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Polymorphisms in PAH metabolising enzyme CYP1A1 in colorectal cancer and their clinicopathological correlations

Running title: CYP1A1 in colorectal carcinoma

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Abstract

CYP1A1 enzyme is integral to the biotransformation of polycyclic aromatic hydrocarbons to carcinogenic compounds. This study aimed to screen mutations in exon 7 (ex7) of CYP1A1 and investigate its clinicopathological correlations in fresh tissue samples from 85 patients (42 women; 43 men) with colorectal carcinoma (CRC). Tumour tissues and matched non-neoplastic mucosa tissues were collected prospectively. Genomic DNA was extracted from all tissues, and subject to high-resolution melt curve analysis for CYP1A1-ex7. Sanger sequencing was employed to detect specific mutations. Three known single nucleotide polymorphisms (SNPs) were identified in both tumour and matched non-neoplastic tissue for the same individual. Of the 85 patients, one third (n=28) harboured either rs1048943, rs1799814, or rs41279188. Patients who had a SNP at ex7 of CYP1A1 were significantly more likely to be over 65 years of age (*p=0.015*). Furthermore, individuals harbouring a SNP at exon7 showed a low incidence of perineural cancer infiltration (*p=0.025*) when compared to the wild-type population. Overall, polymorphisms at exon 7 of CYP1A1 are present in patients with CRC and associated with a few clinicopathological characteristics.

Keywords

CYP1A1, colorectal cancer, genetic variant, polycyclic aromatic hydrocarbon

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds involved in the meat-related carcinogenesis of colorectal carcinoma (CRC) [1]. The term is an umbrella term for a class of compounds containing hydrogen and carbon atoms arranged in more than one aromatic ring structure [2]. These compounds are formed in meats that have undergone processing or high-temperature cooking [1]. Many PAHs are capable of inducing toxicity and mutations leading to cancer in the human body [3]. Red and processed meat intake is an established risk factor for CRC, which is the third most commonly diagnosed cancer [1,4]. After ingestion, parent PAHs (procarcinogen) in meat undergo xenobiotic metabolism resulting in the formation of toxic/mutagenic/carcinogenic metabolites (carcinogen) [5]. The main metabolic pathway involved in PAH transformation is the CYP450/epoxide hydrolase (CYP/EH) pathway [6].

The CYP/EH metabolic pathway is capable of transforming a parent PAH compound to a carcinogenic diol-epoxide [6]. The two key enzymes involved in this pathway regarding CRC are cytochrome P450, family 1, subfamily A (CYP1A1) and microsomal epoxide hydrolase (mEH) [7]. Briefly, CYP1A1 converts the PAH to an epoxide intermediate, which is then converted to a dihydrodiol via epoxide hydrolase, and finally to a dihydrodiol epoxide (carcinogen) by CYP1A1 [6]. Thus CYP1A1 plays a major role in facilitating the formation of a PAH-dihydrodiol epoxide that can covalently bind DNA to create adducts in the colon [8] and increase cancer risk.

Composed of seven exons and six introns, the *CYP1A1* gene is located on chromosome 15q22-q24 [9]. *CYP1A1* is a highly polymorphic gene and much attention has been given to polymorphisms of four single nucleotide polymorphisms (SNPs); T3801C (m1), A2455G (m2), T3205C (m3), and C2453A (m4) [9,10]. M2 and m4 SNPs are located on exon 7 (ex7)

68 and both SNPs result in amino acid changes; isoleucine to threonine at codon 462 and
69 threonine to asparagine at codon 461, respectively [11].

70 Previous studies have indicated the potential roles of CYP1A1 SNPs at exon 7 in breast
71 and lung cancers [12–15]. There is limited research on CYP1A1 variants and its association
72 with CRCs, with most existing studies focusing on CRC risk in case-control populations [16–
73 18]. Therefore, we aimed to investigate the prevalence of *CYP1A1* exon 7 variants and its
74 clinicopathological correlations in patients with CRC.
75

Materials and methods

Patients and clinicopathological data recruitment

Tumour tissues and matched non-neoplastic mucosa tissues (taken adjacent to the margin of surgical resection) from the same patient with CRC, were collected prospectively from 2012-2015 by a colorectal cancer surgeon (CTL). These tissues were collected in fresh from hospitals in Queensland, Australia, and were snap-frozen in liquid nitrogen and stored at -80°C until use. Recruitment of patients for this study was not subject to any bias. Tissue samples that lacked adequate cancer mass or patients who did not return for clinical follow-up were excluded from the study. Ethical approval for this study was obtained from the Griffith University Human Research Ethics Committee (GU Ref No.: MSC/17/10/HREC).

The other portion of the surgical specimen for primary CRC were fixed in formaldehyde and representative blocks were taken for histological examination and pathological staging. Histological sections were sliced from these blocks and stained with haematoxylin and eosin (H&E) for examination via light microscopy. The sections of prospectively collected cancer tissue were reviewed to confirm at least 70% volume was composed of CRC. Only primary adenocarcinoma was included in this study. Pathological features of all cancer tissues were reviewed by an anatomical pathologist (AKL) based on the World Health Organization (WHO) criteria [19]. Additionally, the matched non-neoplastic mucosa tissue collected was reviewed to ensure there was no cancer present. Once the prospectively collected tissues were confirmed as satisfactory for inclusion, they were sectioned in 7µm slices using a cryostat (Leica Biosystems, Mt Waverley, VIC, Australia) for extractions of genetic material.

Clinical management

Clinical management of the patients was performed according to a pre-agreed standardised multi-disciplinary protocol. The use of neo-adjuvant and adjuvant therapy was decided following the latest surgical guidelines [20]. The follow-up period and survival time were calculated as the date of surgery to the date of death or the date of the most recent clinical visit. Post-operative cancer recurrence or presence of residual cancer was also recorded.

DNA extraction

DNA extraction and purification on the cut sections were done using QIAGEN DNEasy Blood and Tissue Kits (QIAGEN, Hilden, Germany) as per the manufacturer's protocol. DNA was quantified using the Nanodrop spectrophotometer (BioLab, Ipswich, MA, USA). Concentrations were recorded as ng/µl and purity were measuring using 260/280 ratio. Samples were stored at -20°C until further analysis.

High-resolution melt (HRM) curve analysis

HRM analysis was performed on genomic DNA on the paired samples of cancer and matched non-neoplastic tissues to detect alterations in sequences at exon 7 of CYP1A1. A 204 base pair fragment on CYP1A1-ex7 was amplified using 5' CTGTCTCCCTCTGGTTACAGGAAGC 3' forward primer and 5' TTCCACCCGTTGCAGCAGGATAGCC 3' reverse primer [21]. Amplification of target sequences was achieved using QuantStudio HRM facility and HRM Software for QuantStudio™ 6 and 7 systems (QuantStudio, ThermoFisher Scientific, Waltham, MA, USA). A total reaction volume of 10µl consisted of 5µl 2x SensiFAST HRM Kit (Meridian Bioscience Inc., Cincinnati, OH, USA), 0.4µl 10µM forward and reverse primers, 1µl of

30ng/µl gDNA, and 3.2µl diethylpyrocarbonate (DEPC, RNase free) treated water. The thermocycling protocol was as per the mastermix guidelines. Melt curve data were obtained every by reading fluorescence at each increment of temperature increase between 65-85°C; the rate of temperature increase was 0.05°C per second. As described previously [22], all runs included a no-template control, and all reactions were performed at a minimum of duplicates. Variant alleles appeared as a distinct melt curve that differed to the curve displayed by wildtypes. HRM results were interpreted as mutant when both replicates were identical to each other, but different to the wild-type melt curve.

Purification of polymerase chain reaction (PCR) products and Sanger sequencing

Potential variant samples detected by HRM analysis was subject to Sanger sequencing to confirm its genomic sequence. PCR products were subject to gel electrophoresis and purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA, USA) following the manufacturer's protocol. These purified products underwent sequencing using Big Dye Terminator (BDT) chemistry version 3.1 (Applied Biosystems, Foster City, CA, USA) under standard PCR cycling conditions. This data was then analysed by the 3730xl Capillary sequencer (Applied Biosystems) at Australian Genome Research Facility (AGRF).

Sequence analysis

Chromatograms were analysed using Chromas 2.31 sequence analyser software. In addition to rigorous manual analysis of chromatograms, variants were identified using NCBI: BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and further confirmed using Indigo: Rapid Indel Discovery (https://www.gear-genomics.com/indigo). In-silico predictions regarding disease causing, damaging, and/or deleterious variants were investigated using bioinformatic

149 tools such as MutationTaster (NCBI 37/Ensembl 69) [23], PROVEAN (Protein variation
150 effect analyser) and SIFT (Sorting intolerant from tolerant). Using PROVEAN and SIFT, the
151 cut-off values of -2.5 and 0.05 were used for the prediction of pathogenic and non-pathogenic
152 mutations, respectively.
153
154 **Statistical analysis**
155 Comparisons between CYP1A1 variants and clinicopathological parameters were
156 performed using the Chi-square test, likelihood ratio, and Fisher's exact test. Survival
157 analysis was performed using the Kaplan-Meier estimator. SPSS Statistics 27 (IBM SPSS
158 Inc., New York, USA) was used for all statistical analyses. Statistical significance was
159 accepted at $p < 0.05$).
160
161

Results

Clinical characteristics

The patient cohort included in this study comprised 85 patients (42 women, 43 men) with CRC. The mean age of the cohort was 68 years, ranging from 24-91 years. 57.6% of patients had stage I/II CRC, while 42.4% had stage III/IV cancers. In addition, 43.5% were proximal (right colon) cancers (located in the caecum, ascending or transverse colon), while the remaining 56.5% were classified as distal (left colorectum) tumours (pertaining to descending colon, sigmoid colon, or rectum). The median survival of the patient cohort is 72 months. There was no loss of patients to follow up and the maximal follow up period was 113 months.

Identification of *CYP1A1* exon 7 variants in colorectal tissue samples

CYP1A1 variants in CRC and matched non-neoplastic tissues were initially suspected based on HRM curve deviation from the wild type. Three variants were suspected via HRM analysis, and then confirmed with Sanger sequencing. All three suspected variants were known SNPs: rs1799814 (m4 variant), rs1048943 (m2 variant), and rs41279188. Out of 85 patients in this cohort, eight patients harboured rs1799814, nine patients had rs1048943, and eleven patients had rs41279188 variants; the remaining 57 patients harboured the wild-type sequence for CYP1A1-ex7 (Table 1). All individuals expressed identical sequences in both cancer and matched non-neoplastic tissues.

The three detected SNPs were expressed heterozygously as confirmed by Sanger sequencing and chromatogram analysis (figure 1). In-silico analysis results presented within table 1 shows that m2 and m4 variants are likely to be neutral polymorphisms that are tolerated, while rs41279188 is predicted to be deleterious and disease-causing that is not tolerated. None of the SNPs caused frameshift mutations.

Clinicopathological correlations of CYP1A1 exon 7 variants

Associations between patients with a wild-type sequence versus those with a polymorphism at CYP1A1-ex7 are presented in table 2. Individuals who harboured a polymorphism at this site were significantly less likely to be less than 65 years of age compared to those with wild-type alleles (*p=0.015*). Patients who detected positive for a polymorphism were significantly less likely to have received pre-operative adjuvant therapy (*p=0.009*), and less likely to have had perineural infiltration by cancer (*p=0.025*). Tumour size, subsite, and presence of other polyp(s) were also noted to be nominally associated with the presence of a SNP at CYP1A1-exon 7, though these did not reach statistical significance. Gender, cancer perforation, lymph node metastasis, metastasis, pathological stage, tumour grade, lymphovascular invasion were not associated with CRC patients harbouring CYP1A1-exon 7 polymorphism.

Analysis of specific polymorphisms (rs1048943, rs1799814, rs41279188) within the entire cohort and these clinicopathological parameters did not reach statistical significance (Table 3). However, age (*p=0.071*) and administration of pre-operative adjuvant therapy (*p=0.079*) are noted to be nominally associated.

Further investigations into individual SNPs are reported in Table 4. CRC patients with rs1048943 were significantly more likely than non-rs1048943 patients (i.e., wildtype, rs1799814, rs41279188) to have a tumour invasion depth at level 3 (*p=0.033*). Additionally, CRC patients with rs41279188 SNP were significantly less likely to have distal colorectal tumours compared to non-rs41279188 (i.e., wildtype, rs1048943, rs1799814) (*p=0.036*). Specifically, rs41279188 patients were less likely to have tumours of the sigmoid colon and rectum (*p=0.022*).

210

211 **Association with patient survival**

212 The patient cohort displayed an expected distribution of survival based on tumour
213 stages, whereby stage IV individuals showed decreased survival and survival times improved
214 as tumour staging decreased. Although not statistically significant, patients with either
215 rs1048943, rs1799814, or rs41279188 showed a slightly improved survival by three months
216 (Figure 2).

Discussion

CYP1A1 is a key enzyme in metabolising xenobiotics such as polycyclic aromatic hydrocarbons into mutagenic substrates that can cause DNA damage [24]. The current study shows that genetic variants in CYP1A1-exon 7 are present and are associated with clinicopathological characteristics in a cohort of patients with colorectal cancer . Recent evidence regarding CYP1A1 and its relationship with CRC is unclear, as some results suggest an association with modified CRC risk and meat intake [16,17], whilst other studies show no relationship between this gene and CRC [25–28]. The m2 variant (rs1048943) has been linked to higher enzymatic activity in patients with lung cancer [29] and thus can confer higher cancer risk due to more carcinogenic metabolites being produced. rs1799814 and rs41279188 have not been extensively studied in CRC. The current results suggest that individuals with either rs1048943, rs1799814 or rs41279188 at ex7 are slightly protected from CRC. The lack of perineural infiltration in CRC from these patients may be in concur with this concept. Additionally, patients with a polymorphism were older, therefore making their cancer more likely a disease of age. Moreover, individuals with a polymorphism at this site were less likely to have associated polyps compared to individuals with wild-type alleles. The association with lack of pre-operative adjuvant therapy with CYP1A1 polymorphisms requires further investigation with a larger patient cohort. Advanced stage rectal cancer are often treated by adjuvant therapy before surgery to increase the chance of complete resection. It is likely that rectal cancer patients with polymorphism are of lower clinical staging and have not been treated by pre-operative therapy. Taken together, our results suggest that patients with a polymorphism exhibit more favourable clinicopathological characteristics when compared with the wild-type cohort.

The activity of CYP1A1 pushes the PAH through a metabolic pathway that ultimately has two fates; excretion or creation of a DNA adduct [30]. As it is uncertain about the

activities of CYP1A1 in individuals with a polymorphism, those with a high CYP1A1 activity resulting from their SNP may also have high rates of PAH-metabolite excretion. This excretion is determined by the biological activities of detoxifying enzymes such as GSTM and UGT families [31], and have not been investigated in this current study.

rs41279188 was predicted to have damaging effects, possibly because it induced a missense mutation. Our initial analysis shows that CRC patients with rs41279188 are less likely to have tumours of the distal colorectum. Distal colorectal cancers tend to present earlier and have a better prognosis [32], thus the predicted damaging effects of rs41279188 could explain this relationship. Further research into this SNP and its role in prediction and causation of proximal/distal CRCs would be helpful to understand this link.

This study is limited due to the low number of patients who harboured each polymorphism. However, our results link the presence of a polymorphism at CYP1A1-exon 7 to age and some tumour characteristics and provide a basis for future investigations into this gene and associated genes in colorectal cancer.

Conclusion

Patients who had SNPs at CYP1A1 exon 7 composed almost one-third of this CRC study population. Three SNPs (rs1048943, rs1799814, and rs41279188) were significantly associated with advanced age and perineural invasion. Such results suggest a protective role for these polymorphisms. However, future studies are warranted to fully understand the molecular pathogenesis of the genetic variants. Overall, the CYP1A1 SNPs are linked to favourable clinicopathological characteristics in our cohort of patients with CRC.

Figure legends

Figure 1. Exonic variants detected in colorectal adenocarcinoma and matched non-neoplastic mucosa tissue. Comparison of wild-type HRM curves and chromatogram to detected variants in CYP1A1 exon 7

Figure 2. Survival distribution in colorectal cancer and perineural invasion correlation to CYP1A1 polymorphism. A) survival rates in patients with colorectal adenocarcinoma as per cancer stage. B) Patients with polymorphism at exon 7 of CYP1A1 have a longer survival time compared to those with wild-type alleles ($p>0.05$). C) Patients without a polymorphism at exon 7 of CYP1A1 are more likely to have perineural invasion.

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