GENE EXPRESSION OF DNA REPAIR PROTEINS IN COLORECTAL CANCER AND MEDULLOBLASTOMA

PhD Thesis

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DECLARATION

I certify that this thesis, which I submit to the University of Salford in partial fulfilment of the requirements for a Degree of Doctor of Philosophy, is a presentation of my own research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. No content of this thesis has been submitted, in part or as a whole, for a higher degree at this or any other university.

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ABSTRACT

DNA repair plays a critical role in maintaining the integrity of the genome and the dysregulation of key DNA repair genes has been implicated in the development, progression and chemotherapeutic resistance of different cancer types. Consequently, many studies have made attempts to identify and quantify the expression of various DNA repair genes and their products in different cancers. It is on a similar note that this research was conceived, to evaluate the expression patterns of the base excision repair genes *Neil1*, *Neil2*, *Neil3*, *Ogg1*, and *Nthl1,* the nucleotide excision repair gene *Ercc1* and the mismatch repair gene *Mlh1* in colorectal cancer (CRC) tumours and matched normal colon tissue. The project was then extended to analyse a further sixteen colon samples that focused on *Neil3, Nthl1* and *Ercc1,* that encode DNA repair proteins that have been implicated in chemotherapy resitance mechanisms. To learn more about mechanisms of genotoxic agent resistance, *Neil3* and *Ercc1* were analysed at the transcriptome and proteome level in DAOY medulloblastoma cells and cisplatin – resistant DAOY cells. Additionally, attempts were made to generate mesothelioma-derived cancer stem cells and preliminary gene expression analyses were undertaken on human embryonic stem cells. Thus, RNA was extracted, complementary DNA synthesized, and RT-PCR performed. Gene expression levels were determined by quantitative PCR using the Sybr green method and analysed using the comparative Ct method and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) as the standard. Of the matched samples investigated, 75% showed increased expression of one or more of the DNA repair genes analysed, however, there was no clear pattern of expression and a wide range of expression levels observed for individual genes in both normal and tumour tissues. For example, the gene encoding the DNA glycosylase Nthl1 was the most frequently highly expressed in both normal and tumour samples with about 75% showing high expression of the *Nthl1* gene with expression levels ranging from 3.3 to over 1400-fold higher than *Gapdh*. DAOY cells were grown in cisplatin and gene expression of *Neil3* and *Ercc1* analysed in the resulting cisplatin - resistant cells. Results indicated that the expression of both these genes may be increased in the resistant cell line and that NEIL3 protein was also increased. Cancer stem cells were derived from parental mesothelioma cells but were still too small a fraction of the cell population to be analysed by these methods. The expression of *Gapdh*, *Neil3* and *Ercc1* was determined in a series of preliminary experiments on human embryonic stem cells.

ABBREVIATIONS

8-oxoG:	8-oxoguanine
AP:	apurinic/apyrimidinic
APC:	adenomatous polyposis coli
APE1:	AP-endonuclease-1
BER:	base excision repair
BLAST:	basic local alignment search tool
bp:	base pair
CAK:	CDK-activating kinase
cDNA:	complementary DNA
CDK:	cyclin dependent kinase
CETN2:	centrin 2
CIMP:	CpG island methylator phenotype
CRC:	colorectal cancer
CSA & B:	Cockayne syndrome complementation group A and B
CSC:	Cancer stem cells
Ct:	threshold cycle
DDR:	DNA damage response
DNA:	deoxyribonucleic acid
DSB:	double-strand break
EDTA:	ethylenediaminetetraacetic acid
EGFR:	epidermal growth factor receptor
EPCAM:	epithelial cell adhesion molecule
ERCC1:	excision repair cross-complementation group 1
ES:	embryonic stem
FBS:	foetal bovine serum
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
GG-NER:	global genome nucleotide excision repair
hMLH1:	human MutL homolog
hNEIL3:	human NEIL3
HNPCC:	hereditary nonpolyposis colorectal cancer
ICL:	interstrand crosslink
mCRC:	metastatic colorectal cancer

MLH1:	MutL homolog 1
MMR:	mismatch repair
MSI:	microsatellite instability
MSI-H:	high-level microsatellite instability
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUTYH:	human mutY DNA glycosylase
NCBI:	National Center for Biotechnology Information,
Nei:	endonuclease VIII
NEIL1/2/3:	endonuclease VIII-like 1/2/3
NER:	nucleotide excision repair
NK:	natural killer
NTHL1:	endonuclease III homolog
NUDT1:	nudix hydroxylase 1
OGG1:	8-oxoguanine DNA glycosylase
PARP-1:	poly (ADP-ribose)polymerase 1
PCR:	polymerase chain reaction
PD-1:	programmed cell death 1
PD-L1:	programmed cell death ligand 1
PMS2:	postmeiotic segregation increase 2
PNK:	polynucleotide kinase
POL:	polymerase
Polβ:	DNA polymerase β
Polδ:	DNA polymerase δ
Polε:	DNA polymerase ε
PUA:	polyunsaturated aldehyde,
qPCR:	quantitative PCR
RNA:	ribonucleic acid
RNAPII:	RNA polymerase II
ROS:	reactive oxygen species
RPA:	replication protein A
RT-PCR:	reverse-transcription PCR
siRNA:	small interfering RNA
SSB:	single-strand break
TFIIH:	transcription initiation factor IIH

TC-NER:	transcription-coupled nucleotide excision repair
VEGF:	vascular endothelial growth factor
UV:	ultraviolet
USP7:	ubiquitin specific-processing protease 7
UVSSA:	UV-stimulated scaffold protein A,
XP:	xeroderma pigmentosum
XRCC1:	X-ray repair cross complementing protein 1

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Chapter 1

1.1. General introduction

In a multicellular organism, the constituent cells undergo cell division as a reproductive mechanism, resulting in the formation of well organised and collaborative entities known as tissues. These cells are subject to a strictly regulated form of collaboration, characterized by selflessness and apparently devoid of competition. Hence, at any point in time, each cell is either, resting, growing, differentiating, dividing, or dying, as needed; and for the benefit of the other cells that made up the organism. Thus, a form of social control network oversees cellular behaviours, including cell-to-cell communication, such as relay, receipt and interpretation of sets of intracellular, as well as extracellular signals in an elaborate manner (Tlsty and Coussens, 2006). Disregard to the sense of community or any attempt to evade the cell cycle system that controls the timing of cell division by a cell, or group of cells, could pose serious problems for the organism. Additionally, molecular disturbances such as genetic mutations, may selectively confer undue advantage on a given cell or group of cells that may result in the cells growing more rapidly, differentiating and dividing more rapidly and evading death signals (Hanahan & Weinberg, 2000). Consequently, the selectively advantaged cells may become the progenitor of a vigorously growing mutant clone, initiating selfishness and encouraging competition. Through repeated mutations, the mutant clones could engage in serious competition, and natural selection could usher in dangerous cell population, and subsequent tumour development. This is known as the mutator phenotype and is probably the main theory of cancer development (Loeb, 2001).

From the foregoing, cancers could be defined as heterogeneous multicellular assemblages, made up of cells of different origins, interacting with each other and the extracellular signals in complex fashions that encourage the dysfunction of cell cycle control. Essentially, cancer is a disease that arises from the disruption of cellular and genetic functions. Although, cancer development is a multistep process, it ultimately results from changes in the genome, in which intrinsic cellular functions including apoptosis, cell differentiation, metabolism, cell cycle check-point control and cell adhesion;-immunological response, as well as status of the vasculature are affected (Hanahan & Weinberg, 2011). In fact, reports from human cancer studies and animal models have clearly demonstrated that during the process of tumorigenesis, a range of genetic changes take place, each conferring a given type a

specialized competitive advantage, resulting in the successive and progressive change of normal human cells into cancer cells (Loeb, 2001). The heterogeneous nature of cancer accounts for the prevalence of many different types, each displaying different combinations of characteristic cellular and genetic changes. Even within a single type of cancer, heterogeneity and tumour subsets that are uniquely defined can be identified (De Sousa *et al*., 2013).

The increasing incidence of cancers is alarming, and cancer has become one of the major factors responsible for disease-related deaths globally (Kanavos, 2006). This high death rate could be attributed to the inherent abilities of cancer cells to resist chemotherapy, metastatic ability and high degree of immortality with concomitant recurrence capacity. Current treatments include conventional therapies such as surgery, radiotherapy and chemotherapy (Banerjee *et al*., 2017; Huang *et al*., 2017). In the past decade, immuno-gene therapy has been introduced as the fourth treatment modality, due to its robustness and promises (Parney & Chang, 2003). Unfortunately, irrespective of the availability of these variety of options for the treatment of cancer, it is still daunting to define an effective treatment regimen to cure patients especially those whose cancers have metastized to distant organs. This can be linked to resistance to therapy and relapse associated with most cancers (Zahreddine & Borden, 2013).

Nevertheless, studies that target the discovery of molecular pathways that promote tumour growth have improved our understanding of this disease and has revolutionized the way cancer cells can be targeted (Sinicrope *et al*., 2016). Today, several mechanisms that contribute to the development of cancer have been elucidated, indicating a difference in the dynamics and characteristics of normal cells compared to cancer cells (Hanahan & Weinberg, 2011; Turkson, 2017). Whereas normal tissues maintain tissue integrity and function by balancing the signals that are involved in cell growth and cell division, cancer cells exhibit both dysregulated proliferative signalling and replicative immortality, thus giving them a growth and survival advantage (Hanahan and Weinberg, 2000, 2011). Unlike cells under normal physiological conditions, cancer cells employ several mechanisms to achieve uncontrolled proliferation and DNA replication, most of which involve a subset of the regulatory instructions transmitted by an activated receptor acting as an oncogene (Vogelstein & Kinzler, 2014). Cancer cells up-regulate growth factor ligands and stimulate normal cells to release factors that can support cancer growth, up-regulate receptor proteins

that render cells hyperresponsive to growth factor ligands and structurally alter receptor molecules that facilitate ligand-independent cell division (Cheng *et al*., 2008).

In the last decades, evaluation of several human tumours has implicated somatic and germline mutations as additional downstream pathways by which cancer cells sustain immortality (Stratton *et al*., 2009; Davies and Samuels, 2010). To contribute to the global quest to conquer cancer, this PhD project sought to investigate the expression patterns of selected DNA repair genes in different cancer types, including colorectal cancer (CRC) and medulloblastoma. However, the thesis begins with a brief review of DNA damage, some of which is pre-mutagenic and therefore thought to be a prerequisite for carcinogenesis. This is followed by a review of DNA repair mechanisms that are found in mammalian cells. Many of the proteins involved in DNA repair are tumour suppressor genes and individuals lacking particular DNA repair functions are often more cancer prone. Next, a synopsis of each of the cancer types employed in this research is presented, followed by an introduction to stem cells. While preliminary experiments presented here were carried out on human embryonic stem (ES) cells, the concept of cancer stem cells is the focus of much research to find more effective treatments (Dawood *et al.,* 2014).

1.2. DNA Damage

DNA is the active molecule of an organism and serves as a repository of genetic information. Since DNA plays a major role in replication and transcription, its integrity and stability at any time is a prerequisite for evolutionary fitness and for the health of the individual organism. An individual cell can receive up to one million DNA alterations daily. Some of these changes are spontaneous, such as the loss of a purine base from the double-stranded DNA molecule (depurination) and deamination, where the amino group is lost from cytosine and adenine (see Pierce, 2017 for review). However, other DNA damage results from exposure to endogenous and environmental genotoxic agents that can cause a multitude of chemical alterations to the DNA molecule. Thus, the resultant effect on the DNA molecule can range from many different forms of DNA base damage, bulky adducts attached to bases, single- and double-strand breaks and intra-strand and inter-strand DNA cross-links. Some of these DNA lesions will be pre-mutagenic, leading to permanent changes in the genomic DNA sequence, while others will be toxic and cause cell death at the next round of DNA replication due to collapse of the replication fork (Friedberg *et al.,* 2005).

While the inherent lability of the DNA molecule can be beneficial, such as in the process of natural selection in evolution, it can also be deleterious to the cell and organism, resulting in altered pathology. Thus, deleterious DNA changes can cause genomic instability and challenge the stability and integrity of the organism (Pierce, 2017).

Several endogenous and exogenous agents responsible for insults on DNA have been described. The most common exogenous or environmental DNA-damaging agents are ultraviolet (UV) radiation, chemical agents and chemotherapeutic drugs. Some of these will result in disease conditions such as cancer. For instance, it is well known that skin cancers are the result of exposure to UV light (Seebode *et al*., 2016) and lung cancer is largely a consequence of cigarette smoke inhalation (Doll and Bradford-Hill, 1950). Related to this, it has also been reported that colon epithelial cells are also prone to exogenous mutagens, many resulting from normal metabolism of ingested material (Greenman *et al*., 2007).

Apart from the afore-mentioned exogenous sources of DNA damage, cellular DNA is also under constant attack from endogenous agents such as reactive oxygen species (ROS) produced as a consequence of normal aerobic metabolism or resulting from inflammatory cytokines that leads to a state of oxidative stress (Federico *et al.,* 2007). However, another source of DNA damage comes from the replicative DNA polymerases. During DNA replication, the enzyme DNA polymerase adds a nucleotide to the strand of the DNA (Pierce, 2017). However, the DNA polymerase can add incorrect nucleotides during DNA replication and although the main replicative DNA polymerases have a 3′ - 5′ "proofreading" exonunclease activity and can recognize and correct many of these errors, inefficient corrections could lead to mutations that can in turn result in disease conditions such as cancer (Pierce, 2017).

As mentioned, endogenous agents such as ROS play an important role in reactions resulting in DNA damage (Durand & Storz, 2017). The key ROS that are of importance include the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical, ($^{\bullet}OH$) (Beckman & Ames, 1997; Hazra *et al*., 2007). Although, the physiological role of ROS in cells is evident in the maintenance of homoeostasis (Hancock *et al*., 2001), damage to cellular macromolecules such as lipids, protein, and DNA may ensue when cellular production overwhelms its antioxidant capacity (Friedberg *et al.,* 2005). Such elevated ROS levels leads to increased levels of DNA damage, causing mutations and ultimately genetic instability and pathological conditions. It has been reported that several cancer types

including, pancreatic (Vaquero *et al*., 2004) prostate (Kumar *et al*., 2008) breast (Hecht *et al*., 2016) and colon (Acharya *et al*., 2010) show an increased level of ROS. Besides its role in cancer, ROS has been implicated in several human diseases including the ageing process, cardiovascular disease, diabetes, sterility, autoimmune diseases and neurological diseases (Sedelnikova *et al*., 2010). The mechanism of action of ROS is by induction of several covalent modifications to DNA including in single base lesions, DNA strand breaks, intra- and inter-strand cross-links.

An example of oxidative DNA damage caused by ROS is 8-oxo-7,8-dihydroguanine (8-oxoG), which although not toxic, is highly mutagenic (Suzuki & Kamiya, 2016). 8-oxoG is known to mismatch with adenine and by so doing results in 'GC' to 'TA' transversions. Several independent reports have confirmed the involvement of this mis-pairing in somatic mutations in lung, CRC, breast, gastric, and ovarian cancer (Fortini *et al*., 2003). Besides causing mutation, ROS is involved in activating transcription factors such as NF-kB, activator protein-1 (AP-1) and hypoxia inducible factor-1 (HIF-1α), whose role in cancer cell growth and survival, angiogenesis, invasion, and metastasis have been well established (Gupta *et al*., 2012).

1.3. DNA Repair Mechanisms

As mentioned earlier, cells are constantly under attack by agents known to generate genomic instability, resulting in structural damage to the DNA molecule (Fortini *et al*., 2003). Mechanistically, living organisms have evolved a plethora of molecular mechanisms to detect and repair the various types of damage that can occur to DNA, irrespective of the source. This they do by employing one of five distinct DNA repair mechanisms that play a critical role in maintaining the integrity of the genome (Friedberg *et al.,* 2005). This, however, does not occur in isolation but is linked to cell cycle regulation; before the replication of DNA and the eventual division of the cell can take place, cell cycle checkpoint mechanisms ensure that a cell's DNA is intact. Therefore, if DNA is damaged, the cell has the ability to stop the cell cycle in G1, remove the lesion and restore the original base sequence, before passing through the cell cycle restriction point and commencing DNA replication, thus maintaining genetic stability (Pierce, 2017). Five DNA repair mechanisms have been described (Friedberg *et al.,* 2005) and these are: (i) mismatch repair (MMR), (ii)

base excision repair (BER), (iii) nucleotide excision repair (NER), (iv) Non-homologous end-joining (NHEJ) and, (v) homologous recombination repair (HRR). Recently, Abbotts *et al.,* (2014) reported that loss or truncation of the efficiency of one or more of the DNA repair pathways can accelerate the accumulation of additional mutations by up to 1000-fold (Abbotts *et al*., 2014). Thus, unrepaired DNA damage is the major source of potentially mutagenic lesions that drive carcinogenesis (Friedberg *et al.,* 2005).

Loss of any one of these pathways can result in serious consequences for the organism. For example, individuals lacking NER are hypersensitive to sunlight and exhibit either accelerated ageing or cancer predisposition (Friedberg *et al.,* 2005). Similarly, several inherited colorectal cancer (CRC) syndromes are associated with genetic defects in both MMR and BER (Weren *et al*., 2015). On the other hand, high expression of DNA repair proteins in cancer cells can confer resistance to certain chemotherapeutic agents used in cancer treatment, such as the platinum-based compounds and alkylating agents.Thus, it has been reported that the excision repair cross-complementation group 1 (*Ercc1*) gene is upregulated in colon cancer cells treated with oxaliplatin, and that the small interfering RNA (siRNA) knockdown of *Ercc1* in these cells sensitises them to the chemotherapeutic effects of oxaliplatin (Seethram *et al*., 2010). The BER DNA glycosylase NEIL3 is also highly expressed in most cancer cell lines and metastatic melanoma compared to normal tissue cells, where its expression is generally restricted to rapidly dividing cells in the thymus and testes (Section 1.3.2; Kauffmann *et al.,* 2008; Hildrestrand *et al.,* 2009). As more biochemical information becomes available on the activity of this enzyme (Martin *et al.,* 2017; Albelazi *et al.,* 2019), it is of increasing interest to determine the levels of expression of *Neil3* in tumour samples and the likely effect this may have on conferring resistance to genotoxic chemotherapeutic agents.

The next three sections review in more detail the mechanisms of MMR, BER and NER. As the double-strand break rejoing pathways (NHEJ and HRR) are outwith the aims of the project, these are not covered in the following sections.

1.3.1. Mismatch Repair

DNA mismatch repair proteins function by recognizing base mismatches and other lesions (*e.g.* nucleotide insertion or deletion) that result from DNA polymerase slippage during DNA replication (Lodish, 2004). As a result, it is sometimes called, post-replication repair and if it fails, the errors induced during DNA replication are fixed in the genome (mutation). As mentioned previously, during DNA replication the enzyme DNA polymerase adds a nucleotide to the growing strand of DNA at the replication fork. MMR is involved in the post-replicative repair of the errors made by DNA polymerases that have escaped proofreading (Umar & Kunkel, 1996). The MMR system will recognise base-base mismatches, insertions or deletions occurring in the double-stranded DNA (Harfe & Jinks-Robertson, 2000). Following this recognition, MMR proteins then initiate the process of resection and specifically degrade the affected region of the newly synthesised strand. A DNA polymerase will then correctly resynthesise the daughter strand of the DNA in a template dependent manner (Lodish, 2004).

Several genes are important in the normal function of MMR including, in human cells: MutS homolog 2 and 6 (*Msh2* and *Msh6*), MutL homolog 1 and 3 (*Mlh1* and *Mlh3*) and post-meiotic segregation increased 1 and 2 (*Pms1* and *Pms2*: Harfe & Jinks-Robertson, 2000). A mutation in any of these genes can result in microsatellite instability (MSI) and predisposes the individual to certain cancers, including colorectal and ovarian cancers (Abbotts *et al*., 2014).

Thus, germline mutations in *Mlh1*, *Msh2* or *Pms2*, or even deletions in the epithelial cell adhesion molecule (*Epcam*) gene that cause allele-specific *Msh2* inactivation has been linked to hereditary nonpolyposis colorectal cancers (HNPCC; Lynch syndrome) that results in early onset CRC. It has been reported that inactivating mutations in any of the MMR genes can be found in up to 70% of HNPCC and that, of these, over 90% occur in the *hMsh2* or *hMlh1* genes and display a high level microsatellite instability (MSI-H) phenotype (Wheeler *et al*., 2000).

The second major mutation of MMR genes found in human cells are the somatic mutations occurring because of promoter methylation of *Mlh1*. Often this methylation can be seen in the context of CpG island methylator phenotype (CIMP). In sporadic cancer, hyper-methylation of the promoter region is the cause of *Mlh1* inactivation especially in CRC with MSI-H (Haydon and Jass, 2002). First-degree relatives of CRC patients with hyper-

methylation of the promoter region are at a higher risk (up to 60%) of developing CRC. In order to reverse *Mlh1*-methylation in colon cancer, the group of Fujita and colleagues (2007) demonstrated that the de-methylating agent 5-aza-2′-deoxycytidine (5-aza-dC) can induce *Mlh1* expression and sensitise cancer cells to 5-fluorouracil (Fujita *et al.*, 2007). Consequently, the expression profile of *Mlh1* will be analysed in the course of this project, particularly in solid colon cancer tumour tissues, with the view to ascertaining the expression pattern at the transcriptional level.

1.3.2. Base Excision Repair

Amongst the DNA repair mechanisms, BER is the most versatile repair mechanism and is involved in repairing the majority of DNA damage arising from both endogenous and exogenous sources (Svilar *et al.*, 2011). These include single-strand breaks (SSB), depurination and deamination, alkylation and oxidation derived base damage. DNA base damage resulting from exposure to environmental factors, ROS as well as alkylation-induced damage especially those from alkylating agents including chemotherapy and radiotherapy are also repaired by BER (Maynard *et al.,* 2009; Whitaker *et al.,* 2017). The broad functionality of BER results mainly from a group of enzymes called DNA glycosylases that recognise and excise a plethora of chemically modified bases from DNA (Mullins *et al.,* 2019). Thus, DNA glycosylases recognize and initiate BER by removing an overlapping subset of damaged bases, leaving an abasic site that is further processed by short-patch BER or long-patch BER that uses different proteins to complete the repair process (Whitaker *et al.,* 2017). Additionally, poly (ADP-ribose) polymerase 1 (PARP-1) and PARP-2 are known to facilitate BER by binding to DNA ends at SSBs and synthesizing poly (ADP-ribose) polymers on acceptor proteins and the DNA itself (Dantzer *et al.,* 1999; Talhaoui *et al.,* 2016). The poly(ADP-ribose) destabilizes the nucleosome structure allowing BER proteins access to the damage site. The role of BER in protecting the colorectal tissue against oxidative DNA damage, caused by high levels of oxygen radicals either generated by bacteria or dietary carcinogens cannot be overemphasized. Steps involved in BER are described in Figure 1.1.

DNA glycosylases are important enzymes in BER (Mullins *et al.,* 2019). These enzymes have been well studied and eleven different proteins have been identified in mammalian cells (Jacobs & Schär, 2012). These eleven enzymes can be subdivided into four structurally

distincy superfamilies, (i) the uracil DNA glycosylases (UDG), (ii) the helix-hairpin-helix DNA glycosylases (HhH), (iii) the alkylpurine DNA glycosylases (APNG or MPG) and, (iv) the endonuclease VIII – like DNA glycosylases (NEIL). These enzymes can be further divided into two classes, depending on whether they are monofunctional (UDG and APNG/MPG), or bi-functional (HhH and NEIL) (Jacobs & Schär, 2012). The monofunctional DNA glycosylases (UDG and APNG/MPG) have only DNA glycosylase activity, catalysing the breakage of the glycosylic bond between the deoxyribose sugar and the damaged base, while bifunctional DNA glycosylases also have an associated apurinic/apyrimidinic (AP) lyase activity (Jacobs & Schär, 2012). The five bifunctional DNA glycosylases in mammalian cells, 8-oxoguanine DNA glycosylase (Ogg1), endonuclease III homolog (Nthl1), and the three endonuclease VIII paralogs (NEIL1, NEIL2 and NEIL3) all recognise and excise oxidised bases from either double-stranded or single-stranded DNA (Jacobs & Schär, 2012).

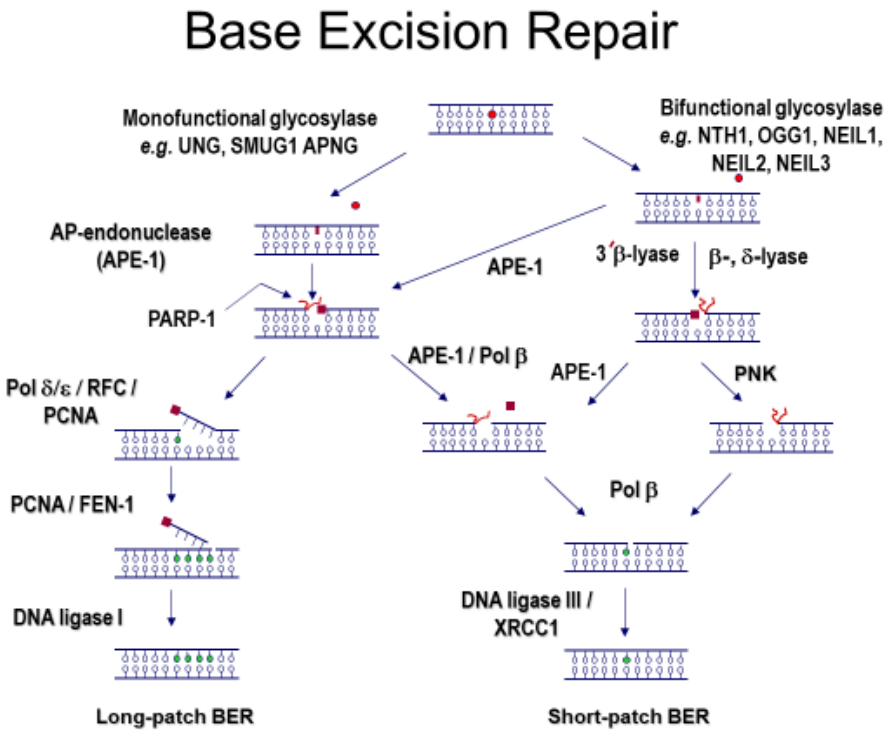


Figure 1.1. A current model for BER in mammalian cells. (Elder, unpublished).

The DNA glycosylases NEIL1, NEIL2 and NEIL3 are mammalian homologs of the *Escherichia coli* Nei protein and have been shown to excise oxidised purine and pyrimidine bases from both single- and double-stranded DNA (Liu *et al.,* 2013; Albelazi *et al.,* 2019).

Amongst these enzymes, NEIL3 is the largest, uniquely containing a long C-terminal region ending with tandem GRF-zinc finger domains (Albelazi *et al.,* 2019). Both NEIL1 and NEIL3 are reported to be cell cycle regulated, with expression peaking in S-phase and S/G2-phase respectively, while NEIL2 is constitutively expressed throughout the different stages of cell cycle (Neurauter *et al.,* 2012; Hazra & Mitra, 2006; Chakraborty *et al.,* 2015). Thus, while evidence from biochemical studies and knockout mice support the idea of an involvement of NEIL2 in transcription-coupled BER (Chakraborty *et al.,* 2015), recent biochemical evidence reinforces previous reports supporting the involvement of Neil1 and NEIL3 at the replication fork (Albelazi *et al.,* 2019; Neurauter *et al.,* 2012). Furthermore, NEIL3 also shows a unique restricted expression pattern in normal cells, being expressed only in highly dividing cells such as those in the developing brain, the thymus and testes (Hildrestrand *et al.,* 2009; Morland *et al.,* 2002). However, high levels of NEIL3 have been observed in metastatic melanoma (Kauffmann *et al.,* 2008) and in cancer cells generally (Hildrestrand *et al.,* 2009; Duweb, 2015). Recently, it was demonstrated that abnormal expression of the *Neil* DNA glycosylase genes is associated with somatic mutation in several human cancers (Shinmura *et al*., 2016).

An ongoing study in our laboratory reported that *Neil3* is highly expressed in the CRC cell line HCT116. However, following siRNA treatment, this expression can be substantially reduced. Further knockdown of *Neil3* can sensitize tumour cells to oxaliplatin treatment (Taylor *et al*., 2015). These results reveal a potentially novel activity for *Neil3* and indicate that it could be a major resistance mechanism to certain chemotherapeutic DNA damaging agents in solid tumours. Consequently, it is one of the objectives of this thesis, to investigate the expression profiles of *Neil3* gene in solid tumours derived from different colorectal cancer patients.

Besides the DNA glycosylases, mutations in the BER genes are associated with several cancer types. Moreover, the observation that mutations in the gene coding for MUTYH, a DNA glycosylase that releases adenine base paired with 8-oxoG predispose to CRC has provided strong evidence that dysregulation of the BER pathway contributes to disease susceptibility (Hazra *et al*., 2007). Furthermore, a report by Weren *et al*. (2015) showed that a germline homozygous mutation in the *Nthl1* gene that codes for a DNA glycosylase that removes oxidized pyrimidines causes adenomatous polyposis and therefore, predisposes to colorectal cancer (Weren *et al.,* 2015). More recently, Grolleman *et al.,* (2019) have reported that biallelic germline mutations in the *Nthl1* gene predispose carriers to tumours at multiple

sites, again reinforcing the importance of unrepaired oxidative base damage in carcinogenesis and of the DNA glycosylases and BER in maintaining genetic integrity.

1.3.3. Nucleotide Excision Repair.

Mammalian nucleotide excision repair (NER) is a constitutive DNA repair mechanism and its impairment can result in several disease conditions including cancer and premature ageing (Marteijn *et al*., 2014). It is usually involved in the repair of DNA lesions usually bulky adducts that destroy the normal double-helical conformation of duplex DNA, irrespective of whether the insult is induced by endogenous or exogenous agents (Friedberg *et al.,* 2005). Bulky adducts are products of different DNA damaging agents including UV radiation and chemicals including alkylating agents. It has been extensively documented that exposing DNA to UV radiation typically results in cyclobutane pyrimidine dimers (CPD) and pyrimidine-(6,4)-pyrimidone products (6-4PP) that are known to be helix distorting lesions (Rastogi *et al*., 2010). When DNA is exposed to chemicals or alkylating agents such as polycyclic aromatic hydrocarbons that are common in cigarette smoke or charcoaled meat, bulky adducts can also be formed (Melis *et al*., 2013). When bulky alterations happen, it typically arrests polymerase progression during DNA replication and transcription resulting in a damaged replication fork or stalled transcription bubble (Gillet and Scharer, 2006). Our understanding of the cellular mechanisms of NER and its relationship with several cellular processes during DNA repair process indicates that NER employs four mechanisms to repair damaged DNA: (i) NER recognizes the extent and location of the damage, (ii) Upon this recognition, NER incises both ends of the damaged strand and removes an oligonucleotide containing the damaged nucleotides, (iii) This is followed by the synthesis of a new DNA strand to fill the gap and restore the DNA duplex that is devoid of damage, and (iv) ligation by DNA ligase to seal the nick at the end of the newly synthesized DNA strand (Pierce, 2017). Functionally, NER can be divided into two related pathways; global genome repair (GG-NER) and transcription-coupled repair (TC-NER). These pathways are known to occur in a divergent manner but can proceed along the same part when they have recognized the damage on the DNA (Melis *et al*., 2013). They both involve complex mechanisms to initiate the repair response, adopting several reaction-reversal steps before incision (Luijsterburg *et al*., 2010). Such a process is necessary to avoid undesirable and irreversible DNA modification and to ensure re-start of transcription.

GC-NER and TC-NER function in a coordinated manner, involving about thirty proteins, to achieve efficient DNA repair (Figure 1.2). The first step in GG-NER is to scan the entire genome for helix distortions; this is done through the recruitment of several damage sensor proteins (Pierce, 2017). In damage caused by UV radiation, recruitment of xeroderma pigmentosum group C protein (XPC) results in a complex with the human homolog of yeast RAD23 (XPC–RAD23B) and centrin 2 (CETN2) (Masutani *et al*., 1994). The principal role of XPC-RAD23B-CETN2 complex is to probe for distorting lesions and to recognize the structural damage in DNA by the activities of the ultraviolet (UV) radiation DNA damage binding protein complex (UV-DDB). Following recognition of this structural damage, XPC will bind to strand opposite to the lesion resulting in the dissociation of RAD23B (Schärer, 2007).

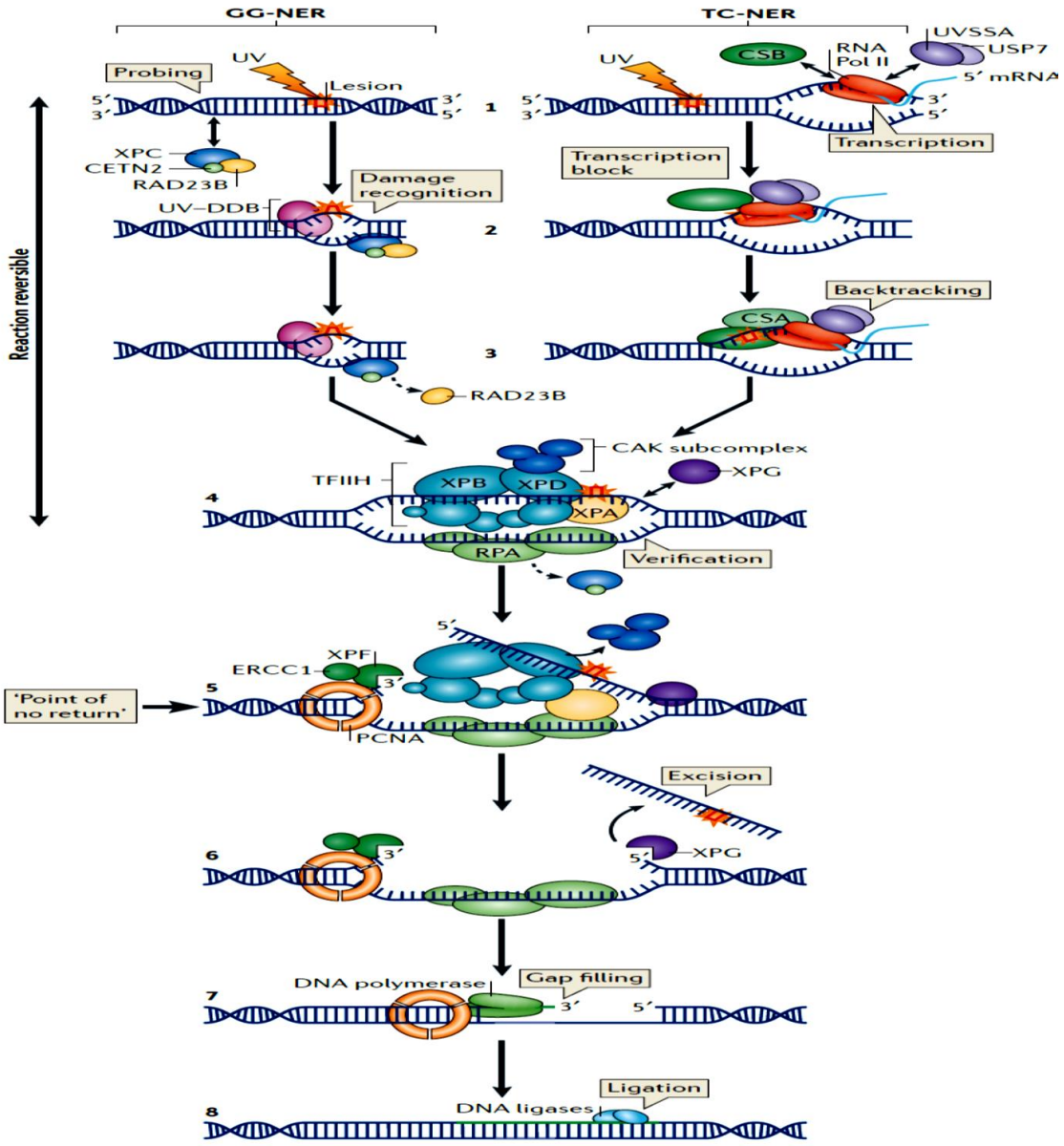


Figure 1.2. A representation of nucleotide excision repair (de Laat et al., 1999)

In contrast to GG-NER where damage is recognized directly, recognition of damage in TC-NER takes an indirect approach. During transcription elongation, the stalling of RNA polymerase II is an indication that there is a lesion in the DNA. When this happens, NER recruits several proteins to remove the lesions. These include UV-stimulated scaffold protein A and ubiquitin-specific-processing protease 7 (Schwertman *et al.*, 2012) and Cockayne syndrome complementation group A (CSA) and B (CSB). The purpose of recruitment of these proteins is to promote DNA repair as well as restart of the transcription elongation process. While CSA is implicated in the elongation process itself, CSB is known for its role

in the displacement of the stalled RNA polymerase via a transient interaction (Kamiuchi *et al*., 2002). Upon stalling at a lesion, it has been documented that the affinity of CSB for RNA polymerase II increases, leading to the formation of CSA-CSB complex. The formed complex is then involved in the reversal of the translocation (backtracking) of RNA polymerase II, thus rendering the DNA lesion accessible for repair. After this step, the GG-NER and the TC-NER converge to continue the repair process of the damaged DNA (Figure 1.2; de Laat et al., 1999).

The dissociation of RAD23B in the GG-NER mechanism and the backtracking step in the TC-NER mechanism will trigger the deployment of the transcription factor II H (TFIIH) complex, that is known to have helicase activities. The presence of two TFIIH basal helicase subunits including XPB and XPD results in the opening of the DNA duplex site where damage has occurred. However, if XPD fails to detect any damage, the DNA repair process can be aborted. This is because the major role of XPD is for damage verification. If there is successful verification of damage by XPD, the helicase activities will proceed successfully. This process will result in a bubble formation, allowing the engagement of XPA and RPA (replication protein A), as well as the assembly of the complexes necessary to initiate incision (Compe & Egly, 2012).

The XPF-*ERCC1* complex is a structure activated endonuclease that incises the DNA strand on the 5′ side of the helix distorting lesion. Similarly, XPG performs a similar function 3′ to the lesion (Figure 1.2). This step leaves a single strand gap of 22-30 nucleotides and it has been suggested that this step is necessary in triggering, a DNA damage signalling reaction (Marteijn *et al*., 2014).

In cancer, several studies have shown that the ERCC1-XPF complex is responsible for conferring resistance to platinum based drugs (Seetharam *et al*., 2010; Baba *et al*., 2012) and in particular it has been demonstrated that the over expression of the *Ercc1* gene is associated with oxaliplatin resistance in metastatic colon cancer (Choueiri *et al*., 2015). Thus, it has been shown that its knock down by siRNA-mediated gene silencing can sensitize the CRC cell lines to oxaliplatin, thereby implicating the role of *Ercc1* in conferring resistance to this crosslinking agent (Seetharam *et al*., 2010).

It is based on the above premise that this PhD thesis seeks to profile the expression of *Ercc1* in different solid tumours derived from CRC with a view to establishing the expression pattern in tumour versus normal colon tissue.

1.4. Colorectal Cancer

Colorectal cancer (CRC) or colorectal adenocarcinoma is one of the most predominant malignant neoplasms and it contributes significantly to cancer-related deaths worldwide (Ferlay *et al*., 2015). In 2014, the number of newly diagnosed patients in the United States alone reached nearly 140,000, ranking this disease in second place as a cause of death due to cancer in adults (Siegel *et al*., 2014). Worldwide, it occupies third place and second place respectively, as the leading cause of deaths relating to cancer in men and women (Ferlay *et al*., 2015). Although 55% of the cases are found across the industrialized world, Australia and New Zealand record the highest rates of CRC with Africa showing the lowest rates (Ries *et al*., 2017). The discrepancy in the documented incidences and death rate can be attributed to poor diagnosis or improper data registry (Ferlay *et al*., 2015). Diagnosis of CRC at the stage when it has not metastized to distant organ usually signals a good prognosis and about 50% of patients have a 5-year survival. However, patients at the metastatic stage have only 12% survival rate at 5 years (Ferlay *et al*., 2015). CRC may be asymptomatic for several years and the American Cancer Society has recently recommended screening from 45 years of age (Mannucci *et al.,* 2019). Detecting blood in the stool and unexplained weight loss have previously been reported to be the only symptoms warranting further exploration for polyps and CRC (Adelstein *et al.,* 2011).

While there is no single, distinct cause of CRC, several risk factors leading to its development have been described (Kupfer & Ellis, 2017). This neoplasm is sporadic with the majority caused by diet, lifestyle, age and only about 15% to 35% linked to hereditary factors (Burt, 2007; Mishra & Hall, 2012). Evidence shows that patients with history of genetic instability have a greater chance of getting the disease and such patients are especially likely to show germline mutations relative to the patients that have spontaneous CRC (Gallagher *et al*., 2010).

Based on their origin, CRC has been traditionally categorized into two biological subgroups, namely a minority (15%) that show microsatellite instability (MSI), which is primarily predominant at the right colon and known to be frequently linked to the CpG island methylator phenotype (CIMP; Popat *et al*., 2005). Additionally, predictive and prognostic information indicate that they exhibit hyper-mutation including mutation at both the *KRAS* and *BRAF* oncogenes. On the other hand, the second group comprising 85% of patients is made up of a subgroup considered to be microsatellite stable but chromosomally unstable

(Marisa *et al*., 2013; Roepman *et al*., 2014). It is worthwhile to mention that the above classifications are largely based on gene expression profiling; thus, might solely focus on single mutations or epigenetic alterations. Therefore, with the advances in genomic technology, scientists are now focussing on whole exome and genome sequencing to cover a wide range of genome analysis. This technique can sequence the entire human coding DNA looking at both the coding and non-coding regions. This will help to provide additional information on all the alterations that might have occurred at a single nucleotide including copy number and structural variants. Recently, screening with high-throughput gene expression profiling including next generation sequencing or expression arrays (microarray) has demonstrated that some CRC types overlap with the above-mentioned groups and cannot be established only by single mutations or epigenetic profiling (Sinicrope *et al*., 2016).

The development of CRC is seen to be a multistep process that involves the development of benign polyps that have the capability to evolve into carcinoma *in situ* by the accumulation of somatic mutations (Shussman and Wexner, 2014). Factors such as age, diet, lifestyle, and family history are associated with the development of polyps and CRC (Rasool *et al.*, 2013). Even though there is a good correlation between polyps and CRC development, three different subtypes of polyps have been described, distinguished on the basis of histology, such as tubular/villous adenoma, hyperplastic polyps and sessile/traditional serrated adenomas (Kalimuthu *et al.*, 2016). Similarly, there is a suggested correlation between the risk of cancer development with the number and size of previously developed polyps (Shussman & Wexner, 2014). This means that multiple colonic polyp development with malignant potential will amount to an increased lifetime risk of developing CRC.

1.4.1. Genetic Predisposition to Colorectal Cancer

Knowledge of the genetics that defines cancer development is critically important in the discovery and development of corresponding therapies for the treatment of any particular cancer. In recent years, cancer research scientists have fully become aware to the reality that genetic mutation is one of the hallmarks of cancer development including CRC (Hanahan and Weinberg, 2000; 2011; Loeb, 2001). Like other cancers, CRC is heterogenous in nature and genome sequencing has identified 24 genes that are predominantly subject to mutation including *APC, TP53, SMAD4, PIK3CA*, and *KRAS* (Cancer Genome Atlas Network, 2012). The overall classification of CRC is categorised into three important tumour subtypes

including hyper-mutated, non-mutated and CIMP subtypes (Rodriguez-Salas *et al*., 2017). The hyper-mutated tumours have been reported to account for up to 16% of all CRC (Dienstmann *et al*., 2017). While only one-quarter display somatic mismatch repair (MMR) gene and DNA polymerase ε alterations, three-quarters of them show high-frequency MSI (MSI-H) (Cancer Genome Atlas Network., 2012). Mutations in the germline account for 2 - 5% of CRC and may be as a result of autosomal dominant syndrome (Gatalica *et al*., 2017). The most prevalent and most studied is termed hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome (Lynch *et al.*, 2015). When compared to the age of the patients, evidence shows that most patients with sporadic CRC are older patients, while patients with Lynch syndrome and other genetic predispositions are usually younger (Mauri *et al.*, 2019). This can be due to loss of *Mlh1* expression, the increase of which is directly proportional with age (Kakar *et al*., 2003). Moreover, sporadic colorectal tumours are also characterized by high *BRAF* (*V600E*) mutation with loss of MLH1 and PMS2 proteins (Kakar *et al*., 2003).

Furthermore, microsatellite instability (MSI) positive CRC can be found at the proximal bowel exhibiting poor differentiation. This can be due to the presence of dense lymphocytic infiltration, suggesting strong anti-tumoural immune responses. Moreover, this is an indicative of positive prognosis (Nosho *et al*., 2010). On the other hand, the non-mutated subtype accounts for 84% of CRC; with the majority characterised by many somatic copy number changes and aneuploidy; exhibiting genetic alterations at the *KRAS* and *PIK3CA* genes. This subtype is also known to possess loss of heterozygosity of several tumour-suppressor genes including *APC* and *TP53* (Cancer Genome Atlas Network, 2012).

The third subtype as mentioned above is the CIMP characterised predominantly by DNA methylation of CPG islands (Hawkins *et al*., 2002; Weisenberger *et al*., 2006). This results in gene silencing and the subtype exhibits deficient MMR, resulting in MSI-H. Moreover, CRC with an MMR/MS-IH phenotype is said to result in a higher proportion of sporadic tumours, accounting for up to two-thirds, while the remaining one-third are linked to a germline mutation in the MMR genes including *Mlh1*, *Msh2*, *Msh6* and *Pms2* (Buchanan *et al*., 2014). Furthermore, evidence has shown that *BRAF/V600E* mutations can be another consequence of the MMR/MS-IH phenotype as well as CIMP. Thus, MSI-H is suggested to be caused by aberrant hyper-methylation that inactivates, principally, *Mlh1* (Domingo *et al*., 2004).

Additionally, two recessive cancer - predisposing genes *MUTYH* and *Nthl1* that both code for proteins that act in base excision repair (BER) have been confirmed to be associated with increased polyposis and adenomatous polyposis with a high risk of CRC respectively (Weren *et al*., 2018). Indeed, carriers of biallelic mutations in *Nthl1* have been shown to have an increaseed risk of other cancer types, including breast cancer (Kuiper & Hoogerbrugge, 2015).

1.4.2. Colorectal Cancer Treatment and Oxaliplatin

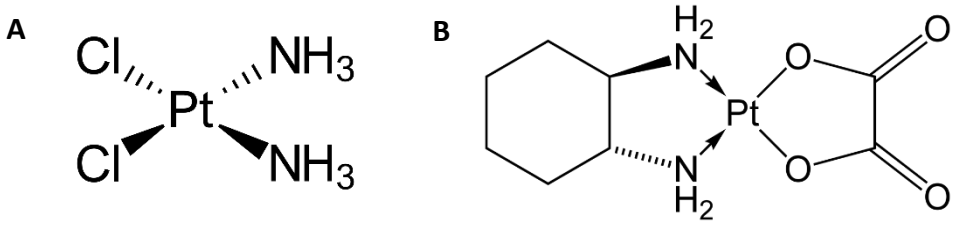


Figure 1.3. Molecular structures of (A) cisplatin and (B) oxaliplatin.

Systemic chemotherapy has been used to control cancer and alleviate its related symptoms at the metastatic stage. For metastatic CRC, it has been reported that a combination of the antimetabolite fluoropyrimidines (intravenous 5-fluorouracil and oral capecitabine), the DNA topoisomerase I inhibitor, irinotecan and the genotoxic platinum – based agent oxaliplatin (FOLFOXIRI) showed an improved survival of these patients (Leal, 2017). However, achieving complete remission at this stage is still daunting, as resistance accounting for nearly 30-50% is still a major obstacle (O'Connell *et al*., 2008).

Oxaliplatin (Figure 1.3B) is a third-generation platinum – based alkylating agent forming primarily N-alkylation products at the N7 of guanine. Similar to cisplatin (*cis*-diamminedichloroplatinum (II); Figure 1.3A), this leads to both intra- and inter-strand cross links (ICLs) in the DNA molecule, effectively disrupting DNA replication and transcription and leading to cell death. For cisplatin, the high extracellular chloride ion concentration maintains the molecule in an inactive state and only when it is transported inside the cell, where the chloride ion concentration is 5 to 30 times lower, are the chloride groups displaced by water molecules to create an effective alkylating agent. The most prevalent products are 1,2-d(GpG) intrastrand crosslinks that make up 90% of the DNA adducts, 1,2-d(ApG) intrastrand crosslinks and ICLs. In the cell, the activated cisplatin has a half-life of around two hours, while the protecting chelating ligands of oxaliplatin give this agent a much longer

half-life, with solutions of oxaliplatin and the related carboplatin being stable in water for a period of weeks to months (Johnstone *et al.,* 2016).

The repair of cisplatin – induced intrastrand crosslinks is thought to be completed by NER and the resistance to DNA repair, and therefore the cytotoxic effectiveness of this agent, is due to the bending of the DNA at the adduct and the resulting binding of the DNA by high-mobility group box proteins that have a great affinity for cisplatin modified DNA and thus shield the lesion from the NER proteins (Awuah *et al.,* 2017). In CRC, increased expression of *Ercc1*, which encodes one half of the ERCC1/XPF lesion specific endonuclease, has been correlated with oxaliplatin resistance (Galluzzi *et al.,* 2012). However, mouse cells lacking the BER DNA glycosylase NEIL3 also showed resistance to cisplatin (Rolseth *et al.,* 2013). Further evidence of a role for DNA glycosylases in ICL repair came from biochemical studies by Couvé and colleagues (2009), which indicated that NEIL1 could excise psoralen – induced ICLs from DNA and more recent work has shown that both NEIL1 and NEIL3 can resolve psoralen - induced ICLs in three- and four-stranded DNA structures (Martin *et al*., 2017) and also that NEIL3 can release ICLs at DNA replication forks (Semlow *et al*., 2016). Therefore, as NEIL3 has been reported to be highly expressed in cancer cells and metastatic tumours (Kauffmann *et al.,* 2008; Hildrestrand *et al.,* 2009) and recent work from my laboratory at the University of Salford had also indicated a role of NEIL3 in the resistance to oxaliplatin (Taylor *et al.,* 2015), it was important to determine the levels of NEIL3 in the CRC tissues and cell lines analysed in this work.

1.4.3. Colorectal Cancer Treatment and Development of Targeted Therapy

The use of conventional therapies for cancer patients is long standing, with the treatment modalities recording substantial improvements over the years. Patients with CRC can benefit from radiotherapy, surgery, chemotherapy or a mixture of surgery and chemotherapeutic agents depending on the stage of the disease (Mishra *et al.,* 2013). Surgery has been successful when diagnosis and therapeutic interventions happen at the initial stages with the tumour displaying adequate surgical margins and no invasive characteristics. Although, there are no curative options for most metastatic CRC, Kopetz and colleagues (2009) reported that an improvement in the median overall survival can be realised through surgical resection of both primary CRC and metastases (Kopetz *et al*., 2009).

Due to the shortcoming of conventional therapies, scientists are focusing on targeting metastatic CRC at the molecular level. Cancers including CRC can be characterized based on the biomarkers they present. This means that profiling tumours can be a watershed event in optimizing therapy to suit an individual patient's need. The key is in identifying reliable biomarkers by performing baseline assessment of tumour gene expression and/or immune profile for the best chance of therapeutic success. With the recent innovations in molecular testing techniques that allow for high throughput genomic analysis, patients can be selected for targeted therapy based on their tumour biology and dispositions (Ohhara *et al*., 2016). So far, techniques involving next generation sequencing and even a much newer technology for detecting a mutation in circulating tumour DNA have been described (Perakis *et al*., 2017). Since these technologies can detect somatic modifications and mutations such as insertions/deletions, copy number variation and rearrangement and base substitutions, molecular intervention strategies can be tailored to target the key molecules involved in CRC proliferation, envasion and metastasis (Diaz & Bardelli, 2014; Sinicrope *et al*., 2016). By implication, targeted therapy directed against the wrong mutation or given to a patient with tumour of unrelated characteristics will not benefit the patient.

Several biomarkers for CRC including *KRAS, NRAS, BRAF* mutation, DNA mismatch repair (MMR), MSI, and CpG island hypermethylation have been evaluated (Sinicrope *et al*., 2016). Consequent upon reports emanating from such findings, treatment modalities such as the use of small molecule inhibitors, antibodies (Baudino, 2015), immunotherapy (Lynch & Murphy, 2016) and RNA-based technologies such as siRNA or small hairpin RNA (Seetharam *et al*., 2010) have been evaluated. Although some of these techniques are still at the preclinical stage, the majority have made their way to the clinic, recording some promising outcomes.

Recent reports show that better patient survival can be achieved by recombinant humanized monoclonal IgG antibody targeting either the EGFR or VEGF pathway. The result of the clinical trial using the above antibodies as summarized by Ohhara *et al*., (2016) indicated that one anti VEGF antibody; bevacizumab, and two EGFR targeting antibodies; cetuximab and panitumumab resulted in significant anti-CRC metastatic control in combination with cytotoxic therapy (Ohhara *et al*., 2016). The clinical trial report showed that they can be used in first line, second line or even in salvage settings to enhance overall patient survival beyond 40 months from the period of initial diagnosis (Van Cutsem *et al*., 2011; Heinemann *et al*., 2014). However, this treatment is not suitable for everyone as only those in a healthy

state can benefit from it due to inherent toxicity associated with chemotherapy. Additionally, patients that have *KRAS/NRAS* mutations are not subject to this therapy as this mutation lies downstream of EGFR. In such instance, mutation at the *KRAS/NRAS* triggers the transcription of the ligand for EGFR; transforming growth factor-alpha (TGF-α). This will in turn create an autocrine signaling loop that contributes to tumoural resistance to anti-EGFR monoclonal antibodies (mAbs) including cetuximab and panitumuab (Lièvre *et al*., 2006).

Immunotherapy for the treatment of cancer has come a long way and has been accepted as the fourth treatment modality besides surgery, chemotherapy and radiotherapy. This treatment modality is currently approved for many solid tumours due to its efficacy in controlling cancer with minimal overall toxicity. The functions of immune cells in the development and progression of tumour has been well documented (Hanahan & Weinberg, 2011) indicating that several cancer types show phenotypic immune cell characteristics. It has been demonstrated that CRC with MSI can be characterized by the presence of a particular immunogenic phenotype. It was further established that this subtype has increasing lymphocytic infiltration which can possibly be due to the creation of tumour-specific neo-antigens during accumulation of mutations (Schwitalle *et al*., 2008). When primary tumour tissues from patients were further characterized an increasing presence of Th1 transcription factors was recorded. This is translated to the presence of activated cytotoxic CD8T cells, Th1 cells producing high levels of IFN-y as well as T-BET expressing T cells (Llosa *et al*., 2015). The presence of immune cell infiltration in the CRC is indicative of a positive prognosis. However, the immune microenvironment of CRC is composed of immune checkpoints that are cytotoxic to activated T cells. In a similar vein, the presence of apoptotic cell death ligands, such as programmed cell death ligand 1 (PD-L1), programmed cell death 1 (PD-1), T lymphocyte associated antigen 4 (CTLA4), lymphocyte activation gene 3 (LAG3) and indoleamine 2, 3-dioxygenses (IDO) have been shown to be the hallmark characteristic of several cancers including CRC (Llosa *et al*., 2015).

Targeting immune checkpoints has been the major focus of immunotherapy. The engagement PD-L1 on the surface of the tumour cells with PD-1 present on the immune cells including T cells, B-cells and natural killer cells produces inhibitory signals that result in T cell exhaustion and energy (Llosa *et al*., 2015). Inhibition of this pathway resulted tumour regression and reversal of T cell exhaustion in majority of cancer types including melanoma, non-small lung cancer and renal carcinoma. This is a pointer to the fact that the

mechanism of tumour regression using this strategy lies in activation of tumour infiltration lymphocyte around the tumour border. Recently, Brahmer and colleagues (2009) showed in their phase II clinical trial that, blocking PD1/PD-L1 interaction in CRC patients with MDX-1106 resulted in complete response for a period more than 21 months (Sui *et al.,* 2015). Looking at the individual treatment modalities, one can see their strengths and weaknesses. Since long-term clinical benefit to more patients is the ultimate goal, future cancer therapy is likely to focus on combinatorial approaches involving targeted inhibitors, immunotherapy, chemotherapy, surgery, radiation as well as novel therapies to achieve success.

1.5. Medulloblastoma

Medulloblastoma is a malignant, embryonal, heterogeneous, and highly aggressive tumour of the central nervous system with a preferential manifestation in children and a marked metastatic tendency via the cerebrospinal fluid (CSF) (Louis *et al*., 2007; Gibson *et al*., 2010; Robinson *et al*., 2012; Gajjar and Robinson, 2014). The development of medulloblastoma is mostly sporadic, originating from the interior fossa because of aberrant cerebellar development (Marino, 2005). In very rare cases, medulloblastoma has been reported to be associated with heritable disorders like LiFraumeni, Turcot or Gorlin syndrome (Parsons *et al*., 2011; Johansson *et al*., 2016). More than 70% of reported cases of medulloblastoma occur in patients under the age of 15 years, with the incidence peak being 3 to 6 years (Peris-Bonet *et al*., 2006). However, medulloblastoma is much less frequent in adults, accounting for less than 3% of primary tumours of the central nervous system (Smoll and Drummond, 2012).

From a US registry analysis obtained from the Surveillance, Epidemiology, and End-Results (SEER) database, it was found that medulloblastoma incidence was 1.5 cases per million in the general population, and children were reported to show over 10 times more likelihood of developing the disease than adults (Rutkowski *et al*., 2010; Smoll and Drummond, 2012). For the period 2000 to 2007, the European annual incidence rate reported 6.8 per million children within the age range of 0 – 14 years, with highest rates recorded in Southern and Central Europe (Massimino *et al*., 2016). In males, the occurrence was sign more relative to females; and the prevalence rate per annum was reported to be

higher in children that are not more than 9 years of age, indicating a frequency of less than 8 in every million. On the other hand, 6 persons per million was reported in infants, an indication of a reduction in the rate of incidence, whereas, in children whose age range is between 10 to 14 years, the incidence rate was reportedly lowest, represented by 4 persons in every million (Peris-Bonet *et al*., 2006). In the case of individuals that were above the age of 14 years but not more than 19 years of age, higher incidence rate was reported at an annual frequency of 2.33 persons per million; with incidence rate declining beyond 19 years of age up to the age of 40 years, depicting an alignment with the embryonal origin of medulloblastoma (Giordana *et al*., 1999). Between 1978 and 1997, the incidence of medulloblastoma was on the rise, with a record 1.3% increase during this period (Peris-Bonet *et al*., 2006). In parts of North-America, the occurrence of medulloblastoma was reported to be 5.07 per million children aged 0 to 19 years (Kohler *et al*., 2011). Comparatively, from 1114 diagnosis of brain tumours at the Egyptian Children's Cancer Hospital from 2007 to 2013, medulloblastoma represented 23.2% of the overall number of diagnosed cases; this shows an agreement with the reported cases of medulloblastoma in North America and Europe (Ezzat *et al*., 2016).

Children diagnosed with medulloblastoma in Europe, between the years 2000 – 2007, showed 81% survival for 1 year, 63% for 3 years and 56% survival was reported for 5 years. Essentially, worst prognosis was found among infants, where 5-year survival was reported to be 33%, but for children aged 1 to 4 years, a relatively improved survival of 47%; whereas, marked prognosis was reported for children aged 5 to 14 years of age, at survival rate of 67% (Kohler *et al*., 2011). From year 1999 and 2007, survival of patients with medulloblastoma remained stable (Gatta *et al*., 2009), while in the nineties, survival significantly improved and the possibility of the patients dying dropped by 30% (Gatta *et al*., 2014).

For children aged above 5 years, the standard of care requires surgical resection, irradiation of the craniospinal region, and CT that have amounted to an enhanced general survival rate, which accounted for approximately 70–75% in clinical setting (Lannering *et al*., 2012). The variation in the results reported across European countries are indicative of the fact that there are challenges in harnessing of effective treatment and/or reach effectively, timely and accurately consistent diagnosis. In Northern Europe, an improved 5-year

survival rate at 64% was reported, while it was relatively lowest in countries within Eastern Europe at 53% (Gatta *et al*., 2014).

Because the highest incidence of medulloblastoma occurs during childhood; it is rational to assert that certain factors operating at very early stage of life might play a key role in determining the disease development (Massimino *et al*., 2016). Birth weight is frequently suggested as an easy but rough indicator of medulloblastoma prenatal exposures. On this note, Harder *et al*. (2008) systematically reviewed the relationships between patients' birth weights and the localisation of primary brain tumours of specific histological features. Interestingly, they observed that high birth weight was significantly associated with increased risk of medulloblastoma. Similarly, other studies have made attempts to speculate on a possible aetiology that is considered infectious. For instance, a study conducted in England, investigated a variety of perinatal factors and their respective impacts on brain tumours in children (Fear *et al*., 2001). The researchers observed that children whose mothers had a documented case of viral infection during pregnancy had over 11 times increased risk of development of malignant tumours of the central nervous system (CNS) (Fear *et al*., 2001). However, this finding was not supported by recently and widely updated reviews (Johnson *et al*., 2014, Crump *et al*., 2015). In a large population-based control-case study, Harding *et al*. (2009) evaluated the profile of day care and social contacts in the first year of life, alongside other indicators of infectious exposure. The authors reported elevated risk of medulloblastoma development in children that have had no social contact with other infants in the first year of life. However, they interestingly noted that analysis of other related hallmarks of infectious exposure such as joint use of bedroom, domestic exposure to school-age children, and sequence of birth; failed to concur with the proposition of a protective effect of infectious exposure (Harding *et al*., 2009).

Diet is another player in medulloblastoma and other tumours of the nervous system, and its implications as risk factor and a positive factor, have been reported in multiple studies. The hypothesis that maternal dietary intake of N-nitroso compounds (NOC) and NOC precursors in the course of pregnancy increases the risk of brain tumour development in offspring, is known to be one of the most comprehensively investigated hypotheses (Dietrich *et al*., 2005; Massimino *et al*., 2016). Based on this premise, a large international collaborative case-control study on childhood brain tumours was conducted to ascertain the relationships between histology-specific risk and consumption of specific food groups

during pregnancy (Pogoda *et al*., 2009). The researchers observed that cured meats, eggs/dairy, and oil products were the main foods regularly linked with increased risk; whereas, foods that are mostly associated with lowered risk were fresh fish, yellow-orange vegetables, and grains (Pogoda *et al*., 2009).

Another risk factor that has been under serious investigation is the maternal occupational exposure. Li *et al*. (2009), while studying the causal relationship between maternal occupational exposure and the incidence of childhood brain tumours, evaluated the implications of extremely low frequency magnetic fields (ELF-MF) just prior to and during pregnancy. They reported a seriously elevated risk for astroglial tumours and for the entire childhood brain tumours investigated (Li *et al*., 2009). Similarly, several epidemiological studies have investigated the correlation between parental exposure to pesticide and childhood brain tumours, and many of the research outcomes were suggestive of affirmative correlation (Massimino *et al*., 2016). It was based on this rationale that Shim *et al*., (2009), conducted a population- based case-control study to evaluate that association between the occurrence of brain cancer in children and parental exposure to pesticides in occupational and residential settings. They reported negligible relationship with medulloblastoma for any of the pesticide subtype or the sources exposure investigated (Shim *et al*., 2009). Additionally, Rosso *et al*., (2008), evaluated the relationship between the hobbies of fathers and medulloblastoma; and they observed an increased risk of medulloblastoma development in children whose fathers' hobbies are linked to household use of chemicals, particularly pesticides. When parental occupation was considered, Cordier *et al*., (1997), reported an increased risk of PNET with parental exposure to polycyclic aromatic hydrocarbons and high maternal exposure to solvent during the five-year period before birth. In another vein, certain genetic abnormalities such as Gorlin, Turcot, and Li-Fraumeni syndromes have been reported as cancer-predisposition syndromes that are known to be defining risk factors of medulloblastoma (Villani *et al*., 2012).

1.5.1. Histology and Pathobiology of Medulloblastoma

To date, the actual cellular origin of medulloblastoma remains subject to debate (Gibson *et al*., 2010). Several reports have shown that the origin of medulloblastoma could be from two different embryonal cell groups: cells from the ventricular zone, which differentiate into various cells of the cerebellum; and cells from the external germinal layer (EGL), which differentiate into cerebella granule cells (Kuzan-Fischer *et al*., 2018). These cell groups are related to different molecular subtypes of medulloblastoma and it has been established that ventricular zone cells give rise to the wingless (WNT) subtype, whereas sonic hedgehog (SHH) medulloblastoma is produced from the EGL cells (Fan and Eberhart, 2008; Rusert *et al*., 2014; Kuzan-Fischer *et al*., 2018). In the subsequent sections, these molecular subtypes will be reviewed in a more elaborate manner.

The 2007 WHO classification of tumours of the central nervous system recognises five major variants of medulloblastoma, including the classic medulloblastoma, desmoplastic/nodular, medulloblastoma with extensive nodularity (MBEN), anaplastic and large cell (Giangaspero *et al*., 2007; Massimino *et al*., 2016; Kuzan-Fischer *et al*., 2018). From these five forms, large-cell medulloblastoma as well as anaplastic variant of the disease have significant overlapping characteristics; consequently, several studies have attempted to group them into large cell/anaplastic (LC/A) medulloblastoma (Gilbertson and Ellison, 2008). The incidence rate of the combined LC/A type of medulloblastoma has been reported to oscillate between 10% and 22%; whereas, anaplastic medulloblastoma is established only in event of severe and diffuse anaplasia, comprising up to 50% of reported cases (Giangaspero *et al*., 2007). Nodular/desmoplastic medulloblastoma constitute about 7%, while MBEN comprises up to 3% of the entire reported cases of the disease, while classic subtype of medulloblastoma make up the remaining (Gilbertson and Ellison, 2008; Massimino *et al*., 2016).

Histologically, the classic subtype of medulloblastoma is made up of tightly packed cells with characteristic oval, round, or carrot-shaped hyperchromatic nuclei encircled by minimal cytoplasm (Massimino *et al*., 2013). The Desmoplastic/nodular form of medulloblastoma is known to have nodular, reticulin-free zones, which are neuronal maturation zones. It is characterised by a small nuclear cytoplasmic ratio, a fibrillary matrix, and homogenous cells with a neurocytic appearance (Eberhart *et al*., 2002a). The densely-packed cells at very active mitotic state surround the constituent nodules, which results in

the production of a dense intercellular reticulin-positive network of fibres (McManamy *et al*., 2007).

Medulloblastoma with extensive nodularity (MBEN) is known to be predominant in infants and it has characteristic better prognosis (Eberhart *et al*., 2002a); and it is distinct from the closely-related nodular/desmoplastic subtype by possessing an expansive lobular conformation because of the unusual elongation of the reticulin-free zones and enriched with neuropil-like tissue (McManamy *et al*., 2003). Such zones are filled with small cells that have characteristic spherical nuclei that bear close semblance to the cells of a central neurocytoma and exhibit a streaming pattern; coupled with the marked reduction of the internodular component in some areas (McManamy *et al*., 2007). Structurally, the large cell medulloblastoma is made up of monomorphic cells with large, round, vesicular nuclei, prominent nucleoli and variably abundant eosinophilic cytoplasm. Groups of these large cells tend to combine with morphologically different cells with characteristic nuclear polymorphism and nuclear conformation; this morphological variant has been termed anaplastic (Massimino *et al*., 2016). Large cell and anaplastic histological forms of medulloblastoma have been reported to show considerable cytological overlap and many studies have attempted to describe the histological alternation between non-anaplastic to anaplastic subtypes over time. However, some studies have reported alternated transition intra-tumour, as deduced from the presence of varying degrees of cytological atypia or anaplasia in any given tumour (Eberhart *et al*., 2002a; Massimino *et al*., 2016).

Clinically, different reports have significantly shown good prognosis for the nodular/desmoplastic medulloblastoma at least in certain age groups as well as risk groups, particularly in children at younger age (Rutkowski *et al*., 2005; McManamy *et al*., 2007). Additionally, classic form of medulloblastoma has been reported to show significantly better prognostic outcome relative to the LC/A histological variant (Massimino *et al*., 2013).

1.5.2. Molecular Subgroups of Medulloblastoma

Increased understanding of the molecular characteristics of medulloblastoma and the advent of molecular diagnostics have resulted in the classification of the disease into distinctive subgroups (Kool *et al*., 2012). From the currently established global understanding, there are four distinct subgroups of tumours of the medulla, including WNT (wingless), SHH

(sonic hedgehog), Group 3 medulloblastoma, and Group 4 medulloblastoma (Taylor *et al*., 2012), as represented in Table 1.1. These four molecular subgroups of medulloblastoma were isolated using series of genomics and molecular studies They are known and identified by properly defined genetic, molecular, clinical, histopathological, and prognostic features (Northcott *et al*., 2012; Ramaswamy *et al*., 2014; Schneider *et al*., 2105; Ramaswamy and Taylor, 2017; Kuzan-Fischer *et al*., 2018). From recent research findings derived from genetic, transcriptional and epigenetic data, it has been suggested the need to further categorise medulloblastoma into subtypes based on molecularly characteristics and such sub-classification is most likely to have positive impact on patient stratification in future clinical trials (Northcott *et al*., 2017; Schwalbe *et al*., 2017; Cavalli *et al*., 2017).

Table 1.1. Molecular Subgroups of Medolloblastoma.

	WNT	**SHH**	**GROUP 3**	**GROUP 4**
Age Group	Children & Adults	Infants, Children & Adults	Infants & Children	Infants, Children & Adults
Metastasis	Rarely M+	Uncommonly M+	Very frequently M+	Frequently M+
Prognosis	Very good	Infants good, others intermediate	Poor	Intermediate
Genetics	CTNNB1 mutation	PTCH1/EMO/SUFU mutation / GL12 amplification / MYCN amplification	MYC amplification	CDK6 amplification
Gene Expression	WNT signalling MYC+	SHH signalling MYCN+	Photoreceptor / GABAergic MYCN+++	Neuronal/Glutamatergic minimal MYC/MYCN

1.5.2.1. WNT (Wingless) Medulloblastoma

This is the least prevalent medulloblastoma molecular subgroup, constituting approximately 11% of the total reported incidences of medulloblastoma (Kool *et al*., 2012). Though, wingless form of medulloblastoma has been reported to occur at all ages, children are predominantly affected, with the highest frequency of occurrence recorded in children of 10 to 12 years (Taylor *et al*., 2012). Unlike other molecular subgroups of medulloblastoma, WNT forms have been reported to have a female preponderance, based on gender ratio. Typically, WNT tumours are known to occur in the mid region of the brain, affecting the IV ventricle and extending to the brain stem (Gajjar and Robinson, 2014).

Based on their histological features, most WNT forms of medulloblastoma belong to the classic histological subgroup; although, instances of wingless medulloblastoma with LC/A histology have been rarely reported (Gajjar and Robinson, 2014). IHC expression of DKK1, Filamin-A, YAP-1, and beta-catenin, particularly the nuclear +/− cytoplasmic expression, have been demonstrated as a very reliable method for the identification of medulloblastoma that have characteristic wingless pathway (Ellison *et al*., 2011). On a positive note, WNT medulloblastoma show much lower metastatic diffusion than other molecular subgroups and they have best prognosis, with reported survival rates of about 95-100% (Salaroli *et al*., 2015). The reason behind the characteristic improved survival rates associated with WNT is still unknown, though, it has been attributed to its increased susceptibility to radiation therapy (Gajjar and Robinson, 2014; Salaroli *et al*., 2015).

More than 75% of tumours in the WNT molecular subgroup of medulloblastoma habour an exon 3 mutation, and in particular a point mutation of the CTNNB1 gene, which encodes beta-catenin (Gilbertson, 2004), resulting in the increased excitation of the wingless pathway through beta-catenin resistant to breakdown and resulting in nuclear localisation of the gene products (proteins) with elevated transcription of genes such as cyclin D1 and MYC, that are concerned with cellular proliferation (Massimino *et al*., 2016; Kuzan-Fischer *et al*., 2018). Cytogenetically, WNT pathway tumours have been reported to show a characteristic monosomy 6 in more than 79% of patients (Shih *et al*., 2014). Besides monosomy 6, the genomic composition of WNT medulloblastoma is comparatively silent and only associated with scarce chromosomal deletion and/or insertion across the genome (Gilbertson, 2004). However, in addition to monosomy 6, other genetic alterations such as copy number variation (CNV) and/or single nucleotide variants (SNV) include mutations

in the gene that promotes cellular proliferation through the increased activating capacity of beta-catenin, SMARCB4, p53 gene alteration, tetraploidy, and MLL2 alterations (Northcott *et al*., 2012; Jones *et al*., 2012). In a clinical trial that assessed medulloblastoma patients with characteristic WNT molecular features, Clifford *et al*. (2015) revealed new insights into the clinical features and further demonstrated that relapses occur at higher frequency in patients of 16 years at diagnosis. This report agrees with the earlier reports from a series of retrospective studies, in which bimodal distribution of age and very poor prognosis were demonstrated in adults with WNT medulloblastoma relative to children (Korshunov *et al*., 2010; Kool *et al*., 2012; Clifford *et al*., 2015).

1.5.2.2. Sonic Hedgehog Medulloblastoma

In normal cerebellar physiology and development, the sonic hedgehog (SHH) pathway plays a significant role, where it is responsible for the initiation of the rapid growth of primordial cells of the neuron, and its proliferation in the growing cerebellum and other brain tissues alike (Massimino *et al*., 2016). The formation and growth of the external germinal layer from the cells of the granule and precursor cells is facilitated by the SHH ligand (McManamy *et al*., 2007). Additionally, paracrine signalling emanating from SHH or the activation resulting from PTCH1 mutations leads to the breakdown of some molecular regulators, especially the serpentine G-protein coupled receptor SMO from PTCH, and its subsequent translocation into the apical section of the cilium, releasing GLI2 from its original repressor called suppressor of fused homolog (Archer *et al*., 2012). From thence, the GLI2 localises to the nucleus, where it modulates genes that are responsible for the excessive growth of the granule cell precursor cells of the cerebellum, resulting in tumour development (Jones *et al*., 2012; Archer *et al*., 2012).

The SHH molecular subgroup of medulloblastoma has been reported to account for approximately 30% of all reported cases of medulloblastoma (Taylor *et al*., 2012). Relative to age, its localisation is bimodal, occurring more frequently in children of less than 3 years of age and adults of 16 years of age and above; and less frequently reported in patients who are 3 years to 16 years old (Gibson *et al*., 2010). The gender ratio has been shown to be 1:1, though incidence rate has been shown to be slightly higher in males (Gajjar and Robinson, 2014). The SHH subgroup of medulloblastoma predominantly occurs in the hemispheric region of the cerebellum; however, some tumours of the SHH pathway have been reported

to originate in the mid vermis (Jones *et al*., 2012). Histologically, SHH medulloblastoma have characteristic nodular cum desmoplastic form with MBEN being exclusively added into this group; though, there have been reports other forms of SHH medulloblastoma which are known to be either of classic or LC/A histology (Ellison *et al*., 2011). Molecular biomarkers like GAB1, SFRP, and GLI1 protein have been reported as the key hallmarks for the identification of SHH medulloblastoma using tumour IHC expression for such molecular features; but diagnosis of the disease at metastatic stage rarely occurs (Ellison *et al*., 2011; Taylor *et al*., 2012).

SHH medulloblastoma has characteristic PTCH1 mutations and approximately 36–54% cases of SHH medulloblastoma have been reported to show PTCH1 mutations (Kool *et al*., 2014). The characterisation of somatic mutations of PTCH1 in patients devoid of Gorlin syndrome has deepened the already established correlation between medulloblastoma and sonic hedgehog signalling, including molecular alterations in SUFU and SMO, elevated expression of SHH, GLI2, and increased expression of MYCN genes (Massimino *et al*., 2016). Previous reports have associated the expression of MYC or MYCN with histological features that are large-cell/anaplastic and poor prognostic features of medulloblastoma. Similarly, tumours with characteristic MYC or MYCN over-expression and tumours harbouring 6q insertion make up subgroups of medulloblastoma which are especially characterised by poor prognosis (Eberhart *et al*., 2002b). Kool *et al*., (2014) observed that mutations affecting PTCH1 gene occurred at an approximate percentage frequency of 36% in infants, 42% in children, and 54% in adults. In their work, which was a large-scale genomic study of sonic hedgehog subgroup of medulloblastoma, they further observed that SUFU mutations were characteristically predominant in infant medulloblastoma patients, and SMO mutations were more frequent in adult medulloblastoma patients (Kool *et al*., 2014).

The SHH subgroup of medulloblastoma has a broader molecular heterogeneity in older children, where they show increased expression of MYCN and GLI2 genes; TP53 mutations predominant in cancers of older children with sonic hedgehog medulloblastoma have also been reported (Zhukova *et al*., 2013). Similarly, over 50% of such children were reported to have Li- Fraumeni syndrome and mutations of genes in germline cells that conferred SHH medulloblastoma in individuals with very poor prognosis (Jones *et al*., 2012). They also reported tetraploidy in about 29% of investigated samples and it was associated with p53 mutations. In the same vein, alterations affecting copy number such as the increased

expression of protein phosphatase, PIK3C2G, IRS2, YAP-1, and PIK3C2B coupled with the deletion of PTEN which are localised on chromosome 10q23.31 and mutations in other related genes, was reported by Northcott *et al*. (2012b).

At the point of diagnosis, patients within the SHH molecular subgroup of medulloblastoma rarely show a disseminated tumour because they are tenable to immediate prognostic propensity, with overall survival of over 75% across a 5-year period, particularly when standard therapy is employed in the treatment (Taylor *et al*., 2012). Although, in a relatively recent study, Min *et al*. (2013) reported that a complete deletion of chromosome 14, increased expression of GLI2, loss of q10, MYCN upregulation, anaplastic and metastatic medulloblastoma at the diagnostic stage identify, have aided the characterisation of further subgroups in sonic hedgehog medulloblastoma patients, worsening their prognostic propensities.

1.5.2.3. Group 3 Molecular Subgroup of Medulloblastoma

This molecular subgroup of medulloblastoma is roughly responsible for 25-28% of the entire diagnosed cases of medulloblastoma and it is predominantly found in children, characteristically affecting more males, increased frequency of metastasis at the point of diagnosis and high incidence of LC/A histology (Northcott *et al*., 2012). In children, medulloblastoma Group 3 has not been shown to be a defining factor and the molecular pathogenesis of the disease remains largely unknown. However, some scientists have identified expression for NPR3 (Natriuretic Peptide Receptor 3) as a confirmatory feature of Group 3 medulloblastoma, however, the validity of this biomarker is in doubt (Taylor *et al*., 2012).

Almost all cases of Group 3 medulloblastoma have aberrant MYC expression, sometimes associated with high-level expression (Northcott *et al.,* 2012). Group 3 medulloblastoma also shows characteristic genomic instability with frequent gains due to the insertion at chromosomes 1q, 7q, and 17q along with loss of 10q, 11, 16, and 17p (Northcott *et al*., 2012). At an early stage in the development of Group 3 medulloblastoma there is a significant occurrence of tetraploidy in up to 54% of all cases of the disease (Jones *et al*., 2102). When there are no p53 mutations, chromothripsis is a frequent event in Group 3 medulloblastoma, resulting in aberrant chromosomal rearrangement or fusions due to

inefficient DNA repair. Similarly, TGF-β signalling appears to be increased because of dysregulation of the genes that are implicated in this signalling pathway and association with downstream target genes including OTX-2 (Northcott *et al*., 2012).

Amongst all the four molecular subgroups of medulloblastoma, Group 3 is known to be associated with the worst prognosis, with less than 50% survival and no survivor exceeding 10 years of follow-up in evaluation of retrospective studies (Kool *et al*., 2012; Massimino *et al*., 2016). This agrees with the findings of the study conducted by Shih *et al*. (2014) that revealed that the existence of i17q, MYC upregulation, and the presence of metastatic medulloblastoma that are responsible for poor prognosis of Group 3 medulloblastoma, and patients without these markers show a relatively better survival.

1.5.2.4. Group 4 Molecular Subgroup of Medulloblastoma

Group 4 medulloblastoma has been reported to be the most predominant subtype, constituting approximately 35% of all diagnosed medulloblastoma. Group 4 medulloblastoma affects patients of all age groups, with male predominance, but it is rarer in infants (Kool *et al*., 2012; Northcott *et al*., 2012). Although, Group 4 medulloblastoma is the commonest of all the molecular subtypes, its molecular pathogenesis is poorly understood (Kuzan-Fischer *et al*., 2018). Histologically, most of the Group 4 medulloblastoma have characteristic classic histology; however, some instances of LC/A have been reported (Massimino *et al*., 2016). Group 4 medulloblastoma have been linked with KCNA1 as its IHC marker but no other report has validated such claim (Ellison *et al*., 2011).

Group 4 medulloblastoma patients have been reported to have a moderate prognosis with standard cytotoxicity regimen. Shih *et al*. (2014) observed that excellent prognostic features are on subset of patients with Group 4 medulloblastoma following the loss of chromosome 11 and the presence of i17q, regardless of the attainment of metastatic stage by the disease at the point of diagnosis. Like Group 3 tumours, Group 4 medulloblastoma is characterised by tetraploidy in at least 40% of cases, as an initial transformation (Jones *et al*., 2012). In over 80% cases of medulloblastoma, the occurrence of isochromosome 17q has been reported in addition to 17p deletion, and MYCN and CDK6 genes are reported to be commonly amplified (Skowron *et al*., 2015). Female patients of Group 4

medulloblastoma predominantly lose one copy of the X chromosome, implying that one or more tumour suppressor genes might be present on this chromosome (Jones *et al*., 2012). Also, in Group 4 medulloblastoma, neural stem cells are kept undifferentiated state due to the excessive amplification of EZH2 (enhancer of Zeste homologue 2) and mutations affecting chromatin-remodelling genes such as KDM6A, which codes for a H3K27 methylase and located on chromosome Xp11.3., ZYMYM3, and CHD7; thus, sustaining tumorigenesis (Massimino *et al*., 2016). In Table 1.2, a synopsis of the key genomic and clinical features of the four molecular subgroups of medulloblastoma is given.

Table 1.2. A synopsis of the key genomic and clinical features of the medulloblastoma molecular subgroups.

	WNT	**SHH**	**GROUP 3**	**GROUP 4**
Histological Feature	Classic, Rarely LCA	Desmoplastic, Classic, LCA	Classic, LCA	Classic, LCA
Rate of Metastasis	Low	Low	High	High
Prognostic State	Excellent	Intermediate	Poor	Intermediate
Alterations Somatic Copy Number	–	MYCN (12%) GLI2 (8%)	MYC (17%) PVT1 (12%) OTX2 (8%)	SNCAIP (10%) MYCN (6%) CDK6 (5%)
Single-Nucleotide Variants	CTNNB1 (91%) DDX3X (50%) SMARCA4 (26%) MLL2 (13%) TPS3 (13%)	TERT (60%) PTCH1 (46%) SUFU (24%) MLL2 (16%) SMQ (14%) TP53 (13%)	SMARCA4 (11%) MLL2 (4%)	KDM6A (13%) MLL (5%)
Broad Events	6 Loss	3q Gain 9q, 10q, 14q Loss	1q, 7, 17q, 18q Gain 8, 10q, 11, 16p, 17p Loss	7, 17q, 18q Gain 8,11p, X Loss
Expression	WNT Signaling	SHH Signaling	MYC/Retinal Signature	Neuronal Signature
Recurrence	–	Local	Metastatic	Metastatic

Adapted from: (Massimino *et al.,* 2016).

1.5.3. Risk-Based Classification of Medulloblastoma Patients

Medulloblastoma patients are traditionally categorized into two key risk strata, namely, the average risk and the high risk medulloblastoma patients. This classification is based on three clinical factors, which includes the patients' age during diagnosis, whether there is leptomeningeal dissemination or not, and extent of residual tumour post-resection (Kuzan-Fischer *et al*., 2018). Patients are classified as average risk if they are older than 3 years of age at the point of diagnosis, with residual tumour size less than 1.5 cm^2, in addition to negative results for macroscopic metastasis on imaging scans as well as CSF analysis for microscopic tumour cells (Massimino *et al*., 2016; Kuzan-Fischer *et al*., 2018). High risk medulloblastoma patients are characterised by the presence of metastasis and/or a postoperative tumour size greater than 1.5 cm^2. Medulloblastoma patients that are infants below the age of 3 years are generally referred to as high risk patients (Northcott *et al*., 2011).

At the 2015 meeting held in Heidelberg, a new medulloblastoma patient risk classification protocol premised on the molecular and prognostic features of the disease was suggested for patients of medulloblastoma that are within the age range of 3 and 17 years (Ramaswamy *et al*., 2016a, b). This proposed reclassification protocol is made up of four risk groups, mainly defined by outcome; and considers the heterogeneous nature of the disease and detailed information of the molecular subgroup (Kuzan-Fischer *et al*., 2018). Based on survival outcome, the protocol considers as very high risk if the patients' survival is less than 50%, high risk patients show 50-75% survival, standard risk patients have survival outcome of 75-90%, and low risk patients have over 90% survival outcome (Ramaswamy *et al*., 2016b). The protocol also opined that individuals suffering from metastatic group 3 medulloblastoma and patients that have sonic hedgehog tumours with characteristic p53 mutations, show appalling prognosis and are to be accorded the class of very high risk (Ramaswamy *et al*., 2016a; Kuzan-Fischer *et al*., 2018). Medulloblastoma patients are also considered high risk if they are diagnosed with metastatic or MYCN amplified SHH tumour; same is applicable to group 4 medulloblastoma patients with leptomeningeal dissemination (Ramaswamy *et al*., 2016b). On the other hand, medulloblastoma patients are considered standard risk if they have unamplified MYCN, unmutated p53 SHH medulloblastoma, group 3 medulloblastoma with unamplified MYCN, and group 4 tumours devoid of chromosome 11 loss, as shown in Table 1.3 (Ramaswamy *et al*., 2016b; Kuzan-Fischer *et al*., 2018).

Importantly, the recently proposed patient risk classification gives room for the assessment of treatment reduction for patients whose outcomes are favourable and facilitates the possibility of identifying as well as testing recent rationale for target-specific therapies in medulloblastoma patients within the range of high risk to very high-risk categories (Ramaswamy *et al*., 2016b; Kuzan-Fischer *et al*., 2018).

Table 1.3. Medulloblastoma patient risk stratification using molecular and survival outcome as criteria.

	Low risk (>90% survival)	**Standard risk (survival rate of 75-90%)**	**High risk (survival of 50-75%)**	**Very high risk (survival = <50%)**
WNT	Non-metastatic			
SHH		Non-metastatic AND TP53WT AND No MYCN amplification	Metastatic AND TP53 WT OR Non-metastatic AND MYCN amplification	TP53 mutation
Group 3		Non-metastatic AND No MYC amplification		Metastatic AND MYC amplification
Group 4	Non-metastatic AND Chromosome 11 loss	Non-metastatic AND No chromosome 11 loss	Metastatic	

Adapted from: Kuzan-Fischer *et al*. (2018).

1.5.4. Current Medulloblastoma Therapies

Currently, the treatment protocols for medulloblastoma patients are mostly hinged on the conventional risk categorisation and patient's age at the point diagnosis (Ramaswamy and Taylor, 2017). Irrespective of the risk group, patients are firstly subjected to tumour resection during diagnosis (Kuzan-Fischer *et al*., 2017). Recently, Thompson *et al*., (2016) conducted a re-evaluation of the prognostic value of the extent of medulloblastoma resection, taking the different subgroups into consideration. Their findings revealed that regardless of molecular subgroup, gross total resection has no benefit subtotal resection in overall survival for patients, but for patients that were subjected to near-total in lieu of gross-total resection, no overall survival or progression-free survival advantage was recorded (Thompson *et al*., 2016). Consequently, it is rational to suggest that maximal removal by surgery stands as the benchmark of care for patients of medulloblastoma. Additionally, there were no obvious practical benefits of surgical resection of minimal residual medulloblastoma that embodies an increased risk of morbidity neurologically (Thompson *et al*., 2016; Kuzan-Fischer *et al*., 2018).

Though, cut-off age varies from one clinical trial to another, Lafay-Cousin *et al*. (2016) reported that patients designated as average risk patients between the age of 3 to 5 years are exposed to the irradiation of craniospinal region at the of 23.4 Gy boosted with an additional dose of 55 Gy to the tumour microenvironment in the posterior fossa followed by a chemotherapeutic regimen that is characteristically cytotoxic. For individuals at the "high risk" category, Holgado *et al*., (2017) suggested craniospinal irradiation of 36-39 Gy dosage, boosted with a further dose of 55 Gy to the bed of the tumour, supported with cytotoxic chemotherapeutic regimen. In this case, cisplatin/carboplatin-vincristine-cyclophosphamide combination regimens constitute the typical chemotherapeutic intervention (Kuzan-Fischer *et al*., 2018).

Due to the debilitating side effects of craniospinal radiation on the neuro-cognitive ability of developing nervous system, infant patients of medulloblastoma that are not up to the age of 3 to 5 years are now treated through approaches devoid of irradiation (Grill *et al*., 2005; Cohen *et al*., 2015; Holgado *et al*., 2017). For such non-radiation medulloblastoma therapies targeting infants within the age group of 3-5 years, a variety of chemotherapy regimens have been under intense research, including etoposide, cisplatin, vincristine, and cyclophosphamide, and a further administration of autologous hematopoietic cell rescue and

methotrexate administered intravenously and intraventricularly, cyclophosphamide, cisplatin analogue (carboplatin), and vincristine (Rutkowski *et al*., 2005; Cohen *et al*., 2017; Kuzan-Fischer *et al*., 2018). Unfortunately, for patients in this age group that are diagnosed with non-desmoplastic histology and macroscopic metastatic medulloblastoma, survival rates and general outcomes have been abysmally poor (Ramaswamy and Taylor, 2017).

1.5.5. Biologically-Informed Medulloblastoma Treatment Strategies: Targeted Therapies

Because of the current knowledge of the fact that medulloblastoma is an aggregation of highly heterogenous tumours, molecularly stratified clinical trials have been executed, with focal interest in molecular subgroups of the disease and a defined knowledge of patient risk stratum (Ramaswamy *et al*., 2016 a; b; Northcott *et al*., 2012b). Owing to the advanced understanding of major molecular and genetic changes within various medulloblastoma subgroups and histological subtypes, researchers have continued to strive to develop novel targeted therapies specific to molecular pathways and risk-adapted treatment protocols that are patient-specific and suitable to a patient's tumour (Triscott *et al*., 2013; Gajjar *et al*., 2014; Holgado *et al*., 2017; Ramaswamy and Taylor, 2017). Recent research efforts are geared towards many scientific trials to develop and experiment and establish small molecular inhibitors, immunotherapies and other therapies that are based on antibodies, with potentials of taking advantage of the molecular vulnerabilities of different molecular subgroups as well as histological subtypes of medulloblastoma (Faria *et al*., 2015; Badodi *et al*., 2017; Holgado *et al*., 2017).

For low risk, non-metastatic WNT medulloblastoma, de-escalation of first-line treatment is one of the key strategies that are introduced in majority of active clinical trials (Holgado *et al*., 2017). These clinical trials are designed with the intent of craniospinal irradiation reduction or elimination and implement reduced dosage of chemotherapeutic regimes (Ramaswamy and Taylor, 2017). Owing to the excellent overall patients' survival that have reported for this class of medulloblastoma, these clinical pre-clinical studies are primarily aimed at reducing deaths emanating from side effects of treatments in patients with survival outcomes that are biologically favourable (Ramaswamy *et al*., 2011; Henrich *et al*., 2014; Ramaswamy *et al*., 2016b).

For more than a decade now, action-specific molecular targeted therapies against SHH medulloblastoma have been under serious investigation; hence, the activation of Hedgehog signalling pathway in medulloblastoma has been identified and established in many preclinical studies and the efficacy of inhibitors of hedgehog pathways in medulloblastoma has been proven in different *in vitro* studies (Berman *et al*., 2002; Taipale *et al*., 2002; Robinson *et al*., 2015). Some of the first action-specific therapies to advance to first stage trials in individuals suffering from medulloblastoma were the competitive antagonists of the smoothened receptor, vismodegib and sonidegib (Robinson *et al*., 2015). Interestingly, whether paediatric or adult, all medulloblastoma patients treated with vismodegib recorded an improvement in the survival of patients with persistent sonic hedgehog type of medulloblastoma, though in other molecular subgroups of medulloblastoma, the survival outcomes were dismal (MacDonald *et al*., 2014; Robinson *et al*., 2015). One of the major determinants of response to SMO (smoothened) inhibition is the defined localisation of hedgehog cascade modifications downstream of SMO, such as SUFU mutations, which negatively regulates hedgehog signalling and the amplification GLI group zinc finger 2 (GLI2) or MYCN upregulation, that result in the conferment of resistance to SMO inhibitors (Pambid *et al*., 2014; Kool *et al*., 2014). Based on these molecular premises, a single therapy involving the use of smoothened inhibition has been reportedly linked to the isolation of therapy-resistant sub-clones through SMO inhibition mutations or increased expression of alternative proteins that are associated with survival (Buonamici *et al*., 2010; Massimino *et al*., 2016); hence, indicative of the possibility that SMO inhibitors cannot achieve durable treatment response independently, but will require the company of additional agents to function and guarantee better treatment response (Yauch *et al*., 2009). Similarly, alternative molecular agents that target downstream components of the hedgehog signalling pathway have been reported to be made up of the trioxide of arsenic and itraconazole, GLI transcription factor inhibitors, that could show effectiveness in a small group of hedgehog-activated medulloblastoma patients that is not determined by SHH-PTCH1-SMO (Kim *et al*., 2013; Rusert *et al*., 2014).

Furthermore, other molecular pathways that may proffer targetable vulnerabilities within the SHH subgroup have been identified and established through a series of preclinical studies (Mille *et al*., 2014; Faria *et al*., 2015). For instance, the significance of the PI3K pathway in SHH medulloblastoma metastasis was demonstrated in a genomic analysis of mouse model of SHH medulloblastoma, in which tumour populations at the stage of metastasis were

enriched for clones through the addition of the PI3K pathway (Wu *et al*., 2012). Mutations affecting TP53 are frequently reported in SHH medulloblastoma subtypes; hence, molecular therapies targeting such pathway could provide a reasonable alternative to irradiation and help overcome radiation resistance associated with these mutations (Tabori *et al*., 2010; Zhukova *et al*., 2014).

For group 3 and group 4 molecular subgroups of medulloblastoma, there are no current specific targeted therapies in existing clinical trials (Kuzan-Fischer *et al*., 2018). However, Holgado *et al*. (2017), while attempting to produce a tailored medulloblastoma therapy through genomics and evaluating one molecular subgroup at a time; designed a trial containing a treatment regimen for patients that have progressed to metastasis during the time of diagnosis, incomplete removal and amplification of MYC or MYCN, that were referred to as high risk patients (Holgado *et al*., 2017). Although patients with Group 3 medulloblastoma molecular subgroup have been shown to have worst prognosis, no current clinical trials are known to be investigating targeted therapies in Group 3 and Group 4 medulloblastoma patients (Faria *et al*., 2015). It is therefore, important that such studies are prioritised.

Group 3 and Group 4 molecular subgroups of medulloblastoma have been shown to demonstrate high degree of heterogeneity as per activated signalling pathways, with the overexpression of MYC being the most predominant cytogenetic marker in Group 3 tumours (Venkataraman *et al*., 2014; Pei *et al*., 2016). Treatment via the combined use of PI3K and molecular inhibitors have shown promising results when preclinical agents in Group 3 MYC-driven medulloblastoma are targeted (Alimova *et al*., 2012; Dubuc *et al*., 2013; Hovestadt *et al*., 2014; MacDonald *et al*., 2014).

1.5.6. Metastatic Medulloblastoma

Typically, medulloblastoma metastasis involves tumour cell movement to the leptomeninges, and the spread and establishment of the disease within the leptomeningeal area constitute the most formidable treatment challenge confronting clinicians (Kuzan-Fischer *et al*., 2018). Across all molecular subgroups of medulloblastoma, the incidence of metastatic tumours at diagnosis has been reported to be approximately 40%, although the frequency of metastasis varies across different subgroups at the point of diagnosis (Wu *et*

al., 2012; Zapotocky *et al*., 2017). In the non-wingless subgroup of medulloblastoma, tumour metastasis during the period of diagnosis is an indicator of bad prognostic feature (Ramaswamy *et al*., 2016b).

Owing to the widely-reported treatment failure that have pervaded the clinical setting of relapsed metastatic medulloblastoma, the current key focus of investigation is treatment of metastatic compartment (Ramaswamy and Taylor, 2017). Significantly, medulloblastoma has been demonstrated to undergo notable clonal selection and evolution during tumorigenesis; by implication, tumour cells from metastatic compartment are have characteristic genetic and epigenetic changes that are uncommon in the primary tumour cells (Wang *et al*., 2015). This assertion is based on reports from genomic analysis via integrated profiling approach such as copy number variation, DNA methylation, and exon sequencing analysis of corresponding tumour samples derived from human medulloblastoma at primary and metastatic phases; in addition to reports obtained from a murine transposon-driven a model of SHH medulloblastoma, where unified transposon insertion locations were shown to be glaringly different between the primary and metastatic tumours (Wu *et al*., 2012). It is therefore, rationale to suggest that molecular pathways determine the survival of metastatic medulloblastoma cells in the metastatic microenvironment or compartment, and such cells are invariably different from the cells of the primary tumours and specialised targeted treatment will be likely required to achieve an improved survival outcome. Currently, the molecular mechanisms that underlie medulloblastoma metastasis is still poorly understood; consequently, preclinical studies that are aimed at identifying such mechanisms, whereas future trials are needed to establish the defining genetic and molecular profiles of medulloblastoma; and analysis of the metastatic tumour microenvironment via tissue biopsy to establish the presence of possible therapeutic target that could help optimize medulloblastoma patients selection for further experimental therapies. It is also important to delve into the implications of DNA damage repair genes in the development of different molecular subgroups of medulloblastoma; given the universal report of frequent mutations in of key cancer-critical genes, especially the tumour suppressor TP53 gene in most of the medulloblastoma subgroups. This is where this PhD is likely to contribute in improving our understanding of the molecular characteristics of medulloblastoma and consequent development of possible molecular targeted therapies.

1.6. Mesothelioma

Mesothelioma is a rare but prolifically aggressive, asbestos-related cancer that develops on the linings (known as the mesothelium) that cover many internal organs such as the lungs, heart and abdomen (Kondola *et al*., 2016). It is often referred to as a cryptic neoplasm owing to its characteristic ability to maintain a prolonged period of latency following contact with asbestos, remaining asymptomatic and undetected in the body for up to 40 years in some cases (Frank, 2012). It originates from the surfaces of mesothelium tissues in the pleural region; though, it is not exclusive to the pleural mesothelium, as it can emerge in the tunica vaginalis as well as the peritoneal (Teta *et al*., 2008). In the US, Surveillance Epidemiology and End Results (SEER) registry data indicated that the number of reported new cases of mesothelioma per annum is approximately 3,300, compared to about 200,000 lung cancer incidences (Frank, 2012).

The risk of mesothelioma development increases with increased exposure to asbestos and disease symptoms could take about 20 years and sometimes, up to 50 years to appear following contact with asbestos. Currently, patients suffering from mesothelioma show a characteristic poor life expectance, because it has no known cure at present. However, the disease stage, histological subtype and the tumour location are the most significant determinants the survival patient. Additionally, the overall health of the patient, age and whether that cancer has assumed metastatic state, are also of critical prognostic significance (Frank, 2012).

1.6.1. Histology and Prognostic Features of Mesothelioma

Mesothelioma is categorized based on location or cell type. Dependent of the location where a tumour first develops, mesothelioma is subdivided into four primary types, namely: pleural (lungs), peritoneal (abdomen), pericardial (heart), and testicular (testis) mesothelioma (Teta *et al*., 2008).

Pleural mesothelioma is the type of cancer that develops in the tissue surfaces of the linings the lungs, known as the pleura. It is the most predominant form of mesothelioma, accounting for over 80% of reported new cases. Pleural mesothelioma is difficult to diagnose because it shows no or very minimal symptoms in the early stages (Kondola *et al*., 2016). However,

at later stages, symptoms tend to worsen significantly; hence, pleural mesothelioma prognosis is very poor, with most patients surviving less than 17 months following the appearance of initial noticeable symptoms (Barreiro and Katzman, 2006; Kondola *et al*., 2016).

Peritoneal mesothelioma is the form of mesothelioma in which the tumour develops in the thin layer of tissues lining the abdomen, the abdominal mesothelium. Because of the proximity between the abdominal lining and vital abdominal organs, peritoneal mesothelioma frequently spreads to such organs as liver, spleen and bowel. It is characterized by severe pain in the abdominal as the most predominant symptom, in addition to discomfort associated with abdominal effusion (fluid buildup) (Raza *et al*., 2014). Although, peritoneal mesothelioma has poor prognosis, the use of hyperthermic intraperitoneal chemotherapy (HIPEC) in recent years, has somewhat impacted positively on prognosis. In this case, outcomes are patient-specific but survivorship, is dependent on the patient's situation and particular diagnostic factors (Barreiro and Katzman, 2006).

Pericardial mesothelioma is the type of mesothelioma that originates from the tissues lining the heart cavity, the pericardium. Pericardial mesothelioma is very rare, accounting for less than 1% of the total reported cases of mesothelioma. As the disease progresses, it limits the rate of oxygen supply to the heart, resulting in further degeneration of patient's health. The symptoms of pericardial mesothelioma resemble those of heart attack, including severe chest pain and breathing difficulty. In most cases, pericardial mesothelioma remains undiagnosed until autopsy is carried out (Sardar *et al*., 2012).

Testicular mesothelioma is the rarest form of mesothelioma affecting the linings of the testicles. Only fewer than 100 cases of testicular mesothelioma have been diagnosed across the globe; hence, the mechanisms of development and the course of treatment are poorly understood (Frank, 2012).

Currently, the only effective way to confirm mesothelioma diagnosis is by the analysis of cell types in the tissue samples obtained via biopsy. Such histological examination provides insight into which types of cell and subset of cells constitute mesothelioma. Consequently, mesothelioma is categorized into three forms based on the type of constituent cells, namely: epithelioid, sarcomatoid, and biphasic mesothelioma (Frank, 2012).

Epithelioid mesothelioma is the commonest cell type prevalent in mesothelioma, accounting

for over 75% of all diagnosed cases. It is easily distinguishable under high magnification, especially with reference to its elongated pattern of shape. Epithelioid mesothelioma is mostly predominant in lung cancer and has the best prognosis of all mesothelioma cell types (Husain *et al*., 2009).

Sarcomatoid mesothelioma cells originate from supportive tissue structures such as bones and muscles, accounting for less than 10% of all diagnosed cases of mesothelioma. They present the worst prognosis among all the cell types and are usually very difficult to treat. Histologically, they appear elongated and spindle-like and arranged in haphazard fashion (Wu *et al*., 2013).

Biphasic mesothelioma is a mixture of both epithelioid and sarcomatoid cell types, coexisting as a single tumour tissue. It constitutes about 40% of all reported cases of mesothelioma. In this type of mesothelioma, the constituent cell types are differentiated, and by implication, the epithelioid cell types exist in separate area from the sarcomatoid cells. Biphasic mesothelioma has better prognosis than sarcomatoid mesothelioma and poorer prognosis relative to epithelioid mesothelioma (Husain *et al*., 2009).

1.6.2. Molecular Pathogenesis of Malignant Mesothelioma

As reviewed in the preceding sections, malignant mesothelioma is a rare but aggressive cancer that essentially develops in the superficial parts of serosal cells of the pleural region, peritoneum, and sometimes the surface linings of the pericardium and the tunica vaginalis of the testis (Tsao *et al*., 2009). About 80% of the entire reported cases of malignant mesothelioma originated from the pleura and are referred to as pleural mesothelioma or malignant pleural mesothelioma (MPM). Malignant mesothelioma development occurs latently in patients, and due to the ineffectiveness of radiological tools in detecting the disease at early stages, mesothelioma is mostly diagnosed at advanced stage; and biomarkers for early stage diagnosis are yet to be established (Sekido, 2013). In malignant mesothelioma, the structural localisation and features of the body cavities in which the disease originated from, aid the spread and invasion of neighbouring cavities by malignant cells (Tsao *et al*., 2009).

Histologically, three major subtypes of malignant mesothelioma have been characterized, namely, epithelioid, sarcomatoid and biphasic; with rare histological variants also included

in this disease entity (Husain *et al*., 2009). Due to its characteristic unresponsiveness to conventional therapy and concomitant poor prognostic features, as indicated by a paltry 9 to 12 months median survival of patients after diagnosis and considering its recalcitrance to recent advances in chemotherapeutic regimens combining cisplatin and pemetrexed (Vogelzang *et al*., 2003), it is therefore, urgent to undertake more studies to understand the molecular pathogenesis of mesothelioma. Although, some new molecular target drugs against the disease have been developed and occasionally demonstrated stabilization of malignant mesothelioma, such treatment modalities have failed to advance to the stage they could be recommended as an optimal/standard treatment regimen (Jakobsen *et al*., 2011).

Because malignant mesothelioma is a relatively uncommon, research to expose its molecular pathogenesis and understand the genetic and epigenetic alterations that aid malignant mesothelioma development has lagged relative to other common cancer types (Jean *et al*., 2012). In recent times, research findings from global genetic and epigenetic evaluations are facilitating the delineation of basic molecular abnormalities of this rare, but extremely aggressive cancer. Recently, several reviews of mesothelioma have made attempts to describe the myriads of genetic, epigenetic and signalling pathway modifications (Jean *et al*., 2012; Sekido, 2013). Considering the foregoing review, this PhD thesis investigated the expression of several DNA repair genes from different DNA repair pathways such as *Neil1*, *Neil2*, *Neil3,* (BER), *Ercc1,* (nucleotide excision repair, NER) and *Mlh1* (MMR) in cell lines derived from malignant mesothelioma and further, attempted to compare gene expression levels in these cells with cancer stem cells derived from the cell line.

1.6.3. Asbestos-Induced Molecular and Genetic Damage

Exposure to asbestos has been shown to be the major cause of malignant mesothelioma and more than 80% of individuals that have this cancer have reportedly had contact with asbestos at one point in their lifetime (Pass *et al*., 2004). Asbestos represents a group of six mineral fibres, categorised into two subcategories, (a) a collection of fibres that are rod-like, known as the amphiboles, with five constituent members, namely, brown asbestos also called amosite, blue asbestos also known as crocidolite, tremolite, actinolite, and anthophyllite and, (b) the serpentine subcategory, made up of white asbestos, which is also known as chrysotile, as the only member of the category (Baumann *et al*., 2013). The correlation between the asbestos of the amphibole category and the tumorigenesis of malignant mesothelioma has

been extensively documented; particularly the blue asbestos, which has been severally reported to be the type of asbestos with the highest carcinogenic propensity (Mossman *et al*., 1990). An asbestos-like mineral known as erionite has also been implicated as a major cause of malignant mesothelioma (Sekido, 2013).

After the inhalation of asbestos fibres deep into the lungs and their subsequent penetration into the pleural space, the interaction between the asbestos fibres with the cells of the mesothelium and inflammatory cells results in the initiation of prolonged cascade and repeated of tissue damage, tissue inflammation and repair, and the eventual development of malignant mesothelioma in a yet to be established mechanism (Liu *et al*., 2000; Pass *et al*., 2004). It is still unknown why parietal pleura is the initial site of development of asbestos-induced malignant mesothelioma, rather than the visceral pleura. Relative to other cell types, cells of the human mesothelium show very high susceptibility to asbestos toxicity; hence, there is a paradoxical question of how asbestos leads to the development of malignant mesothelioma given that the exposure of human cells derived from the mesothelium to asbestos is expected to lead to the death of the cells (Liu *et al*., 2000; Sekido, 2013).

Many possible mechanisms that define the involvement of asbestos fibres in the development of malignant mesothelioma have been suggested (Toyokuni, 2009; Heintz *et al*., 2010). As represented in Figure 1.1, there are four proposed routes by which asbestos fibres initiate molecular and cytological destructions of cells of the mesothelium and severe inflammation that characterize malignant mesothelioma, namely:

(i) **Generation of reactive oxygen species:** DNA damage including DNA strand breaks are caused by reactive oxygen species (ROS) emanating from the asbestos fibres and their exposed surfaces. Macrophages, while attempting to defend the organism phagocytose the asbestos fibres, however, they are unable to digest them, resulting in further production of abundant ROS (Toyokuni, 2009; Sekido, 2013).

(ii) **Engulfment of asbestos fibres by mesothelial cells:** the uptake of asbestos fibres by the cells of the mesothelium can result in its physical interference with the process of mitosis through the disruption of the mitotic spindles. Chromosomal structural aberrations and mesothelial aneuploidy could result from the entanglement of the asbestos fibres with mitotic spindles or

chromosomes (Toyokuni, 2009).

(iii) **Uptake of chemicals and binding of proteins by asbestos fibres:** a variety of chemicals and proteins are adsorbed by the asbestos fibres to the extensive surface of asbestos leading to the accumulation of hazardous molecules including those capable of causing neoplasm of the mesothelium. Additionally, some important proteins have high affinity for asbestos fibres and when such proteins are deficient, mesothelial cells may be negatively affected (Toyokuni, 2009).

(iv) **Release of cytokines and growth factors:** mesothelial cells and macrophages exposed to asbestos release myriads of cytokines and growth factors such as tumour necrosis factor-α (TNFα), interleukin-1β (IL-1β), transforming growth factor-β (TGFβ), and platelet-derived growth factor (PDGF), which induce inflammation and promote tumour development and cellular proliferation (As reviewed by Sekido, 2013). For instance, it has been demonstrated that TNFα activates nuclear factor-κB (NF-κB), resulting in mesothelial cell survival and inhibition of asbestos-induced cytotoxicity (Yang *et al*., 2006). It has also been shown that following the exposure of mesothelial cells to asbestos, they release high-mobility group box 1 protein and then, undergo necrosis, promoting inflammatory response (Yang *et al*., 2010). From the foregoing models of asbestos-induced pathogenesis of mesothelioma, it is rational to suggest that the aberrantly activated molecular signalling network among mesothelial cells, inflammatory cells, fibroblasts and other stromal cells that were exposed to asbestos, may result in a congregation of mutant clones of mesothelial cells, which harbour DNA damage and aneuploidy, and aggregate as cancer cells, forming a tumour microenvironment of mesothelioma as shown in Figure 1.4 (Sekido, 2013)

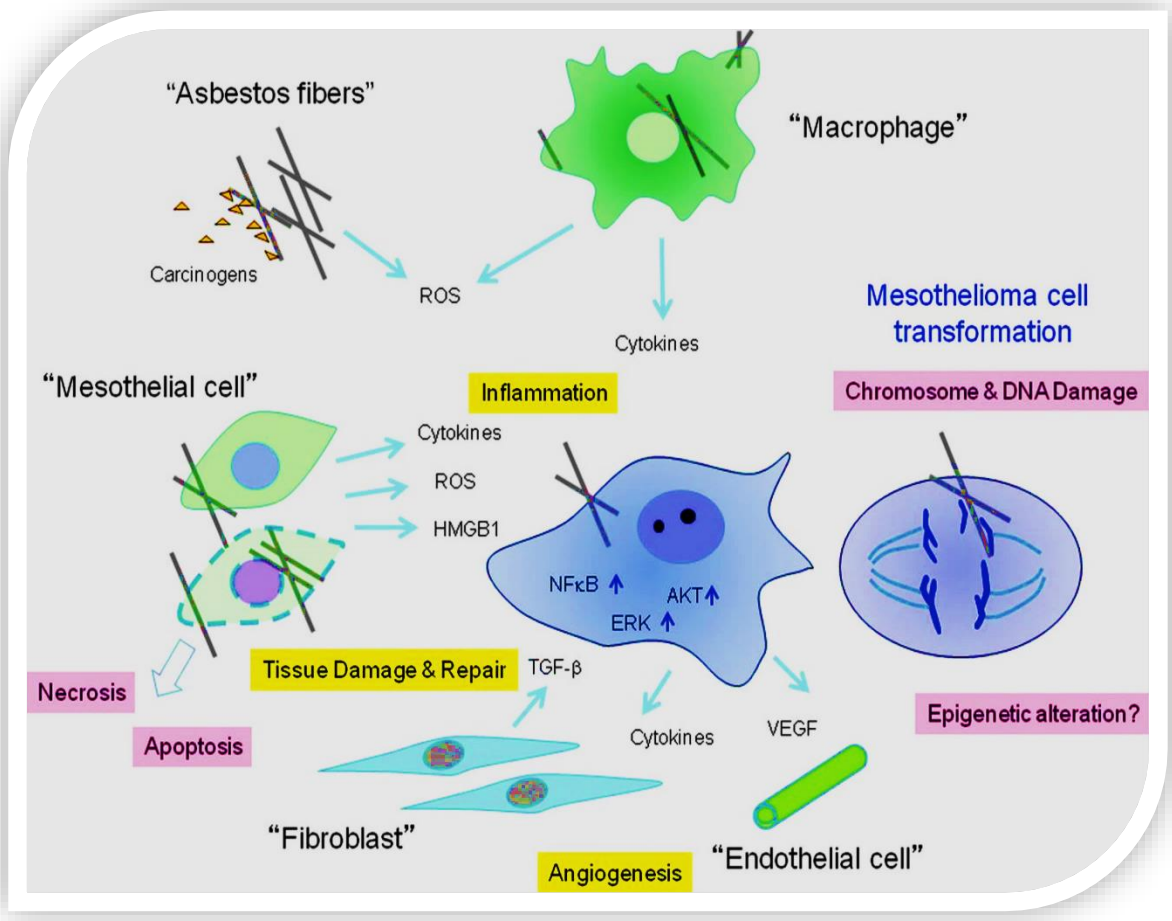


Figure 1.4. A schematic representation of the molecular mechanisms underlying the process of tumour development due to asbestos fibres. High-mobility group box 1 (HMGB1) protein; reactive oxygen species (ROS); transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) (Sekido, 2013).

Asbestos-induced DNA damage in cells of the mesothelium or DNA damage emanating from other factors must be correctly and timely repaired to ensure that DNA integrity in the cells is maintained. Base excision repair (BER), NER, MMR and homologous recombination and non-homologous end-joining (for the repair of double strand breaks) have been reported as the key DNA damage repair mechanisms in mammalian cells (Toumpanakis *et al*., 2011). Each of these DNA repair routes have been variously shown to be significantly overexpressed in malignant mesothelioma, and most prominent of them are genes related to the repair of double-strand breaks (Røe *et al*., 2010). Similarly, genetic polymorphisms in genes that encode BER proteins such as X-ray cross complementing group 1 (XRCC1) have been reported to be overexpressed in malignant mesothelioma (Røe

et al., 2010). It is therefore, rational to hypothesize that the increased expression of some genes involved in DNA repair and their products could be responsible for the observed resistance of mesothelioma to chemotherapy and radiotherapy; hence, the focus of this research. In later sections of this chapter, a detailed review of DNA repair genes and their implications in cancer development and therapy will be presented.

Besides the asbestos-dependent molecular pathogenesis of malignant mesothelioma reviewed above and the models of mesothelioma development represented in Figure 1.4, there are several other molecular events and signalling pathways that determine the development of malignant tumours. These asbestos-independent molecular pathogenetic pathways of malignant mesothelioma are predicated on DNA damage affecting the proto-oncogenes and tumour suppressor genes, resulting in their activation and inactivation respectively. The next sections will attempt to review the molecular pathogenesis of malignant mesothelioma on the basis of oncogenic activation and tumour suppressor gene inactivation.

1.6.4. Activation of Oncogenic Cascades

In malignant cells of most cancer types, there is a characteristic activation of receptor tyrosine kinases (RTK) (Baselga, 2006). Activation of RTKs results in the concomitant overexpression of two major oncogene-dependent cell signalling pathways, the phosphoinositide-3 kinase (PI3K)-AKT pathway and Raf-MEK-extracellular signal-regulated kinase, which are known to regulate the growth, proliferation and survival of malignant cells (Sekido, 2013). The RTK oncogenes such as epidermal growth factor receptor (EGFR) and MET have been shown to be constitutively and simultaneously activated in many cultured malignant mesothelioma cells (Brevet *et al*., 2011). There have also been reports of other receptors of RTK such as AXL, which were suggested to be associated with more malignant phenotypes of mesothelioma (Laurie *et al*., 2011).

In a similar vein, the activation of the mammalian target of rapamycin (mTOR) signalling pathway has been reported to significantly contribute to the pathogenesis of many cancer types, including malignant mesothelioma (Hartman *et al*., 2010). In a related event, Varghese *et al*. (2011) demonstrated that the upregulation of PI3K and mTOR signalling pathways in patients with malignant peritoneal mesothelioma resulted in shortened survival.

In addition to the pathways of mitogen-activated protein kinase (MAPK) and PI3K-AKT, reports have also indicated the aberrant activation and overexpression of the signal transducer and activator of transcription 1 (STAT1) in malignant mesothelioma using a phosphotyrosine proteomic screen (Menges *et al*., 2010). Besides the frequent activation of STAT1, the Src group of kinases have as well, been suggested to be required in the development of malignant mesothelioma (Menges *et al*., 2010).

1.6.5. Inactivation of Tumour Suppressor Genes

In human malignant mesothelioma, three major tumour suppressor genes, including the cyclin-dependent kinase inhibitor 2A (*CDKN2A*), neurofibromatosis type 2 (*NF2*), and BRCA1-associated protein-1 (*BAP1*) genes, are frequently altered, resulting in their inactivation and the aberrant expression of their respective gene product and concomitant dysfunctional substrate interaction; culminating in cancer cell proliferation, survival and resistance to therapy. In this section, the attendant implications of the inactivation of these key tumour suppressor genes will be reviewed, with emphasis on the type of mutation events that produce the DNA damage.

(i) The cyclin-dependent kinase inhibitor 2A/alternative reading frame genes: In malignant mesothelioma, the *CDKN2A* gene is known to be prone to mutation and has been reported as the most frequently mutated and inactivated tumour suppressor gene (Foulkes *et al*., 1997; Guo *et al*., 2015). The gene is localised on chromosome 9p21.3 and encodes two proteins, p16INK4a and p14ARF. Cell cycle fate is determined by p16INK4a through the combined pathways of cyclin-dependent retinoblastoma protein and cyclin-dependent kinase 4 (CDK4). On the other hand, p14ARF modulates the Tp53 pathway by inactivating MDM2 that in turn, controls p53 function (Musti *et al*., 2006). Hence, when *CDKN2A* is deleted in a homozygous fashion, two main tumour-suppressing pathways of retinoblastoma and p53 are deactivated in malignant mesothelioma cells. Over the past decade, several reports have shown that the analysis of malignant mesothelioma tissues or cell lines via fluorescent *in situ* hybridization (FISH) revealed that more than 70% of the analysed samples showed uniform loss of the *CDKN2A* locus (Chiosea *et al*., 2008; Matsumoto *et al*., 2013; Wu *et al*., 2013). Based on histological sub-categorization, malignant mesothelioma that belongs

to the epithelioid type showed approximately 70% of *CDKN2A* homozygous deletions, while sarcomatoid malignant mesothelioma cases displayed approximately 100% homozygous deletion of the *CDKN2A* gene (Matsumoto *et al*., 2013).

(ii) **Neurofibromatosis type 2 inactivation:** the *NF2* gene encodes moesin-ezrin-radixin-like protein (Merlin), a tumour suppressor protein, belonging to the band 4.1 family of cytoskeletal linker proteins (Bianchi et al., 1995). Tumorigenesis of the nervous system such as bilateral vestibular schwannomas at the eighth cranial nerve, spinal schwannomas and meningiomas, are a defining characteristic of NF2 cancer syndrome; and bi-allelic mutations of *NF2* are also reported to occur frequently in sporadic cases of these tumours (Thurneysen *et al*., 2009). In malignant mesothelioma, the *NF2* gene has been variously reported to be the key tumour suppressor gene most affected by the loss of the chromosomal locus 22q12 (Bianchi *et al*., 1995), with 40 – 50% of malignant mesothelioma cases shown to be harboring an inactivation mutation (Murakami *et al*., 2011). The tumour suppression role of Merlin has been reported to be modulated by the E3 ubiquitin ligase CRL4 (DCAF1) in a study that employed malignant mesothelioma cells and immortalized mesothelial cells (Li *et al*., 2010).

(iii) **Deactivation of BRCA1-associated protein-1 (BAP1):** The *BAP1* gene has been shown to be a very important tumour suppressor gene in malignant mesothelioma, where 23% of 53 cases were shown to have somatic mutationsin *BAP1* (Bott *et al*., 2011). Similarly, a study conducted by Yoshikawa *et al*. (2012), using malignant mesothelioma patients of Japanese origin, showed that *BAP1* is frequently mutated. *BAP1* is localized to chromosome 3p21.1, where it is responsible for the production of a nuclear ubiquitin C-terminal hydrolase, which is a member of the deubiquitinating enzymes. The substrate specificity of BAP1 is characteristically broad and it is known for its affinity for several substrates including the host cell factor 1 transcriptional scaffolding subunit, which is an N -acetyl-glucosamine transferase that is O-linked, human orthologs of additional sex combs (ASXL1/ASXL2), and fork-head transcription factors (FOXK1/FOXK2). BAP1 has been reported to have a role in a variety of cellular

processes such as, cell growth control, response to DNA damage, chromatin dynamics, and modulation of the cell cycle (Eletr & Wilkinson, 2011). Additionally, it has also been reported that BAP1 is involved in modulating polycomb target proteins in malignant mesothelioma tissues (Murali et al., 2013). Interestingly, two families with phylogenetic history of high incidence of mesothelioma were shown to have germline mutations of the *BAP1* gene and some carriers of *BAP1* gene mutations in the two families developed other types of cancer (Testa *et al*., 2011).

1.6.6. Chemotherapeutic Treatment of Mesothelioma

Over many years, research has strived to establish the best chemotherapeutic treatment regimen for mesothelioma, however, the results have been disappointing, partly due to the ability of mesothelioma to resist chemotherapy and the non-availability of effective chemotherapeutic agents with minimal cytotoxicity (Frank, 2012). Single anticancer agents such as antimetabolites, anthracyclines, and platinum-based agents have been studied but only show an approximate response rate of 10% (Su, 2009). Oncologists and scientists were previously concerned that chemotherapeutic treatment of mesothelioma failed to produce better patient outcome relative to the lone implementation of best supportive care (BSC). Consequently, the UK Medical Research Council (MRC) tried to develop the key advantages of chemotherapeutic use, when compared to supportive care in a clinical trial in individuals suffering from mesothelioma, who were not previously treated (Muers *et al*., 2008). The research design employed was a three-arm design and participants were randomized to best supportive care with the inclusion or exclusion of one out of two chemotherapeutic regimens: vinorelbine as a single agent or a combination regimen comprising mitomycin, vinblastine, and cisplatin. Upon combination and comparison, the results of the two arms of chemotherapy relative to BSC alone, showed median survival for patients at 8.5 months and 7.6 months respectively; which showed statistical insignificance. Further statistical exploration of the two chemotherapy arms independently showed that patients treated with vinorelbine had a median survival of 9.4 months, but patients in the MVP arm had no significant survival advantage (Muers *et al*., 2008; Frank, 2012).

As a monotherapy, vinorelbine was further tested in a second-line or salvage setting, in

which 63 patients with relapsed or refractory mesothelioma were given the drug weekly. The results produced 16% rate of susceptibility and 9.6 months median survival rate (Stebbing *et al*., 2009). However, a combination therapy regimen of vinorelbine and cisplatin as first-line of treatment in patients with non-resectable malignant pleural mesothelioma, achieved a 30% response rate, median survival rate of 16.8 months and median period of progression of 7.2 months (Sørensen *et al*., 2008).

In 23 patients with untreated cases of malignant pleural mesothelioma, the use of gemcitabine monotherapy achieved a 31% rate of response and improved symptoms were recorded in over 40% of the participants, (Bischoff *et al*., 1998). However, the sample size employed by the researchers was too small and the patients in the trial were all at the early-stage of the disease and promising epithelial histology (Frank, 2012). In other clinical trials, where gemcitabine was used as a monotherapy, the response rates were disappointing, ranging from 0 to 7% and median survival of 4.7 months to 8 months (Kindler *et al*., 2001). Interestingly, clinical trials employing a combination therapy regimen of gemcitabine and cisplatin or carboplatin resulted in response rates of 12% to 48% and median time to progression of 6 months to 9 months (Jackman, 2009).

Berghmans *et al*. (2002) reported that cisplatin was the potent monotherapy to which, un-resectable malignant pleural mesothelioma is susceptible; hence, serves as a standard chemotherapy for the disease. The reliability and potency of cisplatin as a single therapy has made it the backbone of most doublet regimens, over the years. On this note, Vogelzang *et al*. (2003) reported that the dual therapy of cisplatin and pemetrexed is considered the optimal treatment option and opined that it should be regarded as a standard first-line chemotherapy for the treatment of unresectable mesothelioma that has assumed malignancy; and indeed, it has become a treatment benchmark which is recommended as the combination therapy regimen to resectable mesothelioma (Frank, 2012).

Regardless of the improvement recorded with the use of cisplatin and pemetrexed combination therapy regimen against mesothelioma; the treatment of the disease, like most cancer types, is still gravely challenged by chemotherapeutic resistance. Consequently, approximately 67% of the investigated patients failed to demonstrate positive response to the cisplatin - pemetrexed combined therapy approach, and majority of the subjects will progress after first-line therapy and usually die no later than one year after diagnosis (Green *et al*., 2007). On this note, scientists have been gearing efforts towards finding and

developing better chemotherapeutic regimen by exploiting the activities of molecular markers that promote mesothelioma response to cisplatin-pemetrexed combination therapy (Castagneto *et al*., 2007).

Interestingly, one of the genes that formed the focus of this PhD work, the excision repair cross-complementing 1 (*Ercc1*) gene, has been variously shown to enhance cisplatin activity against different cancer types. For instance, *Ercc1*, which is primarily known for the repair of helix-distorting DNA adducts, has been shown to also repair DNA strand damage caused by cisplatin and the expression of *Ercc1* gene correlates with favourable prognosis in ovarian cancer and non-small cell lung cancer patients treated with chemotherapeutic regimen containing cisplatin (Simon *et al*., 2005). Zucali and colleagues (2011) in a similar vein, reported that there is a positive interrelationship between the upregulation of thymidine synthetase (TS) protein and prolonged progression-free survival, and mesothelioma patients' overall survival, in a carboplatin-pemetrexed treatment regimen.

1.6.7. New Approaches to Mesothelioma Treatment: Targeted Therapy

Like most cancer types, successful treatment of patients with malignant mesothelioma remains a challenge, due to the usual chemotherapeutic resistance that bedevils most cancers. In addition to its characteristic resistance to therapy, mesothelioma treatment is further hampered by its inherent latency and consequent late diagnosis and poor prognosis. Currently, treatment approaches are: chemotherapy, and multimodal treatment including surgical resection combined with chemotherapy and/or radiotherapy, photodynamic therapy (PDT), and hyperthermic perfusion of the pleura followed by resection (Zervos *et al*., 2008; Bononi *et al*., 2015). These treatment regimens may positively affect the patient's quality of life; however, only modest effects have been recorded with same treatment modalities in improving overall survival. Increased understanding of the molecular pathogenesis and characteristics of mesothelioma, and the rationale behind investigating novel targeted approaches have resulted in development of novel potential therapeutic strategies aimed at exploiting the molecular features of mesothelioma to produce action-specificity; hence, the name targeted therapy. In the recent past, the identification and characterization of many different forms of mutation, a variety of enzymatic catalases, myriads of growth factors, and glycoproteins that contribute to the inherent refractory and poor prognostic features of

malignant mesothelioma led to the development of targeted therapies including (i) molecular therapies, (ii) immunotherapy, (iii) targeting asbestos-induced inflammation (Bononi *et al*., 2015). However, for the purpose of this thesis, only molecular therapies will be reviewed.

1.6.7.1. Molecular Therapies

Few years ago, it was reported that malignant mesothelioma are polyclonal tumours, formed by the aggregation of different independent subclones, which may account for a high degree of cellular heterogeneity within the tumour microenvironment and contribute to the development of chemo-resistant subpopulations in various *in vitro* experiments conducted (Comertpay *et al*., 2014). The characteristic heterogeneity of cell subpopulation in malignant mesothelioma highlights the importance of simultaneously attacking several different molecular targets to obliterate the different clones, given that each clone may be defined by distinct set of molecular alterations (Bononi *et al*., 2015).

From the foregoing review, the importance of *BAP1* gene as a major tumour suppressor gene, its physiological role in biological processes, as well as its importance in the molecular pathogenesis of malignant mesothelioma cannot be overemphasized. It is therefore, not surprising that *BAP1* gene and its gene products are of key interest in the quest of researchers to develop molecular-based target therapies that are action-specific. For instance, it has been reported that the somatic loss of *BAP1* is associated to a slightly longer survival (Arzt *et al*., 2014). This finding is probably rationalized by the fact that most somatic mutations are frequently detected in epithelioid malignant mesothelioma, which have a better prognosis than sarcomatoid and biphasic malignant mesothelioma (Farzin *et al*., 2015). When malignant mesothelioma occurs in a setting of germline *BAP1* gene mutations, their prognosis is markedly better with survival of over 5 – 10 years (Baumann *et al*., 2015). However, it is surprising that germline *BAP1* mutations promotes malignant mesothelioma on one hand, while they are on the other hand, reported to reduce the disease aggressiveness. The mechanistic underpinnings of this dual role of double-edged sword activity are yet to be elucidated.

BAP1 functions as a tumour suppressor have been ascribed to (i) BAP1 deubiquitination of histone H2A, leading to transcriptional activation of genes that regulate cell growth (Scheuermann *et al*., 2010); (ii) BAP1 functions as a transcriptional coregulator by

interaction to host cell factor-1 (HCF1), Ying Yang 1 (YY1), and E2F1, to induce transcription of genes involved in cell cycle regulation (Yu *et al*., 2010); and (iii) BAP1 contribution to DNA repair (Ismail *et al*., 2014; Yu *et al*., 2014), as depicted in Figure 1.5.

Owing to the implication of BAP1 in chromatin re-modelling and its inherent ability to deubiquitinate H2A, it is very likely that BAP1 mutations promote cellular sensitivity to epigenetic modulators. The epigenetic regulation of tumour suppressor genes via chromatin condensation and de-condensation has been reported to be of mechanistic significance in the development of mesothelioma and most cancers (Bononi *et al*., 2015). For instance, the homeostasis of the acetylated and deacetylated forms of histone proteins is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation is increased by HATs, facilitating greater accessibility of chromatin for gene expression. On the other hand, HDACs inhibitors alter the wrapping of DNA strands around histones, modifying the access of transcription factors and affecting the expression of different genes (Landreville *et al*., 2012).

The therapeutic effect of four different HDAC inhibitors, valproic acid, trichostatin A, LBH-589, and suberoylanilide hydroxamic acid (vorinostat), has been investigated using primary UVM cells and in UVM cell lines. Interestingly, these compounds were shown to reverse H2A hyper-ubiquitination due to BAP1 loss, and initiated differentiation, cell cycle exit, a shift to a differentiated, melanocytic gene expression profile in cultured UVM cells. However, valproic acid inhibited UVM tumour growth in vivo (Landreville *et al*., 2012). In various In vitro experiments to understand the role of HDAC inhibition in malignant mesothelioma, increased apoptosis was predominantly reported in malignant mesothelioma cell lines following treatment with HDAC inhibitors alone or in combination with chemotherapy (Neuzil *et al*., 2004; Bolden *et al*., 2006; Symanowski *et al*., 2009).

Germline mutations resulting in the loss of BAP1 has been shown to alter the sensitivity of malignant pleural mesothelioma cells to HDAC inhibitors through the regulation of HDAC2 transcription. However, established malignant mesothelioma cell lines with low endogenous HDAC2 were found to be resistant to HDAC inhibition (Sacco *et al*., 2015). These reports are indicative of the fact that HDAC inhibitors might be effective in the adjuvant therapy of patients with BAP1 mutated malignant mesothelioma.

Vorinostat is approved by the FDA for the treatment of cutaneous T-cell lymphoma. In a

Phase I clinical trial using patients with advanced malignant pleural mesothelioma, 30% of patients that received vorinostat had a stabilization of their disease lasting more than 4 months (Kelly *et al*., 2005). However, negative results have been reported in phase II and phase III clinical trials of vorinostat and belinostat respectively, in patients with advanced pre-treated malignant pleural mesothelioma (Ramalingam *et al*., 2009; Krug *et al*., 2015) as shown in Figure 1.5.

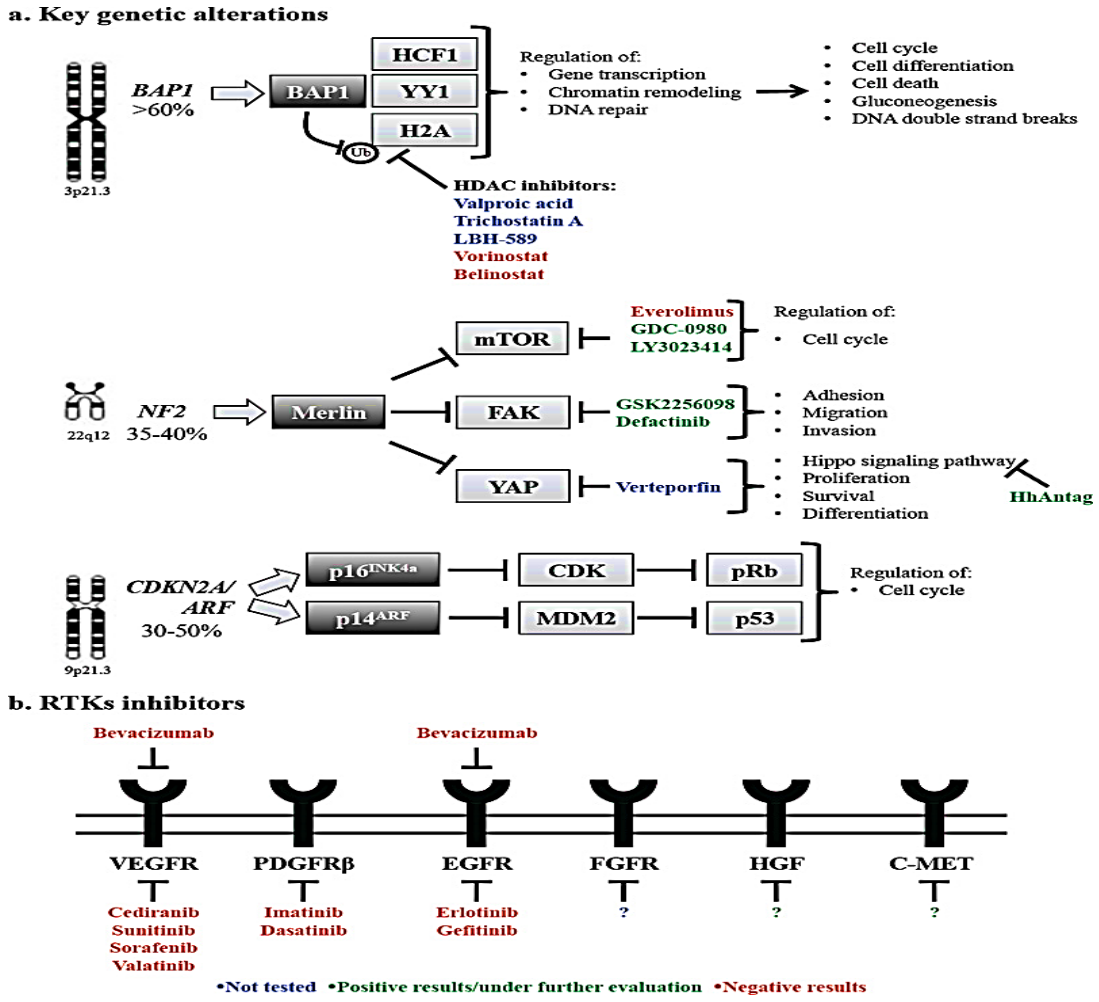


Figure 1.5. Schematic representation of the key molecular and genetic alterations involved in the development of malignant mesothelioma and possible strategies for therapeutic intervention (Bononi *et al.*, 2015).

Besides BAP1, the role of NF2 gene, another tumour suppressor gene, in the pathogenesis of malignant mesothelioma was originally reported by data showing that asbestos-treated $NF2^{+/-}$ mice exhibit a significantly accelerated malignant mesothelioma tumour formation relative to wild-type littermates (Altomare *et al*., 2005). Merlin, which is the gene

product encoded by NF2 gene, interacts with multiple substrates thereby, modulating multiple signal transduction cascades including mTOR, focal adhesion kinase (FAK) and Hippo signalling pathways (Bianchi *et al*., 1995) as shown in Figure 1.5.

Cellular proliferation is mediated by merlin through the inhibition of mTOR, in an AKT-independent manner (Ladanyi *et al*., 2012). Loss of merlin has been shown to result in the activation of mTOR signalling in malignant mesothelioma cells; hence, in merlin silenced tumours, mitogenic signalling is highly upregulated and cellular proliferation is markedly increased. Expectedly, merlin-negative malignant mesothelioma cells were shown to be more sensitive to the mTOR inhibitor rapamycin, relative to merlin-positive cells (Lopez-Lago *et al*., 2009). Owing to this significant observation, mTOR has been identified as a therapeutic target in the large fraction of malignant mesotheliomas that carry NF2 mutations and provided the rationale for the further study of mTOR inhibitors as possible molecular target therapy in malignant mesothelioma. However, it was unfortunate that the oral form of mTOR inhibitor known as everolimus, when tested in a phase II clinical trial as second- and third-line of treatment in unselected pre-treated malignant pleural mesothelioma patients, showed limited clinical activity.

It is imperative to note that mTOR inhibition alone results in compensatory upregulation of PI3K, consequently permitting the restoration of the downstream AKT signalling (Carracedo *et al*., 2008). To address this mechanism of mTOR resistance, GDC-0980, a potent and selective oral dual inhibitor of class I PI3K and mTOR were tested. GDC-0980 demonstrated broad activity in various xenograft cancer models, including malignant pleural mesothelioma (Kanteti *et al*., 2014), but pulmonary toxicity of this class of agents limits their application in a clinical setting. Other molecular alterations that are targeted for therapeutic use against malignant mesothelioma include cyclin-dependent kinase inhibitor 2A (CDKN2A) / alternative reading frame (ARF) and neurofibromatosis type 2 (NF2), VEGF, *etc*.

From the forgoing review, it is evident that scientists are more interested in the molecular alterations that bring about the development of malignant mesothelioma, without recourse to the implication of correctional events that physiologically repair these alterations. Consequently, this PhD thesis places emphasis on the involvement of DNA repair genes and their products in the treatment of malignant mesothelioma; with the view to providing insights into the possible therapeutic significance.

1.7. Stem Cells

Embryonic stem (ES) cells are obtained from the inner cell mass of an embryo, typically during the developmental stage known as a blastocyst (Ramalho-Santos and Willenbring, 2007). They are characterised by two distinguishing features: pluripotency, which is the capacity of embryonic stem cells to generate all the other cell types that constitute the histology of adult organisms (Kim and Orkin, 2011; Takahashi and Yamanaka, 2015); and self-renewal ability, which defines the ability of embryonic stem cells to retain its cellular characteristics whilst maintaining a proliferative state (Choumerianou *et al*., 2008). The realisation of the full potential of ES cells in basic biology, biomedicine and regenerative medicine is critically dependent on understanding these two keys defining characteristics (Kim and Orkin, 2011). Additionally, the applicability of ES cells in regenerative medicine and related fields of science is further determined by knowledge of the molecular and genetic underpinnings of ES cells (Roeder and Radtke, 2009).

For the past two decades, research has been ongoing to decipher the molecular characteristic of ES cells and enhance their usefulness to mankind. Consequently, a group of transcription factors, including the homeodomain protein Oct4, Sox2, and Nanog (Tai and Ying, 2013), were identified and demonstrated in different studies, as major regulatory factors responsible for the control of the pluripotent feature of embryonic stem cells (Mitsui *et al*., 2003; Chambers *et al*., 2003; Avilion *et al*., 2003). This cadre of regulatory transcription factors are known as ES cell core factors (Kim and Orkin, 2011). The relevance of transcriptional regulatory mechanisms to cell fate control and pluripotency (Graf *et al*., 2009) cannot be overemphasised. Interestingly, Yamanaka and Takahashi (2015) reported that the introduction of the transcription factors Oct4, Sox2, Klf4, and Myc (Tan *et al*., 2013), into somatic cells can result in the reprogramming of the somatic cells into ES cell-like cells known as induced pluripotent stem (iPS) cells (Yamanaka and Takahashi, 2006; Takahashi *et al*., 2007; Wernig *et al*., 2007; Yu *et al*., 2007; Park *et al*., 2008a).

Owing to the attendant advancements in high-throughput technologies over the past decade, enormous databases of proteomic and genomic information have been assembled through such technologies like gene expression profiling, microarrays or sequencing

(Hawkins *et al*., 2010), the study of the interactions between and within proteins, the use of affinity purification and mass spectrometry for the identification of members of protein complexes (Gavin *et al*., 2011), and the downregulation of genes by RNA interference (Dykxhoorn and Lieberman, 2005). These new tools have been employed in the dissection of pluripotency and self-renewal control in embryonic stem cells (MacArthur *et al*., 2009; Roeder and Radtke, 2009) and further deepened the comprehensibility of our understanding of cell states at system level (Marks *et al*., 2012).

Delineation of the cells that sustain cancer has been a very critical goal in the context of cancer biology. Consequently, researchers have proposed that upon transplantation, a small population of cells have characteristic ability of re-initiation of tumour formation; and responsible for maintenance of the tumours and their resistance to anticancer therapies (Kim and Orkin, 2011). These tumour-initiating cells otherwise known as cancer stem cells might originate from progenitor cells or adult stem cells or from somatic cells dedifferentiation (Reya *et al*., 2001; Boroviak *et al*., 2014). Scientists have proposed the hypothesis that ES cells and cancer cells share commonalities relating to the regulation of gene expression, which has likely implications and linkage to the embryonic state of tumour initiating cells prevalent in tumour microenvironment (Kim *et al*., 2010). Additionally, evidence deduced from research works that employed somatic cell reprogramming has further underscored key similarities between tumour cell and induced-pluripotent stem cells. Pluripotency acquisition during reprogramming, typically represents a reversal of differentiation suggested for certain cancer types (Daley, 2008; Papatsenko *et al*., 2015). Several researchers have made concerted efforts to explain the inherent characteristic of cancer stem cells to renew self, and as a result, specific expression signatures of embryonic stem cells have been defined, which have also been analysed in diverse cancers (Wong *et al*., 2008; Somervaille *et al*., 2009; Schoenhals *et al*., 2009; Mizuno *et al*., 2010; Shats *et al*., 2011; Radzisheuskaya and Silva, 2014).

In the experimental segments of this PhD, the expression profiles of some key DNA repair genes in ES cells are investigated with the views to gain insights into the shared similarities between ES cells and tumour initiating cells and contributing to the repository of information about the molecular definition of the stem cell like state of tumour initiating cells. Based on this rationale, this part of the Introduction will attempt to critically review the molecular signatures and gene regulations in ES cells, with emphasis on transcriptional factors, regulation of somatic cell reprogramming, and the activities of different genes

encoding DNA repair proteins within the different DNA repair mechanisms. These will be aimed at gaining more insights into the molecular and cellular similarities between embryonic stem cells and cancer cell and furthering our understating of the role of acquired self-renewal ability of cancer cells in development of drug resistance capabilities by tumour cells.

1.7.1. Molecular Signatures and Genomic Regulations in Embryonic Stem Cells

To understand the molecular signature of embryonic stem cells, it is important to present a synopsis of the key modulators of the self-renewal and pluripotent characteristics of embryonic stem cells, and the acquisition of pluripotent features during somatic cell reprogramming. Consequently, Table 1.4 captures these key modulators of pluripotency in stem cells, their physiological relevance, and the molecular techniques employed in the methods of their investigation (Kim and Orkin, 2011).

Table 1.4. Presentation of the genomic studies of self-renewal and pluripotency characteristics of embryonic stem cells.

Regulators	Function	Methods	Reference(s)
Core factors			
Oct4 (Pou5f1)	ES cell core factor	ChIP, MS	Loh *et al*., 2006; Kim *et al*., 2008; van den Berg *et al* 2010; Pardo *et al*., 2010; Radzisheuskaya and Silva, 2014; Dunn *et al*., 2014; Xu *et al*., 2014.
Sox2	ES cell core factor	ChIP, MS	Chen *et al*., 2008; Kim *et al*., 2008; Lopez-Bertoni *et al*., 2014; Wu *et al*., 2014.
Nanog	ES cell core factor	ChIP, MS	Loh *et al*., 2006; Chen *et al*., 2008; Kim *et al*., 2008; Costa *et al*., 2013; Do *et al*., 2013; Gingold *et al*., 2014.
Tcf3 (Tcf7l1)	Wnt signalling	ChIP	Cole *et al*., 2008; Martello *et al*., 2012
Klf4	LIF signalling	ChIP	Chen *et al*., 2008; Kim *et al*., 2008
Stat3	LIF signalling	ChIP	Chen *et al*., 2008; van Oosten *et al*., 2012
Dax1 (Nr0b1)	Negative regulation of transcription	ChIP, MS	Wang *et al*., 2006; Kim *et al*., 2008

Sall4	Self-renewal and pluripotency	ChIP, MS	Wang *et al*., 2006; Yang *et al*., 2008; Lim *et al*., 2008; Rao *et al*., 2010
Polycomb-related factors			
Ezh2	PRC2, repressor	ChIP, MS	Shen *et al*., 2009; Peng *et al*., 2009
Jarid2	Fine-tuning of PRC2	ChIP, MS	Shen *et al*., 2009; Peng *et al*., 2009
Mtf2	Polycomb-like protein	ChIP, MS	Shen *et al*., 2009
Suz12	PRC2, repressor	ChIP, MS	Boyer *et al*., 2006; Lee *et al*., 2006; Shen *et al*., 2009; Peng *et al*., 2009.
Eed	PRC2, repressor	ChIP	Boyer *et al*., 2006; Lee *et al*., 2006.
Rnf2	PRC1, repressor	ChIP	Boyer *et al*., 2006.
Phc1	PRC1, repressor	ChIP	Boyer *et al*., 2006.
Myc-related factors			
Myc	Proliferation	ChIP, MS	Chen *et al*., 2008; Kim *et al*., 2010.
Max	Myc-inter*ACTIN*g	ChIP, MS	Kim *et al*., 2010.
Zfx	Self-renewal	ChIP	Chen *et al*., 2008.
Trrap	Histone acetylation	RNAi	Fazzio *et al*., 2008.
Tip60 (Kat5)	Histone acetylation	ChIP, MS, RNAi	Fazzio *et al*., 2008; Kim *et al*., 2010.
Ep400	Histone acetylation	MS, RNAi	Fazzio *et al*., 2008.
Dmap1	Histone acetylation	ChIP, MS, RNAi	Fazzio *et al*., 2008; Kim *et al*., 2010.
E2F1	Regulator of cell cycle	ChIP	Chen *et al*., 2008.
E2F4	Transcription activator	ChIP, MS	Kim *et al*., 2010.
Cnot3	General transcription regulator	ChIP, RNAi	Hu *et al*., 2009.
Trim28 (Tif1b)	Transcription co-activator	ChIP, RNAi	Hu *et al*., 2009

LIF = leukaemia inhibitory factor.

Adapted from: (Kim and Orkin, 2011; Morgani *et al*., 2017).

1.7.2. Core Transcriptional Regulators in Embryonic Stem Cells

The availability cytokines in conjunction with many other factors regulate pluripotency and self-renewal characteristics of embryonic stem cells in the cell culture environment (Morey *et al*., 2015). An aggregation of regulatory networks called the pluripotency gene regulatory network (PGRN) is responsible for maintaining ES cell pluripotency (Morgani *et al*., 2017); and-pluripotency is believed to exists in many different states, dependent on the stage of the progenitor cells (Davidson *et al*., 2015).

Pluripotency exists transiently in mouse embryo, from the onset of cleavage cycles until late stages of blastocyst (Boroviak *et al*., 2014). This is contrary to the human embryonic stem cells cultivated in vitro, where differentiation requires that the cells become non-pluripotent, whereas conversion of somatic cells to induced-pluripotent cells needs reversal to pluripotent state (Takahashi and Yamanaka, 2015). Embryonic stem cells and induced-pluripotent cells are likely to be subject to the same basic PGRN network; hence, controlling reprogramming and differentiation is dependent on the knowledge of the various network of genes that transcriptionally regulate cell fate transition and lineage determination (Morgani *et al*., 2017).

Genome-wide studies have in recent years, expanded the list of regulatory factors in the embryonic stem cell pluripotency network (Marks *et al*., 2012). For instance, reconstructed networks of transcriptional regulators that demonstrated many similar features were independently produced by Dunn *et al*. (2014) and Xu *et al*. (2014). Multiple positive correlations between the differently reconstructed transcriptional networks were identified when the two constructs were merged as shown in Figure 1.6.

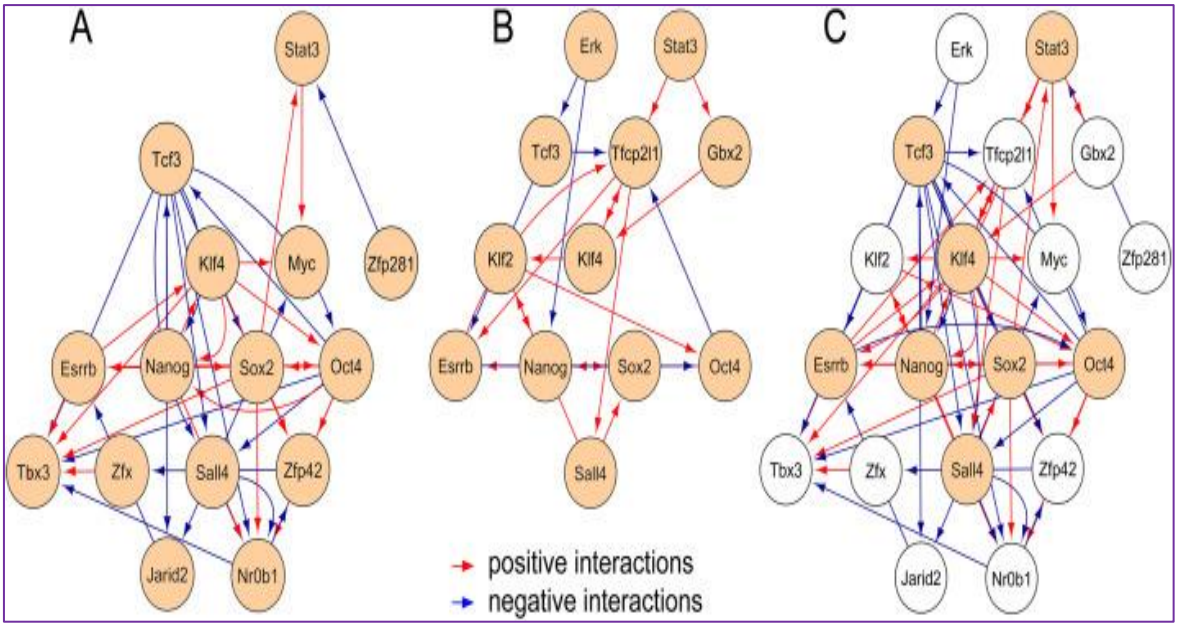


Figure 1.6. A comparative representation of published regulatory networks for embryonic stem cells, showing pluripotency network reconstructions with respect to published reports (A & B) and a combination of both networks to reveal areas of negative and positive interactions (C; Papatsenko *et al*., 2018).

Although, intracellular signalling cascades may intersect and diverge, activation and regulation of specific transcription are typically affected by an inherent effector protein of each pathway, originating from the nucleus (Itoh *et al*., 2014; Lakatos *et al*., 2014). Hence, besides the set of core transcriptional factors represented in Figure 1.6, the next of transcriptional factors are represented by regulatory factors like Stat3, Smads, β-catenin, and ERK, which are correspondingly effectors of LIF, BMP, WNT and FGF signalling routes (Tai and Ying, 2013; Itoh *et al*., 2014; Lakatos *et al*., 2014), as shown in Figure 1.7. Essentially, regulatory signals originating the effectors are relayed to several downstream receptors, inclusive of pluripotency network, which is made up of transcription factors like Oct4, Sox2, Nanog, Esrrb, Tbx3, and c-Myc (Boyer *et al*., 2006). These core transcription factors are responsible for processing inputs from signalling systems and deciding the self-renewal and differentiation propensities of embryonic stem cells (Dunn *et al*., 2014; Xu *et al*., 2014).

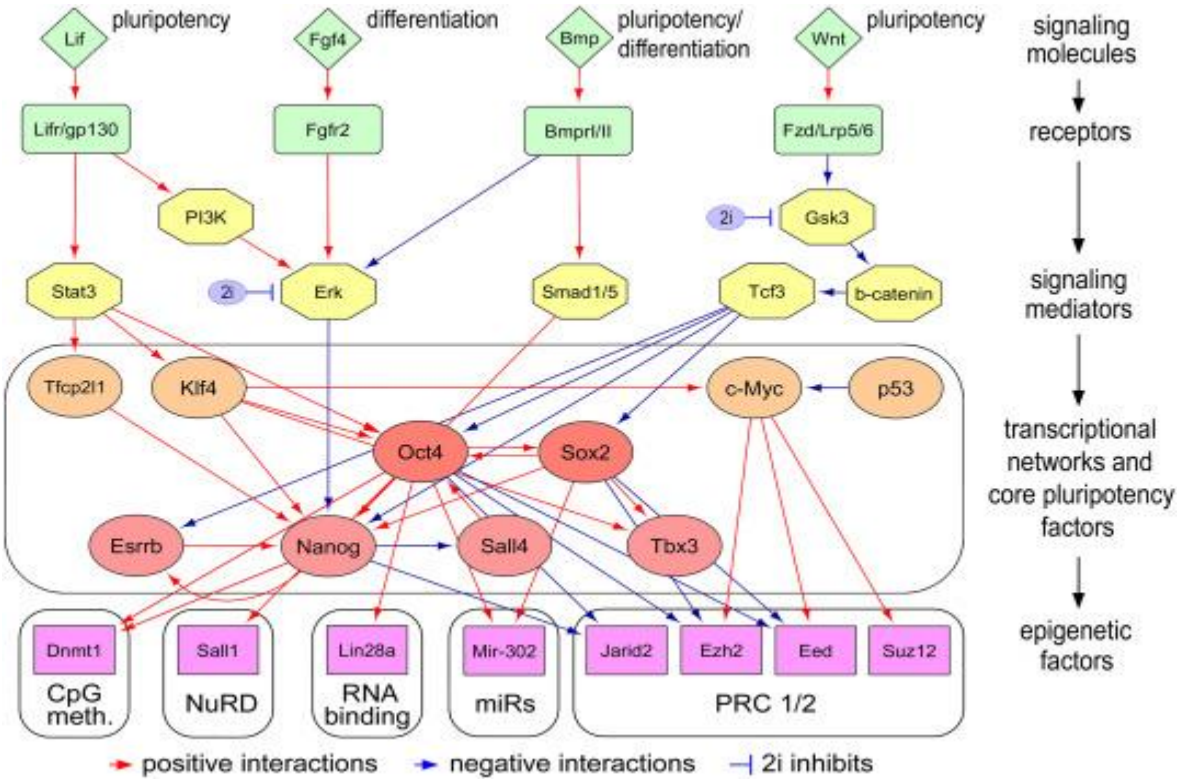


Figure 1.7. A representation of the organization of pluripotency gene regulatory networks in hierarchical fashion. showing signalling pathways (green coloured), the mediators of signalling pathway (yellow colouration), constituents of the transcriptional pluripotency networks (in red shades), and lowest levels of the pluripotency network (shown in violet shades) (Papatsenko *et al*., 2018).

Scientists are of the view that transcription factors constituted by CPN represent a third hierarchical level, downstream of the signalling modulators like Stat3, Smads, etc., and in the embryo several key pluripotency transcription factors are expressed maternally (Zuccotti *et al*., 2011). These set of maternally expressed genes are involved in the regulation of arrangement of the inner mass of the cells and epiblast and are critically important in further embryonic development (Le Bin *et al*., 2014; Radzisheuskaya and Silva, 2014). The upregulation of a simple combination of these embryonic stem cell transcriptional core factors in vitro has been shown to possess the capability of inducing the reprogramming of somatic cells to induced pluripotent stem cells (Takahashi and Yamanaka, 2006). It has been reported that cells re-enter pluripotency state and pluripotency network is re-established during this process (Buganim *et al*., 2012; Takahashi and Yamanaka, 2015). Many of the pluripotent transcription core factors within the CPN are likely to serve as targets of the key signalling effectors either directly or indirectly (Babaie *et al*., 2007). For instance, a core

pluripotency factor Nanog is targeted by Tfcp2l1, which on the other hand, is targeted by Stat3 that emanates from the leukaemia inhibitory factor (LIF) signalling; this is in addition to Nanog which is targeted by Tcf3 repressor, downstream of β-catenin that is part of the WNT signalling (Ye *et al*., 2013). In fact, various studies have shown that thousands of genes within the genome of embryonic stem cells is targeted by the core pluripotency factors (Ivanova *et al*., 2006; Chen *et al*., 2008; Ye *et al*., 2013).

1.7.3. Regulation of Somatic Cell Reprogramming

In the initial report where Yamanaka and colleagues (2006) demonstrated somatic cell reprogramming, terminally differentiated cells represented by mouse fibroblasts, were changed to stem-cell-like cells with pluripotent features via four key pluripotent modulators, comprising two core ES cell transcriptional factors, Klf4 and Myc, and Oct4 and Sox2 (Yamanaka and Takahashi, 2006; Takahashi *et al*., 2007). The eventual success recorded in the reprogramming of human fibroblasts to pluripotent-stem-cell-like cells (Takahashi *et al*., 2007; Yu *et al*., 2007; Park *et al*., 2008a), in addition to the production of pluripotent-stem-cell-like cell lines that are disease-specific with cells derived from genetic disorder patients, gives an avenue for *in vitro* investigations of various diseases in humans (Dimos *et al*., 2008; Park *et al.*, 2008b). Interestingly, as demonstrated by the initial studies conducted by Yamanaka and Takahashi (2006), the four modulators of reprogramming show elevated expression in ES cells; and reprogramming modulators have been variously suggested to play distinctive roles in the initiation and development of different cancer types (Werbowetski-Ogilvie and Bhatia, 2008; Daley, 2008). Drawing inference from these interesting observations, it is rational to hypothesize that there might be common denominators between in the pathways of reprogramming of somatic cells, regulation of pluripotent characteristics in embryonic stem cells, and transformation of the cells (Werbowetski-Ogilvie and Bhatia, 2008).

Another important regulatory factor in embryonic stem cells, which have also been implicated to have essential roles in early development are the polycomb-group (PcG) proteins. Following their initial discovery in Drosophila melanogaster, PcG proteins have been shown to contribute to repression of crucial developmental or lineage-specific regulators through the generation of a repressive histone mark (Margueron and Reinberg, 2011). In both mouse and human embryonic stem cells, interrogating PcG-repressive protein

complex (PRC)1 and PRC2 targets by ChiP revealed that several commonly repressed genes are linked to PRC proteins, including lineage-specific transcription factors (Boyer *et al*., 2006; Lee *et al*., 2006). These studies demonstrated that PRC proteins are responsible for the maintenance of the undifferentiated state of embryonic stem cells through the repression of critically relevant genes that are involved in the regulation of embryonic development. Similarly, in some studies where RNA immunoprecipitation and sequencing were employed, non-coding RNA molecules interactions with the PRC complexes has been shown to be involved in the regulation of target genes (Zhao *et al*., 2010); and PRC proteins have also been reported to play roles in the somatic cell reprogramming process (Pereira *et al*., 2010; Zhang *et al*., 2011).

One of the most frequently reported oncogenes is the activation of Myc, which has been reported in over 70% cases of human cancers (Nilsson and Cleveland, 2003). Myc has been implicated in numerous cellular physiological processes and has been shown to be involved in myriad of important biochemical pathways, of which the regulation of self-renewal capability in embryonic stem cells is inclusive (Meyer and Penn, 2008). In embryonic stem cells, interrogation of the targets of Myc demonstrated that the role of Myc in the maintenance of the pluripotency of embryonic stem cells is independent of and totally different from that of the core factors (Kim *et al*., 2008; Chen *et al*., 2008). Relative to the core factors of embryonic stem cells, Myc has many more chromatin targets, and genes targeted by Myc are enriched in pathways that are involved in protein synthesis and cellular metabolic processes (Chen *et al*., 2008). In contrast, the target genes of the core factors of embryonic stem cells are implicated in transcription and developmental processes (Kim *et al*., 2008; Chen *et al*., 2008). In somatic cell reprogramming, the role of Myc is considered dispensable (Wernig *et al*., 2008; Nakagawa *et al*., 2008); however, the effectiveness and speed of reprogramming by Myc are indicative of the possibility of its involvement in providing a favourable the reprogramming process with the conducive atmosphere via the regulation of chromosome structural alterations (Knoepfler *et al*., 2006; Knoepfler, 2008; Gaspar-Maia *et al*., 2011). In ES cells, proteins that interact with Myc and their target substrates have been identified; where the Myc network has been revealed to be different from the ES cell key survival network or the PRC network (Kim *et al*., 2010). Notably, histone acetyltransferase (HAT) complex proteins, which is known to interact with Myc in embryonic stem cells (Fazzio *et al* 2008; Kim *et al*., 2010), have been reported to play critical

role in the definition of embryonic stem cell identity, control its pluripotent characteristic and somatic cell reprogramming (Fazzio *et al*., 2008).

1.7.4. Embryonic Stem Cell Signatures in Cancer

Because of the commonalities in the characteristics embryonic stem cells and tumour cells, researchers were compelled to investigate the gene whose products are responsible for the regulation of these common denominators (Reya *et al*., 2001). From the foregoing review, it is a common knowledge that one of the key factors that has been shown to aid the reprogramming of somatic cells, Myc, a known oncogene, and p53 pathways deactivation, which has also been demonstrated as a predominant characteristic of numerous cancers, facilitates the efficiency of the reprogramming process (Yamanaka and Takahashi, 2006; Hanna *et al*., 2009; Krizhanovsky and Lowe, 2009; Utika *et al*., 2009). From these findings, the evidence is further glaring that unifying pathways could serve both in the acquisition of pluripotent characteristic and in the process of tumour development. Consequent upon the above premise, it is safe to propose that understanding of the pluripotent characteristic of embryonic stem cells and somatic cell reprogramming could deepen our knowledge of the important shared characteristics of ES cells and cancer cells. This therefore, rationalizes the fact that many ES cell -specific gene sets that have been reported in the investigations of the pluripotent stem cells could serve as important analytic instruments for the analysis of the expression patterns genes in various tumours; hence a key part of the objectives of this PhD is to analyse the expression patterns of DNA damage repair genes in embryonic stem cells as well as in tumours of the colon, central nervous system and mesothelium. On this note, the DNA repair genes of the key DNA repair pathways that are prevalent in ES cells are reviewed in the following section.

1.7.5. DNA Repair in Embryonic Stem Cells

The survival, self-renewal and pluripotent potential of both ES cells and adult stem cells could be limited by endogenous DNA damage due to oxidative metabolism and environmental pollution, and all DNA alterations inclusive of base and backbone modifications, single strand break, and double strand break (Frosina, 2010). In ES cells, maintenance of genomic stability must be stringent and tightly controlled as any genetic alterations affecting the progenitor cells will compromise the functionality and genomic stability of the entire cell lineages (Frosina, 2010). Consistent with this assertion, it has been

shown that mitotic recombination rate and the frequency of mutations are remarkably less in embryonic stem cells than in adult somatic cells (Hong *et al*., 2007). Additionally, it has been shown that mechanisms of mutagenesis also differ in ES cells relative to adult somatic cells. Although, the majority of mutations result in loss of heterozygosity (LOH) in ES cells as well as mouse embryonic fibroblasts, LOH in ES cells is initiated via chromosomal nondisjunction, whereas in mouse embryonic fibroblasts, it occurs through mitotic recombination (Tichy and Stambrook, 2008). Similarly, evaluation of spontaneous mutation at *hprt*, an X-linked locus, revealed that it is latent in ES cells (<10) and ~10 in mouse embryonic fibroblasts. Consequently, several mechanistic approaches must be executed to ensure the robust repression and reversal of spontaneous mutagenesis in embryonic stem cells and DNA repair is likely one of them (Maynard *et al*., 2008; Tichy and Stambrook, 2008; Frosina, 2009). Furthermore, irrespective of the characteristic finite replicative potentials of embryonic stem cells, studies employing murine genetic models have expressly indicated that DNA repair is critically important to the life span and the capability of embryonic stem cells to respond to stress (Warren and Rossi, 2009).

In the research conducted by Maynard and colleagues (2008) using single-cell gel electrophoresis (SCGE), human embryonic stem cells were observed to have more efficient repair of different types of DNA damage than human primary fibroblasts (Maynard *et al*., 2008). Similarly, Maynard and colleagues (2008) further reported that the transcriptional levels of majority of DNA repair genes, inclusive of those involved in BER and inter-strand crosslink repair, were highly upregulated in ES cells relative to human somatic cells (Maynard *et al*., 2008). In agreement with the above findings, the expression of antioxidant and DNA repair genes was markedly reduced, and increased DNA damage was recorded during spontaneous differentiation of two human ES cell lines (Saretzki *et al*., 2008).

In ES cells, replicating chromatin has been shown to be highly vulnerable to strand breaks (Banáth *et al*., 2009). In mammalian cells, including ES cells, two pathways repair double-strand breaks; non-homologous end joining (NHEJ), which is the key repair pathway and homology-directed repair (HDR), which functionally replaces NHEJ in the presence of a sister chromatid (Friedberg *et al.*, 2005). During the differentiation of murine ES cells, the expression of genes involved in double-strand break repair, such as Rad51 has been reported to diminish (Saretzki *et al*., 2004; Saretzki *et al*., 2008; Tichy and Stambrook, 2008). Regardless of the overexpression of O^6-methylguanine-DNA-methyltransferase, murine ES cells have been shown to be characterised by a higher apoptotic frequency relative to

differentiated cells following N-methyl-N-nitro-N-nitrosoguanidine treatment (Roos *et al*., 2007). This is because in ES cells, there is an elevated expression level of the MMR proteins, MSH2 and MSH6, that triggers fruitless cycles of O^6-methylguanine repair/replication (Casorelli *et al*., 2008). Several reports are consistent with the suggestion that the high apoptotic response of murine ES cells may be partly responsible for the reduction of the mutational load in these cells (Roos *et al*., 2007; van der Wees *et al*., 2007). Therefore, from available reports and research evidence, it has been shown that ES cells are characterised by elevated DNA repair capacity relative to their differentiated derivatives (Frosina, 2010).

Bracker and colleagues (2006) investigated the differences in the capabilities of DNA repair and the level of the gene products during the hematopoietic stem cell maturation process; and they observed that the removal of DNA adducts, the repair of strand breaks and the resistance to DNA-reactive drugs were noticeably higher in stem (CD34+ 38−) than in mature (CD34−) or progenitor (CD34+ 38+) cells isolated from umbilical cord from the same individual (Bracker *et al*., 2006). It is therefore, rational to assert that sluggishly dividing stem cells are likely to be under the protection of extensive DNA repair while more differentiated and less valuable cells, in the event of damage, could be removed via apoptosis (Frosina, 2010).

Transcription-coupled NER (TC-NER), has a preference for the repair of the transcribed strand of active genes relative the strand that is not transcribed, thereby providing cells with a mechanism for repairing DNA damage during, and restarting transcription (Hanawalt and Spivak, 2008). Like BER and double-strand break repair, NER genes are under-expressed in the majority of human cells that are in the process of differentiation, as reported in the monocytic primordial cells during their differentiation to macrophages or neural stem cells transformation to neurons (Nouspikel and Hanawalt, 2000; Hsu *et al*., 2007; Nouspikel, 2007). During cellular differentiation, authentication of NER occurs due to the non-ubiquitination of proteins involved in NER, which on the other hand is associated with the disparities in phosphorylation of the ubiquitin-activating enzyme (E1) (Nouspikel, 2007). To make sure that genes that are active are protected from damage and proficiently repaired in the event of any damage, cells are characteristically endowed with a specialised mechanism called differentiation-associated repair (DAR), in addition to transcription-coupled repair (Frosina, 2010). Because of the activities of DAR in differentiated cells, DAR could be regarded as a subcategory of GG-NER, with emphasis on the differentiated cells

chromatin loci within which transcription takes place (Nouspikel *et al*., 2006; Nouspikel, 2007).

Expression of the 8-oxoguanine DNA glycosylase (*Ogg1*) gene, one of the genes that formed the key focus of this PhD, has been reported to show a characteristic overexpression in regions of the neonatal mouse brain containing multiple neural stem cells (Hildrestrand *et al*., 2007). Interestingly, it has been shown that the expression level and activity of *Ogg1* are elevated in neural stem/primordial cells from new-born mice and fades as the animal grows to adulthood and following initiation of cellular differentiation (Hildrestrand *et al*., 2007; Frosina, 2010). It is therefore, safe to assert that elevated expression of *Ogg1* and other BER proteins may be responsible for the protection of neural stem/progenitor cells from oxidative DNA damage (Hildrestrand *et al*., 2007). Another DNA glycosylase gene, Nei endonuclease VIII-like 3 (*Neil3*), another key gene of interest in thisproject, has also been demonstrated to be highly elevated in brain regions harbouring stem cell populations (Rolseth *et al*., 2008; Hildrestrand *et al*., 2009).

1.8. Research Aims

Cancer development involves the complex mechanisms of DNA damage, particularly damage that results in the loss of function of tumour suppressor genes and gain of function by proto-oncogenes. In this instance, the inability of DNA repair genes to immediately code for corrective proteins to restore damaged DNA, probably because of mutation, results in development of mutant cell clones (Loeb, 2001). Accordingly, many genes have been identified that encode proteins that are directly or indirectly involved in the repair of damaged DNA and attempts have been made to classify them into major repair pathways as previously reviewed. Additionally, some of the genes that are responsible for the repair of damaged DNA have been further implicated in tumour development and drug resistance in different cancer types. For instance, high levels of *Ercc1* expression have been reported to result in CRC resistance to oxaliplatin (Baba *et al*., 2012; Choueiri *et al*., 2015). Similarly, high levels of *Neil3* have been found in metastatic melanoma tumours and various cancer cell lines and may also be a resistance factor against cancer chemotherapy (Seetharam *et al*., 2010; Elder, pers. commun.).

Though mammalian cells adopt several mechanisms to proofread DNA sequences and ensure error-free DNA replication before cell division, impairment in the functions of genes

responsible for the repair of DNA damage and correction of replication-associated errors could amount to the accumulation of errors with concomitant genetic instability and consequent development of aberrant cellular clones (Loeb, 2001). These aberrations may occur because of exposure to extrinsic factors such as exposure to carcinogens or mutagens and/or intrinsic factors such as endogenously produced ROS (Stratton *et al*., 2009). Owing to the enormity of significance attached to an error-free replication and the vital roles of DNA repair genes in ensuring that normal cellular physiology is not compromised, many studies have made attempts to identify, characterize and establish various DNA repair genes and their products in different cancer types. It is on a similar note, that this research was conceived, with the view to mapping the expression patterns of different genes that have characteristically been associated with different forms of DNA repair in different cancer types including colorectal cancer, medulloblastoma and mesothelioma with a focus on the BER DNA glycosylase NEIL3, the NER endonuclease ERCC1 and the mismatch repair gene MLH1 (MutL-homolog 1). This will be done with a view to gain insight into the involvement of these genes and their products in cancer development and understanding the causal relationships between their expression and cancer resistance to chemotherapy.

1.9. Research Objectives

To achieve the above aims, this research will key into the following objectives:

1. Extraction of RNA from colon cancer tissues and, where available, matched normal colon tissue.
2. Quantify the expression levels of *Neil1*, *Neil2*, *Neil3*, *Ercc11*, *Nthl1* and *Mlh1* in colorectal cancer tissues relative to normal colon tissues using quantitative PCR.
3. Quantify the expression levels of *Neil1*, *Neil2*, *Neil3*, *Ercc1* and *Mlh1* in medulloblastoma cell lines using quantitative PCR.
4. Extraction of RNA from human embryonic stem cell lines and RT-PCR of DNA repair genes of interest.
5. To determine the growth response of medulloblastoma cell lines following genotoxic insult.

Chapter 2

2.0. MATERIALS AND METHODS

2.1. Materials

The RNeasy Fibrous Tissue Mini kit and QuantiNova Reverse Transcription kit were obtained from Qiagen Ltd, UK. The ISOLATE II RNA Mini Kit, SensiFast SYBR LO-ROX kit and MyTaq RedMix were obtained from Bioline UK. Cisplatin, oxalipatin & *tert*-butyl hydroperoxide were obtained from Sigma-Aldrich. The Aldefluor kit was from Stem Cell Technology and the SuperSignal West Femto Maximum Sensitivity Substrate and TRIzol RNA isolation Reagent were from ThermoFisher Scientific. Tris base, ammonium persulfate and sodium dodecyl sulfate were from Fisher Scientific.

Table 2.1. Agarose Gel Electrophoresis Buffers

Material	Description
Tris-Borate-EDTA (TBE)	5x TBE was prepared by dissolving 54 g of Tris base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA pH8.0 in 1 L of dH_2O. The 5x TBE stock solutions was diluted 10-fold to 0.5 x prior to use.
Agarose Gel Electrophoresis Loading Buffer	4 g of sucrose, 25 mg of bromophenol blue (0.25% w/v), and 2.4 ml of 0.5 M EDTA pH 8.0 was mixed and made up to 10 ml with dH_2O.
DNA Size Marker	2 µl of Hyperladder 1kb or Hyperladder 100 bp (Bioline UK) was made up to 10 µl with dH_2O
GelRed	4 µl of GelRed (Biotium) was added to 100 ml of agarose gel solution at approximately 50ºC.

Table 2.2. Reagents used in Protein Analysis.

Reagent	Description
Acrylamide	Acrylamide (30% acrylamide, 0.8% bis) obtained ready for use (Bio-Rad Laboratories Ltd).
Ammonium Persulfate (APS)	A 10% (w/v) stock solution of APS was prepared by dissolving 1 g of APS in 10 ml dH_2O. Aliquots were stored at -20°C.
Tetramethylethylenediamine (TEMED)	TEMED was obtained from Sigma-Aldrich.
3x SDS-PAGE Loading Buffer	The stock was prepared by mixing, 2.4 ml of 1 M Tris-HCl pH6.8, 3 ml 20% (w/v) SDS, 3 ml, 100% (v/v) glycerol, 1.6 µl 2-mercaptoethanol and 6 mg bromophenol blue and the final volume adjusted to 10 ml with dH_2O and stored at 4°C.
10x SDS-PAGE Running Buffer	The 10x stock was prepared by mixing 30.2 g Tris base, 10 g of SDS and 144 g of glycine in about 900 ml of dH_2O. The pH was adjusted to pH8.3 if required and the volume adjusted to 1 L with dH_2O. The 10x stock was diluted to 1x buffer just before use.
10x Western Blot Transfer Buffer (WTB)	10x western blot transfer buffer (WTB) was prepared by dissolving 144 g glycine and 30.2 g Tris base and the final volume made up to 1 L with dH_2O. A 1x working buffer was prepared by adding 100 ml of 10x WTB to 200 ml of methanol and made up to 1 L with dH_2O and stored at 4°C.
10x Phosphate Buffered Saline (PBS)	10x PBS was prepared by adding 80 g NaCl, 2 g KCl, 7.62 g Na_2HPO_4 and 0.77 g KH_2PO_4 to 800 ml of dH_2O. Once fully dissolved, the pH was adjusted to pH7.4 with concentrated HCl and the total volume made up to 1 L with dH_2O.

PBS-Tween-20	PBS-Tween-20 contained 100 ml of 10x PBS and 1 ml of Tween-20 made up to 1 L with dH_2O.
Blocking buffer	Blocking buffer was prepared using 5% (w/v) non-fat milk in PBS-Tween-20.
Antibody dilution buffer	The antibody dilution buffer contained 2.5% (w/v) non-fat milk in 1x PBS-Tween-20
Protein molecular weight (MW) Standards	5 µl of PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was added to a lane of each SDS-PAGE gel.
RIPA buffer	50 mM Tris-HCl pH8.0, 150 mM sodium chloride, 1% igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS.

2.1.1. Tissue Samples Collection and Designation

Frozen human colon tumour and matched normal colon tissues were kindly provided by Dr. Andrew Povey, University of Manchester. The tissues were designated as N for normal colon tissues and corresponding colon cancer tissues represented by T. In addition, sixteen colon tissue samples were collected from the Biobank at the Central Manchester University Hospital NHS Foundation Trust and stored at -80^{o}C until used.

2.2. Methods

2.2.1. Tissue Preparation and RNA Extraction

Prior to the start of the RNA extraction, the work environment was cleaned with 70% ethanol and DEPC (diethyl pyrocarbonate) water to inactivate RNase enzymes. All equipment and plasticware were disinfected in 3% (w/v) Virkon solution before disposal or re-use.

RNA extraction was carried out from colon cancer and matched normal colon tissue samples using the RNeasy Fibrous Tissue Mini kit (Qiagen), following the manufacturer's instructions. Briefly, 30 mg of frozen colon cancer tissue or corresponding normal colon tissue was transferred to 300 µl Buffer RLT, disrupted and homogenized using a Tissue Lyser (Qiagen). The supernatant was transferred into a sterile 1.5 ml Eppendorf tube for RNA isolation. Subsequently, 590 µl of RNase-free water and 10 µl of proteinase K was added, mixed, incubate at 55^{0}C for 10 min and centrifuged at 10,000 *xg* for 3 min. The

supernatant was transferred to a new tube and 0.5 volumes of 100% ethanol was added and mixed, followed by the transfer of 700 µl of the sample to RNeasy Mini column in a 2ml collection tube and its centrifugation for 15 s at 8000 *xg*. The flow-through was discarded and the step was repeated until complete lysate was produced. Then, 350 µl of Buffer RW1 was added to RNeasy column and centrifuged for 15 s at 8000 xg, and the flow-through was discarded. 10 µl of DNase stock solution was mixed with 70 µl Buffer RDD and this was added to the RNeasy membrane, and incubated for 15 min at 25^0C. 350 µl of Buffer RW1 was added to the RNeasy column and centrifuged for 15 s at 8000 *xg* and the flow-through discarded. Then, 500 µl of Buffer RPE was added to the RNeasy column and centrifuged for 15 s at 8000 *xg* and discarded the flow-through. Another 500 µl of Buffer RPE was added to the RNeasy column and centrifuged for 2 min at 8,000 *xg*. The RNeasy column was placed in new 2 ml tube and centrifuged at 8,000 *xg* for 1 min. Finally, the RNA was eluted by placing the RNeasy column in a new 1.5 ml tube and 30 µl of RNase-free water was added directly onto the centre of the column followed by centrifugation at 8,000 *xg* for 1 min.

2.2.2. RNA Extraction from Tissue Using Trizol Reagent

Fifty milligrams of frozen tissue were homogenized in liquid nitrogen using RNase free mortar and pestle until ground into a fine powder. An RNase - free spatula was used to transfer the powder to 1 ml of Trizol solution in a 2 ml Eppendorf tube. The mixture was vortexed thoroughly and 20% chloroform (200 µl) was added. The mixture was vortexed for 30 s and incubated at room temperature for 2 min, then centrifuged at 12,000 *xg* for 15 min at 4^oC. The clear phase was transferred to a new tube and 200 µl of isopropanol was added to precipitate the RNA. The mixture was vortexed for 30 s and incubated at room temperature for 10 min followed by centrifugation at 12,000 *xg* for 10 min. The supernatant was removed by inverting the tubes and the tubes left to dry on absorbent paper. The RNA pellet was washed with 1 ml of 75% ethanol and vortex for 30 s and centrifuged at 7,500 *xg* for 5 min at 4^oC. Then, the supernatant was discarded, and the pellet was re-suspended in 50 µl of RNase-free water and stored at -20^oC until required.

2.2.3. Determination of the Concentration of RNA

After purification, the RNA concentration of each sample was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA analysis was carried out by first using 1 µl of the elution buffer to blank the Nanodrop, after which 1 µl of the RNA sample was pipetted onto the pedestal on the Nanodrop and then the concentration measured at 260 nm.

2.2.4. Agarose Gel Electrophoresis of the RNA Product

The integrity of the extracted RNA was assessed using 1% agarose gel electrophoresis. The agarose gel was prepared by mixing 0.5 g of agarose powder with 50 ml of 0.5x TBE (Tris-borate-EDTA) in a 250 ml Duran bottle and then heating the mixture in a microwave oven for 2-3 minutes, until the agarose powder had completely dissolved. Once the agarose had cooled sufficiently to be held by hand, 2 µl of GelRed (Biotium) was added and the contents poured into a gel tray containing a gel comb to create wells. Subsequently, 10µl of each RNA sample was loaded into each well along with 10 µl Hyperladder 1kb DNA ladder (prepared by mixing 1 µl Hyperladder 1 kb and 2 µl loading buffer in 7 µl dH_2O). The gel was subjected to electrophoresis at 100V for 70 min. The bands were imaged and recorded by UV-trans illumination and GeneSnap software (Syngene, Geneflow Ltd).

2.2.5. Complementary DNA (cDNA) Synthesis by Reverse Transcription

The extracted RNA was reverse transcribed into complementary DNA (cDNA) using the QuantiNova Reverse Transcription kit (Qiagen) according to the manufacturer's guidelines. Thus, 2 µl of genomic DNA (gDNA) removal mix was mixed with 3 µl of RNA (1.0 ng), 1 µl internal control RNA and 9 µl RNase-free water to make a final volume of 15 µl and incubated at 45°C for 2 min, then placed immediately on ice. The reaction mixture for reverse-transcription was prepared by mixing 1 µl Reverse Transcription Enzyme with 4 µl Reverse Transcription mix and the 15 µl entire genomic DNA elimination reaction mixture, to make a final volume of 20 µl. This was incubated at 25°C for 3 min followed by further incubation at 45°C for 10 min. The reverse transcriptase enzyme was inactivated by incubating at 85°C for 5 min. The cDNA obtained was amplified by the target gene primers using the polymerase chain reaction (PCR) as described in Section 2.2.6.

2.2.6. Primer Design

The primers used to assess the expression of the target DNA repair genes were designed using National Centre for Biotechnology Information software (NCBI/Primer BLAST). The primer pairs were picked from different exons of the genes. The primers were purchased from Eurofins Genomics and reconstituted following the manufacturer's instructions to 100 µM with 10 mM Tris-HCl pH8.0 and 1 mM EDTA (TE) buffer and stored at -20°C. The forward and reverse primers for the different target genes are tabulated in Table 2.3.

Table 2.3. qPCR Gene Specific Primers.

Target Gene	DNA Sequence	Tm (°C)	GC-Content (%)	PCR product (bp)
Gapdh 1014	GGTGGTCTCCTCTGACTTCAACA	61.8	52.2	127
Gapdh 1140	GTTGCTGTAGCCAAATTCGTTGT	60.5	43.5	
Ercc1 884S	CAAAACGGACAGTCAGACCCT	59.8	52.4	146
Ercc1 1029AS	TCAAGAAGGGCTCGTGCAG	58.8	57.9	
Neil1 1071S[@@]	AGAAGATAAGGACCAAGCTGC	57.9	47.6	212
Neil1 1283AS[@@]	GATCCCCCTGGAACCAGATG	61.4	60.0	
Neil1 1079S	AGGACCAAGCTGCAGAATCC	60.0	55.0	125
Neil1 1203AS	GCTCGAAAGGCAGCAAAGTC	60.1	55.0	
Mlh1 2276S[#]	AGGAGTCGACCCTCTCAGG	61.0	63.2	66
Mlh1 2342AS[#]	GTCCACTTCCAGGAGTTTGG	59.4	55.0	
Neil2 631S	GAAGCTTCCCCGTAGAAGAGG	61.8	57.1	122
Neil2 773AS	TGTAGCTTCTTACTGCTGCCC	59.8	52.4	

Neil2 1291S[##]	GCCTTAGAAGCTCTAGGCCA	59.4	55.0	145
Neil2 1436AS[##]	GCACTCAGGACTGAACCGAG	60.2	54.0	
Neil3 1651S	CGCCTCTGCATTGTCCGAGT	62.3	62.3	147
Neil3 1798AS	TGGAACGCTTGCCATGGTTG	61.8	61.8	
Nthl1 679S[@]	GATGGCACACCTGGCTATG	58.8	57.9	165
Nthl1 844AS[@]	CCACAGCTCCCTAGGCAG	60.5	66.7	
Ogg1 1020S	AGCAGCTACGAGAGTCCTCA	59.4	55.0	137
Ogg1 1156AS	CATATGGACATCCACGGGCA	59.4	55.0	

[@@] Obtained from Shinmura *et al*., (2004); [@]Obtained from Goto *et al*., (2009); [#]Obtained from Jensen *et al*., (2013); [##] Obtained from Mandal *et al*., (2012).

2.2.7. Amplification of Genes of Interest in Colon Cancer and Normal Colon Tissues by Reverse Transcription PCR (RT-PCR).

Following the conversion of the RNA extracted from the colon tumours and corresponding colon tissues to complementary DNA (cDNA) by the reverse transcription reaction, the sequences of the target genes were amplified by PCR. The primers were prepared by diluting 1 µl of 100 µM stock with 9 µl of TE buffer. The cDNA sample was mixed with 2x MyTaq RedMix (Bioline UK), forward primer, reverse primer and the total reaction was made up to 25 µl by adding dH_2O (Table 2.4). Denaturation, annealing and extension were run for 30 cycles. Table 2.5 shows the conditions of the PCR reaction.

Table 2.4. Composition of the PCR reaction mixture.

Reagent	Volume
Template cDNA (1.0 ng)	1 µl
2x MyTaq RedMix	12.5 µl
10 µM Forward primer	0.5 µl
10 µM Reverse primer	0.5 µl
dH_2O	10.5 µl
Total	25 µl

Table 2.5. PCR Reaction Conditions.

PCR conditions	Temperature	Time	
Pre-denaturation	95°C	1 min	
Denaturation	95°C	10 s	30 cycles
Annealing	60°C	10 s	
Extension	72°C	10 s	
Final extension	72°C	5 min	
Final Hold	4°C	Hold	

Upon the completion of the PCR reaction, 5 µl from each PCR reaction was mixed with 2 µl of gel loading buffer and 3 µl of dH_2O, then 10 µl was loaded on a 2% agarose gel (prepared by mixing 2.0 g agarose with 100 ml 0.5x TBE and 4 µl of GelRed) along with 10 µl Hyperladder 100bp DNA marker (prepared by mixing 1 µl Hyperladder 100bp, 2 µl loading buffer and 7 µl dH_2O). The gel was subjected to electrophoresis at 100V for 70 min. The bands were imaged and recorded as previously described in Section 2.2.3.

2.2.8. Amplification of Genes of Interest in the Biobank Tumour Samples by RT-PCR

Following the conversion of the RNA extracted from the various colon tumours to cDNA, the DNA sequences of the target genes were amplified by PCR. The primers were prepared by diluting 1 µl with 9 µl TE buffer and the cDNA sample was mixed with OneTaq Hot

Start DNA Polymerase (New England Biolabs) and the total reaction was made up to 25 µl by adding nuclease free water as indicated in Table 2.6. Denaturation, annealing and extension were run for 35 cycles (Table 2.7).

Table 2.6. One Taq PCR reaction mixture.

Reagent	Volume
5x OneTaq Standard Reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
One Taq Hot Start DNA Polymerase	0.125 µl
Template DNA (< 1,000 ng)	Variable
Nuclease-free water	to 25 µl

Table 2.7. PCR Reaction conditions for OneTaq Hot Start Reactions.

PCR conditions	Temperature	Time	
Pre-denaturation	94°C	1 min	
Denaturation	94°C	15 s	35 cycles
Annealing	60°C	15 s	
Extension	68°C	30 s	
Final extension	68°C	5 min	
Final Hold	4°C	Hold	

2.2.9. Quantitative PCR (qPCR)

Quantitative real time PCR (qPCR) was carried out to quantify the expression of the target genes on cDNA from colon cancer tissue samples using the SensiFast SYBR Lo-ROX Kit (Bioline UK), following the manufacturer's instructions. The reference gene for normalization was glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and melting curves were used to confirm the specificity of the PCR primers as well as purity of products.

Accordingly, 5 µl of cDNA from the reverse transcription reaction (20 µl) was added to 45 µl of TE buffer to obtain a final volume of 50 µl and then a standard curve was obtained by a 10-fold serial dilution which was made as follows: 100%, 10%, 1%, 0.1% and 0% cDNA by the following steps:

10%: 8 µl from 100% of cDNA sample was added to 72 µl of RNase-free water.

1%: 8 µl from 10% of cDNA sample was added to 72 µl of RNase-free water.

0.1%: 8 µl from 1% of cDNA sample was added to 72 µl of RNase-free water.

0.01%: 8 µl from 0.1% of cDNA sample was added to 72 µl of RNase-free water.

Subsequently, triplicate aliquots of 5 µl cDNA template were added to the wells of a 96–well reaction plate as shown in Table 2.8. The PCR primers were prepared by diluting 1 µl (100 pmol) with 9 µl TE buffer to give a final concentration of 10 µM. For each PCR reaction mix, 18.5 µl of forward primer, 18.5 µl of reverse primer, 230 µl 2x SensiFast SYBR Lo-ROX (containing SYBR Green l dye and dNTPs) and 78 µl RNase-free water were mixed in a 1.5 ml micro-centrifuge tube and then 15 µl was distributed in each well as shown in Table 2.8. Negative control wells had the following: forward primer, reverse primer, 2x SensiFast SYBR Lo-ROX and RNase-free water. The qPCR reactions were prepared in a 96-well plate and the wells were loaded as shown in Table 2.9. Two-step cycling was used for the PCR reaction and the conditions are given in Table 2.10. Melting curve from 72^{o}C to 90^{o}C, reading every 1°C, hold at 30 s.

Table 2.8. Loading of the 10-fold serially diluted cDNA in triplicate in a 96-well plate.

100%	**10%**	**1%**	**0.1%**	**0.01%**
A1 - A12	C1-C12	D1- D12	E1- E12	F1 –F12
B1 –B12				

Table 2.9. Sample Loading Pattern for qPCR in a 96-well plate.

Primer	Sample	Standard	Negative Control
Gapdh	A1, A2, A3	B1, B2, B3, C1, C2, C3, D1, D2, D3, E1, E2, E3, F1, F2, F3.	G1, G2, G3.
Neil1	A4, A5, A6	B4, B5, B6, C4, C5, C6, D4, D5, D6, E4, E5, E6, F4, F5, F6.	G4, G5, G6.
Neil2	A7, A8, A9	B7, B8, B9, C7, C8, C9, D7, D8, D9, E7, E8, E9, F7, F8, F9.	G7, G8, G9.
Neil3	A10, A11, A12	B10, B11, B12, C10, C11, C12, D10, D11, D12, E10, E11, E12, F10, F11, F12.	G10, G11, G12.

Table 2.10. qPCR conditions

Cycles	Temperature	Time	PCR phase
1	95°C	2 min	Denaturation
40	95°C	5 s	Denaturation
40	60°C	30 s	Annealing/ Extension

Analysis of the results was conducted through MJ Opticon Monitor Version 3.1 software and the relative expression of the target gene in each cell line was calculated by normalization of its mRNA quantitative values with the values of *Gapdh* using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

By plotting fluorescence against the cycle number, an amplification plot that represents the accumulation of product over the duration of the PCR reaction was generated. *Gapdh* was used as a reference gene and it is assumed that its expression level is constant in the cells

from tumour and normal tissues. Each run of qPCR has a standard curve that was generated through serial dilution of cDNA.

2.3. Cell Culture

2.3.1. Cells

The medulloblastoma (DAOY) cell lines, including the cisplatin-resistant subtype and the conventional medulloblastoma cells were kind gifts from Dr Gianpiero Di Leva, University of Salford. The Mero25 mesothelioma cell line was kindly supplied by Professor Luciano Mutti, University of Salford. The human embryonic stem (hES) cell pellets were kindly supplied by Professor Daniel Brison, Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK and were stored at -80˚C until required.

2.3.2. Cell Culture Methods

The medulloblastoma and mesothelioma cell lines were grown at early passage and working stocks were aliquoted in vials and stored in 10% (v/v) dimethyl sulfoxide (DMSO), 90% FBS at -80˚C or in liquid nitrogen for short-term storage. For each experiment, the medulloblastoma (DAOY) cell lines were routinely cultured in RPMI1640 with 0.2 mM L-glutamine (Invitrogen), supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen, UK) and 1% penicillin-streptomycin (Complete medium; Invitrogen, UK) until 70-80% confluent. Similarly, the mesothelioma (MERO-25) cells were cultured in Dulbecco's modified eagle medium (DMEM) with 0.2 mM L-glutamine, supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin (Complete medium;) until 70-80% confluent.

Cells were split 1:10-1:15 when confluency of 70%-80% was reached. The cells were washed with phosphate buffered saline (PBS) and then incubated for up to five minutes at 37°C with 5 ml of 1x trypsin-EDTA (Invitrogen). Once most cells had detached from the flask, the trypsin-EDTA was quenched by the addition of double the volume of complete cell culture medium. Cells were collected and then pelleted by centrifugation for 3 min at 1200 rpm. Cells were then re-suspended in medium and seeded in T75 flasks for further experiments.

2.3.3. Cell Seeding Conditions

Unless otherwise stated, all experiments were performed in triplicate. Cells from flasks that were 70-80% confluent and were seeded in either 96-, 12- or 6-well plates or 10 cm petri dishes or T25 flasks, depending on the experiment. The cells were pelleted (as described in Section 2.3.2) and re-suspended in fresh medium. To accurately establish the number of cells per millilitre, the cells were counted using a haemocytometer and plated at the appropriate density depending on cell type, the plate used and the experiment, as detailed throughout the corresponding Sections.

2.3.4. Cell Viability Assays (MTT Assay)

The genotoxic agents, oxaliplatin, cisplatin and *tert*-butyl hydroperoxide were employed in this project to induce DNA damage and to assess cell viability in relation to the gene expression of selected DNA repair genes. Normal and cisplatin-resistant DAOY medulloblastoma cells were grown in RPMI1640 medium supplemented with 2 mM L-glutamine, 10% FBS and 1% penicillin-streptomycin. The cells were maintained at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were cultured overnight in 96-well plates (2 x 10^3/well) containing 100 µl complete medium prior to incubation with 100 µl of complete medium containing various concentrations of either oxaliplatin, cisplatin or *tert*-butyl hydroperoxide (0.78 µM - 100 µM) in each well and incubated for 72 h at 37°C in a humidified atmosphere and 5% CO_2.

Once incubation was complete, 40 µl of MTT (3 mg/ml) reagent was added into each well using a multi-channel pipette and incubated for 3 h at 37^0C. (Then purple formazan crystals were observed in the wells). The medium was aspirated carefully from each well and 200 µl of DMSO was added to each well using a multi-channel pipette and mixed thoroughly until the crystals dissolved fully. The plate was read at 540 nm and 690 nm using the multi-scan Ascent plate reader (Thermo Scientific) and analysed.

2.3.5. Gene Expression Analysis in Cell Lines

RNA extraction was carried out from the stored pellets of the normal & cisplatin-resistant DAOY cells, Mero25 and human embryonic stem cell lines using the Isolate II RNA Mini kit following the manufacturer's instructions. Cell pellets were thawed on ice for 5 min. Three hundred and fifty microlitres of lysis buffer was added to 3.5 µl of 2-mercaptoethanol and mixed on a vortex in 1.5 ml tubes, then added to the cell pellets. The lysate was transferred to an Isolate II filter (violet) which was then placed in a 2 ml collection tube and centrifuged at 11,000 *xg* for 1 min. The flow-through was transferred to a 1.5 ml microcentrifuge tube, and then 350 µl of 70% ethanol was added and mixed by pipetting up and down 5 times. It was transferred to a 2 ml collection tube which was placed in an Isolate II RNA mini column (blue) and centrifuged at 11,000 *xg* for 30 s. After that, the column was put in a new 2 ml collection tube and the silica membrane was desalted by adding 350 µl of membrane desalting buffer and centrifuging at 11,000 *xg* for 1 min before the membrane was dried through centrifugation at 11,000 *xg* for 1 min.

The DNase I reaction mixture was prepared by adding 10 µl of reconstituted DNase I to 90 µl of reaction buffer for DNase I and mixed by gently flicking the tube. Genomic DNA was digested by adding 95 µl DNase I reaction mixture directly on the centre of the silica membrane and incubating at room temperature for 15 min. The silica membrane was then washed several times, first by adding 200 µl of wash buffer RWI, followed by centrifugation at 11,000 *xg* for 30 s, and then the column was placed into a new collection tube (2 ml). The second wash was carried out by adding 600 µl wash buffer RW2, then it was centrifuged at 11,000 *xg* for 30 s, the flow- through was discarded and the column was placed back into the collection tube. The third wash was carried out by adding 250 µl wash buffer RW2 followed by centrifugation at 11,000 *xg* for 2 min, the flow-through discarded and the column placed into a nuclease free 1.5 ml collection tube. Finally, the RNA was eluted by adding 60 µl of RNase-free water directly onto the centre of the silica membrane and incubated for 1 min at room temperature followed by centrifugation at 11,000 *xg* for 1 min.

Following the successful extraction, the RNA concentration was measured, and RNA quality was assessed, cDNA was synthesised, RT-PCR and qPCR were conducted as previously described in Sections 2.2.7 and 2.2.8.

2.3.6. Preparation of cancer stem cells.

2-hydroxyethyl methacrylate (polyHEMA) was prepared by adding 12 g into 1 L of 98% (v/v) ethanol and mixed on a magnetic stirrer at 45-50°C for about 8 h to dissolve complete dissolution. After this, 1.5 ml of this solution was added into each well of a 6-well tissue culture plate and incubated in an oven at 55°C for 5 days. Before seeding of the cells, the coated plate, without the lid, was placed in a sterile cabinet under a UV light for 10 – 15 min. Two millilitres of freshly grown Mero25 mesothelioma cells was mixed with 4 ml of cancer stem cell medium (DMEM/F-12) in a 15 ml tube and centrifuged at 1,250 rpm for 5 min. The supernatant was discarded, and 2 ml of cancer stem cell medium was added to resuspend the cells. A sterile syringe with a 25G needle was used to pipette the cells up and down once to make a single cell suspension. Then, 5000 cells were seeded in 2 ml of DMEM/F-12 medium in each well of the 6-well plate and incubated for 5 days at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. Thereafter, the cells were harvested for flow cytometry analysis and RNA extraction.

2.3.7. Flow Cytometry Analysis of Cancer Stem Cells Derived from Mesothelioma Cell Line Mero-25.

Cancer stem cells (CSC) were confirmed by flow cytometry analysis of viable cells using an Aldefluor kit (Stem Cell Technologies). Cancer stem cells derived from the mesothelioma cell line Mero-25 were grown to 80% confluence in DMEM/F-12 medium All the cells were transferred to a 1.5 ml tube and centrifuged at 1300 *xg* for 5 min. The supernatant was discarded and 100 µl of 1x trypsin-EDTA was added and incubated for one minute. Two hundered microlitres of DMEM/F12 medium was added, pipetted up and down and the cells centrifuged at 1200 *xg* for 5 min, after which the supernatant was discarded. Following this, 400 µl of DMEM/F12 medium was added, pipetted up and down with a syringe to separate the cells. Then, a "test" and a "control" tube for each sample to be tested were labelled and 400 µl of the cell suspension was placed into each of the sample tube (test). Five microlitres of Aldefluor DEAB reagent was added to the control tube and recapped immediately. 5 µl of the activated Aldefluor reagent was added into the tube containing 400 µl of cell suspension. 200 µl of the mixture was immediately added to the DEAB tube (control). The test and control sample were incubated for 60 min at 37°C. After incubation, all of the tubes were centrifuged for 5 min at 250 *xg* and the supernatant was discarded. The cell pellet was

re-suspended in 0.5 ml of Aldefluor Assay Buffer and kept on ice. The cells were analysed for viable cancer stem cells following mixing with Adefluor. Analysis of derived cancer stem cells was carried out using a FACSCalibur flow cytometer (BD Biosciences).

2.4. Protein Analysis

2.4.1. Protein extraction and Separation by SDS-PAGE

Medulloblastoma DAOY cells (Normal & cisplatin-resistant) were grown in T25 flasks up to 80% confluence, as previously described in Section 2.3.2. Each cell line was lysed directly in flasks with ice-cold RIPA buffer containing 50 mM Tris-HCl pH8.0, 150 mM sodium chloride, 1% igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS); and protease inhibitor cocktail (Invitrogen, UK). Protein concentrations were determined using the Bradford method (Bradford, 1976), and lysates were snap-frozen in liquid nitrogen before storage at -80^{o}C. Cell lysates were thawed on ice and mixed at the ratio of 1:1 with Laemmli buffer containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, and 0.125 M Tris-HCl pH6.8. The mixtures containing 15 µg, 30 µg or 60 µg of total protein were heated for 5 min at 95^{o}C before separating the proteins through a 10% SDS-polyacrylamide gel (see Table 2.11) at a constant voltage of 80V for 30 min, then at 120V for 1 h.

Table 2.11. Composition of SDS-polyacrylamide gels.

Percentage gels	**10%**	**5%**
	Resolving	**Stacking**
dH_2O	10.94 ml	6.73 ml
30% acrylamide	9.33 ml	1.67 ml
1.5 M Tris-HCl (pH 8.8)	7 ml	-
0.5 M Tris-HCl (pH 6.8)	-	1.25 ml
0.2 M EDTA	280 µl	100 µl
10% SDS	280 µl	100 µl
10% APS	157 µl	157 µl
TEMED	17 µl	17 µl

2.4.2. Western blotting

Following separation by SDS-PAGE, the proteins were transferred onto PVDF membranes at constant current of 400 mA for two hours, using ice-cold 1x western transfer buffer (WTB). The membranes were subsequently blocked for 1 h in blocking buffer (5% non-fat milk in PBS-Tween-20). This was followed by overnight incubation at 4^{o}C with primary antibody specific for the target protein under investigation in 2.5% non-fat milk in PBS-Tween-20 at the dilution indicated in the manufacturer's data sheet. The blots were then washed three times with 0.1% PBS-Tween for ten-minute intervals, before further incubation with secondary antibody - horseradish peroxidase (HRP) conjugate inantibody dilution buffer for 1 h at room temperature, at the dilution specified in the manufacturer's data sheet. Afterwards, the blots were washed three times for 10 min with PBS-Tween-20. Finally, the membrane was washed in PBS for 5 min to remove excess Tween-20 and placed on a paper towel to drain off excess liquid. Then, the chemiluminescence substrate reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Thermos Scientific) was prepared with 500 µl of each femto reagent and poured over the membrane. Finally, the membrane was incubated on a shaker for 1 – 2 min at room temperature and the protein bands were viewed on a G-box image analyser (Syngene).

Chapter 3

RESULTS

3.1. Preliminary experiments

To ensure that the RNA extraction procedures and RT-PCR reactions were robust and working as expected, preliminary experiments were carried out on two cancer cell lines, HCT116, a human colorectal carcinoma cell line and Mero25, a mesothelioma - derived cell line. Figure 3.1 shows that the two bands of 28S and 18S rRNA were obtained from both cell lines using the Bioline ISOLATE II RNA mini kit.

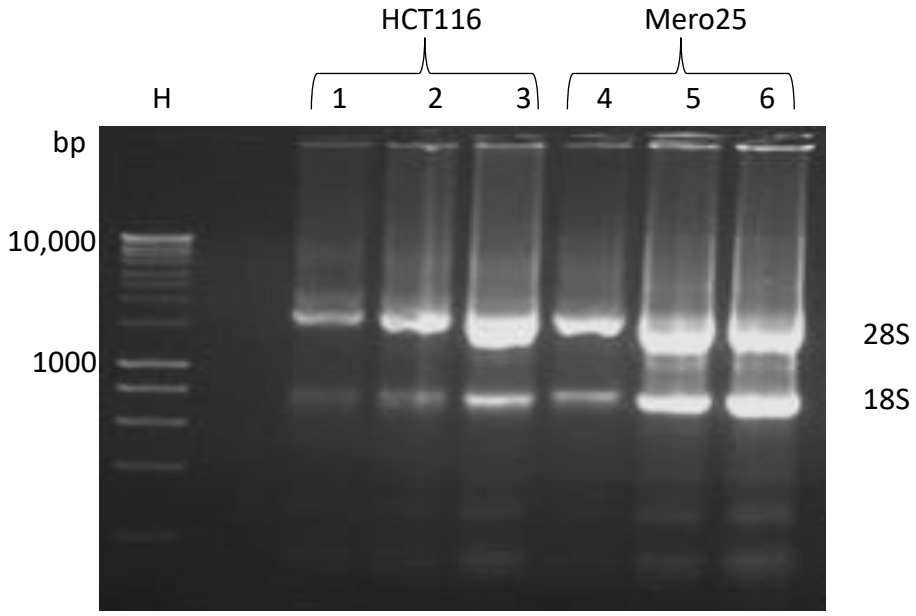


Figure 3.1 Agarose gel electrophoresis of RNA extracted from HCT116 and Mero25
Lane H: Hyperladder 1kb; lanes 1, 2 µl; lane 2, 5 µl; lane 3, 7 µl; lane 4, 2 µl; lane 5, 5 µl; lane 6, 7 µl.

Following this, the PCR primer pairs obtained for each target gene (Table 2.3) were tested on cDNA prepared from the two RNA samples. The results are presented in Figure 3.2 - Figure 3.3.

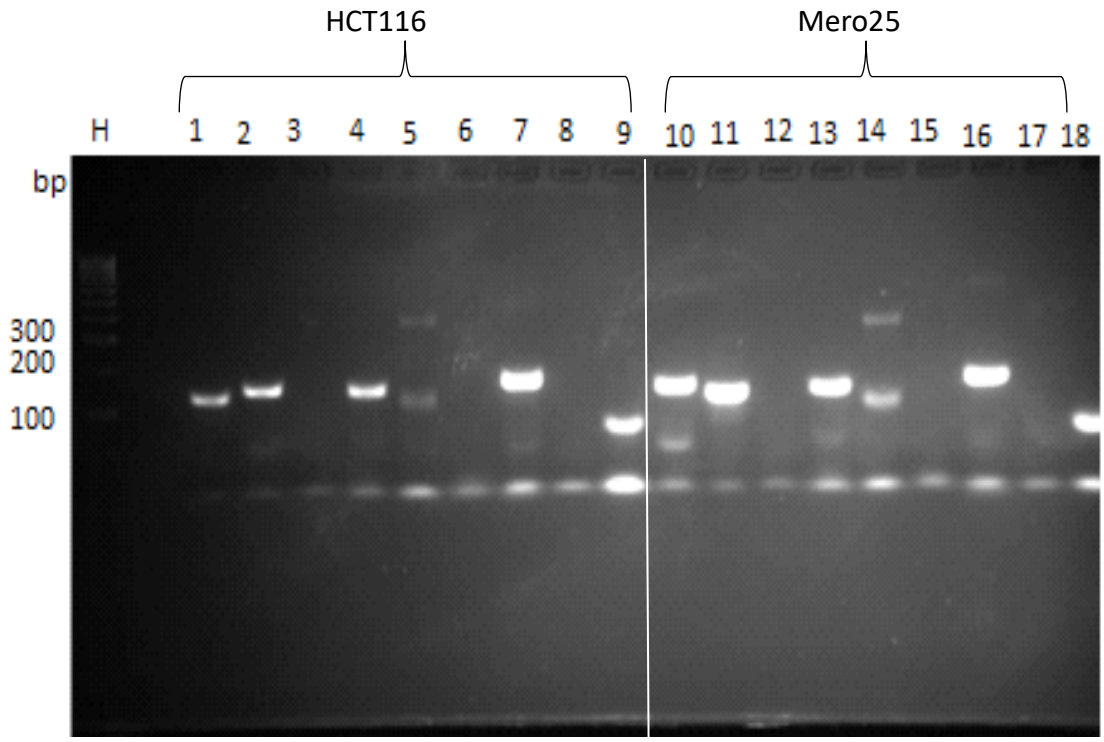


Figure 3.2 Agarose gel electrophoresis of RT-PCR products of *Gapdh, Ercc1, Ogg1*, *Nthl1* and *Mlh1* genes for HCT116 and Mero-25 cell lines.

Lane 1, *Gapdh*; lane 2, *Ercc1* (884S); lane 3, *Ercc1* (994S); lane 4, *Ogg1* (1020S); lane 5, *Ogg1* (1258S); lane 6, *Nthl1* (349S); lane 7, *Nthl1* (679S); lane 8, *Mlh1* (1814S); lane 9, *Mlh1* (2276S); lane 10, *Ercc1* (884S); lane 11, *Gapdh*; lane 12, *Ercc1* (994S); lane 13, *Ogg1* (1020S); lane 14, *Ogg1* (1258S); lane 15, *Nthl1* (349S); lane 16, *Nthl1* (679S); lane 17, *Mlh1* (1814S); lane 18, *Mlh1* (2276). H represents Hyperladder 100bp.

The results of the RT-PCR reactions shown in Figure 3.2 indicate that the majority of gene specific primer pairs amplified the target section of cDNA uniquely, resulting in a single band in each lane. However, there were exceptions and no PCR product were obtained from primer pairs 994S for *Ercc1* (lanes 3 and 12), 349S for *Nthl1* (lanes 6 and 15) or 1814S for *Mlh1* (lanes 8 and 17) in cDNA prepared from either cell line. Thus, where more than one set of primers for a particular gene was used, the primer pair showing the more robust activity was chosen.

Figure 3.3 shows a similar result for the three *Neil* genes, although this time there is a marked difference in expression levels between the two cell lines, with the exception of *Gapdh* (lanes 1 and 7) and *Neil3* (lanes 6 and 12) using this qualitative method.

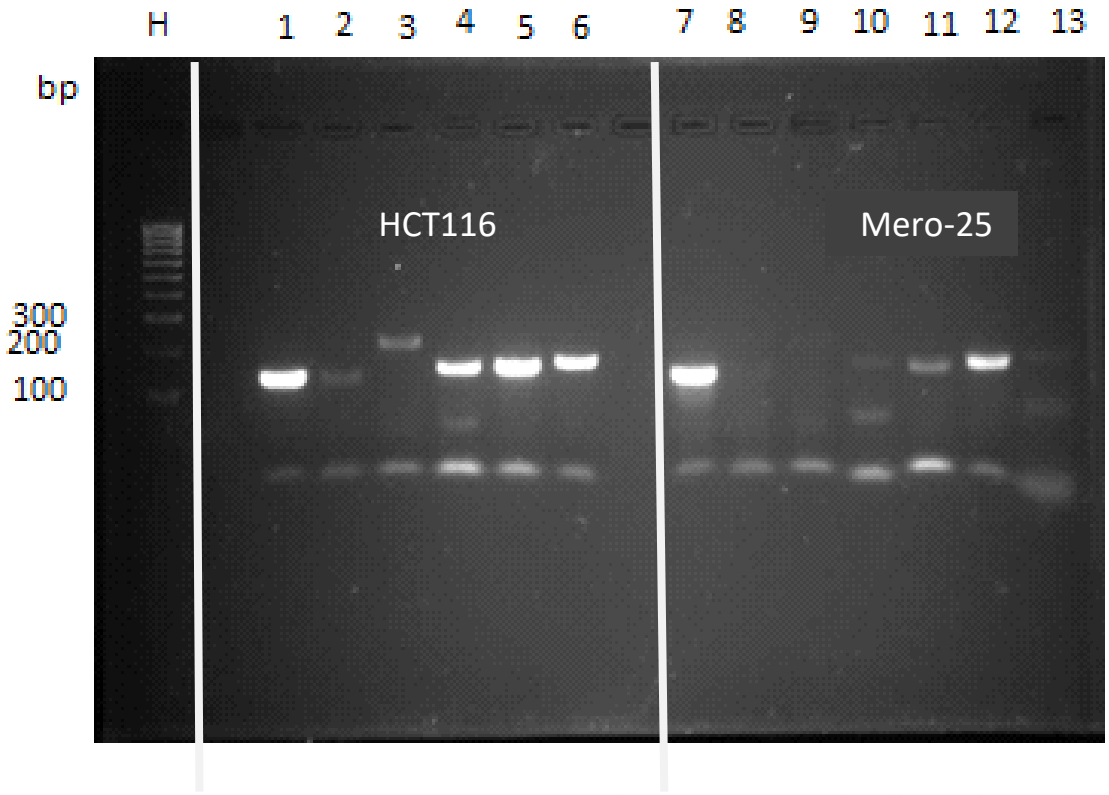


Figure 3.3 Agarose gel electrophoresis of RT-PCR products of *Gapdh, Neil1, Neil2* and *Neil3* for HCT116 and Mero-25 cells.

Lanes 1 – 6, HCT116 and lanes 7-13, Mero-25. Lane 1, *Gapdh*; lane 2, *Neil1* (1079S); lane 3, *Neil1* (1071S); lane 4, *Neil2* (631S); lane 5, *Neil2* (1291S); lane 6, *Neil3* (1651S); lane7, *Gapdh*; lane 8, *Neil1* (1079S); lane 9, *Neil1* (1071S); lane 10, *Neil2* (631S); lane 11, *Neil2* (1291S); lane 12, *Neil3* (1651S); lane 13, *Neil2* (631S). H: Hyperladder 100 bp.

Following these preliminary experiments to gain experience in working with RNA and to confirm the specificity and suitability of the PCR primers, the project could now focus on the expression of the selected DNA repair genes in CRC tumour samples (Section 3.2), the change in expression of a subset of these genes in response to cisplatin treatment in medulloblastoma cells (Section 3.4) and an analysis of gene expression in putative cancer stem cells derived from a mesothelioma cell line (Section 3.5) and in human ES cells (Section 3.6).

3.1.1. Colon tissue samples

In this project, colon tissue samples were obtained from two sources. The first twelve samples included matched control tissue (Table 3.1) and were obtained from the laboratory of Dr Andrew Povey (University of Manchester, UK), where they had been kept in long-term storage at -80°C. Despite many attempts and change of protocols the total RNA obtained from these samples rarely produced the characteristic double rRNA bands that had been expected, indicating that the rRNA, and by inference, the mRNA may have been degraded (Figure 3.4). While RT-PCR results were still obtained for these samples and a control gene (*Gapdh*) consistently produced similar results across all samples, it was decided to obtain fresh samples from the biobank at the Central Manchester University Hospital NHS Foundation Trust and these are listed in Table 3.2. These samples also had the benefit of having some basic patient information that may have been useful. However, again, despite all efforts to avoid RNA degradation, the two rRNA bands were rarely observed in the total RNA samples extracted from these tumour samples (Figure 3.5). The analysis of the gene expression of selected DNA repair genes from these samples is described in the following sections.

Table 3.1 Nomenclature of matched tumour and normal colon samples

Sample Number	Colon Tumour Samples	Matched Normal Colon Tissue Samples	Designation
1	2T	1N	Pair 1
2	4T	3N	Pair 2
3	6T	5N	Pair 3
4	8T	7N	Pair 4
5	10T	10N	Pair 5
6	11T	11N	Pair 6
7	12T	12N	Pair 7
8	13T	13N	Pair 8
9	33T	33N	Pair 9
10	34T	34N	Pair 10
11	35T	35N	Pair 11
12	36T	36N	Pair 12

Table 3.2 Description of the sixteen biobank colon tissue samples

Sample Number	Tissue ID	Tissue Sample Site	Gender	Age	Tissue Status
1	1538	Colon	Male	72	Normal
2	1545	Colon	Female	63	Normal
3	1573	Colon	Female	70	Normal
4	1573T	Colon	Female	70	Tumour
5	1580	Not Specified	Male	69	Normal
6	1581	Not Specified	Female	62	Normal
7	1581T	Not Specified	Female	62	Tumour
8	1597	Caecum	Male	54	Normal
9	1597T	Caecum	Male	54	Tumour
10	1604	Colon	Female	79	Normal
11	1604T	Colon	Female	79	Tumour
12	1610	Caecum	Male	59	Tumour
13	1620	Colon	Male	65	Tumour
14	1626	Colon	Female	73	Tumour
15	1627	Colon	Male	64	Tumour
16	1630	Colon	Male	64	Tumour

3.1.1.1. Assessment of Yield and Integrity of RNA Extracted from Samples.

To establish the quality of the RNA purified from the different colon cancer tissue samples and their corresponding normal colon tissues, agarose gel electrophoresis was conducted. For total RNA extracted from human cells, it was expected that the gels should show two distinct bands representing 18S and 28S rRNA. However, as can be seen in Figure 3.4, the extracted RNA was often degraded and the rRNA bands were not observed, thus Figure 3.4 typifies the banding patterns of RNA extracted from the colon cancer and normal colon tissues, with the thickness of the bands serving as indicators of RNA yield. A DNA ladder was included in all gels solely to confirm that the electrophoresis had occurred correctly.

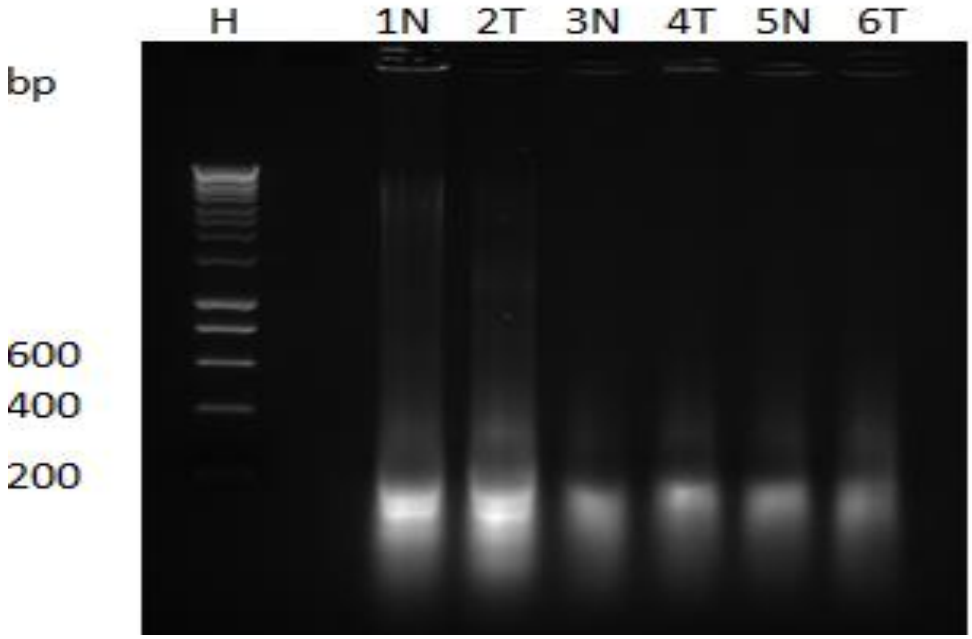


Figure 3.4 Agarose gel electrophoresis of RNA extracted from different colon tumour tissue samples and matched normal colon tissue.

Lane H, Hyperladder 1kb; lane 1N, normal colon tissue from sample 1N; lane 2T, colon tumour tissue from sample 2T; lane 3N, normal colon tissue from sample 3N; lane 4T, colon tumour tissue from sample 4T; lane 5N, normal colon tissue from sample 5N; lane 6T colon tumour tissue from sample 6T.

Following many unsuccessful attempts to reduce RNA degradation, sixteen tissue samples were obtained from a biobank as described in Section 3.1.1. The source of these sixteen samples and available patient information is given in Table 3.2.

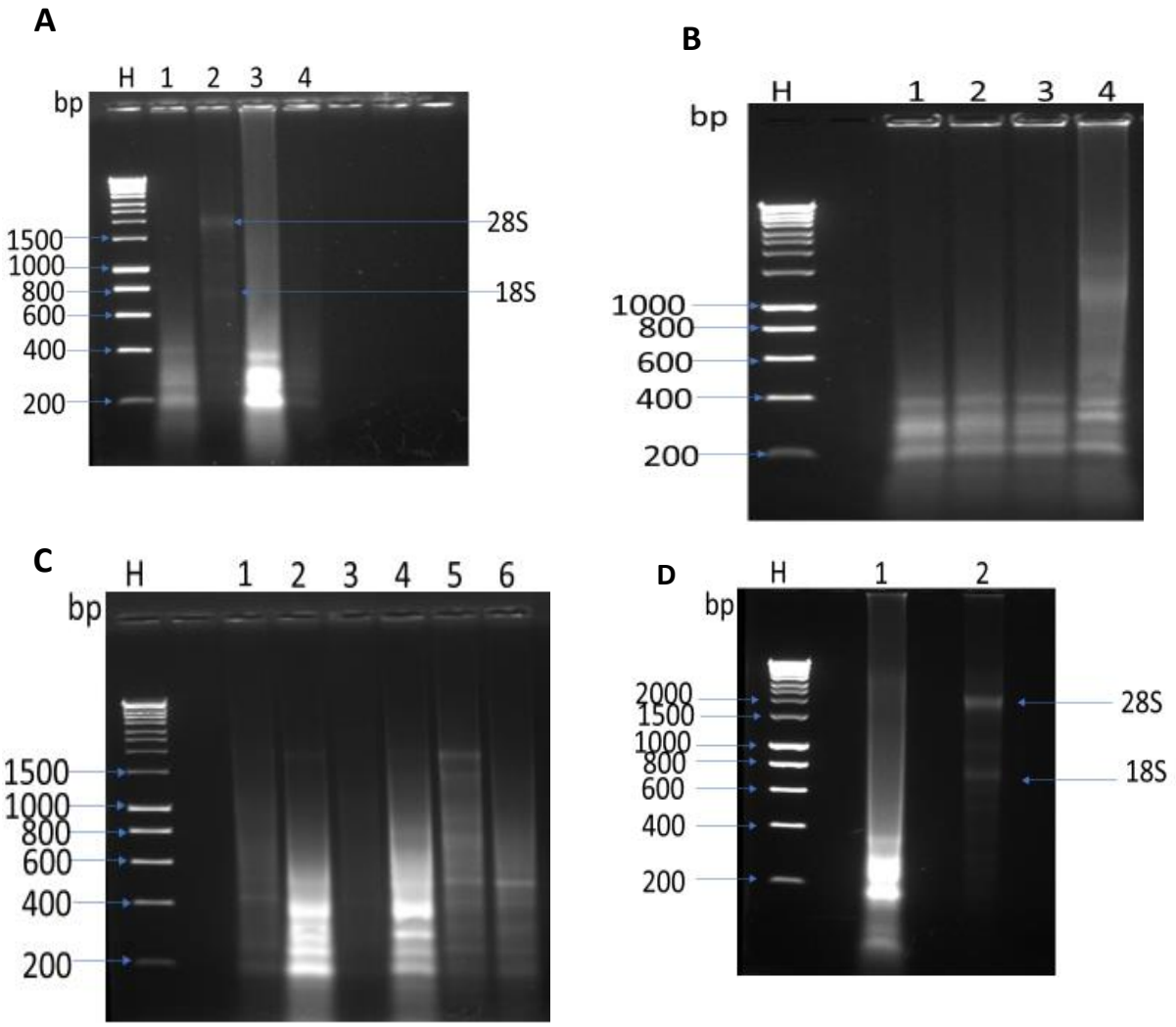


Figure 3.5. Agarose gel electrophoresis of RNA extracted from the 16 colon tissue samples obtained from the biobank (see Table 3.2 for full details).

A: Lane H, Hyperladder 1kb; lane 1, Sample 1538; lane 2, Sample 1545; lane 3, Sample 1604T; lane 4, Sample 1610. **B**: Lane H, Hyperladder 1kb; lane 1, Sample 1573; lane 2, Sample 1573T; lane 3, Sample 1581; lane 4, Sample 1620. **C**: Lane H, Hyperladder 1kb; lane 1, Sample 1580; lane 2, Sample 1581T; lane 3, Sample 1597; lane 4, Sample 1626; lane 5, Sample 1627; lane 6, Sample 1630. **D**: Lane H, Hyperladder 1kb; lane 1, Sample 1597T; lane 2, Sample 1604.

The results presented in Figure 3.5 again indicate ribosomal RNA degradation in the majority of samples, only lane 2 in Figure 3.5A (sample 1545) and lane 2 in Figure 3.5D (sample 1604) showing the expected 28S and 18S rRNA bands. However, there is no evidence of a high molecular band in any of the samples that would indicate genomic DNA contamination and therefore the DNase step in the RNA extraction procedure appears to

have been successful. As shown in subsequent sections, the lack of intact rRNA did not prevent the amplification of specific mRNA sequences from any of the samples, although it is acknowledged that there is no way of knowing the effect of the observed rRNA degradation on the mRNA component and it is assumed that if degradation occurred, it affected all mRNA molecules equally.

3.2. Reverse Transcripion-PCR

3.2.1. Primer Specificity

RT-PCR was carried out on cDNA from tissue samples using specific primers of the target genes. To confirm the primer specificity and ensure that genes of interest only were amplified, agarose gel electrophoresis of the RT-PCR products was conducted, with particular attention given to ensuring a unique product of the correct size was obtained. Thus, Figure 3.6 is a representative gel from the matched tissue samples listed in Table 3.1, while Figure 3.7 to Figure 3.13 show the results for the different genes analysed from each of the RNA samples from the different CRC tissue samples listed in Table 3.2.

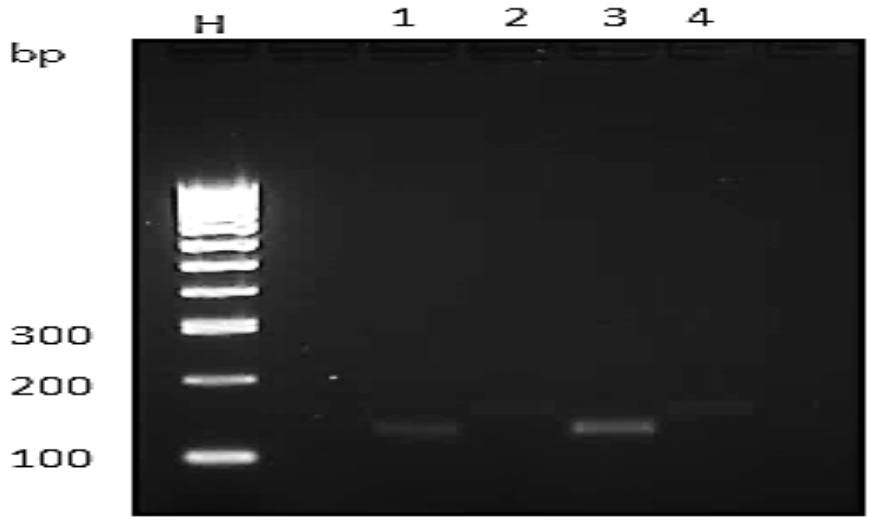


Figure 3.6. Agarose gel electrophoresis of RT-PCR products corresponding to *Gapdh* and *Neil3* from tissue samples 10N and 10T.

Lane H, Hyperladder 100bp; lane 1, *Gapdh* from sample 10N; lane 2, *Neil3* from sample 10N; lane 3, *Gapdh* from sample 10T; lane 4, *Neil3* from sample 10T.

Figure 3.6 shows that a single band of the expected size was obtained when PCR primers specific for *Gapdh* (127 bp) and *Neil3* (147 bp) were used. There appears to be little observable difference between the RNA sample extracted for the normal tissue (lanes 2 and 3) and the RNA extracted from the CRC sample (lanes 3 and 4) and for these tissues, and in the semi-quantitative nature of the assay, *Neil3* expression was less than that of *Gapdh.*

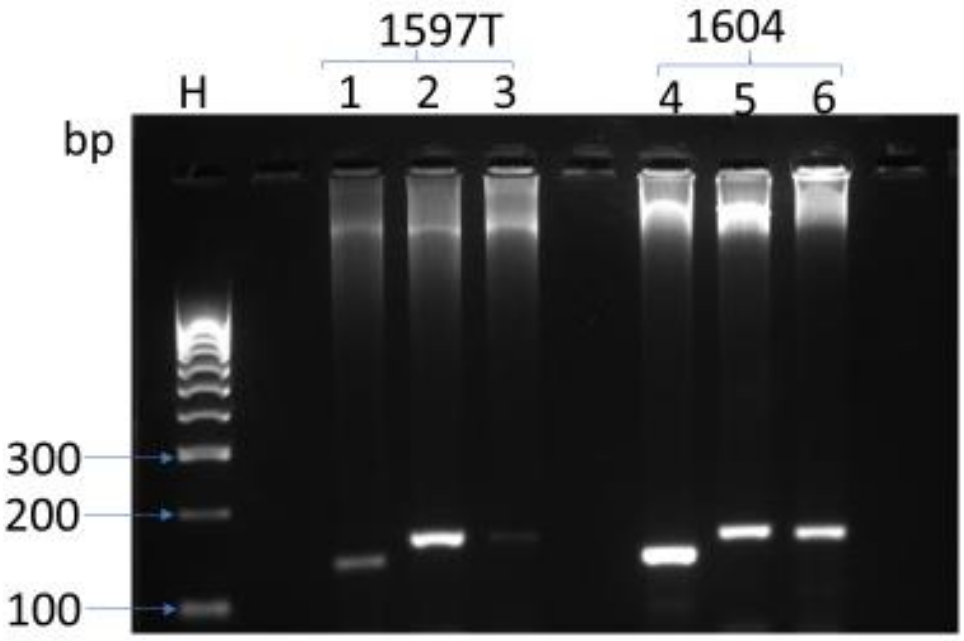


Figure 3.7. Agarose gel electrophoresis of RT-PCR products of tissue samples 1597T and 1604.

Lane H, Hyperladder 100 bp; lanes 1 & 4, *Gapdh* (127 bp); lanes 2 & 5, *Neil3* (147 bp); lanes 3 & 6, *Ercc1* (146 bp).

Figure 3.7 is the first of seven gels showing the RT-PCR results from the sixteen biobank tissue samples listed in Table 3.2. Sample 1597T was obtained from the tumour of a 54 year-old male, while 1604 was from a 79 year-old female. The results of the PCR from each sample look quite different, with all bands with the exception of *Neil3* much stronger in 1604. It is worth noting that the *Gapdh* band is much stronger in 1604 and, if reproducible, will have a bearing on the qPCR results, where the amount of PCR product was compared with that of *Gapdh*. However, all reactions again show just a single band of the correct size indicating that the PCR primers can be used for qPCR.

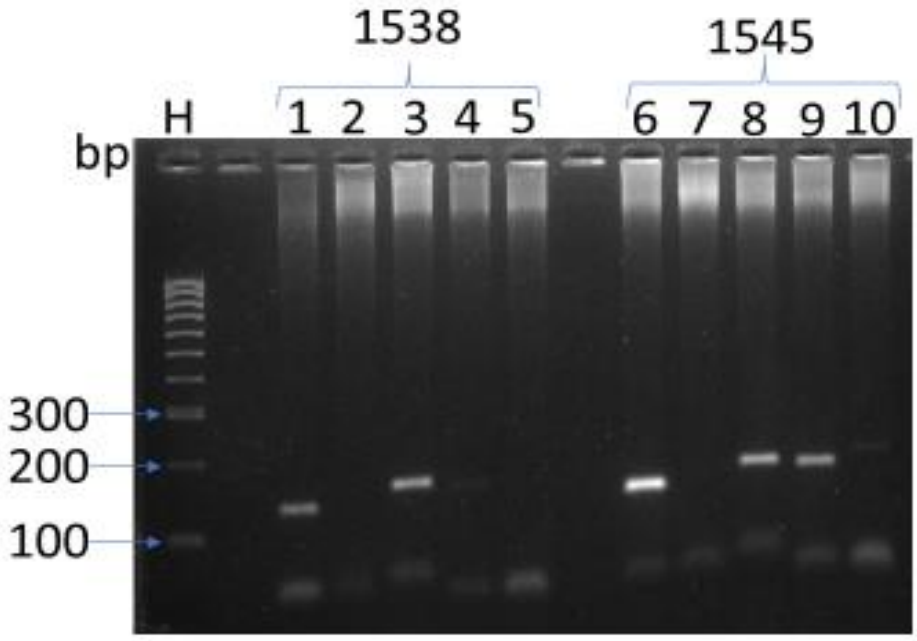


Figure 3.8. Agarose gel electrophoresis of RT-PCR products of tissue samples 1538 & 1545 for *Gapdh*, *Neil1*, *Neil3*, *Ercc1* and *Nthl1*.

Lane H, Hyperladder 100 bp; lanes 1 & 6, *Gapdh*); lanes 2 & 7, *Neil1*; lanes 3 & 8, *Neil3*; lanes 4 & 9, *Ercc1*; lanes 5 & 10, *Nthl1*.

In Figure 3.8, cDNA prepared from RNA extracted from samples 1538 and 1545 were subjected to RT-PCR for *Neil1* (212 bp), *Neil3* (147 bp), *Ercc1* (146 bp) and *Nthl1* (165 bp) in addition to *Gapdh* (127 bp). Sample 1538 was obtained from a 72 year-old male and sample 1545 from a 63 year-old female. Again a single band was obtained from each sample where the PCR produced a band and only the reactions for *Neil1* failed to yield a product in either sample (Figure 3.8, lanes 2 and 7). Again, the intensity of the band for *Gapdh* varies between the samples (lanes 1 and 6). Only *Neil3* is at a similar intensity in both samples (lanes 3 and 8). *Ercc1* appears more highly expressed in sample 1545 (lanes 4 and 9) and *Nthl1* is also more pronounced in 1545 (lanes 5 and 10).

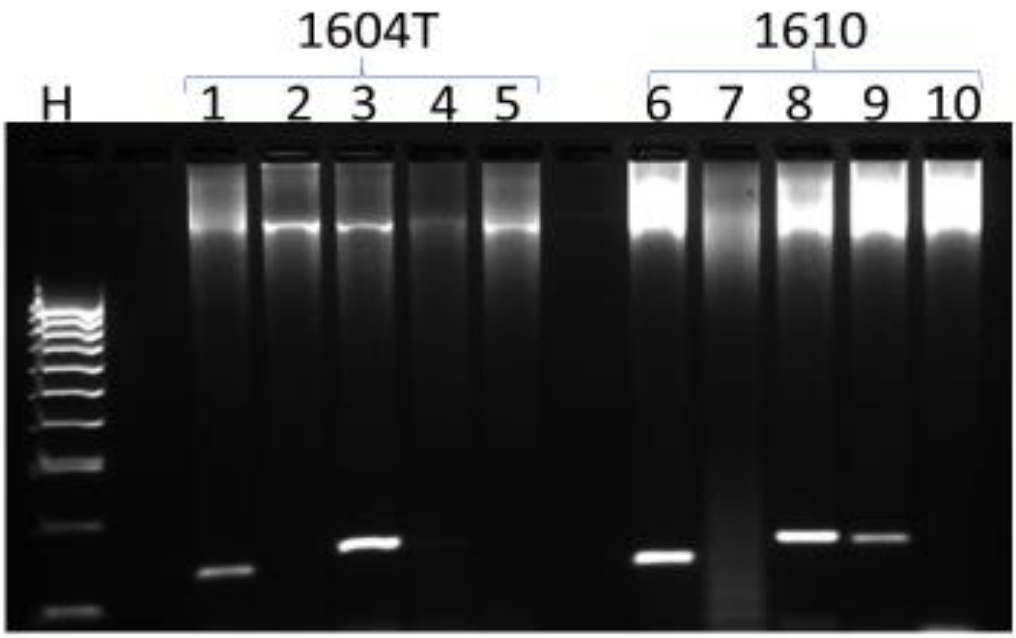


Figure 3.9. Agarose gel electrophoresis of RT-PCR products of tissue samples 1604T & 1610 for *Gapdh*, *Neil1*, *Neil3*, *Ercc1* and *Nthl1*.

Lane H, Hyperladder 100 bp; lanes 1 & 6, *Gapdh*; lanes 2 & 7, *Neil1*; lanes 3 & 8, *Neil3*; lanes 4 & 9, *Ercc1*; lanes 5 & 10, *Nthl1*.

In Figure 3.9, cDNA prepared from RNA extracted from samples 1604T and 1610 were subjected to RT-PCR for *Neil1*, *Neil3*, *Ercc1* and *Nthl1* in addition to *Gapdh*. Sample 1604T was obtained from a 79 year-old female and sample 1610 from a 59 year-old male. The results are similar to those presented in Figure 3.8 in that no band was obtained for *Neil1* (lanes 2 and 7) and that the intensity of the band for *Gapdh* is different between the two samples (lanes 1 and 6). *Neil3* is again present as a high intensity band in both samples and while *Ercc1* is only faintly visible in 1604T, it is more intense in 1610. However, no band was obtained for *Nthl1* from either tumour sample (lanes 5 and 10).

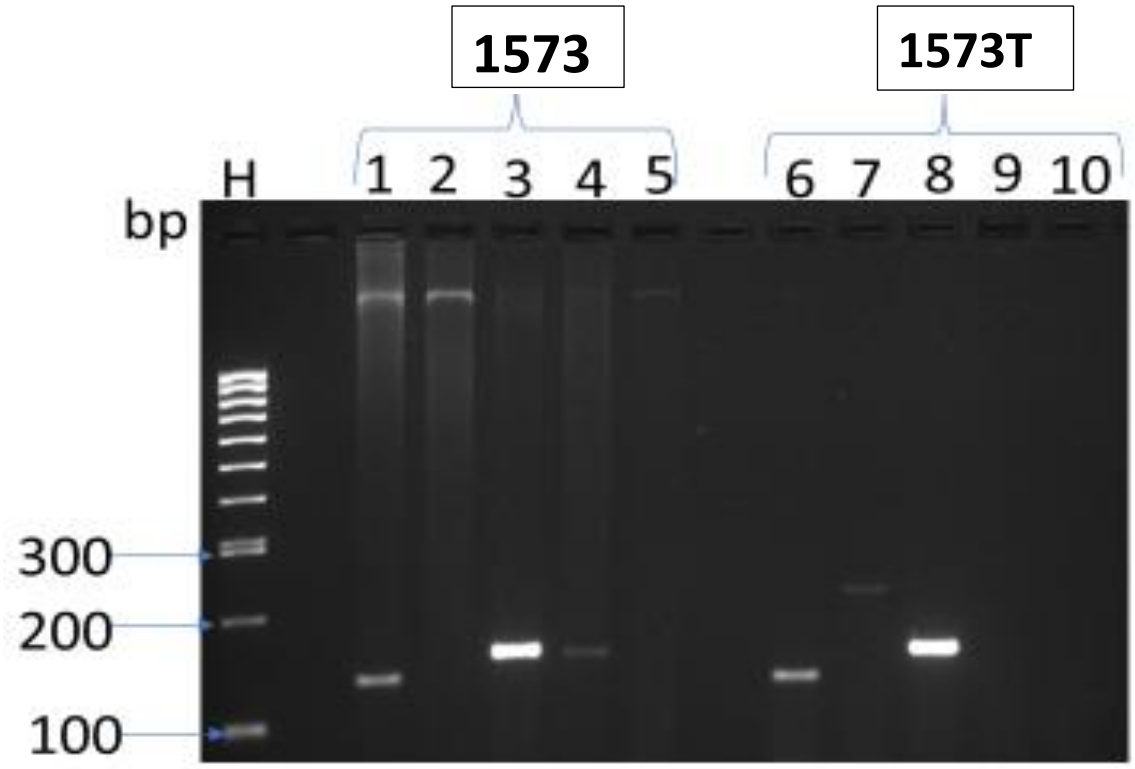


Figure 3.10. Agarose gel electrophoresis of RT-PCR products of tissue samples 1573 & 1573T for *Gapdh, Neil1*, *Neil3, Ercc1* and *Nthl1*.

Lane H, Hyperladder 100 bp; lanes 1 & 6, *Gapdh*; lanes 2 & 7, *Neil1*; lanes 3 & 8, *Neil3*; lanes 4 & 9, *Ercc1*; lanes 5 & 10, *Nthl1*.

In Figure 3.10, cDNA prepared from RNA extracted from samples 1573 and 1573T were subjected to RT-PCR for *Neil1*, *Neil3*, *Ercc1* and *Nthl1* in addition to *Gapdh*. Sample 1573 was obtained from the colon of a 70 year-old female and sample 1573T also from the colon of a 70 year-old female. The results again indicate high expression levels of *Neil3* (lanes 3 and 8) and similar levels of expression of the control gene, *Gapdh* (lanes 1 and 6). While *Neil1* is again absent in sample 1573 (lane 2), a band is observed for Neil1 in sample 1573T (lane 7). Conversely, *Ercc1* expression is observed in sample 1573 (lane 5) but not in 1573T (lane 10). Expression of *Nthl1* was not observed in either sample (lanes 5 and 10).

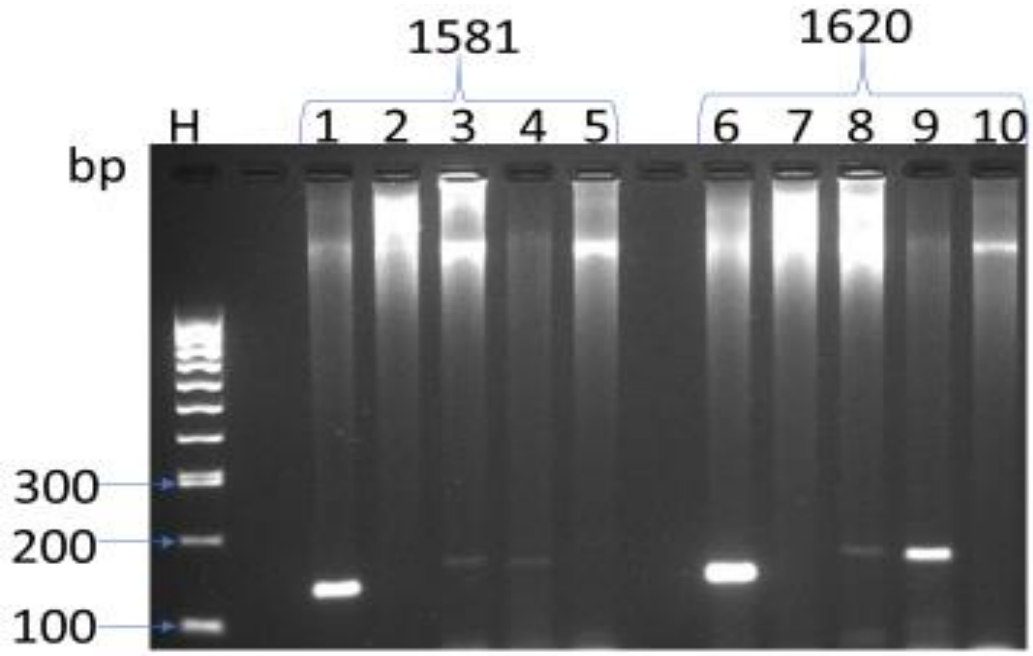


Figure 3.11. Agarose gel electrophoresis of RT-PCR products of tissue samples 1581 and 1620 for *Gapdh*, *Neil1*, *Neil3*, *Ercc1* and *Nthl1*.

Lane H, Hyperladder 100 bp; lanes 1 & 6, *Gapdh*; lanes 2 & 7, *Neil1*; lanes 3 & 8, *Neil 3*; lane 4 & 9, *Ercc1*; lanes 5 & 10, *Nthl1*.

In Figure 3.11, cDNA prepared from RNA extracted from samples 1581 and 1620 were subjected to RT-PCR for *Neil1, Neil3, Ercc1* and *Nthl1* in addition to *Gapdh*. Sample 1581 was obtained from a 62 year-old female and sample 1620 from the colon of a 65 year-old male. In both these samples, *Gapdh* is an intense band, indicating that there was nothing in the PCR mixture inhibiting the reaction (lanes 1 and 6). However, the only other intense band observed is that for *Nthl1* in 1620 (lane 9). *Neil3* is again present in both samples but the bands are much reduced in intensity with respect to *Gapdh* as seen previously (lanes 3 and 8). No bands for *Neil1* or *Nthl1* were observed in either sample.

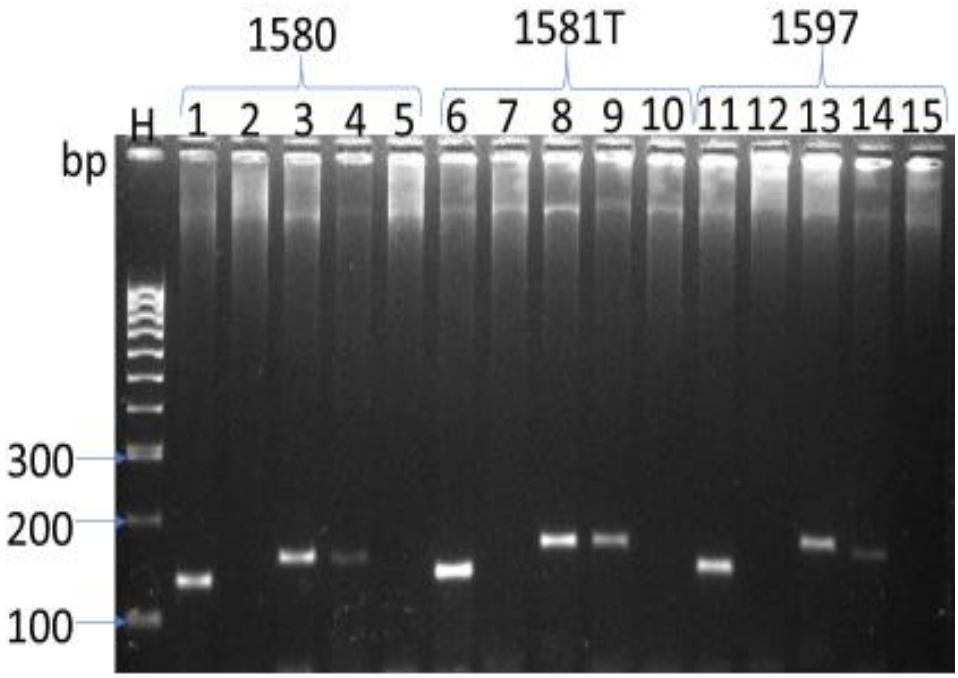


Figure 3.12. Agarose gel electrophoresis of RT-PCR products of tissue samples 1580, 1581T & 1597 for *Gapdh*, *Neil1*, *Neil3*, *Ercc1* and *Nthl1*.

Lane H, Hyperladder 100 bp; lanes 1, 6 & 11, *Gapdh*; lanes 2, 7 & 12, *Neil1*; lanes 3, 8 & 13, *Neil3*; lanes 4, 9 & 14, *Ercc1*; lanes 5, 10 & 15, *Nthl1*.

In Figure 3.12, cDNA prepared from RNA extracted from samples 1580, 1581T and 1597 were subjected to RT-PCR for *Neil1*, *Neil3*, *Ercc1* and *Nthl1* in addition to *Gapdh*. Sample 1580 was obtained from a 69 year-old male, sample 1581T from a 62 year-old female and sample 1597 from the caecum of a 54 year-old male. The results are strikingly similar for all three samples: *Gapdh*, *Neil3* and *Ercc1* PCR products all show a similar pattern of intensity while no bands arre observed for *Neil1* or *Nthl1*.

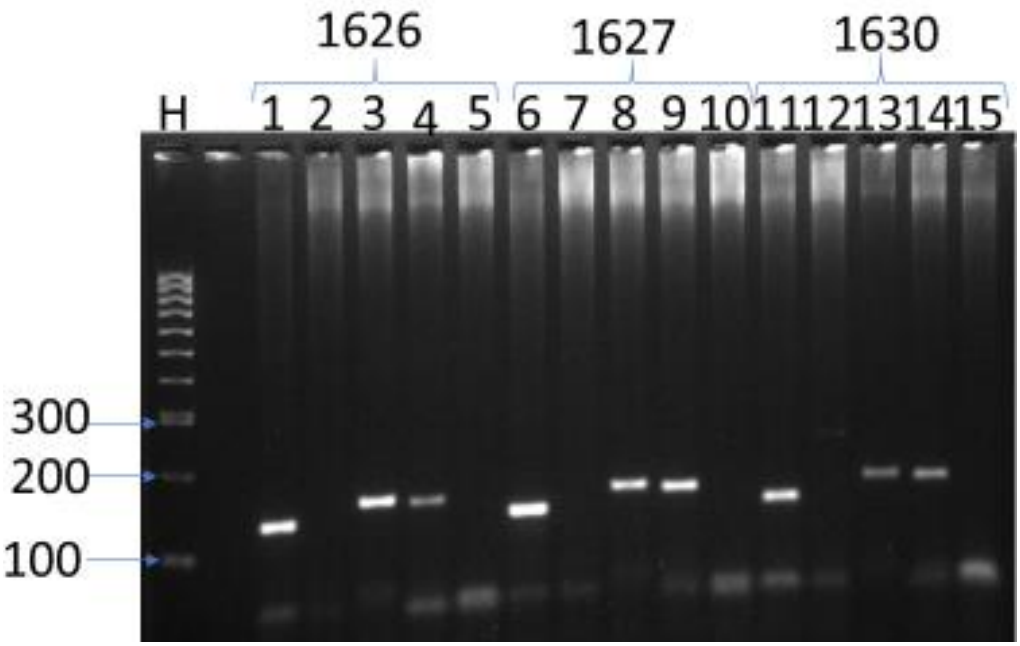


Figure 3.13. Agarose gel electrophoresis of RT-PCR products of tissue samples 1626, 1627 & 1630 for *Gapdh*, *Neil1*, *Neil3*, *Ercc1* and *Nthl1*.

Lane H, Hyperladder 100bp; lanes 1, 6 & 11, *Gapdh*; lanes 2, 7 & 12, *Neil1*; lanes 3, 8 & 13, *Neil3*; lanes 4, 9 & 14, *Ercc1*; lanes 5, 10 & 15, *Nthl1*.

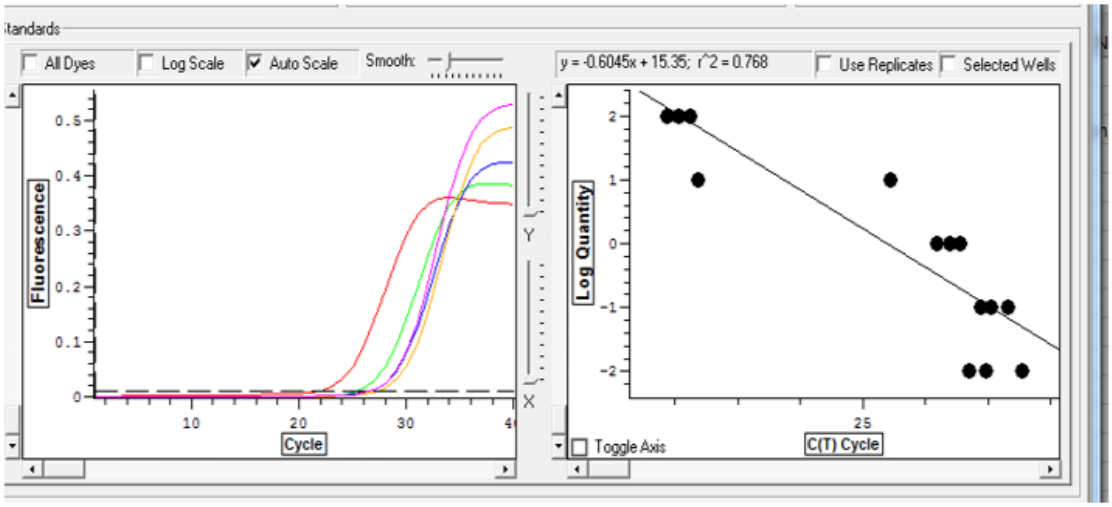
In Figure 3.13, cDNA prepared from RNA extracted from samples 1626, 1627 and 1630 were subjected to RT-PCR for *Neil1*, *Neil3*, *Ercc1* and *Nthl1* in addition to *Gapdh*. Sample 1626 was obtained from the colon of a 73 year-old female, sample 1627 from a 64 year-old male and sample 1630 from the colon of a 64 year-old male. As in Figure 3.13, the pattern of bands is similar across all the tumour samples with *Gapdh*, *Neil3* and *Ercc1* expressed in all three tumour samples. A faint band for *Neil1* is observed in sample 1630 (lane 12), but *Nthl1* is absent from all tumour samples (lanes 5, 10, 15).

3.2.2. Quantitative PCR

Quantitative PCR (qPCR) is one of the most powerful technologies in molecular biology. It is a fluorescent based technique used to measure the level of the mRNA of target genes with the aid of fluorescent dye such as SYBR Green 1. By plotting fluorescence against the cycle number, an amplification plot that represents the accumulation of product over the duration of the PCR reaction was generated. Appropriate normalization is required in qPCR to sort out experimental errors that may arise from extraction of RNA and generation of

cDNA. *Gapdh* was used as a reference gene, as it is assumed that its expression level is constant in cells. Each run of qPCR has a standard curve that was generated through serial dilution of the cDNA. The melting curves are indicators of the purity and specificity of primers for target genes as shown in Figure 3.14 to Figure 3.17.

A B



C

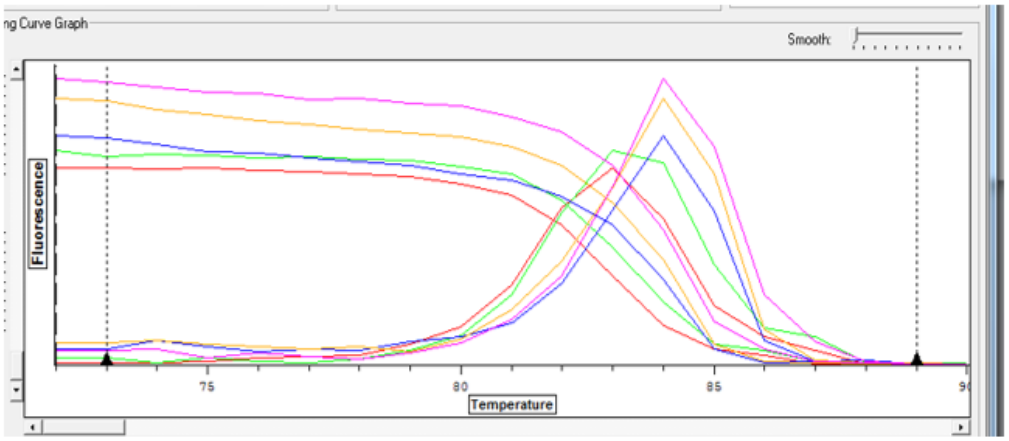
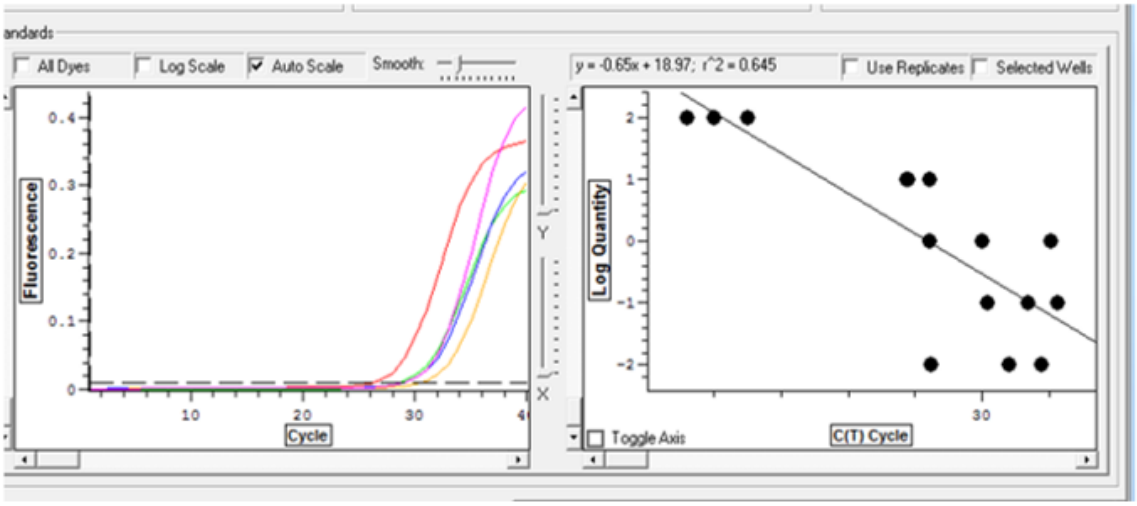


Figure 3.14. Analysis of *Gapdh* expression in tissue sample 10T.

In Figure 3.14A, each coloured line represents a single PCR reaction of a 10-fold serial dilution of the cDNA. The threshold cycle (Ct) was set on the graph at a point where the Ct and the log quantity became linear. In this example, the sample reaches the Ct value at 22 cycles (Figure 3.14A). The linear regression curve (the standard curve for use with *Gapdh*) represents the relation between the Ct cycle and the log quantity and r^2 showing a

value of 0.768 (Figure 3.14B). The melting curve with a single peak shows that a single product is amplified (Figure 3.14C).

A B



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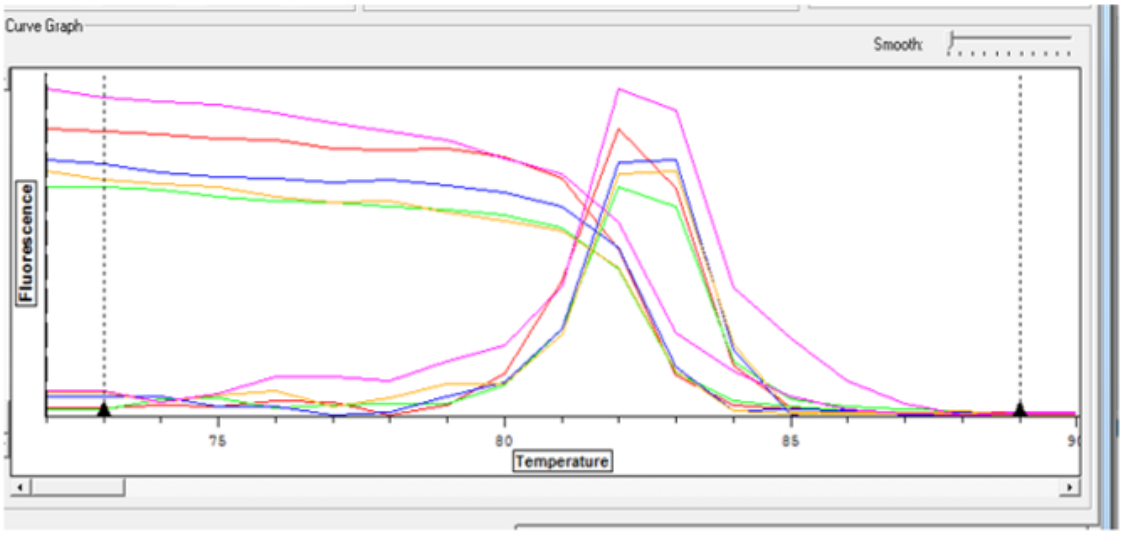
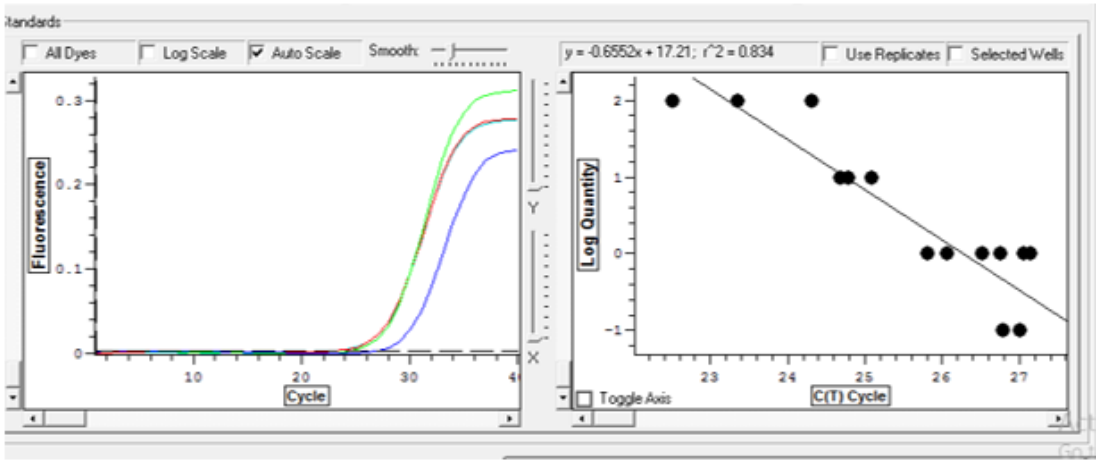


Figure 3.15. Analysis of *Neil3* expression in tissue sample 10T.

Figure 3.15A, each coloured line represents a single PCR reaction of a 10-fold serial dilution of the cDNA. The threshold cycle (Ct) was set on the graph at a point where the Ct and the log quantity became linear. In this example, the sample reaches the Ct value at 26 cycles (Figure 3.15A). The linear regression curve (the standard curve for use with *Gapdh*) represents the relation between the Ct cycle and the log quantity and r^2 showing a value of 0.645 (Figure 3.15B). The melting curve with a single peak indicatess that a single

product is amplified (Figure 3.15C).

A B



C

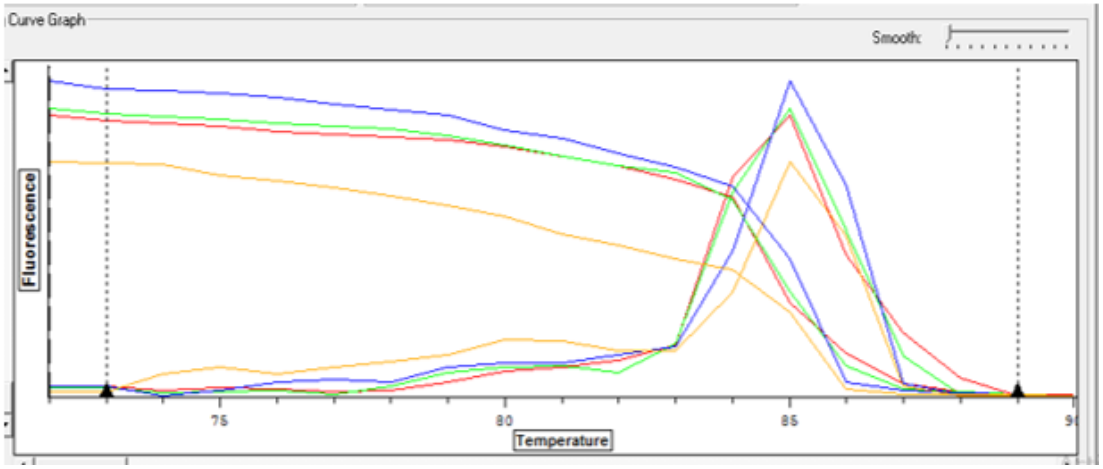


Figure 3.16. Analysis of *Ercc1* expression in tissue sample 13N.

In Figure 3.16A each coloured line represents a single PCR reaction of a 10-fold serial dilution of the cDNA.The threshold cycle (Ct) was set on the graph at a point where the Ct and the log quantity became linear. In this example, the sample reaches the Ct value at 26 cycles (Figure 3.16A). The linear regression curve (the standard curve for use with Gapdh) represents the relation between the Ct cycle and the log quantity and r^2 showing a value of 0.834 (Figure 3.16B). The melting curve with a single peak shows that a single product is amplified (Figure 3.16C).

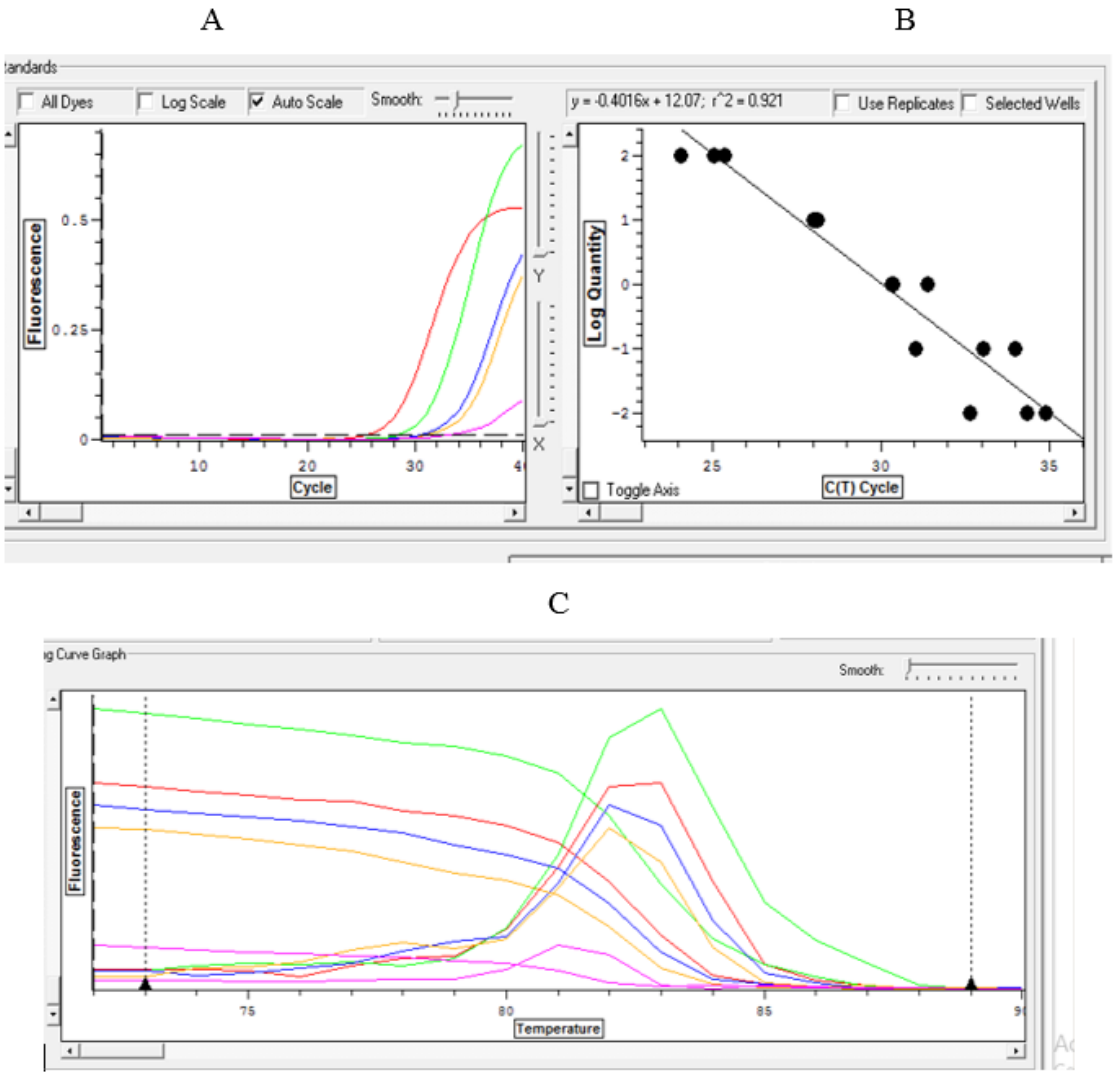


Figure 3.17. Analysis of *Mlh1* expression in tissue sample 34T.

In Figure 3.17A each coloured line represents a single PCR reaction of a 10-fold serial dilution of the cDNA. The threshold cycle (Ct) was set on the graph at a point where the Ct and the log quantity became linear. In this example, the sample reaches the Ct value at 26 cycles (Figure 3.17B). The linear regression curve (the standard curve for use with *Gapdh*) represents the relation between the Ct cycle and the log quantity and r^2 showing a value of 0.921 (Figure 3.17B). The melting curve with a single peak indicates that a single product is amplified (Figure 3.17C).

3.2.2.1. Analysis of the Expression Patterns of Selected DNA Repair Genes in Different Colon Cancer Tissues Relative to Normal Colon Tissue.

Following the RT-PCR experiments detailed in Figure 3.6 to Figure 3.13, which confirmed that one band of the correct length was obtained for each primer pair, and the control experiments detailed in Figure 3.14 to Figure 3.17, qPCR was carried out, firstly to compare gene expression between CRC tumour and matched normal colon tissue (Figure 3.18 to Figure 3.19; Table 3.3) and then to compare expression of the selected DNA repair genes against that of the control *Gapdh* (Figure 3.20 to Figure 3.26; Table 3.5). Initially, the expression patterns of seven different DNA repair genes, *Neil1*, *Neil2*, *Neil3*, *Ogg1*, *Nthl1*, *Ercc1* and *Mlh1* were quantified with *Gapdh* employed as the housekeeping gene (control; Figure 3.18), however, this was reduced due to practical considerations to just three CRC relevant target genes from three different DNA repair pathways, BER, NER and MMR: *Neil3, Ercc1* and *Mlh1* (Figure 3.19).

Results are presented as bar graphs and summarized in two tables, where the fold difference in expression is clearly assigned. As the range of fold-increase in gene expression varied widely, and in order to show the results in a meaningful way, the y-axis range has also been varied between graphs, as it was not possible to show all results using just one axis (Figure 3.20 to Figure 3.22). Furthermore, the results obtained for the sixteen CRC samples are also shown based on the target gene of interest in order to give a better visual appreciation of the spread of results obtained (Figure 3.23 to Figure 3.26).

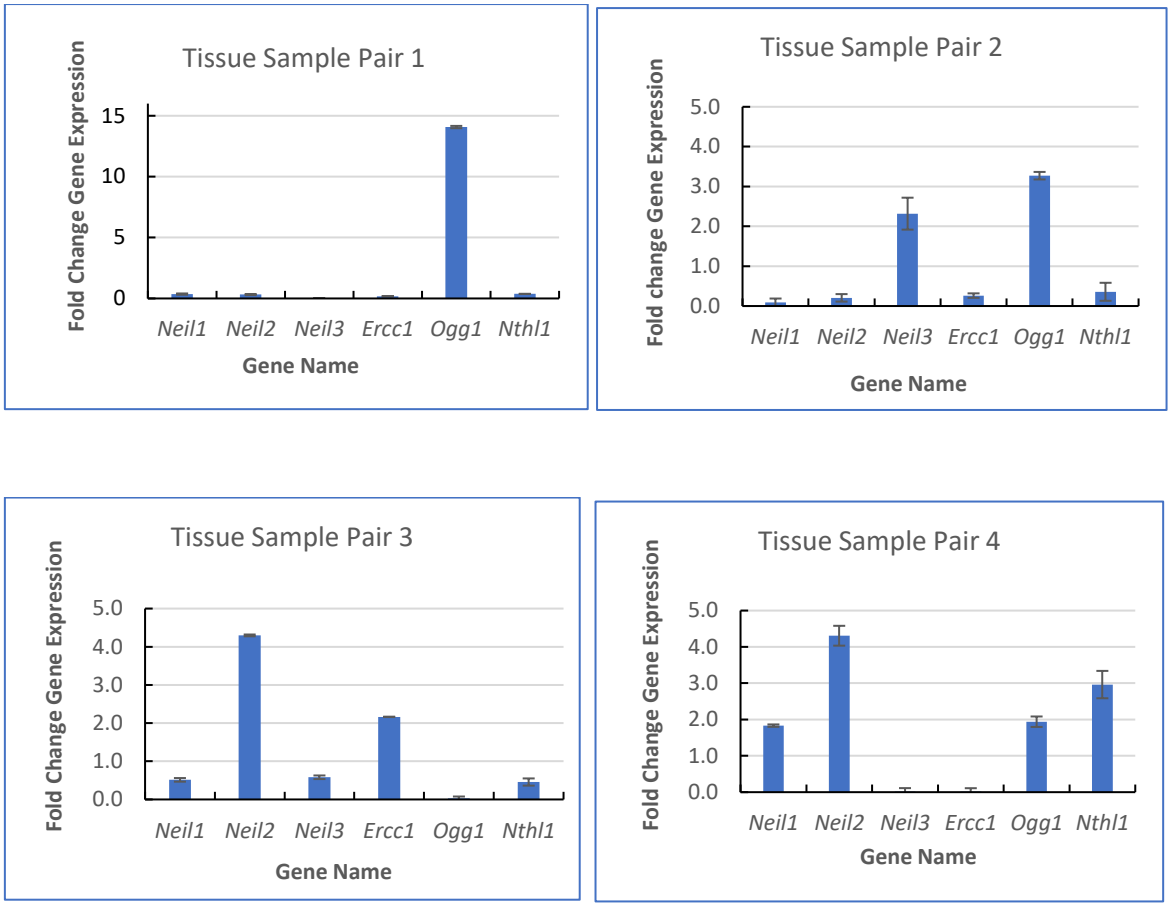
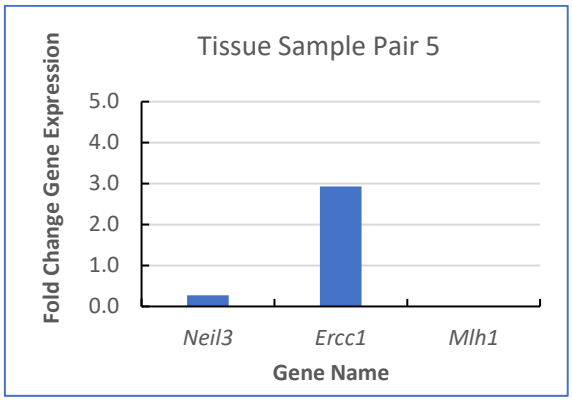


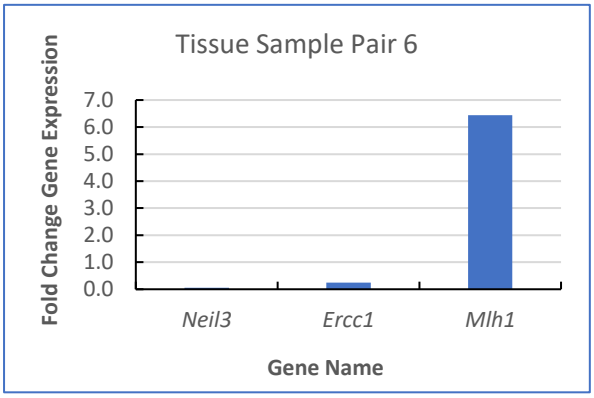
Figure 3.18. Gene expression levels of six DNA repair genes in colon tumour tissue compared to matched normal tissue

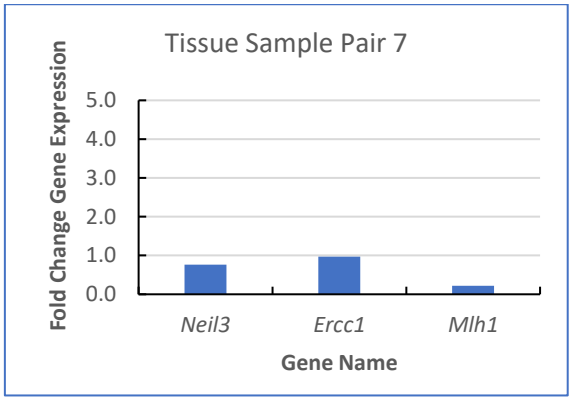
Figure 3.18 shows the gene expression of the DNA glycosylase genes, *Neil1*, *Neil2*, *Neil3*, *Ogg1* and *Nthl1* and the NER gene *Ercc1* in colon tumour samples when compared with matched normal colon tissue. Overall, there is no clear pattern of expression in the different tumour samples, with different genes being most highly expressed in each of the four paired samples. For example, in tissue sample pair 1, the expression of *Ogg1* was the only gene to be induced in the tumour sample with a 14-fold increase. In the other tumour samples increased expression of one or more genes was more modest. In tissue pair 2, the expression of *Neil3* and *Ogg1* were induced 2.3- and 3.3-fold respectively, while in tissue pair 3, *Neil2* (4.3-fold) and *Ercc1* (3.3-fold) were induced in the tumour sample. In tissue pair 4, *Neil2* (4.3-fold) and *Nthl1* (3.0-fold) expression was induced, with *Neil1* and *Ogg1* just below the 2.0-fold induction level.

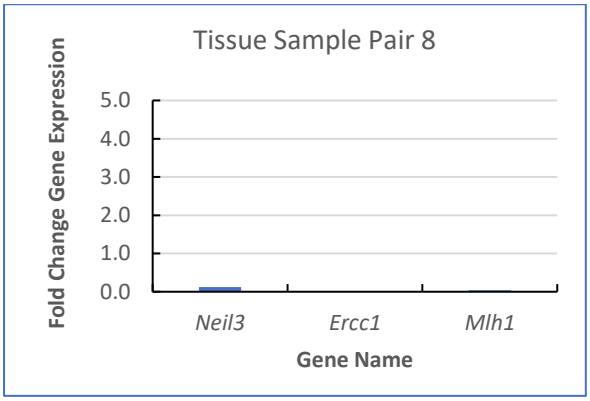
Following analysis of these results it was decided to focus on the expression of *Ercc1*, which is thought to be a contributing factor in the resistance of colon tumours to oxaliplatin

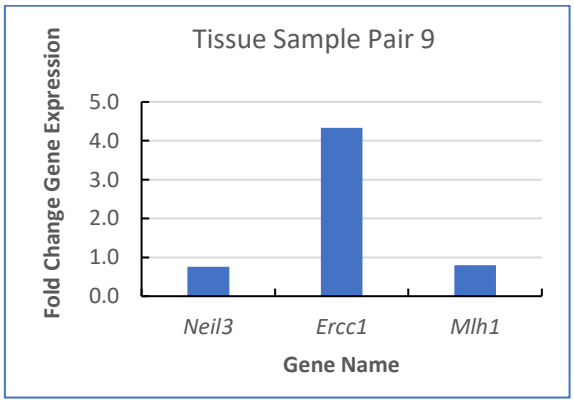
(Sheetharam *et al.*, 2010) and *Neil3*, as there is increasing evidence from our laboratory and others that it too may be involved in the repair of ICLs and therefore resistance to oxaliplatin treatment (Martin *et al.*, 2017; Semlow *et al.*, 2016). Finally, the expression of the MMR gene *Mlh1* was also studied because a lack of MMR and *Mlh1* in particular, is often associated with CRC (Kheirelseid *et al.*, 2013).

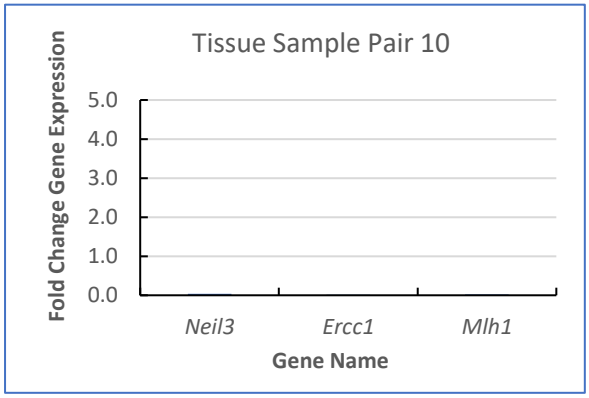












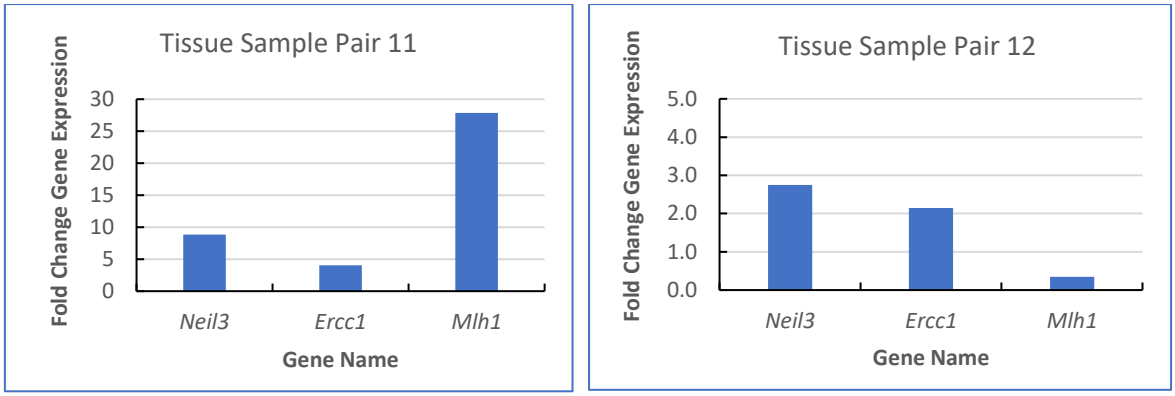


Figure 3.19 Gene expression levels of three DNA repair genes in colon tumour tissue compared to matched normal tissue

Similar to the results obtained in Figure 3.18, Figure 3.19 shows no clear pattern of gene expression in the eight tissue pairs analysed. *Neil3* was induced in only two of the sample pairs (Pair 11, 8.8-fold; Pair 12 2.7-fold), while expression of *Ercc1* was induced in four of the eight samples, ranging from 2.1-fold (Pair 12) to 4.3-fold (Pair 9). *Mlh1* also showed highly variable expression levels with an induction of 27.8-fold observed in Pair 11. None of the genes analysed were induced in tissue pairs 8 and 10.

As no obvious pattern was emerging form these samples, it was decided to obtain fresh samples from a biobank in Manchester. For this study, the expression of genes encoding proteins likely to be involved in the repair of ICLs (*Neil*, *Neil3*, and *Ercc1*) was studied, along with *Nthl1*, which encodes a DNA glycosylase, the lack of which is known to be a risk factor in early onset CRC (Weren *et al.*, 2018).

Table 3.3. Summary of the expression patterns of selected DNA repair genes in colon tumours versus matched normal colon tissue.

Samples	**≤ 1-fold**	**1-2 fold**	**2-10 fold**	**≥ 10-fold**
Pair 1	*Neil1*, *Neil2*, *Neil3*, *Ercc1* & *Nthl1*			*Ogg1*
Pair 2	*Neil1*, *Neil 2*, *Ercc1* & *Nthl1*		*Neil3* & *Ogg1*	
Pair 3	*Neil1*, *Neil3*, *Ogg1* & *Nthl1*		*Neil2* & *Ercc1*	
Pair 4	*Neil3* & *Ercc1*	*Neil1* & *Ogg1*	*Neil2* & *Nthl1*	
Pair 5	*Neil3* & *Mlh1*		*Ercc1*	
Pair 6	*Neil3* & *Ercc1*		*Mlh1*	
Pair 7	*Neil3*, *Ercc1* & *Mlh1*			
Pair 8	*Neil3*, *Ercc1* & *Mlh1*			
Pair 9	*Neil3* & *Mlh1*		*Ercc1*	
Pair 10	*Neil3*, *Ercc1* & *Mlh1*			
Pair 11			*Neil3* & *Ercc1*	*Mlh1*
Pair 12	*Mlh1*		*Neil3* & *Ercc1*	

3.2.3. Analysis of the Expression Patterns of DNA Repair Genes in Different Colon Tumour Samples Relative to *Gapdh.*

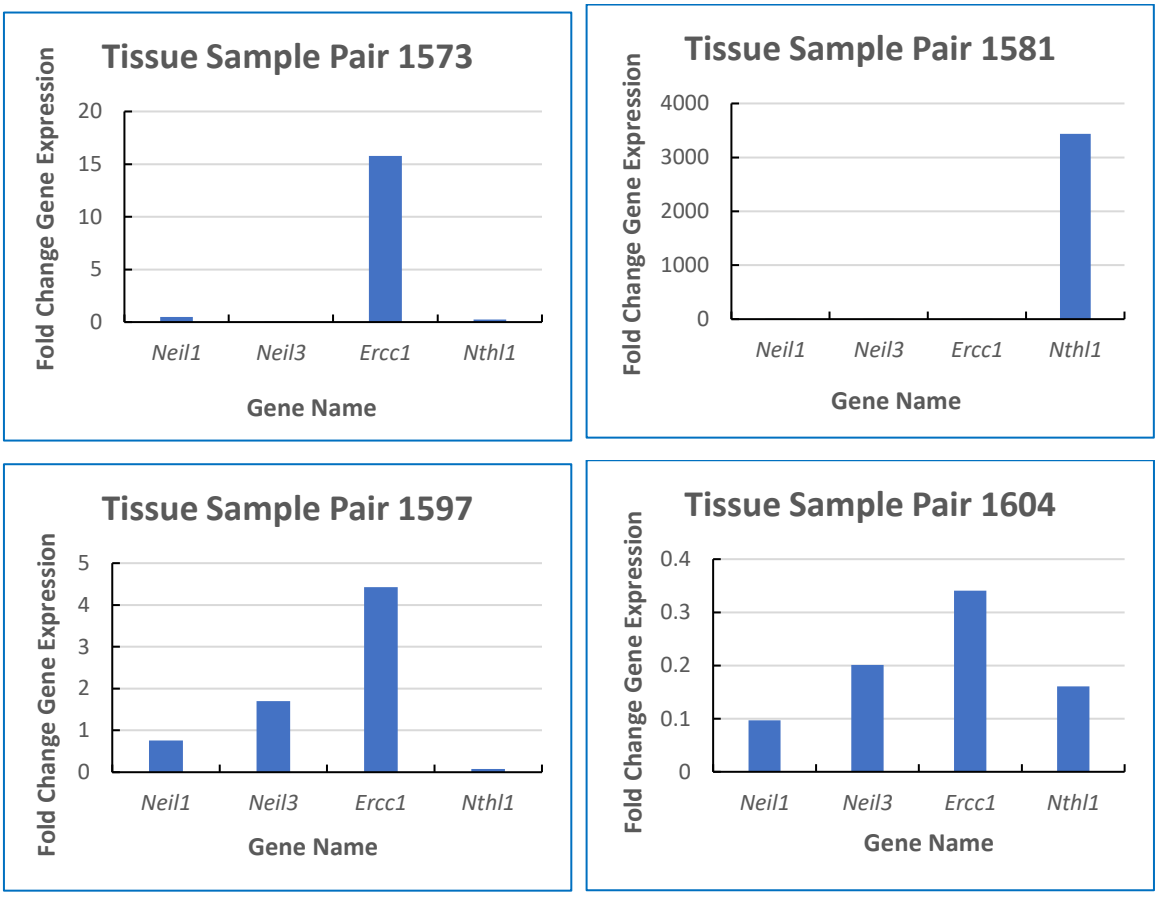


Figure 3.20. Gene expression levels of four DNA repair genes in biobank colon tumour tissue compared to normal colon tissue samples

Table 3.4. Summary of the expression patterns of four selected DNA repair genes in colon tumours versus matched normal colon tissue (Biobank samples).

Samples	≤ 1-fold	1-2 fold	2-10 fold	≥ 10 fold
Pair 1573	*Neil1*, *Neil3* & *Nthl1*			*Ercc1*
Pair 1581	*Neil3*	*Neil1*	*Ercc1*	*Nthl1*
Pair 1597	*Neil1* & *Nthl1*	*Neil3*	*Ercc1*	
Pair 1604	*Neil1*, Neil3, *Ercc1* & *Nthl1*			

Figure 3.20 and Table 3.4 shows the expression of the DNA repair genes, *Neil1*, *Neil3*, *Ercc1* and *Nthl1* in colon tumour samples when compared with matched normal colon tissues. It should be noted that tissue sample pair 1581 exhibited abnormally high levels of *Nthl1* (a 3436-fold increase) that masks the behaviour of the other genes tested. The results show that

the expression of *Neil1* did not change substantially between normal and tumour samples, with the highest fold difference occurring in tissue pair 1581 (1.3 fold). Similarly, the change in *Neil3* expression was less than 2-fold in all tissue pairs (0.03 fold in pair 1581). However, higher levels of *Ercc1* were observed in three out of the four tissue pairs, with a 3.4-fold increase being observed for pair 1581, compared with 15.8-fold for 1573 and 4.4-fold for pair 1597. With the exception of pair 1581, the expression of *Nthl1* did not change substantially in the tumour samples. As no obvious pattern is evident, and the scale of fold change also differs markedly between samples, Figure 3.23 to Figure 3.26 present the data by gene and also where necessary using different scales on the y-axis to present the data more clearly.

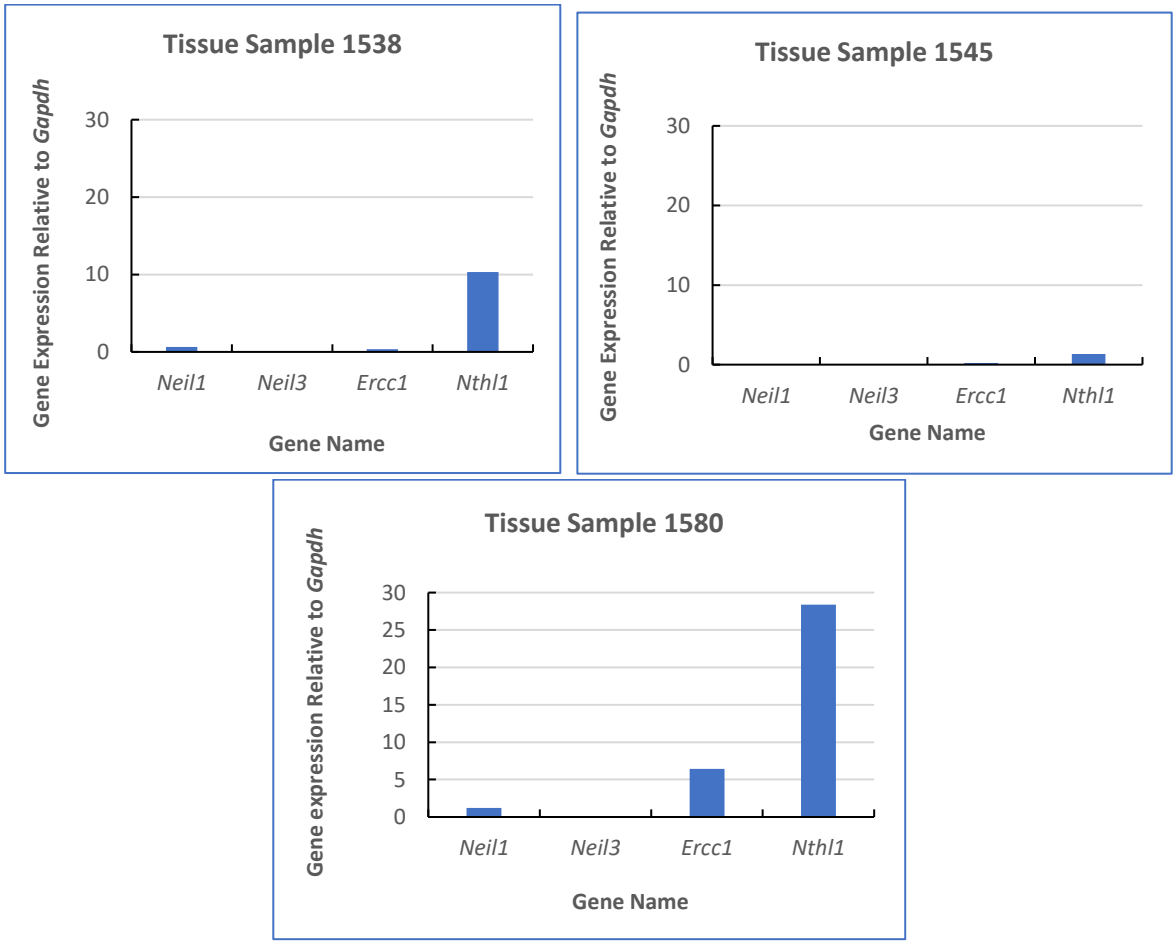


Figure 3.21. Gene expression levels of four DNA repair genes in biobank normal colon tissue samples.

In Figure 3.21, three normal colon tissues were examined for the expression of the DNA repair genes. *Neil1*, *Neil3*, *Ercc1* and *Nthl1* in comparison with *Gapdh*. The results show that most of the genes were not highly expressed in comparison with *Gapdh* in the three

normal colon tissue samples. The exception was sample 1580 that showed high expression of *Nthl1* (28-fold). Low level of *Ercc1* was also observed in majority of the samples, the exception being sample 1580, where expression was 6-fold greater than that of *Gapdh.*

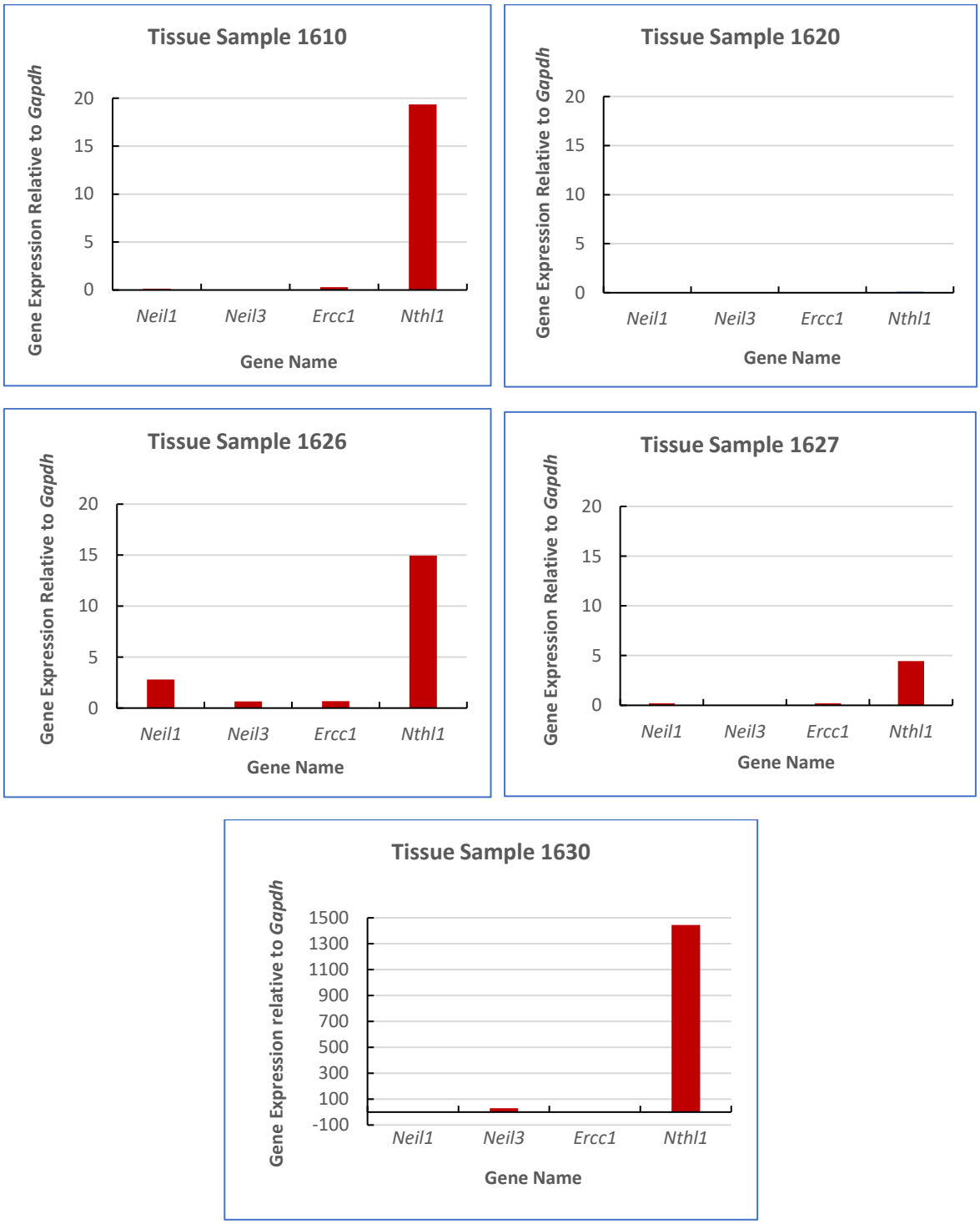
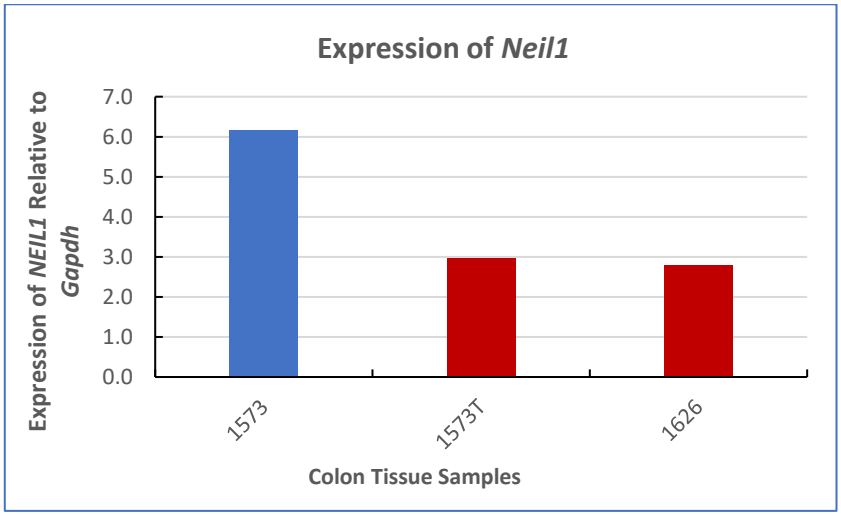


Figure 3.22. Gene expression levels of four DNA repair genes in biobank colon tumour tissue samples.

In Figure 3.22, five colon tumour tissues were examined for the expression of the DNA repair genes, *Neil1*, *Neil3*, *Ercc1* and *Nthl1* in comparison with *Gapdh*. The results show that, similar to the normal tissue samples, most of the genes were not highly expressed relative to *Gapdh* in most of the five colon tumour samples. The exception is *Nthl1*, which was highly expressed (more than 4-fold) in four out of the five tumour samples and abnormally highly expressed in sample 1630.

A.



B.

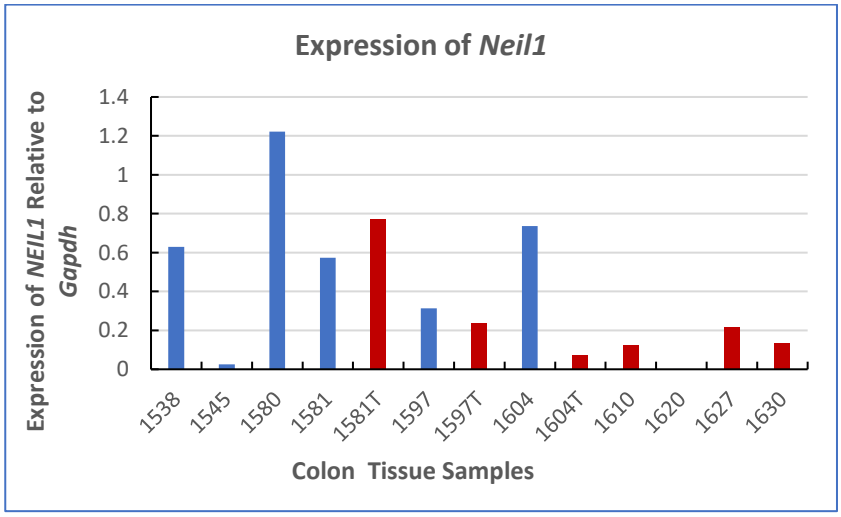


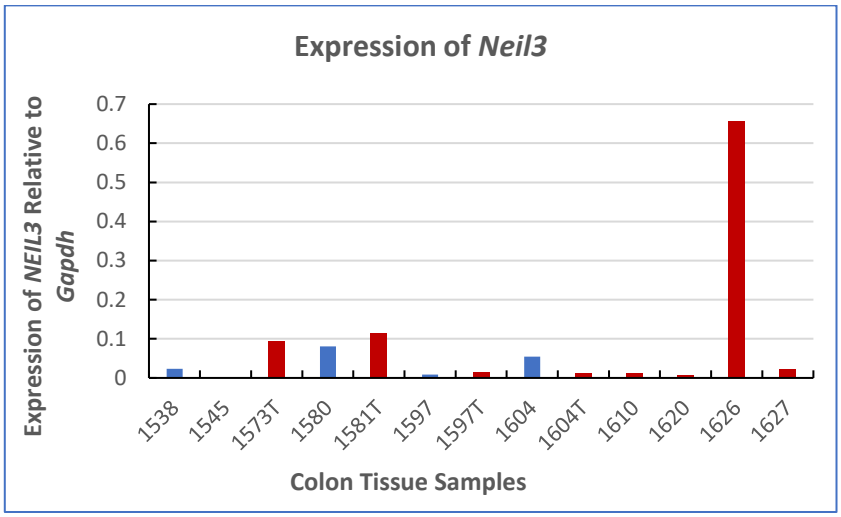
Figure 3.23. Gene expression levels of *Neil1* in sixteen colon tissue samples.

Normal tissue samples are shown in blue and tumour samples in red.

In Figure 3.23, the sixteen colon tissue samples were examined for the comparative expression of the DNA repair gene *Neil1*. In this instance, all of the samples showed higher expression levels of *Neil1* when compared with *Gapdh*. However, the range of gene

expression varied from 0.0006-fold in sample 1620 (tumour) to 6.2-fold in sample 1573 (normal).

A.



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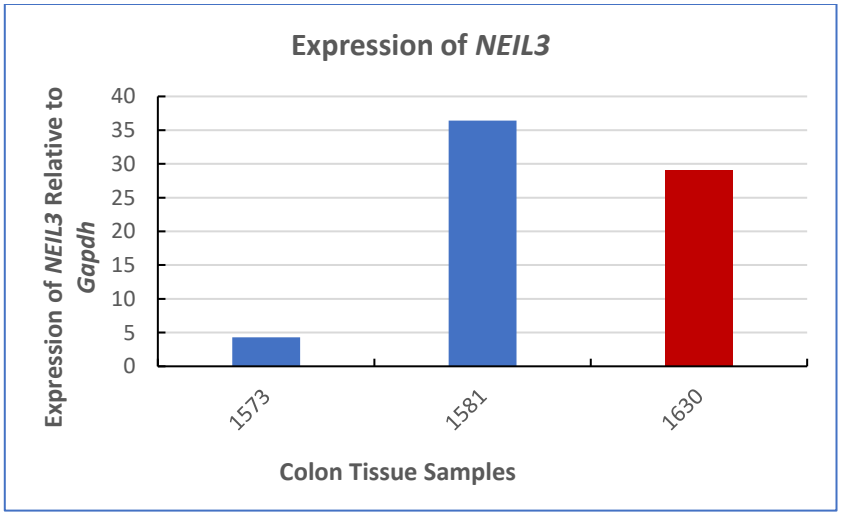
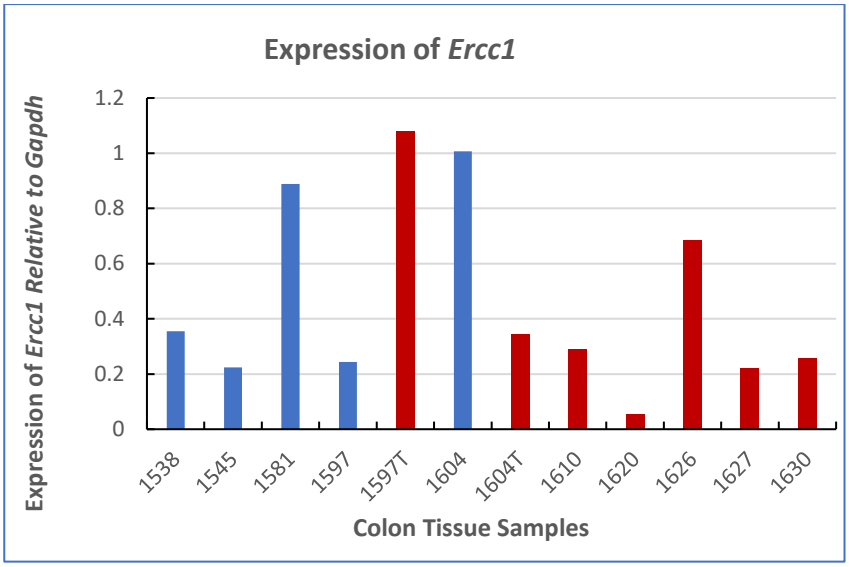


Figure 3.24. Gene expression levels of *Neil3* in sixteen colon tissue samples.

Normal tissue samples are shown in blue and tumour samples in red.

Figure 3.24 is a representation of the expression of *Neil3* DNA repair gene for the sixteen colon tissue samples following qPCR analysis. Evidently, the expression of *Neil3* gene were very low in virtually all the samples except samples 1573 (normal), 1581 (normal) and 1630 (tumour; 4.3-fold, 36.4-fold and 29.1-fold respectively) which showed high expression of *Neil3* relative to *Gapdh.* Clearly there seems to be no substantial increase in the gene expression of *Neil3* in the colon tumour samples compared with the normal colon tissue.

A.



B.

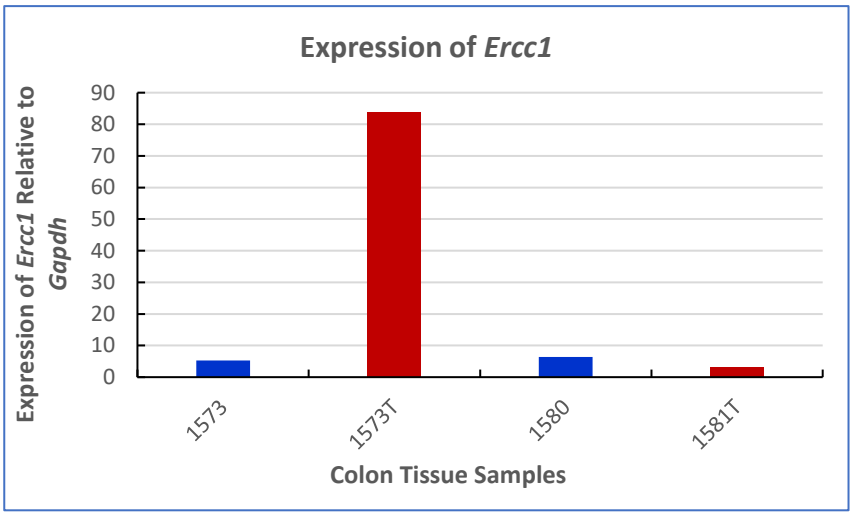


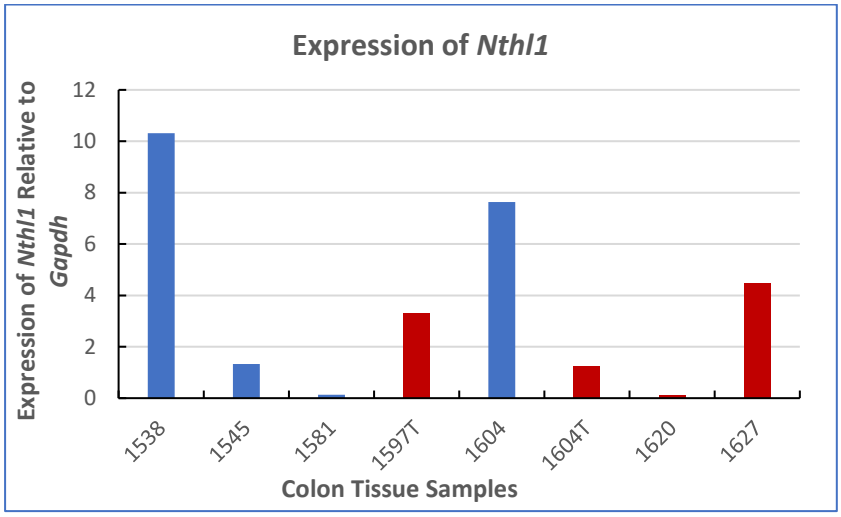
Figure 3.25. Gene expression levels of *Ercc1* in sixteen colon tissue samples.

Normal tissue samples are shown in blue and tumour samples in red.

The gene expression of *Ercc1* was assessed in the sixteen colon tissue samples and is shown in Figure 3.25A & B. In all samples, the expression of *Ercc1* was higher than that of *Gapdh*, however Figure 3.25A indicates that in ten of the samples the level of expression was less than 1-fold greater than *Gapdh*, and in two others (1597T and 1604), just over one-fold greater than *Gapdh*. On the other hand, Figure 3.25B shows that three samples showed relatively high (3 – 6-fold) levels of *Ercc1* expression, while one (1573T) had abnormally high levels of expression (84-fold). Interestingly, the matched normal colon tissue sample of the latter, 1573, had a high expression basal level of *Ercc1* (5.3-fold over *Gapdh*)

indicating that expression levels of *Nthl1* in this patient were elevated compared with the majority of patient samples.

A.



B.

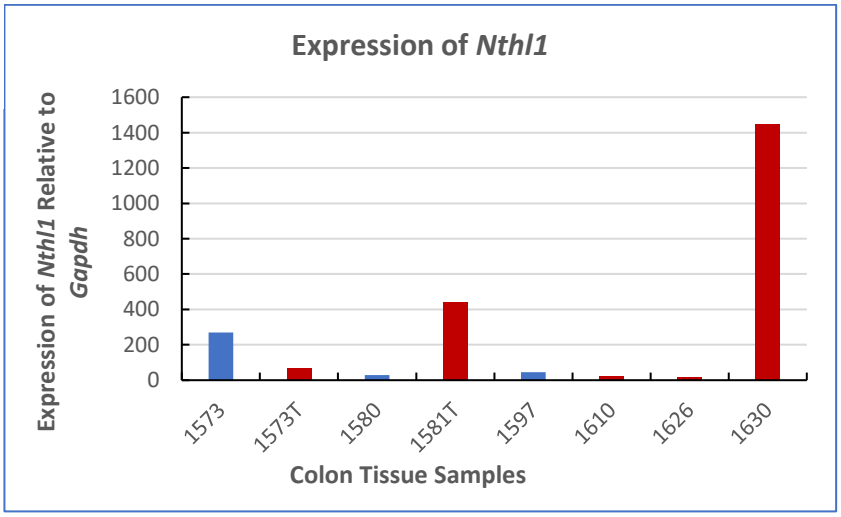


Figure 3.26. Gene expression levels of *Nthl1* in sixteen colon tissue samples. Normal tissue samples are shown in blue and tumour samples in red.

In Figure 3.26, sixteen colon tissue samples were examined for the comparative expression of the DNA damage repair gene, *Nthl1*. Again, gene expression of *Nthl1* is very heterogeneous across samples and, with the exception of 1630 (tumour), higher expression levels are observed in both normal and tumour samples.

The gene expression data for the sixteen colon tissue samples is summarized in Table 3.5. From Table 3.5 it can be seen that the expression of *Nthl1* is most frequently highly expressed in cells of the colon, but that this can occur in normal cells as well as tumour cells.

Similarly, *Ercc1* was moderately highly expressed (>2 fold) in four tissue samples, but this was split 50:50 between normal and tumour samples. Thus overall, the data in Table 3.5 does not indicate any increased expression of any of the four DNA repair genes tested in a tumour – specific manner.

Table 3.5 Summary of the expression patterns of DNA repair genes for the sixteen colon tissue samples obtained from the biobank. Figures represent fold difference to the expression of Gapdh.

Sample No.	Status	≤1-fold	1-2 fold		2-10 fold	≥ 10 fold
1538	Normal	*Neil1*, *Neil3* & *Ercc1*				*Nthl1*
1545	Normal	*Neil1*, *Neil3* & *Ercc1*	*Nthl1*			
1573	Normal				*Neil1*, *Neil3* & *Ercc1*	*Nthl1*
1580	Normal	*Neil3*	*Neil1*		*Ercc1*	*Nthl1*
1581	Normal	*Neil1*, *Ercc1* & *Nthl1*				*Neil3*
1597	Normal	*Neil1*, *Neil3* & *Ercc1*				*Nthl1*
1604	Normal	*Neil1* & *Neil3*	*Ercc1*		*Nthl1*	
1573T	Tumour	*Neil3*	*Neil1*			*Ercc1* & *Nthl1*
1581T	Tumour	*Neil1* & *Neil3*			*Ercc1*	*Nthl1*
1597T	Tumour	*Neil1* & *Neil3*	*Ercc1*		*Nthl1*	
1604T	Tumour	*Neil1*, *Neil3* & *Ercc1*	*Nthl1*			
1610	Tumour	*Neil1*, *Neil3* & *Ercc1*				*Nthl1*
1620	Tumour	*Neil1*, *Neil3*, *Ercc1* & *Nthl1*				
1626	Tumour	*Neil3* & *Ercc1*			*Neil1*	*Nthl1*

1627	Tumour	*Neil1*, *Neil3* & *Ercc1*			*Nthl1*	
1630	Tumour	*Neil1* & *Ercc1*				*Neil3* & *Nthl1*

3.3. Medulloblastoma: DAOY Normal and Cisplatin-Resistant Cell Lines.

Medulloblastoma SHH group DAOY cells were grown in cisplatin as described in Section 2.3.2 and the surviving resistant cells used in the following experiments and compared with the parental cells.

3.4. Cell Viability Assay on Medulloblastoma cell lines.

To check that the surviving cells were in fact more resistant to cisplatin than the parental cell line, MTT assays were carried out to determine the IC50 values of each cell line for cisplatin, oxaliplatin and *tert*-butyl hydroperoxide, a chemical that induces ROS and therefore oxidative DNA damage in the cells. Figure 3.27 shows that the cisplatin-treated cells were more resistant to the genotoxic effects of cisplatin, with an IC50 value of 11.6 µM compared with 3.8 µM for the parental cells (Table 3.6). The result of the MTT assay with oxaliplatin was not clear cut and no difference between the cell lines was observed (

Figure **3.28**). Lastly, the cells were treated with an agent known to induce oxidative stress within cells. As NEIL3 has DNA glycosylase activity and is known to be active on oxidized pyrimidines (Albelazi *et al.*, 2019), it was thought that if NEIL3 was induced in response to cisplatin, the cells may also be more resistant to *tert*-butyl hydroperoxide, an agent known to induce oxidative DNA damage in mammalian cells (Duweb, 2015). Figure 3.29 shows that this hypothesis was correct and that the cisplatin – resistant cells were also more resistant to this oxidizing agent (4.9 and 3.9 µM respectively), giving circumstantial evidence at least, that the process of repairing cisplatin lesions in DNA also requires proteins involved in removing oxidised (base) damage from DNA.

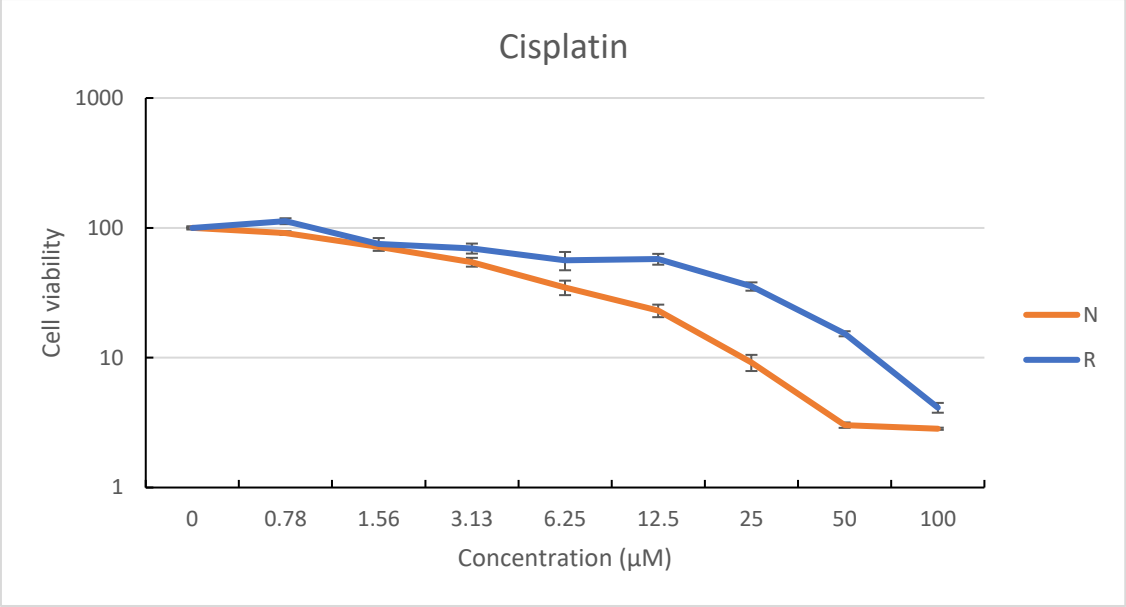


Figure 3.27. Growth response of DAOY normal and cisplatin - resistant cell lines to cisplatin.

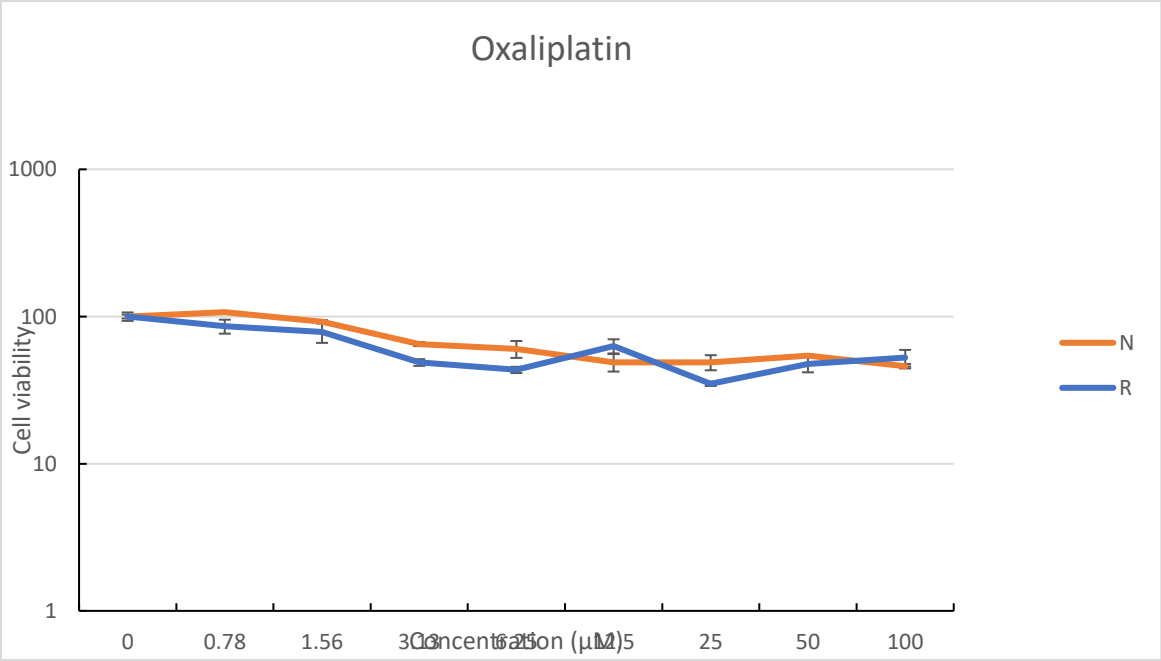


Figure 3.28. Growth response of DAOY normal and cisplatin - resistant cell lines to oxaliplatin.

In Figures 3.27 and 3.28, the orange line represents the normal, and the blue line the cisplatin - resistant DAOY cell line.

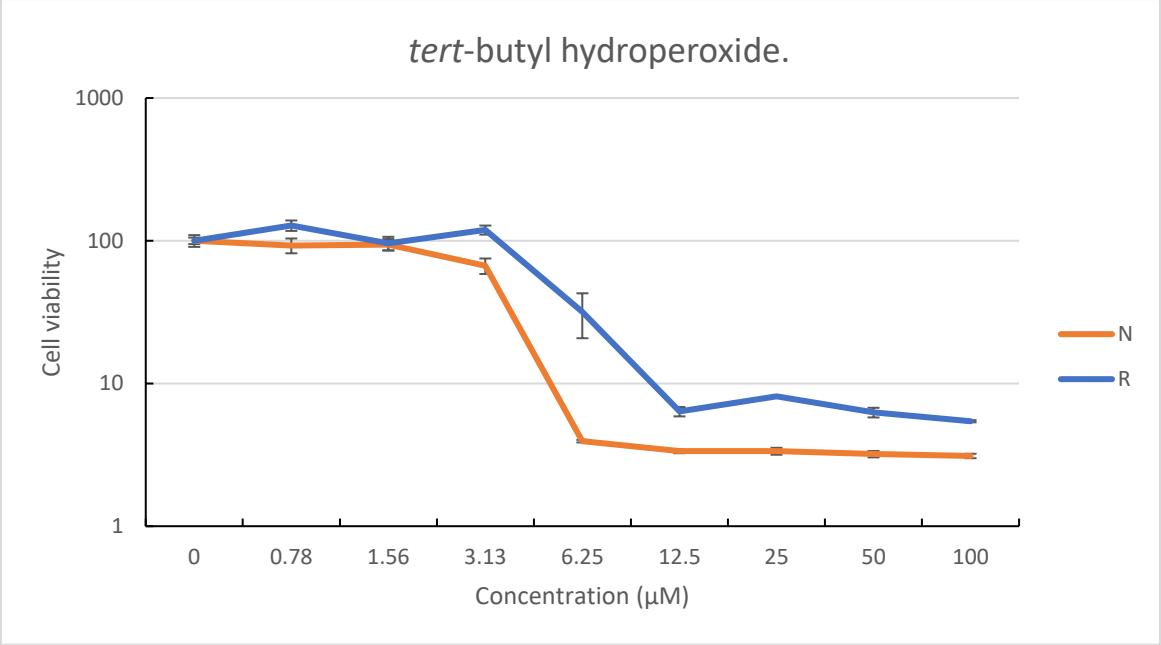


Figure 3.29. Growth response of DAOY normal and cisplatin - resistant cell lines to *tert*-butyl hydroperoxide.

The orange line represents the normal, and the blue line the cisplatin - resistant DAOY cell line.

Table 3.6. IC50 concentrations for three genotoxic agents used to treat the DAOY cell lines.

Drug	Normal (µM)	Cisplatin - resistant (µM)
oxaliplatin	25.74	27.34
cisplatin	3.78	11.61
tert-butyl hydroperoxide	3.97	4.93

3.4.1. Assessment of Yield and Integrity of RNA Extracted from Medulloblastoma DAOY normal and cisplatin - resistant cell lines.

RNA extraction was performed to assess the yield and integrity of RNA extracted from DAOY medulloblastoma cell (normal) & cisplatin-resistant DAOY cells. The aim was to establish the quality of the RNA purified from the cell lines. The RNA was extracted and separated by agarose gel electrophoresis producing two distinct bands of rRNA (Figure

3.30). This confirmed the presence of the intact ribosomal RNA bands (28S &18S) for total RNA extracted from the cells which indicated that the mRNA was most likely intact in the samples.

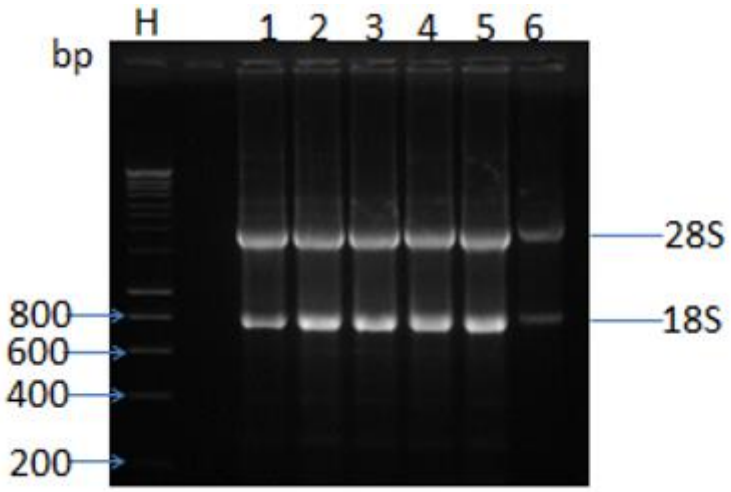


Figure 3.30. Agarose gel electrophoresis of RNA extracted from DAOY medulloblastoma cells (Normal & cisplatin - resistant).

Lane H, Hyperladder 1kb; lanes 1 - 4, RNA from DAOY cells; lanes 5 & 6, RNA from cisplatin - resistant DAOY cells.

3.4.2. Confirmation of Target Product and Primer Specificity.

RT-PCR was carried out on cDNA from the two medulloblastoma cell lines using specific primers for the target genes. To confirm the primer specificity and ensure that genes of interest were amplified, agarose gel electrophoresis of the RT-PCR products was conducted, with particular attention to the band size and to ensure only a single product was obtained (Figure 3.31).

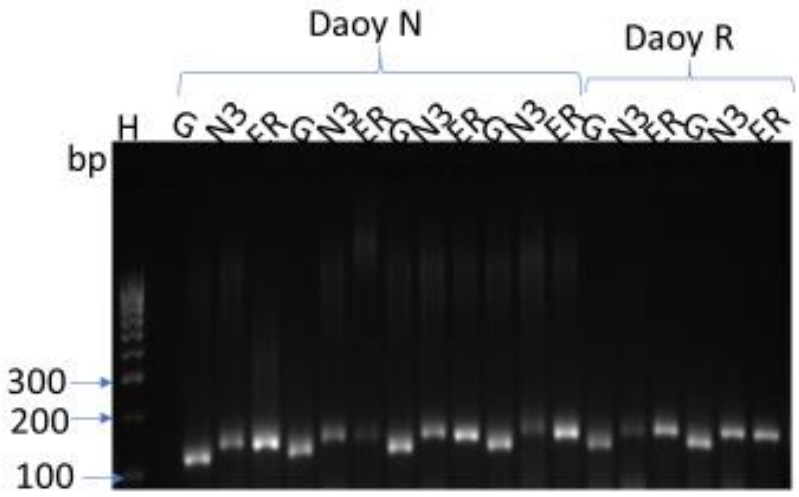


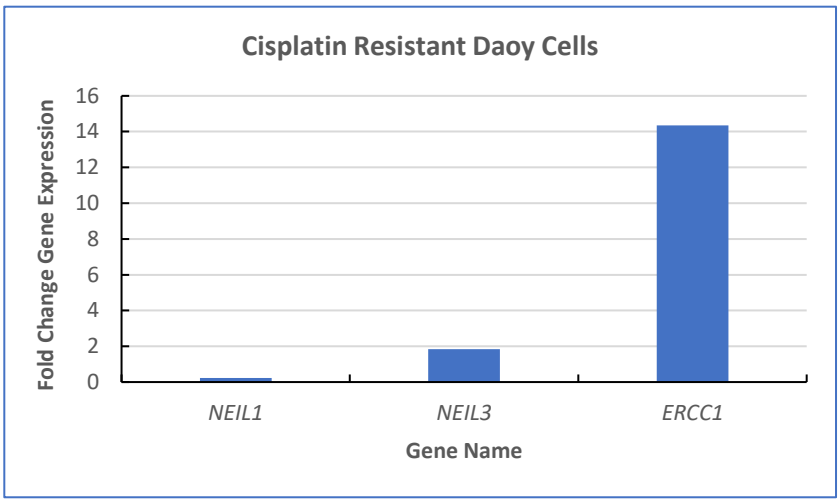
Figure 3.31. Agarose gel electrophoresis of RT-PCR products for *Gapdh, Neil3* and *Ercc1* from DAOY normal and cisplatin-resistant cells.

Lane H, Hyperladder 100 bp; G represents *Gapdh* (127 bp); N3 represents *Neil3* (147 bp); ER represents *Ercc1* (146 bp).

Figure 3.31 shows the PCR products for *Gapdh*, *Neil3* and *Ercc1* from four different RNA samples from DAOY cells and two different samples of RNA from cisplatin – resistant DAOY cells. Clearly, both cell lines express all three genes with inter-sample variation evident between the different RNA samples. However, as only one band of the correct size was obtained for each sample, the primer pairs and cDNA samples could now be assessed by qPCR.

3.4.3. Expression pattern of selected DNA repair genes in medulloblastoma DAOY cells.

A.



B.

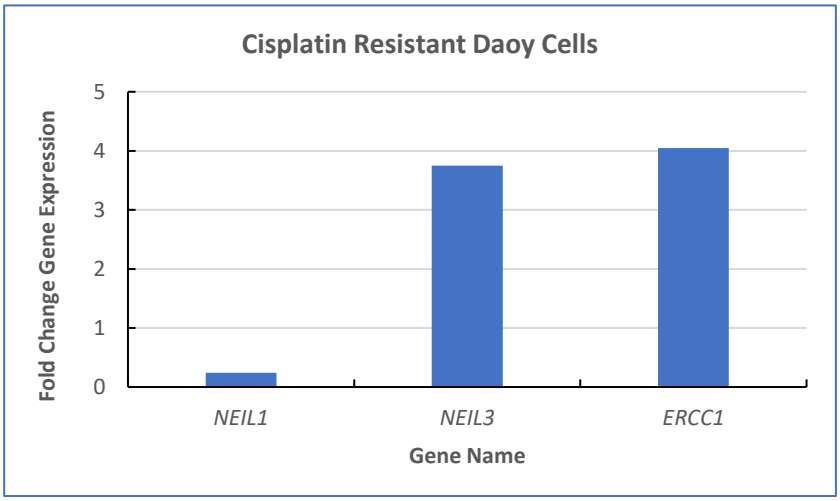


Figure 3.32. Increased gene expression levels of *Neil1, Neil3* and *Ercc1* in cisplatin resistant DAOY cell lines. (A) and (B) are representations of the same data, with outlyers removed in (B).

Figure 3.32 shows the fold increase in the expression of *Neil1, Neil3 and Ercc1* in the cisplatin –resistant DAOY cell lines. As the experiment was only performed once and the raw data shows a high level of variability, it was decided to present the data in two froms. In Figure 3.32A, the means were taken from all three replicates, the -ΔΔCt values obtained, and the fold change calculated. This clearly shows a more than 14-fold increase in *Ercc1* expression and only about a 2-fold increase in *Neil3*. However, when the outlying data was removed, and the means calculated from the remaining, consistent, figures, the results were

substantially different (Figure 3.32B). Now, the increase in gene expression for *Ercc1* and *Neil3* is similar, with both showing about a 4-fold increase over that in the control cells. However, in both scenarios, the expression of *Neil1* is largely unaffected by the chronic cisplatin treatment (Figure 3.32).

Of course, to be confident in the result, the qPCR experiment would have to be repeated, however, the results presented in Figure 3.32B do more reflect the western blot results shown below (Figure 3.33 and Figure 3.34), where NEIL3 protein does seem to be increased in the cisplatin – resistant cells.

3.4.4. Protein Analyses

Following separation by SDS-PAGE, the proteins were transferred onto PVDF membranes and probed with specific antibodies (Figure 3.33 and Figure 3.34). Figure 3.33 shows a western blot that has been probed with antibodies specific for NEIL3 and β-actin, as the loading control. It is clear that the amount of NEIL3 is greater in the protein samples extracted from the cisplatin treated DAOY cells. This correlates with induction of *Neil3* gene expression observed in Figure 3.32B. However, an induction of ERCC1 was not observed by western blot (Figure 3.34) unlike the gene expression levels observed in Figure 3.32. Due to time constraints, these experiments were only performed once, and would need to be repeated to obtain a definitive answer.

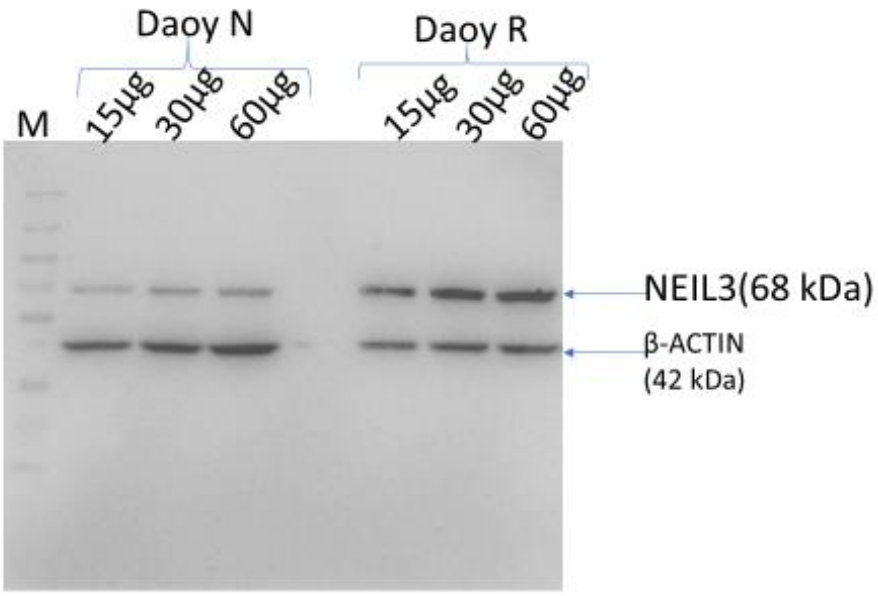


Figure 3.33. Western blot analysis of NEIL3 and β-actin in DAOY normal (DAOY N) and cisplatin - resistant (DAOY R) cell lines.

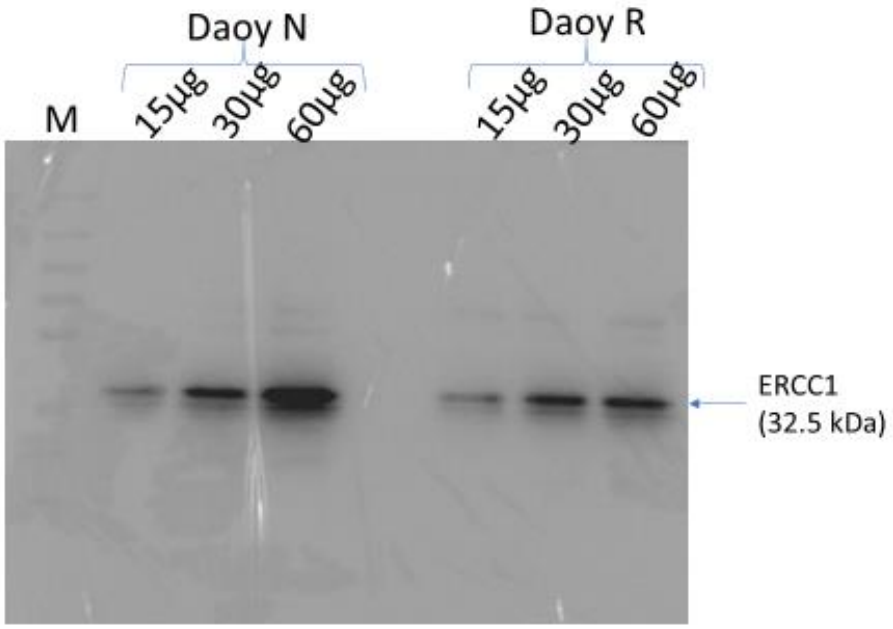


Figure 3.34. Western blot analysis of ERCC1 in DAOY normal and cisplatin - resistant (DAOY R) cell lines.

3.5. Mesothelioma

3.5.1. Assessment of Integrity of RNA Extracted from Mesothelioma Cells.

The Mero25 cell line was cultured as described in Section 2.3.2 and total RNA extracted using the Bioline Isolate II kit as described in Section 2.3.5. Figure 3.35 shows that the two rRNA bands were consistently obtained from both the Mero25 and putative Mero25 – derived cancer stem cells.

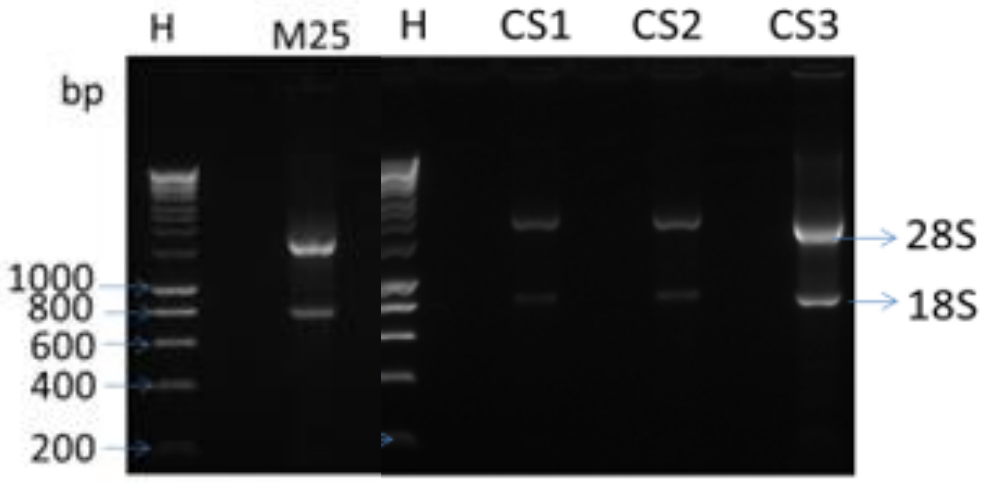


Figure 3.35. Agarose gel electrophoresis of RNA extracted from Mero25 cells.

H, Hyperladder (1kb); M25, Mero25 mesothelioma cell line; CS1, cancer stem cell pellet 1; CS2, cancer stem cell pellet 2; CS3, cancer stem cell pellet 3.

3.5.2. RT-PCR from Mero25 – derived RNA.

Following first-strand synthesis, the cDNA was subjected to RT-PCR to detect *Gapdh, Neil3* and *Ercc1* gene expression in the Mero25 cells. Figure 3.36 shows that all three genes were detected in these cells, with *Gapdh* showing the highest level of expression (lane 1). Following this, RT-PCR was also carried out on cDNA prepared from RNA extracted from the putative Mero25 – derived cancer stem cells. Figure 3.37 shows that a very similar patteren of gene expression for these three genes was obtained from three independent cell pellets of these cells. *Mlh1* was also tested for and the cell lines proved to be positive for expression of this MMR gene (Figure 3.37, lanes 4, 8 and 12).

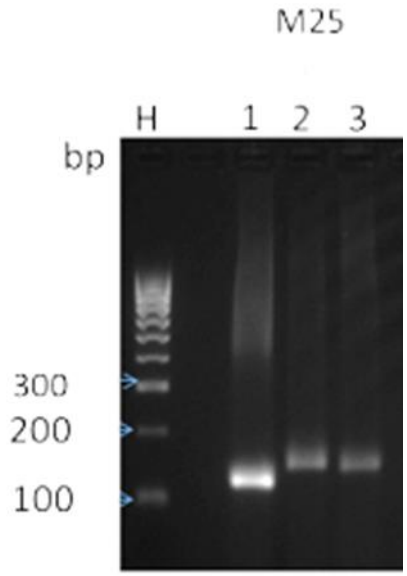


Figure 3.36. RT-PCR of *Gapdh, Neil3,* and *Ercc1* from Mero25 mesothelioma cells.

Lane H, Hyperladder 1kb; lane 1, *Gapdh*, lane 2, *Neil3*, lane 3 *Ercc1*.

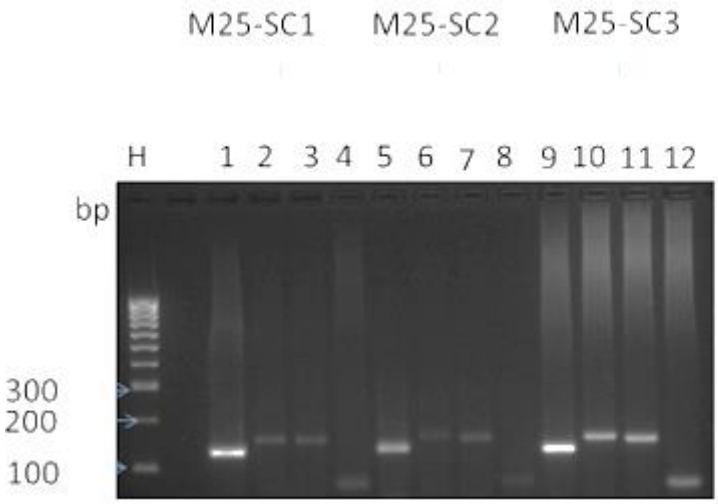


Figure 3.37. RT-PCR of putative cancer stem cells from Mero25 mesothelioma cells.

Lane H, Hyperladder 1kb; lanes 1, 5, 9, *Gapdh*, lanes 2, 6, 10, *Neil3*; lanes 3, 7, 11, *Ercc1*; lanes 4, 8, 12, *Mlh1*.

As the RT-PCR results showed little or no difference in the gene expression of the target genes between the parental Mero25 cells and the Mero25 – derived cancer stem cells, it was decided to analyse the putative cancer stem cells for their 'stem cellness' based on the expression of aldehyde dehydrogenase, a well known cancer stem cell marker (Tomita *et al.*, 2016). Using a commercial kit and flow cytometry, the results shown in Figure 3.38 indicate that while cancer stem cells were produced as described in Section 2.3.7, they still made up less than 10% (7.4%) of the cell population being analysed. As it was beyond the scope of the project to improve the yield of stem cells in the population, or to attempt to purify the aldehyde dehydrogenase – expressing cells from the cell population, it was decided not to proceed further with this analysis.

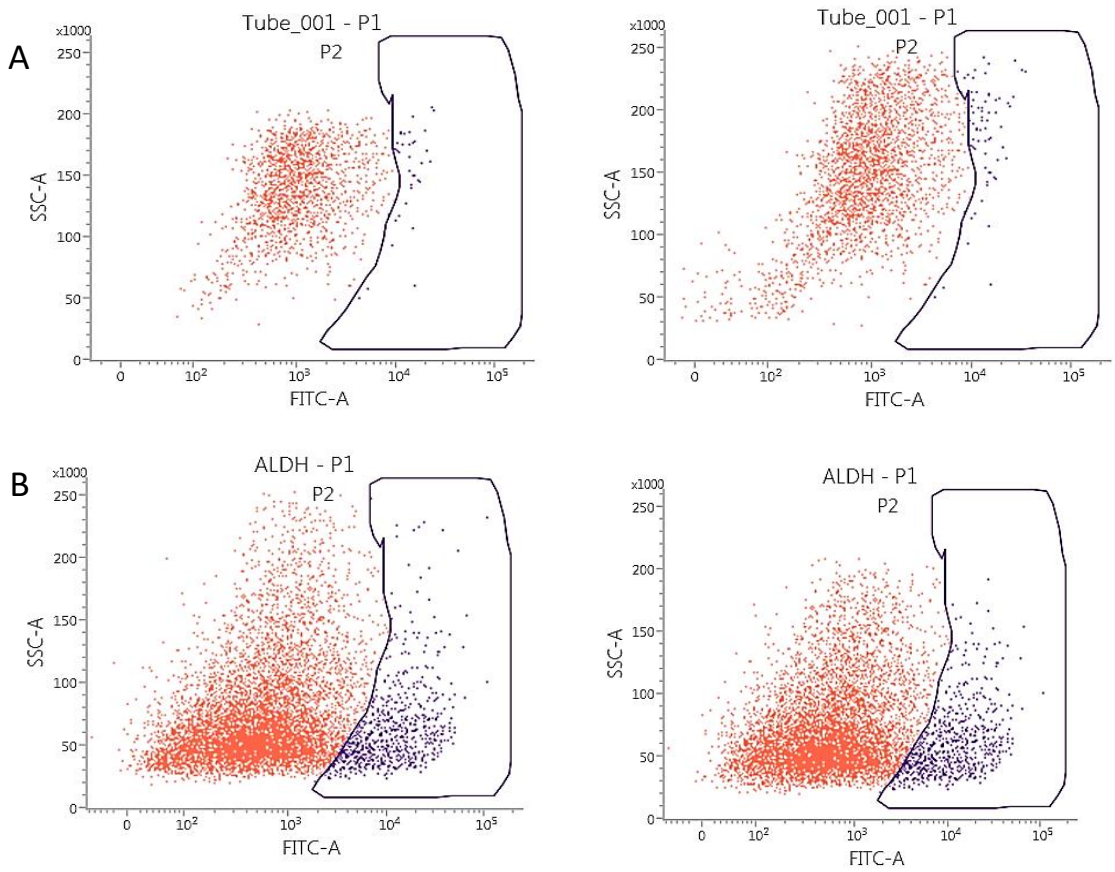


Figure 3.38. Flow cytometry analysis of (A) Mero25 and (B) Mero25 - derived cancer stem cells.

Cells expressing aldehyde dehydrogenase are delimited and shown in blue.

3.6. Human Embryonic Stem Cells

3.6.1. Assessment of RNA Integrity

An RNA extraction experiment was performed to assess the yield and integrity of RNA extracted from human ES cells. The aim was to establish the quality of the RNA purified from the cell. The RNA was extracted and analysed by agarose gel electrophoresis. The results shown in Figure 3.39 reveal that all extractions produced two distinct bands confirming the presence of intact ribosomal RNA (28S and 18S) in the total RNA extracted from the cells and indicating that the mRNA component is most likely intact in the samples.

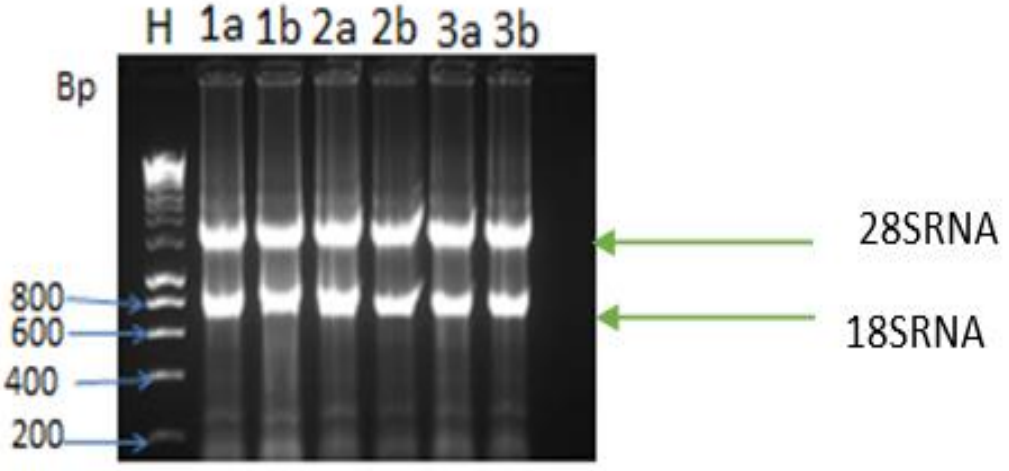


Figure 3.39. Agarose gel electrophoresis of RNA extracted from human ES cells.

Lane H, Hyperladder 1kb; lanes 1a - 3b contain RNA purified from three different human ES cell pellets, with each sample in duplicate (a and b).

3.6.2. Reverse-transcription PCR.

RT-PCR was carried out on cDNA prepared from human ES cells using specific primers for the target genes. To confirm the primer specificity and ensure that genes of interest were amplified, agarose gel electrophoresis of the RT-PCR products was conducted. Five separate ES cell pellets were processed and the RT-PCR results for *Gapdh, Neil3* and *Ercc1* are shown in Figure 3.40. The results are extremely consistent and indicate that all three genes are expressed by human ES cells.

It had been hoped to extend this work to human oocytes as little is known about the DNA repair capacity of of these female germ cells (Stringer *et al.*, 2018) and it is becoming increasingly important to determine the ability of germ cells to maintain genetic integrity and mainitaing fertility as the use of *in vitro* fertilization continues to rise (IVF more popular…., 2019). However, as this was essentially outwith the scope of the project, it was decided to focus on the other cancer – related topics described earlier.

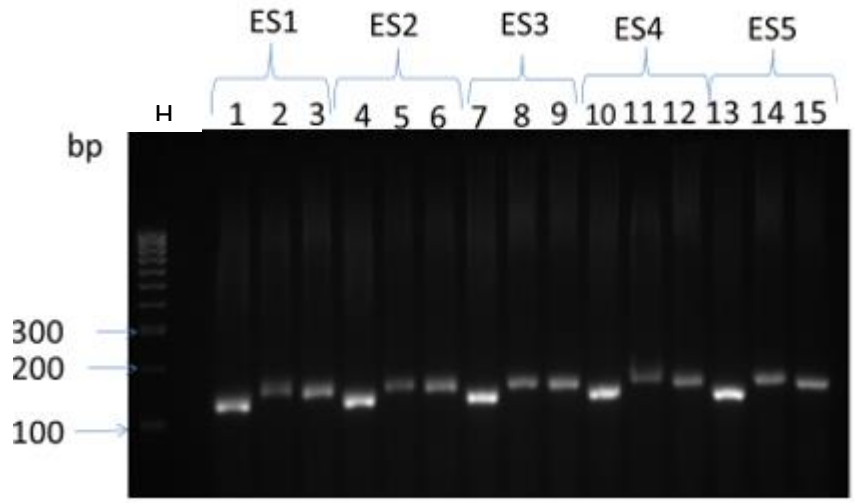


Figure 3.40. Agarose gel electrophoresis of RT-PCR products of *Gapdh, Neil3 and Ercc1* from human ES cells.

Lane H, Hyperladder 100 bp; lanes 1, 4, 7, 10 & 13 *Gapdh*; lanes 2, 5, 8, 11 & 14 *Neil3*; lanes 3, 6, 9, 12 & 15, *Ercc1*.

Chapter 4

4.1. DISCUSSION

The molecular mechanisms of cancer development, progression, metastasis and resistance to therapy revolve around genetic instability. Consequently, sustained DNA damage affecting key genes coding for proliferation supporting proteins (oncogenes), anti-proliferative proteins (tumour suppressor genes) and DNA repair proteins, could enhance the acquisition of immortal characteristics leading to tumour development and subsequently facilitate chemotherapeutic resistance. Essentially, damage to the DNA molecule that results in the loss of function of tumour suppressor genes and the gain of function by proto-oncogenes is exacerbated by the ablation or dysregulation of DNA repair genes that code for proteins to restore the damaged DNA. Ultimately, this results in the development of mutant cell clones and the subsequent acquisition of the various hallmarks of cancer (Hanahan & Weinberg, 2000; Loeb, 2001). Accordingly, many genes have been identified that encode proteins that are directly or indirectly involved in the repair of damaged DNA and they have been classified into the major repair pathways previously reviewed (Section 1.3). Additionally, some of the genes that are responsible for the repair of damaged DNA have been further implicated in tumour development and drug resistance in different cancer types. For instance, high levels of *Ercc1* expression have been reported to result in poor survival of CRC patients receiving a combination of oxaliplatin and 5-fluorouracil (Shirota *et al.,* 2001). Similarly, high levels of *Neil3* have been found in melanoma tumour and various cancer cell lines and may also be a resistance factor against oxaliplatin chemotherapy (Shinmura *et al*., 2016; Taylor *et al.,* 2015).

Thus, the primary objective of this project was to determine the gene expression levels of specific DNA repair genes in colon cancer tissues. Initially, the focus was on a set of CRC tissue and matched normal colon tissue samples that had been stored for some years at -80°C at the University of Manchester. However, it soon became clear that despite employing several different methods to extract and purify the RNA and the addition of RNase inhibitors to the buffers, it was not possible to obtain non-degraded RNA from these samples, as determined by the presence of the 28S and 18S rRNA bands (Figure 3.4). The question then was, was the RNA already degraded in the frozen tissue samples, either through length of storage at -80°C, or inappropriate handling and length of time to freezing at the time of

surgery? A further problem with these samples was the lack of information on the patients and site of the tumour. Had the results of the gene expression analyses between the tumour and normal samples been significantly and reproducibly different, the analysis of the samples would still have been worthwhile, but with no clear pattern emerging, a full analysis of the data was not possible.

For these reasons, it was decided to obtain fresh tumour samples from a biobank at a hospital in Manchester. These samples had the benefit of not being stored for an extended period before RNA extraction and came with some basic patient information (Table 3.2). However, these samples were also refractory to RNA extraction and few of the samples showed intact 28S and 18S rRNA (Figure 3.5A - Figure 3.5D). This was in marked contrast to the RNA extraction from cells, where the ribosomal RNA bands were routinely seen following RNA extraction (Figure 3.1). Therefore, either much greater care is needed at the point of collection at surgery, with rapid snap-freezing of the samples along with the use of reagents such as RNase AWAY (Sigma-Aldrich) to pre-treat plasticware and/or more modifications need to be made to the RNA extraction procedure in the investigating laboratory.

In the majority of CRC tissue samples, gene expression levels of the BER DNA glycosylase genes *Neil1*, *Neil3*, *Nthl1*, and *Ercc1* one part of the XPF/ERCC1 lesion specific endonuclease in NER were analysed. These particular DNA repair genes were chosen for the following reasons: (i) *Neil3* gene expression has been found to be increased in cancer cells and in certain metastatic tumours, (ii) *Ercc1* expression has been shown to be increased following oxaliplatin treatment, a clinically relevant agent used to treat CRC, and (iii) ERCC1 and NER are involved in the repair of platinum-based drug – induced DNA damage. A lack of NEIL3 has been shown to sensitize mouse cells to the related agent cisplatin (Rolseth *et al.*, 2013) and recent reports from two independent laboratories indicate that NEIL3 (and NEIL1) can repair ICLs in DNA (Semlow *et al.*, 2016; Martin *et al.*, 2017). As the tissue samples were from patients who had not been exposed to oxaliplatin, it was hoped that the expression levels of these genes would give some indication of the likely success of oxaliplatin treatment with the chance that more than one subset of tumours could be identified, albeit with a small number of samples. *Nthl1* was also included because high expression has also been linked to cisplatin resistance (Guay *et al.*, 2008) and deletion mutations lead to a CRC prone syndrome (Weren *et al.*, 2015).

Analysis of the results from the matched tissue samples given in Table 3.3 show that most of the tumour tissues did not show high increases (< 1-fold increase) in any of the DNA repair genes tested. However, there were exceptions and *Ercc1* was moderately increased in five out of the twelve tissue pairs tested and *Neil3* in three. In one tissue pair, *Ogg1* was highly expressed in the tumour sample (one of three samples tested) and the MMR gene *Mlh1* in another (one of eight samples tested). Thus, there is no clear pattern of gene expression of the target genes and instead the results indicate the heterogeneous nature of different tumours and the random nature of mutation accumulation (Loeb, 2001).

For the sixteen biobank tissue samples, expression of the target genes was based on the expression of the housekeeping gene *Gapdh* (Table 3.5). These tissues were categorized into three groups. (i) Four colon tumour tissues with corresponding matched normal colon tissue samples (sample pairs 1573, 1581, 1597 and 1604), (ii) Three normal colon tissue samples (samples 1538, 1545 and 1580) and, (iii) Five colon tumour samples with no matched controls (samples 1610, 1620, 1626, 1627 and 1630). Therefore, it must be assumed that the expression of *Gapdh* was constant between the different tumour samples. Accepting this, Table 3.5 and Figure 3.20 – Figure 3.26 again indicate a wide range of gene expression of the different DNA repair genes. However, *Nthl1* stands out, as it is highly expressed (>10-fold) in both normal (four out of seven samples) and tumour tissue (five out of nine samples; Table 3.5) and moderately expressed (2 – 10-fold) in tumour sample 1627. In contrast, *Ercc1* is highly expressed in only one tumour sample pair (1573) and moderately expressed in sample pairs 1581 and 1604 and sample 1580, while *Neil3* is highly expressed only in sample 1630. The expression of *Neil1*, which has also been shown to resolve ICLs in DNA was also analysed in these samples but was the least highly expressed of the four target genes and its expression was not substantially increased (>2-fold) in the match paired tumour samples analysed (Table 3.3 and Table 3.4).

These results again indicate the heterogenous nature of CRC with no obvious pattern of gene expression emerging. However, the exceptionally high level of *Ercc1* expression observed in sample pair 1573 (Table 3.4) and perhaps the high level of *Neil3* in sample 1630 (Table 3.5) would suggest a poor response to oxaliplatin treatment. There does not appear to be any correlation with site of tumour, or sex and age of patient. However, from another perspective, the predominant over-expression of the *Nthl1* and to a lesser extent *Neil3* may not be unconnected to high levels of ROS, which has been reported to potentiate mutation and accumulation of genetic instability in different cancer types including, pancreatic

(Vaquero *et al*., 2004), prostate (Kumar *et al*., 2008), breast (Hecht *et al*., 2016) and colon (Acharya *et al*., 2010).

While the RT-PCR and qPCR results show the expected results, it must be remembered that most of the RNA samples appeared degraded, with the exception of samples 1581T and 1604 (Figure 3.5A and Figure 3.5D), both of which show high *Nthl1* expression (Table 3.5). Thus, this perhaps does give credence to the overall results and that while overall the mRNA pool may be reduced by degradation, the ratio of individual mRNA species has not been altered. Therefore, basing the expression levels on an internal control, *Gapdh*, should give a true reflection of the relative amounts of each target gene in the mRNA pool. Going forward, it would be interesting to compare the expression of these genes before and after treatment with oxaliplatin and to extend the gene expression studies to proteomics and determine the level of protein by western blotting.

The experiments on medulloblastoma cells that had acquired resistance to cisplatin further indicated that NEIL3 is involved in the repair of cisplatin and oxidative lesions in DNA (Section 3.4). Although the cisplatin – treated cells were indeed more resistant to cisplatin and an ROS inducing agent (Figure 3.27& Figure 3.29), it was nevertheless surprising that a similar resistance was not observed after treatment with oxaliplatin (

Figure **3.28**). Good quality RNA was obtained from both DAOY cell lines (Figure 3.30) and reproducible results obtained from RT-PCR (Figure 3.31). Unfortunately, however, the data obtained from the qPCR experiment for the normal DAOY cells in particular was not of the expected quality and there was no time to repeat the experiment as required. Therefore, two possible outcomes are given in this thesis (Figure 3.32). In Figure 3.32A, all the triplicates were used to calculate a mean and the -ΔΔCt values plotted, using *Gapdh* as the control. However, in Figure 3.32B, obvious outliers were removed from the data set before calculating the -ΔΔCt values. From both calculations, *Ercc1* gene expression is induced following cisplatin treatment, however, Figure 3.32B shows that *Neil3* gene expression is also increased following cisplatin treatment. While the experiment would need to be repeated to determine which of these outcomes is correct, Figure 3.33 does show an increase in NEIL3 protein in the cisplatin – resistant cells. While this could be the result of an increase in the stability of the protein, it is likely that even if this is correct, it occurs in conjunction with increased gene expression, suggesting that the result shown in Figure 3.32B is likely to be correct. However, rather surprisingly, no similar increase in ERCC1 protein was observed

in these experiments (Figure 3.32) and therefore this set of experiments needs to be repeated to obtain reproducible data.

Cancer stem cells (CSC) are a slow growing sub-population of a tumour that are intrinsically resistant to conventional chemotherapy regimen (Pattabiraman and Weinberg, 2014). Like ES cells, CSC are characterized by a capability of self-renewal, *i.e.* one of the daughter cells remains a stem cell. While resistance to radiotherapy and chemotherapy appears to be related to the induction of an epithelial-mesenchymal transition that leads to chemoresistance, a lower proliferation rate and higher quiescence and an increase in anti-apoptotic signalling (Pattabiraman and Weinberg, 2014), in the context of this project, it was thought worthwhile to study the gene expression of the DNA repair genes of interest. For these experiments it was decided to use a cell line derived from a mesothelioma patient as the model, as these were being studied by another group at the University of Salford.

However, when the initial results of the RT-PCR experiments showed no difference between the parental Mero25 cells and the putative CSC population (Figure 3.36 & Figure 3.37), it was decided to check the percentage of the latter cell population that showed CSC characteristics. This was achieved using a commercial kit to analyse the amount of aldehyde dehydrogenase, a well-known CSC marker, in the cell population (Figure 3.38). The results indicated that the CSC population was only a minor fraction (7.4%) of the total cell population. Therefore, to obtain useful data from this set of experiments would take much longer than the time available to obtain the correct conditions to achieve a cell population predominantly made up of CSC and this particular project was not taken further.

As part of a larger project studying the underlying causes of infertility, human ES cells were obtained as the first step to studying gene expression of DNA repair genes in human. oocytes. As with CSC, ES cells have an unlimited proliferation potential and exhibit a rapid cell cycle, due to a shortened G1 phase compared to differentiated cells (Jang *et al.*, 2017). Due to their rapid proliferation and role in carrying the genetic information to the next generation, it would be assumed that these cells had a generally high expression of DNA repair genes to correct DNA damage quickly along with an efficient DNA damage response network to remove genetically vulnerable cells from the population. Figure 3.40 shows that the expression of *Neil3* and *Ercc1* was observed in human ES cells. Had time allowed, this work would have been extended to study the expression of a larger panel of DNA repair genes. However, the ultimate aim of the work was to compare the expression of DNA repair

genes in different populations of oocytes, some known to be fertile and some infertile and to see if DNA repair capacity could be a useful biomarker of female fertility with regard to IVF treatment.

4.2. CONCLUSIONS

From the results presented in this report, there is no clear pattern of gene expression of the target genes and instead the results indicate both the apparent varied basal expression levels in normal colon tissue of the DNA repair genes analysed and the heterogeneous nature of different tumours, presumably due to the random nature of mutation accumulation (Loeb, 2001). However, it would still be most interesting to compare the expression of these genes before and after treatment with oxaliplatin. If initial results were promising, a whole transcriptome approach using RNA-seq would be appropriate, to determine global changes in gene expression occurring following chemotherapy. Suitable target genes could then be chosen, and the analysis extended to proteomics to determine the level of specific proteins by western blotting.

There is need for the future research to focus on the relationship between the DNA repair genes and stage of tumour development. Furthermore, there is a need to analyze the expression patterns of DNA repair genes in other tumour types and their characterization as a common feature in different cancer types for potential use as biomarkers for cancer diagnosis.

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APPENDIX

cDNA of genes showing sites of PCR primers

1. *NEIL1* gene qPCR primers (NM_001256552.1)

PCR product size

212bp

Primers from published work: Shinmura *et al*., (2004).

RT 1071 S: AGA AGA TAA GGA CCA AGC TGC

RT 1283 AS: GAT CCC CCT GGA ACC AGA TG

1 cctttagtgg ctgcagtggc gacgagcgga acggagtgcg ggttcccggg gagggggcgt
61 gcaggatcgg gttgtggggg gtgcgctgta gggctaagga gaagatgagc tttggggggc
121 cctgaggagg aggggagtca ggattaaccg aggacagggt cgcagcgggg gc**atg**gggag
181 gacgggcccg agagcgccgc tcctccccaa aactaggatt tggagcctca cgttctcccg
241 caaaacgagc cctgtgggtg ccaggccagg ggcggctccc aggtgtggag ggggcaaggt
301 acgggcgtct gccgtcgcta agtgcccccc tccacttaag gcacaccttc gcctccgatt
361 ccgaaccgcc cggggacaga ggcaagtggc aaagcaggga gggcggagga ctctgccacc
421 ctccctcagg atgcctgagg gccccgagct gcacctggcc agccagtttg tgaatgaggc
481 ctgcagggcg ctggtgttcg gcggctgcgt ggagaagtcc tctgtcagcc gcaaccctga
541 ggtgcccttt gagagcagtg cctaccgcat ctcagcttca gcccgcggca aggagctgcg
601 cctgatactg agccctctgc ctggggccca gccccaacag gagccactgg ccctggtctt
661 ccgcttcggc atgtccggct cttttcagct ggtgccccgc gaggagctgc cacgccatgc
721 ccacctgcgc ttttacacgg ccccgcctgg cccccggctc gccctatgtt tcgtggacat
781 ccgccggttc ggccgctggg accttggggg aaagtggcag ccgggccgcg ggccctgtgt
841 cttgcaggag taccagcagt tcagggagaa tgtgctacga aacctagcgg ataaggcctt
901 tgaccggccc atctgcgagg ccctcctgga ccagaggttc ttcaatggca ttggcaacta
961 tctgcgggca gagatcctgt accggctgaa gatccccccc tttgagaagg cccgctcggt
1021 cctggaggcc ctgcagcagc acaggccgag cccggagctg accctgagcc agaagataag
1081 gaccaagctg cagaatccag acctgctgga gctatgtcac tcagtgccca aggaagtggt
1141 ccagttgggg ggcaaaggct acgggtcaga gagcggggag gaggactttg ctgcctttcg
1201 agcctggctg cgctgctatg gcatgccagg catgagctcc ctgcaggacc ggcatggccg

1261 taccatctgg ttccaggggg atcctggacc gttggcaccc aaagggcgca agtcccgcaa

1321 aaagaaatcc aaggccacac agctgagtcc tgaggacaga gtggaggacg ctttgcctcc

1381 aagcaaggcc ccttccagga cacgaagggc aaagagagac cttcctaaga ggactgcaac

1441 ccagcggcct gaggggacca gcctccagca ggacccagaa gctcccacag tgcccaagaa

1501 ggggaggagg aaggggcgac aggcagcctc tggccactgc agaccccgga aggtcaaggc

1561 tgacatccca tccttggaac cagaggggac ctcagcctct **tag**caggagg ctctccttgc

1621 ttgcactcac cctttcttat tgtcttgccc tgcatctggg ggtctgaatt tttgggagca

1681 ggcaatatct gaaggtgcaa acaggcccta cggctgttcc ctgcacaact ctcatggttt

1741 taattgtacc ccatcttcca catctttaaa gctcatgtga aaaatgctgc atttttaata

1801 aactgataca tttgaacttc

2. human *NEIL2*

PCR product size

145bp

Primers from published work: Mandal *et al*., (2012)

1291 S: GCCTTAGAAGCTCTAGGCCA

1436 AS: GCACTCAGGACTGAACCGAG

1 ggggcgtcccctaaggggacggaggccgcatgggccgccgagccgggaaatctccgcccc

61 cagctggagcggctgtgcgggctgcgtagcggtgctgggtcgggccgacgtgccacccac

121 ccggagccggtgagtgcagccgcccgccctccggtagatctgcggcctggcggagaagtc

181 gggaggggacaggaagggagggcgggccccgggccctcctccgtctcagccgcctgcgga

241 ggtgctgcccacgcctggaggccccccactgaccctcagacccgcgtctgcgcccctctcc

301 ccgcaccccgaggcagagttgggaaagcagtggtcttagacccccacctcgggcactcg

361 gaagagaacggcggagacaacccctcctcttccctggctggcgcagcgccagcctcgagc

421 tcctcggtagcccccgggcagggagggccggagggtgggcgcggcatcttcagcgactct

481 tcgaagtcccttccgcgtctcatctttcaaggctgttgcagaggcggcttgcttcccacc

541 tgtccatctccataaaaatccctaaacgaaacatgcccacgtgtccggagattttcagga

601 cttggtgcatttcagatgaaggcttttccagaagcttccccgtagaagaggatcaggcat

661 ccaactggttaagggatgccagaagggccgttggtgaggaaatttcaccatttggtctcc

721 ccctttgtgggtcagcaggtggtcaagacaggggcagcagtaagaagctacagcccgcc

781 agcctgcagtctctgtggctccaggacacccaggtccatggaaagaaattattccttaga

841 tttgatctagatgaagaaatggggccccctggcagcagcccaacaccagagcctccacaa

901 aaagaagtgcagaaggaaggggctgcggacccaaagcaggtcggggagcccagcgggcag

961 aagacccttgatggatcctcacggtctgcagagctcgtcccccagggcgaggatgattct

1021 gagtatttggagagagacgcccctgcaggagatgctgggaggtggctgcgtgtcagcttt

1081 ggtttgtttggcagcgtttgggtgaacgatttctccagagccaagaaagccaacaagagg

1141 ggggactggagggaccttccccgaggttggtcctgcactttggtggtggtggcttcctg

1201 gcattttataattgtcagttgtcttggagctcttccccagtggtcacacccacctgtgac

1261 atcctgtctgagaagttccatcgaggacaagccttagaagctctaggccaggctcagcct

1321 gtctgctatacactgctggaccagagatacttctcagggctagggaacatcattaagaat

1381 gaagccttgtacagagctgggatccatccccttctctcggttcagtcctgagtgcctcg

1441 cgtcgggaggtcctggtggatcacgtggtggagttcagtacagcctggctgcagggcaag

1501 ttccaaggcagaccgcagcacacacaggtctaccagaaagaacagtgccctgctggccac

1561 caggtcatgaaggaggcgtttgggcccgaagatgggttacagaggctcacctggtggtgc

1621 ccgcagtgccagccccagttgtcagaggagccagagcagtgccagttctcctaaggagct

1681 ggtggtgctcctcacggaaccttgccgcttggggaacctgacgtctaagtgtccagaaag

1741 gaggatgtgggcagggacggggtacagaggatagtgtgggtcagaggtgccagtagtata

1801 atattcgtctccctggagttatgttgaaggcagagttttcatagggttagatttttttta

1861 tccttttctagttcagttaattcatcctgttgaattgcaccatcgtgaaagatgggaaaa

1921 atcgtgatgatgggtaaggggaaaacttcccggaaggcaatggggcaaggaaaaagaaag

1981 cctatgggaaatggctgtgctcccaacatagctttgcagatgatgtgggttttttttttt

2041 tttttggttgtttgttttgagagagagtcttgctctgtctccctggctagggtgtggtgg

2101 tgtgatcttggctcacggcagccttgccctccctggctcaagcagtcttcatctcagcct

2161 ccagagtagctgggactacaggcatgtgatatgatgctcggctgatttttgtttacttttt

2221 tagagagatggggtcttgccatatttgccaggctggtttgaactcctacaactcaagcat

2281 tcctcccaccttggtctcccaaaatgttgggaccacgggtgtgagccaccgcgcccagct

2341 agctcctgtgttttgtttttgttttgtaactttggttgatgttaaggccctccattttgg

2401 aaagcaggaaaacaggatttttttttttttatcttgttccctggaggatccagggatgag

2461 gatagagtggcctgagagcagtgcttggattcagcctcctgctgggtccttctgctggat

2521 acaggcaccaagaggcggtcgtggagcagggagctgcgccttcctggggtgcccggtggt

2581 gtgtagagaaaagctgcttgtttactccttaagtcaatgtattggtgactgttgatttgt

2641 tgaacaattcaggaatcaagggctgtggagaaactccctcatgttgttggcaacaggtga

2701aacctagagcggtgacatgaaaataaagctcactgttactcgc

3. ***ERCC1*** **gene qPCR primers (**NM_001983.3)

PCR product size

ERCC1**:** **884S:** CAAAACGGACAGTCAGACCCT 146 bp

ERCC1**: 1029AS:** TCAAGAAGGGCTCGTGCAG

Primers from published work: Seetharam *et al*., (2010).

1 ccggaagtgc tgcgagccct gggccacgct ggccgtgctg gcagtgggcc gcctcgatcc

61 ctctgcagtc tttcccttga ggctccaaga ccagcaggtg aggcctcgcg gcgctgaaac

121 cgtgaggccc ggaccacagg ctccag**atg**g accctgggaa ggacaaagag ggggtgcccc

181 agccctcagg gccgccagca aggaagaaat ttgtgatacc cctcgacgag gatgaggtcc

241 ctcctggagt ggccaagccc ttattccgat ctacacagag ccttcccact gtggacacct

301 cggcccaggc ggcccctcag acctacgccg aatatgccat ctcacagcct ctggaagggg

361 ctggggccac gtgccccaca gggtcagagc ccctggcagg agagacgccc aaccaggccc

421 tgaaacccgg ggcaaaatcc aacagcatca ttgtgagccc tcggcagagg ggcaatcccg

481 tactgaagtt cgtgcgcaat gtgccctggg aatttggcga cgtaattccc gactatgtgc

541 tgggccagag cacctgtgcc ctgttcctca gcctccgcta ccacaacctg cacccagact

601 acatccatgg gcggctgcag agcctgggga agaacttcgc cttgcgggtc ctgcttgtcc

661 aggtggatgt gaaagatccc cagcaggccc tcaaggagct ggctaagatg tgtatcctgg

721 ccgactgcac attgatcctc gcctggagcc ccgaggaagc tgggcggtac ctggagacct

781 acaaggccta tgagcagaaa ccagcggacc tcctgatgga gaagctagag caggacttcg

841 tctcccgggt gactgaatgt ctgaccaccg tgaagtcagt caa**caaaacg gacagtcaga**

901 ccctcctgac cacatttggatctctggaac agctcatcgc cgcatcaaga gaagatctgg

961 ccttatgccc aggcctgggc cctcagaaag cccggaggct gtttgatgtc **ctg**cacgagc

1021 ccttcttgaa agtaccc**tga** tgaccccagc tgccaaggaa acccccagtg taataataaa

1081 tcgtcctccc aggccaggct cctgctggct gcgctggtgc agtctctggg gagggattct

1141 gggggtgtca ccttctggtg gcccaggtgg gcaccttcag ctttctttag ttcctcagtt

1201 tcccgggggc agactacaca ggctgctgct gctgctgctt ccgcttcttg tcccggcctg

1261 tgggagcctc ctccccagac tctgaattca gtggcggccc tggcatctcc tcttggggca

1321 ctgtctctgg catccggctt tcctgactct gcttcttcct cttcttggtg gatcccggag

1381 ttgccctggc ttcaggctgt ccctcccctg gcagttcagg ctctagtggc tgaattggct

1441 cagtcactgt gtgacctctc tctttcttct tcttcttctt cttggtggat gtgggagctg

1501 cctgaggctc aaggtcatcc ggcagctcag gccccaccac ctctgtctct ggctccactg

1561 tggcatcttg ctgtttttct ttcttcgtct tctttttggg agctgccaga gctgcctggg

1621 cctgaggctt cgctccttct ggctgttgag gcgccatggt ccccccctggg gactccagag

1681 gcttcatctc cggctccact ggctccatcg cctccgtccc tggctccatc attgccatct

1741 gtccctttc ttttttcctc ttcttcgtag ggggcagagg gatggcttcc tccagtggct

1801 ccaccttcac ctgtggctga gactcaactg tcacccccctc ctctggctcc atcccttccg

1861 tcccctttg cctctttctc tttttggtcg gggacaggac tgtgtcttct agaggctcag

1921 tgttaatctg ttcctgcttc actgtcttgt cttctggctc gaaggtttct ttccctttgg

1981 gcttcttcct cttcttggtg gtggacggga acagcactcc cagaggctcc agtgtctcca

2041 ctgtgggctc tgtccccaca ggccctgctg cctctggttc tttcagctgc tgatttttt

2101 tcttcttctt cttccgcaca tccatttctg gcgaccccaa agccatgtcc acctccaggg

2161 ccccgtgccc attcactgcc tcctgagtga ctggggcctc tgtcacctgc atctccttt

2221 tcttcttccc tgaggtgagc aggttggggg ccaaggctga cctaggccct gtgactggtg

2281 ggttgccccc aaaggcacag aaccgaggcc tcaggccagg agggatctgt ggtgggggac

2341 ttgctgggat gggctgcaga gggctccctg acagggattg ctggggaccc tcaaggatcc

2401 ttagggtgcc ctgggggct gaggcacagg tgagtccacc tcctgcctcc gttgaggggg

2461 ccagcagggt cgcttctcca gcttggggac agctgctgag gactcgatag cggtgccgct

2521 tgcctgccaa tttgcccttg acgatctggg agccagagag aggcacatgc cgcccattga

2581 agctacagag agaaacaggg agggcagagg cttaagtgga acaggagagg gaaggttttt

2641 tgatttttt tttgtttttt tttgagagag tcttgctctg ttgcctaggc tggagtgcag

2701 tggcatgatc tcggctcact gcaatgtcca cctcctgggt tcaagcgatt ctcctgcctc

2761 agcctctcaa gtagctggga ttacaggcac ctgccaccac gcccagccaa tttttgtatt

2821 tttagtagag acaatttcac tatgttggcc aggctggtct tgaactcctg acctcaagtg

2881 atctgctcgc ctcggcctcc caaaggatgg gattacaggc accagccact gcgcctggct

2941 ggcctctggt ttttaataaa acatgactag agtgactcca tcttaaagtg agtagctagg

3001 cacttacaag gttcatgctt atggcctgaa aataaccaca tcccaggctg accaccaatt

3061 ataattacag aatatttatg gccatacaga acatgttcca ccaagcctgc agaatgtcca

3121 aatgtcctaa gaatgcagcc cccattactt aaatataaca taaatgagca agcttaggtt

3181 gcaggattaa tggtcgtgga taacaccaat agcccctacc tttagtgagc ttatctgcac

3241 actccaagtt taactatagt tccttatagt ttcttataag tagaaatact aacaaagggc

3301 tgtgggtttc tccccctgct ttctgaggac actctctct gtaaaggagt agtttccaat

3361 aaacttgttt ctttcactgt gcaaaaaaaa aaaaaaaaaa

4. *NTH1* gene qPCR primers (NM_002528.5)

PCR product size

165 bp

Primers from published work: Goto *et al*., (2009).

RT 679 **S:** GATGGCACACCTGGCTATG

RT 844**AS**: CCACAGCTCCCTAGGCAG

1 ggccgcatgg gccgccggg**a tg**tgtagtcc gcaggagtcc ggcatgaccg ccttgagcgc

61 gaggatgctg acccggagcc ggagcctggg acccggggct gggccgcggg ggtgtaggga

121 ggagcccggg cctctccgga gaagagaggc tgcagcagaa gcgaggaaaa gccacagccc

181 cgtgaagcgt ccgcggaaag cacagagact gcgtgtggcc tatgagggct cggacagtga

241 gaaaggtgag ggggctgagc ccctcaaggt gccagtctgg gagccccagg actggcagca

301 acagctggtc aacatccgtg ccatgaggaa caaaaaggat gcacctgtgg accatctggg

361 gactgagcac tgctatgact ccagtgcccc cccaaaggta cgcaggtacc aggtgctgct

421 gtcactgatg ctctccagcc aaaccaaaga ccaggtgacg gcgggcgcca tgcagcgact

481 gcgggcgcgg ggcctgacgg tggacagcat cctgcagaca gatgatgcca cgctgggcaa

541 gctcatctac cccgtcggtt tctggaggag caaggtgaaa tacatcaagc agaccagcgc

601 catcctgcag cagcactacg gtggggacat cccagcctct gtggccgagc tggtggcgct

661 gccgggtgtt gggcccaaga tggcacacct ggctatggct gtggcctggg gcactgtgtc

721 aggcattgca gtggacacgc atgtgcacag aatcgccaac aggctgaggt ggaccaagaa

781 ggcaaccaag tccccagagg agacccgcgc cgccctggag gagtggctgc ctagggagct

841 gtggcacgag atcaatggac tcttggtggg cttcggccag cagacctgtc tgcctgtgca

901 ccctcgctgc cacgcctgcc tcaaccaagc cctctgcccg gccgcccagg gtctc**tga**tg

961 gccgcatggc tctggccgag gtgccgctgt ggccaccgtc tgtgaagtgg ctttacgctt

1021 caggaagcca cgcctgttga ataaagcttt ggtgtgtttg cagatgg

5. *MLH1* gene qPCR primers (NM_000249)

PCR product size

66 bp

Primers from published work: Jensen *et al*., (2013).

RT 2276 S: AGGAGTCGACCCTCTCAGG

RT 2342 AS: GTCCACTTCCAGGAGTTTGG

1 gaagagaccc agcaacccac agagttgaga aatttgactg gcattcaagc tgtccaatca

61 atagctgccg ctgaagggtg gggctggatg gcgtaagcta cagctgaagg aagaacgtga

121 gcacgaggca ctgaggtgat tggctgaagg cacttccgtt gagcatctag acgtttcctt

181 ggctcttctg gcgccaaa**at g**tcgttcgtg gcaggggtta ttcggcggct ggacgagaca

241 gtggtgaacc gcatcgcggc gggggaagtt atccagcggc cagctaatgc tatcaaagag

301 atgattgaga actgtttaga tgcaaaatcc acaagtattc aagtgattgt taaagaggga

361 ggcctgaagt tgattcagat ccaagacaat ggcaccggga tcaggaaaga agatctggat

421 attgtatgtg aaaggttcac tactagtaaa ctgcagtcct ttgaggattt agccagtatt

481 tctacctatg gctttcgagg tgaggctttg gccagcataa gccatgtggc tcatgttact

541 attacaacga aaacagctga tggaaagtgt gcatacagag caagttactc agatggaaaa

601 ctgaaagccc ctcctaaacc atgtgctggc aatcaaggga cccagatcac gtggaggac

661 ctttttaca acatagccac gaggagaaaa gctttaaaaa atccaagtga agaatatggg

721 aaaattttgg aagttgttgg caggtattca gtacacaatg caggcattag tttctcagtt

781 aaaaaacaag gagagacagt agctgatgtt aggacactac ccaatgcctc aaccgtggac

841 aatattcgct ccatctttgg aaatgctgtt agtcgagaac tgatagaaat tggatgtgag

901 gataaaaccc tagccttcaa aatgaatggt tacatatcca atgcaaacta ctcagtgaag

961 aagtgcatct tcttactctt catcaaccat cgtctggtag aatcaacttc cttgagaaaa

1021 gccatagaaa cagtgtatgc agcctatttg cccaaaaaca cacacccatt cctgtacctc

1081 agtttagaaa tcagtcccca gaatgtggat gttaatgtgc accccacaaa gcatgaagtt

1141 cacttcctgc acgaggagag catcctggag cgggtgcagc agcacatcga gagcaagctc

1201 ctgggctcca attcctccag gatgtacttc acccagactt tgctaccagg acttgctggc

1261 ccctctgggg agatggttaa atccacaaca agtctgacct cgtcttctac ttctggaagt

1321 agtgataagg tctatgccca ccagatggtt cgtacagatt cccgggaaca gaagcttgat

1381 gcatttctgc agcctctgag caaacccctg tccagtcagc cccaggccat tgtcacagag

1441 gataagacag atatttctag tggcagggct aggcagcaag atgaggagat gcttgaactc

1501 ccagcccctg ctgaagtggc tgccaaaaat cagagcttgg agggggatac aacaaagggg

1561 acttcagaaa tgtcagagaa gagaggacct acttccagca accccagaaa gagacatcgg

1621 gaagattctg atgtggaaat ggtggaagat gattcccgaa aggaaatgac tgcagcttgt

1681 acccccccgga gaaggatcat taacctcact agtgttttga gtctccagga agaaattaat

1741 gagcagggac atgaggttct ccgggagatg ttgcataacc actccttcgt gggctgtgtg

1801 aatcctcagt gggccttggc acagcatcaa accaagttat accttctcaa caccaccaag

1861 cttagtgaag aactgttcta ccagatactc atttatgatt ttgccaattt tggtgttctc

1921 aggttatcgg agccagcacc gctctttgac cttgccatgc ttgccttaga tagtccagag

1981 agtggctgga cagaggaaga tggtcccaaa gaaggacttg ctgaatacat tgttgagttt

2041 ctgaagaaga aggctgagat gcttgcagac tatttctctt tggaaattga tgaggaaggg

2101 aacctgattg gattacccct tctgattgac aactatgtgc ccccttgga gggactgcct

2161 atcttcattc ttcgactagc cactgaggtg aattgggacg aagaaaagga atgttttgaa

2221 agcctcagta aagaatgcgc tatgttctat tccatccgga agcagtacat atctgaggag

2281 tcgaccctct caggccagca gagtgaagtg cctggctcca ttccaaactc ctggaagtgg

2341 actgtggaac acattgtcta taaagccttg cgctcacaca ttctgcctcc taaacatttc

2401 acagaagatg gaaatatcct gcagcttgct aacctgcctg atctatacaa agtctttgag

2461 aggtgt**taa**a tatggttatt tatgcactgt gggatgtgtt cttctttctc tgtattccga

2521 tacaaagtgt tgtatcaaag tgtgatatac aaagtgtacc aacataagtg ttggtagcac

2581 ttaagactta tacttgcctt ctgatagtat tcctttatac acagtggatt gattataaat

2641 aaatagatgt gtcttaacat aa

6. *OGG1* gene qPCR primers (NM_002542)

PCR product size

RT 1020 S: AGCAGCTACGAGAGTCCTCA 137 bp

RT 1156 AS: CATATGGACATCCACGGGCA

Primers from published work: Santos *et al*., (2014).

1 ctacttccgg tggtgctgtg gtctgcccct ggagaaccca gaagaacaca gctgtgcgcg

61 cccacaggct ctgggggcgg gagaagataa gtcgcaagga ggggggcggga cctacacctc

121 aggaaagccg gagaattggg gcacgaagcg gggctttgat gacccgcaaa gggcgaggca

181 tgcaggaggt ggaggaatta agtgaaacag ggaaggttgt taaacagcac cgtgtgggcg

241 aggccttaag ggtcgtggtc cttgtctggg cggggtcttt gggcgtcgac gaggcctggt

301 tctgggtagg cggggctact acggggcggt gcctgctgtg gaa**atg**cctg cccgcgcgct

361 tctgcccagg cgcatggggc atcgtactct agcctccact cctgccctgt gggcctccat

421 cccgtgccct cgctctgagc tgcgcctgga cctggttctg ccttctggac aatctttccg

481 gtggagggag caaagtcctg cacactggag tggtgtacta gcggatcaag tatggacact

541 gactcagact gaggagcagc tccactgcac tgtgtaccga ggagacaaga gccaggctag

601 caggcccaca ccagacgagc tggaggccgt gcgcaagtac ttccagctag atgttaccct

661 ggctcaactg tatcaccact ggggttccgt ggactcccac ttccaagagg tggctcagaa

721 attccaaggt gtgcgactgc tgcgacaaga ccccatcgaa tgccttttct cttttatctg

781 ttcctccaac aacaacatcg cccgcatcac tggcatggtg gagcggctgt gccaggcttt

841 tggacctcgg ctcatccagc ttgatgatgt cacctaccat ggcttcccca gcctgcaggc

901 cctggctggg ccagaggtgg aggctcatct caggaagctg ggcctgggct atcgtgcccg

961 ttacgtgagt gccagtgccc gagccatcct ggaagaacag ggcgggctag cctggctgca

1021 gcagctacga gagtcctcat atgaggaggc ccacaaggcc ctctgcatcc tgcctggagt

1081 gggcaccaaggtggctgact gcatctgcct gatggcccta gacaagcccc aggctgtgcc

1141 cgtggatgtc catatgtggc acattgccca acgtgactac agctggcacc ctaccacgtc

1201 ccaggcgaag ggaccgagcc cccagaccaa caaggaactg ggaaactttt tccggagcct

1261 gtggggacct tatgctggct gggcccaagc ggtgctgttc agtgccgacc tgcgccaatc

1321 ccgccatgct caggagccac cagcaaagcg cagaaagggt tccaaagggc cggaaggc**ta**

1381 gatggggcac cctggacaaa gaaattcccc aagcaccttc ccctccattc cccacttctc

1441 tctccccatc cccacccagt ctcatgttgg ggaggggcct ccctgtgact acctcaaagg

1501 ccaggcaccc ccaaatcaag cagtcagttt gcacaacaag atggggtggg ggatattgag

1561 ggagacagcg ctaaggatgg ttttatcttc cctttattac aagaaggaac aataaaatag

1621 aaacatttgt atggaaaaaa aaaaaaaaaa aa