RESEARCH ARTICLE

Association between Circulating Vitamin D, the *Taq1* Vitamin D Receptor Gene Polymorphism and Colorectal Cancer Risk among Jordanians

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Abstract

Background: The physiological role of vitamin D extends beyond bone health and calcium-phosphate homeostasis to effects on cancer risk, mainly for colorectal cancer. Vitamin D may have an anticancer effect in colorectal cancer mediated by binding of the active form 1,25(OH)$_2$D to the vitamin D receptor (VDR). The *Taq1* VDR gene polymorphism, a C-to-T base substitution (rs731236) in exon 9 may influence its expression and function. The aim of this study wass to determine the 25(OH)D vitamin D level and to investigate the association between circulating vitamin D level and *Taq1*VDR gene polymorphism among Jordanian colorectal cancer patients. Materials and Methods: This case control study enrolled ninety-three patients and one hundred and two healthy Jordanian volunteers from AL-Basheer Hospital/Amman (2012-2013). Ethical approval and signed consent forms were obtained from all participants before sample collection. 25(OH)D levels were determined by competitive immunoassay Elecsys (Roche Diagnostic, France). DNA was extracted (Promega, USA) and amplified by PCR followed by VDR *Taq1* restriction enzyme digestion. The genotype distribution was evaluated by paired *t-test* and *chi-square*. Comparison between vitamin D levels among CRC and control were assessed by odds ratio with 95% confidence interval. Results: The vitamin D serum level was significantly lower among colorectal cancer patients (8.34 ng/ml) compared to the healthy control group (21.02ng/ml). Patients deficient in vitamin D (less than 10.0 ng/ml) had increased colorectal cancer risk 19.2 fold compared to control. Only 2.2% of CRC patients had optimal vitamin D compared to 23.5% among healthy control. TT, Tt and tt *Taq1* genotype frequencies among CRC cases was 35.5%, 50.5% and 14% compared to 43.1%, 41.2% and 15.7% among healthy control; respectively. CRC patients had lower mean vitamin D level among TT (8.91±4.31) and Tt (9.15±5.25) genotypes compared to control ((21.3±8.31) and (19.3±7.68); respectively. Conclusions: There is significant association between low 25(OH)D serum level and colorectal cancer risk. The VDR*Taq1* polymorphism was associated with increased colorectal cancer risk among patient with VDR*Taq1* TT and Tt genotypes. Understanding the functional mechanism of VDR*Taq1* TT and Tt may provide a strategy for colorectal cancer prevention and treatment.

Keywords: Colorectal cancer - vitamin D receptor - gene polymorphism - vitamin D

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Introduction

Colorectal cancer (CRC) is a major public health problem; it is the third most common cancer in men and the second in women worldwide (Kang et al., 2011) that arises from abnormal growth in the lining or epithelium of the colon, rectum or appendix (Bia et al., 2012). CRC is usually asymptomatic at early stages; and the classic warning signs are nonspecific, such as change in bowel habits, fecal occult blood, unexplained weight loss and feeling tired (Labianca et al., 2010). CRC risk factors include: obesity, lifestyle, age, physical activity, family history and racial or ethnic back ground (Cunningham et al., 2010; Natrah et al., 2012).

In Jordan, CRC is considered the most common cancer among Jordanian males, and the second most common (after breast cancer) among Jordanian females (Jordan Cancer Registry, 2010). Moreover, it is the second most frequent leading cause of death, after heart diseases, among Jordanians (www.khcc.jo).

Vitamin D is a group of fat-soluble secosteroids that is synthesized under skin or ingested from diet (Biancuzzo et al., 2013). To be activated, it undergoes two hydroxylations: the first hydroxylation occurs in the liver where vitamin D is converted to 25-hydroxyvitamin D (25(OH)D) (Jones, 2008). The second hydroxylation occurs in the kidney where 25(OH)D is converted to the active form 1, 25 dihydroxyvitamin D (1,25(OH)$_2$D) (Holick et al., 2011). The main role of 1, 25(OH)$_2$D is calcium-phosphate homeostasis (Verstuyf et al., 2010),

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regulation of cell cycle (Pereira et al., 2012), and nowadays it has anticancer effect especially among colon cancer (Holick etal., 2011; Welsh, 2012).

1, 25$(OH)_2$D initiates its biological responses by binding to vitamin D receptor (VDR) (Haussler et al., 2011). VDR is a protein consists of 427 amino acids, with a molecular weight of about forty eight KDa (Bouillon et al., 2008). It is also an endocrine member of nuclear receptor (NRs) super-family which can enhance gene transcription and regulate gene expression in a ligand-inducible manner (Deeb et al., 2007). VDR forms a heterodimer with Retinoid X receptor (RXR) and its ligand (9 cis-retinoic acid) (Carlberg and Seuter, 2009). When 1, 25$(OH)_2$D is absent, VDR-RXR heterodimer binds to a specific sequence within its target vitamin D-responsive gene named vitamin D response elements (VDREs) (Byers et al., 2012). Also it binds to transcriptional co-repressors and silencing mediators which maintain a repressed transcriptional state (Pereira et al., 2012).

VDR is encoded by the vitamin receptor gene that is located on chromosome 12q12-q14 (Raimondi et al., 2009). It is quite large gene about 75 kb (Uitterlinden et al., 2004) of 14 exons (Jehan et al., 2007), that regulates the expression of about 500 genes in the human genome (Carlberg, 2003), in almost all body tissues, mainly in metabolic tissues, such as intestine, kidney, skin and thyroid gland (Carlberg and Seuter, 2009). Only few tissues have low VDR expression, such as RBCs, mature striated muscles, and some highly differentiated brain cells (Bouillon et al., 2008). VDR is expressed on colon mucosa and is associated with colorectal epithelial proliferation (Bai et al., 2012). VDR is also expressed in most malignant proliferative cells (Carlberg and Seuter, 2009), which is necessary for cell-cycle arrest and apoptosis within the cancerous cells (Zinser et al., 2003, Zheng et al., 2012). VDR polymorphisms play an important role in the etiology, incidence and prognosis of cancers (Ingles et al., 1997), and trials on VDR-deficient mice showed an enhanced susceptibility of tumorigenesis within many tissues (Bouillon et al., 2008).

More than four hundreds and seventy polymorphisms within the VDR gene have been reported (Slattery, 2007; Ashktorab et al., 2011). The most extensively studied polymorphisms are a 5` *Fok1* (rs10735810) in exon 2, changes in intron 8 which generate *Bsm1* (rs1544410) and *Apa1* (rs7975232), change in exon 9 (codon 352) that generates *Taq1* (rs731236), *Tru91* (rs757343) in entron 8, and poly (A) mononucleotide repeat in the 3` flanking region (Ashktorab et al., 2011). Polymorphism effect is dependent on its location within the VDR gene. Cdx2 polymorphism in VDR promoter modify VDR intestinal transcription and regulation of calcium absorption (Arai et al., 2001). Polymorphism in 3` VDR gene regulatory region within *Bsm1*, *Apa1*, and *Taq1*, are involved in VDR gene regulation and mRNA stability (Richetta et al., 2014).

VDR gene polymorphisms influence the binding of 1,25$(OH)_2$D to the VDR, that affect the VDR-mediated signaling pathways of vitamin D (Bai, 2012), including the regulatory effect of vitamin D on cell cycle, proliferation, apoptosis, invasion and angiogenesis. VDR gene polymorphisms is associated with increased susceptibility to cancer, including colorectal cancer (Raimondi et al., 2009). *Taq1* VDR gene polymorphism (*Taq1*, rs731236) resulted in T-to-C substitution at codon 352 within exon 9 (Kostner et al., 2009). Frequencies of the different *Taq1* genotypes vary widely across different population and ethnic groups most likely due to different evolutionary processes (Kostner et al., 2009). TT genotype has been shown to be associated with lower circulating levels of vitamin D (Kostner et al., 2009). Many genetic studies evaluated the association between *Taq1* VDR gene polymorphisms and colorectal cancer (Slattery et al., 2001; Peters et al., 2004; Flugge et al., 2007; Yaylim-Eraltan et al., 2007). Results were contraversial, and the anti-cancer mechanism of vitamin D (Ashktorab et al., 2011; Gunduz et al., 2012; Pereira et al., 2012; Vuolo et al., 2012) is unclear.

Up to our knowledge, no report evaluated the association between circulating vitamin D level and VDR *Taq1* gene polymorphism among Jordanian population. So the aim of this study is screen *Taq1* VDR gene polymorphism, C to T base substitution in exon 9 among Jordanian CRC patients, to determine 25-hydroxyl vitamin D serum level among CRC patients and to compare them with healthy control.

Materials and Methods

This case-control study enrolled ninety three colorectal cancer patients (47 males and 46 females) recruited from the gastrointestinal cancer clinic at Al-Basheer Hospital/ Amman (2012-2013). One hundred and two healthy control volunteers (52 males and 50 females) were also recruited from Jordanian society. Ethical approval was obtained for all patients from the Institutional Review Board at Hashemite University. Consent forms were signed by all participants before interviewing and sample collection. Blood samples (EDTA and plain tube) were obtained for all participants; serum was collected in plain tubes by centrifugation and storage at 60ºC for 25-hydroxyvitamin D determination using Elecsys vitamin D total assay kit (Roche Diagnostics) by competitive enzyme immunoassay. 25(OH)D level were classified (according to Thacher and Clarke., 2011) into deficient 25(OH)D level less or equal 10.0ng/ml, insufficient if 25(OH)D level between 11 and 20ng/ml and optimal if 25(OH)D level more than 20ng/ml.

DNA was extracted from EDTA tubes by Wizard Genomic DNA extraction and purification kits (Promega, USA), then amplified (Bio Rad iCycler, USA) using: forward 5'-CAGACCATGGACAGGGAGCAA-3' and reverse 5'-GCAACTCCTCATGGCTGAGGTCTC-3' primers that detect VDR*Taq1* polymorphism SNP (rs731236) within exon 9 of the VDR gene. PCR amplification was carried out in 50μl reaction volumes using 25μl Go Taq® Green Master Mix, containing bacterially derived Taq DNA polymerase, dNTPs (400μM of each dATP, dGTP, dCTP, dTTP), 3mM $MgCl_2$ and reaction buffers.

DNA samples were amplified using programmed PCR protocol: Initial denaturation step at 94˚C for 3min, followed by 30cycles at 94ºC for 45s; 58ºC for 60s; and

72ºC for 90s, then a reaction is carried out at 73ºC for 5min, Finally the refrigeration cycle at 4ºC for ∞. Amplification products then visualized by gel electrophoresis on a 2% agarose gel stained with ethidium bromide. SNPs *Taq1* (rs731236) in VDR gene was detected by restriction enzyme digest using the restriction endonuclease *Taq1* digestion (Promega, USA) at 65°C for two hours. *Taq1* TT genotype digestion produce 494bp+251bp fragments, *Taq1* Tt genotype produce 494 bp+293 bp+251 bp+201bp fragments and *Taq1* tt genotype produce 293 bp+251 bp+201bp fragments. Digested PCR products then loaded on 2.5% agarose gels and electrophoresed at 100V for 45min. DNA fragments visualized using the "100bp" DNA ladder (Promega, USA) as size rollers. All gels were photographed using the IP-010-SD photo documentation system program (Vilber Lourmat, EEC, UK).

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 17.0. Means and standard deviations were calculated and compared among study groups using *t-test* to assess the significance of difference (p value). Mean 25(OH)D level among deficient, insufficient and optimal CRC and control were determined by odd ratios (OR) and 95% confidence intervals (CI) using "Mantel Haenszel" method. Statistical significance was defined as p value≤0.05.

Results

The mean serum level of 25(OH)D among CRC patients was significantly (p value≤0.05) lower than control (Table 1). Among CRC group, the mean level was 8.34±5.98 ng/ml, while 25(OH)D level in the control group was 21.02±8.66ng/ml. Vitamin D status is assessed by measuring the prohormone 25(OH)D, which is the most stable metabolite of vitamin D in human serum. Statistical difference was found in the mean level of 25(OH)D among CRC patients (Mean±standard deviation) compared to control (21.02±8.66), respectively. Seventy percent of CRC patients are deficient, twenty eight percent were insufficient and only two percent are optimal. Compared to 12.7, 63.75 and 23.55; respectively (Table 1). No statistical differences were found between TT, Tt and tt among CRC patients and control (Table 2). Comparing the mean 25(OH)D levels among CRC and control groups for different genotypes showed that there was statistical significant difference among TT (p value=0.01) and Tt (p value=0.04) genotype between CRC and control (Table 3).

Table 1. Classification of 25(OH) D Vitamin D Status Among Colorectal Cancer Patients

25(OH) status	CRC patients (%)	Control (%)	OR(95%CI)	p value
Deficient	65(70%)	13(12.7%)	19.71(6.81-23.38)	0.001
Insufficiency	26(28%)	65(63.7%)	0.32(0.01-0.62)	0.003
Optimal	2(2.2%)	24(23.5%)	0.2(0.13-0.27)	0.005

*Deficient: less or equal 10.0ng/ml; Insufficiency: between 10.0 and 20ng/ml; Optimal: more than 20ng/ml

Table 2. Frequencies of *Taq1* genotypes TT, Tt and tt Among CRC and Control

VDR *Taq1* genotype	CRC n(%)	control n(%)	p value
TT	33(35.5%)	44(43.1%)	0.534
Tt	47(50.5%)	42(41.2%)	0.175
Tt	13(14%)	16(15.7%)	0.297)
Total	93	102	

Table 3. Differences in the Mean 25(OH) D Levels between Different Genotypes and Control

Genotype	N	Mean±Std(ng/ml)	p value
TT CRC patient	33	8.91±4.31	
TT control	44	21.32±8.31	0.01
Tt CRC patients	47	9.15±5.25	
Tt control	42	19.31±7.68	0.04
tt patients	13	9.53±6.39	
tt control	16	20.32±5.73	0.084

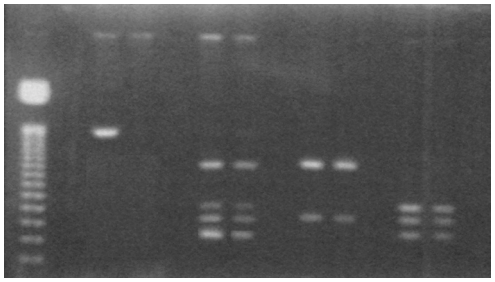

Figure 1. Agarose Gel Electrophoresis for the Genotypes Resulted from *Taq1* Enzyme Digestion for the PCR Product. Lane M: 50bp DNA ladder. Lane 1: negative control (745bp) band. Lane 5: positive control for TT wild type (494bp, 251bp). Lane3: positive control for Tt (heterozygous) genotype (494bp, 293bp, 251bp, 201bp) Lane 7: positive control for tt variant (293bp, 251bp, 201bp). Lanes 4, 6 and 8: randomly selected samples with Tt, TT and tt genotypes; respectively

Discussion

Many studies showed the anti cancer role of vitamin D in colorectal cancer (Zinser et al., 2003; Bouillon et al., 2008; Pereira et al., 2012; Zheng et al., 2012; Rasool et al., 2013; Yu et al., 2014), which is mediated by binding of the active form 1,25(OH)$_2$D to the VDR (Marcinkowska. 2001). In advanced CRC stages VDR expression decreases (Larriba and Mun˜oz., 2005; Matusiak et al., 2005; Anderson et al., 2006), and blocks the anti-cancer action of 1, 25(OH)$_2$D (Pereira et al., 2012). Both 25 (OH) D and 1, 25(OH)$_2$D have localized effect in colon tissue mediated by reducing cell proliferation and inducing cell differentiation (Seifert et al., 2009).

Studying vitamin D level and VDR genetic polymorphisms among CRC Jordanian is an important issue because of the high incidence of CRC among Jordanian which account for 11.5% of all newly diagnosed cancer cases (Jordan Cancer Registry, 2010) and because of high prevalence of vitamin D deficiency levels among them that is accounted for 76% of Jordanian males and 90% of Jordanian females (Mallah, 2011). Up to our knowledge, this is the first study that correlates VDR-*Taq1* gene polymorphism, CRC and circulatory vitamin D level among Jordanian.

Classification of CRC and control according to 25(OH)D vitamin D serum levels showed that most CRC

had vitamin D deficiency, while most control showed insufficiency (Table 1). Only about one quarter of normal control have optimal vitamin D level compared to three percent among CRC. This may be due to biological and behavioral factors interfere with normal vitamin D synthesis (Tsiaras and Weinstock, 2011), such as skin pigmentation, in door working, using sun screen, clothing habits and lifestyle related factors (Mallah, 2011). Or due to other factors including nutritional, food fortification, supplement use and the degree of urbanization (Lips, 2007).

This study shows significant decrease in serum 25(OH) D level among CRC compared to control ($p \leq 0.05$), and 25(OH)D concentration less than 10.0ng/ml (deficiency) is associated with about 19 times increased CRC risk. Many previous studies from different ethnic groups showed similar inverse association (Lee et al., 2011). High serum 25(OH)D levels were associated with decreased colorectal adenoma risk, reduced proliferation and increased differentiation in CRC patients among Turkish (Flugge et al., 2007), European (Jenab et al., 2010), USA (Freedman et al., 2007), and among diverse population of Japanese, Latino, African-American, Caucasian and Native Hawaiian participants (Woolcott et al., 2010).

According to this study, TT *Taq1*genotype, Tt and tt genotypes frequencies among the healthy Jordanian participants are 43.1 %, 41.2 % and 15.7%, while the frequencies among CRC are 35.5%. 50.5% and 14%, respectively. *Taq1* gene polymorphism genotypes frequencies differ among different ethnicities, different cancer types (Bid et al., 2005), and methodology by which *Taq1* VDR polymorphism was investigated, sample size and vitamin D serum levels (Onen et al., 2008). Among Indian individuals the frequencies of *Taq1* variants were 49%, 43% and 8% (Bhanushali et al., 2010) while Japanese study showed 77%, 22% and 1% (Minamitani et al., 1998) and a Turkish, study showed frequency of 40.8%, 47.9% and 11.2% (Dilmec et al., 2009) for TT, Tt and tt respectively.

Our results also showed significant difference in vitamin D level among CRC and control groups within TT and Tt genotypes (Table 3). T allele of VDR *Taq1* may be an important modifier of colorectal cancer among Jordanians. Previous studies showed that T allele VDR TaqI was associated with lower circulating levels of vitamin D (Kostner et al., 2009; Bhanushali et al., 2010). The possible explanation for this association may be *Taq1* polymorphism influence calcium metabolism, which plays an important role in feedback mechanism of Vitamin D levels (Bhanushali et al., 2010). Additional investigation in different populations and among large study groups of how the genotypes affect the functional mechanism VDR may provide better understanding CRC mechanism and treatment

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