Global Methylation Patterns in Genomic and Cell Free DNA in Uveal Melanoma

Sarah Tadhg Ferrier

Dr. Julia Burnier's Laboratory

Department of Pathology

McGill University

Montreal, Quebec, Canada

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Abstract :

Background: Uveal Melanoma is the most common primary intraocular malignancy in adults, but despite effective local treatments, outcomes have not improved in decades. Because of this, new methods for monitoring the disease are necessary, as well as new avenues to explore for adjuvant treatments. Liquid biopsy – the analysis of biofluids such as blood, saliva, and urine – has the potential to track tumour features non-invasively, and without the need for a primary tumour to sample.

Methods: Genetic and epigenetic data for 80 UM cases from the Cancer Genome Atlas (TCGA) was analysed using Minfi and Limma in order to determine significantly differentially methylated loci. Differentially methylated loci were mapped to a human genome assembly, and differentially methylated genes were analysed for changes in DAVID gene ontology and KEGG pathways between cases. 4 UM cell lines were grown in the presence of increasing doses of azacytidine. The effects of azacytidine on each of the cell lines was monitored by incucyte live cell imaging and CCK8 assays. Genomic and cell free DNA from cells with and without azacytidine (0, 1, 5, and 20 μM) was extracted using the Qiagen DNA mini kit and Qiagen cell free nucleic acid kits, and methylation was analysed using the Illumina MethylationEPIC arrays.

Results: Hierarchical clustering divided UM patients into two distinct groups based on methylation pattern. These groups differed significantly in terms of clinical outcomes, with metastatic disease present almost exclusively in one group. The differentially methylated cases showed differences in levels of methylation at specific genes that are known to be important in cancer progression and cell signaling. Treatment of UM cells with azacytidine led to changes in the pattern of methylation, with different alterations seen depending on the cell line. UM cell lines show different patterns of methylation based on their molecular profiles, especially BAP1 protein expression, and matched primary and metastatic UM tumours additionally showed similar patterns of DNA methylation.

Conclusion: UM cases can be highly differentially methylated depending on clinical and genomic characteristics. Targeting these changes in methylation may be an appropriate method to develop neoadjuvant treatments for metastatic UM, by altering the pathways that are significantly affected in cases that have poor outcomes.

Résumé :
Contexte : Le Mélanome Uvéal (MU) est la tumeur maligne intraoculaire primitive la plus commune chez les adultes, mais malgré des traitements locaux efficaces, les résultats a long-terme ne se sont pas améliorés au cours des dernières décennies. À cause de cela, de nouvelles méthodes de suivi de la maladie sont nécessaires, ainsi que de nouvelles pistes à explorer pour des traitements adjuvants. La biopsie liquide - l'analyse des biofluides tels que le sang, la salive et l'urine - a le potentiel de suivre les caractéristiques tumorales de manière non invasive et sans la nécessité de prélever une tumeur primaire.
Méthodes : Les données génétiques et épigénétiques de 80 cas d'UM du Cancer Genome Atlas (TCGA) ont été analysées à l'aide des programmes Minfi et Limma afin de déterminer des régions méthylées de manière significativement différente. Des régions à méthylation différentielle ont été alignées sur un assemblage de génome humain, et les gènes à méthylation différentielle ont été analysés pour les changements dans le programme DAVID et les voies KEGG entre les cas. 4 lignées cellulaires MU ont été cultivées en présence de doses croissantes d'azacytidine. Les effets de l'azacytidine sur chacune des lignées cellulaires ont été surveillés par imagerie de cellules à temps réel incucytes et dosages de CCK8. L'ADN génomique et acellulaire de cellules avec et sans azacytidine (0, 1, 5 et 20 μM) a été extrait à l'aide du kit A.D.N. mini de Qiagen et des kits d'acide nucléique sans cellule de Qiagen. Le degré de méthylation a été analysée à l'aide des puces Illumina MethylationEPIC.
Résultats : Le regroupement hiérarchique a divisé les patients atteints du MU en deux groupes distincts en fonction de leurs motifs de méthylation. Ces groupes différaient significativement en ce qui concerne les résultats cliniques, avec une maladie métastatique présente presque exclusivement dans un groupe. Les cas de méthylation différentielle ont montré des différences dans les niveaux de méthylation au niveau de gènes spécifiques qui sont importants dans la progression du cancer et la signalisation cellulaire. Le traitement des cellules MU avec de l'azacytidine a mené à des changements dans le motif de méthylation, avec différentes altérations observées selon la lignée cellulaire. Les lignées cellulaires MU présentent différents motifs de méthylation en fonction de leurs profils moléculaires - en particulier l'expression de la protéine BAP1 - et les tumeurs MU primaires et métastatiques correspondantes ont montré un motif similaire de méthylation de l'ADN.
Conclusion : les cas de MU peuvent être fortement méthylés de manière différentielle en fonction des caractéristiques cliniques et génomiques. Cibler ces changements dans la méthylation pourrait être une méthode appropriée pour développer des traitements

néoadjuvants pour le MU métastatique, en modifiant les voies qui sont significativement affectées dans les cas qui ont un risque élevé de métastases.

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Contribution of Authors

This is to certify that I have completed all the work and experiments being presented in this thesis under the supervision of Dr. Julia Valdemarin Burnier. The Illumina methylation arrays were performed by Genome Québec. The computational work done in bumphunter for differentially methylated regions in the second manuscript was performed by Marvin Li under the supervision of myself and Amadou Barry from the McGill initiative in Computational Medicine.

List of Abbreviations

AML	Acute myelogenous leukemia
AMPK	AMP-activated protein kinase
BAP1	BRCA1-associated protein 1
BRCA1	Breast and Overian Cancer Susceptibility Protein 1
cAMP	cyclic adenosine monophosphate
cfDNA	Cell-free DNA
CM	Cutaneous Melanoma
CNV	Copy Number Variation
ctDNA	Circulating tumour DNA
CYSLTR2	Cysteinyl Leukotriene Receptor 2
DMP	Differentially Methylated Probe
DMR	Differentially Methylated Region
DMSO	dimethyl sulfoxide
DNMT	DNA Methyltransferase
ECM	Extracellular matrix
EFS	Embryonic fyn-associated substrate
EIF1AX	Eukaryotic Translation Initiation Factor 1A X-Linked
ERK	Extracellular signal regulated kinase
FNA	Fine-needle aspiration
GEP	Gene expression profiling
GNA11	Guanine nucleotide-binding protein subunit alpha 11
GNAQ	Guanine nucleotide-binding protein G(q) subunit α
GO	Gene ontology
HDAC4	Histone deacetylase 4
hTERT	Telomerase reverse transcriptase
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL12	Interleukin 12
IL12RB2	Interleukin 12 receptor subunit beta 2
MAPK	Mitogen Activated Protein Kinase
MGMT	O-6-methylguanine-DNA methyltransferase

MeDIP-Seq	Methylated DNA Immunoprecipitation Sequencing
MEK	Mitogen-activated protein kinase kinase
NFIA	Nuclear factor 1A
PI3K	Phosphoinositide 3-kinase
PLCB4	Phospholipase C Beta 4
PTEN	Phosphate and tensin homolog
Rap1	Ras-proximate 1
RASSF1A	Ras association domain family member 1A
RING	Really Interesting New Gene
SF3B1	Splicing Factor 3b Subunit 1
SNP	Single Nucleotide Polymorphism
TCGA	The Cancer Genome Atlas
UM	Uveal Melanoma
UV	Ultraviolet
WNT	Wingless-related integration site
YAP	Yes-associated protein
ZNF	Zinc Finger

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(**B**) the *HDAC4* gene and (**C**) the *IL12RB2* gene. For each probe, *p < 0.05, **p < 0.01, ***p < 0.001. Selected methylation beta values vs. mRNA expression z-scores relative to all samples (mRNA expression data obtained through the cBioPortal for Cancer Genomics as log RNA Seq V2 RSEM) for (**D**) the *NFIA* gene (**E**) the *HDAC4* gene, and (**F**) the *IL12RB2* gene.

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Figure 1. Azacytidine treatment inhibits proliferation and reduces viability of UM cells in a concentration-depending manner. A-D Growth curves obtained through Incucyte live cell imaging system, tracking cellular confluency over 3-5 days after treatment with 0-80μM Azacytidine. E. Graph represents the level of viability as detected via a CCK8 colorimetric assay by relative percent metabolic activity, measured after 72 hours and calculated as absorbency of the treated samples by the absorbency of the untreated samples. CCK8 data was analysed using two-way ANOVA, while Incucyte data was analysed using repeated measured ANOVA with Tuckey's post-hoc test. Differences between the treatments were additionally calculated using a t-test for the effect of the drug at one doubling time, with $p<0.05$ being considered as significant in all cases.

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Figure 3. Azacytidine treatment did not impact BAP1 protein expression. Representative immunocytochemistry images are shown for anti-BAP1 monoclonal antibody (1:2000) staining for MP41 (A), Mel270 (B), and OMM2.5 (C), and MP46 (D) at a dilution of 1:2000 following 72-hour Azacytidine treatments of 0, 5 and 20 μM. Images taken at 10x magnification using the (Invitrogen EVOS XL Core Imaging System)

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Figure S1. Figure S1. Hierarchical clustering of differentially methylated probes for 4 cell lines (Mel270, OMM2.5, MP41, and MP46, labeled on graph) compared to 80 primary UM cases from the TCGA database using SWAN normalized Beta Values, with dark red indicating a locus is fully hypermethylated and dark blue indicating a locus is fully hypomethylated.

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1. Introduction

1.1. Uveal Melanoma

1.1.1. Overview of Uveal Melanoma

Uveal Melanoma (UM) is a rare intraocular malignancy with an annual incidence of 3.75 cases per million per year in Canada [1] and 5.1 per million per year in the US [2]. UM arises in the choroid, ciliary body, or iris. It occurs more frequently in people with fair skin, light eyes, ocular melanocytosis, ocular nevi, and familial BRCA1-associated protein 1 (BAP1) mutations [3]. Unlike with cutaneous melanoma, exposure to ultraviolet (UV) radiation is not associated with UM incidence, though occupational exposure to blue light has been associated with increased development of UM [4, 5]. While effective local disease management is available – commonly with radiotherapy but potentially also through surgical techniques including resection and enucleation – almost 50% of UM cases will lead to metastatic disease, which is fatal [2, 6]. Metastatic disease in UM is mostly to the liver but can also occur in the lungs and bones, and even when receiving treatment for metastases, median survival in patients with diagnosed metastatic disease is 1.07 years [7]. There are currently no effective treatments that allow for long term survival of patients with metastatic UM.

1.1.2 Current treatment options for UM

Treatment of primary UM includes radiation – notably plaque brachytherapy for medium tumours and external beam radiotherapy for large tumours – and either local resection or enucleation as surgical options [8]. Since data has shown similar rates in metastasis and death between patients undergoing local radiation or enucleation, the most common first line treatment for primary UM is radiation therapy [6, 8, 9]. While local control is generally achieved, treatments targeting metastatic UM have been largely unsuccessful, though some options can extend survival by a few months. Specifically, liver targeted treatments have the potential to improve patient outcomes; hepatic resection, hepatic intra-arterial chemotherapy, and hepatic arterial chemoembolization are used in patients given the overwhelming predilection of UM metastases for the liver [10]. More recent clinical trials include the use of combined immune checkpoint inhibitors which, while effective for some cutaneous melanomas, have not shown similar improvements in outcome in UM [11]. Other treatments that have been tried in UM include Mitogen-activated protein kinase kinase (MEK) inhibitors, which were not shown to be effective, and drugs including tebentafusp, as

well as liver directed therapies [11]. Of all options available for metastatic UM, liver directed therapies currently are the only option that seem to potentially prolong survival, though limited conclusive data exists, and patient survival remains short [12]. Despite attempts at improving survival for patients with liver metastasis, the current guidelines encourage clinical trials for patients with metastatic UM, as there are no treatments that have been shown to improve significantly survival [8].

1.1.3 Driver events

UM is characterized by initiating mutations in the Gα11/Q pathway, with 57% of UM tumours showing hotspot mutations in Guanine nucleotide-binding protein G(q) subunit α (GNAQ) and 41% of tumours having Guanine nucleotide-binding protein subunit alpha 11 (GNA11) mutations. In samples that do not contain these hotspot mutations in GNAQ or GNA11, there are usually mutations in other genes related to the same pathway, generally Phospholipase C Beta 4 (PLCB4) or Cysteinyl Leukotriene Receptor 2 (CYSLTR2) [13]. These mutations are present in almost all UM cases and are unrelated to the likelihood of developing metastatic disease. Additionally, these mutations are also seen in ocular nevi that do not progress to melanoma, and transfection with GNAQ in melanocytes has been shown to be insufficient in inducing anchorage independent growth in UM cells, suggesting that these mutations are not sufficient for malignant transformation in UM [14, 15]. On the other hand, there are three mutations in UM that have been associated with the likelihood of developing metastatic disease: BRCA1-associated protein 1 (BAP1), Splicing Factor 3b Subunit 1 (SF3B1), and Eukaryotic Translation Initiation Factor 1A X-Linked (EIF1AX). These are associated with a high risk of early metastasis, a moderate risk of generally late metastasis, and a low risk of metastasis, respectively [16]. These mutations have additionally been associated with different gene expression profiles across UM cases [17]. Inactivating BAP1 mutations in UM can occur as single nucleotide polymorphisms (SNPs) or insertions/deletions (indels) along with more distant mutations affecting the regulation of BAP1 expression, with alterations often being long or complex [18]. Loss of BAP1 protein expression as well as confirmed loss-of-function BAP1 mutations are highly associated with metastasis in UM, and are associated with a molecular profile that is accompanied by a high risk of metastasis [18, 19]. Due to its localization on

chromosome 3 (3p21.1), the loss of BAP1 protein expression is also highly associated with monosomy 3, a frequent genomic event seen in metastasizing UMs.

1.1.4 Important molecular pathways in UM

There are many cancer-associated pathways that have been found to be commonly altered in UM, including the Ras-Raf-MEK-Extracellular signal Regulated Kinase- Mitogen Activated Protein Kinase (Ras-Raf-MEK-ERK-MAPK) cascade, which is important in the regulation of cell growth and proliferation. Along with this, there are also alterations in G-protein coupled receptor-related cascades, with mutations causing constitutive activation of signaling leading to the increased transcription downstream, including through ERK1/2, Rho/Rac/ Yes-associated protein (YAP), and Phosphoinositide 3-kinase (PI3K)/Akt pathways [20]. The initiating mutations in UM involving constitutive activation of G-protein-alpha subunits leads to activation of the Ras/Raf/MEK/ERK signaling pathway, which promotes cellular proliferation and tumour progression [10]. In general, the retinoblastoma tumour suppressor pathway and p53 pathway can be disrupted in UM cases – though rarely through mutations – leading to decreased ability to block cell cycle progression, along with constitutive activation of the PI3K/Akt and Ras/Raf/MEK/ERK pathways, which promote cell proliferation [14].These factors all play into the ability of these tumours to grow and eventually spread, as is the case in other malignancies. Along with this, more recent evidence has suggested that YAP signaling may also play a role in the progression of UM independently of GNAQ/11 mutations [21].

1.2. BAP1 mutations

1.2.1 Importance of BAP1 mutations in cancer

BAP1 mutations were first found to be related to cancer through a correlation of increased susceptibility in some families of developing mesothelioma, along with a number of other malignancies including atypical spitz tumours as well as uveal and cutaneous melanomas [22, 23]. These families were later discovered to share germline mutations in the BAP1 gene, leading to a loss of BAP1 protein expression [24]. Following this, in 2010, Harbour et al. reported that loss of function mutations in the gene encoding for the BAP1 protein were a common occurrence in metastasizing UM [19]. Because of this, loss of function BAP1 mutations were found to not only be present

in familial cancer syndromes, but indeed also seem to play a large role in the development of metastatic UM.

1.2.2 Role of BAP1 in tumour suppression and epigenetic regulation

BAP1 was first discovered due to its interaction with the Really Interesting New Gene (RING) finger domain of Breast and Overian Cancer Susceptibility Protein 1 (BRCA1), hence its name BRCA1-associated protein 1 [22]. Despite this, further work in elucidating the roles of BAP1 have shown that the BAP1 protein has larger roles as a deubiquitylase. It also regulates many cellular functions, with negative effects on cell viability and growth [22, 25]. In lung cancer cells, this protein has been shown to have tumour suppressor activity, with cells expressing wild type BAP1 showing signs of early apoptosis and late apoptosis/necrosis independently of BRCA1 [25]. While BAP1 mutations are a very strong indicator of poor outcome in UM, these alterations in the BAP1 sequence are often long or complex, making them potentially difficult to detect in samples as they do not occur as point mutations such as those seen with GNAQ or GNA11 [18]. As a response to this, BAP1 protein staining has been looked at as an alternative method of determining which UM samples have lost BAP1 expression, though this is only relevant in cases where samples of the tumour are available [26].

1.3. Epigenetic Changes

1.3.1 Overview of epigenetic changes

For the last few decades, epigenetic changes – heritable alterations in gene expression that do not affect the DNA sequence itself – have been shown to occur to a great degree in cancer. These changes can include additions to histones such as methylation and acetylation on specific histone tails, but also can occur directly onto DNA, as is the case with DNA methylation [27]. DNA methylation occurs through the addition of methyl groups directly onto cytosine residues by DNA methyltransferases [28]. Methylation in promoter regions of genes is known to alter gene expression by directly preventing the binding of transcription factors from their binding sites or by recruiting chromatin protein complexes that hinder transcription [29]. Both these factors can lead to the same final effect, which is to prevent transcription and eventually alter gene expression. While methylation at CpG islands has been repeatedly shown to block gene expression, its effect in the rest of the genome is not as clear; DNA methylation in the gene body, unlike methylation at transcription start sites, has the potential to

stimulate transcriptional elongation and potentially affect splicing, while methylation in repeat regions plays an important role in genomic stability overall [30].

1.3.2 Alterations in methylation across cancer

Tumour cells are marked by an overall pattern of global hypomethylation along with specific hypermethylation of certain genes [31, 32]. In humans, cytosines that precede guanosines in DNA sequences – known as CpG dinucleotides – can be methylated to form methylcytosine by DNA methyltransferases (DNMTs) [33]. Specific hypermethylation is seen in cancer at CpG islands, which are present at transcription start sites; this hypermethylation is known to block transcription at these sites, influencing gene expression [32, 33]. Cytosine methylation in promoter regions has specifically been shown to lead to the inactivation of both tumour suppressor genes and DNA repair genes in certain types of cancer, which can lead to tumour progression and, alongside other events, potentially act as a "second hit" in cancer development [29, 34]. On the other hand, the global pattern of hypomethylation in cancer occurs largely at repeat sequences such as retrotransposons and endogenous retroviruses, and promotes heterochromatin decondensation and DNA recombination at repeat sequences [32].

1.3.3 Role of methylation in gene expression changes

In cancer cells, DNA methylation has also been shown to be associated with certain histone modifications, wherein reversing hypermethylation at promoter regions using DNMT inhibitors can also lead to alterations in histone modifications [35]. Overall, this points to an interdependence of histone modifications on DNA methylation, and shows that some changes in gene expression are specifically related to the alterations in promoter methylation [35]. Since histone modifications additionally play an important role in heterochromatin formation, this relationship between DNA methylation and histone alterations furthers the idea that DNA methylation plays an important role in the regulation of gene expression in normal tissues as well as in disease processes [36]. Alongside this, hypermethylation at CpG islands near transcription start sites is known to cause loss of gene expression by blocking the initiation of transcription [30].

1.3.4 Specific methylation changes associated with cancer

Numerous specific changes in methylation have been seen across tumours, many of which share the common characteristics of contributing to effects in pathways that are related to the hallmarks of cancer described by Hanahan and Weinberg [37, 38].

These changes include effects on genes involved in cell cycle regulation, DNA repair, promotion of apoptosis, or signaling cascades involved in tumour progression [39]. Additionally, there are many promoters that have been investigated in cancer specifically, such as O-6-methylguanine-DNMT (MGMT), which has been shown to be silenced through hypermethylation in many carcinomas [40]. Additionally, $p16^{INK4a}$ and $p15^{INK4A}$ are important in cell cycle regulation are can often be silenced through DNA methylation in cancer [40, 41]. Overall, depending on the tumour type, there are numerous genes that can be inactivated by hypermethylation. Depending on the case, these methylation changes can potentially be used for prognostication or monitoring of various malignancies.

1.3.5 Detection of DNA methylation changes for cancer monitoring

Alterations in methylation have already been used for the monitoring of cancer in a number of malignancies, both in solid tumours as well as in blood. In glioblastoma multiforme (GBM), both single point DNA methylation – in this case at the MGMT promoter – as well as array based techniques have been used to stratify cases [42]. Indeed, since DNA methylation occurs early in tumour development and remains stable over the disease course, it is often possible in tumours with a large epigenetic component to detect important methylation patterns that are indicative of outcomes in disease [43, 44]. This has been done across many tumour types, where either specific tumour suppressor genes or global patterns of methylation can give insight into patient prognosis, and may help guide treatment along with other factors.

1.3.6 Use of DNA demethylating agents in cancer treatment

Since alterations in DNA methylation can have such a profound effect on the behaviour of cells, including tumour cells, reversing these alterations has the potential to make a tumour easier to treat. The use of DNA demethylating agents, which are generally nucleoside analogs of cytidine, lead to promoter demethylation, gene re-expression, and changes in the histone code [35]. Indeed, DNMT inhibitors are currently used in the treatment of acute myelogenous leukemia (AML) and myelodysplastic syndrome, where they increase patient survival with relatively minimal side effects and improved quality of life [45, 46]. They have also been tested in other tumours including UM, where they have been shown to improve the efficacy of MEK inhibitor treatment in patients [47].

1.3.7 Methylation changes in UM

Over the past few years, there has been some work into the epigenetic alterations in UM, including DNA methylation. First, as in many other tumours, specific changes in methylation were examined, including hypermethylation of hTERT , Ras association domain family member 1A (RASSF1A), and Embryonic fyn-associated substrate (EFS), amongst other genes, being seen across UM tumours [48-50]. Following this, the cancer genome atlas (TCGA) study of UM showed that monosomy 3 tumours showed a unique pattern of global methylation, and that disomy 3 tumours showed some differences in methylation depending on the mutations present within the tumour [18]. Additionally, they observed that these methylation changes led to alterations in gene expression, including for microRNAs (miRNA) [18]. In cell lines, BAP1 loss was then shown to be associated with methylomic repatterning in UM cells, with alterations in genes involved in axon guidance and melanogenesis [51]. Overall, these works have pointed to a role of large-scale epigenetic changes occurring in the progression of UM, and to a role of the BAP1 protein in these alterations. Additionally, work showing that RASSF1 hypermethylation can be reverted using azacytidine [52], as well as data that has shown that the DNMTi decitabine limits escape from MEK inhibition in UM cells [47] suggests that altering methylation patterns may be a viable technique to explore in the treatment of UM.

1.4. Liquid Biopsy

Genetic and epigenetic profiling of tumours generally requires either a biopsy or surgical specimen, which can be especially difficult to access in tumours such as UM. Additionally, since metastatic UM often occurs as micrometastases in the absence of a primary tumour – with the primary tumour often being removed years prior – obtaining specimens can be especially difficult [10]. For this reason, less invasive methods such to monitor these genetic and epigenetic alterations would be of great use in these cases where there biopsies are not feasible.

1.4.1 Challenges in molecular analysis of biopsies

Traditionally, biopsy techniques such as fine needle aspiration (FNA) have been used to diagnose and guide treatment of tumours [53]. Biopsies have been of great use because they allow for histological analysis, and in more recent history, have given us the opportunity to perform genetic profiling of tumour cells [54]. In the last decades,

the availability of cost-effective sequencing techniques has allowed us to examine the cancer genome as a whole, and has consequently led to a new era in work on personalized medicine based on specific genomic alterations [55]. Despite these advances, there are issues in using mutation-specific pharmacological treatments, as single biopsies can only give an image of the tumour in one location and at one time. Specifically, it has been shown that 63-69% of all somatic mutations are not detectable across every region within a tumour, which can lead to biopsies showing an incomplete picture of the tumour at the time of sampling [56]. In addition, since tumours change over time, it would be ideal to obtain samples from multiple regions within tumours at different time points across treatment, but this is not feasible due to the discomfort for patients, associated risks, and economic considerations in capturing serial biopsies [54]. This is especially relevant in the context of biopsies that are in locations that are more difficult to sample. For example, in UM, biopsies are typically not performed for diagnosis and are instead only used in specialized centers for prognostication [8, 57]. Instead, follow up for patients will not involve serial biopsies, but will instead be largely centered around liver imaging as it is the most common site for metastasis [8].

1.4.2 Liquid biopsy

Because of this, liquid biopsy – the examination of bodily fluids to monitor characteristics of systemic disease – is an ideal option for non-invasively capturing a picture of the disease landscape as a whole in real time.

1.4.3 Liquid biopsy in cancer monitoring

Depending on the tumour of interest, liquid biopsy in cancer can involve many different biofluids, including blood, urine, saliva, and cerebrospinal fluid, amongst others. Liquid biopsy has the potential for being better at guiding treatment options than traditional biopsy because of its non-invasiveness, and because it is not confounded by intra- and inter-tumour heterogeneity in the same way as traditional biopsies [56]. Additionally, there are many features of tumour DNA that can be interrogated through liquid biopsy, such as mutations, methylation patterns, and detection of viral DNA, all of which can be used in different tumour types to monitor cancer progression [58]. Along with these specific changes in the genome, cell free DNA within cancer patients will differ from cell free DNA in healthy individuals in features such as fragment length, with cancer patients showing more fragmented patterns of cfDNA [59].

1.4.4 Detection of circulating tumour DNA using liquid biopsy

Cell-free DNA (cfDNA) are short segments of nucleic acids that are released from cells into circulation and have a very short half-life in circulation, making them an up to date picture of DNA release into circulation. Circulating tumour DNA (ctDNA) is a form of circulating cell free DNA that has been reported as lasting an hour or less in blood – allowing for a real-time assessment of tumour DNA release at the time of sample collection [60]. cfDNA is believed to be released from cells through apoptosis, necrosis, and potentially active secretion, and this DNA will maintain genetic and epigenetic modifications present on their cell of origin [61]. Overall, the data on cfDNA indicates that these short fragments will reflect cell turnover processes at the time of sampling.

1.4.5 Methylation to detect differences in cells of origin for tumour monitoring

As discussed with other liquid biopsy assays, dying cells release pieces of fragmented DNA into circulation which retain the features of the cell of origin. Since all cell types have unique methylation patterns which remain stable even in cancer, there is the potential to determine the cell of origin on the basis of the methylation pattern of cell free DNA [59, 62]. In recent years, there has been more exploration into this field, using computational techniques to determine cell types of origin based on methylation patterns within samples. Cell type deconvolution algorithms can exploit the differentially methylated regions between cell types in order to infer the proportions of different cell types present within a sample [63]. Because tumour cells will maintain unique features depending on their origin, it is possible to use deconvolution in order to determine which cell types are shedding a higher proportion of circulating DNA. This has been shown in prostate cancer where patients who have successful treatment will show decreases in the levels of prostate cell free DNA compared to pre-treatment levels [64]. In addition to this, it has been shown that plasma cell free DNA methylation – when analysed by Methylated DNA Immunoprecipitation Sequencing (MeDIP-Seq) – can show enrichment in the same sites that are known to be hypermethylated in primary tumours of the same type, including pancreatic ductal adenocarcinoma (PDAC), colorectal carcinoma (CRC), AML, and breast, lung, renal, and bladder cancers [65]. Because cells have characteristic methylation patterns that relate to their cellular identity, changes in the methylation patterns of cell free DNA could therefore show a

wide array of issues within the body; deviations from the normal cellular composition can be an indicator of an increase in cell death originating from any part of the body, and can therefore determine the likely site of origin of a number of health issues [64]. Additionally, since these methods of cell type deconvolution give predictions of the proportion of circulating DNA originating from different cell types, they can be useful in estimating tumour burden [59]. Still, these methods which look at full methylation patterns of cells can be expensive and can sometimes require larger amounts of starting DNA. Therefore, using fewer loci for the analysis of methylation in ctDNA would be a more cost-effective and potentially clinically relevant method for monitoring ctDNA through methylation profiles.

Aims:

Given the current lack of treatment options for metastatic UM, as well as well as the potential importance of methylation in tumour growth and dissemination, we aimed to gain insight into the specific methylation events that may be driving UM towards metastasis, and to determine the ways in which methylation patterns may be important in determining outcomes in UM. The goal of this work was to offer new avenues for potential treatments in UM, along with determining whether these changes might be detectable in cell free DNA, which would allow us to monitor patients long after primary tumour treatment.

In part 1 of this work, we aimed to detect the methylation changes that were occurring in UM patients, to see whether specific alterations might be associated with poor outcome.

In part 2 of this project, the goal was to determine if the changes that were seen in UM patients could be recapitulated in UM cell lines. Further to this, we aimed to see whether changes induced by the DNA demethylating agent azacytidine were occurring in pathways that could be targeted pharmacologically in other ways, in order to open up new avenues to revisit for the treatment of metastatic UM.

Finally, the goal was to see whether the changes in genomic DNA methylation could be tracked in cell free DNA from UM cells, and whether treatment with azacytidine had an effect on global methylation patterns in either genomic or cell free DNA.

Chapter 2.

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Linker to Article #1

For this first portion of the project, we were interested in seeing whether there were specific changes in methylation that might promote metastasis and tumour progression in UM. We hypothesized that, because UM has a relatively low mutational burden, methylation events may be responsible for driving metastatic progression in this disease, and that UM cases that develop into metastatic disease would show a different pattern of DNA methylation. To examine this, we studied data from the TCGA database, which contains genetic, epigenetic, and transcriptomic data for many tumour types, including 80 UM samples. The idea behind this was to compare the methylation across UM cases to see whether methylation pattern alone is a good predictor of disease outcome, and if these changes led to alterations in gene expression. Finally, the goal of this work was to see whether there were potential pathways that are affected by methylation in UM that might point to therapeutic targets. In this work, we looked into specific genes and pathways modified by methylation in UM patients to see whether these particular changes could potentially allow for more targeted treatment of high-risk UM, as well as to see whether a small number of methylation loci could stratify cases based on patient outcomes.

Novel Methylation Patterns Predict Outcome in Uveal Melanoma

Sarah Tadhg Ferrier [1] and Julia Valdemarin Burnier [1,2,3,*]

[1] Cancer Research Program, Research Institute of the McGill University Health Centre, Montreal, QC, Canada, H4A 3J1; sarah.ferrier@mail.mcgill.ca

[2] Experimental Pathology Unit, Department of Pathology, McGill University; Montreal, QC, Canada, H3A 0G4

[3] Department of Oncology, McGill University; Montreal, QC, Canada, H3A 0G4

* Correspondence: julia.burnier@mcgill.ca

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Abstract: Uveal melanoma (UM) is the most common intraocular tumour in adults. Despite effective local treatments, 50% of patients develop metastasis. Better ways to determine prognosis are needed as well as new therapeutic targets. Epigenetic changes are important events driving cancer progression; however, few studies exist on methylation changes in UM. Our aim was to identify methylation events associated with UM prognosis. Matched clinical, genetic, and methylation data for 80 UM cases were obtained from The Cancer Genome Atlas (TCGA). Top differentially methylated loci were sorted through hierarchical clustering based on methylation patterns, and these patterns were compared to tumour characteristics, genomic aberrations, and patient outcome. Hierarchical clustering revealed two distinct groups. These classifications effectively separated high and low-risk cases, with significant differences between groups in patient survival ($p < 0.0001$) and correlation with known prognostic factors. Major differences in methylation of specific genes, notably *NFIA*, *HDAC4*, and *IL12RB2*, were also seen. The methylation patterns identified in this study indicate potential novel prognostic indicators of UM and highlight the power of methylation changes in predicting outcome. The methylation events enriched in the high-risk group suggest that epigenetic modulating drugs may be useful in reducing metastatic potential, and that specific differentially methylated loci could act as biomarkers of therapeutic response.

Keywords: epigenetics; DNA methylation; uveal melanoma; BAP1; prognostic markers; metastasis

1. Introduction

Uveal melanoma (UM) is the most common primary intraocular tumour in adults and the most common non-skin form of melanoma, with a reported incidence of 5.1 cases per million in the US [1]. Unlike cutaneous melanoma (CM), UM arises from melanocytes located in the uveal tract, most commonly in the choroid, and displays different genomic mutations and molecular profile than the more common CM [2]. Despite effective methods for treating the primary tumour–either through local radiotherapy or less commonly enucleation [3]–there is currently no effective treatment for metastatic disease, which occurs in approximately 50% of patients regardless of primary ocular treatment [4]. Unfortunately, metastatic UM is associated with high mortality within 6–12 months. Prognostic factors of UM include features such as cell type, with a poorer prognosis in patients with epithelioid cell tumours and better prognosis for spindle cell tumours as well as tumour size. Additionally, tumours with increased mitotic activity, closed vascular loops in the tumour, increased tumour-infiltrating lymphocytes, and extrascleral extension all show poorer outcomes [5]. Genetically, UM is characterized by a set of chromosomal aberrations and somatic mutations. Most notably, monosomy in chromosome 3 is an important prognostic marker related to metastasis [5,6]. Copy number variations in chromosome 6 and 8 are also observed, with gain of chromosome 8q being associated with poor prognosis [7]. More than 80% of UMs harbour mutually exclusive mutations in *GNAQ* or *GNA11*, which lead to constitutive activation of signaling pathways such as the Ras-ERK and PI3K/Akt/mTOR pathways. *GNAQ* and *GNA11* mutations are initiating mutations in UM, and despite their presence in almost all of these tumours, the presence of these mutations is not generally related to the development of metastasis or to prognosis [8]. There are three mutations that have been shown to be associated with prognosis in UM; *EIF1AX*, *SF3B1*, and *BAP1* mutations can be used to classify UMs into low, intermediate, and high-risk, respectively. *EIF1AX* mutations are generally an indicator of good prognosis and are associated with low risk of metastasis. *SF3B1* mutations have been associated with late metastases, while *BAP1* mutations are associated with the development of early metastases [9]. While there is research on the genetic changes that underlie metastasis in UM, little is known about the mechanisms by which systemic progression occurs, and more work is needed to uncover the mechanisms through which the genetic aberrations seen in UM lead to metastasis.

DNA methylation is an important and well-studied epigenetic modification in mammals that is normally responsible for the regulation of gene expression, especially in developing cells. This process is regulated by DNA methyltransferases, which are responsible for maintaining DNA methylation on the genome. DNA methylation is associated with alterations in chromatin structure, and methylation occurring specifically on CpG islands is highly associated with the silencing of gene expression [10]. DNA methylation is recognized as an important event in cancer, where a pattern of global hypomethylation leading to genomic instability is often seen [11]. Along with this, many tumour types also show a specific pattern of hypermethylation at CpG islands which can be important in tumour progression, for example leading to the silencing of tumour suppressor genes [12]. While the mechanisms underlying tumour methylation are not fully understood, changes in methylation are maintained throughout cell replication and play an important role in the progression of multiple tumour types [13,14]. Because epigenetic processes such as DNA methylation are mitotically heritable, they can play similar roles as genetic alterations in the development of cancer, making them an important target in both prognostication and drug development. This is especially important as epigenetic events can confer growth advantages to cells by disrupting gene expression similarly to genetic events, but can exert its effects much more rapidly than mutations [15].

In UM, several known changes in promoter methylation have been studied, such as on the *RASSF1A* gene [16], and a global pattern of methylation has been associated with molecular subtype and overall prognosis [16,17]. Additionally, a recent study has shown that BAP1 knockdown in UM cells is associated with methylomic reprogramming in these cells, pointing to a link between the genetic mutations seen in UM and large scale changes in methylation pattern [18]. Despite these observations, few studies have investigated whether the changes in methylation pattern may contribute to the metastatic phenotype. As such, further investigation into the specific changes in methylation seen in these tumours are needed to more completely uncover the events that dictate outcome in UM and to uncover new therapeutic targets. Given the promise that epigenetic-targeting agents have shown in many tumour types, either through targeting specific modifications directly or through targeting epigenetic regulators, the reversal of epigenetic alterations may be a promising avenue for preventing metastasis in UM [19,20]. Furthermore, monitoring the specific changes in UM methylation that are

associated with a high risk of metastasis would indicate tumour response to different therapeutic agents. This is particularly important given the high rate of metastasis in UM and its poor prognosis.

In this study, we sought to investigate in detail potential epigenetic biomarkers in UM that could be related to prognosis. Using the Cancer Genome Atlas (TCGA) data, we were able to differentiate two groups with prognostically significant patterns of methylation, and to highlight some potential targets for high risk of metastasis in UM. We demonstrate the importance of specific methylation changes in UM on tumour progression by looking at both the overall promoter methylation pattern as well as at specific loci which are highly differentially methylated depending on the risk level of the patient. The data represents an important step in determining promising targets for better prognostication and treatment in this deadly ocular malignancy.

2. Methods

2.1. Dataset

Raw methylation intensity values from the Illumina 450k methylation array were obtained directly from the TCGA legacy archive for 80 UM cases. Along with this, the *TCGA biolinks* package was used to extract extended clinical data, RNAseq, and copy number variation (CNV) files. The Illumina 450k array annotation was obtained with the information from hg19 human genome assembly to map the loci on the array to their genomic location.

2.2. Removal of Poor-Quality Probes

The *Minfi* program was used to read the raw IDAT files and to calculate the detection *p* value for every genomic position in each of the 80 samples. Positions with *p* values >0.01 were discarded from further analysis to remove any probes where both the methylated and unmethylated channels reported background signal level, as determined by the negative control positions in the array. The mean detection *p* values across all samples were also calculated in order to ensure that there were no poor quality samples [21]. Probes that failed in one or more samples were removed from further analysis (total of 6480 removed), as well as probes on sex chromosomes, in order to remove some of the variation in methylation pattern caused by sex differences (11,004 removed). A total of 467,668 probes were kept for further analysis.

2.3. Normalization of Samples

The normalization was performed through both Quantile and SWAN normalization for further comparison [22,23]. These values were compared to the raw data, obtained using the *preprocessRaw* function, which brings together the methylated and unmethylated channels into beta values without further normalization. Quality Control reports were produced using Minfi and the different methods of normalization were visually compared using these reports.

2.4. Hierarchical Clustering of the Top Differentially Methylated Probes (DMPs)

The standard deviation across samples for each probe was calculated, and they were subsequently sorted by degree of differential methylation, as defined by the points which had the highest standard deviation across samples. The top 10,000 of these DMPs were selected for further analysis. These probes were inputted into Minfi in order to perform hierarchical clustering.

The results of this hierarchical clustering were compared to selected clinical and genetic data in order to determine whether the groups were clustering based on prognostically relevant data. The two groups were compared based on the sex and age of the individuals, survival and the development of metastatic UM, *GNAQ*, *GNA11*, and *BAP1* mutations, and CNVs.

2.5. Analysis of DMPs between the Two Groups

DMPs were also analysed using limma, which compared these DMPs across the previously determined groups from the hierarchical clustering, with the probes aligned to the hg38 human genome assembly. The highly differentially methylated probes, considered as those with a log fold change of ±1.5 or more in between the groups, were submitted into the DAVID Functional Annotation Clustering Tool.

Custom JavaScript code was used to separate CpGs for genes of interest in order to gather the quantile normalized beta values for all DMPs on the CpGs associated with these genes in all cases.

3. Results

3.1. Similar Levels of Overall Methylation Are Seen in 80 Cases of UM through Analysis of TCGA Data

A total of 80 UM cases from the TCGA were analysed for methylation profiling. Patient characteristics can be seen in Table 1. After removal of poor-quality probes and

normalization of data, the overall level of methylation at the remaining sites was analysed. Overall, the cases showed very similar levels of overall methylation, with an average ratio of intensities between methylated and unmethylated alleles (beta value) of 0.48 (range = 0.42–0.5) (Figure 1). This suggests that any differences between these samples are not due to higher or lower overall methylation at all probes, but instead are caused by hypermethylation and hypomethylation at specific CpG islands. Of the probes studied, 1708 probes were found to have a log fold change value of at least 2.0 (range = 2.00 to 4.73), while 785 probes were found to have log fold change values of -2.0 or less (range = -2.00 to 4.17).

Table 1. Patient characteristics.

Number of Patients	**80**
Sex (M:F)	45:35
Number of Deaths	24
Age at diagnosis (average, years (range))	62 (22–86)
Follow up (average, days (range))	767 (4–2600)

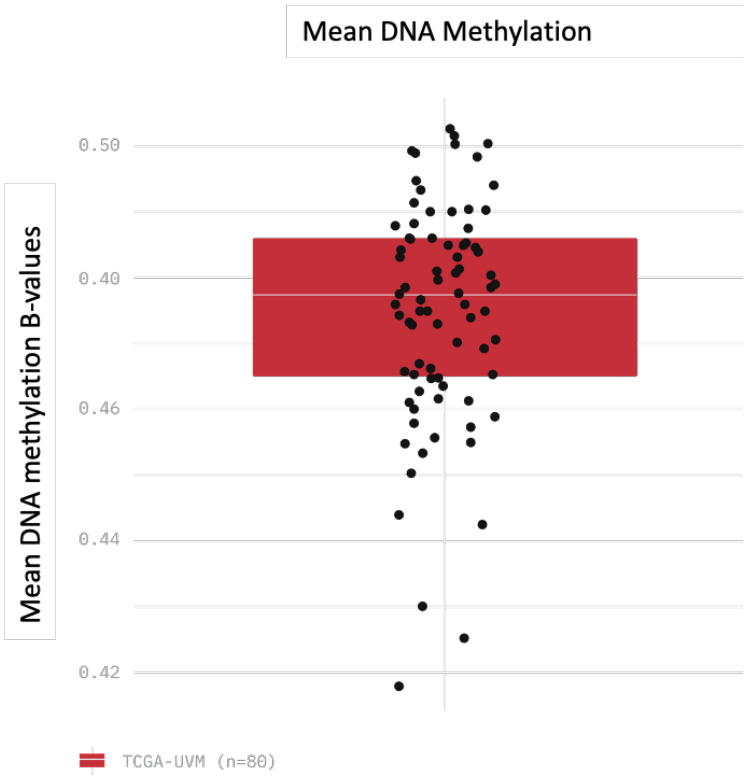


Figure 1. Total mean DNA methylation values for each patient sample (n = 80) as calculated by mean methylation beta values (ratio of intensities between methylated and unmethylated alleles) for all probes in the Illumina 450k methylation array.

3.2. Unsupervised Clustering Analysis Reveals Two Main Methylation Patterns in This Cohort

As overall degree of methylation was similar across cases (Figure 1), we aimed to determine whether patterns of methylation at specific sites would reveal important differences in the patient cohort. Unsupervised clustering of these 80 cases of UM based on the pattern of differential methylation revealed two major groups as shown in a heat map (Figure 2A) and principal component analysis (Figure 2B).

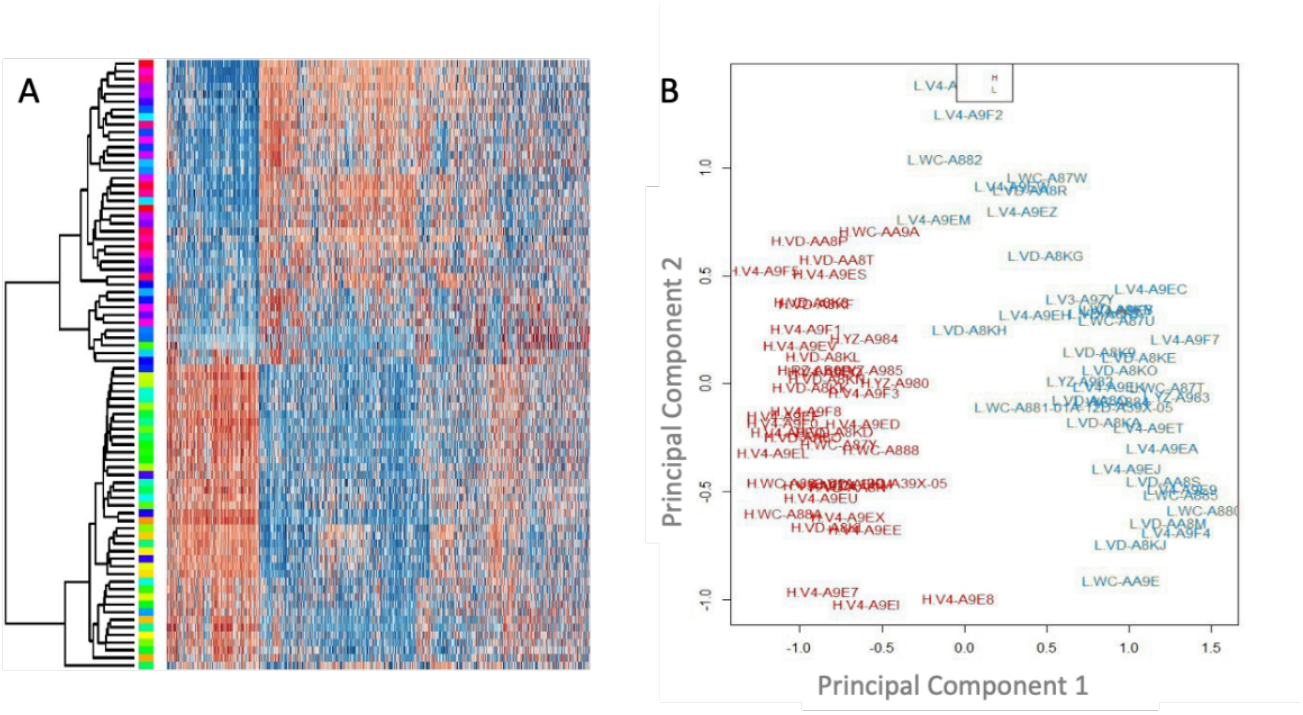


Figure 2. Unsupervised clustering analysis of 80 Uveal Melanoma (UM) cases from TCGA. (**A**) Heatmap showing the top 10,000 loci for all the patients using quantile normalized beta values, with dark red being fully hypermethylated and dark blue being fully hypomethylated for each locus. (**B**) Principal component analysis for cases, labeled by case number and risk group (as determined by hierarchical clustering).

3.3. Methylation Patterns Are Significantly Associated with Outcome and Effectively Stratify Patients into High and Low Risk Groups

Upon further investigation into these groups, no statistically significant differences were seen in clinical features that are generally prognostically insignificant, with similar M:F sex ratios ($p = 0.822$) and mean age at diagnosis (59 vs. 65 years, $p = 0.423$). In contrast, the two groups identified by unsupervised clustering analysis differed significantly in terms of outcome, and we therefore termed them "high-risk" and "low-risk" based on the clinical outcomes of the patients in each group (Table 2). Importantly, overall survival varied very significantly between the two groups as shown by a Kaplan-Meier curve (Figure 3). While 23 (58%) of the 40 high risk patients developed metastasis, only 4 (10%) of the low risk patients did ($p < 0.00001$, Table 2). Of these 4 patients, only 1 died, while 22 of 23 metastatic patients in the high-risk group died ($p < 0.00001$; average survival of 1.79 years before death from metastatic UM, Table 2).

Table 2. Patient characteristics by group.

	Low-Risk	**High-Risk**	
Number of Patients	40	40	
Sex (M:F)	22:18	23:17	* X^2: 0.0508, $p = 0.822$
Age at diagnosis (average, years (range))	59 (22–79)	65 (41–86)	** $p = 0.423$
Follow up (average, days (range))	973 (6–2600)	560 (4–1862)	
Metastasis	4	23	* X^2: 20.18, $p < 0.00001$
Death (from metastasis or unspecified)	1	23	* X^2: 28.81, $p < 0.00001$

* Chi Square test: n = 80 df = 1, ** Fisher's exact test.

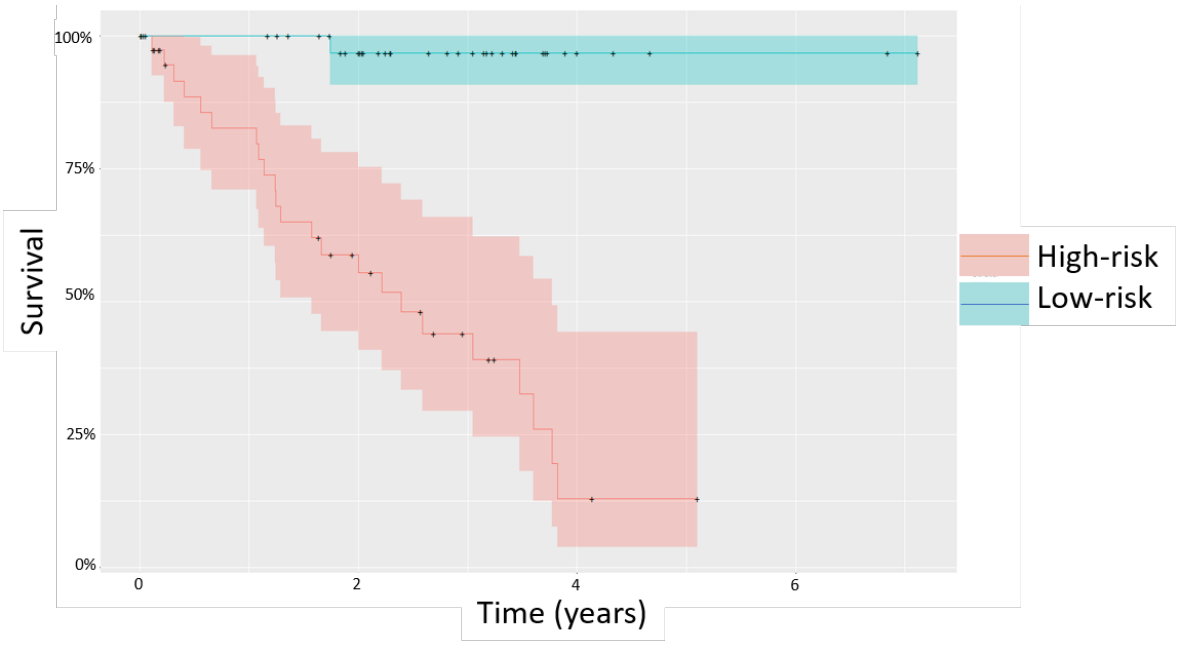


Figure 3. Comparison of the two groups of patients determined by hierarchical clustering. Kaplan–Meier survival function for the patients based on methylation risk groupings.

3.4. The High and Low-Risk Groups Differed in Clinical and Histopathological Features of the Ocular Tumours

Like in many malignancies, clinical and histopathological features correlate with patient outcome. In UM, increasing ocular tumour size has been shown to be associated with decreased survival [5]. Moreover, UM can be classified according to cell type: epithelioid, spindle, or mixed cell tumours. While spindle cell tumours have better prognosis, tumours composed of epithelioid cells are associated with worse prognosis [5]. As such, metastatic UM tumours tend to be predominately composed of epithelioid- or mixed-cell populations. Here, the two groups identified in the methylation analysis were compared on the basis of tumour features, including size and cell type. In terms of cell type, the clinical information from TCGA was classified by approximate percentages for each cell type as well as by the number of epithelioid or spindle cell predominant tumours in each group. While both groups contained epithelioid and spindle tumours, the high-risk group showed a higher proportion of epithelioid cell type tumours (20 vs. 3 epithelioid or epithelioid-predominant tumours). In terms of tumour size, tumours of the low-risk group were smaller in both thickness (average thickness = 9.99 mm, range = 5–15.5 vs.

10.85 mm, range = 4–16) and average basal diameter (average diameter = 16.15 mm, range = 7.8–23.6 vs. 17.72 mm, range = 10.6–25), with only diameter showing significance between groups ($p < 0.05$, Table 3). The number of tumours with closed connective loops were also significantly different between groups ($p < 0.0005$, Table 3).

Table 3. Tumour characteristics by group.

	Low-Risk	High-Risk	Significance
Tumour thickness (mm)	9.99	10.9	$p = 0.171$
Tumour diameter (mm)	16.15	17.72	$p = 0.044$
Cell type (% spindle:epithelioid)	~80:20	~50:50	
Presence of closed connective loops	13	30	$X^2 = 14.5$, $p = 0.00013$
Extraocular extension	2	5	$X^2 = 1.4$, $p = 0.23$

3.5. Methylation Stratification Highly Correlated with Genomic Factors Associated with Metastasis

Given the relatively short follow up (average of 2.35 years for the patients who did not succumb to metastasis), it is not possible to determine which patients would develop metastasis. Because of this, we compared our methylation groups to known markers of poor prognosis, such as mutations and chromosomal aberrations, to determine the likelihood of metastasis (Figure 4A).

The occurrence of *GNAQ* and *GNA11* mutations, which are initiating events in UM and not generally believed to be prognostically significant, differed between the two groups, with more *GNAQ* mutations in the low risk group (25 vs. 15, $p < 0.05$) and slightly more *GNA11* mutations in the high risk group (22 vs. 14, $p = 0.07$, Table 4). *BAP1* mutations are the most prognostically significant genomic alteration in UM, and are associated with high risk of metastasis [24]. The presence of a *BAP1* mutation was significantly associated with survival in this cohort ($p = 0.00022$, Figure 4B). The two groups differed by the number of cases with presence/absence of a *BAP1* mutation ($p < 0.00001$), with all confirmed *BAP1* mutations (24/80) found in the high-risk group (Figure 4A).

In addition to *BAP1* mutations, chromosomal changes are significantly associated with risk of metastasis and survival in UM [7]. Monosomy 3 is associated with high risk of

metastasis and is the strongest cytogenetic indicator to predict UM metastasis [6,7]. Indeed, loss of chromosome 3 was significantly associated with survival (Figure 4B). Loss of chromosome 3 was significantly more common in the high-risk group than in the low risk group (35 cases vs. 3 cases, $p < 0.00001$) (Table 4, Figure 4A).

Moreover, amplification of chromosome 8q, which is found in 40% of UMs, is also associated with poor prognosis [6]. Cases with gains in chromosome 8q in this cohort were associated with high-risk grouping ($p < 0.00001$), while gain of chromosome 6p were associated with the low-risk grouping ($p < 0.00001$), Table 4). Another frequent alteration is chromosome 1p loss (found in 25% of UMs), which occurs frequently with monosomy 3. Loss of chromosome 1p is not associated with decreased disease-free survival except in instances where this loss is combined with a loss of chromosome 3 [7]. Loss of chromosome 1 was similar between risk groupings (Table 4), although it was more commonly seen alongside a loss of chromosome 3 in the high-risk group (10 cases of concurrent loss of chromosomes 1 and 3 in the high-risk group vs. 1 case in the low-risk group).

Table 4. Mutations and chromosomal aberrations by group.

Genetic Alteration	Low-Risk	High-Risk	Significance
GNAQ mutations	25	15	* X^2: 5, $p = 0.0253$
GNA11 mutations	14	22	* X^2: 3.23, $p = 0.0722$
BAP1 mutation	0	35	** $p < 0.00001$
Chromosome 3 loss	3	35	* X^2: 51.33, $p < 0.00001$
Chromosome 6p gain	33	12	* X^2: 22.4, $p < 0.00001$
Chromosome 8q gain	23	37	* X^2: 13.06 $p = 0.0003$
Chromosome 1 loss	7	11	* X^2: 1.147, $p = 0.284$

*Chi Square test: n = 80 df = 1, **Fisher's exact test.

Importantly, all cases in the high-risk group had at least one important marker of poor outcome (either chromosome 3 loss, confirmed *BAP1* mutation, and/or development of metastasis) (Figure 4A) [7,24]. Additionally, the methylation groupings were more

accurate in predicting death from metastasis in this cohort than other known prognostic indicators of UM, including chromosome 3 loss, *BAP1* mutations, and clinical features (Figure 4B).

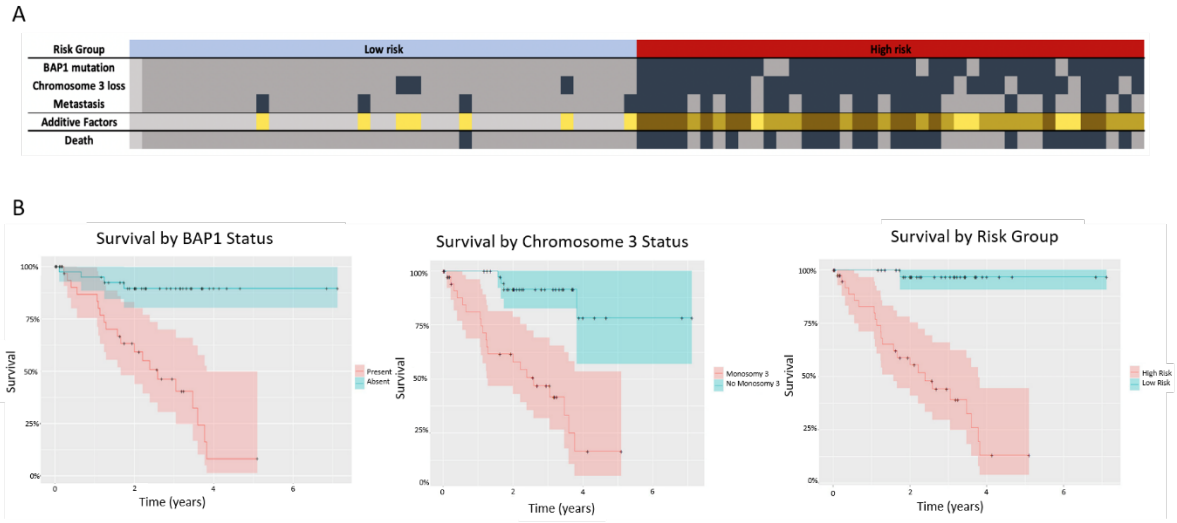


Figure 4. (**A**) Prognostic factors (BAP1 mutation, chromosome 3 loss, and development of metastasis) separated by risk grouping. Total number of these prognostically significant factors present for each patient represented in yellow scale. Death (due to confirmed metastatic disease or deaths with no specified cause) is also shown. Dark grey represents the occurrence of the event. (**B**) Comparison of survival for major prognostic factors BAP1 ($p = 2.47 \times 10^{-6}$), chromosome 3 loss ($p = 2.98 \times 10^{-6}$), and methylation risk groupings ($p = 5.35 \times 10^{-8}$).

3.6. Gene Ontology (GO) Analysis Reveals Enrichment for Genes Involved in Signal Transduction Pathways in the High-Risk Group

We conducted a GO analysis to determine gene classes that were commonly differentially methylated between the two risk groups. Our analysis revealed that the most significant DMPs were especially enriched for genes involved in signal transduction, including genes associated with pathways in cancer (KEGG pathways) (Figure 5A, Table S1) and in tumour suppressor genes (Figure 5A, Table S2). In the high-risk group, DAVID GO revealed that many of the genes with the highest log fold change in methylation levels between the two groups were involved in signal transduction such as for mTOR

signaling, PI3K/Akt signaling, and Ras signaling (Figure 5B, Tables S3–S5). Along with this, the analysis showed hypermethylation of a number of genes involved in the negative regulation of ERK1/2 in the high-risk group, as seen by analysis of DAVID biological process GO (Table S6). This category includes the hypermethylation of 10 probes associated with in the *PTEN* gene in the high-risk group (Table S7).

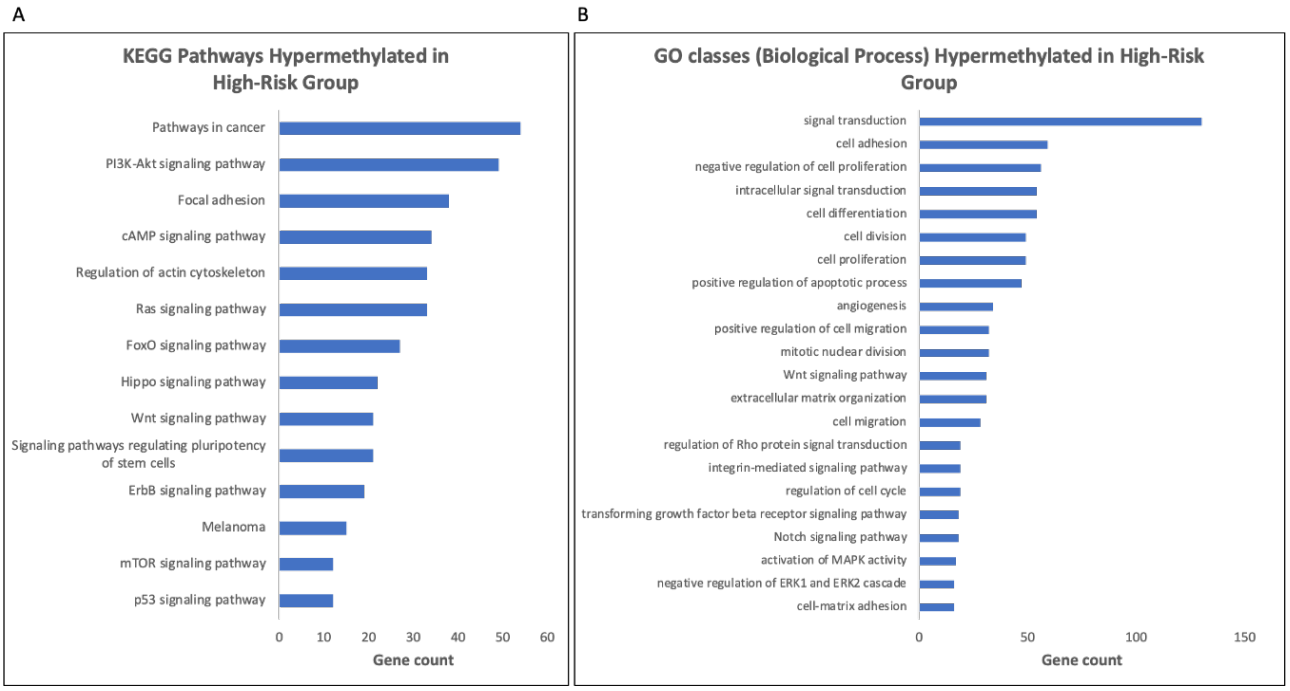


Figure 5. Selected list from the most highly affected Gene Ontology (GO) pathways between the two groups based on differentially methylated genes (log fold change of 1.5× or more between the high and low risk groups) for (**A**) KEGG pathways and (**B**) GO biological process classes. Pathways sorted by number of genes in each class, analysis done in DAVID using EASE score of 0.05.

3.7. Hypermethylation of Tumour Suppressor Genes and Transcriptional and Epigenetic Regulators Are Seen in the High-Risk Group

Among the many DMPs, several tumour suppressor genes and transcriptional regulators appear to be methylated in the high-risk group. Additionally, genes coding for one of the IL12 receptor subunits, IL12RB2, were found to be hypermethylated in the high-risk group (Table S8). Of interest, a high degree of differential methylation between the two

groups was seen at nine probes (with a log fold change of more than ±1.5) thought to be involved in coding of *NFIA* (Figure 6A, Table S9). *RASSF1*, a gene known to be inactivated through methylation in UM, was also found to be highly differentially methylated at multiple probes between the high- and low-risk groups (Table S10). Similarly, hypermethylation in the high-risk group in multiple *ZNF* genes, especially in ZNF358 (three probes with a log FC > 1.5, range: 3.49–4.37, Figure 6B) and ZNF532 (nine probes with a log FC > 1.5, range: 1.63–4.04) was seen (Tables S11 and S12).

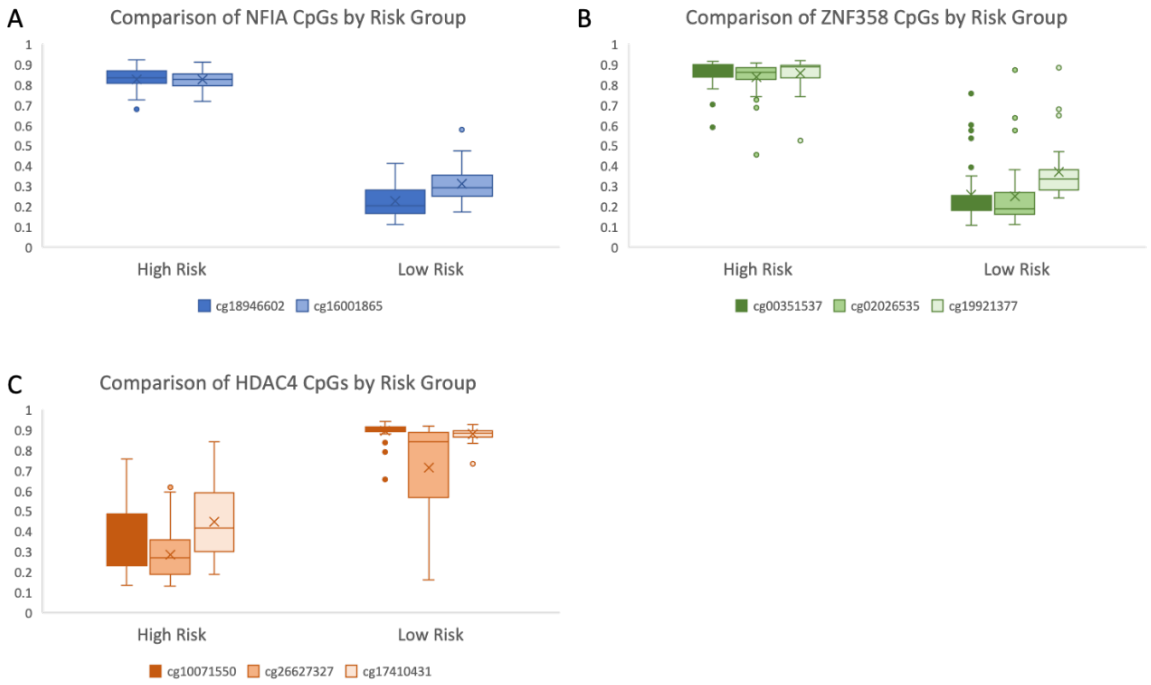
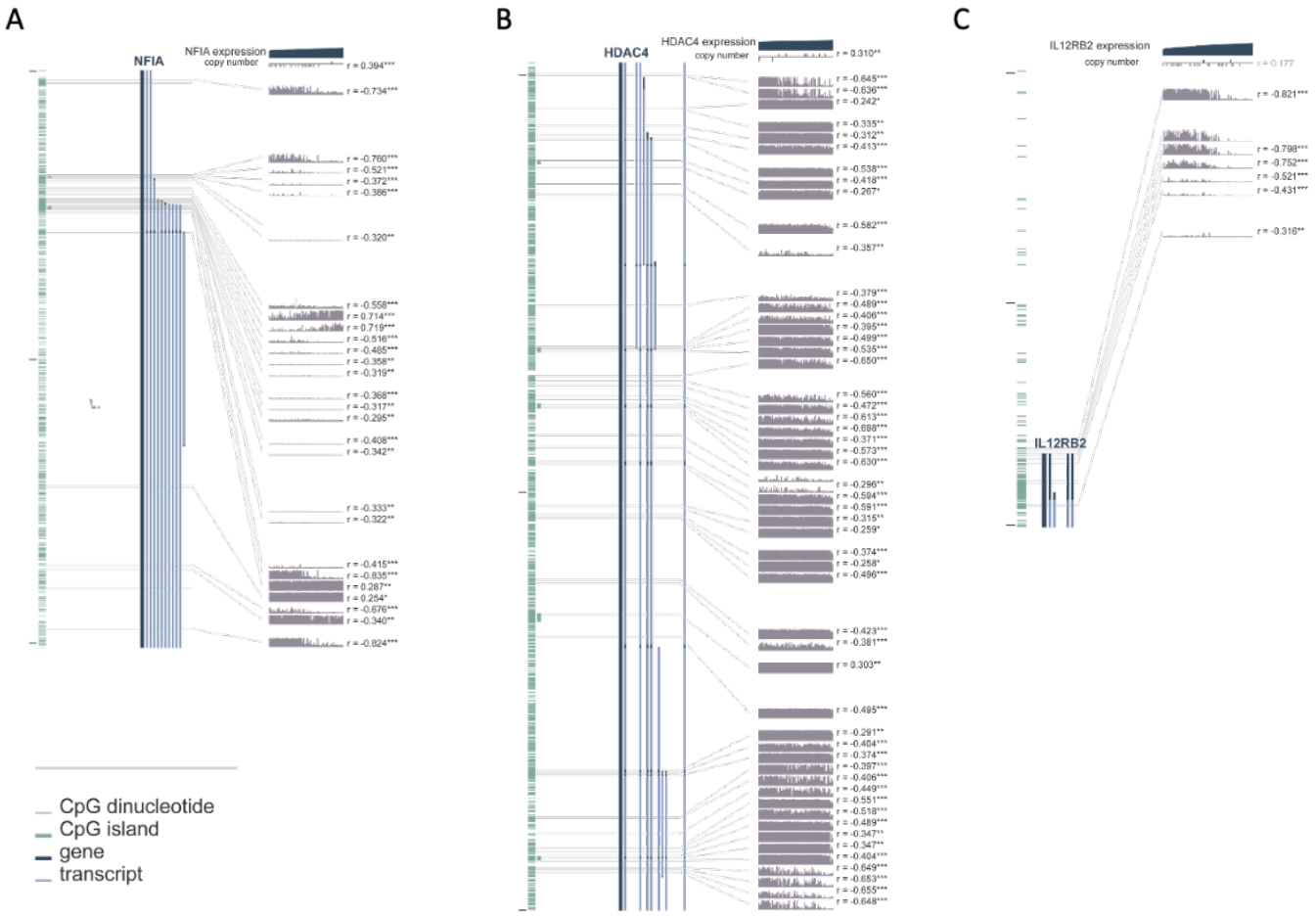


Figure 6. Box and whisker plots of average Beta values for all cases across selected highly differentially methylated probes, separated by high and low risk group, for (**A**) the *NFIA* gene (designated in blue and orange), (**B**) the *ZNF358* gene (designated in blue, orange, and grey) (**C**) the *HDAC4* gene (designated in blue, orange, and grey). Full list of differentially methylated probes for each of these genes available in supplementary Tables S9, S11, and S13.

3.8. Changes in Methylation are Found in Genes with a Role in Epigenetic Modifications

HDAC inhibitors are currently being investigated in UM for their potential to reverse the phenotypic effects of loss of BAP1 expression [25]. Interestingly, 49 probes associated with HDAC were found to be hypomethylated in the high-risk group, including probes with a log fold change of up to 4.08 between the two risk groups (sample of differentially methylated CpGs, Figure 6C, Table S13).

Moreover, we assessed the RNA sequencing data to determine if methylation at these genes may be associated with differences in gene expression, which showed that for certain CpG sites for these genes, hypermethylation was associated with reduced expression (Figure 7).



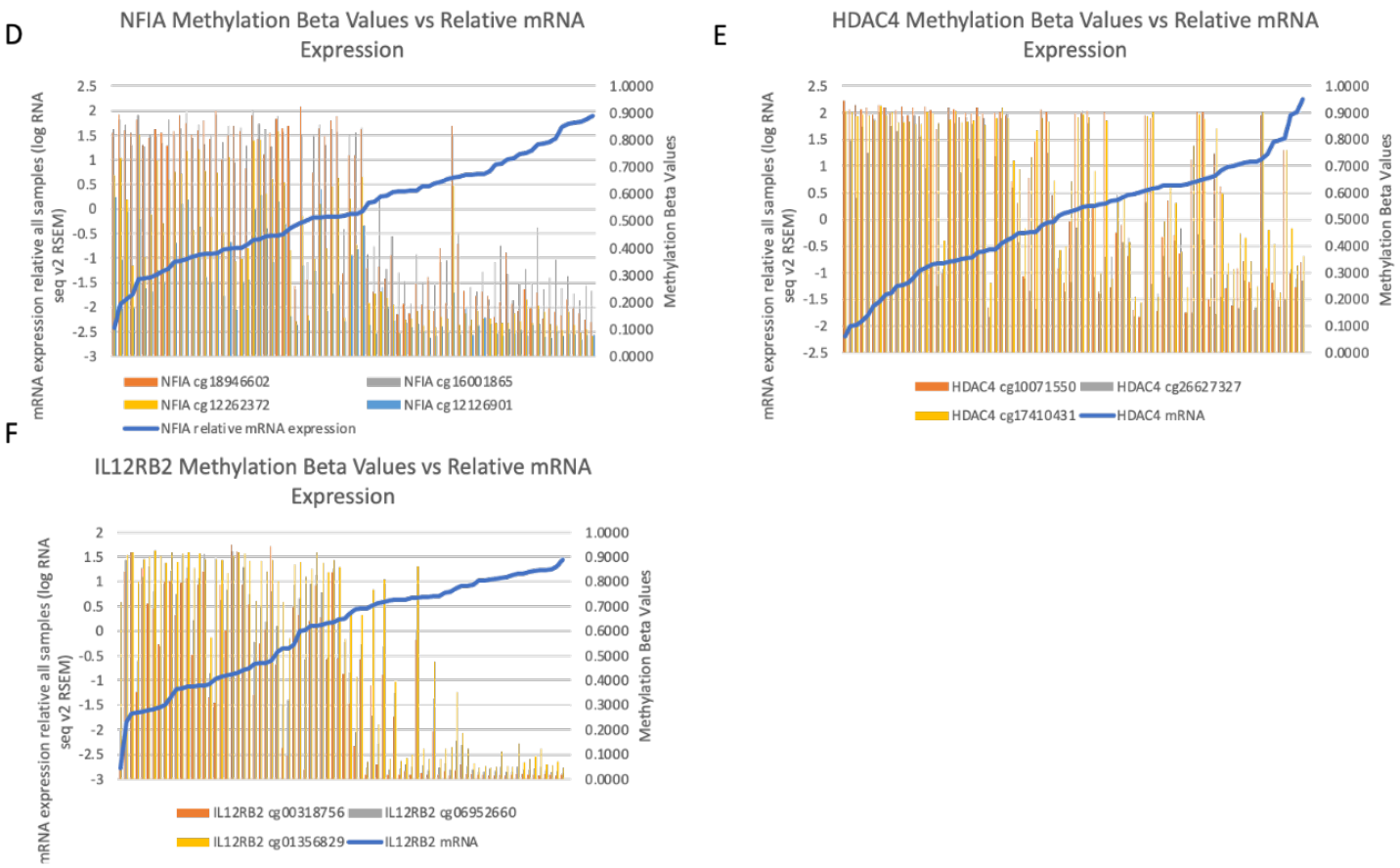


Figure 7. Degree of methylation at selected loci across all cases (n = 80), sorted in order of increasing gene expression (lowest to highest gene expression) for (**A**) the *NFIA* gene (**B**) the *HDAC4* gene and (**C**) the *IL12RB2* gene. For each probe, *p < 0.05, **p < 0.01, ***p < 0.001. Selected methylation beta values vs. mRNA expression z-scores relative to all samples (mRNA expression data obtained through the cBioPortal for Cancer Genomics as log RNA Seq V2 RSEM) for (**D**) the *NFIA* gene (**E**) the *HDAC4* gene, and (**F**) the *IL12RB2* gene.

4. Discussion

In this study, we conducted an analysis to investigate potential epigenetic biomarkers in UM that could be related to prognosis. Unsupervised clustering analysis of 80 UM cases showed separation of patients into two groups based on methylation changes. These groups were very significantly associated with outcome, highlighting the importance of

methylation profiling in this tumour type. The data suggest that differential methylation may act as a predictor of prognosis in UM (Figure 3).

A loss of function mutation in *BAP1* is a very strong predictor of metastasis, and was found in 24 cases of this cohort, all of which were placed into the high-risk group. All cases in the high-risk group had one or more important markers of poor outcome (either confirmed metastatic UM, chromosome 3 loss, and/or confirmed *BAP1* mutation), suggesting that these cases all show high metastatic potential as compared to the cases designated into the low-risk group (Figure 4).

Additionally, the methylation groupings were more accurate in predicting the development of metastasis in this cohort than other significant markers of UM progression, including chromosome 3 loss, *BAP1* mutations, and tumour features used to predict prognosis (Figure 4B). This is clinically significant because of the limitations of known prognostic markers in UM. *BAP1* mutations are difficult to detect as they can occur on multiple locations in the gene, therefore requiring whole-gene sequencing. While immunohistochemistry has been used for BAP1 protein expression profiling, further work remains to be done in this field to confirm the usefulness of BAP1 IHC for prognostication in UM [26]. Additionally, while there are clinical features such as epithelioid cell type, presence of lymphocytic infiltrate, increased mitotic activity, and tumour size that are useful in prognostication, these features can show intra-tumour heterogeneity and depend on the biopsy specimen that is analysed [27]. As such, methylation patterns may provide a powerful alternative or complementary biomarker of prognosis, requiring small amounts of input DNA. Furthermore, techniques such as liquid biopsy are gaining more interest in detection of tumour biomarkers, and even with fragmented and low abundance DNA, can be used to detect methylation patterns [28]. Our group is currently developing such an approach in UM. In recent years, gene expression profiling (GEP)-based testing has become more important for prognostication of UM, based on the development of a 15-gene panel validated by Onken et al. This test has been shown to be a strong indicator of prognosis, dividing 446/459 of studied patients into class 1 and class 2 GEP, with 1.1% of class 1 patients and 25.9% of class 2 patients developing metastases after a median follow-up of 17.4 months [29].

Three of the four patients in the low-risk group who developed metastasis were alive as of the most recent updates in the TCGA database. One of these three patients did not have metastasis at the time of the study, although they had developed metastasis at the

time of the latest update. The fourth patient was the only patient in the low-risk group who died from metastatic disease. Incidentally, this patient did not show either a *BAP1* or *SF3B1* mutation, nor did they have any changes in copy number for chromosome 3, all of which would be expected signs of poor prognosis. For the remaining three patients in the low risk group who developed metastasis, two showed *SF3B1* mutations, which have been associated with the development of late metastases, suggesting that methylation patterns are altered in patients who develop earlier metastases, but may be different in patients who will develop later metastases. Supporting this, 15 of the 18 patients with *SF3B1* mutations were classified in the low-risk group. As none of the patients in the low-risk group who developed metastasis showed *BAP1* mutations or copy number alterations at chromosome 3, other factors may be contributing to metastasis in these patients and warrant further examination.

For the patients in the high-risk grouping, metastasis tended to occur early (average of 1.86 years), and was generally, but not always, associated with the presence of alterations in chromosome 3 or *BAP1* mutations. As these features are associated with early metastasis, this is a potential indicator that there are alterations in methylation that may promote the development of metastasis through changes in gene expression. Additionally, as inactivating BAP1 mutations can occur across multiple regions of the gene, it is possible that not all BAP1 mutations have been detected in this study, and that changes in methylation in the high risk group may be related to changes in the BAP1 gene that were not discovered in the TCGA study.

We conducted a GO analysis to determine which genes are most commonly differentially methylated in the high-risk group. Differential methylation at a number of genes involved in cancer (KEGG pathways) and for tumour suppressors (Tables S1 and S2) may point to differential regulation of cell proliferation and tumour dissemination occurring through changes in methylation in UM in genes that are not mutated. Our analysis revealed differential methylation in multiple signaling pathways that have specifically been involved in UM progression (Figure 5), including the PI3K/Akt signaling pathway (Figure 5A, KEGG pathways, Table S4), which has been implicated in UM [30]. Particularly of interest was the hypermethylation of a number of genes involved in the negative regulation of ERK1/2 in the high-risk group (Figure 5), and the hypermethylation of 10 probes associated with the PTEN gene in the high-risk group (Table S7). PTEN has previously been demonstrated to act as a tumour suppressor in UM,

and is known to have a role in the regulation of both the PI3K/Akt and ERK1/2 pathways [31]. While previous data has suggested that genomic alterations are responsible for changes in PTEN signaling in UM, methylation may also play a role in regulating PTEN expression [32]. While the MAPK pathway has been shown to be commonly activated in UM, this effect does not occur through mutations in Ras genes [33]. Instead, the constitutive activation of GNAQ/GNA11 is believed to lead to the activation of this signaling cascade [34]. Despite the lack of mutations in Ras family genes, the differential methylation at numerous genes involved in Ras signaling (Figure 5A, Table S5) indicate a potential impact of methylation on signaling in this pathway in UM. In the same vein, the *GNAQ*/*GNA11* mutations in UM lead to increased phosphorylation of ERK, driving growth in UM cells through the Ras/Raf/MEK/ERK pathway [35]. The hypermethylation of multiple genes involved in the negative regulation of ERK1/2 signaling in the high-risk group implies the existence of alternative mechanisms that might also be working to increase ERK activation in UM. Along with this, UM cells show upregulation of PI3K/Akt/mTOR pathway, and the methylation of genes involved in mTOR and PI3K signaling (Figure 5A, Tables S3 and S4) points to yet another example of a signaling pathway known to be altered in UM potentially being epigenetically regulated to some extent. Additionally, genes coding for one of the IL12 receptor subunits, IL12RB2, were found to be hypermethylated in the high-risk group. IL12RB2 forms a receptor with high affinity for IL12 along with IL12RB1, leading to activation of signaling. IL12RB2 is the subunit in the IL12 receptor that is required for IL12-dependent signaling [36]. Hypermethylation at these sites in the high-risk group may suggest decreased receptor activity in the high-risk cases, especially given that hypermethylation was associated with reduced gene expression in these cases (Figure 7C,F). IL-12 has shown anti-tumour activity in other cancer types. In lung cancer cell lines that are negative for IL12RB2, use of the demethylating agent 5-aza-deoxycytidine was able to restore expression of the receptor [36]. Additionally, IL12RB2 knockout mice have been shown to develop spontaneous tumours (B cell and lung epithelial), and restoring the IL12RB2 leads to reduction of these tumours (in terms of proliferation, size, and microvessel formation) [37]. Endogenous IL-12 was shown to exert antitumour effects only in IL12RB2+ tumours, suggesting that changes in gene expression associated with DNA methylation may also have an impact on host antitumour response mechanisms.

Hypermethylation at genes encoding its receptor could point to a new therapeutic avenue in UM [38].

While large-scale epigenetic changes have been documented in UM tumours, and these changes have been significantly related to prognosis, there are currently no approved treatments using these agents in UM. In vitro, treatment with epigenetic modifying drugs has been shown to reduce growth and invasiveness in UM cell lines, and decitabine (a DNMT inhibitor) in combination with MEK inhibition has been shown to suppress growth in UM cells [39,40]. Additionally, decitabine has been used safely in clinical trials via hepatic arterial infusion in patients with unresectable liver metastases (NCT02316028), which is promising in the context of high risk UM patients [41].

While these agents hold promise, identification of the exact methylation events responsible for development of metastatic disease are needed to uncover clinical targets. Methylation of specific genes likely has significant impact on gene expression in UM, either through direct silencing or through affecting different pathways such as histone acetylation or ubiquitination. For example, hypermethylation of apoptosis-related genes and hypomethylation of growth promoting genes in the high-risk group may indicate potential targets for therapeutic avenues to explore. In our study, the specific DMPs associated with NFIA, HDAC4, and IL12RB2 correlated with the level of gene expression of these genes as seen on RNA sequencing data (Figure 7), with hypermethylation at these probes being associated with lower expression of transcripts for these genes [42,43]. Additionally, genes with very consistent differences in methylation between the two groups in this study such as NFIA and HDAC4 may be useful prognostic indicators for UM, and their methylation and expression patterns warrant further study. Because these genes appear to be epigenetically regulated in UM, the use of epigenetic modifying drugs could be a powerful strategy. This is consistent with the findings of Field et al. [18], showing that many specific genes and functional pathways are altered through methylation in certain UM cases, and that this information may help in the future to find potential therapeutic avenues for UM patients that have a high risk of developing metastases. As suggested, BAP1 mutations seem to be importantly associated with the altered methylation patterns, though the lack of BAP1 mutations in some patients in the high-risk group implies that there are also potentially other events that can lead to the same downstream effects on methylation patterns.

Among the many DMPs, one of the most highly differentially methylated genes was found to be NFIA. Since this gene is ubiquitously expressed in many tissues [44], this significant difference in methylation is of interest. We identified hypermethylation at CpG shores and islands along with hypomethylation at CpG shelves in the high-risk cases, suggesting that methylation may be impacting gene expression of NFIA (Figure 7A,D). The NFIA gene encodes a member of the nuclear factor 1 (NF1) family of transcription factors, and has been associated with cancer prognosis in some cancers, such as astrocytomas [45], and may be associated positively or negatively with prognosis depending on the tumour type [46]. NF1 family genes have been shown to play a role in epigenetic regulation, via remodeling of chromatin structure to alter gene expression. Depending on the tumour type, NF1 genes may act as either an oncogene or tumour suppressor, potentially through their regulatory effects on gene expression [46]. The high degree of differential methylation of this gene in the high-risk group suggests methylation of the NFIA gene as both a potential biomarker and therapeutic target in UM. In this cohort, the most highly differentially methylated NFIA probes were also fully segregated based on risk group, highlighting that methylation of this single gene may be able to classify patients into high and low-risk groups (Figure 6A). Our data show that, for these two loci, all cases in the high-risk group had methylation beta values above 0.5, and all cases in the low-risk group had methylation beta values below 0.5 (average of 0.83 vs. 0.23 for the first DMP and average of 0.83 vs. 0.31 for the second DMP).

RASSF1 was also found to be highly differentially methylated at multiple probes between the high and low-risk groups (Table S10). This gene has been investigated in UM and has been shown to be inactivated through methylation. Induction of RASSF1 expression has been shown to reduce tumorigenicity of UM cells in vitro [47]. Previously, 5-aza-2-deoxycytidine was shown to reverse RASSF1 methylation in a UM cell line, suggesting that the presence of hypermethylation in this region is a potentially reversible change that increases tumorigenicity in UM [47].

Zinc finger (ZNF) proteins are gaining interest in cancer studies due to their potential roles as either tumour suppressors or oncogenes [48]. In the present study, hypermethylation was seen in multiple ZNF genes in the high-risk group, especially ZNF358 (three probes with a log FC > 1.5, range: 3.49–4.37) and ZNF532 (nine probes with a log FC > 1.5, range: 1.63–4.04). ZNFs can function as tumour suppressors, and are inactivated in some tumour types through promoter hypermethylation [49]. ZNF358

specifically is expressed in neural folds during neural crest differentiation, and acts as a transcription factor [50]. Similarly to NFIA, multiple probes for ZNF358 segregate very strongly by risk groupings (Figure 6B). Overall, these large-scale differences in methylation of sites associated with these ZNFs suggest the potential of methylation at these sites as a biomarker of disease progression, as well as a target for epigenetic modifying drugs.

HDAC inhibitors are currently of strong clinical interest in UM due to their potential to reverse the phenotypic effects of loss of BAP1 expression, specifically inducing growth arrest and differentiation in UM [25]. Additionally, recent work by Kuznetsov et al. noted a relationship between BAP1 loss and HDAC4, where BAP1 mutant UM cells showed a change in HDAC4 expression pattern from cytoplasmic to nuclear. It was hypothesized that this change was at least partially responsible for restricting the function of HDAC4 in these cells [51]. This furthers the idea that HDAC4 specifically might be an important histone deacetylase in UM progression. In our analysis, 49 probes associated with HDAC were found to be hypomethylated in the high-risk group, including probes with a log fold change of up to 4.08 between the two risk groups (sample of differentially methylated CpGs, Figure 6C). Of note, hypomethylation of several sites associated with HDAC4 was also seen. While current studies involving use of valproic acid, an HDAC inhibitor, as adjuvant therapy in UM are underway (NCT02068586), inhibitors which selectively target HDAC4 should also be investigated to determine whether all HDAC inhibitors are equally promising options as possible adjuvant treatments in UM.

Given the relatively short patient follow up, this study was not able to show a pattern of methylation that predicts late metastasis. Further studies with longer follow up times should be performed to determine whether late metastasis shows a separate methylation pattern.

5. Conclusions

To conclude, patterns of DNA methylation in this cohort were significantly related to prognosis. Our analysis reveals that hierarchical clustering of methylation values separates 80 UM cases into two major groups that differ very significantly in terms of the development of metastasis and overall survival. Additionally, there are very significant differences between the two groups in methylation of specific genes known to be

important in UM and in cancer progression in general, including PTEN, NFIA, IL12RB2, RASSF1, and HDAC4. These observed changes point to a role of methylation analysis, both for wide scale changes and for individual loci, as being potentially useful for prognostication of patients as well as offering insight into the potentially reversible changes that are driving UM towards a metastatic phenotype. Furthermore, the observation that specific loci were able to effectively separate the two groups in the same manner as the hierarchical clustering based on 10,000 loci suggests that changes in methylation may be observable in samples that contain very small amounts of DNA. Given the high rate of metastasis and its associated poor prognosis, such data provide important insight into novel and clinically useful biomarkers and therapeutic targets in UM.

Supplementary Materials: The following are available online at www.mdpi.com/2075-1729/10/10/248/s1, Table S1: Differentially methylated genes in the Pathways in Cancer KEGG pathway with a log FC ≥ 1.5, Table S2: Differentially methylated genes in the Tumour Suppressors KEGG pathway with a log FC ≥ 1.5, Table S3: Differentially methylated genes in the mTOR signaling KEGG pathway with a log FC ≥ 1.5, Table S4: Differentially methylated genes in the PI3K/Akt KEGG pathway with a log FC ≥ 1.5, Table S5: Differentially methylated genes in the Ras signaling KEGG pathway with a log FC ≥ 1.5, Table S6: Differentially methylated genes in the Negative Regulation of ERK1/2 Signaling KEGG pathway with a log FC ≥ 1.5, Table S7: Log Fold Change for all differentially methylated probes associated with the PTEN gene, Table S8: Log Fold Change for all differentially methylated probes associated with the IL12RB2 gene, Table S9: Log Fold Change for all differentially methylated probes associated with the NFIA gene, Table S10: Log Fold Change for all differentially methylated probes associated with the RASSF1 gene, Table S11: Log Fold Change for all differentially methylated probes associated with the ZNF358 gene, Table S12: Log Fold Change for all differentially methylated probes associated with the ZNF532 gene, Table S13: Log Fold Change for all differentially methylated probes associated with the HDAC4 gene

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Chapter 3.

Linker to Article #2

In Chapter 2, we presented compelling evidence for a correlation between methylation patterns and patient outcome in UM. Our next goal was twofold: (1) to see whether these changes could be potentially monitored in patients through liquid biopsy, and (2) to determine whether specific changes in methylation could be reversed using DNA demethylating agents. To address these questions, we sought to determine whether changes in genomic DNA methylation could be successfully tracked using cell free DNA in an *in-vitro* system, and whether the methylation changes occurred in specific pathways that may indicate potential therapeutic targets. Changes in methylation were observed by treating the cells with different doses of Azacytidine (an epigenetic modifier), and then analyzing genomic and cell free DNA using methylation arrays. To represent different patient populations, the *in-vitro* work was performed in a panel of four human UM cells: a primary UM cell line that expresses the BAP1 protein (MP41), a primary cell line with loss of BAP1 protein expression (MP46), and a set of paired primary and metastatic UM cell lines (Mel270 and OMM2.5, respectively). Methylation profiling was using Illumina MethylationEPIC arrays (Genome Quebec), and changes in our in vitro model were compared to the data generated in Chapter 2. Ultimately we aimed to determine whether the DNA methylation patterns examined in patients were recapitulated in cell lines, and whether these changes could be reverted to "lower risk" patterns through treatment with DNMT inhibitors. Finally, the goal of this work was to see whether DNMT inhibitor treatment would elucidate further avenues for UM treatment with drugs targeting the specific altered molecular pathways.

Azacytidine on Methylation Patterns of Genomic and Cell-Free DNA in Uveal Melanoma

Sarah Tadhg Ferrier[1,2], Marvin Li[1], and Julia Valdemarin Burnier[1,2,3]

[1] Cancer Research Program, Research Institute of the McGill University Health Centre, Montreal, QC H4A 3J1, Canada

[2] Experimental Pathology Unit, Department of Pathology, McGill University, Montreal, QC H3A 0G4, Canada

[3] Department of Oncology, McGill University, Montreal, QC H3A 0G4, Canada

Abstract:

Background: Uveal melanoma (UM) is the most common primary intraocular tumour in adults, but despite effective local treatment, patient outcomes have not improved in many decades. Approximately 50% of patients develop metastasis, which is often associated with loss of BAP1 expression in the tumour. Epigenetic changes are an important factor in cancer progression, potentially leading to major alterations in gene expression. We have previously demonstrated differential methylation profiles associated with metastatic risk in UM patients, and separately shown that cell-free DNA (cfDNA) from a blood-based liquid biopsy can be tracked in patients. Here, we aimed to determine whether the specific changes in methylation profiles could be altered using a DNA methyltransferase (DNMT) inhibitor in UM cell lines, and whether these could be tracked through cfDNA.

Methods: To determine the effect of an epigenetic modifying drug, four primary and metastatic UM cell lines (primary: MP41, MP46; matched primary and metastatic: Mel270, and OMM2.5) were treated with different concentrations of azacytidine (DNMT inhibitor) and analysed for cell proliferation, colony formation, and BAP1 protein expression. Genomic and cfDNA methylation from untreated and treated cells was profiled by Illumina MethylationEPIC arrays, and results of the arrays were analysed using hierarchical clustering, principal component analysis, and bumphunter to uncover alterations in methylation patterns across the genome.

Results: In all UM cell lines, azacytidine treatment resulted in a dose-dependent reduction in proliferation at higher concentrations. Low concentrations of azacytidine

significantly affected colony formation in all cell lines except for the metastatic OMM2.5, which only showed significant effects at higher doses. Methylation profiling revealed differences in methylation patterns between cell lines depending on BAP1 expression status, while matched primary and metastatic cell lines from the same patient showed very similar patterns. Azacytidine treatment led to significant changes in methylation at higher concentrations, and alterations were seen in pathways known to be important in UM progression, such as PI3K/Akt signaling and MAPK signaling, as well as in pathways involved in cancer progression more generally, such as in the regulation of stemlike potential and pathways regulating cell motility and invasion. Finally, cfDNA maintained the same pattern of methylation seen in genomic DNA. Conclusion: Azacytidine treatment resulted in anti-proliferative and anti-clonogenic effects on primary and metastatic UM cells. Moreover, methylation profiling revealed changes affecting pathways important in UM cell proliferation and tumorigenesis. Importantly, the methylation patterns in genomic DNA were maintained in cfDNA, suggesting that liquid biopsy-based methylated ctDNA monitoring could reflect the changes seen in the tumour cell of origin. The effects in cell lines combined with the specific changes in methylation of molecular pathways points to the potential for DNMT inhibitors to be used in combination with other treatments targeting specific pathways in UM.

1. Introduction

Uveal Melanoma (UM) is the most common primary intraocular tumour in adults, and the most common non-cutaneous melanoma, with a reported incidence of 5.1 cases per million per year in the US and 3.75 cases per million in Canada [1, 2]. While effective local treatments – including radiation, resection, and enucleation – exist for the primary tumour, there is currently no effective treatment for UM metastasis, which occurs in almost 50% of patients and is fatal [2]. UM metastasis occurs most frequently to the liver, with ~90% of patients having liver metastases at the time of death from metastatic disease, but can also occur in the lungs and bones [3]. While many attempts have been made to manage metastatic UM, including various chemotherapeutic agents, liver-directed therapies, and MEK inhibitors amongst others, there is currently no treatment for UM that can extend life significantly once metastasis has occurred [3, 4].

UM is characterized by initiating mutations in the *GNAQ* or *GNA11* genes in the majority of patients, with alterations in the Ras-Raf-MEK-ERK-MAPK cascade leading to proliferation of tumour cells [3, 5]. Additionally, there are also mutations associated with prognosis, such as in *EIF1AX*, *SF3B1*, and *BAP1*, which are linked to low, moderate and high risk of metastasis, respectively [6]. As UM tumours are associated with a relatively low mutational burden – especially when compared to their cutaneous counterpart – there are likely other genomic events that are driving tumour progression and metastasis in UM [7]. Indeed, alterations in methylation have been demonstrated to be associated with outcome in UM patients, and we have previously shown that specific pathways are altered through methylation in UM and may be useful for developing targeted therapies [8].

Methylation – the addition of a methyl group to cytosine residues that precede guanosines in DNA sequences – is an epigenetic modification that has a profound impact on gene expression and is known to be significantly altered in many cancers [9]. Cancer is often marked by a pattern of global hypomethylation leading to genomic instability paired with specific hypermethylation of certain genes such as tumour suppressors [10]. Hypermethylation at CpG islands within the promoter regions of genes, specifically at transcription start sites, is highly associated with transcriptional silencing [9, 11]. In tumours, this can lead to decreased expression of tumour suppressor genes along with other genes, leading to unregulated cellular proliferation and potentially triggering genomic events that drive tumour growth and dissemination [12]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which are responsible for regulating the methylation levels on the DNA sequence. This action may be altered through the use of drugs known as DNMT inhibitors, which can act on the different classes of DNMTs to reduce DNA methylation. Included in this list is azacytidine, a nucleoside analog of cytidine that acts as a general DNMT inhibitor, blocking all classes of DNMTs.

Previous work has shown that there are large scale alterations in methylation in UM associated with outcome [6]. Indeed, our recent study revealed patterns that are associated with prognosis and specific genomic pathways altered in UM patients through methylation [6, 8]. However, it remains unclear how treatment with DNMT inhibitors may impact UM cells, as different epigenetic and genetic profiles in these cells may lead to different treatment responses. Specifically, loss of BAP1 protein

expression has also been associated with large changes in methylation profile in UM, and work by Field et. al has suggested that BAP1 may be epigenetically regulated in UM [13].

While DNMT inhibitors in isolation are not an effective treatment option for UM [14], they may have the potential to alter methylation profiles in a manner that makes these tumours more amenable to pre-existing therapies. Understanding the molecular mechanisms underlying the epigenetic changes associated with DNMT inhibitor treatment may allow us to identify specific combinations of drugs that could be used in conjunction with DNMT inhibition. Indeed, decitabine – a DNMT inhibitor – has recently been shown to limit escape from MEK inhibition in UM cell lines, pointing to the possibility of DNMT inhibition in combination with MEK inhibition as adjuvant treatment in UM [15].

Cell-free DNA (cfDNA) is a form of circulating DNA that has a very short half-life in circulation [16] . This DNA can originate as a result of cell death or through active secretion from normal cells or from tumour cells, where it is referred to as circulating tumour DNA (ctDNA) [17]. ctDNA can be isolated from blood and other fluids (liquid biopsy), offering an important alternative to traditional tissue biopsy. In UM, serial biopsies are not routinely performed and disease progression is often detected only after macroscopic signs of metastatic disease; as such, non-invasive liquid biopsy-based sampling could provide very powerful way to detect and monitor disease. ctDNA has been detected in multiple tumour types to monitor disease progression and treatment response even in cases with relatively low disease burden (reviewed in Nature Reviews Clinical Oncology and Molecular Diagnosis and Therapy) [18, 19], and indeed our group has shown that tracking ctDNA based on initiating mutations in *GNAQ* and *GNA11* may be a biomarker in UM [20]. However, these initiating mutations are not related to patient outcomes [21], and the presence of metastasis-promoting mutations, such as BAP1 mutations are difficult to target as they occur at multiple regions throughout the gene and even at distant regulatory sites [6]. Due to these difficulties in tracking specific markers, and given the potential role of epigenetic changes in tumour growth and dissemination, we sought to examine changes in methylation that may be maintained in cell free DNA and could eventually be tracked in liquid biopsy.

Identifying the specific changes in methylation that are associated with increased risk in UM, as well as gaining insight into the molecular pathways altered with DNMT

inhibitor treatment, has the potential to guide neoadjuvant treatment in this difficult disease.

2. Methods

2.1 Cell lines

Four human UM cell lines were used: MP41 (primary), MP46 (primary), Mel270 (primary), and OMM 2.5 (metastatic matched to Mel270). MP41 and MP46 were purchased from American Type Culture Collection (ATCC). Mel270 and OMM2.5 were kindly provided by Dr. Vanessa Morales (University of Tennessee). All cells were grown in RPMI-1640 media (Gibco, cat. 11875093) supplemented with 10% fetal bovine serum, 2mM GlutaMAX, 1mM sodium pyruvate, 10mM HEPES, 0.1% 10 U/mL penicillin and 10 μg/mL streptomycin, and 10μg/mL insulin. Cell media was changed every 48-72 hours and cells were split using 0.05% trypsin-EDTA when they reached 80-90% confluency.

2.2 Azacytidine treatment

Azacytidine was fully dissolved in warmed dimethyl sulfoxide (DMSO) to a concentration of 40mM, then mixed with complete media to obtain final concentrations between 0.5-80 μM by serial dilution. DMSO concentrations in the cell culture medium were equalized to 0.05% by adding additional DMSO depending on the drug concentration. Cells were treated in 96 well plates, with triplicates for each concentration for each cell line (0.5μM, 1μM, 2.5μM, 5μM, 10μM, 20μM, 40μM, 80μM). Treatment was conducted for 72 hours before cellular assays and/or DNA extraction.

2.3 Cell viability and proliferation

Cell viability after treatment was examined using the CCK8 assay – with relative metabolic activity being used as a stand-in for cell proliferation – as well as a confluence mask from the Incucyte live cell imaging system over the course of a 72-hour treatment. The effect of azacytidine was assessed based on relative cell confluency after one doubling time. Cells were plated in order to have equivalent cell densities with a final confluency of ~90% at the time of DNA extraction, calculated based on the respective doubling rates of the cell lines in culture.

2.4 Cell preparation and DNA extraction

After 72 hours of Azacytidine treatment, supernatant from the cells was transferred to tubes and centrifuged at 2000*g* for 15 min to separate any potential cells from the cell

free DNA. Cells were washed with PBS and then treated with 0.05% trypsin for 3-5 min, until they detached. Trypsin was neutralized with complete media containing 10% FBS. Cells were spun at 230*g* for 5 min, and cells were resuspended in PBS for DNA extraction.

Genomic DNA was extracted using the Qiagen DNA mini kit, according to the standard protocol. DNA was eluted using 30μL buffer AVE followed by a second elution of 20μL. For each elution step, buffer AVE was added to the samples and then left for 10 minutes before spinning for 2 minutes at 20000*g*.

Cell free DNA was extracted with the Qiagen Circulating Nucleic Acid Kit using a modified version of the protocol for extraction of DNA from 5mL serum or plasma, as routinely performed in our lab [20], with alterations to allow for extraction of larger quantities of cell free DNA from supernatant. Briefly, each sample was treated with 100 μL proteinase K per mL of supernatant (~2mL per sample), and then mixed with 16mL Buffer ACL with carrier RNA before being pulse vortexed for 30 seconds. Samples were incubated for 30 min at 60°C. Each sample was removed from heat and divided into 4 tubes. 9mL buffer ACB was added to each sample tube, and then incubated on ice for 5 min. Samples were pulled through QIAamp Mini columns using a vacuum connector with a 20 mL tube extender. The 4 tubes for each sample were run sequentially through the same column in order to collect the total DNA from each sample onto a single membrane. DNA was extracted using a two-step elution, first with 20 μL buffer AVE followed by 10 μL buffer AVE. For each elution step, elution buffer was left in the tubes for 20 minutes and then spun for 2 minutes at 20000*g*.

Genomic and Cell free DNA quantification was performed using the Qbit fluorometer. DNA samples were briefly vortexed and centrifuged before quantification using 1uL of DNA.

2.5 Methylation arrays for genomic and cell free DNA:

Genomic and cell free DNA were analysed using the Illumina MethylationEPIC array. For this array, DNA was quantified using PicoGreen (Quant-iTTM PicoGreen® dsDNA Products, Invitrogen, P-7589) and read on the SpectraMAX GeminiXS Spectrophotometer. From this, bisulfite conversion was performed with 500ng of DNA using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, D5007). Cells and treatments were randomized in the array positioning to reduce the effects on intra-array variability on results. The Illumina Methylation 850K kit was used as described by the

manufacturer's protocol, except that 8µl of bisulfate converted material was utilized to initiate the amplification step. An Illumina Hybridization oven was used for incubating amplified DNA (37°C) and for BeadChips hybridization (48°C). A Hybex incubator was used for the fragmentation (37°C) and for the denaturation (95°C) steps. The X-stain step was carried out with a Tecan Freedom evo robot with a Te-Flow module. Arrays were scanned with an Illumina iScan Reader. Arrays were analysed using the Minfi package.

2.6 Cell staining for BAP1 expression:

Azacytidine treated cells were plated on 8 well glass slides at a concentration of 15,000 cells/well. 24 hours after plating, concentrations of 0µM, 1µM, 5µM, and 20µM were administered for 72 hours for MP41, MP46, Mel270, and OMM2.5 cells. After the drug treatment was finished, cells were fixed in 4% paraformaldehyde for 30 minutes, and then immunocytochemistry was performed using the Vector Laboratories ImmPRESS HRP staining kit with the anti-BAP1 antibody (C-4) from SCBT (cat. sc-28383) at concentrations of 1:200 and 1:2000.

2.7 Colony formation assay:

Cells were plated in triplicates in 6 well plates with 1000 cells/well (2000 cells/well for MP46) with and without azacytidine (1µM, 5µM, and 20µM) and grown for ~6 doubling cycles (9 days for MP41, Mel270, and OMM2.5, and 27.5 days for MP46). Media was added as needed during the experiment, in equal amounts to both the azacytidine treated and untreated wells. Cells were stained by crystal violet and number of colonies was counted manually in all plates, with a colony being defined as a group of 50+ cells. For MP46, colonies were counted as groups of 30+ cells.

2.8 Radiosensitivity assay:

UM cells (MP41, MP46, Mel270, OMM2.5) were plated in 24 well plates and treated with azacytidine (0, 1, and 2µM) for 72 hours before being exposed to 20Gy of radiation (MultiRad225, 0.5mm Al filter, 225.0 kV(Max), 13.0 mA, exposure time 3:57 min). 96 hours after exposure to irradiation, relative cell viability was measured using the CCK8 assay (100uL/well), with absorbance at 450nm being measured after 3 hours of incubation.

2.9 Sample and probe quality control for Illumina MethylationEPIC arrays

The Minfi program [22] with an additional function to adapt the package to the Illumina MethylationEPIC arrays [23] was used to read the raw IDAT files and to

calculate the detection p value for every genomic position in each of the 32 samples. Positions with p values >0.01 were discarded from further analysis to remove any probes where both the methylated and unmethylated channels reported background signal level, as determined by the negative control positions in the array. The mean detection p values across all samples were also calculated in order to ensure that there were no poor quality samples. Probes that failed in one or more samples were removed from further analysis. Raw mean methylation values were calculated as the arithmetic mean after removing the poor quality probes. SWAN normalization was used on samples in order to decrease the effect of technical variation present in the Illumina arrays, as it reduce the technical variability between Infinium type I and II probes and allows for better detection of differential methylation [24].

Normalization was performed using Quantile, SWAN, and functional normalization (*funnorm*). These values were compared to the raw data, obtained using the *preprocessRaw* function. Normalization methods were visually compared using quality control reports generated through Minfi.

CpG sites from the MethylationEPIC arrays were aligned to the hg19 human genome annotation (IlluminaHumanMethylationEPICanno.ilm10b4.hg19). β Values, defined as $\beta = M/(M+U+100)$, where M and U are the raw methylated and unmethylated intensities from the array were used for plotting and illustrative purposes. *Mvalues*, defined as $\log(M/U)$ were used for statistical analysis, as they are preferred for these purposes due to the heteroscedasticity of β values [25].

For statistical analysis, three variables were included for the samples: type of sample (genomic vs cell-free DNA), concentration of azacytidine (0, 1, 5, and 20μM), and cell line (MP41, MP46, Mel270, and OMM2.5). Type was analysed as a binary variable, cell-line was dummy coded, and drug concentration was analysed as a continuous variable.

2.10 Analysis of differentially methylated regions

The bumphunter algorithm in the minfi package was applied to samples to find candidate differentially methylated regions, using *Mvalues*. This program is used to detect differentially methylated regions (DMRs) from genomics data while modelling batch effects by surrogate variable analysis [26]. This function assumes continuous coefficients of the variable of interest (continuous or binary) while accepting other covariates. Smoothing should therefore improve the precision of this analysis [27]. The cutoff of the algorithm is the minimum percentage difference (above or below) required

for candidate regions of the outcome of interest. The algorithm then computes the statistical quantities for the candidate regions with bootstrapping. The algorithm starts with a cutoff of 0.1 (10% difference), with increases of 0.05 until the number of candidate regions is below 30,000 [22]. The coefficients of the outcome of interest are smoothed by local regression smoothing with gaussian kernel. The number of bootstraps was set to 1000 for analysis. Candidate regions were filtered by a maximum of 5% family-wise error rate for the region.

2.11 Analysis of differentially methylated probes

Hierarchical clustering was performed using the Minfi program for the probes with the highest degree of standard deviation in methylation values across samples. The top 10000 differentially methylated probes (DMPs) were then inputted into minfi to perform hierarchical clustering.

The global methylation profiles of samples were then compared using the limma program for linear models to compare different groups within the sample. These groups were then compared using principal component analysis (PCA). PCA was performed based on the global methylation pattern to compare samples by sample type (genomic vs. cell free DNA), cell type (MP41, MP46, Mel270, and OMM2.5), and concentration of azacytidine (0, 1, 5, and 20μM)

2.12 Differential Methylation of KEGG pathways

For comparisons that showed differential methylation in limma analysis, differentially methylated CPGs were mapped to the hg19 human genome assembly, and genes with an adjusted $p<0.05$ were sorted by log fold change. The top 20000 probes with the highest log fold of differential methylation (both hypomethylation and hypermethylation, logFC: -10.23 - -2.79 and 4.0 - 9.79) were analysed by DAVID Functional Annotation. Probes that were not mapped to any gene as well as duplicate genes were removed (3780 hypomethylated genes and 2841 hypermethylated genes submitted for final DAVID analysis)

3. Results

3.1 Azacytidine treatment resulted in significant decreases in UM cell proliferation and viability

In our previous study, we demonstrated differential patterns of methylation associated with overall survival in UM patients [8]. Here, we aimed to determine whether a

DNMT inhibitor, azacytidine, affected cellular behaviour in a panel of human primary and metastatic UM cells (**Table 1**). Cells were examined by Incucyte live cell imaging, over the course of 72-120 hours to determine the effect of treatment on the rate of cellular proliferation. Additionally, a colorimetric assay in which reduction of water-soluble tetrazolium salt (WST-8) produces orange formazan, was utilized as a means to determine metabolic activity of UM cells upon treatment, acting as a stand-in for relative cell proliferation at the time of the assay. Azacytidine treatment caused dose-dependent reduction in cell proliferation (**Figure 1A-D**) and in cellular dehydrogenase activity (**Figure 1E**) in all cell lines tested at concentrations of 5μM and higher. Reduced effects of azacytidine on proliferation was noted in the MP46 cell line. While MP41, Mel 270, and OMM2.5 have a doubling time in the range of 30-36 hours, MP46 only doubles once every ~104 hours. Since azacytidine is incorporated into DNA upon cell division, the global effects on cell proliferation may only be captured after longer periods of time.

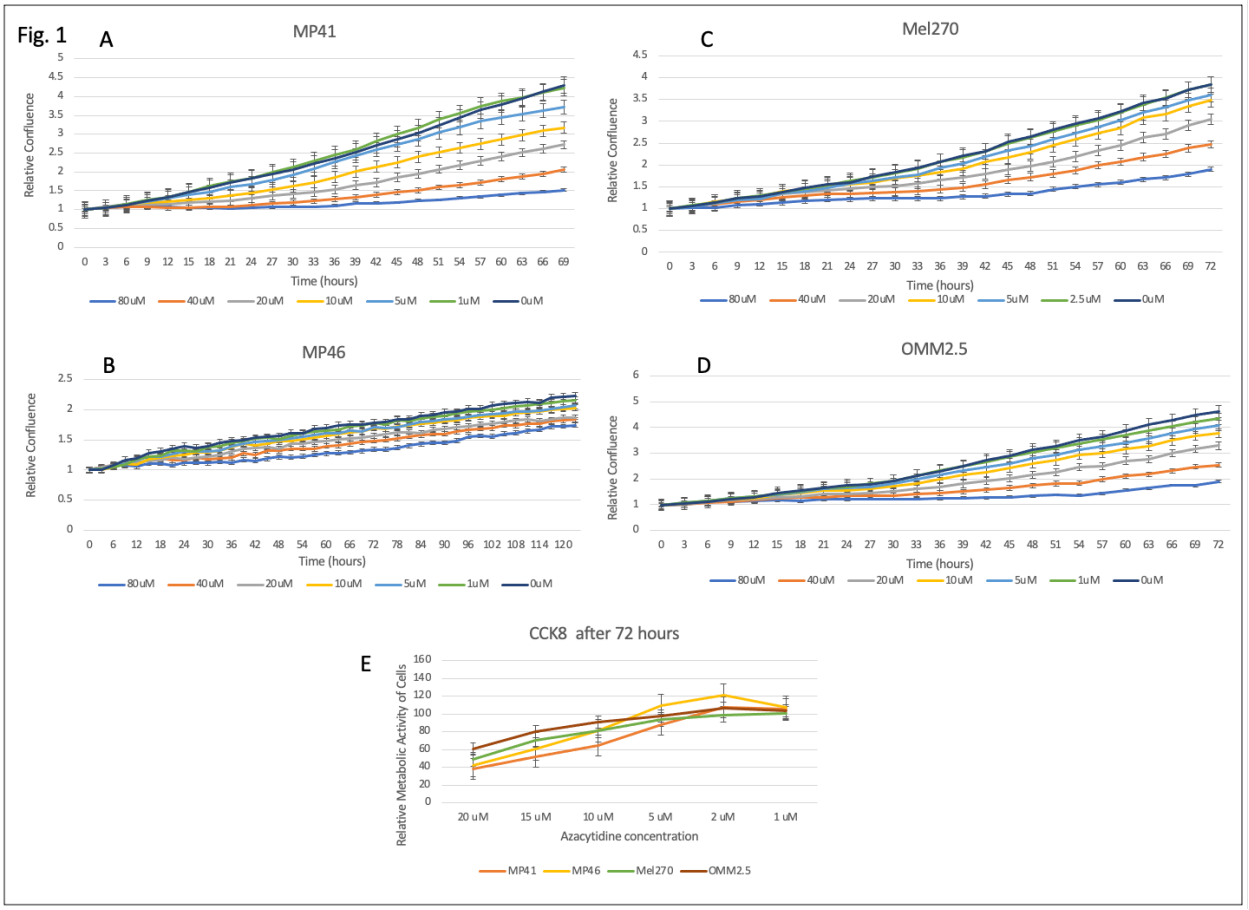


Figure 1. **Azacytidine treatment inhibits proliferation and reduces viability of UM cells in a concentration-depending manner**. **A-D** Growth curves obtained through Incucyte live cell imaging system, tracking cellular confluency over 3-5 days (depending on the doubling time of the cell line) after treatment with 0-80μM Azacytidine. **E**. Graph represents the level of viability as detected via a CCK8 colorimetric assay by relative percent metabolic activity, measured after 72 hours and calculated as absorbency of the treated samples by the absorbency of the untreated sample**s.** CCK8 data was analysed using two-way ANOVA, while Incucyte data was analysed using repeated measured ANOVA with Tuckey's post-hoc test. Differences between the treatments were additionally calculated using a t-test for the effect of the drug at one doubling time, with $p<0.05$ being considered as significant in all cases. Graphs represent assays done with 3 replicates, including standard error across wells.

	MP41	MP46	Mel270	OMM2.5
Gα11/Q alteration	GNA11 c.626 A>T	GNAQ c.626 A>T	GNAQ c.626 A>C	GNAQ c.626 A>C
Tumor type	Primary	Primary	Primary	Metastatic
Chr.3	Loss chr.3	Isodisomy 3	Disomy 3, loss of 3p24, 3q21.2-3q24	Disomy 3, loss of 3p24, 3q21.2-3q24
Chr. 6	Gain 6p, loss 6q	Gain 6p, loss 6q	Tetrasomy 6p	Tetrasomy 6p
Chr. 8	L8p; G8q;	Loss 8p, gain 8q	Disomy 8q, extra 8	Disomy 8q, extra 8
BAP1 expression	Yes	No	Yes	Yes
Chr. 3 LOH	Yes	Yes	Not Reported	Not reported
Doubling time (experimental)	~30 hours	~104 hours	~36 hours	~33 hours

Table 1. Selected genomic characteristics of MP41 and MP46 cell lines [28] and the Mel270 and OMM2.5 cell lines [29].

3.2 Effects of azacytidine on colony formation in UM cells

The ability of cancer cells to form colonies represents an important characteristic of carcinogenesis, as it measures their ability to show limitless replicative potential – a major hallmark of cancer [30, 31]. The measurement of colony formation in response to drug treatment has long been used as a method to screen anti-cancer drugs, offering valuable insight into the potential of these drugs to affect the replication of tumour cells [32]. Azacytidine treatment at both cytotoxic (5μM and 20μM) and non-cytotoxic (1μM) concentrations led to reduced clonogenic potential for all UM cell lines, with increasing reductions seen at higher concentrations (**Figure 2A**). Primary UM cell lines MP41 and Mel270 showed significantly reduced number of colonies at concentrations of 1μM, 5μM, and 20μM (MP41 1μM: $p = 0.046$, 5μM: $p = 0.012$, 20μM: $p = 0.0015$, Mel270 1μM: $p = 0.010$, 5μM: $p = 0.0036$, 20μM: $p = 0.00027$) , while metastatic OMM2.5 showed a significant reduction of colonies at concentrations of 5μM and 20μM ($p = 0.015$, $p = 0.0027$) (**Figure 2B**). MP46 showed a trend of reduction in colonies at every dose, but this effect was not significant (**Figure 2B**). This may be due to the much longer doubling time of this cell line (**Table 1**). As primary UM tumours are often treated with radiation as a first line therapy, treatments that enhance the radiosensitivity of UM cell lines may be useful as adjuvants at the time of primary tumour treatment [33, 34]. All cell lines showed reduced proliferation in response to irradiation when treated with sublethal doses of Azacytidine (1μM and 2μM), though this effect was only significant in the MP41 and MP46 cell lines (**Figure 2C**). MP46 cells formed colonies at a lower rate (27.5 days for colony formation in MP46 cells vs. 9 days for the other three cell lines). Despite this, MP46 maintained the trend of reduction of cell clonogenicity seen in other cell lines.

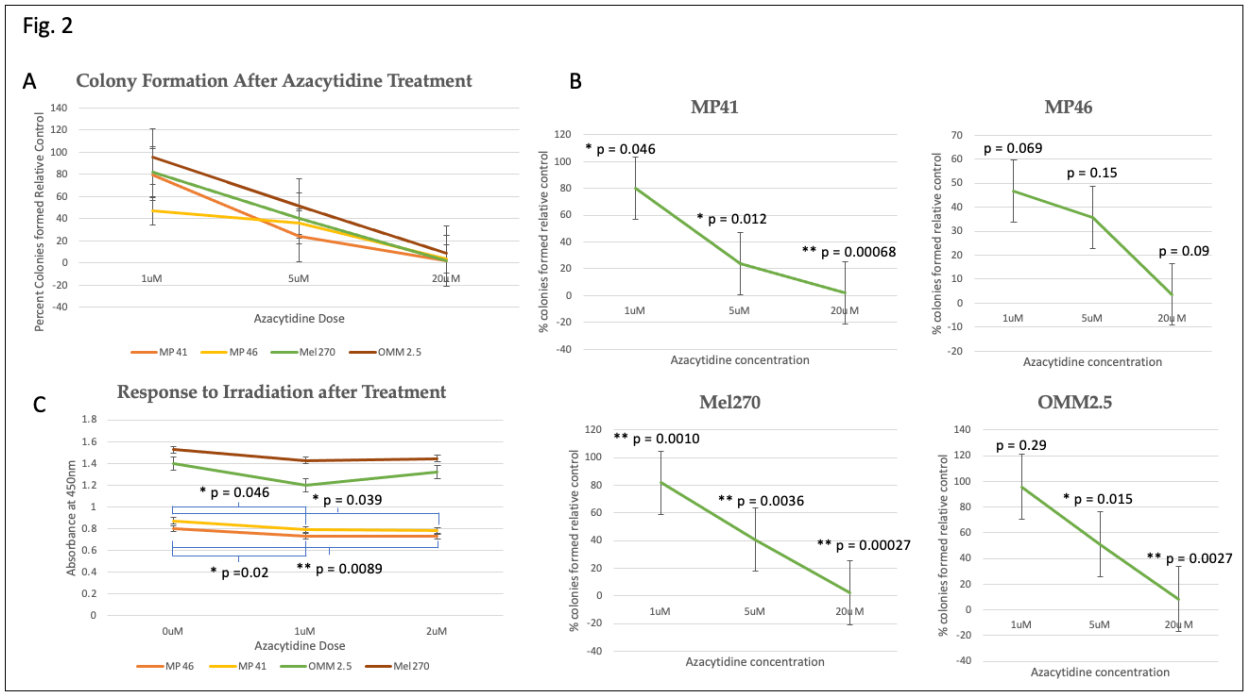


Figure 2. Azacytidine treatment leads to reduced clonogenic potential and increased radiosensitivity in UM cells. A) Comparison of colony formation across all four cell lines at doses of 1, 5, and 20 μM, calculated as percent of colonies formed relative control. p<0.01: MP41 20μM, Mel270 5μM Mel270 20μM p<0.05: MP41 5μM, 1μM, Mel270 1μM, OMM2.5 5μM, 20μM B) Response to irradiation in all cell lines measured using level of viability as detected via a CCK8 colorimetric assay. C) Colony formation in each cell line at doses of 1, 5, and 20 μM, calculated as percent colonies formed relative control. Graphs represent assays done with 5 replicates, including standard error across wells. P values were calculated using t-test, legend: * = p<0.05, ** = p<0.01

3.3 BAP1 protein expression is not reverted by the use of DNA demethylating agents

Loss of expression mutations in *BAP1* are an important determinant of metastatic risk in UM, and are highly associated with epigenomic alterations in UM [13, 35]. While *BAP1* mutations are thought to be early events, data suggests that epigenetic regulation of BAP1 may be responsible for some cases of loss of BAP1 expression, and also contribute to metastatic risk in the same manner as mutations in this gene [13]. Here, we aimed to determine whether treatment with azacytidine could restore loss of BAP1 expression in the MP46 cell line, which has shown loss of BAP1 protein expression with no

discovered BAP1 mutations (**Table 1)**. Three of our cell lines (MP41, Mel270, and OMM2.5) have been reported to express BAP1 expression by immunocytochemistry [28, 29], while MP46 has a loss of BAP1 expression [28]. Here, we tested the presence of BAP1 protein expression immunocytochemically in all cells before and after 72 hour treatment. All cell lines maintained their reported BAP1 status; MP41, Mel270, and OMM2.5 were BAP1 positive (**Figure 3A-C**) while MP46 was BAP1 negative (**Figure 3D**). Treatment with azacytidine did not affect BAP1 protein expression in any of these cell lines up to concentrations of 20μM.

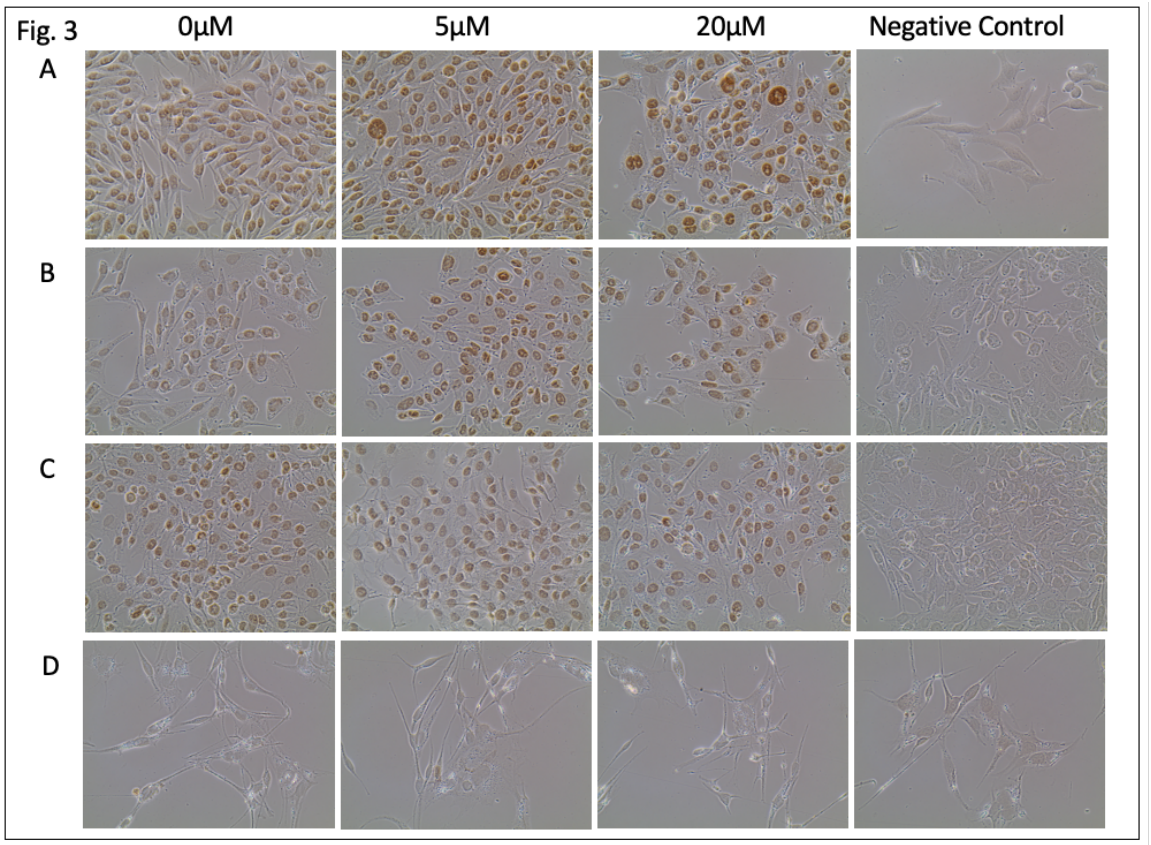


Figure 3. Azacytidine treatment did not impact BAP1 protein expression. Representative immunocytochemistry images for anti-BAP1 monoclonal antibody (1:2000) staining for MP41 for **(A)**, Mel270 **(B)**, and OMM2.5 **(C)**, and MP46 **(D)** at a dilution of 1:2000 following 72-hour Azacytidine treatments of 0, 5 and 20 μM. Images taken at 10x magnification using the (Invitrogen EVOS XL Core Imaging System). Negative control performed using the same staining procedure with normal horse serum in place of primary antibody.

3.4 Methylation profiling indicates that cell-line specific differences are the most impactful determinant of methylation profile based on unsupervised clustering

To determine the impact of azacytidine treatment on the methylation profile of UM cells, we conducted methylation profiling using the Illumina MethylationEPIC arrays on four human UM cell lines (MP41, MP46, Mel270, and OMM2.5) following treatment with four concentrations of azacytidine (0, 1, 5, and 20 μM). Moreover, as our goal was to also examine the potential use of cfDNA for monitoring methylation patterns, we extracted DNA from both the cells and cell-free conditioned media (**Figure 4**).

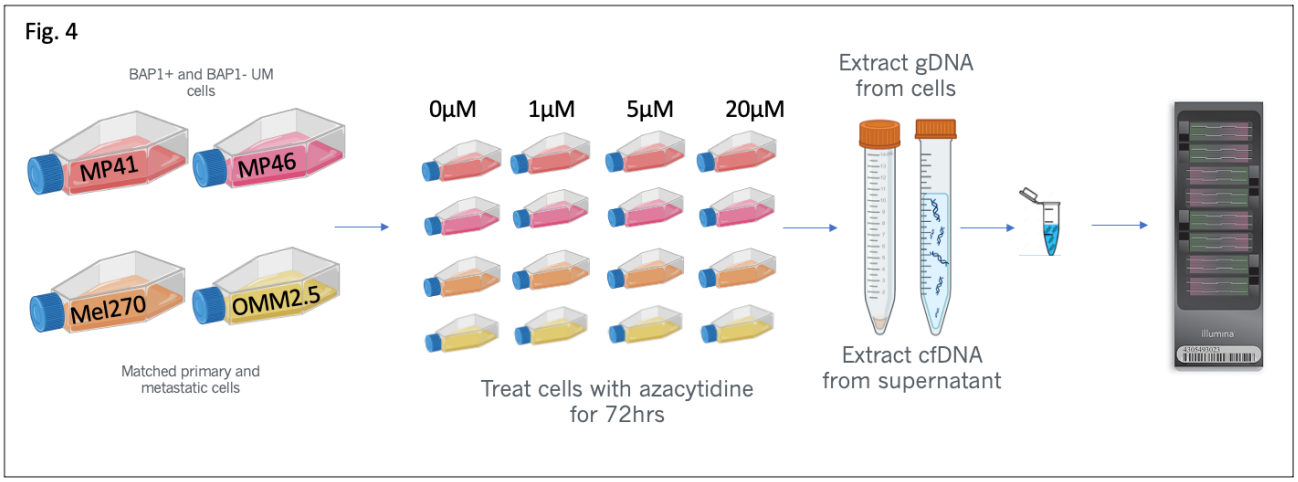


Figure 4. Schematic representing the assay design for extraction and analysis of cell free and genomic DNA from samples treated with different doses of azacytidine

Quality control for the Illumina arrays indicated that all samples were of high quality based on the control probes included in the array, and that the large majority of probes were of sufficient quality to be used in the final analysis. Based on the detection p-value, 22,949 low quality probes with $p>0.01$ were removed from further analysis, with an overall mean detection p-value below 0.003 for most samples (**Figure 5A**). As such, a total of 842,910 probes were deemed to be high quality and were included for the final analysis. Moreover, all samples (cell-free and genomic DNA for all four cell lines across all four azacytidine treatments) were found to be of good quality, with no failed reads in the quality control step, therefore leaving 32 samples for final analysis (**Figure 5B**). M-values mapped as expected to beta values (**Figure 5C**). SWAN normalization – which is performed to reduce the technical variability between Infinium type I and II probes to

allow for better detection of differential methylation [24] – maintained differences present between samples seen in the raw beta values. Because of this, M-values and SWAN normalised beta values were used for final analysis. This method was selected as a final normalization step due to unexpected changes in overall beta values being seen with the use of functional normalization.

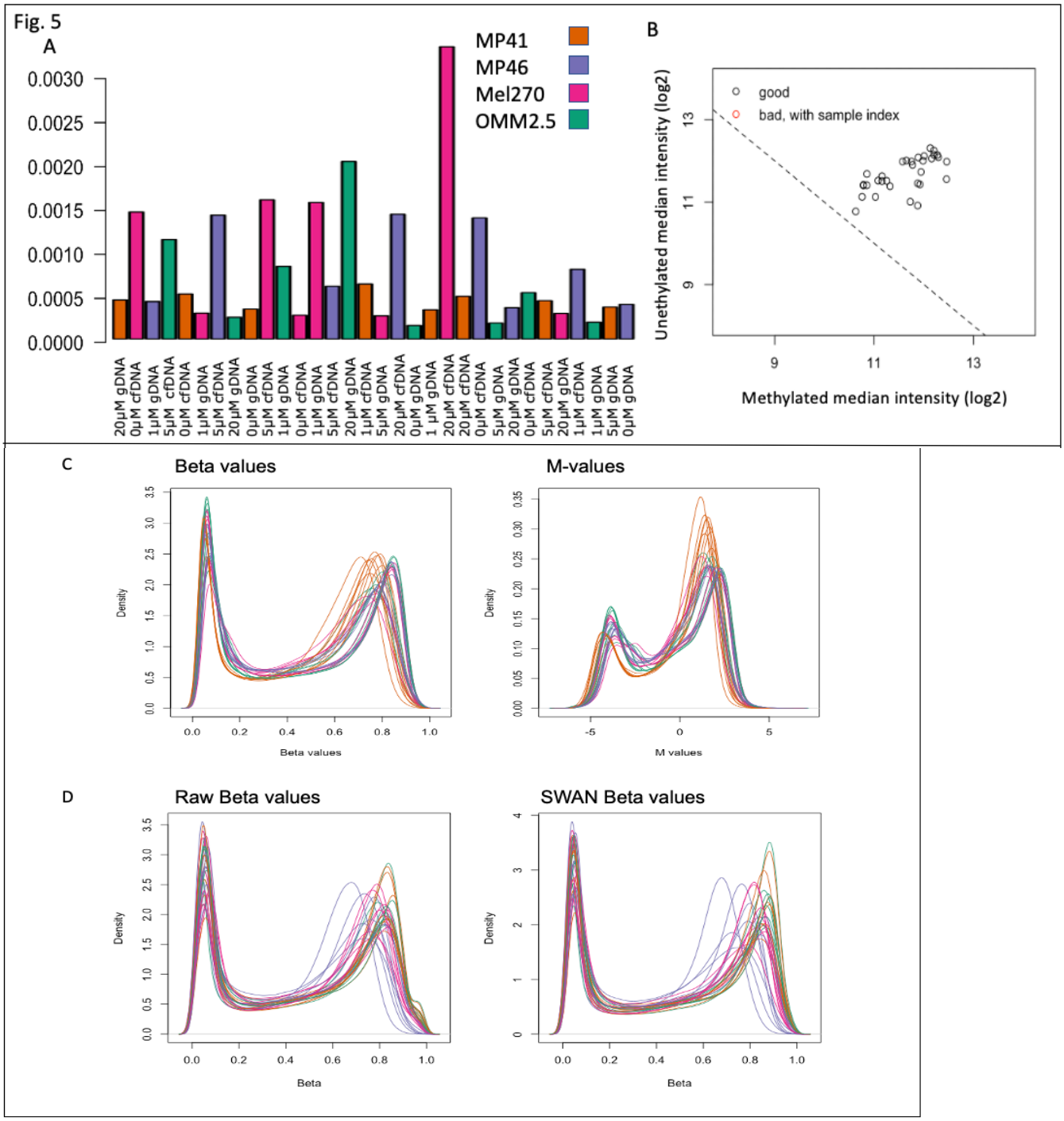
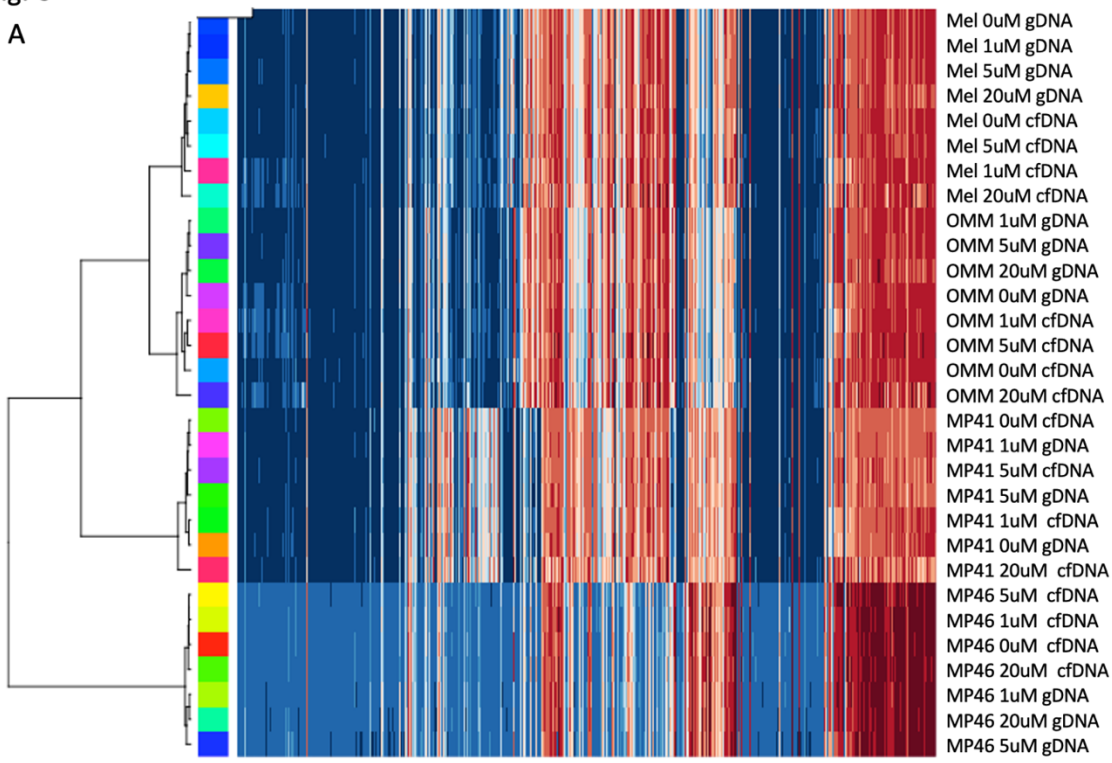


Figure 5. Methylation arrays yielded high quality samples with few low-quality probes (A) Mean detection P values shown across all samples. **(B)** Quality control plot shown for all samples. **(C)** Comparison of Beta Values to M values for all samples, coloured by cell line. **(D)** Comparison of raw beta values to SWAN normalised beta values for all samples, coloured by concentration of azacytidine.

Hierarchical clustering analysis was conducted in Minfi with the aim of identifying similarities and differences in methylation profiles across samples. Samples from the same cell line clustered together regardless of the origin of the DNA (genomic or cell-free) or the concentration of azacytidine used, as shown in a heat map (**Figure 6A**) and in a principal component analysis (**Figure 6B**). These data demonstrated that different cell types clustered separately, with different sample types and treatments maintaining the differences between these cell types (**Figure 6A**). Mel270 and OMM2.5, the matched primary and metastatic UM cell lines, showed a high degree of similarity, branching together in the hierarchical clustering (**Figure 6A**). Moreover, principal component analysis showed a higher degree of similarity between Mel270 and OMM2.5, while MP41 and MP46 clustered separately from the other cell lines (**Figure 6B)**.
In order to determine whether the methylation pattern of cells was similar to those seen in patients, the methylation patterns of untreated genomic DNA samples were compared to the patterns from methylation arrays for 80 primary human UM samples from The Cancer Genome Atlas (TCGA) study. As shown in our previous work, UM samples cluster into two distinct groups depending on methylation pattern, with these groups being highly associated with outcome in patients [8]. When compared to patient samples, UM cell lines clustered separately from both groups of patients (high vs low risk), though they showed some similarities in methylation patterns (**Figure S1**). When examined by Uniform Manifold Approximation and Projection (UMAP), the differences between patient samples and cell lines were seen to be similar in some dimensions, with cell lines clustering along with patient samples for certain dimensions (**Figure S2**)

Fig. 6

A


Mel 0uM gDNA
Mel 1uM gDNA
Mel 5uM gDNA
Mel 20uM gDNA
Mel 0uM cfDNA
Mel 5uM cfDNA
Mel 1uM cfDNA
Mel 20uM cfDNA
OMM 1uM gDNA
OMM 5uM gDNA
OMM 20uM gDNA
OMM 0uM gDNA
OMM 1uM cfDNA
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MP41 0uM cfDNA
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MP46 5uM cfDNA
MP46 1uM cfDNA
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Fig. 6

B

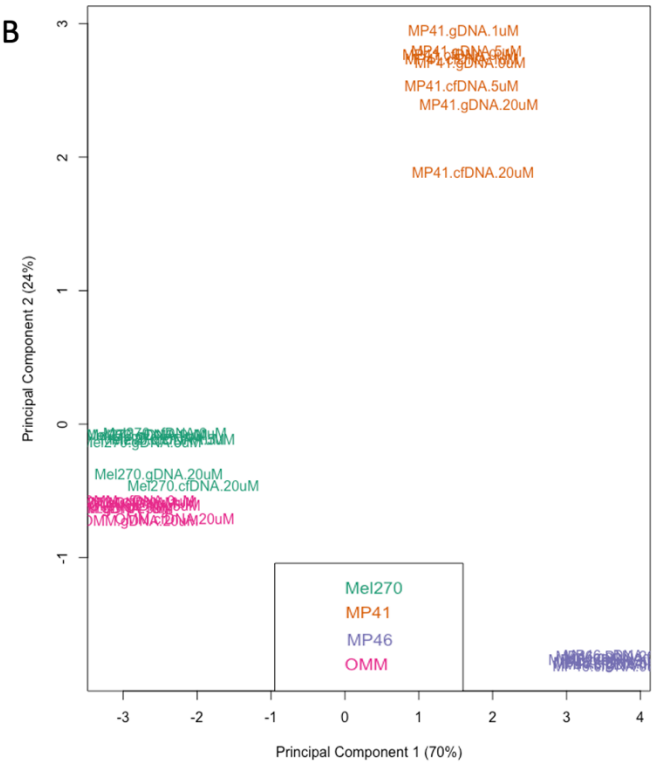
MP41.gDNA.1uM
MP41.cfDNA.5uM
MP41.gDNA.20uM
MP41.cfDNA.20uM
Mel270.gDNA.20uM
Mel270.cfDNA.20uM
Mel270
MP41
MP46
OMM
Principal Component 2 (24%)
Principal Component 1 (70%)
3
2
1
0
-1
-3
-2
-1
0
1
2
3
4

Figure 6. Hierarchical clustering revealed similarities between cell lines. (A) Heat map representing the hierarchical clustering conducted on SWAN normalised methylation array data for all samples: MP41, MP46, Mel270, and OMM2.5, including cfDNA and gDNA for 0, 1, 5, and 20μM azacytidine. **(B)** Principal component analysis for samples, coloured by cell line.

Further analysis by bumphunter showed that MP41 and MP46 had many dissimilarities in methylation patterns across the genome (2855 significantly differentially methylated regions, **Figure 7A**), while OMM2.5 and Mel270 were highly similar (192 significantly differentially methylated regions, **Figure 7B**). The differences between both these pairs of samples were seen across introns, exons, and intergenic regions for these samples (**Figure 7A and 7B**). When the most significantly differentially hypermethylated and hypomethylated genes between the MP41 and MP46 cell lines were submitted to DAVID gene ontology analysis (DAVID GO, KEGG Pathways), we found that the differentially methylated genes between the two groups were related to a number of signaling, cell survival, and cell motility regulation pathways (**Figure 7C and 7D**). The differences between MP41 and MP46 cell lines included a pattern of both hypomethylation and hypermethylation in genes related to pathways with a known importance in UM progression and cancer progression more generally.
Hypomethylation was seen in the MP41 cell line for genes related to general cancer-related pathways, focal adhesion, regulation of the actin cytoskeleton, and axon guidance amongst others (**Figure 7C**). Hypermethylation in the MP41 cell line was seen in many signaling pathways such as PI3K/Akt, RAP1, cyclic adenosine monophosphate (cAMP), and MAPK, along with changes in pathways related to neuroactive ligand-receptor interactions, cell adhesion molecules, axon guidance, and extracellular matrix (ECM) interaction, along with pathways regulating the pluripotency of stem cells, amongst others (**Figure 7D**).
Interestingly, given that the epigenetic regulation of BAP1 in UM has been suggested but not yet shown, 38 probes associated with BAP1 were found to be differentially methylated between MP41 and MP46. Additionally, our previous work demonstrated that *NFIA* and *HDAC4* were highly differentially methylated depending on patient outcomes, with differentially methylated probes for these genes being sufficient to separate patients based on their relative risk of metastasis [8]. Along with these results

in patients, this alteration was maintained in cell lines between the MP41 and MP46 samples, with 88 probes associated with *NFIA* and 241 probes associated with *HDAC4* being significantly differentially methylated in between MP41 and MP46 (adjusted $p<0.05$).

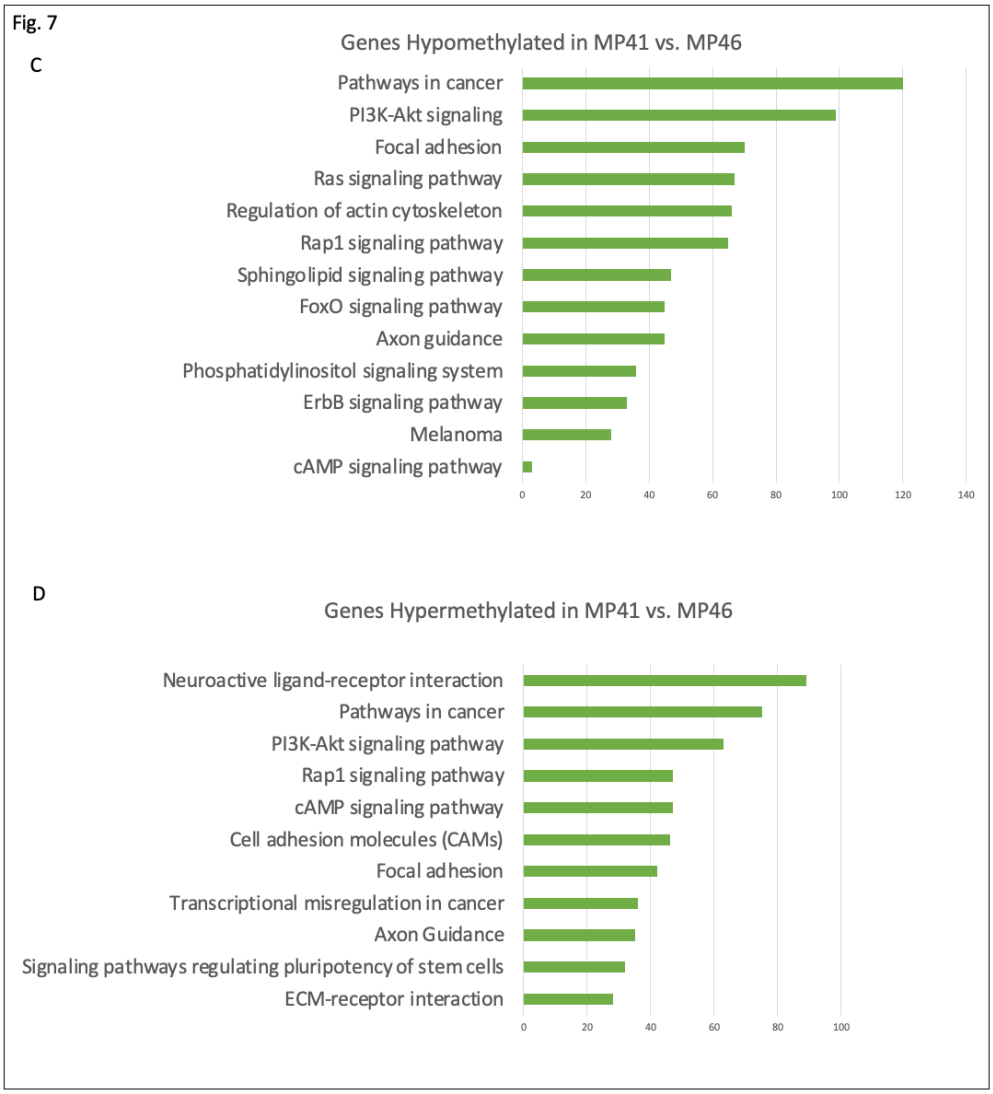

Figure 7. Large scale changes in important molecular pathways were seen between cell lines that differed in BAP1 status, while matched primary and metastatic samples maintain similar degrees of methylation across the genome (A) Plot of differentially methylated regions across chromosomes for the MP41 cell line compared to the MP46 cell line. **(B)** Plot of differentially methylated regions across chromosomes for the OMM2.5 cell line compared to the Mel270 cell line. **(C)** Selected significantly altered KEGG pathways (Bonferroni $p<0.05$) for the top 20000 hypomethylated probes between MP41 and MP46 (measured probes with an adjusted $p<0.05$ with the greatest log fold

change between samples) **(D)** Selected significantly altered KEGG pathways (Bonferroni $p<0.05$) for the top 20000 hypermethylated probes between MP41 and MP46 (measured probes with an adjusted $p<0.05$ with the greatest log fold change between samples).

3.5 Drug treatment affects methylation patterns across genomic regions

Unsurprisingly, treatment with azacytidine showed large-scale changes in terms of DMRs across cell lines, though many of the alterations were not statistically significant, with only two DMRs found overall depending on concentration (cell line included as a covariate). When the samples were separated, OMM2.5 and Mel270 showed 95 DMRs ($p<0.05$) in response to azacytidine treatment (**Figure S3 A and C**). On the other hand, when the MP41 and MP46 samples were analysed separately, there were only two significant DMRs ($p<0.05$) that remained (**Figure S3 B and D**), implying that the DMRs induced by treatment may differ between these cell lines. These alterations appear to be occurring across the genome, with many changes not reaching statistical significance. This effect is likely due to the cutoff used in bumphunter, which may conceal more minor changes in methylation induced by treatment. Given the lack of changes in the overall mean methylation (**Figure 8A**), these changes were likely to include a number of alterations in methylation across the genome including hypermethylation at certain sites and hypomethylation at other sites.

Additionally, the effect of azacytidine can be seen in the density plots of beta values (**Figure 8B**), wherein increasing concentrations of azacytidine shifted the methylation curves downwards and to the left, indicating alterations in the methylation of the cells across the genome. This effect was most prominent in MP41 cells (**Figure 8C**), which additionally showed the strongest response to azacytidine in terms of reduction in cell proliferation after treatment (**Figure 1A**). Despite these alterations, there was no clear pattern of reduced mean DNA methylation with azacytidine treatment in any of the cell lines (**Figure 8A**)**,** with cell lines showing both decreases and increases in degree of methylation depending on the locus, though MP41 cells once again showed a slight reduction in mean methylation at all concentrations (1, 5, and 20μM).

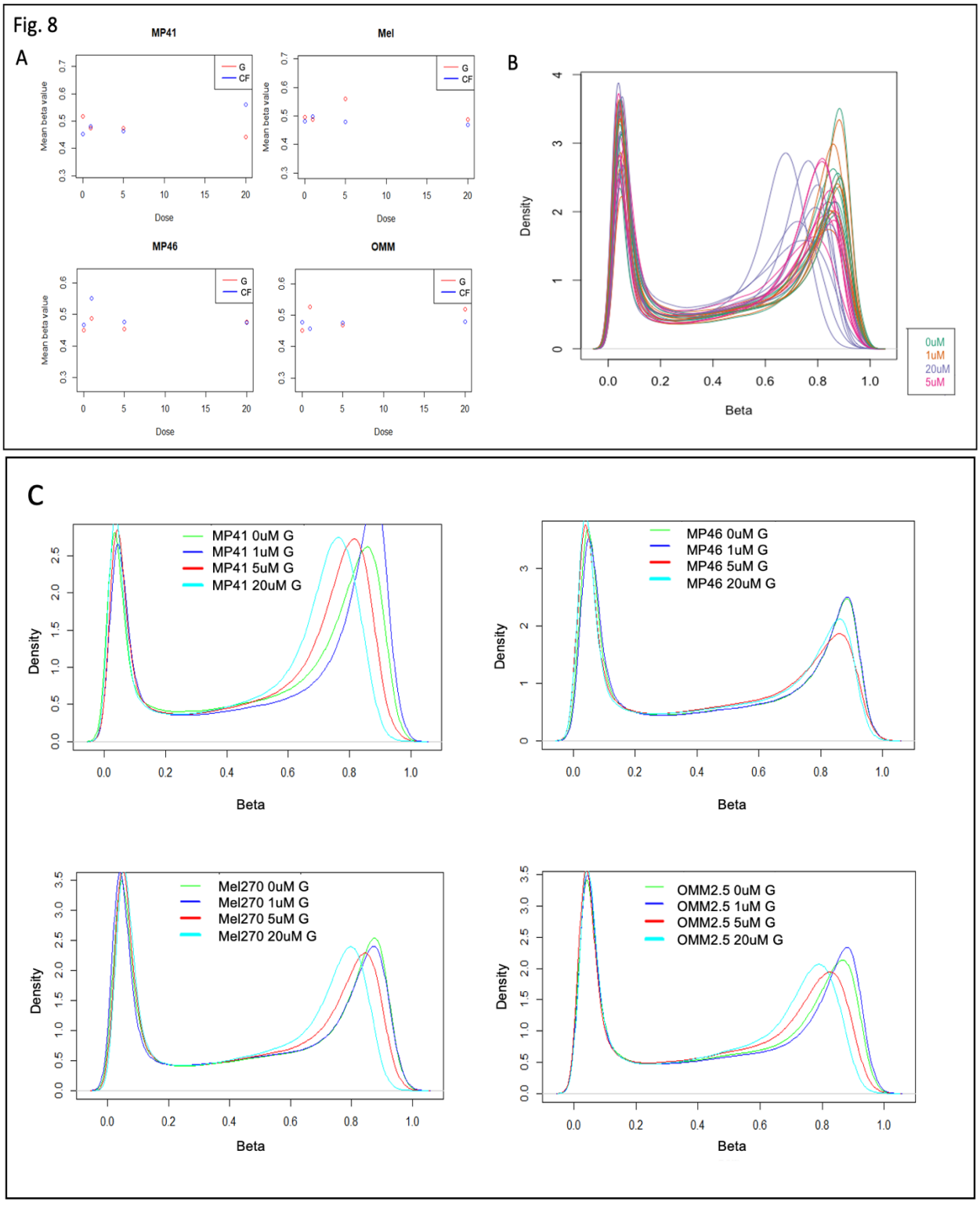

Figure 8 Treatment with azacytidine leads to alterations in methylation patterns that is generally not associated with a reduction in overall mean methylation (A) Comparison of mean methylation of samples for each cell type at the 4 concentrations of

azacytidine tested (0, 1, 5, and 20μM) for cfDNA (CF for cfDNA) and genomic DNA (G for gDNA). **(B)** Plot of methylation beta values by concentration of azacytidine. **(C)** Comparison of mean Genomic DNA methylation at each concentration of azacytidine, by cell line.

3.6 Azacytidine treatment causes significant effects in major signaling pathways

Azacytidine resulted in significant large scale genomic effects in terms of differentially methylated probes at high drug concentration (20μM). Overall, 10842 CpG sites were significantly differentially methylated between the 0μM and 20μM concentrations (adjusted $p<0.05$), with both hypermethylation and hypomethylation seen in response to treatment (log fold change: -2.52 – 2.49).

High dose azacytidine was associated with alterations in genomic pathways associated with many important features in cancer, including general pathways in cancer, signaling pathways including PI3K/Akt, RAP1, MAPK, Wingless-related integration site (Wnt), and AMP-activated protein kinase (AMPK), and pathways regulating the pluripotency of stem cells amongst others (KEGG pathways, **Figure 9D**).

Interestingly, such large scale changes were not seen at 5 and 10 μM. However, while not many significant changes were seen at low doses, there were a very large number of non-significant regions that were found in bumphunter between the untreated samples and the samples treated at 1μM. Moroever, far fewer differences were seen amongst the treated samples (5143 non-significant regions altered between 1 and 5μM and 120 non-significant regions altered between 1 and 20μM), implying that the changes that are seen at 20uM may be occurring to a lesser extent at the lower doses of azacytidine (**Figure 9 A-C**). This is reinforced by the fact that altering the cutoff for candidate regions led to an increase in the number of significant DMRs, showing 49 DMRs ($p<0.05$) occurring in cell lines with azacytidine treatment.

In our previous study revealing a strong correlation between methylation patterns and patient survival, we showed that methylation at specific genes was sufficient to separate patients into high and low risk group [8]. Here, we wanted to determine if specific genes were highlighted in our cell culture model. Interestingly, treatment with azacytidine led to alterations in methylation at specific genes including *HDAC4* (39 CpG sites with adjusted $p<0.05$), which was also identified in our previous study.

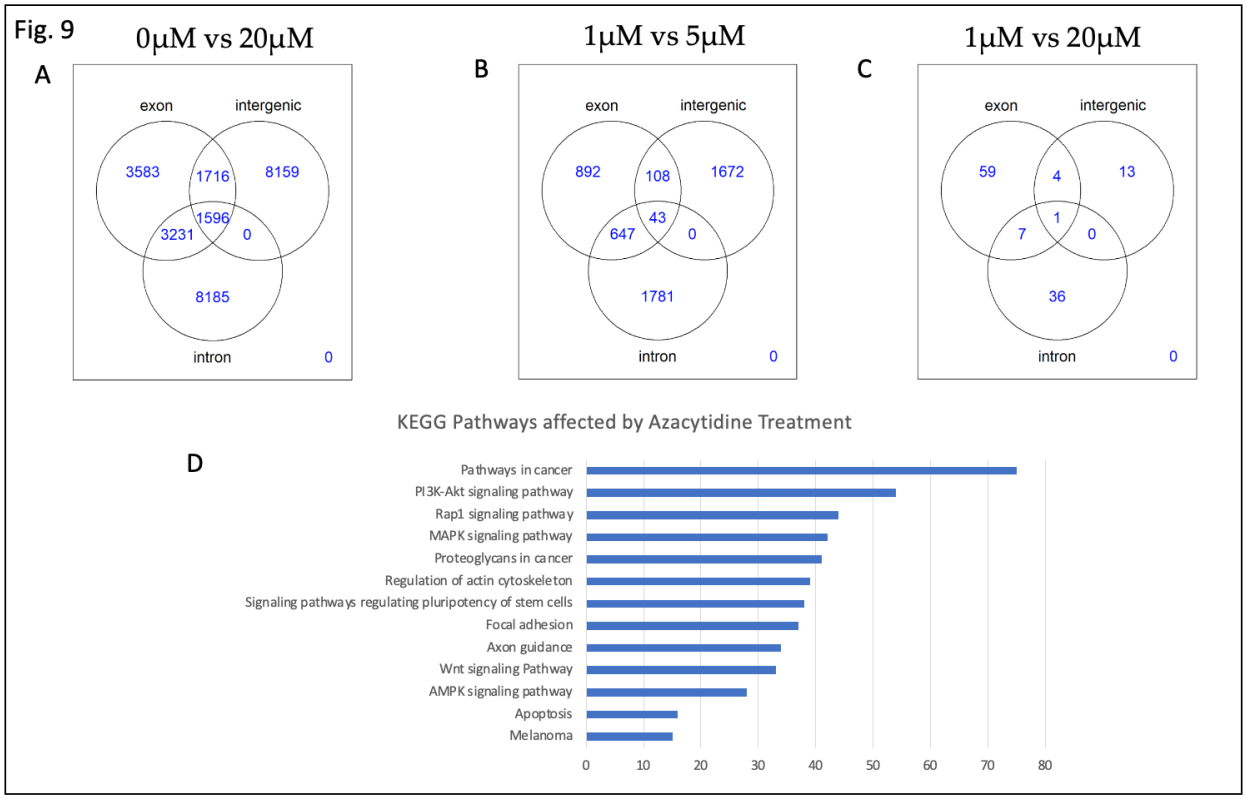

Figure 9. Multiple genomic regions and important molecular pathways are affected by azacytidine treatment. Number of regions found to be differentially methylated depending on type of genomic region (includes non-significant regions) for **(A)** 0 vs 20μM azacytidine, **(B)** 1 vs 5μM azacytidine, and **(C)** 1 vs 20μM azacytidine. **(D)** Selected KEGG pathways found to be significantly differentially methylated between all 0 and 20μM samples (Bonferroni $p<0.05$).

3.7 Genomic and cell free DNA maintain similar patterns of methylation

Cell free DNA is a promising analyte within liquid biopsy that has the potential to provide real time information of cancer cells. Indeed, we have previously demonstrated that blood-based ctDNA detected through UM-driver mutations can be used to monitor disease *in vitro*, in an animal model and in patient samples [20]. Here, our goal was to determine whether cfDNA maintained methylations events of the tumour, and whether it could potentially be a liquid biopsy-based method to detect tumour epigenetic changes. We analysed methylation data from cell free DNA extracted from the conditioned media compared to genomic DNA of UM cells, across cell lines and treatment concentrations. Our data demonstrated very similar methylation profiles

between gDNA and cfDNA from the same cell lines (**Figure 6B**). Only 9 significantly differentially methylated regions across the genome were found between matched genomic and cell free DNA samples across any of the cell lines (MP41, MP46, Mel270, OMM2.5) or concentrations (0, 1, 5, and 20 μM). **(Figure S4)** (considered as regions that are significantly differentially methylated at $p<0.05$, with cell line and drug concentration as covariates)

4. Discussion

Methylation changes have been shown to be associated with outcome in UM [6]. Indeed, our previous study showed that methylation of specific genes and in specific signaling pathways is altered depending on outcome in UM patients [8]. In this study, we aimed to assess the direct effects of DNA demethylating agents – specifically azacytidine – on the functionality and methylation profile of a panel of primary and metastatic human UM cells. Moreover, we sought to determine whether cfDNA reflected the methylation profile of gDNA with and without drug treatment. Overall, this work aimed to determine whether certain genomic pathways involved in UM progression may be specifically altered with azacytidine treatment, which might allow for the potential of combining DNMT inhibitors with medications that affect these pathways, amplifying their overall effects.

Epigenetic modifiers have been investigated in the context of UM with DNMT inhibitors showing moderate anti-tumorigenic effects on their own in primary UM cell lines [36], and synergistic effects when used in combination with other treatments including IFN- γ and the MEK inhibitor trametinib [15, 37]. Additionally, DNMT inhibitors have been shown to be safe to administer to UM patients with unresectable liver metastases [38]. Here, we showed significant reduction in viability and proliferation of all primary and metastatic UM cells following treatment with azacytdine, as well as a reduction in clonogenic potential and increased response to radiotherapy at sublethal doses. This is in line with previous studies showing that azacytidine exerts anti-tumour effects both through direct cytotoxicity at higher doses as well as through modulation of methylation leading to alterations in gene expression which affect the tumorigenic potential of cells [39].

While the response to azacytidine treatment was variable across UM cell lines, reduction in viability was seen in all cell lines studied, indicating that this drug is

capable of affecting a range of UM subtypes, including metastatic UM cells. Despite significant effects on proliferation only being seen at concentrations 5μM and higher in all cell lines, effects on colony formation and radiosensitivity at 1μM suggest anti-tumour effects at even sublethal doses of the drug. This implies that, while the higher doses of azacytidine used may be potentially affecting cells through the direct cytotoxic action of the drug, the specific effects of azacytidine on DNA methylation in UM cells can cause anti-tumour effects. Importantly, the metastatic OMM2.5 cell line was the only cell line that did not show significant reductions in colony formation at 1μM azacytidine, but did show effects at higher concentrations, suggesting that these cells are also sensitive to treatment but may require higher doses.

Loss of function in the *BAP1* gene is a major factor leading to metastasis in UM tumours [6, 35]. While mutations have been identified, they occur across the *BAP1* gene, making their detection challenging. Moreover, previous work by Field et al. implied that BAP1 may be epigenetically regulated in UM, which could potentially be a cause of loss of BAP1 expression in tumours without a *BAP1* mutation [13]. In our current study, DNA methylating agent azacytidine did not revert BAP1 expression in MP46 cells, regardless of drug concentration (1, 5, and 20μM). However, despite this lack of effect, differences in *BAP1* methylation between the MP41 and MP46 cell lines suggest that BAP1 expression may indeed be regulated by DNA methylation. In support of this, 38 DMPs associated with BAP1 expression were found to be differentially methylated between these cell lines. This could be an important feature to explore in tumours with no confirmed *BAP1* mutations but lacking BAP1 protein expression and sharing outcomes with BAP1-mutant tumours.

Overall, while the effects in methylation pattern upon azacytidine treatment only became significant are higher drug concentrations, the large degree of non-significant changes between the 0 and 1μM concentrations imply potential wide-scale effects that potentially differed between cell lines so as to make each individual change non-significant. Changes became more consistent at 20μM, showing many significant differences between the treated and untreated samples for both DMRs and DMPs. Because azacytidine acts as a general DNMT inhibitor, the lack of effect at lower doses is more likely to be related to the relatively low number of cell types studied as opposed to a lack of effect on DNA methylation. Interestingly, when we analysed the specific genes that were differentially methylated, alterations were seen in genes

involved in Wnt signaling, as well as in pathways regulating the pluripotency of stem cells. Such genes are likely to be partially
responsible for the changes in colony-forming capabilities of these cell lines after treatment, showing a reduction in the stem-like property of self-renewal [40].
In order to determine similarities in our cell line data and patient samples, we compared the methylation profiles from our *in vitro* model to those of patients reported in the TCGA [6].While we did not find that UM cell lines and UM cases clustered together, this effect was largely to be expected as cells in culture can undergo global alterations in degree of methylation. Nonetheless, we found that the BAP-negative cell lines MP46 clustered separately from the BAP-positive cells, which is in agreement with the patient data we previously reported showing that BAP1 mutations are a major factor affecting the methylation patterns of UM cells [8]. Additionally, the similar pattern of methylation between the matched primary and metastatic cell lines (Mel270 and OMM2.5) points towards a largely maintained pattern of methylation in UM metastases with regards to the primary tumour in the same patient. Because of this, it would appear that treatment based on epigenetic modifications found in the primary tumour could be useful not only as an adjuvant at the time of primary tumour removal, but also as a method for controlling the development of metastatic disease. This has previously been seen in UM, where Decitabine, another DNMT inhibitor, was shown to limit escape from MEK inhibitor treatment in UM cell lines [15]. While MEK inhibitors had previously been a promising avenue for treatment in UM, they were found to have only minor effects on improving progression-free survival in patients and no effects on overall survival [41]. However, in the present study, alterations in several central signaling cascades were noted upon DNMT inhibitor treatment, lending support for the combination targeting of key signaling cascades. For example, azacytidine treatment impacted genes involved in MAPK signaling, which is in agreement with recent data by Gonçalves et al. that alterations upstream in this signaling cascade may increase the effect of MEK inhibition on UM cells [15]. In the same vein, other signaling pathways shown to be affected by azacytidine treatment may offer insight into pathways that could be targeted along with DNMT inhibitor treatment, including PI3k/Akt, Wnt, Rap1 and MAPK signaling pathways.

PI3K/Akt has been previously implicated in UM oncogenesis, with previous findings showing activation of this pathway in at least 50% of UM [42, 43]. Our data showed hypermethylation of genes related to PI3K/Akt in MP41 cells compared to MP46 cells. As the major difference between MP46 and the other cell lines is its lack of BAP1 protein expression, it additionally points to the idea that these pathways might have different degrees of importance depending on the UM subtype.

Importantly, Wnt plays a central role in cell differentiation, polarization, and migration in normal cells, and mutations in the Wnt gene have been found across cancers [44, 45]. In UM, previous data has shown the potent effects of artesunate, a drug which inhibits Wnt/β- catenin pathway signaling, in reducing viability, colony formation, and migration of UM cells [46]. The effect of azacytidine treatment on this pathway points to a potential impact of epigenetic regulation of Wnt signaling in UM, which could help to revert cells towards a less migratory, less stemlike phenotype.

Rap1 signaling is additionally an important pathway in cancer progression, acting as a regulator of functions including formation and control of cell adhesions and junctions, cellular migration, and cell polarization, giving it an important potential role in cell migration events leading to metastasis [47]. Rap1 has also been shown to have effects on many other pathways which can potentially drive tumour invasion and metastasis, including integrin or cadherin-mediated cell adhesion, activation of PI3K/Akt signaling, and activation of ERK [48]. Effects on genes involved in Rap1 signaling further suggests the potential differential regulation of signaling cascades depending on molecular subtype in UM, as well as the potential impact of azacytidine treatment on these pathways.

Additionally, signaling in PI3K/Akt, MAPK, and mTOR signaling are known to be altered in UM, and targeted therapies against these pathways have been attempted in the treatment of UM metastases [49]. While these strategies have shown promising results *in vitro*, none of these treatments were found to be effective clinically against metastatic UM [3, 28]. Because the activation of these molecular pathways is associated with the development of UM, it is possible that combination therapy with DMNT inhibition would enhance the efficacy of these drugs and provide novel treatment options.

Overall, the modulation of these pathways through specific targeting drugs could potentially be enhanced by DNMT inhibitor treatment, as was shown through the synergistic effects of DNMT inhibition with other therapies in UM cells [15, 37]. This is a promising approach given the regulation of GPCR signaling in UM patients has largely been unsuccessful to date [5] despite the central role of constitutive activation of this pathway in UM oncogenesis. Taken together, the data in this paper reveal new avenues for potential combination approaches that could benefit this patient population. Importantly, the data presented herein revealed that DNA methylation patterns were similar in cfDNA and gDNA, regardless of treatment. This reinforces existing studies showing that cfDNA mimics epigenetic patterns in the parental cell [17]. Previously, cfDNA methylation has been used to monitor different tumour types, and has been shown to be able to identify cell type of origin as well as monitoring treatment response in different tumour types [50]. cfDNA is a promising analyte that can be isolated from liquid biopsy such as blood, allowing it to monitor disease non-invasively. Given the lack of intraocular biopsies required for diagnosis, other less invasive means of determining prognosis are needed in UM. Moreover, with the alarming 50% rate of metastasis, monitoring of patients and early diagnosis of disseminated disease is essential to improve outcome. The use of cfDNA to identify changes in methylation could be a powerful approach to monitor patients through blood-based liquid biopsy. Indeed, we have previously demonstrated that ctDNA monitoring through driver UM mutations is feasible and correlates with clinical disease [20]. The potential for the discovery of not only these driver mutations, but also alterations in methylation which appear to be highly related to outcomes in UM could allow for better methods to track response to treatment and potential recurrence in patients in a non-invasive manner.

5. Conclusions

In this study, we showed strong inhibitory cellular effects of a DNMT inhibitor on a panel of primary and metastatic cells. Azacytidine treatment resulted in anti-proliferative and anti-clonogenic effects in UM cells of different genomic backgrounds, as well as epigenetic regulation of genes involved in signaling, proliferation and migration of cells, especially in pathways related to stem-like properties. Our data strongly suggests that UM cell lines have alterations in DNA methylation that, as we have previously demonstrated in patient samples, impact major cancer-related

signaling pathways. These alterations point to the use of azacytidine as a potential adjuvant treatment – to be used with other drugs targeting the affected pathways – in the management of UM. Moreover, results in the OMM2.5 and Mel270 cell lines suggest a high degree of maintenance of methylation patterns in matched primary and metastatic tumours. Finally, highly similar methylation profiles in gDNA and cfDNA lend support for the use of liquid biopsy-based cfDNA as a tool to monitor UM.

Author contributions:

S.T.F. designed the study and methodology, conducted cell culture experiments (cell culture and azacytidine treatment, Incucyte proliferation assays, CCK8, Radiosensitvity assays, Colony formation, DNA isolation and quantification), and performed computational analysis for unsupervised clustering and the comparisons of sample groups (hierarchical clustering, PCA). M.L. performed computational analysis, including methylation analysis for large genomic regions (bumphunter). J.V.B designed the study, supervised the experiments and analyses and funded the work. All authors contributed and reviewed the manuscript.

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Supplementary Figures:

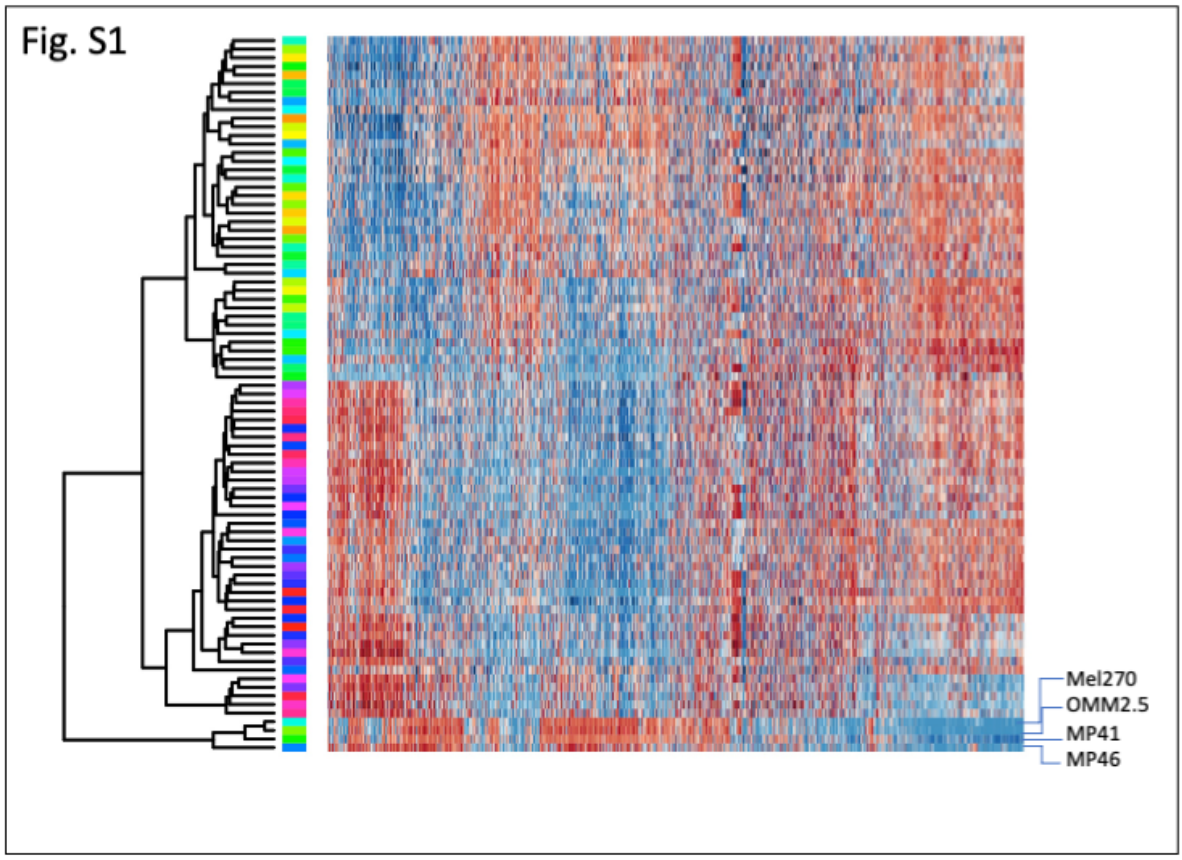

Figure S1. Figure S1. Hierarchical clustering of differentially methylated probes for 4 cell lines (Mel270, OMM2.5, MP41, and MP46, labeled on graph) compared to 80 primary UM cases from the TCGA database using SWAN normalized Beta Values, with dark red indicating a locus is fully hypermethylated and dark blue indicating a locus is fully hypomethylated.

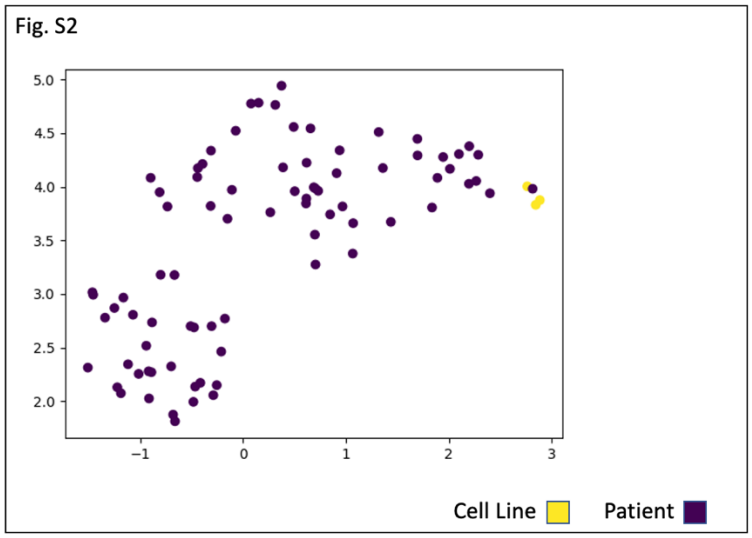

Figure S2. Uniform Manifold Approximation and Projection (UMAP) along the YZ axis, showing the differences in this dimension between genomic DNA from the 4 cell lines (MP41, MP46, Mel270, and OMM2.5) for 80 UM patients from the TCGA database

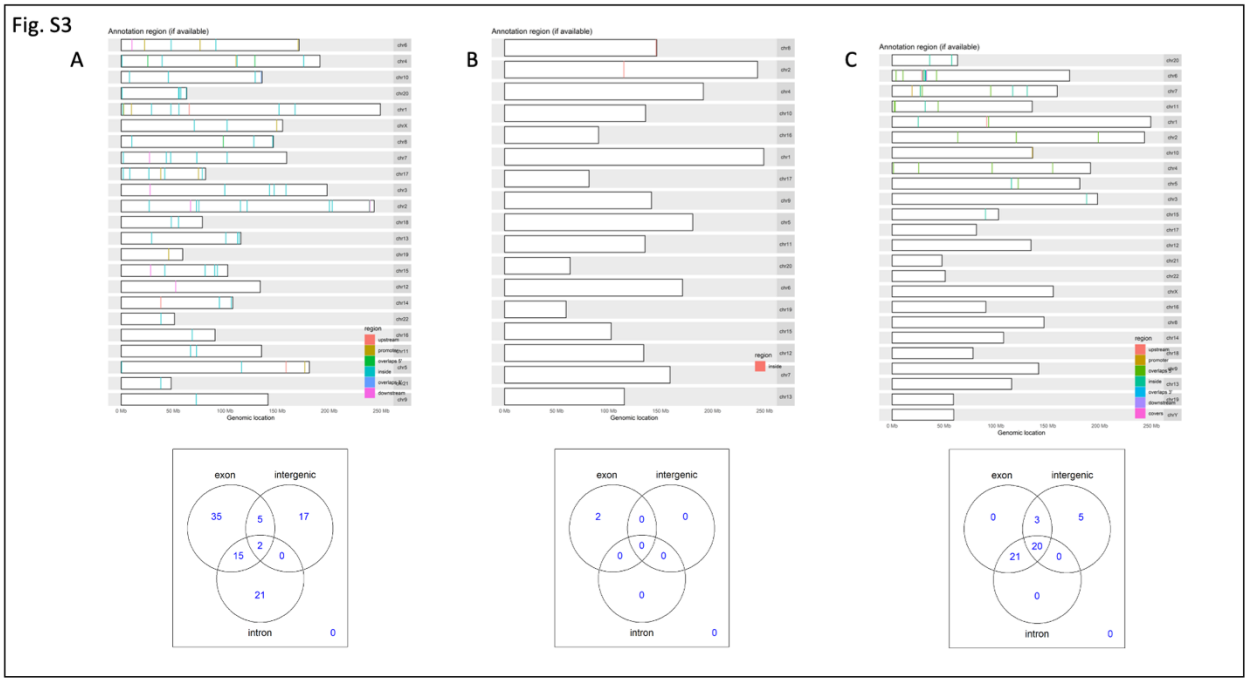

Figure S3. Significantly differentially methylated regions across the genome based on dose for all samples, with sample type and cell line as covariates with venn diagrams for the genomic locations found to be significantly differentially methylated for **(A)** the Mel270 and OMM2.5 cell lines and **(B)** The MP41 and MP46 cell lines, and **(C)** All cell lines using a lower cutoff value.

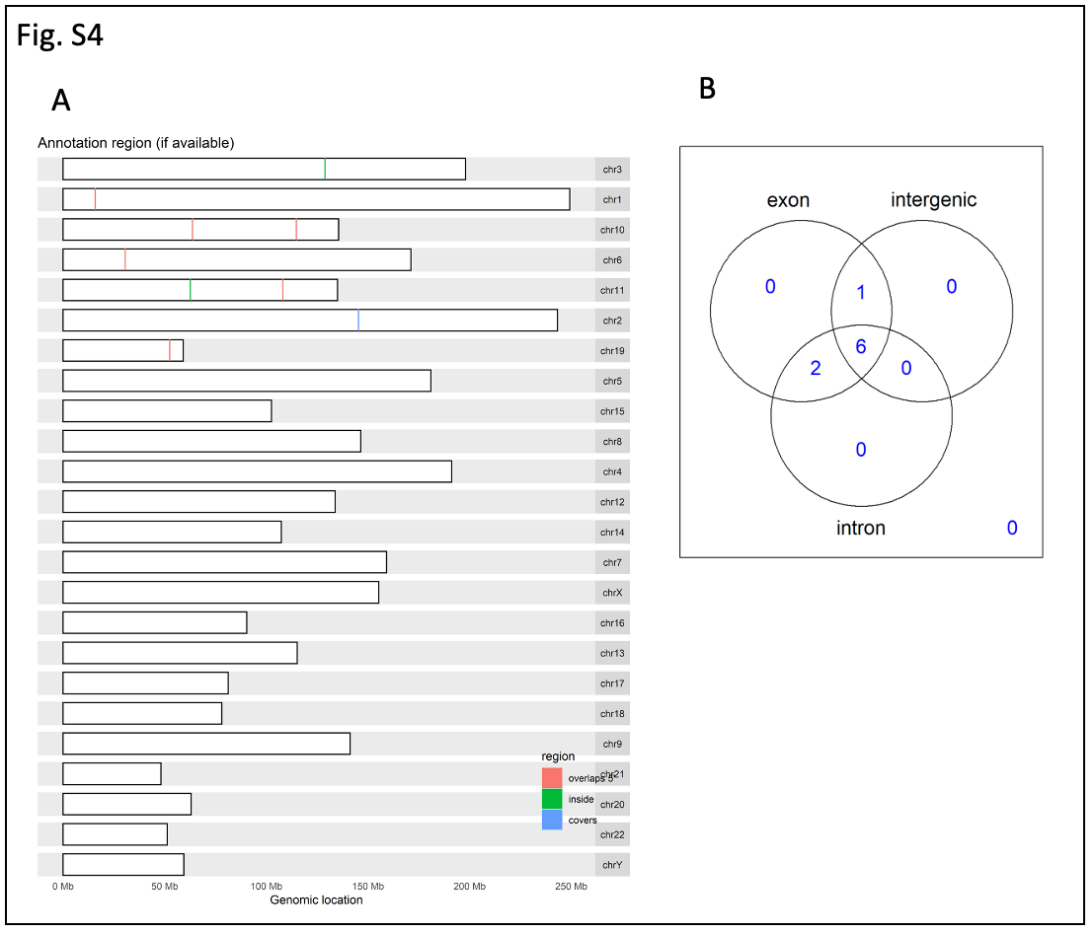

Figure S4. Significantly differentially methylated regions across the genome based on dose for all samples, with sample type and cell line as covariates with venn diagrams for the genomic locations found to be significantly differentially methylated between cell-free and genomic DNA based on A) chromosomal location and B) type of genomic region

References:

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Chapter 4.
General Discussion

DNA methylation, along with other epigenetic modifications, are gaining interest in the study of many malignancies because of their potential to be modified, along with their profound impact on gene expression and the behaviour of tumour cells. In this extremely difficult to treat disease, new adjuvant treatments for UM metastasis could help bring hope for people who otherwise do not have treatment options.

As discussed previously, primary UM tumours can be treated effectively, but treatment options become very limited once the tumour has disseminated. Additionally, evidence suggests that tumour spread can occur early on in the disease process [66], making it unlikely to catch the primary tumour before haematogenous dissemination has occurred. Because of this, there is a direct need for options both in the monitoring of metastatic progression, as well as new treatment avenues based on the specific molecular changes that are occurring.

While UM has a relatively low mutational burden when compared to other cancer types including cutaneous melanoma, we show the presence of large-scale differences in methylation patterns in genes related to cancer progression. Moreover, our data shows a strong correlation between methylation patterns and outcome in analysis of TCGA data from 80 patients. Such changes in DNA methylation may be contributing to tumour progression, potentially through the inactivation of tumour suppressors or the activation of pathways that increase tumour spread and growth. The analysis of these pathways may be an important strategy in understanding the processes underlying tumour progression and the development of metastasis. This hypothesis is strongly supported by our results (Chapter 2) showing that a number of genes with roles in cancer progression were differentially methylated according to patient outcome in UM; alterations were seen in specific genes such as NFIA, IL12RB2, and RASSF1, along with several genes related to signaling cascades such as PI3K/Akt, mTOR, ErbB, and Wnt. Together, these data point to methylation events as potentially important for driving UM progression. Combined with the *in vitro* experimental data (Chapter 3) showing that DNMT inhibition has effects on some of these same pathways, this acts as a further indication that epigenetic modulation might be a very useful adjuvant in the treatment of UM.

Additionally, as we know, tumours can change across time and express different molecular profiles depending on the location sampled within the tumour. Because of this, liquid biopsy approaches may offer a more representative and real-time means of monitoring the disease. Our lab has previously demonstrated an example of ctDNA isolation by liquid biopsy using tumour-specific mutations [67]. This work adds to the field by investigating the methylation patterns in an *in vitro* model of ctDNA. Indeed, in this thesis, I have shown that methylation patterns can be maintained in cfDNA, representing the patterns of the parental cells. This data provides compelling evidence for the tracking of methylation using liquid biopsy to monitor disease progression in UM patients.

Overall, we saw alterations in many important signaling and metastasis-related genes in UM cells upon treatment with a DNMT inhibitor. The methylation profiles and corresponding gene expression changes seen in UM patients in Chapter 2 – combined with the changes in signaling pathways seen in UM cells in Chapter 3 – suggest that methylation-mediated inactivation of genes related to tumour progression could potentially be reversed by methylation-targeting treatments. Additionally, our analysis revealed specific pathways affected by DNA demethylating agents, giving insight into potential targets for adjuvant treatment in UM. For example, in UM cells, MEK inhibitors were shown to be most effective when used in combination with DNMT inhibitors, which may be due to the effects of the DNA demethylating agents on the Ras-Raf-MEK-ERK-MAPK cascade [47]. In the same vein, our data showing changes in methylation on genes coding for histone deacetylases or interleukins point to the potential of DNMT inhibitors for use in combination therapy; DNMT inhibitors could potentially reactivate or inactivate a pathway synergistically with a separate targeted drug such as an HDAC inhibitor or immune modulating drug. This is reinforced by our cell culture data pointing to alterations in many signaling pathways with DNMT inhibitor treatment. While many of the pathways seen in our analyses have been shown in other studies to be altered in UM, the mechanism behind these alterations was previously unclear. Our data suggests that DNA methylation may be responsible for many of the alterations in gene expression and signaling seen in UM cases. For this reason, it would be of great value in future studies to examine the impact of DNMT inhibitors on the expression of different genes within signaling cascades in order to elucidate the exact effects of DNMT inhibition on signal transduction in UM.

Our analysis of patient data pointed to a strong correlation between methylation patterns and gene expression levels. Such changes in gene expression caused by DNMT inhibition alters the molecular profile of the tumour cells. This means that the alterations in gene expression induced by DNMT inhibition within tumour cells could allow us to re-examine a number of treatments that have been ineffective in UM, but to which the cells may now by sensitised. Given that many treatments have been unsuccessful for this malignancy, the potential for synergistic action of other medications with DNMT inhibitors greatly expands the number of avenues that can be explored. In depth knowledge about the exact effects of DNMT inhibitors on these cells shows us which tumour-related pathways might be altered. This in turn reveals targets for combination with methylation-altering treatments. For example, we identified changes in the PI3K/Akt, Rap1, MAPK, Wnt, and mTOR signaling pathways, which are known to be very important in UM progression. The targeting of such pathways offers the potential for many new treatment modalities for UM.

Especially interesting are the alterations in HDAC4 that we saw in both the TCGA patient data as well as the cell culture model, suggesting epigenetic events related to patterns of histone acetylation mediated specifically by HDAC4. These patterns may indeed also have widespread effects and require further study to assess HDAC inhibitors that block different classes of histone deacetylases for their potential to reduce the spread of UM. While changes in chromatin modifications have not been greatly studied in UM, our results in both patient samples and cell lines point to this being a topic that warrants future exploration.

Importantly, our data are the first to demonstrate the corroboration of methylation patterns in cell-free DNA and genomic DNA in UM. This points to a useful clinical application of methylation detection in UM through liquid biopsy**.** In most UM patients who develop metastases, this progression of the disease occurs years after diagnosis and treatment of the primary tumour, making it difficult to predict. Since we showed that 1) DNA methylation differs in patients according to outcome and risk of metastasis, and 2) methylation patterns are maintained in cell free DNA, such patterns could be used to predict metastasis and monitor patients after removal of the primary tumour. This finding is especially relevant for disease monitoring because high levels of differentially methylated DNA may indicate a higher disease burden. Earlier detection of relapse in high risk patients is important despite the current lack of systemic

treatment options, as it gives them more time to enroll in clinical trials or attempt neoadjuvant treatment options. Additionally, the high degree of similarity between the methylation patterns in the matched primary and metastatic UM cell lines may indicate that these methylation changes could be targeted in metastatic disease as well as at the time of diagnosis.

Interestingly, we noted a lack of major differences in mean methylation levels across patient samples, as well as across cell lines and treatment conditions. Moreover, we found many sites of differential methylation across the genome. These observations demonstrate patterns of hypermethylation and hypomethylation which are likely to lead to specific downstream effects on gene expression in these tumours. While azacytidine acts as a DNMT inhibitor, we found similar levels of mean methylation across treatments, as well as a pattern of both hypomethylation and hypermethylation. This indicates that, at the probes studied, azacytidine is having specific effects on UM cells as opposed to more general demethylation, fitting with a previous report in colon cancer and leukemic cell lines [68]. This has important implications for cancer therapy, as specific changes can be targeted, whereas more general alterations in methylation levels may require different approaches. The results from differentially methylated probe analysis show that these specific changes are occurring in pathways that regulate signaling in cancer and pathways in cancer more generally, as well in multiple genes that regulate the movement of cells. Reverting these changes in methylation could reduce the motility and metastatic capabilities of these cells, potentially playing a role in preventing the development of metastatic disease.

Finally, in the analysis of TCGA data, we saw a strong relationship between BAP1 mutation status and differential methylation patterns. This effect was recapitulated in our cell culture model, where the BAP1 negative cell line MP46 clustered separately from the BAP1 positive cell lines. Overall, these findings suggest that loss of BAP1 expression is related to major changes in methylation patterns that may be driving tumour dissemination. These differences may once again point to therapeutic avenues, as BAP1 loss is associated with especially aggressive disease in UM; changes seen in the BAP1 negative cells in signaling cascades such as ErbB, cAMP, PI3K/Akt, and Rap1 signaling, as well as alterations in cell motility and adhesion, suggest useful targets. Many of these alterations were also amongst those most affected by DNMT inhibition,

implying that the changes seen in high risk UM are potentially reversible with treatment.

Conclusion

To conclude, we aimed to understand the role of methylation patterns in UM. Herein, we showed that methylation patterns at specific genes in UM are highly correlated to patient outcome, as well as to other recognized markers of prognosis. These genes are relevant in cell signaling, as well as in other genetic pathways that are important in cancer progression and development of metastasis, including cell adhesion and regulation of cell proliferation. Along with the differences seen between patients, human UM cell lines that recapitulate different facets of the disease were shown to differ in terms of methylation patterns. Moreover, treatment with the DNMT inhibitor azacytidine affected these patterns, through both hypomethylation and hypermethylation. In cell lines, pathways related to cell adhesion and motility, as well as signaling pathways such as MAPK, PI3K/Akt, RAP1, and mTOR were affected by DNMT inhibitor treatment, pointing to the potential clinical relevance of combining DNMT inhibitors with medications that are also relevant in these pathways.

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