMSF, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml Leupeptin, phosphatase inhibitor) on ice. After clearing the cell lysate with agarose bead, it was incubated at 4 °C for overnight with PTEN antibodies and then 30 μl protein A/G agarose beads were added and incubated for 2 h at 4 °C. Beads were washed three times with ice cold RIPA buffer and centrifuged for 5 min at 5000 × *g* between each wash. Protein was eluted from beads with 50 μl 1 × Laemmli dye and subjected to Western blotting as described above.

2.7. Comet assay

The alkaline single-cell gel electrophoresis assay (comet assay) was performed according to Singh et al. [24] with minor modifications. Briefly, U87MG cells were seeded in a 35 mm tissue culture plates for overnight. The cells were then transfected with various PTEN clones or empty vector and then treated with etoposide for 3 h. The U87MG cell suspension was mixed with 37 °C low-melting

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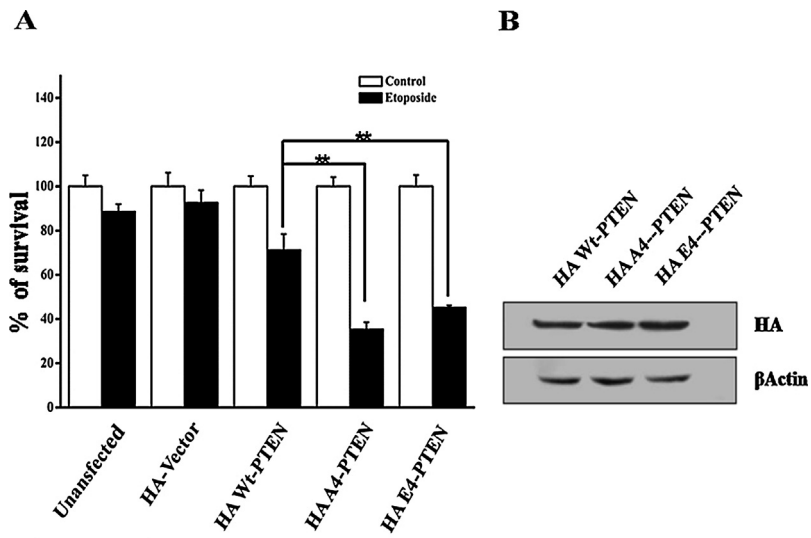

Fig. 1. (A) Untransfected U87MG cells or transiently transfected with any one of wt-PTEN, A4-PTEN, E4-PTEN and empty vector, incubated in the absence or presence of etoposide (25 μM for 3 h) and allowed to recover for 24 h in fresh medium. Percentage survival was determined by MTT assay. Untreated controls of each set were taken as 100%. Values are the mean ± SD of three independent experiments. $^{**}P < 0.005$. (B) Equal expression of different PTEN clones was confirmed by western blotting with anti HA antibody. β actin was taken as loading control.

point agarose and transferred to normal-melting point agarose-coated slides. The slides were then covered with a cover slip and incubated at 4 °C for 20 min for agarose solidification. The cover slips were removed, and cells were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton X-100) for 1 h at 4 °C. After washing with neutralization buffer, the slides were then electrophoreses at 25 V and 300 mA for 20 min. Then the slides were washed with neutralization buffer (0.4 M Tris, pH-7.5) for 20 min at 4 °C. Immediately before the analysis, the slides were stained with ethidium bromide (20 μg/ml) and scored using a fluorescence microscope (Leica, Germany). The percentages of tail DNA were evaluated using Komet Assay software 5.5 at 40× magnification. 50 randomly chosen nuclei were analyzed per treatment, and a total of three independent experiments was performed.

2.8. Statistical analysis

A Student's *t*-test was used to calculate the statistical differences between the groups. $P < 0.05$ and 0.005 was considered as statistically significant. Data analysis was performed using the Origin Pro v.8 software (Origin Lab).

3. Results

3.1. Phospho-mutants of PTEN chemosensitize PTEN null cells

To understand the role of PTEN in DNA damage response, we used three plasmids, HA-wt-PTEN, HA-A4-PTEN (where C-terminal Ser 380, Thr 382/383, Ser 385 are replaced with Alaline) and phospho mimicking HA-E4-PTEN (where C-terminal Ser380, Thr 382/383, Ser 385 are replaced with glutamic acid). At first we transfected these plasmids in PTEN null U87MG cells separately and equal expression of all PTEN clones was confirmed by western blotting of U87MG cell extract with anti HA antibody (Fig. 1B). After etoposide treatment (25 μM for 3 h), the cells were allowed to recover for 24 h in fresh medium and survivability was measured by MTT assay. Significantly higher percentage of survivability in HA-wt-PTEN transfected cells compared to HA-A4-PTEN or HA-E4-PTEN transfected cells during 24 h recovery after DNA damage were observed (Fig. 1A). However compared to untransfected cells, mortality of wt-PTEN transfected cell increased possibly due to the activation of PI3K in untransfected cells. So phospho-mutant PTEN sensitized the cells toward etoposide. This was expected as mutant PTEN lacking phosphorylation site, catalytically more active and down-regulate p-Akt mediated cell survival as previously reported [11] and our western blot experiment also validated it (Fig. S2). Further it was shown that E4-PTEN and A4-PTEN posses greater phosphatase activity than wt-PTEN. Phosphatase activity of E4 is almost similar with A4.

3.2. Wild type PTEN efficiently repair damage DNA

To address whether reduced survivability of phospho-mutant PTEN transfected cells were due to the lack of DNA repair, we carried out alkaline comet assay to estimate DNA damage and repair (Fig. 2). At first PTEN null background of U87MG cells was validated by immunoblotting total cell extract of mock-treated or etoposide treated cells with PTEN antibody (see supplementary Fig. S1). As expected no PTEN bands were observed and additionally the level of phospho-Akt was increased after etoposide treatment. For estimation of DNA repair activity, we separately transfected PTEN clones or empty vector in U87MG cells and after etoposide treatment for 3 h, DNA repair was measured at three different time points, 0 h (immediately after DNA damage), and subsequent 8 h and 24 h recovery period. As seen in Fig. 2A length of tail DNA for wild type PTEN transfected U87MG cells after 24 h recovery is less compared to two other plasmids transfected cells. Quantitatively, the percentage of tail DNA significantly decreases (Fig. 2B) in wild type PTEN transfected cells at 8 h (36.42 ± 2.49–27.20 ± 2.07) and 24 h (17.53 ± 2.69) recovery time compared to A4 (0 h = 37.31 ± 2.08, 8 h = 40.18 ± 1.4, 24 h = 31.04 ± 2.53) and E4 PTEN (0 h = 36.17 ± 1.34, 8 h = 37.38 ± 2.66, 24 h = 32.83 ± 1.93), indicating C-terminal phosphorylation of PTEN is essential for DNA repair.

3.3. Increase phosphorylation in C-terminal PTEN tail in response to DNA damage.

Given that phospho-mutant PTEN expressing PTEN null cells rendered reduce DNA repair capacity, we further proceed to evaluate the phosphorylation status of PTEN in response to DNA damage.

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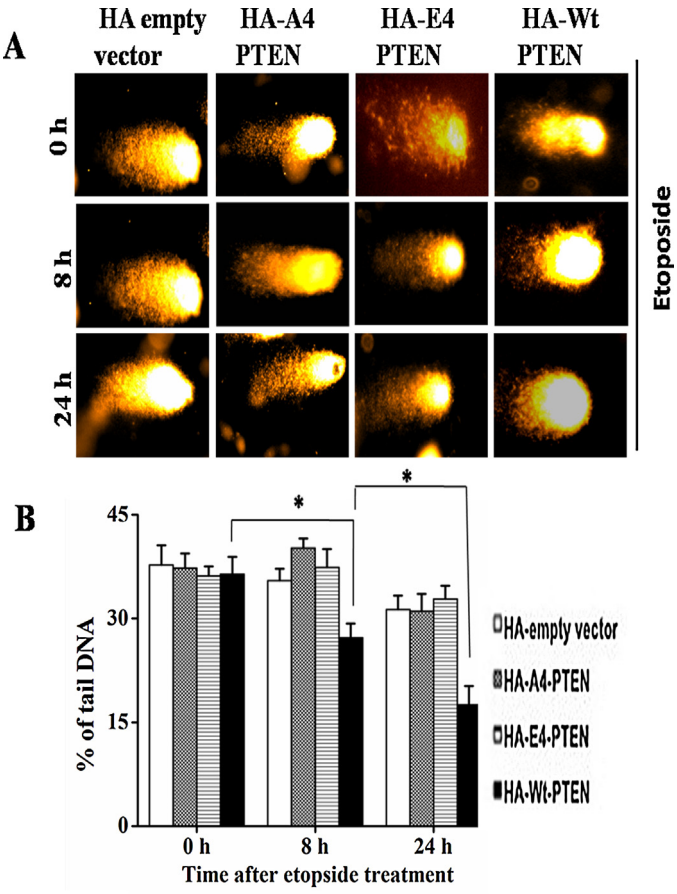

Fig. 2. (A) Representative photomicrographs of comet assay of wt-PTEN, A4-PTEN, E4-PTEN or empty vector transfected PTEN null U87MG cells. The transfected cells were incubated in the presence of etoposide (25 μM for 3 h) and allowed to recover for 8 h and 24 h in fresh medium. 0 h represents immediately after DNA damage. (B) Graph represents the percentage of tail DNA (measured by Komet 5.5 software) at different time points. 50 nuclei per slide were counted. Values are the mean ± SD of three independent experiments. $^{*}P < 0.05$.

U87MG cells were transfected with wild type PTEN and after DNA damage whole cell extract were prepared and immunoblotted against phospho PTEN Ser 380, phospho PTEN Ser 380,Thr382/383, HA, β actin antibodies respectively (Fig. 3A). We found that after DNA damage the phosphorylation of PTEN increases (approx 2 fold) compared to corresponding cell lysate from untreated control. Quantitative data are shown in the side panel (Fig. 3B). To check this for indigenous PTEN, we prepared whole cell lysate of both untreated and etoposide treated HEK293T cells (having wild type PTEN) and cell lysates were immunobloted with total PTEN and phospho PTEN antibodies (Fig. 3C). Here we also observed increase phosphorylation of indigenous PTEN after etoposide treatment. Quantitative analysis of the data is presented in Fig. 3D. Further total PTEN was immunprecipitated from the whole cell lysate of control and etoposide treated HEK 239T cells, and precipitated protein was immunobloted with antibodies against phospho PTEN. As seen in Fig. 4 both phospho Ser 380 and phospho Thr 382/383 increased after etoposide treatment. Quantitative analysis of the blot estimating the ratio of phospho PTEN with total PTEN is shown in Fig. 4B.

3.4. Phospho PTEN localized to nucleus after DNA damage

Phosphorylation of PTEN is associated with its nuclear localization and our data suggest that PTEN is phosphorylated after DNA damage. Thus it is expected that phospho PTEN localization may be associated with DNA damage. To check it, we transfected U87MG cells separately with different PTEN clones and immunolabeled with HA antibody. As seen in Fig. 5A wild type PTEN after DNA damage migrated to nucleus but mutant PTEN was evenly distributed. In order to check this for indigenous PTEN we used HEK293T cells. HEK293T cells were treated with etoposide for 3 h and then immunolabeled with phospho-PTEN or total PTEN antibodies (Fig. 5B). Here we also found that phospho PTEN migrates to nucleus after DNA damage.

3.5. Phospho PTEN colocalized with γH2AX foci

We further investigated if phospho PTEN is recruited to repair foci after DNA damage. So we irradiated cells with ionizing radiation with two different doses (2 Gy and 4 Gy), incubated for 30 min or treated with etoposide (25 μM for 1 h) and then processed for observation in fluorescence microscopy. Here we found that pPTEN(ser 380) develop foci which were colocalized with γH2AX, a universal marker for DNA damage [25] (Fig. 6). This observations indicated that phospho PTEN is recruited to the DNA broken end and may be involved in successful DNA repair event.

3.6. Persistence of DNA damage induced γH2AX, Mre 11and KU70 foci during recovery after DNA damage in cells trasfected with phospho-mutant PTEN.

Next we asked the status of DNA damage induced Mre11 foci during recovery period after etoposide treatment in U87MG cells transfected separately with HA-wt-PTEN, HA-E4-PTEN, HA-A4-PTEN and HA-empty vector. The cells were treated with etoposide for 3 h and after removal of etoposide the cells were allowed to recover for three different time points (0 h, 8 h and 24 h). As shown in Fig. 6, the number of Mre11 foci positive cells peaked at 0 h after etoposide treatment, in all the plasmid transfected U87MG cells (wild type PTEN = 77.42% ± 3.16, A4-PTEN = 82.13% ± 3.43, E4-PTEN = 81.78% ± 2.85, HA = 80.87% ± 3.85), but in the wild type PTEN expressing cells the number of Mre11 foci positive cells decreased during subsequent recovery time (8 h = 57.93% ± 6.2, 24 h = 24.81% ± 3.99), The corresponding values for A4-PTEN (8 h = 78.9%3.86, 24 h = 76.8% ± 4.2) and for E4 PTEN (8 h = 75.01% ± 4.1, 24 h = 72% ± 2.6) demonstrated persistence of Mre11 foci. Thus the cells transfected with the plasmids lacking phosphorylation sites were impaired in restoration of Mre11 localization (Fig. 7B) indicating absence or delayed DNA repair. We also measured the γH2AX foci at 0 h and 24 h recovery time point after etoposide treatment in phospho-mutant and wt PTEN transfected U87MG cells. Almost equal amount of γH2AX foci were seen for all the clone at 0 h time point but during recovery period of 24 h this foci significantly reduced in wt-PTEN transfected cells compared to phosphor mutant transfected cells (Fig. 7A). PTEN deficient cells and cell expressing phospho-mutant PTEN were also defective in resolving the Ku70 foci. Here we also observed (Fig. 7C) that the number of Ku70 foci containing cells significantly decreased after 24 h recovery in wild type PTEN transfected cells (0 h = 75.01% ± 6.8, 24 h = 42.4 ± 4.6) compared to A4-PTEN (0 h = 85% ± 7.2, 24 h = 70.8% ± 4.9) and E4-PTEN (0 h = 78% ± 6.1, 24 h = 62.7% ± 7.2).

4. Discussion

The ability of DNA repair in cells is crucial for maintaining genomic integrity and normal functioning of cells. PTEN, a well known tumor suppressor protein is now emerging as a DNA repair protein apart from its role in down regulating oncogenic PI3K/AKT pathway, cell cycle and apoptosis. Though the structural

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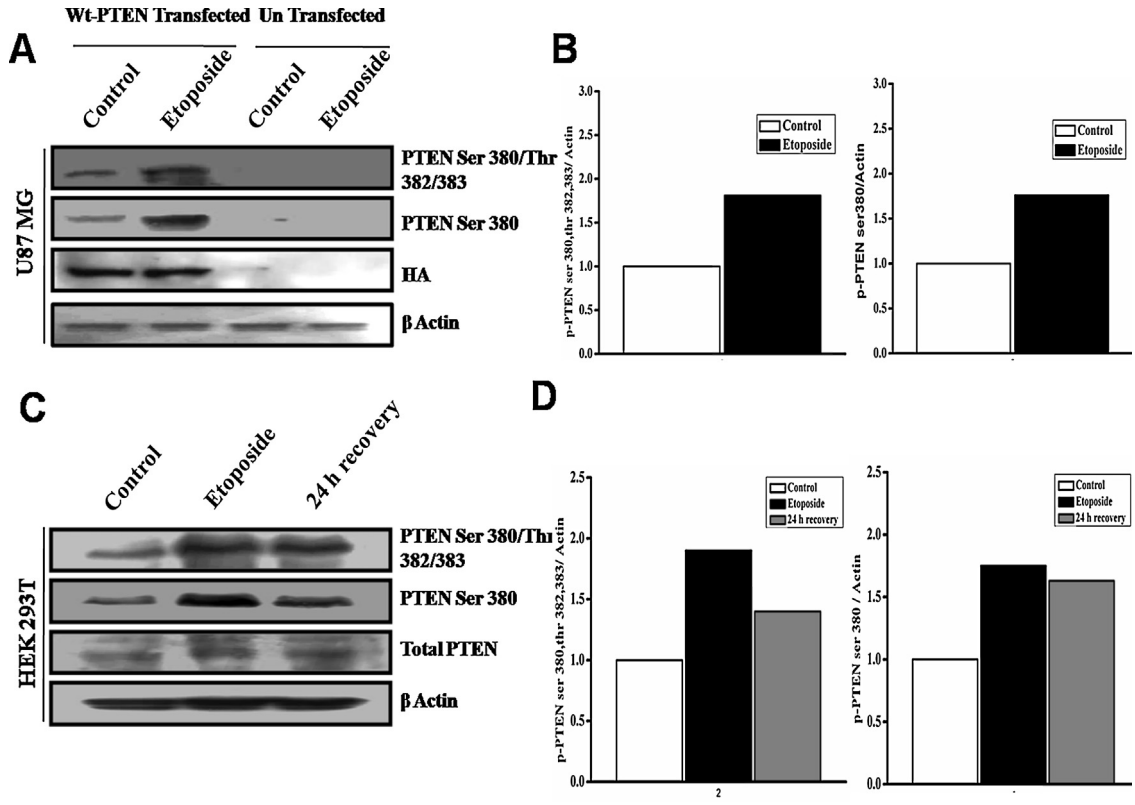

Fig. 3. DNA damage induced phosphorylation of wt-PTEN. (A) Growing U87MG cells were incubated without or with etoposide (25 μM for 3 h) and the cellular lysates were probed with phospho-PTEN specific antibodies or total PTEN. β actin was taken as loading control. (B) and (D) Band intensity was measured by Image.J software. Control is normalized to 1. (C) HEK293T cells were treated without or with etoposide and allowed to grow in fresh medium for 24 h. Then cell lysate was prepared, blotted with indicated antibodies.

and functional relationship of PTEN has been explored in previous literatures [1–3,5,9], very limited studies were carried out to explore the exact role of phosphorylated PTEN. The 50 amino acid residues at the C-terminal of PTEN are the sites of major phosphorylation of PTEN, which largely contributes for its stability, nuclear localization and phosphatase activity. In this report we have established that phosphorylation at the STT motif of PTEN is essential for its DNA repair activity and contributes for cell survival. We have designed experiments with PTEN null cells U87MG and express different plasmid consisting wild type PTEN, phospho-deficient PTEN and phospho-mimic PTEN in this cells to study cell survival and DNA repair. C-terminal phosphorylation results in stability of PTEN and simultaneously compromising its phosphatase activity. It has been reported that several human cancers are associated with C-terminal mutation of PTEN [26]. Moreover it is also reported that C-terminal is essential for both phosphatase and tumor suppressor activity [9]. Thus regulation of diverse PTEN activity is mediated by C-terminal of PTEN and phosphorylation of STT motif is not merely responsible for its stability but may also contributes to its other activities. In this report we have clearly shown that DNA repair

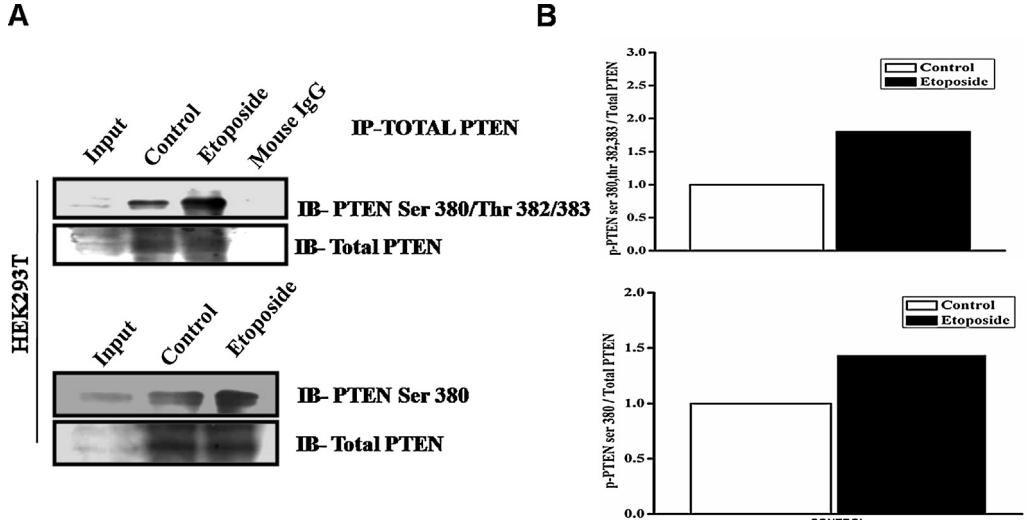

Fig. 4. (A) Whole cell lysate of etoposide treated (25 μM for 3 h) or untreated HEK293T cell was immunoprecipitated with total PTEN antibody and immune blotted with phospho specific antibodies as indicated. (B) Graph represents the relative intensity measured by Image.J software.

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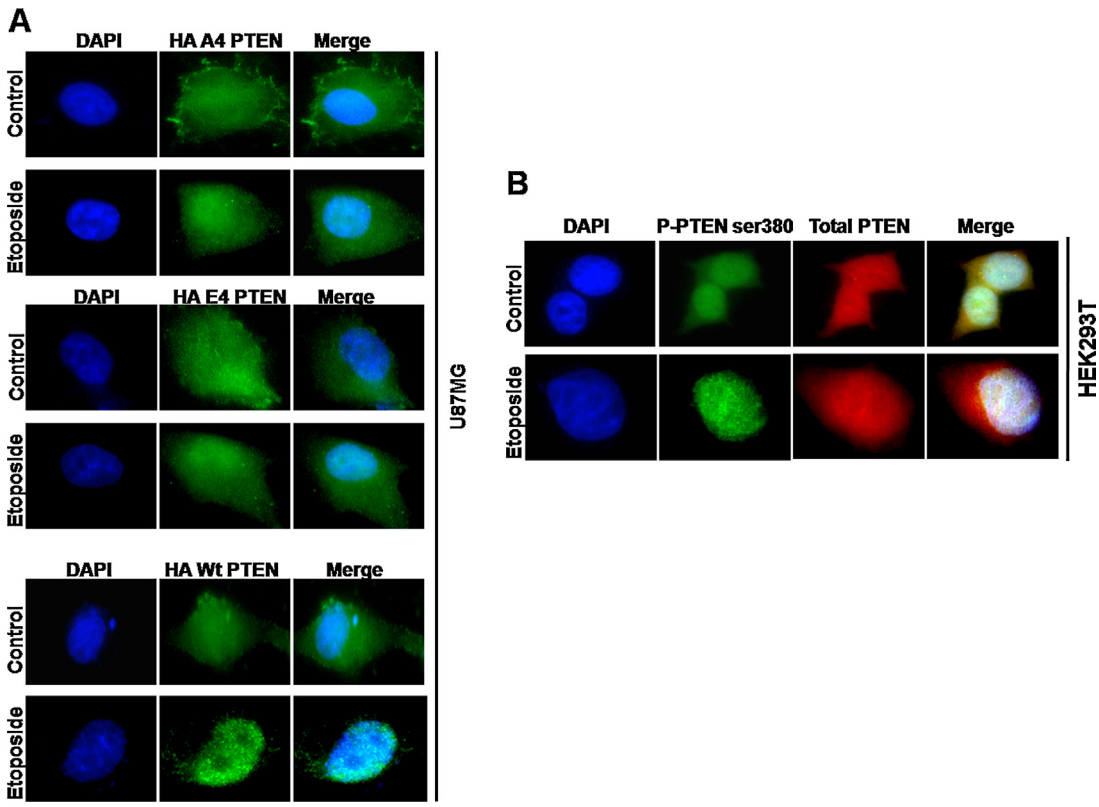

Fig. 5. (A) Representative photomicrographs of U87MG cells after transient expression of HA-tagged wt-PTEN or phospho-mutant PTEN in absence or presence of etoposide (25 μM for 3 h) treatment. Cells were immunolabeled with anti-HA (green) for PTEN and DAPI for nuclear staining. (B) Representative photomicrographs of control and etoposide treated HEK293T cells-immune labeled with PTEN-Ser380 (green) or total PTEN(red).

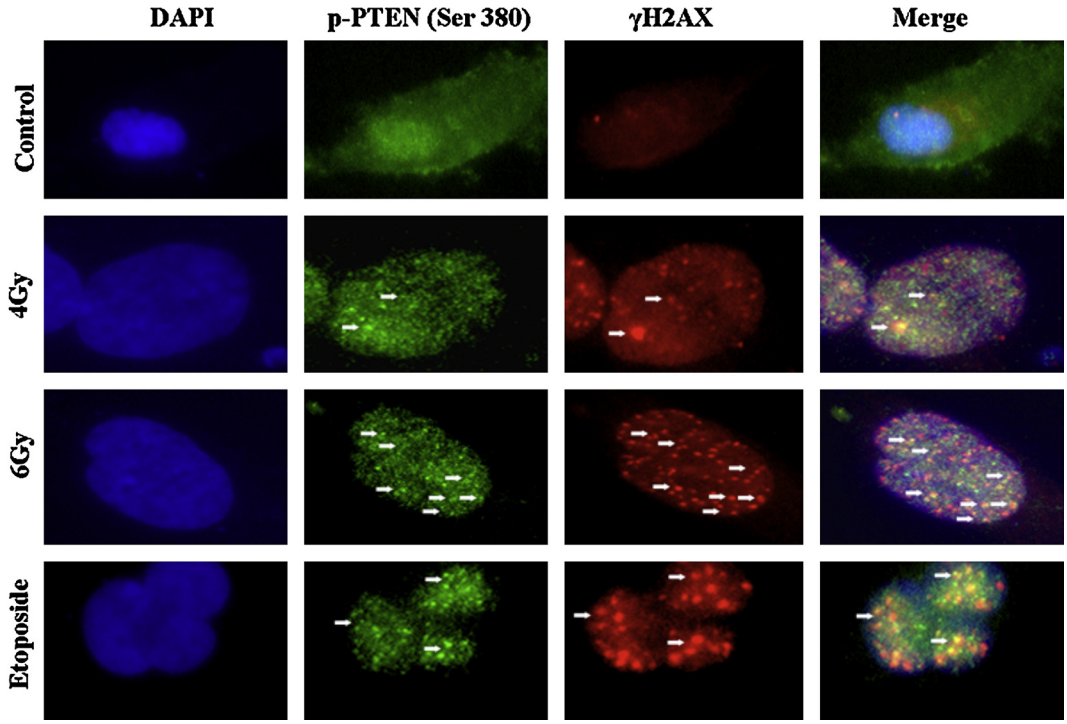

Fig. 6. DNA damage induced foci of phospho PTEN. Cells were irradiated with γ rays or etoposide and then immunolabeled with PTEN Ser 380 (green), γH2AX (red) and DAPI for nuclear stain. Yellow dots and arrows indicate colocalization.

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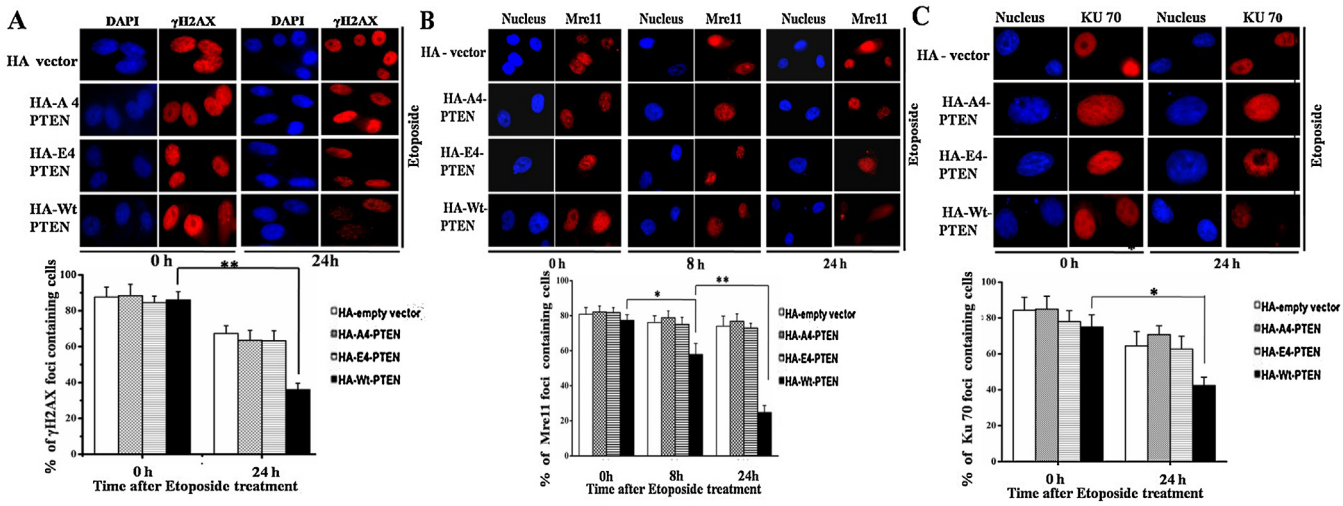

Fig. 7. Representative photomicrographs of etoposide (25 μM for 3 h) treated γH2AX, Mre11 and Ku70 foci of U87MG cells transiently transfected with the indicated PTEN vectors at indicated recovery time point. (A) Representative microscopic image and quantitative data of γH2AX foci at different time points. Values are the mean ± SD of three independent experiments. $^{*}P < 0.05$ (B). Representative microscopic image and quantitative data of Mre11 foci containing cells at different time point. Values are mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$. (C) Representative microscopic image and quantitative data of Ku70 foci containing cells at different time points. Values are the mean ± SD of three independent experiments. $^{*}P < 0.05$.

activity of U87MG decreases when tranfected with A4-PTEN or E4-PTEN plasmid compare to wild type PTEN. Thus reduced survival of U87MG cells transfected with A4-PTEN or E4-PTEN compared to wild type PTEN is likely to be associated with DNA repair. In the same condition we have seen that exogenous as well indigenous PTEN is phosphorylated after DNA damage and lack of phosphorylation reduced DNA repair. Taken together it is quite possible that PTEN phosphorylation is linked with DNA repair. Additionally we have also established that after DNA damage phospho PTEN translocates to nucleus. PTEN lacks classical nuclear localization signal but contain some motif that promotes its nuclear entry [20]. It has been reported that PTEN enters into nucleus after genotoxic stress [17], though its exact function in nucleus is not known clearly but there is only one report demonstrating that CREB is the nuclear target of PTEN [27]. Here we have demonstrated that translocation of PTEN to nucleus depends on its phosphorylation which is associated with DNA repair. The exact role of PTEN in DNA repair is not known but it seems PTEN phosphatase activity is not required for DNA repair as phosphorylated PTEN is inactive phosphatase. Recently it has been demonstrated that PTEN C terminus is required for genomic stability and tumor suppression and phosphatase activity is not important [28]. Additionally it has been shown that for translocation of PTEN into nucleus, phopshorylation of Ser 380 is not essential [29] rather phosphorylation of, Ser 366/Thr 370 is absolutely necessary. On the contrary, it has been also demonstrated that phosphorylation of Ser 380 is closely associated with accumulation of PTEN on chromatin [30]. However our results are consistence with the observation where it has been shown that oxidative stress induces PTEN translocation to nucleus which depends on phosphorylation of Ser 380 residue [23]. In A4-PTEN and E4-PTEN construct phosphorylation of Ser 366/Thr 370 is possible but we repeatedly observed that the protein corresponding to E4-PTEN and A4-PTEN are not confined to nucleus after etoposide treatment. One possible explanation is that phosphorylation of STT motif, either in all residues or part of it is prerequisite for the phosphorylation of Ser 366/Thr 370 residues. Further, it is possible that phospho-mutant PTEN may have the DNA repair activity but lack of phosphorylation prevents their entry into the nucleus and thus the cells carrying such mutation in PTEN show reduced DNA repair. This was our observation in comet assay. In our indirect immunolabeling we have also demonstrated that phosphorylation of PTEN is necessary for their entry into the nucleus. The exact role of PTEN and the point where it contributes to the DNA repair is not known but from our data it seems likely that it involves later in the DNA repair process. Thus, γH2AX, Mre11 foci forming ability after DNA damage is independent of PTEN, as we have observed in this work. Mre11 complex is a key component of the immediate early response to DNA damage and its catalytic activities are required for the recruitment of various DNA repair proteins. The disappearance of such foci indicates successful repair events. There is no difference in the Mre11 foci formation in PTEN phospho mutant transfected cells compare to wild type PTEN transfected cells. But the restorations of Mre11 in PTEN phospho mutant transfected cells are much slower than wild type PTEN transfected cells. This indicates that phophorylation of PTEN is required not only for PTEN entry to the nucleus but also for its association with DNA repair machinery to carry out successful DNA repair as has been suggested recently [29]. Additionally we have also observed that indigenous phospho PTEN colocalized with γH2AX after introduction of DNA damage by ionizing radiation. Though preliminary, our observation also raised the issue that phospho PTEN may also participate in NHEJ. Previous study indicates that PTEN is associated with HR and chromosomal stability [16]. Here we have shown that etoposide induced Ku70 foci retained longer in phospho-mutant PTEN transfected U87MG cells compare to wt-PTEN transfected cells. In the recent study [31] DNAPK foci has been implicated as a DNA damage signaling and regulates DNA repair activity. If this be the case then our observation with Ku implicates that PTEN phospho-mutant fail to translate DNA damage signaling to DNA repair, suggesting the role of PTEN as an intermediate component of DNA repair. This also suggests that lack of such translocation resulted in persistence of DNA damage sensor protein at the DNA broken end. However the role of PTEN in DNA repair is still in its infancy and needs further studies. But our results, for the first time demonstrated that PTEN phoshorylation is essential for successful DNA repair.

Conflict of interest

All authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrfmmm.2014.08.008.

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