13.1 Introduction

PI3K is an evolutionarily conserved family of signal transducing enzyme which regulates cell proliferation, cell survival, differentiation, apoptosis and angiogenesis (Vogt et al., 2010; Kok et al., 2009). Frequent hyperactivation of PI3K pathway is common in human cancer (Osaki et al., 2004; Yuan and Cantley, 2008; Arcaro and Guerreiro, 2007). Class I PI3K has a long history of association with cancer. It is a heterodimer composed of a catalytic subunit P110α and regulatory subunit p85α (Maurya and Vinayak, 2015; Maurya and Vinayak, 2015; Berenjeno and Vanhaesebroeck, 2009; Zhao and Vogt, 2008; Geering et al., 2007). PI3K is essential for AKT activation through multiple factors, involving membrane translocation and phosphorylation as common process (Dibble and Manning, 2009). AKT is phosphorylated by PDK which is a crucial kinase that functions downstream of PI3K and parallel to AKT. Both PDK and AKT are required for normal mammalian development and play a major role in regulation of cell survival and angiogenesis (Gagliardi et al., 2015; Fyffe and Falasca, 2013; Osaki et al., 2004; Maurya and Vinayak, 2015; Testa and Bellacosa, 2001). AKT promotes cell survival by phosphorylation and inhibition of pro-apoptotic protein BAD (Balmanno and Cook, 2009; Weyhenmeyer et al., 2012; Datta et al., 1997). Inactivation of BAD is also mediated through phosphorylation by ERK activated p90 ribosomal S6 kinase (Roskoski R Jr, 2012). ERK is widely expressed signaling molecule that participates in regulation of a large variety of processes including cell adhesion, cell cycle progression, cell migration, survival, differentiation, metabolism and proliferation (Roskoski R Jr, 2012).

ROS is a group of highly reactive molecules produced in cells which oxidize different biological targets. This group includes H₂O₂, O₂⁻, OH⁻ etc. Initially ROS was considered as destructive molecules resulting in cell death by damaging lipids, proteins and DNA but recently it is reported to participate in cell survival, proliferation and migration (Schieber and Chandel, 2014; Finkel et al., 2011; Groeeger et al., 2009). Moreover, ROS serves as signaling molecule to regulate cell physiology (Finkel et al., 2011; Veal et al., 2007; Rhee, 2006). Accumulation of ROS (oxidative stress) has been linked to hyperactivation of signaling pathways and metabolic adaptations of tumor microenvironment (Schieber and Chandel, 2014). Cancerous cells achieve higher level of ROS than their non-transformed counterparts. Elevated ROS level leads to genomic instability and promotes tumorigenesis (Groeeger et al., 2009).
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H$_2$O$_2$ is most commonly used as source of ROS for oxidative stress preconditioning (Groeger et al., 2009; Pronskato et al., 2012). It is formed by dismutation of superoxide (O$_2$•−) spontaneously or enzymatically, catalyzed by SOD. H$_2$O$_2$ is more stable as compared to other ROS having half life of 1ms and serves as inter as well as intracellular signaling molecule as it easily diffuses from site of production and crosses membrane (Bienert et al., 2006; Sousa et al., 2004). Present study evaluates the impact of H$_2$O$_2$ as a source of ROS on modulation of PI3K-AKT pathway in DLA cells to explore its role in cell survival.

### 13.2 Results

#### 13.2.1 H$_2$O$_2$ induces ROS accumulation

Elevated level of ROS formation has been observed in different types of cancer including DL, which results in oxidative stress (Maurya and Vinayak, 2015; Das and Vinayak, 2014). Therefore, effect of H$_2$O$_2$ was analyzed on ROS level in terms of absorbance (fluorescence/mg protein) using green fluorescent dye, H$_2$DCFDA. ROS level was increased significantly by approximately 5.70 fold with H$_2$O$_2$ in DLA cells (Fig 13.1a). Increased absorbance of DCF with H$_2$O$_2$ was further confirmed through flow cytometry (Fig 13.1b). Amount of ROS generation was indicated by shift in fluorescence. Result suggested that H$_2$O$_2$ induces ROS accumulation several fold in DLA cells.

![Figure 13.1 Effect of H$_2$O$_2$ on ROS accumulation](image)

(a) Homogenates of DLA cells from 3 set of each group were pooled separately and used for total ROS measurement
(b) Cells were loaded with H$_2$DCFDA (10µM) for 30min and amount of ROS generated was indicated by shift in fluorescence as detected by flow cytometry. Data represent as mean ± S.E.M. of three independent experiments. ### denote significant differences at the level of p < 0.001 between H$_2$O$_2$ treated and control groups

#### 13.2.2 H$_2$O$_2$ modulates PI3K signaling

PI3K signaling cascade has been linked to cell proliferation, survival and angiogenesis. Effect of H$_2$O$_2$ was investigated on p85α (regulatory subunit of PI3K), PTEN and PDK1. Phosphorylation
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of p85α was significantly increased by approximately 1.93 fold with H₂O₂; however protein level of p85α was found unchanged (Fig 13.2a). Further, H₂O₂ increased phosphorylation of PDK1 significantly by approximately 34.4% (Fig 13.2b), whereas tumor suppressor PTEN was observed to be decreased by approximately 35% in H₂O₂ induced DLA cells as compared to control (Fig 13.2c).

Figure 13.2 Effect of H₂O₂ on PI3K signaling
(a) Western analysis of p85α, phospho p85α and respective densitometric scanning of band after normalization with β-actin (b) Western analysis of phospho PDK1 and respective densitometric scanning of band after normalization with β-actin and (c) Western analysis of PTEN and respective densitometric scanning of band after normalization with β-actin. Data represent as mean ± S.E.M. of three independent experiments. ### denote significant differences at the level of p < 0.001 between H₂O₂ treated and control groups.

13.2.3 H₂O₂ elicits phosphorylation of AKT at Thr-308 & Ser-473
AKT is an essential downstream target of PI3K signaling pathway that provides a survival signal. AKT activation involves membrane translocation and phosphorylation. H₂O₂ induced phosphorylation of AKT at Ser-473 and Thr-308 significantly by approximately 93.4% and 6.63 fold respectively as compared to control DLA cells (Fig 13.3). However, level of AKT1 was found to be unaffected.
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Figure 13.3 Effect of H₂O₂ on phosphorylation of AKT at Thr-308 & Ser-473
(a) Western analysis of phospho AKT Thr-308, phospho AKT Ser-473 and AKT1 (b) Densitometric scanning of respective band after normalization with β-actin; data represent as mean ± S.E.M. of three independent experiments. ### denote significant differences at the level of p < 0.001 between H₂O₂ treated and control groups

13.2.4 H₂O₂ induces phosphorylation of BAD and ERK1/2
BAD phosphorylation forms BAD - (14-3-3) protein heterodimers which leaves Bcl-2 free to inhibit Bax-triggered apoptosis. Therefore, BAD phosphorylation is considered as anti-apoptotic, whereas dephosphorylation of BAD as pro-apoptotic signal. H₂O₂ increased phosphorylation of BAD significantly by approximately 80% in DLA cells as compared to control (Fig 13.4a). In addition, H₂O₂ increased phosphorylation of ERK1/2 significantly in DLA cells (Fig 13.4b). However, phosphorylated level of ERK1/2 was negligible in control group. Total level of ERK1/2 was unaffected by H₂O₂ (Fig 13.4b).
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Figure 13.4 Effect of H$_2$O$_2$ on phosphorylation of BAD and ERK1/2
(a) Western analysis of phospho BAD and densitometric scanning of band after normalization with β-actin (b) Western analysis of ERK1/2, phospho ERK1/2 and densitometric scanning of band after normalization with β-actin; data represent as mean ± S.E.M. of three independent experiments. ### denote significant differences at the level of p < 0.001 between H$_2$O$_2$ treated and control groups.

13.2.5 H$_2$O$_2$ induces protein expression of TNFR1
The receptor TNFR1 is known to promote cancer growth via PI3K and NF-κB dependent pathways. Level of TNFR1 was increased significantly by approximately 40% in H$_2$O$_2$ induced DLA cells as compared to control (Fig 13.5).

Figure 13.5 Effect of H$_2$O$_2$ on level of TNFR1
(a) Western analysis of TNFR1 and β-actin (b) Densitometric scanning of TNFR1 band after normalization with β-actin; data represent as mean ± S.E.M. of three independent experiments. ### denote significant differences at the level of p < 0.001 between H$_2$O$_2$ treated and control groups.

13.3 Discussion
ROS is involved in many cellular processes that positively and negatively regulate cell fate. It is reported to promote cell proliferation and survival in several cell types (Groeger et al., 2009). On the other hand, it has been shown to induce apoptosis in certain cell types and organs (Niwa et al., 2003; Zhuang et al., 2007). Short time exposure of DLA cells with H$_2$O$_2$ is shown to accumulate ROS which causes oxidative stress leading to genomic instability, and promotes tumorigenesis by hyperactivation of signaling pathways and metabolic adaptation of tumor microenvironment (Groeger et al., 2009).
Effect of H$_2$O$_2$ is analyzed on signaling pathway of proto-oncogene PI3K. Under normal condition PI3K is inactive as its regulatory subunit p85α stabilizes catalytic subunit p110α and inhibits its catalytic activity. p85α is phosphorylated on stimulation and activates PI3K by alleviation of inhibition (Geering et al., 2007). Hyperactivation of p85α has been observed in various cancers (Vanhaesebroeck et al., 2010; Berenjeno and Vanhaesebroeck, 2009; Geering et al., 2007). H$_2$O$_2$ is found to increase phosphorylation of p85α in DLA cells without affecting its level. Result reflects that H$_2$O$_2$ activates PI3K at post translational level by covalent modification without affecting at translational level. Upregulation of PI3K by oxidative stress may be correlated with our earlier report showing downregulation of PI3K by antioxidant QUE in DL mice (Maurya and Vinayak, 2015). Important target of PI3K is Ser/Thr kinase AKT, which is regulated by direct binding to PIP3 and phosphorylation by PDK. AKT are phosphorylated at Thr-308 and Ser-473. PDK1 is responsible for phosphorylation of Thr-308 (Chan et al., 2014; Liao and Hung, 2010). Our result demonstrates increased phosphorylation of both Ser-473 and Thr-308 region of AKT by H$_2$O$_2$ without affecting its protein level in DLA cells. Further, implication of H$_2$O$_2$ on activation of PDK1 is demonstrated by increased phosphorylation of PDK1. PDK1 is frequently elevated in cancer with parallel increased phosphorylation of downstream kinase AKT at Thr-308. This is consistent with our finding of declined level of tumor suppressor PTEN in DLA cells exposed to H$_2$O$_2$. PTEN acts as a negative regulator of PI3K. PTEN is frequently lost or mutated in cancer (Papa et al., 2014; Chalhoub and Baker, 2009). Our finding is supported by earlier report of PTEN inactivation by H$_2$O$_2$ (Leslie et al., 2003). Result supports H$_2$O$_2$ mediated activation of PI3K signaling pathway via PDK1 and AKT.

AKT promotes cell survival via regulation of BAD. BAD is a member of Bcl-2 family which promotes cell death by displacing Bax from binding to Bcl-2 and Bcl-xL (Balmanno and Cook, 2009; Weyhenmeyer et al., 2012; Howells et al., 2011; Yang et al., 1995). Dephosphorylated BAD forms a heterodimer with Bcl-2 and Bcl-xL, inactivating them and allowing Bax/Bak triggered apoptosis. Phosphorylation of BAD leads to its binding to 14-3-3 protein, which leaves Bcl-2 free to inhibit Bax-triggered apoptosis. H$_2$O$_2$ increases phosphorylation of BAD in DLA cells. Our result supports earlier report of AKT-BAD mediated cell survival in vivo and in vitro (Datta et al., 1997). Inactivation of BAD is also mediated through phosphorylation by ERK activated p90 ribosomal S6 kinase. Activation of ERK1/2 inhibits apoptosis by inactivation of
caspase 8. PI3K activation is responsible for ERK1/2 phosphorylation (Bell et al., 2004). Our result shows increased phosphorylation of ERK1/2 in H₂O₂ induced DLA cells without affecting its level, suggesting the role of H₂O₂ in PI3K-ERK-BAD signaling pathway towards cell survival. Thus, H₂O₂ promotes cell survival by inhibiting apoptosis via both pathways. Apart from this, abundance of TNFR1 is known to promote cell survival via PI3K and NF-κB dependant pathway (Oshima et al., 2014; Wajant and Scheurich, 2011; Leslie et al., 2003; Ozes et al., 1999; Gu et al., 2006). Increased level of TNFR1 by H₂O₂ exposure suggests induced survival of DLA cell. The result is supported by earlier report of ROS mediated TNFR1 expression in neuronal cells (Ma et al. 2009).

13.4 Summary
The results of current study suggest that H₂O₂ activates survival factors via increasing ROS level and hyperactivating PI3K. Hyperactivated PI3K promotes cell survival via PI3K-AKT-BAD signaling pathway. In addition, PI3K-ERK-BAD pathway and TNFR1-PI3K-BAD pathway are also activated by H₂O₂ to promote cell survival in T-cell lymphoma. Findings may provide the base for implication of PI3K-AKT signaling in cancer targeting.