AHR gene expression and the polymorphism rs2066853 are associated with clinicopathological parameters in colorectal carcinoma

Running title: AHR in colorectal carcinoma.

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Abstract

The relationship between red and processed meat and its risk towards colorectal carcinoma (CRC) is not fully explored in literature. Polycyclic aromatic hydrocarbons (PAHs) are pro-carcinogenic molecules that are ingested with meat cooked at high temperatures. The metabolic conversion of PAHs to carcinogenic diol epoxides is in part mediated by the aryl hydrocarbon receptor (AhR)-dependent induction of CYP1A1. This study aims to examine and expression profiles and polymorphisms of the *AHR* (*aryl hydrocarbon receptor)* gene which is involved in the metabolic conversion of PAHs in patients with CRC. Genetic analysis was done in matched cancer and non-neoplastic tissues from 79 patients diagnosed with CRCs. Low *AHR* mRNA expression was associated mucinous colorectal adenocarcinoma. Exon 10 of *AHR* showed that 27% of patients had the rs2066853 single nucleotide polymorphism resulting in an arginine to lysine change at codon 554. This variant was significantly associated with a lower likelihood of perineural invasion, presence of synchronous cancer, and multiple colorectal polyps. Furthermore, rs2066853 individuals were significantly more likely to be of more advanced age and have a more favourable tumour grade and pathological stage. These results imply the pathogenic roles of *AHR* in PAH-associated colorectal carcinogenesis.

Keywords

Colorectal cancer, dioxin receptor, clinical, pathological, polycyclic aromatic hydrocarbons, meat

1.0 Introduction

Colorectal cancer (CRC) remains the world's third most deadly and fourth most diagnosed cancer and its rising incidence is attributed to the adoption of a Western lifestyle [1]. Of particular interest, the consumption of red and processed meats is one of many lifestyle risk factors driving the development of CRCs [2,3]. The International Agency for Research on Cancer (IARC) postulated polycyclic aromatic hydrocarbons (PAHs) to be a molecule found in red and processed meats that could be the causative link to CRC [4]. In addition to CRC, PAHs are reported to be associated with lung [5–7], breast [8], gastric [9], oesophageal [9–11], pancreatic [12], skin, bladder [13], and prostate [14,15] cancers. PAHs primarily exert their carcinogenic effects as PAH diol epoxides that are formed via a pathway mediated by the aryl hydrocarbon receptor (AhR).

PAHs are molecules that are ubiquitous in the environment and human exposure can be through ingestion, inhalation, and dermal contact [16]. Ingestion of PAHs via contaminated food products is the route that most highly exposes humans to these compounds [17]. Following ingestion, the metabolic transformation from pro-carcinogenic PAHs to carcinogenic PAH diol epoxides is mediated by enzymatic reactions via the diol epoxide (same as cytochrome P450/epoxide hydrolase (CYP/EH)) pathway [18]. The CYP/EH pathway of PAH activation involves a three-step enzymatic transformation by two enzymes, CYP1A1 and epoxide hydrolase [19]. Importantly, *CYP1A* gene expression is inducible by PAHs via the AhR [20].

AhR has been implicated in the proper functioning of many systems, including the immune, hepatic, cardiovascular, and reproductive systems [21]. Moreover, its functional association with signalling pathways that regulate cell cycle and proliferation, cellular morphology, and cell adhesion and migration suggest a role for AhR in cancer [21]. The AhR protein/dioxin receptor is encoded by the *AHR* gene that consists of 11 exons located on

chromosome 7p21.1 [22]. AhR resides in the cytoplasm of resting-state cells, but upon binding of PAH that acts as a ligand, AhR complexes with auxiliary proteins such as heat shock protein 90 (Hsp90), p23 (co-chaperone), and aryl hydrocarbon receptor interacting protein (AIP) that allows its translocation into the nucleus [23]. Activated AhR in the nucleus heterodimerises with aryl hydrocarbon receptor nuclear translocator (ARNT) (also known as hypoxia-inducible factor 1β (HIF1β)) and binds aryl hydrocarbon response elements (AhRE), to induce transcription of target genes i.e. CYP1A1 [21,23].

Relationships between functional polymorphisms of *AHR* and cancer, particularly CRC, are not well established and requires further investigation [24]. rs2066853 is the most studied single nucleotide polymorphism (SNP) of *AHR*, and describes a G to A transition in exon 10 at position 1661(c.1661G>A) on the coding DNA strand [25]. This nonsynonymous SNP results in an arginine to lysine change at codon 554 (Arg554Lys) in the transcriptional activation domain of AhR [25]. Rs2066853 has been shown to interact with N-acetyltransferase 1 (*NAT1*) genes and meat intake to increase the risk of colorectal polyps [26] and also with glutathione S-transferase pi 1 (*GSTP1*) gene resulting in a higher risk of colonic polyps in patients with acromegaly [27]. Individuals with rs2066853 who were also smokers were more likely to have a higher intestinal permeability in patients with Crohn's disease [28].

Research into rs2066853 and its associations in patients with colorectal cancer and clinicopathological correlations is lacking. Therefore, this study aims to investigate the *AHR* gene in the context of CRC. Specifically, we explore possible changes in *AHR* gene expression in non-neoplastic colon cells and in tissues from CRC patients. Additionally, this study analyses mutations in exon 10, including rs2066853 in non-neoplastic and matched neoplastic tissues of patients with colorectal carcinoma and its associations with clinicopathological parameters.

2.0 Materials and Methods

2.1 Cell culture

CCD841 CoN (ATCC CRL-1790) non-neoplastic colon epithelial-like cell line was used in this study (American Type Culture Collection (Manassas, VA, USA)). Cells were cultured following manufacturer's guidelines in EMEM (Eagle's Minimum Essential Medium, ATCC) supplemented with 10% FBS (fetal bovine serum, Scientifix, Clayton, VIC, Australia). CCD841 CoN was maintained at 37°C with 5% CO_2. Cells used for this study were within passages 4-10.

2.2 PAH treatments of CCD841

Benzo[a]pyrene (B[a]P), phenanthrene (PHEN), fluorene (FLU), and anthracene (ANTH) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and prepared as 50mM stock solutions in dimethylsulfoxide (DMSO) and stored in the dark at 4°C. Working concentrations of 50µM, 25µM, 10µM, and 1µM were created by diluting stock solutions with EMEM+10% FBS. Control treatments utilised using EMEM+10% FBS in place of PAH, while vehicle control treatments contained 0.2% DMSO in EMEM+10% FBS.

2.3 Patient samples

Colorectal cancer tissues and non-cancer tissues taken from the same patient were collected prospectively from 2012 to 2015 by a colorectal surgeon (CTL) at the time of resection of primary cancer from hospitals in Queensland, Australia. These tissues were snap-frozen and stored at -80°C until use and in formalin to process in paraffin for microscopic examination to ensure the adequacy of the tissue for genomic studies. Tissues that lacked adequate cancer mass were excluded from this study. Patients were recruited chronologically

and without bias. The Griffith University Human Research Ethics Committee has granted ethical approval for this work (GU Ref No: MSC/17/10/HREC).

The surgical specimens were then processed for pathological examination and clinical management by the authors (AKL and CTL). The size of the tumour, the location of the tumour as well as the presence of synchronous cancer and many co-existing polyps (more than 10) were documented. Haematoxylin and eosin (H&E) staining of these blocks taken from the surgical specimens was performed to investigate pathological features such as grading, histological subtypes and staged following the World Health Organisation (WHO) criteria [29] as determined by immunohistochemistry. Presence of tumour perforation, lymphovascular permeation, perineural infiltration by carcinoma and microsatellite instability were also noted. Only adenocarcinomas were included in this study. A total of 79 patients (40 women and 39 men) with colorectal adenocarcinomas were included in this study.

2.4 Extraction of DNA and RNA

Sections of 7µm were sliced using a cryostat (Leica Biosystems, VIC, Australia) from the fresh frozen CRC tissues to extract genetic material. DNA extraction was performed using Qiagen DNeasy Blood and Tissue kits (QIAGEN, Hilden, Germany) following the manufacturer's protocol. According to manufacturer's guidelines, RNA was extracted from cells and fresh frozen tissue using miRNeasy Mini Kit (QIAGEN). DNA and RNA purity (260/280 ratio) and concentrations (ng/µL) were measured using a NanoDrop spectrophotometer.

2.5 Quantitative real-time PCR (qPCR) analysis

For quantitative real-time PCR, cDNA was generated from RNA using SensiFAST cDNA synthesis kit (Meridian Bioscience, Cincinnati, OH, USA), following the

manufacturer's guidelines. cDNA concentration was measured using Nanodrop and diluted to a working concentration of 100ng/µL. qPCR was performed using the SensiFAST SYBR No-ROX kit (Meridian Bioscience). Amplification efficiencies were normalised to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase.

Primer sequences for qPCR are as follows:

GAPDH F: 5' – CTTTTGCGTCGCCAG

GAPDH R: 5' – TTGATGGCAACAATATCCAC

AHR F: 5' – TAACAGATGAGGAAGGAACAG

AHR R: 5'- TTGAGAGAGTCCTTGCTTAG

2.6 High-resolution melt (HRM) curve analysis

Genomic DNA (gDNA) was extracted following the protocol for 79 cancers and matched non-cancer tissues. This gDNA was subject to HRM analysis to screen for possible mutations in AHR. HRM curve analysis and target gene amplification were performed on QuantStudio 6/7 Flex systems using HRM software (ThermoFisher, Waltham, MA, USA). Exon 10 of *AHR* were amplified using SensiMIX HRM mastermix (Meridian Bioscience, Cincinnati, OH, USA) following the manufacturer's protocol. This exon was amplified using primer sequences: forward 5'- TTTCAAGATAGTAAAAACAGTGACTTGT-3′ and reverse 5′-ATTTCATCCGTTAAGTCAATGTCT-3′ [30]. Each run included a negative (no template) control. The melt curve data was measured at each incremental increase of 0.05°C/s between temperatures 65-85°C for all assays.

2.7 Purification of PCR products and Sanger sequencing analysis

PCR products from HRM analysis were subject to gel electrophoresis to purify the sample. DNA was extracted from the gel using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Duren, Germany). At the Australian Genome Research Facility (AGRF):

purified DNA was sequenced using Big Dye Terminator (BDT) chemistry version 3.1; this data was analysed using a 3730xl capillary sequencer; and sequences were analysed using Sequence Scanner 2 software. All variants detected via HRM analysis was further investigated using Sanger Sequencing analysis

2.8 In silico analysis

Possible consequences of identified mutations were analysed using computer modelled in-silico analysis. Mutation taster from the National Center of Biotechnology Information [31], Protein Variation Effect Analyser (PROVEAN), and SIFT (Sorting Intolerant From Tolerant) were the chosen bioinformatic analysers used in this study.

2.9 Statistical analysis

Statistics for qPCR results from CCD841 was performed on GraphPad Prism 9.0 (San Diego, CA, USA) comparing differences of mean delta C_t values, whilst presenting graphical data using fold change values. mRNA expression and sequencing data were analysed against clinicopathological parameters on the patient cohort was performed using IBM SPSS statistics 27 (International Business Machines, Armonk, NY, USA).

3.0 Results

3.1 A high concentration of phenanthrene treatment increases AHR gene expression

Exposure of varying concentrations of B[a]P, FLU, and ANTH to CCD841 colon cells did not result in statistically significant changes in *AHR* mRNA expression (Figure 1). High dose of PHEN (50µM) showed 1.5 times fold change increase when compared to the control cells ($p = 0.047$). 50µM PHEN treatment, when compared to vehicle control treatment, did not yield a statistically significant increase.

3.2 AHR mRNA expression in CRC tissue and matched non-neoplastic tissue and clinicopathological significance

Thirty-one cases of CRC (12 females, 19 males), each with matched non-cancer mucosa tissue, underwent qPCR analysis for the level of *AHR* gene expression. The study population had a mean age of 65 years (range 31-88 years). Low *AHR* mRNA expression was defined as the tumour tissue having equal to or less than -2-fold change, normal expression was greater than -2 but less than 2-fold change in *AHR*, whilst high *AHR* expression was equal to or greater than 2-fold change. Approximately 68% (21/31) of this cohort showed low AHR expression, 19% normal, and 13% high expression (Figure 2).

Analysis against clinicopathological parameters showed that older individuals (≥ 50 years of age) were more likely to have low or normal *AHR* expression whilst younger patients (<50 years of age) were significantly more likely to have high *AHR* mRNA expression (likelihood-ratio *Chi*-square = 0.021). Moreover, individuals with mucinous colorectal adenocarcinoma were more likely to have low *AHR* expression (likelihood-ratio *Chi*-square = 0.037). When high and normal expression cohorts were grouped together, patients with high/normal *AHR* expression were less likely to have cancer perforation ($p = 0.021$). All individuals in this cohort who had cancer perforation also had low *AHR* expression. Patients'

gender, site and size of the tumour, tumour differentiation, presence of lymphovascular invasion, distant metastasis, and pathological staging of the tumour did not have significant associations with *AHR* expression.

Individuals whose tumour tissues had similar *AHR* expression compared to their non-neoplastic colonic mucosa tissue seemed to show an improved survival rate. Those with a high level of *AHR* expression had a relatively lower survival rate. Nevertheless, survival analysis was not statistically significant.

3.3 Identification AHR exon 10 variants in colorectal cancer and matched non-neoplastic tissues and clinicopathological correlations

Cancer and matched non-neoplastic mucosa tissues from 79 patients with CRC underwent HRM analysis at exon 10 for *AHR* gene. Genetic variants of *AHR* were initially detected through melt curve deviation during HRM analysis and genotype confirmation was achieved via Sanger sequencing (Figure 3). The SNP rs2066853 heterozygous (GA) and homozygous (AA) alleles were identified. All non-neoplastic mucosa and tumour tissues from the same patient harboured the same genotype.

All patients in this cohort who had synchronous cancers (n=7), perineural infiltration (n=6) and presence of more than 10 polyps (n=6) did not harbour the rs2066853 SNP (p = 0.033, 0.049, 0.049 respectively) (Table 1). Patients with this polymorphism were significantly more likely to have an early tumour (T) stage and a pathological stage of 1 or 2 (p = 0.017 and p = 0.039, respectively).

Although not statistically significant, cancers with a microsatellite instability phenotype were more likely not to harbour this polymorphism (p = 0.071). Moreover, those with rs2066853 were tended to be older (over 65 years of age) (p = 0.084).

Patients' gender, cancer perforation, site and size of tumour, and distant metastasis were not significantly associated with rs2066853. In silico analysis predicted rs2066853 to be a tolerated, non-deleterious polymorphism.

4.0 Discussion

This study investigated various aspects of the *AHR* gene in colorectal cancer. The first part of this study sought to understand changes in *AHR* mRNA expression in colon epithelial-like cells after treatment with PAHs for 72 hours. This chosen treatment time aimed to mimic maximal food transit time. It is well established that PAHs act as ligands to induce transcription of xenobiotic response elements via an AhR-dependent pathway [32–34] in the intestines [35]. However, our results did not indicate any significant changes in *AHR* mRNA levels after PAH treatment after comparing to both control and vehicle control treatments. A similar study in hepatic cells utilised a treatment period of 24hrs [36], thus it is possible that 72hrs was too lengthy and resulted in *AHR* mRNA returning to baseline levels post-PAH treatment. Furthermore, it is possible that PAH treatments increased *AHR* mRNA, but this was translated into a protein to result in an insignificant change in mRNA expression level.

This study involves a cohort of CRC patients who underwent qPCR analysis comparing AHR gene expression in their matched cancer and non-neoplastic mucosa tissues. Of note, most individuals (68%) showed lower *AHR* mRNA levels in their tumour tissue when compared to their matched non-neoplastic mucosal tissue. Tumours expressing low *AHR* were more likely to be mucinous colorectal adenocarcinoma, a specific subtype of CRC characterised by over 50% tumour volume composed of extracellular mucin [37]. These results implicate *AHR* in CRC subtypes and supports mucinous adenocarcinoma as a distinct and separate subtype that differs from conventional adenocarcinoma. *AHR* expression in the intestinal epithelium has been shown to contribute to intestinal integrity by modulating inflammatory processes [38], and thus the lowered expression in tumour tissues could be due to dysregulation of immune function. AhR has also been shown to activate protective pathways to generate an antioxidant defence response [39]. A decreased level of this would be expected in tumour tissues with poorly controlled levels of oxidative stress.

Moreover, in this study, perforation of the tumour through the bowel was only observed in patients whose tumour tissue expressed low *AHR*, and those with low expression were more likely to be younger. Taken together, these results suggest that higher AHR mRNA expression in the tumour tissue is more favourable as there is less likelihood of perforation and more advanced age of requiring surgery. In contrast, survival analysis suggested the opposite as those with low or high expression have less favourable survival rates than normal expression individuals. However, the difference was not statistically significant, and this is a small cohort; thus, additional studies would help confirm these findings.

Further inquiry into exon 10 of the *AHR* gene revealed that all the patients' matched non-neoplastic mucosa and CRC tissue shared the same DNA sequence, thus suggesting that this locus is not a common mutational site in CRC. We also explored the associations between rs2066853 polymorphism (Arg554Lys) and clinicopathological parameters in 79 patients with CRC. Individuals harbouring this polymorphism were less likely to have unfavourable characteristics such as perineural infiltration, increase number (>10) of associated polyps, and presence of synchronous cancer. Additionally, those cancers with the polymorphism were more likely to be less aggressive, lower cancer stage, and exhibit a stable MSI phenotype. These results suggest that the rs2066853 polymorphism have more favourable clinicopathological outcomes in CRC.

Similarly, individuals with rs2066853 are less likely to develop lung cancer [40], but smokers with the variant conferred a higher risk of lung cancer [41]. Rs2066853 does not confer colorectal polyp [42] or colorectal cancer [43] risk, nor was it associated with pancreatic cancer [44] or Crohn's disease [45]. A meta-analysis showed that rs2066853 was not associated with breast, lung, or overall cancer risk [24]. In addition, rs2066853 has been associated with increased disease aggressiveness in patients with acromegaly [46], increased

likelihood of oligoasthenospermia [25], and a higher risk or glioma [47]. Overall, current literature focuses on the risk of disease and has returned mixed results.

In contrast, the current study investigates the relationship of rs2066853 with clinicopathological characteristics of CRC. Whilst this variant may not be associated with disease risk when compared to a healthy population, its association with more favourable parameters could be partly explained by the nonsynonymous nature of this SNP. Individuals with the variant harbour a lysine residue, which could result in the loss of part of the helix structure of the transcriptional activation domain of AhR and affect post-translational modifications, leading to altered AhR target genes expression [48]. Further *in vivo* and *in vitro* studies would help better understand the role of *AHR* and rs2066853, and its observable effects on protein structure, and its role in both protective and harmful signalling pathways in disease states. Future studies could benefit from using multiple cell types, such as immune, endothelial, and neuronal cells, to better mimic the intestinal environment and a series of shortened treatment periods to elucidate the temporal relation between PAH treatments and *AHR* mRNA expression.

5.0 Concluding remarks

Based on these results, *AHR* gene is associated with several aspects of colorectal cancer. Individuals whose tumour tissues express lower levels of *AHR* mRNA were more likely to have mucinous colorectal adenocarcinoma. Moreover, this study shows *AHR* exon 10 is unlikely to be a mutational hotspot in CRC, except for the known SNP rs2066853. In these patients with CRC, rs2066853 was significantly associated with more beneficial tumour characteristics. These results integrated with the current literature suggest a need to further research *AHR* and its genetic variants in CRC as it could be a predictor of clinical outcomes.

Figure Legends

Figure 1: mRNA expression changes of AHR in response to varying treatment concentrations of benzo[a]pyrene (B[a]P), phenanthrene (PHEN), fluorene (FLU), and anthracene (ANTH). * = $p<0.05$.

Figure 2: AHR messenger RNA expression, survival trends, and AHR expression in correlation to mucinous adenocarcinoma in CRC patients. A) fold change of CRC tissue calculated as relative expression to matched non-neoplastic mucosa tissue. AHR gene expression was normalised by GAPDH housekeeping gene. B) Survival analyses of patients with differing low, normal, or high relative AHR expression. C) Tumours expressing high/normal AHR are more likely to be conventional adenocarcinomas. D) Tumours expressing low AHR are more likely to be mucinous adenocarcinomas.

Figure 2: rs2066853 heterozygous and homozygous genotypes as seen via high-resolution melt and Sanger sequencing analysis versus wildtype at the same position. A) depicts wildtype, homozygous and heterozygous HRM curves together and the wildtype chromatogram (GG allele), b) shows the heterozygous HRM curve deviation and chromatogram (GA allele), and c) illustrates the homozygous HRM curve deviation and its respective chromatogram (AA allele).

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Conflicts of Interest

The authors declare no conflict of interest.

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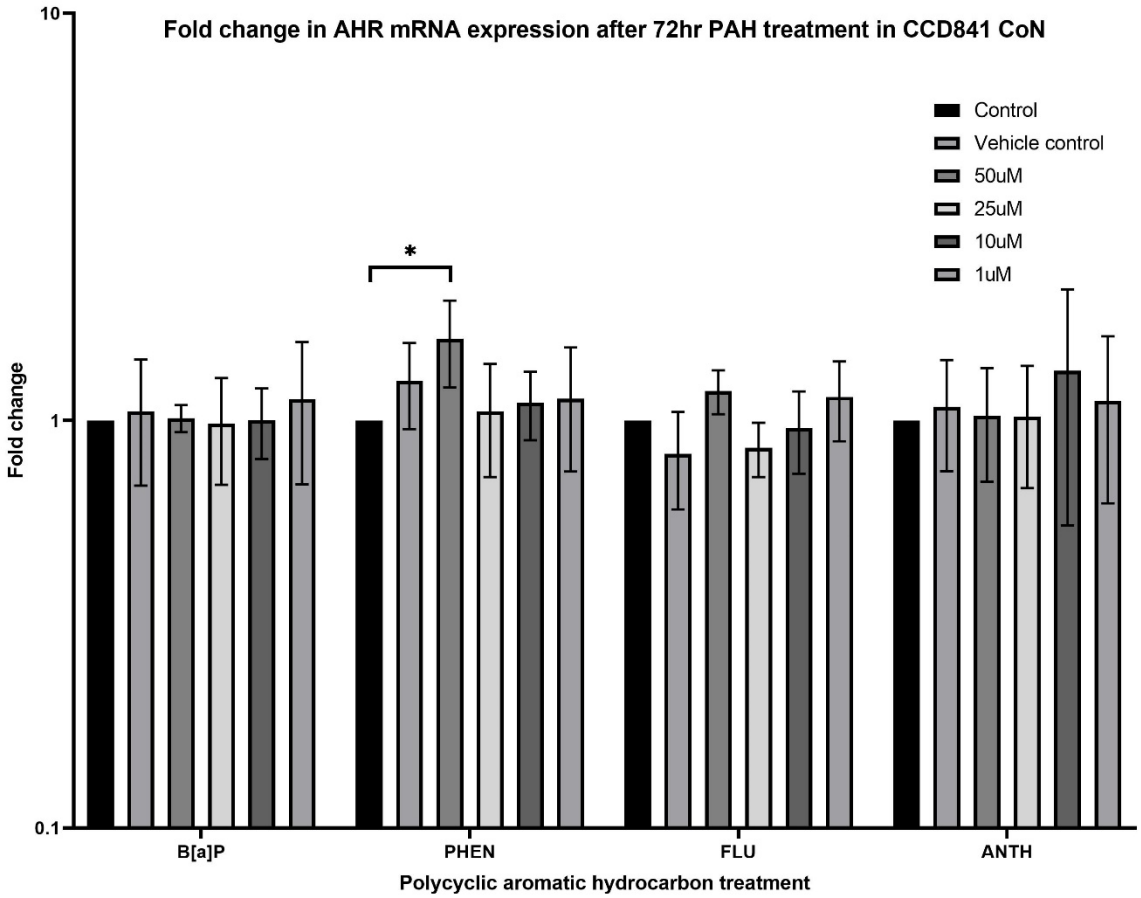
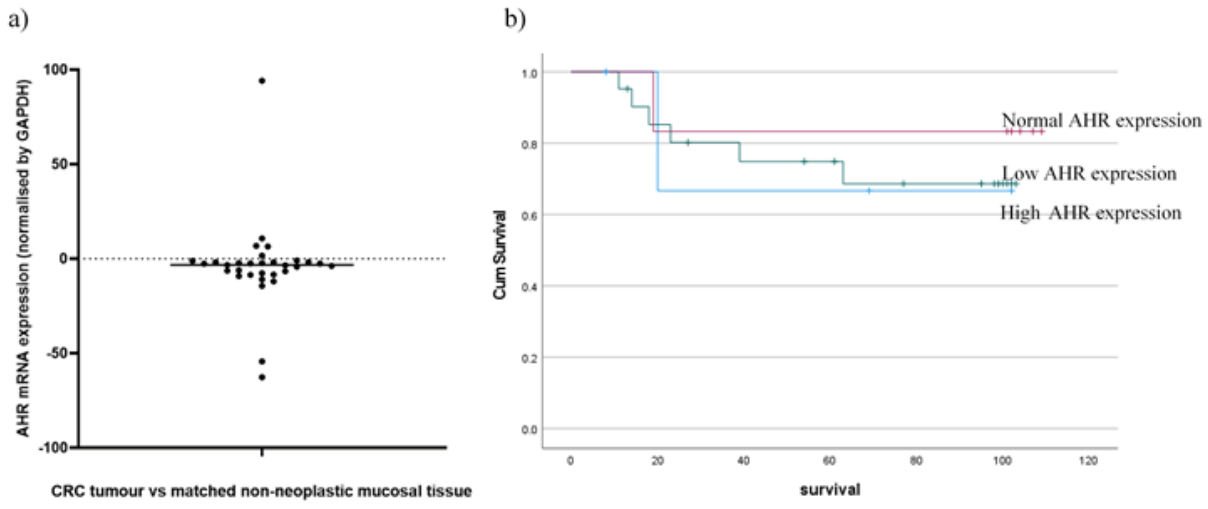
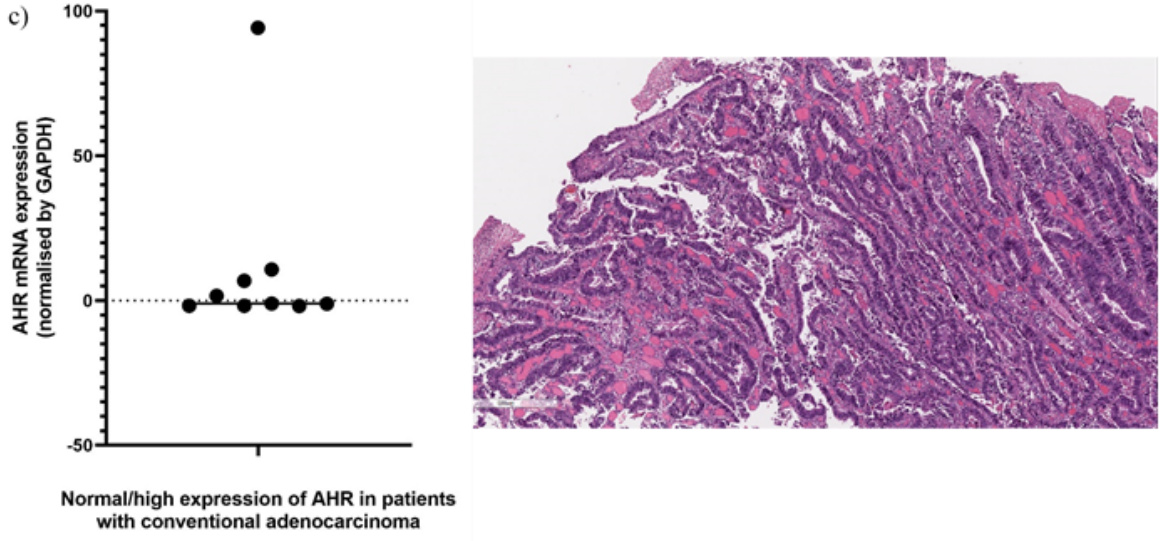
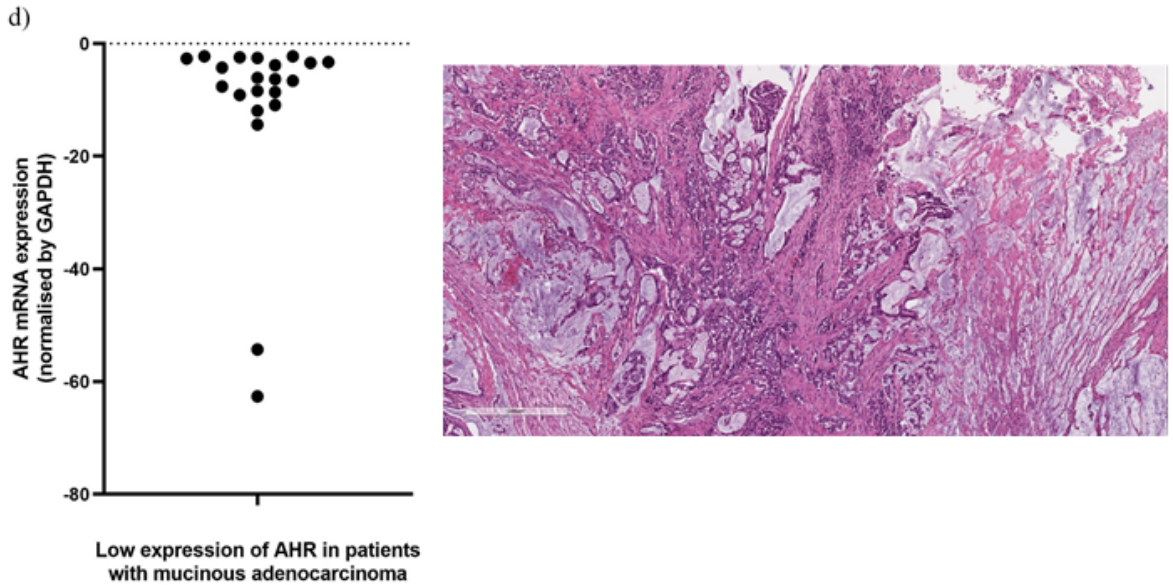
Table 1: Clinicopathological associations between wildtypes and rs2066853 positive variants against CRC characteristics

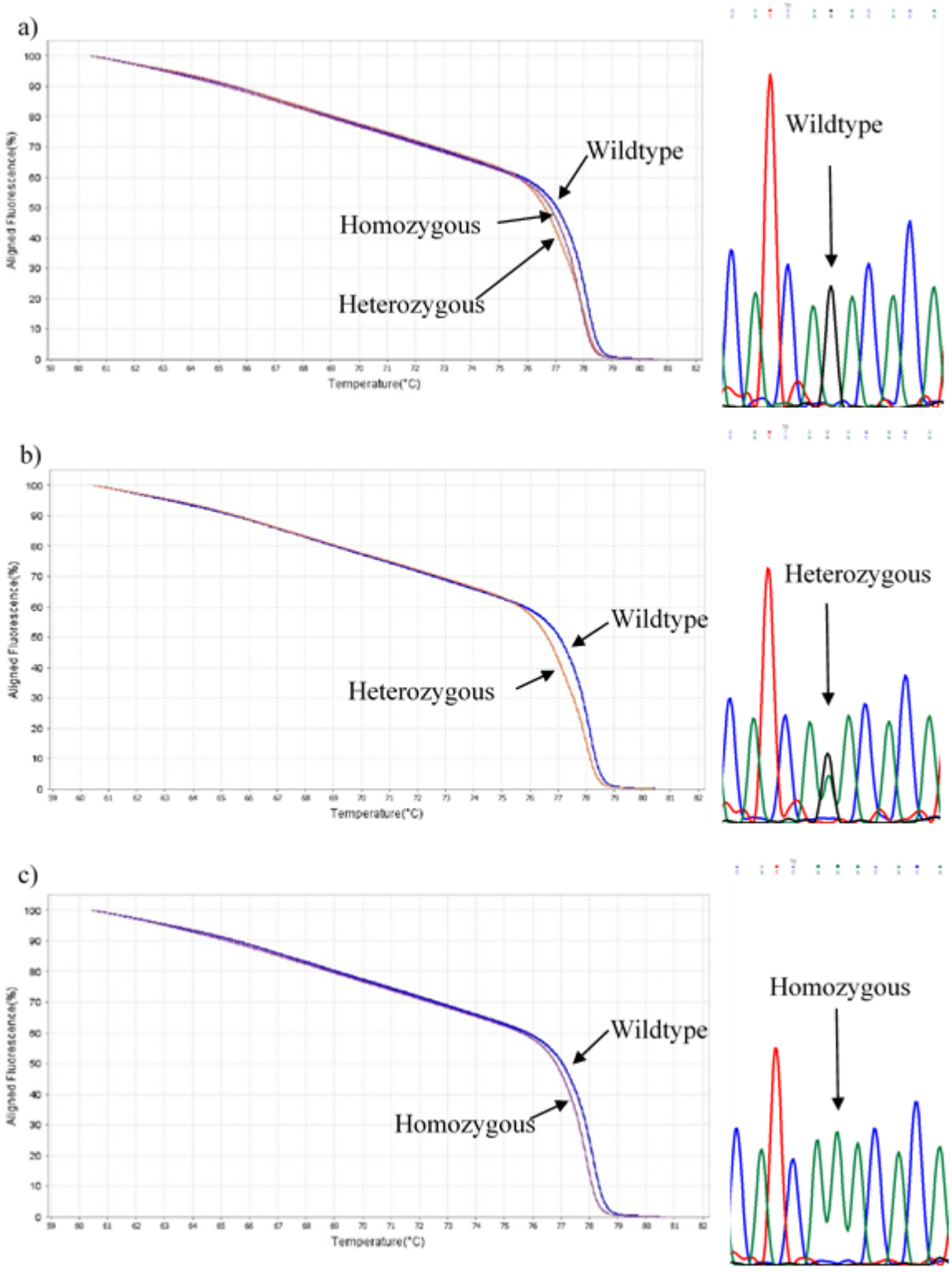
	Wildtype/rs2066853 absent (%)	rs2066853 present (%)	*p*-value
Gender			
Female	29 (37)	29 (37)	
Male	11 (14)	10 (12)	0.852
Age			
≤65	26 (33)	32 (41)	
>65	5 (6)	16 (20)	0.084
Site			
Proximal colon	28 (34)	30 (38)	
Distal colorectum	9 (11)	12 (15)	0.669
Size (maximum dimension)			
≤40mm	27 (34)	31 (39)	
>40mm	13 (16)	8 (10)	0.226
Tumour depth of invasion (T)			
Level 1	1 (1)	2 (3)	
Level 2	6 (7)	4 (5)	
Level 3	34 (43)	13 (16)	
Level 4	17 (22)	2 (3)	**0.017***
Pathological stage			
I	6 (7)	6 (7)	
II	24 (30)	10 (13)	
III	14 (18)	2 (3)	
IV	14 (18)	3 (4)	**0.039***
Perineural infiltration			
Absent	52 (66)	6 (7)	
Present	21 (27)	0 (0)	**0.049***
Presence of numerous polyps (>10)			
10 or less	52 (66)	6 (7)	
Greater than 10	21 (27)	0 (0)	**0.049***
Tumour grade			
Well	5 (6)	4 (5)	
Moderate	43 (55)	15 (19)	
Poor	10 (12)	2 (3)	0.169
Tumour perforation			
Absent	49 (62)	20 (25)	
Present	9 (11)	1 (2)	0.167
Synchronous cancer (second primary cancer diagnosed within 6 months)			
Absent	51 (65)	21 (26)	
Present	7 (9)	0 (0)	**0.033***
Distant metastasis			
Absent	53 (67)	19 (24)	
Present	5 (6)	2 (3)	0.901
Microsatellite instability (MSI)			
Stable	42 (53)	16 (20)	
High	19 (24)	2 (3)	0.071


Fold change in AHR mRNA expression after 72hr PAH treatment in CCD841 CoN
Control
Vehicle control
50uM
25uM
10uM
1uM
*
10
1
0.1
Fold change
B[a]P
PHEN
FLU
ANTH
Polycyclic aromatic hydrocarbon treatment


a)
AHR mRNA expression (normalised by GAPDH)
100
50
0
-50
-100
CRC tumour vs matched non-neoplastic mucosal tissue
b)
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
survival
Normal AHR expression
Low AHR expression
High AHR expression


c)
AHR mRNA expression (normalised by GAPDH)
100
50
0
-50
Normal/high expression of AHR in patients with conventional adenocarcinoma


d)
AHR mRNA expression (normalised by GAPDH)
0
-20
-40
-60
-80
Low expression of AHR in patients with mucinous adenocarcinoma


a)
Aligned Fluorescence(%)
Temperature(°C)
Wildtype
Homozygous
Heterozygous
Wildtype
b)
Aligned Fluorescence(%)
Temperature(°C)
Wildtype
Heterozygous
Heterozygous
c)
Aligned Fluorescence(%)
Temperature(°C)
Wildtype
Homozygous
Homozygous