AMERICAN UNIVERSITY OF BEIRUT

THE ARYL HYDROCARBON RECEPTOR REPRESSOR (*AhRR*) METHYLATION STATUS AS A SMOKING BIOMARKER IN UROTHELIAL BLADDER CANCER TUMORS

by
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...may her soul rest in peace...

ABSTRACT
OF THE THESIS OF

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Title: The Aryl Hydrocarbon Receptor Repressor (*AhRR*) Methylation Status as a Smoking Biomarker in Urothelial Bladder Cancer Tumors

Urinary Bladder Cancer (BCa) is the 10th most incidental malignancy worldwide, with higher rates in industrialized countries. Smoking is considered one of the most important risk factors for BCa. In addition, studies have shown that cigarette smoking results in the demethylation of the *AhRR* gene in blood cells, establishing *AhRR* demethylation as a specific serum smoking biomarker. This study aimed to investigate the value of this biomarker in the target tissue and its possible role in bladder carcinogenesis. We sub-selected 180 tumor-based DNA samples from 263 histologically confirmed BCa patients diagnosed between 2013 and 2017 in two major medical centers in Beirut. *AhRR* % methylation in tumor DNA was determined by bisulfite conversion, pre-amplification by PCR, and differential methylation by droplet digital PCR. Associations between *AhRR* % methylation, patient's smoking status, and tumorigenic outcome indicators were examined using the two-tail Student's t-test. Our results show that muscle-invasiveness is significantly associated with a higher *AhRR* % methylation compared to non-muscle invasive tumors (42.86 ± 23.85% vs. 33.98 ± 20.36%; p=0.011). In addition, oncogenic *FGFR3* E7 C248 mutant genotype was also found to be significantly associated with a lower *AhRR* % methylation compared to wild-type (28.11 ± 9.44% vs. 37.87 ± 22.53%; p=0.036). All other tested associations were not statistically significant. Further research investigating the role of *AhRR* in muscle-invasive bladder cancer tumors (MIBC) and bladder carcinogenesis is recommended.

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ABBREVIATIONS

ACS	American Cancer Society
AhR	Aryl Hydrocarbon Receptor
AhRR	Aryl Hydrocarbon Receptor Repressor
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASR	Age-Standardized Rate
AUB	American University of Beirut
BCa	Urinary Bladder Cancer
BH	Bahman Hospital
CI	Confidence Interval
CO	Carbon Monoxide
CYP	Cytochrome P450
ddPCR	Droplet Digital Polymerase Chain Reaction
DME	Drug-Metabolizing Enzyme
DNA	Deoxyribonucleic Acid
FFPE	Formalin-fixed Paraffin-embedded
FGFR3	Fibroblast growth factor receptor 3
GST	Glutathione S-transferase
HG	High Grade
IRB	Institutional Review Board
LG	Low Grade
MIBC	Muscle-Invasive Bladder Cancer
MoPH	Ministry of Public Health
NAT1	N-acetyltransferase 1

NAT2	N-acetyltransferase 2
NLM	US National Library of Medicine
NMIBC	Non-Muscle Invasive Bladder Cancer
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated biphenyl
PCR	Polymerase Chain Reaction
$PM_{2.5}$	Particulate Matter <2.5µm
qPCR	Quantitative Polymerase Chain Reaction
RB1	RB transcriptional corepressor 1 (previously known as Retinoblastoma 1)
RNA	Ribonucleic acid
SAS	Statistical Analysis Software
SD	Standard Deviation
SGHUMC	St. George Hospital University Medical Center
SNP	Single Nucleotide Polymorphisms
TCC	Transitional Cell Carcinoma
TNM	Tumor-Nodes-Metastasis Staging System
TP53	Tumor protein 53
US/USA	United States of America
WHO	World Health Organization
XRE	Xenobiotic Response Element

CHAPTER 1

INTRODUCTION

1.1. Background Information

Urinary Bladder Cancer (BCa) is the 10th most incident malignancy worldwide, accounting for 3% of all cancers (Ferlay et al., 2018). Globally, the most common type of BCa is urothelial carcinoma, also known as transitional cell carcinoma (TCC), which begins in the cells lining the bladder wall (American Cancer Society [ACS], 2019b). Other much less common types of BCa include squamous cell carcinoma, adenocarcinoma, small cell carcinoma, and sarcoma (ACS, 2019b). Two TCC subtypes are papillary carcinoma and flat carcinoma. Unlike flat carcinoma, which grows within the wall layer of bladder cells, papillary carcinoma grows in a finger-like structure, starting from the wall of the bladder, and expanding towards the bladder's inner hollow space (ACS, 2019b). Flat carcinomas are more likely to invade the detrusor muscle, known as MIBC, whereas papillary carcinoma tends to be non-muscle invasive (NMIBC) (McConkey & Choi, 2018). NMIBC does not usually metastasize and is less likely to cause death but tends to recur frequently; whereas MIBC tends to progress and metastasize rapidly and aggressively, reaching other organs in the body and showing poorer prognosis (McConkey & Choi, 2018).

1.1.1. Tumorigenic Outcome

Grade, stage, and molecular characteristics are important outcomes assessed at diagnosis of BCa tumors. The most recent tumor grading system, updated by the World Health Organization (WHO) in 2016, classifies BCa tumors as low grade (LG) or high

grade (HG) (Comperat et al., 2019). Around 30% of BCa tumors are classified as HG at diagnosis, while about 70% are diagnosed as LG, of which 10-15% may progress to HG (Apollo et al., 2019). Another important clinical aspect at diagnosis of BCa, is the tumor stage, which defines the progress of the disease as well as tumor's invasiveness. The main pathologic stages are pTa and pT1 – pT4 (Kirkali et al., 2005). The tumor stage can also be identified as per the tumor-nodes-metastasis (TNM) staging system, with T qualifying the tumor size, "N" a qualifier for reaching lymph nodes, and "M" qualifying metastasis (Kirkali et al., 2005; Knowles & Hurst, 2015; Sanli et al., 2017). In principle, stages pTa-pT1 are considered non-invasive tumors (NMIBC), while stages pT2-pT4 are classified as MIBC (Apollo et al., 2019). Figure 1 illustrates the main types, grades, and stages of BCa (Knowles & Hurst, 2015).

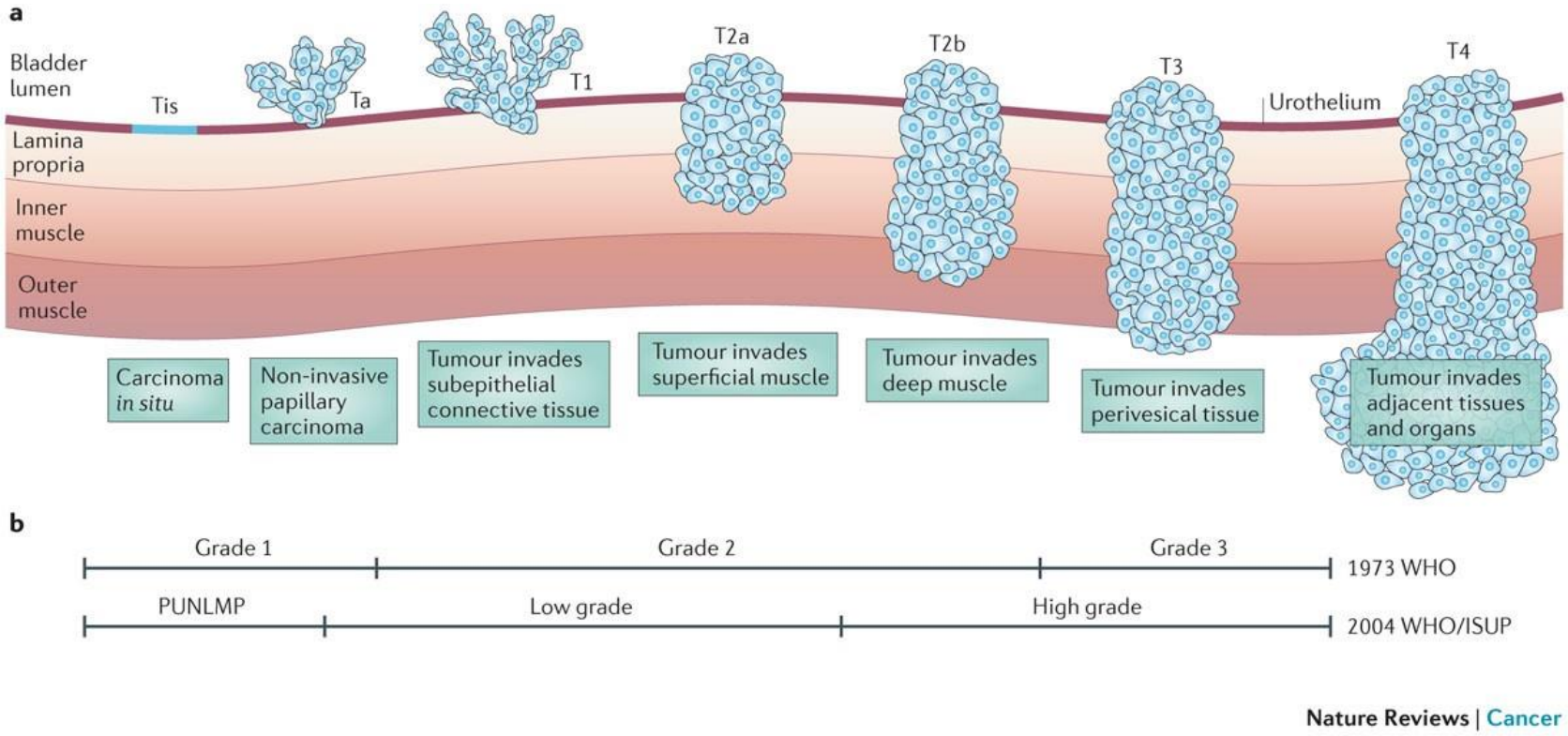

Figure 1. Types, stages, and grades of bladder cancer

Adopted from (Knowles & Hurst, 2015)

1.1.2. BCa Molecular Characteristics

MIBCs are further sub-grouped into two major intrinsic categories, luminal and basal, and very recently six MIBC molecular subtypes have been identified based on an international consensus on gene expression and mutation load patterns; these include the luminal papillary, luminal non-specified, luminal unstable, stroma-rich, basal/squamous, and neuroendocrine-like subtypes (Kamoun et al., 2020). These subtypes were determined based on a large group of molecular determinants, including frequency of mutations in key tumor markers, such as *TP53* (tumor protein 53), *FGFR3* (fibroblast growth factor receptor 3), and *RB1* (RB transcriptional corepressor 1) genes (McConkey & Choi, 2018). *TP53*, *FGFR3*, and *RB1* are genes that code for proteins, which regulate cell growth and division. In particular, *TP53* and *RB1* are tumor-suppressor genes that code for the tumor protein p53 and the retinoblastoma protein, respectively, both of which act as tumor-suppressor proteins that help in preventing uncontrolled cell division (National Library of Medicine (US) [NLM], 2020b; 2020c). The *FGFR3* gene codes for a fibroblast growth factor receptor, which is a receptor that signals cells to grow through interacting with growth factors from outside the cell (NLM, 2020a). Mutations in those genes may result in activation or deactivation of gene expression, which subsequently affects cell growth and progression towards a tumor. Frequency of mutations in the three genes mentioned above is associated with invasiveness, grade, and stage of the tumor. In fact, numerous studies have shown that NMIBC tumors are associated with a higher prevalence of *FGFR3* mutations (Comperat et al., 2019; Smal et al., 2014; Tomlinson et al., 2007). *FGFR3* activating mutations were found to be higher in low grade and in primary stage (pTa) papillary NMIBC

tumors (Geelvink et al., 2018; Lamy et al., 2006; Smal et al., 2014; Tomlinson et al., 2007). This shows that *FGFR3* mutation plays an essential role in NMIBC tumors.

On the other hand, studies have found that MIBC tumors are more likely to have mutations in *TP53* and *RB1*, which deactivates their expression (Comperat et al., 2019; Gallucci et al., 2005; Lamy et al., 2006; Mitra et al., 2007). Lamy et al. (2006), Neuzillet et al. (2012), and Smal et al. (2014) showed that, unlike *FGFR3*, mutant *TP53* was more common in high grade and high stage invasive BCa. Additionally, in a study on MIBC specimens and adjacent normal mucosa, Gallucci et al. (2005) found an association between *RB1* heterozygous deletion and stages pT3-pT4. These findings show that *TP53* and *RB1* play a fundamental role in predicting the invasiveness of BCa tumors. Mutations in key genes are also associated with recurrence. In a study on 57 BCa patients with superficial cancer, van Rhijn et al. (2001) found that patients with the wild-type *FGFR3* were more likely to develop recurrent BCa than those with the mutant type. However, another study found that BCa was more likely to relapse sooner in patients with *FGFR3* mutation at diagnosis (Couffignal et al., 2015). Similarly, a more recent study by Zhu et al. (2019) found that mutant *FGFR3* and mutant *TP53* were more likely to be found in patients with recurrent NMIBC. This inconsistency in the reported results may be a result of different patient characteristics or different follow-up times and requires further investigation (Couffignal et al., 2015). Identifying genetic characteristics of BCa is essential not only for diagnostic and prognostic reasons, but also for better understanding of bladder carcinogenesis, which would provide insights into etiology and prevention.

1.1.3. Bladder Cancer Epidemiology

BCa is common around the world, constituting 3% of all cancer cases worldwide, with an estimate of 199,922 deaths and 549,393 new cases reported in 2018, ranking as the 10th most incident and 13th most fatal malignancy in the world (Ferlay et al., 2018). Common gender differences in BCa have been observed, whereby males have a 3 to 4 times higher risk of acquiring the disease compared to females (Geavlete et al., 2016). A worldwide epidemiological pattern marks BCa, where the highest BCa incidence rates are found in industrialized countries in Europe and North America, while a much lower incidence is observed in developing countries (Bray et al., 2018; Khazaei et al., 2020; Mahdavifar et al., 2016). This epidemiological pattern is not observed in Lebanon, where BCa incidence is globally one of the highest (Khazaei et al., 2020). Despite the relative declines reported in a study by Khachfe et al. (2020), BCa incidence in Lebanon is globally the highest in females (ASR=9.4/100,000) and the second highest in males (ASR=40/100,000) (Bray et al., 2018; Ferlay et al., 2018; MoPH, 2015). In fact, males constitute about 85% of cases with a mean age of 66-68 years old, with little knowledge about the underlying factors (Kobeissi et al., 2013; MoPH, 2015). BCa is also the second-fastest-growing cancer in Lebanon among men over 65 years of age (Haddad et al., 2018). Interestingly, Lebanon is the only country where TCC incidence surpasses lung cancer in males in some years. Egypt is also an exception to the global patterns, historically with a high prevalence of squamous cell carcinoma, which was associated with a high rate of schistosomiasis (Kyritsi et al., 2018; Zheng et al., 2012). Figure 2 shows the ASRs (Age-Standardized Rates) for the top 10 cancers in Lebanon between both genders.

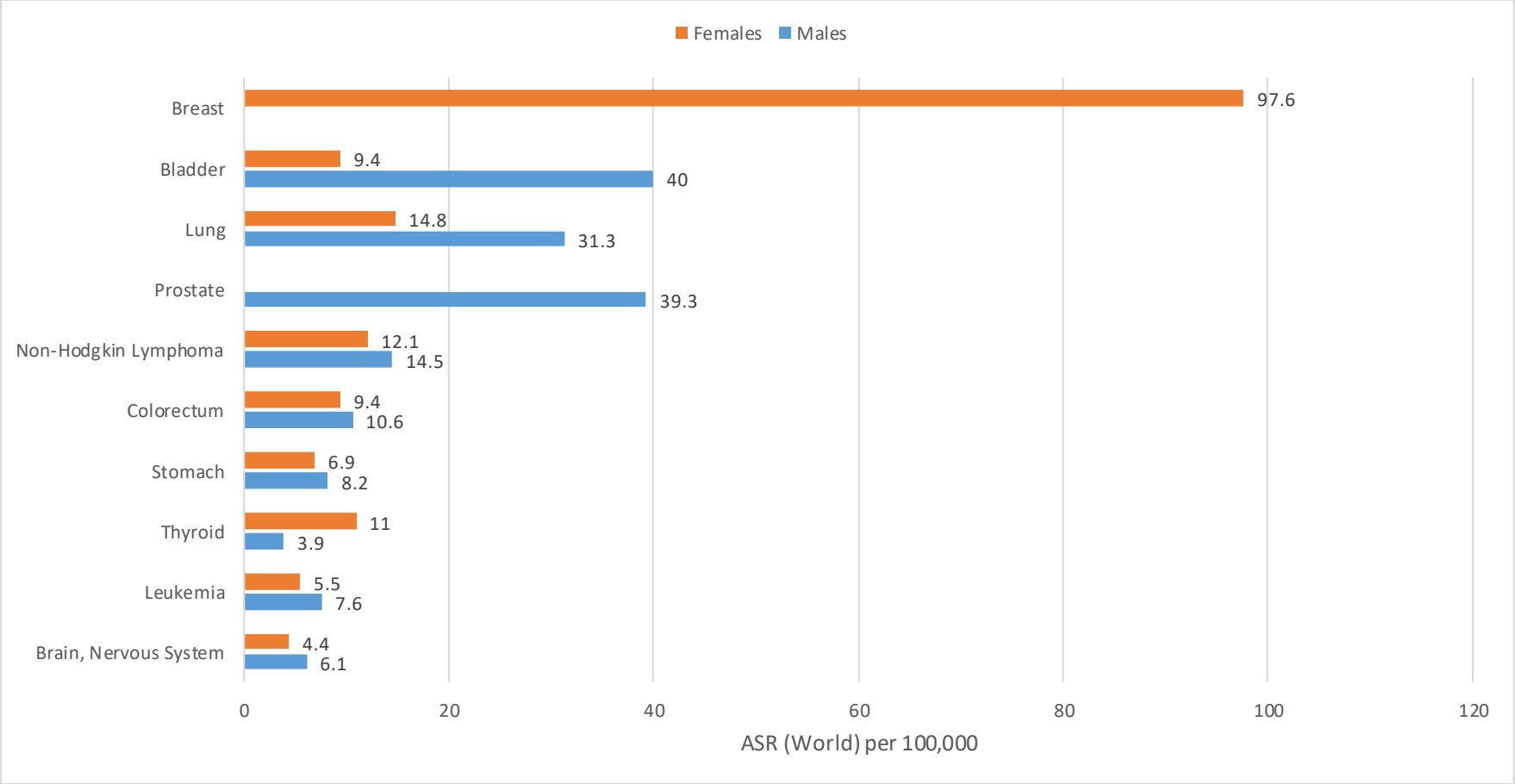

Figure 2. Age standardized incidence rate by gender for the top 10 cancers in Lebanon

Adapted from (Ferlay et al., 2018)

1.1.4. Bladder Cancer Etiology

Most research on BCa etiology suggests that it is an acquired malignancy from environmental and occupational exposures, including aromatic amines, polycyclic aromatic hydrocarbons (PAHs), nitrosamines, water chlorination disinfection by-products, and tobacco smoke (Burger et al., 2013; Cumberbatch et al., 2018; Deb et al., 2019; Lakkis et al., 2018). Genetic predisposition may also increase BCa risk. While some people inherit gene variations that increase the risk of BCa, most BCa-related gene mutations are acquired from carcinogen exposures rather than inherited at birth (ACS, 2019a). Several studies report that variations in N-acetyltransferase 1 (*NAT1*), *NAT2*, and Cytochrome P450 (*CYP*), Glutathione-S-Transferases (*GST*s), among others, increase the risk of BCa (Dhaini et al., 2018; El Kawak et al., 2020; Kumondai et al., 2016; Lukas et al., 2017; Nasr et al., 2017; Yin et al., 2018). Many affected genes are drug-metabolizing enzymes (DMEs) involved in the metabolism of carcinogens. Boada et al. (2015) suggest that genetic polymorphism in genes related to DMEs may play a role in BCa associations with PAH exposure. Therefore, a genetic variant may increase the susceptibility of an individual to develop BCa, by either reducing detoxification or increasing bioactivation of certain carcinogens in the body (ACS, 2019a; Cui et al., 2013). Studies have also shown that prostate cancer patients receiving radiotherapy and radical prostatectomy treatments are more likely to acquire BCa shortly after prostate cancer diagnosis (Abern et al., 2013; Liauw et al., 2006; Wallis et al., 2016), suggesting that radiation may be reaching bladder tissues and causing DNA damage. On the other hand, some infections, such as that of the *Schistosoma haematobium*, have also been linked with BCa, particularly Squamous-Cell Carcinoma (Nesi et al., 2019). *S.*

haematobium is a parasite that causes schistosomiasis (or bilharzia) in humans (Colley et al., 2014).

Most of the environmental risk factors for BCa, such as PAHs, $PM_{2.5}$, 4-aminobiphenyl, nitrosamines, among a mosaic of other components, are major constituents of tobacco smoke (Rodgman & Perfetti, 2013). In fact, epidemiologic evidence shows that tobacco smoking is one of the most important risk factors for BCa (Burger et al., 2013; Kispert et al., 2019; van Osch et al., 2016). The number of smokers has been declining in most Global North regions (Americas and Europe), but not in Global South regions (African region and the Eastern Mediterranean Region), where an increase in smokers is observed in most low-income countries (WHO, 2019). Lebanon, located in the Eastern Mediterranean Region, has a high prevalence of both cigarette and water-pipe smoking. According to the WHO Global Health Observatory database, overall, the prevalence of smoking, for persons aged 15 years and older, in Lebanon was 45.4% in 2015 and is projected to reach 55.4% in 2025, marking among the highest rates globally (WHO, 2015).

Genetic predisposition of DMEs, such as *CYP 2E1* and *NAT1*, have also been suggested to increase BCa risk in Lebanon (Basma et al., 2013; Yassine et al., 2012). Temraz et al. (2019) also considered water chlorination by-products, as contributing risk factors for BCa in the Lebanese population. However, there are still huge data gaps on BCa etiology, particularly in the context of Lebanon (Dhaini & Kobeissi, 2014). Tobacco smoking severity was previously reported to be a major risk factor for BCa in Lebanese men (Kobeissi et al., 2013; Temraz et al., 2019).

1.1.5. Smoking Biomarkers

Various biomarkers have been found to indicate exposure to tobacco smoking, including cotinine levels, exhaled carbon monoxide (CO), hemoglobin arylamine adducts, and Aryl Hydrocarbon Receptor Repressor (*AhRR*) methylation status (Andersen et al., 2017; Benowitz, 1999; Chang et al., 2017). Most of these measures have been proven to have low to moderate specificity or sensitivity (Benowitz, 1999). Moreover, the majority of smoking biomarkers are serum-based, and therefore reflect acute exposure to tobacco smoke with little insight into former exposure for quitters. Although commonly considered a gold standard smoking biomarker, cotinine, the main metabolite of nicotine that is tested in blood serum, urine, or saliva, is limited by its short half-life (about 16-18 hours) and its potential false positives during nicotine replacement therapies for smoking quitters (Andersen et al., 2017; Benowitz et al., 2009; Hukkanen et al., 2005). Exhaled CO is also limited by its very short half-life of 4-5 hours and its other potential environmental sources (Florescu et al., 2009). Arylamine hemoglobin adducts also have moderate specificity and sensitivity (Benowitz, 1999). Moreover, hemoglobin resides on red blood cells, which have relatively short lifespans of about 120 days (Arias & Arias, 2017). Thus, cotinine, exhaled CO, and hemoglobin arylamine adducts are not highly sensitive to detect intermittent or former smoking.

Studies have also clearly established the methylation status of the *AhRR* gene at the cg05575921 locus as a serum biomarker for cigarette smoking in whole blood (Andersen et al., 2017; Bojesen et al., 2017; Dawes et al., 2020; Mikeska & Craig, 2014; Philibert et al., 2019; Philibert et al., 2013; Shenker et al., 2013; Zeilinger et al., 2013). Specifically, cigarette smoking has been shown to demethylate *AhRR* (Andersen et al., 2017). Zhang et al. (2016) found a strong association between gradual *AhRR*

demethylation and both cotinine levels and the number of cigarettes smoked daily. Dawes et al. (2019) and Philibert et al. (2019) have also attributed *AhRR* demethylation in saliva samples to smoking. Some other studies have reported that PAHs, $PM_{2.5}$, and polychlorinated biphenyls (PCBs) are also possible factors that cause *AhRR* demethylation (Alhamdow et al., 2018; Su et al., 2019; Tantoh et al., 2019). However, given that all these substances are components in the tobacco smoke mosaic (Invernizzi, 2004; Lloyd & Denton, 2005; Lu & Zhu, 2007), smoking is currently established as a causative agent for *AhRR* demethylation in blood cells. For these reasons, *AhRR* methylation status is considered a smoking biomarker in blood (Philibert et al., 2019).

However, DNA in blood is mostly contained in white blood cells that have a lifespan of about 13-20 days (Manik et al.). This short lifespan may revert smoking-related demethylation in blood, where research has shown that *AhRR* methylation levels in blood may revert to normal after reducing or ceasing smoking (Philibert et al., 2016; Philibert et al., 2020). Based on this evidence, blood-based assays for *AhRR* methylation may not be highly effective in detecting former smoking. Since most available studies on smoking biomarkers are limited by being blood-based, they are restricted to acute exposures and do not give any insight into causal factors and mechanisms of action at the target tissue. This dearth of research on biomarkers in target tissues stresses the importance of identifying smoking biomarkers in various cells and tissues, including tumor tissues (Gao et al., 2015). Epigenetic biomarkers in the target tissue can potentially overcome the limitations associated with blood-based biomarkers in detecting exposure to tobacco smoke (Andersen et al., 2017).

1.1.6. AhRR Role in Metabolism

Drug-metabolizing enzymes (DMEs) facilitating the elimination or biotransformation of most xenobiotic compounds entering the body (Larigot et al., 2018) are induced through the aryl hydrocarbon receptor (AhR), which plays a key role in various xenobiotic pathways (Andersen et al., 2017). AhR, when activated by these compounds, is translocated into the nucleus where it forms a complex heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Larigot et al., 2018). This complex then acts as a transcription factor binding to DNA at xenobiotic response elements (XRE) of target genes, ultimately inducing expression of a wide panel of genes, including many DMEs (Larigot et al., 2018). As part of the regulatory process, another molecule, the *AhR* repressor (*AhRR*), acts as a feedback modulator by repressing AhR-dependent gene expression (Oshima et al., 2007). However, this regulatory process may be disrupted with *AhRR* demethylation due to smoking exposure. In fact, the demethylation of the *AhRR* gene results in its increased expression, which affects AhR-dependent gene expression and interferes with the metabolism of different toxicants and carcinogens, including tobacco smoke components (Andersen et al., 2017).

Some studies have found that *AhRR* demethylation is a risk factor for lung cancer (Baglietto et al., 2017; Fasanelli et al., 2015). Moreover, a recent study evaluating smoking among postmenopausal women reported that differential methylation of *AhRR* and the G-protein receptor 15 gene mediate the effects of smoking on BCa, potentially revealing downstream effects of smoking and a role in bladder carcinogenesis (Jordahl et al., 2019). The study postulates that increased expression of *AhRR* promotes chronic inflammation in bladder tissue, one of the risk factors for BCa.

The study also calls for more investigation of underlying mechanisms to expand knowledge on the relationship between BCa and smoking as the strongest known risk factor (Jordahl et al., 2019).

1.1.7. AhRR Methylation Analysis

Researchers have been studying DNA methylation for decades, and they have developed various methods to detect methylation levels (Kurdyukov & Bullock, 2016; Yu et al., 2018). Chip-based assays have been widely used in studies exploring epigenetic smoking biomarkers (Philibert et al., 2012; Philibert et al., 2013). However, this method has been criticized due to high cost and long process (Andersen et al., 2017). Pyrosequencing has been found to be suitable for differentially methylated samples, making it feasible for cancer samples; however, it requires specialized equipment and a PCR amplification step, which may result in amplification bias that affects the accuracy and precision of methylation detection (Alghanim et al., 2018; Han et al., 2020; Kurdyukov & Bullock, 2016; Shenker et al., 2013; Vidaki & Kayser, 2018). On the other hand, more affordable and easily performed methods to assess DNA methylation, such as quantitative PCR (qPCR) were developed; nevertheless, qPCR methods are limited in accuracy, precision, and requirement of external reference standards (Hayden et al., 2013). The recent emergence of droplet digital polymerase chain reaction (ddPCR) has helped in reducing the limitations associated with DNA methylation analysis approaches (Andersen et al., 2017; Han et al., 2020; Philibert et al., 2018; Yu et al., 2018). The ddPCR process entails fractioning a sample of bisulfite converted and amplified DNA into 15,000 – 20,000 hydrophobic droplets, thus breaking down the sample into thousands of separate reactions assessed individually in a droplet

reader (Andersen et al., 2017; Hindson et al., 2011; Yu et al., 2018). Assuming a Poisson distribution, the fractional abundance of methylated and demethylated alleles in the sample is measured based on the number of positive and negative droplet reactions (Andersen et al., 2017; Philibert et al., 2018; Yu et al., 2018). Compared with qPCR, ddPCR offers much higher precision, accuracy, and technical simplicity without requiring any external references (Andersen et al., 2017; Hindson et al., 2011). It also allows for lower median errors and more precise and accurate results, which reduced the amplification bias resulting from qPCR and pyrosequencing techniques (Han et al., 2020; Vidaki & Kayser, 2018). Moreover, ddPCR has shown higher accuracy when assessing DNA methylation in archival tumors, for both high and low-DNA input samples, regardless of bisulfite treatment efficiency (Lissa et al., 2018; Van Wesenbeeck et al., 2018). Many studies have already used the ddPCR approach successfully in assessing *AhRR* methylation in blood to determine smoking status (Andersen et al., 2017; Philibert et al., 2018). These studies have found that a methylation value within a population "set point" of 80-90% indicates an individual is a non-smoker, whereas individuals with lower than 80% methylation are more likely to be smokers (Andersen et al., 2017; Philibert et al., 2018). However, so far, no studies have examined the *AhRR* methylation status in bladder tumor tissues.

1.2. Research Question and Objectives

The overarching goal of this study is to investigate the value of *AhRR* methylation status as a possible smoking biomarker and contributor to BCa. The objectives of this study are to:

(1) Assess the correlation between *AhRR* methylation and self-reported smoking status in archival urothelial bladder cancer tissues.

(2) Examine the association between *AhRR* methylation and tumorigenic outcomes.

CHAPTER 2

METHODOLOGY

2.1. Study Population and Sampling

This work is part of a larger ongoing study, which aims at comprehensively investigating BCa etiology in the context of Lebanon (Basma et al., 2013; Dhaini & Kobeissi, 2014; Dhaini & Levy, 2000; El Kawak et al., 2020; Kobeissi et al., 2013; Lakkis et al., 2018; Yassine et al., 2012). Originally, the study population consisted of 263 histologically confirmed urothelial bladder cancer patients diagnosed between 2013 and 2017. BCa patients were identified from the archives of two major medical centers in Beirut, specifically 127 samples from St. George Hospital University Medical Center (SGHUMC), a major referral center in Achrafieh, and 136 samples from Bahman Hospital (BH), a midsize medical center in the Southern Suburbs of Beirut. Out of the total pool of identified samples, 180 tumor-based DNA samples were sub-selected for this thesis, based on the availability of DNA for downstream applications.

The originally recruited cases included Lebanese male patients above 50 years of age, starting with the most recently diagnosed. Non-Lebanese patients, those under the age of 50, subjects with additional cancer types, and those with missing archival tissues were excluded from the study. Given the low BCa incidence in females, enrollment focused on male patients to maintain statistical power. In addition to biospecimens, medical records were used to obtain information on grade and stage of the tumors, as well as patients' self-reported smoking status.

2.2. Ethical Consideration and IRB Approval

An IRB approval, with a waiver of consent, was previously obtained from the American University of Beirut (AUB) and from collaborating medical centers, prior to collecting samples and data. All acquired biospecimens and collected data were obtained as a de-identified set.

2.3. *AhRR* DNA Methylation Testing

Previously extracted tumor DNA had concentrations ranging between 20 and 706 ng/µL. All samples were standardized at a DNA concentration of 40 ng/µL to facilitate downstream applications. *AhRR* methylation at the cg05575921 locus in tumorigenic DNA was determined by performing (1) bisulfite conversion, (2) pre-amplification by polymerase chain reaction (PCR), and (3) DNA methylation assessment by droplet digital polymerase chain reaction (ddPCR). The following sections describe in detail each of the steps taken to determine *AhRR* methylation.

2.3.1. Bisulfite Conversion of Extracted DNA

Complete bisulfite conversion and cleanup of the extracted DNA for methylation analysis was performed in a 96-well setup, using the EpiTect 96 Bisulfite Kit (Qiagen, Valencia, Ca) according to the manufacturer's instruction protocol for 'sodium bisulfite conversion of unmethylated cytosines in DNA isolated from FFPE (formalin-fixed paraffin-embedded) tissue samples using a centrifuge'. The 180 samples were divided into 96-well plates for bisulfite conversion. For each sample, the DNA was dissolved in a bisulfite mix and RNase-free water, with the addition of DNA protect buffer, in a total volume of 140 µL using the provided EpiTect conversion plate.

Bisulfite conversion was then performed using a thermocycler with a heated lid. Thermal cycler conditions consisted of an initial 5-minute denaturation step at 95°C, a 25-minute incubation step at 60°C, a 5-minute denaturation step at 95°C, an 85-minute incubation step at 60°C, a 5-minute denaturation step at 95°C, a 175-minute incubation step at 60°C, and finally an indefinite hold at 20°C. The samples were then transferred to an EpiTect 96 plate for cleanup and elution using carrier RNA buffer, desalting buffer, de-sulfonating buffer, ethanol, and elution buffer. Multiple washing steps were performed before centrifugation at 40°C to ensure the evaporation of residual ethanol and final elution.

2.3.2. Pre-amplification by PCR

After bisulfite conversion, the *AhRR* region in the bisulfite converted DNA was amplified according to the manufacturer's instructions using a Smoke Signature Assay kit (IBI Scientific, Iowa). For each sample, around 3 – 5 µL of bisulfite-converted DNA was mixed with 5 µL of 2X Pre-Amp Master Mix in a 10 µL-volume reaction. Pre-amplification was performed in 96-well plates. The samples were amplified under the following thermocycler conditions: an initial 3-minute denaturation step at 95°C, followed by 20 cycles of 95°C for 15 seconds and 60°C for 60 seconds, and a final indefinite hold at 20°C. The plates were then stored at -20°C until ddPCR was performed.

2.3.3. DNA Methylation Assessment by ddPCR

Prior to performing ddPCR, an initial dilution step was performed to achieve an optimal number of independent strand templates (≅ 20,000 DNA strands) in the final

PCR solution. An average concentration of the pre-amplified samples per plate was determined by measuring a few random samples using a NanoDrop spectrophotometer (ThermoScientific, Waltham, MA). Accordingly, dilution ratios were optimized depending on the average concentration of each plate. Dilutions ranged between 1:35 and 1:3500. The percent methylation at the *AhRR* locus in each sample was quantified using Bio-Rad QX-200 Droplet Digital PCR System. Samples were run in duplicates, in 96-well ddPCR plates, with the addition of non-template controls (water blank), and both methylated ("C") and unmethylated ("T") plasmid controls to each batch. The reactions were prepared by adding ddPCR supermix (Bio-Rad) and *AhRR* primers and probes to obtain a final volume of 22 µL per sample replica for droplet generation. Droplets were then generated by adding 22 µL of DNA mix or controls along with 70 µL of droplet generation oil per sample replica to the droplet generator cartridge (8 wells per cartridge). Gaskets were used to cover the loaded cartridges, which were then placed into the Bio-Rad QX200 droplet generator. About 40 µL generated droplets were then transferred to 96-well PCR plates, which were sealed with an aluminum foil.

Once sealed, the plates were amplified by PCR according to the following thermocycler conditions: an initial 10-minute denaturation step at 95°C, 40 cycles of 95°C for 15 seconds and 55°C for 60 seconds, a 10-minute step at 98°C, and finally an indefinite hold at 12-20°C. After amplification, plates were read using Bio-Rad QX-200 ddPCR reader, which quantifies the number of droplets containing at least one methylated "C" allele, at least one unmethylated "T" allele, as well as at least one of both alleles and no amplifiable alleles. Droplet counts, concentrations, 2D amplitude graphs, and fractional abundances were then assessed using QuantaSoft Analysis Pro Software (version 1.0.596). The percentage methylation at the *AhRR* locus was then

determined using the average fractional abundance between duplicates of each sample. Samples with no amplifiable alleles and those with more than 30% difference between duplicates were discarded (Bojesen et al., 2017).

2.4. Statistical Analysis

2.4.1. Study Variables

The variables of the study included self-reported smoking status, *AhRR* methylation percentage, and tumorigenic outcome.

a. Patients' self-reported smoking status was classified into two categories as 'Ever Smoker' or 'Never Smoker'.
b. *AhRR* methylation percentage at the cg05575921 locus was assessed as a continuous variable.
c. Tumorigenic outcome consisted of three main outcomes: presence of mutations in key tumor markers (*TP53* codons 72 and 248, *RB1* exon 23, and *FGFR3* exon 7 codons 248 and 249), with occasional grouping of homozygous and heterozygous mutants together; Tumor grade assessed as 'Low Grade' or 'High Grade'; Muscle-invasiveness determined as 'Non-Invasive' (PTa-PT1) or 'Invasive' (pT2-pT4).

2.4.2. Analysis

Descriptive statistics were used to summarize the characteristics of the tumors with their distribution frequencies. Boxplot and histogram were generated to check normality in the study population. Univariate analysis consisted of frequency and

percentage distributions for the different categorical variables in the study. Means, standard deviations (SDs), 95% Confidence Intervals (CIs), and ranges were computed for the continuous variable. Associations between *AhRR* methylation and each of the variables (smoking status, tumor grade, invasiveness, *TP53* codon 72, *FGFR3* exon 7 codon 248, and *FGFR3* exon 7 codon 249) were then tested using the two-tail Student's t-test. A Folded-F test was run for each test, and either the Pooled (assuming equal variances) or Satterthwaite method (assuming unequal variances) was used to assess significance of *AhRR* methylation between groups. Specifically, when the Folded-F test resulted in a p-value > 0.05, the Pooled method was used, while for a significant Folded-F test p-value <0.05, the Satterthwaite method was used. Further analysis with stratification by invasiveness was performed using the Wilcoxon Rank Sum test to examine the association between *AhRR* % methylation and both *FGFR3* codons 248 and 249. For all the conducted tests, a $p < 0.05$ was considered as statistically significant. All analyses were performed using Statistical Analysis Software (SAS).

CHAPTER 3

RESULTS

During *AhRR* methylation assessment, 11 samples were discarded due to lack of any methylated or demethylated droplet clusters in ddPCR results. A sample was also rejected due to a difference of more than 30% methylation between duplicates (Bojesen et al., 2017). Therefore, a final sample size of 168 was reached.

3.1. Study Population Characteristics

Out of 168 samples, the majority of the tumors (150) were HG, while only 7 were LG. On the other hand, 68 samples were muscle-invasive, while 99 were non-muscle invasive (Table 1). With respect to smoking status, 74.4% of the patients were ever smokers, while 20.2% had reported they were never smokers. Frequencies of *TP53*, *FGFR3*, and *RB1* genotypes are shown in Table 2. For *TP53* C72, 47.6% showed the wild-type genotype, while around 49.3% presented with at least one mutation. Around 95.8% of samples had wild-type *FGFR3* E7 C248, whereas only 11.9% had wild-type *FGFR3* E7 C249. The remaining samples showed presence of mutant allele (4.1% for *FGFR3* E7 C248 and 88.1% for *FGFR3* E7 C249). On the other hand, mutations in *TP53* C248 and *RB1* E23 were almost totally absent.

Table 1. Frequency distribution of study population characteristics (tumor grade, invasiveness, and patient smoking status) in the total sample (N=168)

Characteristic	N	Frequency (%)
Tumor Grade		
High Grade	150	89.2
Low Grade	7	4.1
Unknown	11	6.5
Invasiveness		
Invasive	68	40.4
Non-Invasive	99	58.9
Unknown	1	0.5
Smoking Status		
Ever Smoker	125	74.4
Never Smoker	34	20.2
Unknown	9	5.35

Table 2. Frequency of *TP53*, *FGFR3*, and *RB1* genotypes in the total sample (N=168)

SNP/Mutation*	-/-	+/+	-/+	Undetermined
TP53 C72	80 (47.6%)	34 (20.2%)	49 (29.1%)	5 (2.9%)
TP53 C248	161 (95.8%)	1 (0.6%)	2 (1.2%)	4 (2.4%)
FGFR3 E7 C248	161 (95.8%)	1 (0.6%)	6 (3.5%)	0 (0.0%)
FGFR3 E7 C249	20 (11.9%)	2 (1.2%)	146 (86.9%)	0 (0.0%)
RB1 E23	138 (82.1%)	0 (0.0%)	0 (0.0%)	30 (17.9%)

*(-/-: Homozygous Wild-Type, +/+: Homozygous Mutant, -/+: Heterozygous)

AhRR methylation ranged between 0 and 99.65% with a mean *AhRR* methylation of 37.46 ± 22.21% (mean ± SD) and was relatively normally distributed across the 168 tumor samples (Figure 3).

3.2. Bivariate Analysis

In the bivariate analysis, results showed that never smokers had a higher mean *AhRR* % methylation of 40.16 ± 23.26% compared with that in ever smokers (36.88 ± 22.52%) (Table 3). However, independent t-test results revealed that the difference in *AhRR* % methylation levels between ever smokers and never smokers was not statistically significant (p=0.456) (Table 3). Similarly, HG tumors had a mean *AhRR* % methylation of 37 ± 22.18%, which was higher than that of LG tumors at 26.59 ± 17.07%. However, no significant association was found between tumor grade and *AhRR* % methylation (p=0.223). On the other hand, results showed that muscle-invasive tumors had a higher *AhRR* % methylation than non-muscle invasive tumors, and the association was statistically significant (p=0.011), with a mean methylation at 42.86 ± 23.85% for invasive tumors versus 33.98 ± 20.36% for non-invasive tumors. Figure 4 shows the distribution of *AhRR* % methylation among invasive and non-invasive tumors. Histograms and Q-Q plots show *AhRR* % methylation to have a normal distribution among invasive and non-invasive tumors, with slight deviations at the tails.

With respect to the key tumor marker genes, mutant *FGFR3* E7 C248 had a lower mean *AhRR* % methylation of 28.11 ± 9.44% compared to 37.87 ± 22.53% for wild-type genotypes, and the association was statistically significant (p=0.036). Samples with mutant *TP53* C72 had a mean *AhRR* % methylation of 39.69 ± 22.77% versus 34.28 ± 20.55% in wild-type genotype. In addition, *FGFR3* E7 C249 genotypes

had a relatively similar mean *AhRR* % methylation level with mutant genotypes at 37.30 ± 22.15% compared to wild-type genotype at 38.66 ± 23.19%. However, these associations were not statistically significant. Figure 5 shows the distribution of *AhRR* % methylation among mutant and wild-type *FGFR3* E7 C248 genotypes. Histograms and Q-Q plots showed that *AhRR* % methylation was roughly normally distributed among the wild-type genotype with slight deviations at the tails.

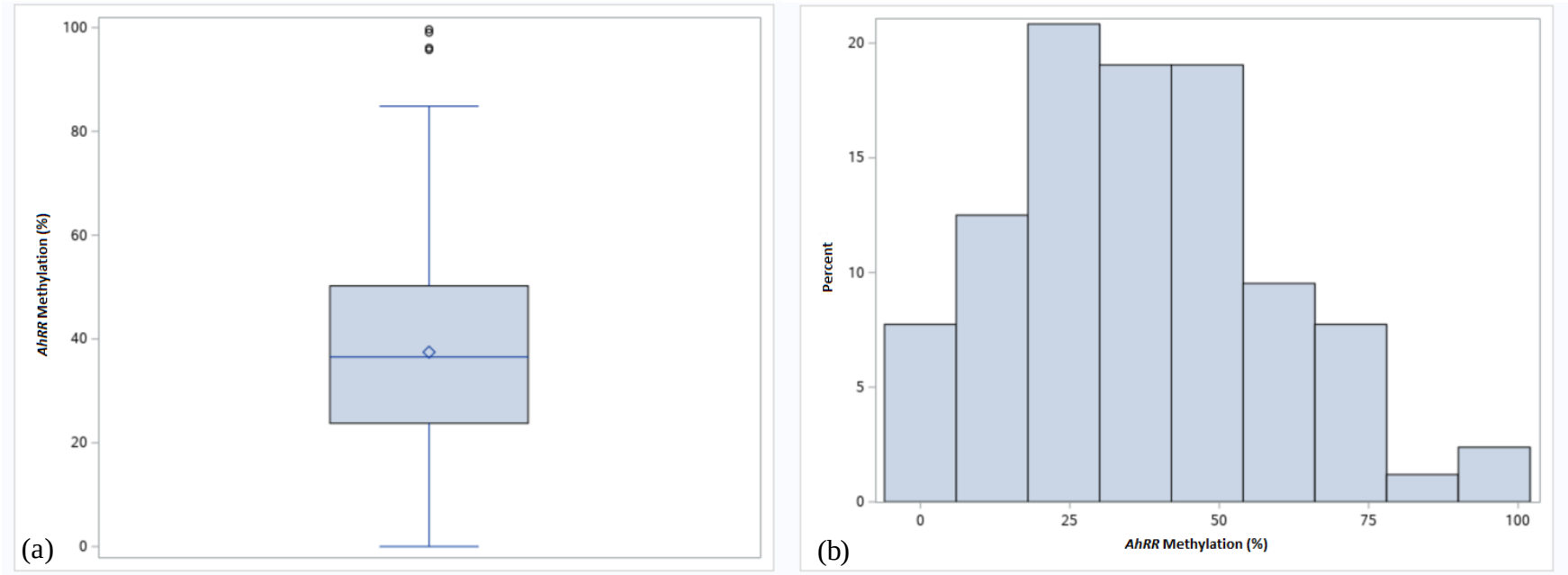

Figure 3. Boxplot (a) and histogram (b) showing the distribution of *AhRR* methylation (%) across the total sample (N=168)

Table 3. *AhRR* % methylation distribution across the different variables (smoking status, tumor grade, muscle-invasiveness, *TP53*-C72, and *FGFR3*-Codons 248 and 249 in the total sample (N = 168)

Variable	**Mean *AhRR* % Methylation (95% CI)**	**Standard Deviation**	**p-value**
Smoking Status (n=159)			
Ever Smoker	36.88 (32.89, 40.86)	22.52	0.456
Never Smoker	40.16 (32.04, 48.28)	23.26	
Tumor Grade (n=157)			
High Grade	37.00 (33.42, 40.58)	22.18	0.223
Low Grade	26.59 (10.80, 42.38)	17.07	
Invasiveness (n=167)			
Invasive	42.86 (37.05, 48.63)	23.85	0.011*
Non-invasive	33.98 (29.92, 38.04)	20.36	
***TP53* C72 (n=163)**			
Mutant	39.69 (34.72, 44.66)	22.77	0.114
Wild-type	34.28 (29.71, 38.85)	20.55	
***FGFR3* E7 C248 (n=168)**			
Mutant	28.11 (19.38, 36.85)	9.44	0.036*
Wild-type	37.87 (34.36, 41.38)	22.53	
***FGFR3* E7 C249 (n=168)**			
Mutant	37.30 (33.71, 40.90)	22.15	0.799
Wild-type	38.66 (27.81, 49.51)	23.19	

*p-value < 0.05 is considered significant

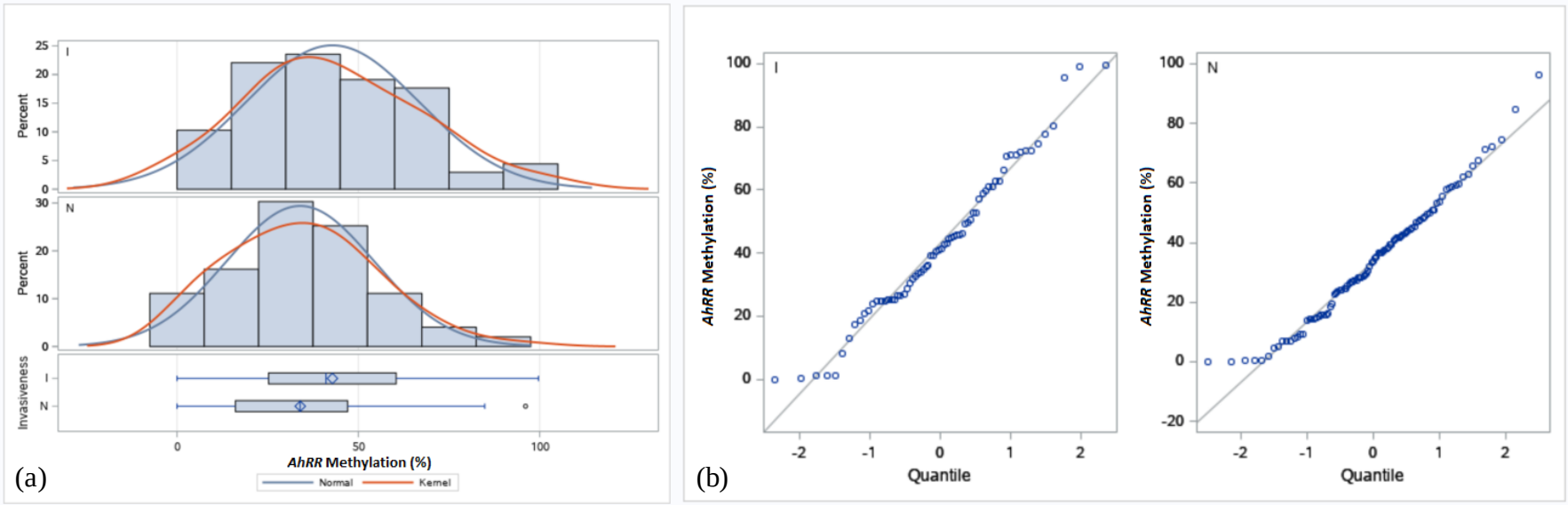

Figure 4. Boxplot and histogram (a) and Q-Q plot (b) showing distribution of *AhRR* methylation (%) among invasive (I) and non-invasive (N) tumors

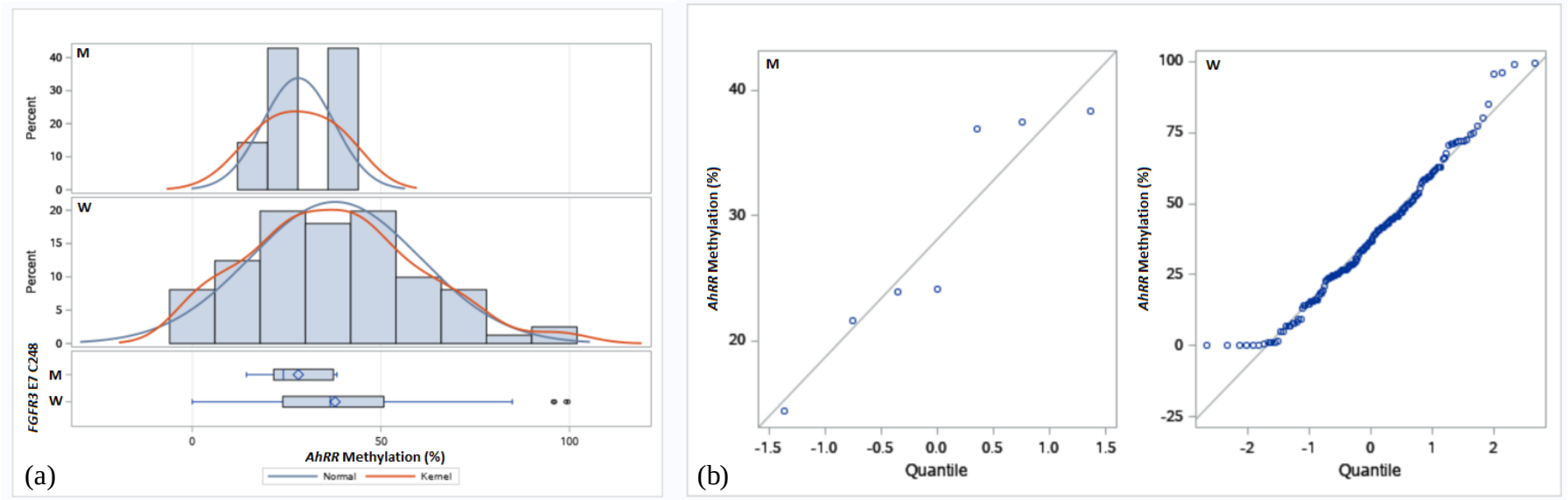

Figure 5. Boxplot and histogram (a) and Q-Q plot (b) showing distribution of *AhRR* methylation (%) among mutant (M) and wild-type (W) *FGFR3* E7 C248 genotypes

3.3. Stratified Analysis

Further analysis using Wilcoxon's rank sum test with stratification by invasiveness (Table 4) showed that for muscle-invasive samples, a wild-type *FGFR3* C248 genotype had a higher mean *AhRR* % methylation compared to a mutant genotype (43.17% vs. 21.60%; p=0.231). Similarly, mean *AhRR* % methylation was higher for the wild-type *FGFR3* C248 compared to the mutant in non-muscle invasive samples (34.29% vs. 23.20%; p=0.491). On the other hand, wild-type *FGFR3* C249 genotype had a lower mean *AhRR* % methylation compared to a mutant genotype for muscle-invasive samples (31.35% vs. 44.39%; p=0.159), whereas for non-muscle invasive samples, the wild-type *FGFR3* C249 showed a higher mean *AhRR* % methylation than the mutant genotype (43.53% vs. 32.66%; p=0.125). However, none of the above tested associations was statistically significant.

Table 4. *AhRR* % methylation distribution for *FGFR3* Codons 248 and 249 stratified by invasiveness using Wilcoxon Rank Sum test

	***FGFR3* C248**		***FGFR3* C249**	
	Invasive	Non-Invasive	Invasive	Non-Invasive
Mutant				
N	1	6	60	87
Mean % *AhRR*	21.60	29.20	44.39	32.66
Wild-type				
N	67	93	8	12
Mean % *AhRR*	43.17	34.29	31.35	43.53
p-value	0.231	0.491	0.159	0.125

CHAPTER 4

DISCUSSION & CONCLUSION

4.1. *AhRR* Methylation and Smoking Status

The first objective of this research was to investigate the value of *AhRR* methylation as a possible smoking biomarker in BCa tumors. Unlike studies which report strong associations between smoking and *AhRR* demethylation in blood (Andersen et al., 2017; Bojesen et al., 2017; Dawes et al., 2020; Philibert et al., 2019), our study found no significant associations between *AhRR* methylation and smoking status in the BCa tissue. Therefore, our findings provide a preliminary basis to suggest that *AhRR* methylation in bladder tumor tissues is not a biomarker of smoking. On the other hand, smoking could be acting on different *AhRR* CpG-sites in the target tissues. De Vries et al. (2018) found that compared to never smokers, current smokers exhibited considerably lower *AhRR* methylation levels at cg21161138 in lung tissues, which is in agreement with other studies assessing *AhRR* demethylation as a smoking biomarker in blood at the common cg05575921 locus (Andersen et al., 2017; Philibert et al., 2019). Thus, assessing tumor-based methylation levels at different *AhRR* CpG-sites may provide further insight into relationships between tobacco smoking and *AhRR* methylation in BCa tumor tissues.

4.2. *AhRR* Methylation and Tumorigenic Outcome

Another objective of this study was to examine the association between *AhRR* methylation and tumorigenic outcomes. Interestingly, we showed that muscle-invasiveness is significantly associated with higher *AhRR* methylation. In fact, this

finding is consistent with evidence from other studies suggesting that *AhRR* plays a key role in tumor suppression and that *AhRR* expression is downregulated in various cancerous tissues (Kumar et al., 2021; Vacher et al., 2018; Vogel & Haarmann-Stemmann, 2017; Zudaire et al., 2008). Zudaire et al. (2008) reports that *AhRR* inhibition, due to increased methylation, contributes to aggressive tumorigenic phenotype, including increased migration and invasiveness and reduced apoptosis in cancer cells. Recent research suggests that *AhR* is associated with BCa progression (Matheus et al., 2020). Given that *AhRR* represses *AhR* function, our finding suggests that higher *AhRR* methylation is causing its downregulation, which may be contributing to disease progression to muscle-invasiveness. One study postulates that the effect of smoking on BCa is mediated through reduced blood *AhRR* methylation (Jordahl et al., 2019). Research has suggested that increased *AhRR* expression in white blood cells could result in promoting chronic inflammation, a risk factor of BCa, thus potentially revealing downstream effects of smoking and a role in bladder carcinogenesis (Jordahl et al., 2019; Wan et al., 2018; Zhang et al., 2017). Moreover, a recent study suggests a dual role for *AhR* in BCa (Yu et al., 2020). Thus, given the close relationship between *AhR* and *AhRR* suggested by Brandstätter et al. (2016), a dual role for *AhRR* in carcinogenesis might also be the case.

Alternatively, *AhRR* normal levels of expression may vary between different tissues given the concept of tissue-specific gene expression and regulation (Brandstätter et al., 2016; Sonawane et al., 2017; Wan et al., 2018). *AhR* induces expression of *AhRR* in a tissue-specific manner, which in turn inhibits *AhR*-induced genes' expression through multiple mechanisms (Dougherty & Pollenz, 2010; Yamamoto et al., 2004). *AhR* tends to induce DME genes, including *CYP450*, such as *CYP1B1*, which contribute

to tumor formation (Yang et al., 2008). *CYP450* genes have also been associated with bladder carcinogenesis (Al-Saraireh et al., 2021; Androutsopoulos et al., 2013; Basma et al., 2013), which further reinforces that *AhRR* may act as a protective factor against BCa tumors. In addition, *AhR* mediates cancer-associated genes in different pathways involved in development and progression of cancer, such as *MMP-9* through the c-Jun-dependent pathway, ERK-FAK-Rac-1 pathway, as well as *Snai1, Twist1, Twist2,* and *Vim* in E-cadherin-related pathways (Wang et al., 2020). Interestingly, varying expression of *Snail, Twist,* E-cadherin, *Vim* and *MMP-9* have been linked to BCa (Lobo et al., 2020; Yu et al., 2010; Zeng et al., 2016), which further elucidates the role of the *AhR-AhRR* relationship in bladder carcinogenesis.

At the same time, no associations were found between tumor grade and *AhRR* methylation. This may be related to the fact that the majority of samples are HG (89.2%). Nevertheless, HG tumors showed a notably higher average methylation compared to LG tumors. This is in agreement with one study where *AhRR* methylation percent was higher in HG pre-cancerous specimen of the cervix and HG dysplasia in the esophagus compared to LG (Zudaire et al., 2008).

In addition, our results showed that *FGFR3* E7 C248 genotype was significantly associated with *AhRR* methylation, where homozygote and heterozygote mutant genotypes appeared to have lower *AhRR* methylation percent levels compared to wild-type genotypes. *FGFR3* activating mutations play an important role in the development of several cancers, including lung, bladder, blood, and cervical cancers (Duperret et al., 2014). According to the Reference SNP (rs) Report by the NLM, mutation at *FGFR3* E7 C248 (rs121913482) is a missense mutation in which nucleotide 'C' in the 5'-GCG-3' nucleotide sequence is substituted with 'T', subsequently resulting in a 5'-GTG-3'

sequence (Sherry, 2001). Given that the AhR-ARNT transcription complex has affinity to bind 5'-GTG-3' sequence (Bacsi et al., 1995; Swanson et al., 1995), we postulate that increased expression of *AhR* is related to overexpression of *FGFR3* when mutated. We also propose that *AhRR* may be acting through a yet-to-be-determined regulatory pathway to suppress tumors that are likely induced by overexpressed *AhR* and *FGFR3*, thus explaining the association between *AhRR* lower methylation (overexpression) and *FGFR3* mutation. This association also reveals potential roles for *AhRR* in bladder carcinogenesis.

Interestingly, upon stratification by invasiveness, our findings showed that the mutant *FGFR3* C248 still had lower *AhRR* methylation levels in both muscle-invasive and non-muscle invasive subgroups. However, these associations were not statistically significant. However, given that only one sample with mutant *FGFR3* C248 was within the invasive subgroup, the observed results may be due to lack of power. With respect to *FGFR3* C249, average *AhRR* methylation levels appeared to be higher in muscle-invasive samples with the mutant genotype and the non-muscle invasive samples with the wild-type genotype. These associations were also not statistically significant. The findings from *FGFR3* C249 are consistent with the literature, where *FGFR3* activating mutations are reported as drivers of muscle-invasiveness in BCa tumors (El Kawak et al., 2020; Foth et al., 2018). The mechanisms specifically involving *AhRR* contribution to BCa progression need to be further investigated in a larger sample.

4.3. Limitations

Several limitations may have affected the findings of this study. Given the low BCa incidence in females, enrollment focused solely on male patients to maintain

statistical power, restricting the possibility of stratifying the analysis by gender. Another limitation is that the study relied on patients' self-reported smoking status, which may be biased by misreporting or underreporting. In fact, research has shown that self-reported smoking status may be inaccurate, especially since smoking may be a socially unaccepted behavior in various contexts (Gorber et al., 2009; Morales et al., 2013). The ability of contacting surviving patients to obtain blood samples to validate smoking status through assessing *AhRR* methylation in blood DNA was limited due to the de-identified nature of the data acquired from the medical centers. Similarly, data on smoking extent or cessation were not obtained from the sampled patients. In addition, the association between *AhRR* methylation and smoking exposure extent and dosage was not possible due to the lack of detailed smoking information. Furthermore, data on other potential occupational and environmental exposures that may affect patients' *AhRR* methylation and bladder carcinogenesis was unavailable, limiting the ability to adjust for any potential confounders.

In addition, DNA was extracted from FFPE tumor samples, which may have affected DNA integrity, resulting in potential degradation of *AhRR* methylation sites. This would explain the lack of ddPCR clusters in 11 samples in this study. Nevertheless, ddPCR has shown better accuracy when assessing DNA methylation in FFPE samples, when compared to other methods of DNA methylation testing (Lissa et al., 2018; Van Wesenbeeck et al., 2018).

4.4. Conclusion

In summary, our study shows that both muscle-invasiveness and the oncogenic *FGFR3* E7 C248 mutant genotype are significantly associated with higher and lower

AhRR methylation levels, respectively. These findings are novel and may help improve knowledge of different BCa mechanisms of carcinogenesis in non-muscle invasive (NMIBC) and muscle-invasive bladder cancer (MIBC) and may make the basis for potential methods of treatment targeting AhRR. Our study provides a preliminary baseline for future research investigating the relationship between differential *AhRR* methylation at multiple loci and mechanisms of bladder carcinogenesis in a large cohort of smokers versus non-smokers. Such studies should ensure validating exposure to smoking by assessing *AhRR* methylation in blood, as well as in BCa tumors and the surrounding non-cancerous bladder tissues. Studying these relationships and mechanisms would improve understanding of BCa etiology.

Despite mentioned limitations, our study strengths lie in several aspects. First, the study targets a population with a high BCa incidence and a high exposure prevalence, which provides power to reported associations. Second, the study is original in evaluating *AhRR* methylation as a biomarker in the target tissue (BCa) and in relation to specific tumorigenic outcome indicators in order to get an insight into the mechanisms of carcinogenesis. Moreover, given that samples were collected from two medical centers situated in different parts of the city with distinct sectarian identity, the examined total pool of samples has better representativeness of the target population, ensuring genetic diversity. Another strength of this study is that *AhRR* % methylation was measured using ddPCR, a highly sensitive technique that partitions DNA strands into around thousands of droplets that are assessed individually, hence providing a highly accurate assessment of % methylation. The validity of the data obtained was further enhanced when we ran samples in duplicates and reported the average % methylation for each patient, while discarding samples with outlying results.

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