CHAPTER 2

REVIEW OF LITERATURE

2.1. Lung Cancer Incidence and Risk Factors

Lung cancer is the most commonly diagnosed cancer worldwide, which carries a greater mortality rate than any other cancers such as breast, colorectal and prostate cancer (Ferlay et al., 2010). According to World Health Organization (WHO), 1.69 million people died in 2015 from the lung cancer only. Lung cancer trend varies according to age, sex, ethnicity and geographical location (Torre et al., 2016). As per current record, out of 5 million deaths in the world per year, 2.41 million are attributed to the developing countries due to lung cancer, which is further estimated to rise to 10 million by 2030. Lung cancer is indeed a major problem in India with 63,000 cases detected per year (Ganesh et al., 2011). The statewide incidence of lung cancer in India has been reported to be highest in the north-eastern states than western states (Rath et al., 2014). Also, the incidence rates are more in males than females. The National Cancer Registry Program (NCRP) of the Indian Council of Medical Research (ICMR) showed varying numbers of cases in different areas, depicted from the data collected from 6 parts of the country (Behera 2012). Lung cancer has been pronounced as the leading cancer in Delhi, Kolkata, Bhopal, Chennai and Mumbai (Ganesh et al., 2011). Lung cancer has been also reported to be more prevalent among farmers as compared to those engaged in other occupations. Lung cancer is categorized depending upon its origin from certain cell types. The most prevalent is non-small cell lung cancer (NSCLC) accounting for 80% which includes 44.73% squamous cell carcinoma and 30.26% adenocarcinoma (Krishnamurthy et al., 2012). Approximately all cases are diagnosed at an advanced stage that is not responsive to surgical intervention. As a result, chemotherapy and radiotherapy remain the only available options, thus constitute a source for clinical research.
2.1.1. Risk Factors for Lung Cancer

Genomic instability as a consequence of interplay between genetic factors and environmental factors contribute to lung cancer, as DNA is frequently damaged by endogenous metabolites and exogenous carcinogens. There are various risk factors that can be ascribed to the development and progression of lung cancer. The major established factor is tobacco use and smoking as it accounts for 80-85% of lung cancer cases worldwide. The remaining 15-20% cases can be attributed to heterogenous reasons such as lifestyle, genetic and environmental factors viz. exposure of chemical pesticides, radiation, radon gas and others (Ferlay et al., 2010). Also, the fact that lung cancer risk is proportioned for both smokers and non-smokers, other prospective risk factors come into the picture. As mentioned earlier, lung cancer cases are more among farmers, may be due to extensive exposure to chemical pesticides as a share of their work to purge pest problems. Farmers have been reported to use maximum percentage of pesticides produced annually for crop production (Wilson et al., 2001).

2.1.2. Environmental Risk Factors

In the present time, environmental toxicity has become a health burden. Due to the widespread growth of population, in order to meet the increasing demands, usage of chemical fertilizers and pesticides for industrialization, agricultural productivity and improving our living is not surprising. These toxic chemicals have potential to become causative factors for health problems as well as to become an obstacle for a cure. People get exposed to these chemicals through daily habits like breathing, drinking and eating with indoor/outdoor air and food as biggest sources. These toxic pollutants build up in our body over a time course (Ragnarsdottir, 2000).

Annually, 5 billion pounds of pesticides are used worldwide, among which organophosphate (OP) and carbamate insecticides account for 34%, dithiocarbamate fungicides for 18% and phenoxy herbicides for 12% (Ye et al., 2013). In India, we have an agriculture-based economy. Organophosphate
pesticides (OPPs) are extensively used in agriculture and are ubiquitous in our environment including residential settings. Besides agriculture, OPPs are also used in other occupational and residential settings such as fishery, food industry, home, gardens, and others (Jaga et al., 2003). Besides this, exposure of pesticides can also take place during production, preparation, transportation, and application in the workplace. The pesticide poisoning, a potential hazard to human health, manifests into respiratory problems, such as coughing and airway inflammation (Jaga et al., 2003). Various population-based studies have investigated the association between occupational pesticide exposures and chronic respiratory pathologies including lung cancer (Mostafalou et al., 2013). Research indicates increased cancer risk including lung cancer for exposure to OPPs (Alavanja et al., 2012; Alavanja et al., 2004; Alavanja et al., 2013; Jaga et al., 2005; Lee et al., 2004; Pesatori et al., 1994).

2.2. Organophosphate Pesticides (OPPs): Structure, Route(s) of Exposure and their Toxicity

OPPs include all insecticides containing phosphorous products derived from phosphoric acid, are considered as most toxic of all pesticides. The OPPs focused in our study are chlorpyrifos (CP) and monocrotophos (MCP), because they are mostly used and sold OPPs. CP and MCP are extensively used throughout the world including India. They are efficient, cheap and easily available. Chemical structures and brand names of OPPs Chlorpyrifos (CP) and Monocrotophos (MCP) are presented in Table 2.1.

2.2.1: Route(s) of Exposure

OPPs are quickly absorbed by inhalation and ingestion. Respiratory route is fastest and oral route is either accidental or suicidal. The other routes of exposure to OPPs are through dermal penetration and also, subsequent absorption differs with the specific pesticides. Exposure to the same OPPs by multiple routes or to multiple OPPs by multiple routes may cause serious additive toxicity. The extent of
poisoning also depends on the rate at which the OPP is absorbed (Kamanyire et al., 2004).

Table 2.1. Chemical structure and brand names of CP and MCP

<table>
<thead>
<tr>
<th>Name of the OPP</th>
<th>Chemical formula</th>
<th>Chemical Structure</th>
<th>Brand Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos (CP)</td>
<td>C₉H₁₁Cl₃NO₃PS</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>Brodan, Dowco 179, Empire, Dursban, Scout, Chlorpyrifos-ethyl, Detmol UA, Eradex, Lorsban, Paqeant, Piridane, Tricel</td>
</tr>
<tr>
<td>Monocrotophos (MCP)</td>
<td>C₇H₁₄NO₅P</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>Azodrin, Crotos, Bilobrin, Crisodrin, Glore Phos36, Monocil, Monocron, More-Phos, Plantdrin, Susvin, Monocron, Monocrotophos 60 WSC</td>
</tr>
</tbody>
</table>

2.2.2. OPPs-induced Acute and Chronic Toxicity

OPPs are classified as neurotoxins as they cause acute, subacute and chronic neurotoxicity in humans by inhibiting acetylcholinesterase (AChE) activity via its phosphorylation. As a result, there is a loss of available AChE which leads to overstimulation of the effector organ by the accumulated excess acetylcholine (ACh) neurotransmitter. Accumulation of ACh at a synapse impairs normal control of nerve impulse transmission, causes respiratory failure, paralysis and eventually death (Ballantyne et al., 2013). Although the toxic effects of OPPs on a cellular and molecular level have been studied in cultured cells by using standard
cytotoxicity and genotoxicity endpoints, the information on the specific pathway(s) involved in OPPs-induced toxicity is still required to be evaluated.

2.2.3. OPPs-induced Oxidative Stress and Inflammation

Reactive oxygen species (ROS) viz. superoxides, hydroxyl radicals and peroxides are associated with damage to cellular macromolecules such as lipids, proteins and nucleic acids. Free radicals and other ROS generated as a result of normal metabolism in the human body or from external stressors, such as exposure to ozone, pesticides, radiations, industrial chemicals etc. are involved in a variety of biological processes such as mutation, inflammation, carcinogenesis, aging, degenerative disorders and other human pathologies (Lukaszewicz-Hussain, 2010). Various studies support the idea that OPPs induce oxidative stress via multi-step pathway as a mechanism of their toxicity in the body (Kovacic, 2003). Large amounts of ROS are produced as a result of inhibition of oxidative phosphorylation coupled with their metabolism by various subunits of cytochrome P450s. It has been reported that OPPs induce oxidative stress in experimental animals and tissue culture models (Giordano et al., 2007; Yu et al., 2008). OPPs-induced brain damage and seizures associated with oxidative stress have been also demonstrated (Chen, 2012). Status of oxidative stress is determined in terms of lipid peroxidation, altered total antioxidant capacity (Altuntas et al., 2002; Gultekin et al., 2000). Reactive nitrogen species (RNS) generated from the nitric oxide (NO) and superoxide via enzymatic activity of inducible nitric oxide synthase (iNOS/NOS2) and NADPH oxidase results in nitrosative stress and DNA damage. NO not only results in DNA damage but also acts as a signalling molecule involved in pro-inflammatory pathways (Dhiman et al., 2015; Patel et al., 1999).

2.3. Metabolism of OPPs Chlorpyrifos (CP) and Monocrotophos (MCP)

Breakdown of OPPs take place mainly by hydrolysis in the liver. The rate of hydrolysis differs from one pesticide to another. The enzymes involved in OPPs metabolism are esterases and cytochrome P450s subunits (CYPs), which differs
in activity between individuals. For organophosphates, breakdown process is relatively slower and hence, significant temporary storage takes place in body fat. Monocrotophos, \([\text{dimethyl(E)-1-methyl-2-(methyl carbamoyl) vinyl phosphate}](\text{MCP})\), a broad-spectrum systemic pesticide is metabolized into primary products such as dimethyl phosphate, \(O\)-desmethyl monocrotophos, and \(N\)-desmethyl monocrotophos; \(N\)-desmethyl monocrotophos being more toxic than MCP (Lee et al., 1990). CP is one of those OPPs which require bioactivation e.g. CYPs 2D6, 3A5, 2B6 and 3A4 are involved in the bioactivation of CP to CP-oxon (Mutch et al., 2006). Another most important reported enzyme involved in metabolism of OPPs is paraoxonase (PON1). This enzyme hydrolyzes the oxon form of CP (Mutch et al., 2006).

2.4. Genotoxic Effects of Monocrotophos (MCP) and Chlorpyrifos (CP)

MCP and CP are widely used OPPs in India (Mathur et al., 1999). MCP, being acutely toxic to birds, was reported to cause mass mortality of Swainson’s hawk in Argentina (Goldstein et al., 1999). MCP, commonly known by the name of Azodrin, is out of patent and thus easily affordable at low cost. It is mainly applied against cotton pests. MCP is not banned for manufacture and import; and the production and demand is relatively higher. MCP is still in use owing to its properties like being cheaper and effective than other alternatives. According to the WHO, swallowing 1,200 milligrams - less than a teaspoon - of MCP can be fatal to humans (Joint et al., 2001). But in India with 1.2 billion populations, where vast numbers are hungry, uneducated and live in far-flung rural districts, loss in agricultural produce is not affordable and that too when MCP possess high efficacy of crop protection at low cost; thus, implementation of a complete ban cannot be ensured. Therefore, despite warning from WHO, MCP is still in use. Various incidents advocate the hazardous effect of MCP use; one of them is recent incidence in Gandaman village, Bihar, India, that reported death of 23 children due to MCP poisoning (Krause et al., 2013). Such incidence advocates the need for better understanding of OPPs toxicity and their mechanisms.

Next, the association studies regarding MCP genotoxicity also points towards the need for further investigations. MCP genotoxicity has been explored in mice
(Zhou et al., 2009), and shown to induce breast cancer cell proliferation in MCF7 cell lines (Isoda et al., 2005). It was found in a study that exposure to MCP induces production of ROS and decreased glutathione (GSH) levels. In addition, MCP is found to induce apoptosis in neuronal PC12 cells (Kashyap et al., 2011; Kashyap et al., 2010), as shown in Table 2.2.

After MCP, CP is other widely used OPPs. It is also used both in residential and agricultural settings to control pests such as termites. CP has the same mode of action as other OPPs i.e. inhibition of AChE. Although studies have shown that CP causes oxidative stress in experimental animal models and cell culture models, data regarding the mutagenic potential remains controversial. Gollaupdi and group concluded that CP did not induce DNA damage in Chinese hamster cells (Gollapudi et al., 1995). However, a recent investigation by Cui and group suggests that CP induces an increase in a number of micronuclei in mouse hepatocytes (Cui et al., 2011). Another study reported that CP causes DNA damage in mice leucocytes in a dose-dependent manner, using alkaline comet assay (Rahman et al., 2002). Another study by Salazar-Arredondo and group has demonstrated sperm DNA damage by various OPPs including CP and CP-oxon in healthy human spermatozoa exposed to 50–750 μM CP and CP-oxon (Salazar-Arredondo et al., 2008). However, all the concentrations of CP were not reported as cytotoxic as assessed by eosin-Y exclusion test. Another study investigated the DNA damage and apoptotic potential of CP in Drosophila melanogaster (Gupta et al., 2010). The parameters studied were ROS generation, DNA damage, oxidative stress markers and apoptosis. Elevated casapase-3 and caspase-9 activities along with depolarization in mitochondrial membrane potential were observed when exposed to CP. Thus, the study advocated ROS-induced apoptosis and DNA damage in Drosophila larvae when exposed to CP. A recent study also demonstrated that OPPs cypermethrin (CMR) and CP causes significant ROS production, chromosome aberrations, apoptosis, micronuclei induction and alterations in the cell cycle in bone marrow cells derived from Swiss albino mice (Chauhan et al., 2016). A significant increase in ROS generation was observed both in vivo and in vitro models. CP genotoxicity has been also evaluated in HepG2 cell line using comet assay, micronuclei assay, Hoechst and acridine orange/ethidium bromide (AO/EB) staining. The decrease in live cell count was
observed at 15-70 mg/L CP using Hoechst 33342 staining. Nuclear fragmentation along with an increase in comet tail length was also seen. The DNA fragmentation index in comet assay was found to be 93.3±2.08 and the average number of micronuclei in the assay was 62.6±1.52 per 1000 cell nuclei at 70 mg/L CP (Patnaik et al., 2016).

Table 2.2. Studies demonstrating the genotoxic effects of MCP and CP using different experimental models.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Pesticide</th>
<th>Model</th>
<th>Inference</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MCP</td>
<td>MCF7 (in vitro)</td>
<td>Induces breast cell proliferation</td>
<td>(Isoda et al., 2005)</td>
</tr>
<tr>
<td>2.</td>
<td>MCP</td>
<td>PC12 (in vitro)</td>
<td>ROS generation and Reduced levels of GSH</td>
<td>(Kashyap et al., 2011)</td>
</tr>
<tr>
<td>3.</td>
<td>MCP</td>
<td>Mice (in vivo)</td>
<td>Induces SSDB/DSDB</td>
<td>(Mahboob et al., 2002)</td>
</tr>
<tr>
<td>4.</td>
<td>MCP</td>
<td>Human lymphocytes culture</td>
<td>Induces SSDB and chromosomal aberrations</td>
<td>(Chakravarthi et al., 2009)</td>
</tr>
<tr>
<td>5.</td>
<td>CP</td>
<td>Rat (in vivo)</td>
<td>Induces oxidative stress</td>
<td>(Mansour et al., 2009; Yu et al., 2008)</td>
</tr>
<tr>
<td>6.</td>
<td>CP</td>
<td>Rat (in vivo)</td>
<td>Modifies antioxidant levels &amp; causes oxidative stress</td>
<td>(Bebe et al., 2003)</td>
</tr>
<tr>
<td>7.</td>
<td>CP</td>
<td><em>Hyalella azteca</em></td>
<td>Chlорpyrifos and methyl mercury exposure causes toxicity.</td>
<td>(Steevens et al., 2001)</td>
</tr>
<tr>
<td>8.</td>
<td>CP</td>
<td><em>Drosophila melanogaster</em></td>
<td>Induces ROS, DNA damage and apoptosis</td>
<td>(Gupta et al., 2010)</td>
</tr>
</tbody>
</table>
Although studies demonstrate that MCP and CP have genotoxic potential, still there is a controversy within the available literature due to different methods being used to assess the DNA damage. Apart from investigating the ability of MCP and CP to cause DNA damage using genotoxic biomarkers or comet assay, the detailed study of activation and regulation of DNA damage signalling pathways are needed to be explained further.

2.5. Types of DNA Damage

DNA damage can be categorized into various types such as spontaneous endogenous damage caused by replication errors, depurination, deamination, and oxidation etc. or exogenous damage by environmental factors such as radiation, chemicals etc. DNA can be damaged via mechanisms viz. simple adducts caused by alkylation, oxidation, and hydrolysis; other forms are cross-linkages due to chemicals like polycyclic aromatic hydrocarbons, mismatches due to faults in DNA replication, direct DNA damage by UV-B radiations, depurination by thermal disruption, indirect DNA damage by UV-A radiations etc.

![Diagram of DNA damage types](image)

**Figure 2.1. Types of DNA damage** (adapted from: Helleday *et al.*, 2014).

Oxidation to DNA bases is caused by ROS, producing a variety of lesions and formation of base adducts (Evans *et al.*, 2004). Most common and abundant adduct known as 8-oxo deoxyguanosine (8-oxodG) is formed by hydroxyl radical. During replication, 8-oxodG lesion leads to the pairing of guanine with adenosine instead of cytosine, which results in guanine to thymine transition mutations, that's
why it is also known as a marker of cellular oxidative stress and DNA damage (Helbock et al., 1999).

Double strand breaks (DSBs), induced by ionizing radiation or chemicals, can cause genetic recombination and DNA replication breakdowns, culminating into apoptosis. IR cause DSBs using two mechanisms; first, directly by breakage of the phosphodiester backbone or indirectly by mediating ROS-induced cellular oxidative stress (Figure 2.1).

Another frequent type of DNA lesion is apurinic or apyrimidinic (AP) site, which is caused by depurination and depyrimidation resulting from cleavage between the DNA base and deoxyribose sugar (Loeb et al., 1986). A study has indicated that normal human liver cells contain 50,000 AP sites per genome (Boiteux et al., 2004; Nakamura et al., 1999). These AP sites block the DNA replication and transcription. Cleavage of AP sites by AP endonucleases (APE1) or by DNA glycosylases (DG) generates single-strand breaks (SSBs) with 3’- or 5’-blocked termini, accumulation of which can be converted into double-strand breaks (DSBs) after DNA replication (Boiteux et al., 2004).

2.6. Cellular Responses to Genotoxic Stress: DNA Repair Pathways

Maintenance of genomic stability is essential as it prevents the onset of carcinogenesis and helps the cells to survive. In cellular response to threat to genomic stability i.e. genotoxic stress, DNA repair pathways get activated to repair the DNA damage lesions and thus, maintains the genomic stability. Various DNA repair pathways are involved in DNA damage response, which responds differently to different forms of DNA damage. SSBs ensue via base excision repair, nucleotide excision repair, and mismatch repair (Figure 2.2). DSBs involve homologous/non-homologous and microhomology mediated end joining.
Figure 2.2. DNA repair pathways in mammalian cells (adapted from: Blanpain et al., 2011).

2.6.1. Nucleotide Excision Repair (NER)

The nucleotide excision repair (NER) pathway mainly deals with bulky DNA adducts, crosslinks and helix-distorting regions. It is a 5-step pathway, 1st step is sensing the DNA lesion; 2nd step is making single strand incision at both flanks of the lesion; 3rd step is then excising of the single stranded nucleotides; 4th step is DNA repair synthesis by replacing the damaged oligonucleotides, and finally 5th step is ligating the remaining single-stranded nick (Figure 2.3). Any defect in NER pathway has been linked with the human pathologies such as Xeroderma Pigmentosum, which is characterized by sensitivity to UV radiation and susceptibility to skin cancer (Costa et al., 2003).
Figure 2.3. Schematic representation of Nucleotide Excision Repair (NER) pathway (adapted from https://oncohemakey.com).

2.6.2. Homologous Recombination (HR)

Homologous end joining pathway is based on homologous recombination (HR). HR pathway along with non-homologous end joining (NHEJ) is for the repair of DNA DSBs. Rad54 participates in HR pathway in multiple stages in concert with Rad51. HR starts by exonucleolytic processing of the 5’ ends of the DSBs to 3’ single ssDNA by a specific nuclease. Then it progresses as an invasion of undamaged sister chromatid by 3’ ssDNA tail to form a D-loop structure. DNA polymerase helps to synthesize missing DNA sequence information in DSBs, keeping the both 3’ ends as primers using the intact duplex as a template. As a result, a four-stranded branched structure known as holiday structure is formed after ligation, which culminates into the cleavage of ‘holiday junctions’ by the enzyme resolvase (Sonoda et al., 2006) [Figure 2.4].
2.6.3. Non-homologous End Joining (NHEJ)

NHEJ is another multi-step pathway for repairing DNA DSBs. NHEJ is mediated by the Ku70/80 proteins and DNA-dependent protein kinase (DNA-PK) complex. NHEJ involves the major role of Ku protein complex starting from identification of damaged DNA lesion, binding to the ends of the DNA DSBs, alignment of DNA strands to their protection from the nucleases (Figure 2.5). Ku protein also contributes by acting as scaffold causing recruitment of DNA-PK catalytic subunit, which orients the DNA for ligation and, via XRCC4 phosphorylation mediates the recruitment of DNA ligase IV, thus allowing the ligation of DNA ends (Lieber, 2010).
2.6.4. Base Excision Repair (BER)

BER-pathway is a frontline DNA repair mechanism against DNA damage caused by oxidation, deamination or alkylation, such as oxidized bases, AP sites, SSBs generated endogenously via cell metabolism or exogenously due to environmental factors UV light, radiation, viruses, chemotherapeutic agents, and others. It is initiated by DNA glycosylase (monofunctional), which removes the damaged base keeping the sugar-phosphate backbone intact and generates AP site; or bifunctional DNA glycosylase such as OGG, NTH1, which due to additional AP lyase activity cut the phosphodiester bond creating SSBs, by leaving unsaturated abasic terminus, which is then finally processed by the APE1. Whereas, AP site generated by monofunctional DNA glycosylase [such as thymine DNA glycosylase (TDG)] is identified by APE1 which incise the phosphodiester bond on immediate 5' site, leaving 3'-OH and 5'-phosphate termini and conscribe
other downstream BER-pathway enzymes to complete the repair process (Figure 2.6). Two sub-pathways work for BER-pathway, short-patch/single nucleotide (SN-BER) or long-patch repair (LP-BER), with different length of repair patch and different set of downstream enzymes involved. The sub-pathway to be followed depends upon the type of DNA glycosylase (monofunctional or bifunctional) and physiological state of the cell (Fortini et al., 1999). Other important enzymes of BER-pathway are X-ray repair cross-complementing 1 (XRCC1), DNA polymerase β (DNA pol β), DNA ligase I/IIIα and poly-(ADP-ribose) polymerase-1 (PARP1). PARP1 poly (ADP)-ribosylates various proteins (including itself) and plays key role by acting as DNA damage sensor and recruiter of downstream DNA repair proteins. XRCC1 act as scaffold protein for other BER-pathway protein-protein interactions. DNA pol β and DNA ligase are involved in repair synthesis and gap-filling (Hegde et al., 2008; Krokan et al., 2013; Seeberg et al., 1995; Thakur et al., 2014).

Poly (ADP-ribose) polymerase 1 (PARP1) is an additional enzyme implied in BER-pathway. PARP1 act as a DNA damage sensor as well as signalling molecule. Stimulated by its binding to DNA damage site, PARP1 results in covalent modification of itself and other proteins by catalyzing the production of long poly (ADP-ribose) polymers. It helps in relocation of downstream repair proteins such as XRCC1 to the DNA damage site. BER-pathway implies PARP1-dependent or PARP1-independent pathway depending upon the type of DNA lesion induced (Reynolds et al., 2015). A study has shown that PARP1 knock down displays efficient SSB repair activity (Godon et al., 2008). Impairment of DNA SSB repair after using PARP1 inhibitors was actually due inhibition of PARP1’s polymeric activity, which trapped the enzyme at the SSB and physically blocked the further repair process (Godon et al., 2008). This explains that PARP1 has no immediate role in BER-pathway, but somehow it affects the repair process.
Figure 2.6. The two sub-pathways of BER-pathway; short-patch (SN-BER) and long-patch (LP-BER) sub-pathways. APE1 functions as an AP endonuclease in SN-BER, initiated by monofunctional DNA glycosylase, and as a 3’ phosphodiesterase in LP-BER, initiated by bifunctional DNA glycosylase. DNA pol β, XRCC1 and DNA ligase IIIα are required for SN-BER to complete single nucleotide repair, PCNA, Pol δ/ε , FEN-1 and DNA ligase I are used in LP-BER to complete multi-nucleotide repair in mammalian cells (Thakur et al., 2014). 

Apart from the indispensable role in DNA repair of normal cells, BER-pathway is also the prime DNA repair system in cancer cells owing to fact that it counteracts the destructive effect of radiotherapeutics and chemotherapeutics. Therefore any imbalance in BER-pathway protein expression and efficiency will not only be responsible for the process of carcinogenesis, but will also affect the cancer treatment efficiency. Dysregulation of BER-pathway has been linked with various human diseases including cancer (Hofseth et al., 2003; Maynard et al., 2009). A study has reported an association between defective adenine DNA glycosylase MYH and colorectal cancer risk (Croitoru et al., 2004). Decreased activity of 8-oxoguanine DNA glycosylase OGG1 has been also linked with lung cancer risk progression (Paz-Elizur et al., 2003). Polymorphism in BER-pathway
enzymes can also affect the BER-pathway efficiency. Epidemiological studies have demonstrated the association of polymorphism within BER proteins with many cancers including lung cancer (Zhang et al., 2006; Zienolddiny et al., 2006). In addition, mutations in BER proteins also have been also linked with the neurological disorders (Wilson et al., 2007).

2.7. APE1/Ref-1: A Multifunctional BER-pathway Enzyme

Apurinic/apyrimidinic endonuclease 1 (APE1) is the central multi-functional enzyme of BER-pathway. The two prime functions of APE1 are: (i) repair enzyme, and (ii) redox-regulator of various transcription factors (TFs) involved in cell’s pro-survival pathways.

2.7.1. Repair Function of APE1

APE1’s repair function can be explained on the basis of two factors: first, it act as a main endonuclease in BER-pathway via repairing damaged bases in DNA by incising phosphodiester backbone at AP site, when BER-pathway is initiated by the monofunctional DNA glycosylase. Also, it possesses 3’ and 5’-exonuclease activity, 3’ phosphatase and 3’ diesterase activity for removal of β-unsaturated aldehyde and 3’ termini phosphate generated by the action of bifunctional DNA glycosylase. In AP endonuclease function, APE1 exerts its function by first inserting its loops into the major and minor grooves of DNA, then flipping of the abasic site into APE1’s hydrophobic pocket and finally kinking of the DNA helix. The C-terminus of APE1 mediates the endonuclease activity on the AP sites of the DNA (Fung et al., 2005). Another most important function of APE1 employs its protein-protein interaction with the downstream enzymes of BER-pathway viz. PARP1, XRCC1, DNA pol β and DNA ligase IIIα that plays a very important role in completion of repair process as reviewed elsewhere in detail (Hegde et al., 2008; Tell et al., 2009).
2.7.2. Redox Regulation Function of APE1

APE1 acts as redox-regulator of various TFs such as c-jun (AP-1), NF-κB, Nrf2, HIF-1α, and p53 etc., which are associated with the process of cancer development and progression. For this reason, APE1 is also known as redox effector factor-1 (Ref-1). The N-terminus of APE1 containing the nuclear localization signal (NLS) region is responsible for the redox regulation of TFs (Bhakat et al., 2009; Tell et al., 2009) [Figure 2.7]. Till date, seven Cys residues have been reported to be conserved in mammalian APE1, out of which three residues viz. Cys65, Cys93 and Cys99 are considered crucial for redox activity. Only Cys99 is solvent accessible, rest Cys65 and Cys93 are surface inaccessible because they are buried (Bhakat et al., 2009; Thakur et al., 2014). Studies have shown that Cys65 variant does not behave like WT-APE1. However, another study has challenged this concept of Cys65-mediated APE1’s redox function by using Cys-to-Ala point mutation and demonstrating that Cys64 (in humans, Cys65) is not responsible for DNA-binding activity of AP-1 (Ordway et al., 2003).

![Figure 2.7. Representation of APE1/Ref-1 protein.](image)

C-terminal region is responsible for repair function and N-terminal region is responsible for redox-function.

APE1 plays its role by acting as a transcriptional co-activator or co-repressor, directly or indirectly, by redox-dependent and redox-independent pathways. Redox-independent pathway includes post-translational modifications such as...
acetylation. By virtue of its role in regulation of various TFs, it is not surprising that it has become a target for means to treat various human pathologies, such as cancer and neurodegenerative disorders. In cancer treatment, targeting APE1 will help in restraining cancer cells from surviving chemotherapy (Luo et al., 2004), whereas, in neurodegenerative disorders, APE1’s role towards neuroprotection will be exploited. Recently, emerging evidence also supports the role of APE1 in RNA quality control (Barnes et al., 2009; Vascotto et al., 2009). So, it can be said that APE1’s regulation takes place at all the three levels; DNA, RNA and proteins. Thus, APE1 is an indispensable factor with the potential to affect cell functions at each level. Owing to this, the role of APE1 in various human pathologies need revision in the light of more detailed studies unveiling the functions of APE1.

2.7.3. APE1 and its Protein-Protein Interactions

APE1 is involved in various canonical and non-canonical protein-protein interactions. The canonical protein-protein interactions involve specific protein-protein interaction(s) in BER-pathway that defines the efficiency and fate of BER-pathway. Such interactions help the BER-pathway proteins to recruit, stimulate and mutually stabilize each other (Fan et al., 2005). For example, it has been reported that APE1 stably interacts with TDG and MutY repair protein (MYH) and thus, enhances their activity to incise damaged bases as reviewed (Luncsford et al., 2013; Thakur et al., 2015). APE1 has been observed to form complexes with DNA pol β to reproduce intermediates of DNA repair (Prasad et al., 2011). Direct interaction between XRCC1 and N-terminal region of APE1 has been found to stimulate APE1’s 3’-5’ exonuclease and 3’-dRP activities (Vidal et al., 2001). PARP1 has been also reported to compete with APE1 for AP sites (Cistulli et al., 2004). Protein-protein interactions between flap endonuclease 1 (FEN1), proliferating cell nuclear antigen (PCNA), and APE1 has been demonstrated to be involved in LP-BER-pathway (Dianova et al., 2001).

The other significant protein-protein interactions of APE1 involve proteins from other DNA transaction pathways. AP-1 is one such protein which was first
reported to get reductively activated by APE1, via redox-regulation of DNA-binding domain of c-jun and c-fos (Xanthoudakis & Curran 1992). Proteins involved in cell survival pathways and other DNA transaction pathways have been reported to interact with APE1 viz. p53, NF-κB, thioredoxin (Trx), hypoxia inducible factor 1 α (HIF-1α), early growth response-1 (Egr-1), signal transducer and activator of transcription 3 (STAT3), cAMP response element-binding protein (CREB) and tissue-specific proteins paired-box 8 and 5 (Pax-8 and Pax-5) (Bhakat et al., 2009; Tell et al., 2009). Mechanism of redox regulation takes place by regulating redox state of critical cysteine (Cys) residues of DNA-binding domain of TFs and N-terminal domain of APE1 (Bhakat et al., 2009). Cys65 and Cys93 residues in APE1 have been considered important for redox regulation (Mol et al., 2000). In addition to direct APE1-mediated reductive activation of some TFs, APE1-induced GSH or thioredoxin-mediated activation also takes place indirectly, which points out the redox chaperone role of APE1 (Ando et al., 2008).

As mentioned earlier, APE1 activates various TFs which are important for cell survival viz. c-jun, NF-κB and Nrf2. Studies have reported for the direct involvement of APE1 in reductive activation of c-jun, NF-κB and Nrf2 (Nishi et al., 2002; Shan et al., 2015; Xanthoudakis & Curran 1992; Xanthoudakis et al., 1992). Also, a study demonstrated the redox chaperone role of APE1 in NF-κB (Ando et al., 2008). Given this, it can be said that APE1 exerts both directly and indirectly various cellular biological roles via redox-regulation.

Various factors involved in cell survival signalling pathways viz. NF-κB, NOS2 and Nrf2, have been recognized as crucial players in the process of cancer initiation and progression (Chaturvedi et al., 2011; Dolcet et al., 2005). One such TF is nuclear factor NF-κB which gets activated upon genotoxic stress. ROS not only activates NF-κB, but also get itself regulated by NF-κB. Upon genotoxic stress, nuclear translocation and transcriptional activation of NF-κB takes place after release of inhibitory IkB molecules through IKK complex activation. The target genes of NF-κB activation are anti-apoptotic genes (Bcl-2, ciAPs etc.), cytokines (IL-1, IL-6, IL-8 etc.), and adhesion factors (VCAM1, ICAM-1 etc.), which
advocates the plausible central role of NF-κB in initiation and progression of cancer (Hoesel et al., 2013) [Figure 2.8].

![Figure 2.8. Activation and signalling mechanism of NF-κB and its crosstalk with other signalling processes (adapted from: Hoesel et al., 2013).](image)

NF-κB induces the activation of iNOS/NOS2. NOS2 is responsible for high level sustained production of NO, which gets oxidized to form RNS which mediates inflammatory effects (Coleman 2001). NOS2 has been also known as angiogenesis modulator. NOS2 upregulates angiogenic factors such as vascular endothelial growth factor (VEGF), VEGF-regulated kinases and mitogen-activated protein kinases, and hence, have a role in tumor promotion (Marrogi et al., 2000).

Another redox-sensitive TF reported to have involvement in the process of carcinogenesis is Nrf2. Nrf2 regulates antioxidant response element- (ARE-) mediated expression of several antioxidant, cytoprotective and metabolic enzymes (phase II detoxification enzymes). Under normal conditions, Nrf2 interacts with and, gets sequestered in cytoplasm by its inhibitor or negative regulator Kelch-like ECH-associated protein 1 (KEAP1), which when gets oxidized upon oxidative
stress releases Nrf2, mediates its translocation to the nucleus, where Nrf2 stimulates the transcription of its target genes, as mentioned earlier. Studies have found Nrf2 overexpression in various cancers including lung cancer (Ohta et al., 2008; Singh et al., 2006). Nrf2 confers pro-tumorigenic effects via increased cytoprotection, proliferation, decreased cell death and increased detoxification, in initial stages of cancer such as promotion and progression (Alexander, 2013). Increased Nrf2 expression and activity is not involved in initiation of cancer or cancer development, but once a cell acquires uncontrolled proliferative property, Nrf2 confers survival advantages such as proliferation and stress resistance. When activated upon oxidative stress in quiescent cells, Nrf2 stimulates the transcriptional activation of antioxidant, cytoprotective and detoxification enzymes to regulate cellular redox homeostasis; however, along with sustained activation of cell survival PI3K-Akt pathway, increased Nrf2 activation upon oxidative stress, stimulates the antioxidant, cytoprotective, detoxification and metabolic enzymes such as Glucose-6-phosphate dehydrogenase (G6PDH), transketolase (TKT) etc., thus promoting the metabolic activities which are advantageous for cell proliferation (Mitsuishi et al., 2012) [Figure 2.9].

Figure 2.9. Function of Nrf2 in proliferating cells (adapted from: Mitsuishi et al., 2012).
2.8. APE1 Perspectives on Lung Cancer: Expression, Subcellular Localization and Polymorphism

In view of APE1’s imperative functions, as repair and redox-regulator enzyme, it may not be unpredictable that APE1’s altered expression levels and activities have been linked with various types of cancers. Various studies have reported association between APE1 and cancer (Herring et al., 1998; Kelley et al., 2001; Wang et al., 2009). Studies have also linked APE1 with lung cancer (Wang et al., 2009; Yoo et al., 2008). Studies suggest that expression and subcellular localization of APE1 are coordinated (Tell et al., 2005). The APE1’s expression pattern and its subcellular localization differ among different cell types. Generally, APE1 is predominantly expressed in the nucleus. But in response to oxidative stress, expression within the cytoplasm has also been reported in cell types with active metabolism, associated with the cell organelles such as mitochondria and endoplasmic reticulum (Tell et al., 2005). APE1 subcellular distribution such as mixed localization or predominant nuclear or increased cytoplasmic localization has been demonstrated in various cancers (Di Maso et al., 2007; Sheng et al., 2012). A study indicated that APE1’s subcellular localization plays a significant role as a prognostic indicator in NSCLC patients (Puglisi et al., 2001).

A recent study has shown that after binding to the AP sites in chromatin, APE1 repairs AP sites via post-translational modification acetylation (AcAPE1). It was demonstrated that acetylation-mediated neutralization of the basicity of the lysine residues in the APE1’s N-terminal region induces a conformational change which stimulates the APE1’s AP endonuclease activity. It was also shown in the same study that cells tend to accumulate more AP sites in the absence of APE1 acetylation (Roychoudhury et al., 2017).

The associations between APE1 polymorphisms and cancer have been well documented by population-based studies (Chang-Claude et al., 2005; Ito et al., 2004). The possible mechanism underlying the link between APE1 mutants and cancer risk is that these single nucleotide polymorphisms (SNPs) lead to amino
acids (aa) substitutions resulting in altering the functions of APE1 and consequently producing altered DNA repair phenotype. One of the commonly found and frequent variant of APE1 is D148E (T/G, codon 148, exon 5, Asp to Glu) located in DNA repair region of APE1 gene (Illuzzi et al., 2013). It is reported to be found among different populations with high frequency and also linked to different tumors (Cao et al., 2011; Chang-Claude et al., 2005; Zhou et al., 2011). One study reported that D148E SNP affects neither the DNA binding activity nor the AP endonuclease activity (Hadi et al., 2000). However, it was found significantly associated with increased DNA damage level as measured by comet assay on peripheral blood lymphocytes (PBMCs) of healthy individuals. The mutant D148E APE1 gene is found to be associated with the response to chemotherapy and radiotherapy in non-small lung cancer (Yin et al., 2011). Some reports have suggested for the role of Asp148Glu polymorphism in lung cancer risk, whereas, some studies found no association between lung cancer risk and Asp148Glu polymorphism (De Ruyck et al., 2007; Zienolddiny et al., 2006). So far, published studies have been performed on a population of China, Japan, Germany, and Finland. However, results from population-based studies have been conflicting due to variables like heterogeneity across populations and dietary lifestyles, environmental exposures which act as effect modifiers.

Other non-synonymous APE1 genetic variants identified in human population that may play a role in carcinogenesis are L104R, R237C and D283G (Wilson et al., 2011). L104R and D283G APE1 polymorphisms have been linked with neurodegenerative disease like amyotrophic lateral sclerosis, (ALS) (Hadi et al., 2000; Illuzzi et al., 2013). R237C variant has been reported to be associated with endometrial cancer (Pieretti et al., 2001). The fact that some polymorphisms occur in the N-terminal region of APE1, which are responsible for post-translational modifications (PTMs) and protein-protein interactions, it is suggested that these APE1 variants may indirectly affect the protein functions and consequently, cellular outcomes (Bhakat et al., 2003). A very recent study demonstrated that non-synonymous APE1 variants viz. D148E, L104R, R237C and D283G present in the human population may increase cancer risk by virtue of
their altered endonuclease activity or ability to interact with BER-pathway proteins (Lirussi et al., 2016).

While these variations are shown to associate with cancer risk, less is known how these mutations actually affect the normal function of APE1. Therefore, investigation of these variant’s biochemical and functional properties is of greater importance in order to find their association with lung cancer incidence. Study addressing what makes these mutant proteins susceptible for higher cancer risk is not conducted so far at the molecular and biochemical level.

2.9. DNA Damage Responses (DDRs): A Plethora of Cell Signalling Events

DDR is a cumulative term for many cell signalling pathways and enzymatic activities, stimulated by oxidative stress or DNA damage. DDGs comprises of events that lead to DNA repair and replication regulation, cell death mechanisms (such as apoptosis, necrosis etc.) or cell-cycle arrest. We have already discussed the DNA repair regulation and cell survival signalling mechanisms in the earlier sections. Tumorigenesis is a function of uncontrolled cell proliferation which results due to disorganization of cell cycle via dysregulated cell cycle mechanisms that control cell cycle progression and checkpoint signalling. Cell proliferation depends upon three phases of cell cycle viz. G0/G1, S and G2/M, and is inhibited by the cell cycle arrest. However, these events are generally conserved due to the cell cycle factors viz. extracellular growth factors, cyclins, cyclin dependent kinases (Cdkks) and Cdk inhibitors (CKI) (Golias et al., 2004). Techniques like flow cytometry helps to quantify and determine cell cycle and cell proliferation. Cell cycle, cell survival and proliferation is mediated by a factor named proliferative cell nuclear antigen (PCNA) those expression indicates the rate of DNA synthesis during the cell cycle, thus acts as a reliable marker for tumor cell proliferation (Yang and Zou, 2009).
Not only cell proliferation, but also cell death determines the cell number. DDRs affect the downstream cell fate decisions such as cell death. Mode of cell death depends upon the extent of oxidative damage, and ROS induces apoptosis. Apoptosis, an orderly and complex cell deletion, is a genetically-controlled removal of damaged cells. Escaping apoptosis is one of the major factors involved in the process of carcinogenesis.

Figure 2.10. Schematic representation of mechanism of apoptosis (adapted from: Ghatage et al., 2012).

Apoptosis can be categorized into two pathways, caspase-dependent and caspase-independent pathways (Figure 2.10). The former one comprises of specific proteases known as caspases, whose activation depends upon proteolytic cleavage or auto-activation; whereas, the latter one comprises of mitochondrial oxidoreductase apoptosis inducing factor (AIF). Capase-9 and caspase-3 act as initiator and effector caspases, respectively, when induced by ROS or cytotoxic agents or pro-apoptotic signals (Mazumder et al., 2008; Thornberry et al., 1998). In caspase-independent pathway, the factor involved is AIF, whose nuclear translocation is lethal for the cell (Daugas et al., 2000). Other apoptosis-related
factors are Bcl-2 and Bax. Bcl-2 prevents apoptosis and its over-expression has been reported in cancer (Amundson et al., 2000). However, Bax is a pro-apoptotic factor. Defect in apoptosis contributes to the process of carcinogenesis by helping the cancer cells to survive and by allowing them to accumulate genetic alterations that may further lead to dysregulation of cell proliferation (Reed 1999). It has been shown that OPPs CP confers its genotoxic effects by inducing apoptosis in human HeLa and HEK293 cells (Li et al., 2015).

In our study, considering the relevant literature, we are hypothesizing that CP and MCP causes: (i) increased cellular oxidative stress and oxidative DNA base damage, which (ii) triggers alterations in BER-pathway and cell survival signalling pathways; and (iii) modulates APE1’s subcellular localization and redox-regulation of TFs, that may ultimately lead to increased risk for lung cancer progression.