The association between two genetic polymorphisms in \textit{ITGB3} and increase risk of venous thromboembolism in cancer patients in Eastern Province of Saudi Arabia

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\textbf{Abstract}

Venous thromboembolism (VTE) is one of the major complications in most cancer patients leading to poor prognosis and short survival. Several common clinical risk factors coexist in cancer patients are used as risk predictive biomarkers to help in the management and prevention of VTE. These include cancer site and stage, chemotherapy regimen and elevated biological markers. However, Genetic polymorphisms in genes controlling coagulation and fibrinolysis are significantly associated with VTE if detected, then they might be more sensitive individual predictive biomarkers for VTE risk assessment. This study was conducted to evaluate the association between \textit{ITGB3} rs3809865 and rs5918 with VTE risk as well as monitor the effect of VTE on overall survival of these cancer patients. In this retrospective case-control study, 195 cancer patients’ formalin-fixed paraffin embedded tissue (FFPE) samples were collected (controls n = 157, case n = 38) using the stored data through Jan 2010 to Sep 2018 from King Fahad Specialist Hospital in Dammam. Samples were genotyped using TaqMan genotyping assay, then logistic regression analysis and Chi-square were used to predict the association between risk factors and VTE. Survival Comparison was tested by the log-rank test. Genetic polymorphisms in \textit{ITGB3} (rs3809865 and rs5918) found not to be associated with VTE increasing risk in cancer patients ($p > 0.05$). While the advanced stage was potentially increasing the risk of VTE events (OR 5.1 CI 2.01–12.9 \textit{p} = 0.001). Patients with VTE showed a poor overall survival reflected by the median survival rate of only three years compared to seven years for cancer patients without VTE. This study highlighted the potential influence of VTE on prognosis and survival of cancer patients and raised the importance of exploring risk predictive biomarkers in our population. This will improve the risk prediction biomarkers leading to implementing safe and effective thrombosis prophylaxis strategies.

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1. Introduction

Venous thromboembolism (VTE) is reported to be a relatively common complication in cancer patients (Donnellan and Khorana, 2017). Cancer increases the risk of venous thromboembolism by four to six folds leading to poor prognosis as well as increased rates of mortality, morbidity, and health care costs (Khalil et al., 2015, Eichinger, 2016, Angelini and Khorana, 2017; Hiraide et al., 2020). Recent study on the 499 092 patients revealed ninefold higher and increased risk of VTE in cancer patients compared to the general population (Mulder et al., 2021). Recent studies are suggesting development of specific recommendation for...
treatting cancer types complicated with VTE (Zheng et al., 2021). It was estimated that 1.6–20% of cancer patients encounter VTE at any time during the disease course (Gran et al., 2018, Mohammed et al., 2015). Also, it was shown that VTE increases the rate of death in cancer patients by more than four folds as it is considered the second leading cause of death in this group of patients after cancer itself (Timp et al., 2013, Khalil et al., 2015, Donnellan and Khorana, 2017). These findings highlighted the importance of exploring potential factors that increase the risk of venous thromboembolism in cancer patients to manage and prevent VTE by establishing thrombosis prophylaxis plan (Iorga et al., 2019). Integrin Subunit Beta 3 (ITGB3) encodes for the beta subunit (IIla) of the glycoprotein GPIIb/IIIa (αIIbβ3 integrin) (Database, 2019). This glycoprotein is considered the main receptor having a crucial role in platelet aggregation (Huang et al., 2019). Intracellular coagulation cascade events is activated upon the latest signalling process leading to irreversible aggregation and stable thrombus formation (Fullard, 2004). Current studies are directed towards identifying genetic markers specifically associated with an individual cancer patient to be used as individual risk factors predictors. Factor V Leiden mutation, prothrombin mutation (G20210A), methylenetetrahydrofolate reductase (MTHFR-A1298C), and (MTHFR-C677T) are studied extensively with conflicting results regarding the significant association with VTE. Most studies found that these polymorphisms increased the risk of venous thromboembolism in both cancer and non–cancer patients in the same proportion and did no show a significant difference in association in either group (Connolly and Francis, 2013). Conversely, a case-control study on breast cancer patients demonstrates a possible association between FV polymorphism that showed to be linked to increase Factor V expression and increase the coagulation activity when investigating polymorphisms in F5 other than FV Leiden (Tinholt et al., 2014). Interestingly, one of the recently studied genetic risk factors is ITGB3 polymorphisms of the Glycoprotein IIIa (Bianconi et al., 2015). Three single nucleotide polymorphisms (SNPs) such as rs4642, rs5918 and rs3809865 in the ITGB3 were studied for the association with VTE (Bianconi et al., 2015). The same study demonstrated a significant association between rs3809865 and VTE in colorectal cancer patients with (p < 0.05), while statistical analysis did not show any significant association between the other polymorphisms and the risk of VTE (Bianconi et al., 2015). Moreover, platelet bearing the glycoprotein IIIa with rs5918 polymorphism showed an increased risk for aggregation as it is assumed to have an increased affinity to fibrinogen. On the other hand, (Khatami et al., 2016, Xiang et al., 2016) reported contradictory conclusions in the association between rs5918 and venous thromboembolism. Allele frequency based on TOPMed (Trans–Omics for Precision Medicine) for SNPs rs3809865 and rs5918 are T = 0.304061 (80482/264690, TOPMed) and C = 0.123586 (32712/264690, TOPMed), respectively (Sherry et al., 2001). This research aim was to investigate the association between two SNPs rs3809865 and rs5918 in the ITGB3 for the GPma of platelets with increased risk of venous thromboembolism in cancer patients in the Eastern Province of Saudi Arabia and to study the overall survival rate of cancer patients and to identify the median time of occurrence of VTE after the initial diagnosis of cancer.

2. Materials and methods

A retrospective case-control study was conducted in Imam Abdulrahman bin Faisal University in accordance with the Declaration of Helsinki. This research protocol was approved by Imam Abdulrahman bin Faisal University Institutional review board (IRB-PCS-2018-03-136) and King Fahad Specialist Hospital (LA80311). The study samples for this research were archived formalin-fixed paraffin embedded tissue (FFPE) samples. For that, waiver consent was sufficient for approval of the study sample collection. The study population for this research was 195 cancer patients diagnosed at King Fahad Specialist Hospital in Dammam (KFSD). The inclusion criteria for controls were as follow, Saudi and non-Saudi of both gender patients older than 18 years with a confirmed diagnosis of cancer by histopathological studies. Additional inclusion criteria for cases were venous thromboembolism VTE which was confirmed by radiological studies. However, the exclusion criteria for both cases and controls were paediatric cancer patients, patients with haematological malignancies and cancer patients with known FV Leiden, prothrombin, MTHFR mutation carriers.

Cancer patients confirmed using histopathology were proven VTE using routine diagnosis. Radiological reports of cancer patients from Jan 2010 to Sep 2018 were collected using ultrasound venous doppler as code for VTE cases that fit the inclusion criteria of the study. A total of 45 cases were available and out of them only 38 (Breast cancer 24; Colon cancer 7; Ovarian cancer 1; Brain cancer 3; Lung cancer 2; and Bladder cancer 1) were included in the research for one of these reasons either the tissue blocks were used for another research or only one block was available for the case and using these blocks for research is against IRB rules. Simple random sampling technique that was done using IBM SPSS Statistics 21 software (Hong Kong, China) was used to select 157 (Breast cancer 76; Colon cancer 41; Ovarian cancer 8; Brain cancer 10; Lung cancer 6; and Bladder cancer 16) control samples. Demographic data and clinical history were collected for selected cases. Data collected include age, gender, date of initial cancer diagnosis, site and stage of cancer, date of VTE occurrence, history of hospitalization, chemotherapy regimen used and date of death.

Tissue blocks containing soft tissue, medium-sized, low fat content and perfect tissue embedding were accepted. From each selected FFPE tissue, four sections with 10 μm thickness were cut using the Rotary Microtome (LEICA RM 2255 Rotary Microtome by Leica Biosystems (IL, USA) then placed in a 1.5 ml sterile microcentrifuge tube. Melting the paraffin using hazard solvents such as ethanol and xylene described by was performed in this research with minor modifications (Sikora et al., 2011). Silicon membrane-based DNA extraction was the method of choice provided by DNeasy Blood and Tissue extraction kit (Qiagen, Hilden, Germany). DNA concentration and purity were measured by Nanodrop 8000 spectrophotometer by Thermo Scientific (DE, USA) and samples were stored at −80 °C to be used for further analysis.

2.1. Genotyping of the studied variants

TaqMan based SNP Genotyping assay was conducted by 7500 Fast Real-Time PCR System (Applied Biosystems®). The flanking sequences of SNP rs3809865 in ITGB3 was obtained from the national centre for biotechnology information (NCBI) with sequence reference (GenBank accession number: NC_0000171.11). Forward and reverse primers flanking the SNP of interest were selected to have an amplicon length between 700 and 800 bp with a C/G content between 40 and 60 %. Complete primers design was achieved by NCBI primer blast together with reverse complement tools. Amplification was optimized using primers, A9865F [(Forward) 5’ ATGGTTCTCTCTCGCAAGG 3’] and As9865R [(Reverse) 5’ GTGACTTCCTGTAATGAT 5’] through gradient PCR. Agarose gel electrophoresis was used for DNA fragments separation according to the fragment size. Agarose gel was then transferred to the Molecular Imager® ChemiDoc Doc® XR System, Bio-Rad (CA, USA) to visualize DNA under UV light. PCR products were purified from impurities like as primers, nucleotides, enzymes, and salts using QiAquick® purification kit. Amplicons
were sequenced using 3500 genetic analyser (Applied Biosystems™ HITACH!, Austin, USA) through Sanger sequencing technology. Purification of PCR products after cycle sequencing was done to remove unbound BigDye® terminators and salts. This step was archived by Big dye terminator™ purification kit, Applied biosystem. The sequencing analysis software mutation surveyor® software was used for ITGB3 sequencing analysis.

IBM SPSS v.21 software was used in this study for descriptive statistical analysis. Mean and standard deviation as well as median and interquartile ranges were used to present the continuous variables. Nominal variables frequencies were presented as percent and numbers. Chi square and logistic regression test were used to compare the frequencies between cases and controls clinical history. It was also used to assess the association between rs3809865 and rs5918 and VTE. Kaplan-Meier Curve and log rank test were used to compare survival time and assess its significance between the study groups. A p-value < 0.05 within 95% confidence interval used to compare survival time and assess its significance between the study groups. A p-value < 0.05 within 95% confidence interval was considered statistically significant Hardy Weinberg equilibrium and minor allele frequency were calculated using Haploview 4.2 software (MA, USA).

### 3. Results

Study population detailed demographics characteristics and clinical data were presented in Table 1, they were divided into two groups: case (n = 38) and controls (n = 157). The mean age of the study population in both cases and controls was 58 years with a mean BMI for cases and controls of approximately 29.2 and 28.1, respectively. The predominance of female patients was demonstrated in both case and control groups accounting for 72.3% of the total study population. Hospitalization status which included any hospital stay more than 4 days as well as patients under palliative care presented 69% of the study population with no significant difference between cases and controls. More than 70% of cases were dead compared to only 17% in controls. In this study, two cancer treatments data known to be a risk factor of VTE were collected which included chemotherapy and hormonal therapy. Most cases were under chemotherapy presenting 92.1%, while for hormonal therapy only 44.7% were under this type of treatment. Advanced stage cancer patients were higher in cases compared to controls accounting for 84%, 50%, respectively. Chemotherapy as well as advanced stage of cancer showed a statistically significant p-value of 0.04 and 0.01, respectively by univariate analysis. But, with logistic regression chemotherapy showed statistically insignificant p-value of 0.56 while advanced stage showed p-value of 0.001 (OR 5.1 CI 2.0–12.9) which indicates advanced stage significant association with VTE occurrence. The samples used for this research were subjected to TaqMan genotyping analysis without any exclusion. As known that the effect of DNA quality will be demonstrated by the success or failure in the final analysis. Only three samples were excluded from the research as they were below the accepted DNA concentration. A total of 192 samples were genotyped using the TaqMan® Genotyping assay. Genotypes were then analyzed by the TaqMan software. The software fluorescence calls were automatically plotted on the allelic discrimination plot which discriminates each allele as a specific cluster. The allelic discrimination plot was divided into three clusters. Lower right cluster (red) represents homozygous for allele 1, upper left cluster (blue) represents homozygous for allele 2, while the center cluster represents heterozygous for allele 1 / allele 2 Allele specifications were determined by rs3809865A (VIC™) / rs3809865T (FAM™) and rs5918C (VIC™) / rs5918T (FAM™).

Allele distribution for the study SNPs differs between cases and controls. For rs3809865, higher frequency of heterozygous genotype AT was demonstrated in the cases showing 51.4% compared to 36.4% in controls. On the other hand, homozygous genotype AA showed a higher frequency in controls compared to only 31.4% in cases. However, these differences in frequency were statistically insignificant by the bivariate analysis showing a p-value of 0.31 for genotype AA and a p-value of 0.98 for genotype AT. Homozygous genotype TT showed the least frequency in cases and controls being 17% and 11%, respectively. For rs5918, homozygous TT genotype showed the highest frequency, 69.7% and 73% for cases and controls, respectively. Heterozygous genotype CT was slightly higher in controls being 22% compared to 27% in case. Lowest frequency was the carriers of homozygous genotype CC. The bivariate analysis reflected statistically insignificant differences between the cases and controls represented by a p-value of 0.53 and 0.64 for genotypes CT and CC, respectively. Genotypes frequency distributions are shown in Table 2. The minor allele for both SNPs was (T). Minor allele frequency (T) of rs3809865 was found to be 0.317, while for rs5918 was 0.162 in the study population (Table 3). Hardy Weinberg equilibrium was calculated for these SNPs by comparing the differences between predicted and observed heterozygosity of these SNPs and found to obey the Hardy Weinberg equilibrium (HWE). Three known samples were sequenced to be used as positive quality control samples for the SNP rs3809865. Sequenced samples analyzed by mutation sur-

### Table 1

| Variable                  | Case (n %) | Control (n %) | Total (n %) | p-value<sup>**</sup> (χ²) |
|---------------------------|-----------|---------------|-------------|-----------------------------|
| Age (year)                | Mean 58 ± 12.4 | Mean 58 ± 13.1 |              |                             |
| BMI                       | Mean 29.2 ± 10 | Mean 28.1 ± 6 |              |                             |
| Gender                    | Female 31 (81.6%) | 110 (70.1%) | 141 (72.3%) | 0.15                        |
|                           | Male 7 (18.4%) | 47 (29.9%) | 54 (27.7%) |                             |
| Cancer treatment          | Chemotherapy 35 (92.1%) | 122 (77.7%) | 157 (80.5%) | 0.04                        |
|                           | Hormonal therapy 17 (44.7%) | 47 (29.9%) | 64 (32.6%) | 0.08                        |
| Stage                     | Local 6 (16.2%) | 78 (40.7%) | 84 (43.3%) | 0.01                        |
|                           | Advanced / metastasis 31 (83.8%) | 79 (50.3%) | 110 (56.7%) |                             |
| Hospitalization           | 29 (76.3%) | 103 (67.3%) | 132 (69.1%) | 0.2                         |
| Mortality                 | Dead 27 (71.1%) | 27 (17.4%) | 54 (28.0%) | <0.001                    |
|                           | Alive 11 (28.9%) | 128 (82.6%) | 139 (72.0%) |                             |

<sup>**</sup> p < 0.05 is statistically significant.
veyor® software results reflected the specific genotype result from the TaqMan® genotyping assay. The sequence results are shown with an example of a sequence chromatogram in Fig. 1. To investigate the association between rs3809865 and VTE, binary logistic regression analysis was used for this investigation. The Genotype TT was used as the reference for both SNPs according to the NCBI reference sequence. Crude logistic regression analysis of rs3809865 showed an odds ratio of 2.6 (95% CI 0.845–7.995) for genotype AA with a \( p \)-value of 0.096, reflecting a statistically insignificant association between VTE and genotype AA in the study population. Genotype AT for the same SNP also did not show any significant association with a \( p \)-value of 0.86 and odds ratio of 1. Investigating the association between rs5918 and VTE reveals a lack of association of both CC and CT genotypes which was reflected by statistically insignificant of \( p \)-value for both genotypes. Genotype CC regression analysis resulted in odds ratio 1.6 (95% CI 0.198–13.9, \( p \)-value 0.64) and for genotype CT the odds ratio was 0.76 (95% CI 0.321–1.8, \( p \)-value 0.53). Table 4 presents crude regression analysis data details for both SNPs.

The duration between the initial diagnosis of cancer and VTE occurrence for the cases was estimated by the calculated median and interquartile range. The median of VTE occurrence was about 22 months after the initial diagnosis of cancer with 25% of cases who experience VTE within five months or less since the initial diagnosis of the cancer. On the other hand, 25% of cases were diagnosed with VTE after 51 months or more since the first cancer diagnosis. Survival probabilities between cases and controls were examined by Kaplan Meier survival plots. Two samples were excluded from this analysis due to the unknown death date. As demonstrated in Kaplan Meier survival plot of Fig. 2 the probabilities of cases survival were low compared to survival probability in controls which clearly indicates a poor survival of cases. A 2-year survival probability in cases was approximately 64% compared to higher survival in controls which was 90%. The log rank (Mental-Cox) test was also used to explore this difference in survival which was statistically significant with \( p \)-value < 0.001. Overall mean survival was around three years for cases and seven years for controls.

### 4. Discussion

Cancer patients encounter multiple complications during the disease course. One of the most common complications is VTE...
that the presence of rs5918 in cancer patients does not carry any risk for VTE occurrence. Similarly, (Xiang et al., 2016) had explored 29 variants in the ITGB3, including rs5918 in the Chinese population, to analyse their influence on GPIib/IIIa receptor expression and their impact on platelet function. The study demonstrated no effect on platelet function and thus no association with thrombosis. Likewise, (Bennett et al., 2001) showed no impact of this SNP on platelet function. However, Komsa-Penkova et al., 2017 explored if rs5918 polymorphism carriers exhibit higher risk for developing VTE. They found that it significantly contributes to increased risk of VTE in the general population only as no cancer patients were included in their study. Also, they noted the increased risk in relation to this polymorphism to be higher in females compared to males. Cancer patients with metastasis or advanced-stage cancer comprising 84% of cases showed an increased risk of VTE in our study. Similarly, Abdul Razak et al., 2018 reported that the risk of VTE in cancer patients with metastasis could reach 58-fold increased risk compared to patients with local cancer. (Khalil et al., 2015) also considered the cancer patients with distant metastasis as VTE high-risk group. Conversely, Khorana and Connolly, 2009, indicated that the stage of cancer was not predictive of VTE risk occurrence in the case of ambulatory cancer patients. Chemotherapy carries an adverse effect on the cancer patients by causing endothelial damage initiating an abnormal thrombosis response which may cause VTE (Maia et al., 2019). In our study, chemotherapy had no impact on increasing VTE risk. Other studies declared that the occurrence of VTE is directly connected to the initiation of chemotherapy (Chen et al., 2018). This finding was also reported by Maia et al., 2019, who found that the risk of VTE in lung cancer patients increases after six months following the chemotherapy initiation. Wang et al., 2019 similarly documented a significant association of chemotherapy with VTE as well. Moreover, (Abdol Razak et al., 2018) stated that the VTE risk associated with chemotherapy differs according to cancer type; highlighting stomach and pancreatic cancer to carry a high VTE risk compared to other cancer types. (Khorana and Connolly, 2009) explored the risk of the specific combination of chemotherapy drugs with increasing VTE events and noted that each chemotherapy drug carries a different risk of causing VTE in cancer patients. As demonstrated above, our results did not match the literature findings, and this may arise from the retrospective nature of the study or different chemotherapy regimens followed in our hospital that may not have any influence on the thrombosis system. Venous thromboembolism risk is distinct in each type of cancer depending on specific cancer mechanisms causing thrombosis (Abdol Razak et al., 2018). In our study, cancer types were not defined as a separate VTE risk factor due to the limited cases of VTE in our study population. Aleem et al., 2012 revealed that breast, lung cancer and non-Hodgkin’s lymphoma was the most common types showed high VTE risk. While Abdul Razak et al., 2018 stated that lung, brain, pancreas and kidney carry the highest risk. Furthermore, Sheth et al., 2017 demonstrated that brain, pancreas and stomach were the highest at risk. As noted, that reports indicating the common primary site of cancer with the highest risk were controversial, this may be influenced by the frequency of each cancer in the population studied. In this study, we compared the survival of cancer patients who encounter VTE with cancer patients without VTE (controls). The results revealed 79% of cases with one-year survival of compared to a 94% for controls. Similarly, a two-year survival was lower by more than 10% in cases but still around 90% in controls. As known that survival may be affected by many factors besides the VTE itself. In our study, the mortality was significantly associated with VTE according to multivariate analysis after covariates adjustments. Also, Mohammed et al., 2015 noted a one-year survival of cases to be only 18.5% with a median survival of 12.4 months in a retrospective study conducted at King Abdullah
Medical city in the western region. Similarly, A recently published retrospective study by Maia et al., 2019 on lung cancer patients confirmed a poor prognosis and reduced overall survival related to VTE occurrence, which was 29.1% survival rate with a median survival of 1.5 months. Conversely, in a study comprising pancreatic cancer patients with advanced-stage, Chen et al., 2018 reported no differences in survival between cancer patients with VTE and without VTE, however, they observed a poor survival in patients with early VTE events with a median survival of 1.8 months compared to late VTE. Chen et al., 2018 included only pancreatic cancer patients with advanced-stage and this may explain the insignificant difference in survival. Also, they confirmed the association of advanced-stage cancer with an increased risk of VTE which was found in our research as well. The discrepancies between these studies may be influenced by sample size, study design, generalized vs. specific cancer sites which highlight the need for further research. Comorbidities like cancer disease progression, renal disease, pulmonary disease, infections, and other factors that may add risk to poor survival should also be considered when monitoring overall survival. As observed by Chen et al., 2018, the early-onset venous thromboembolism was linked with poor survival. This observation highlighted the importance of VTE screening since the initial diagnosis of cancer as the risk increase in the first months after diagnosis. In this study, 25% of cases encountered VTE in the first six months while the remaining had VTE after one year with a median duration of 22 months from cancer diagnosis to VTE occurrence. On the other hand, it was observed by Agnelli et al., 2014 who monitored the VTE risk prospectively, that the risk was 2-folds higher in the first 12 months after an initial cancer diagnosis. Similarly, a study published by Wang et al., 2019 found that the VTE cases increased significantly in the first three months affecting 50% of cases and the risk of death increases after three months of VTE event.

A recent systematic review published by Mulder et al., 2019, analysing the most validated risk assessment tools named Khorana risk assessment tool for ambulatory cancer patients, revealed that most VTE events occurred within the low and intermediate-risk groups. This observation reflects that more studies are required to validate more predictive risk factors to establish precise assessment tools. In King Faisal Specialist Hospital in Riyadh, the Caprine VTE assessment tool is now followed as a mandatory measure for all hospitalized patients before admission considering cancer patients as a very high-risk group. In ambulatory cancer patients the Khorana risk assessment tool is used but as an optional measure to be decided according to the case of the patients by the physician. The retrospective design of the study was a significant limitation causing the enrolment of only a small sample size of the VTE cases due to the difficulty of radiological reports retrieval from the system. The chance of a repetition of any failure of the extraction procedure was limited because of the FFPE samples used in the study. Also, cancer cases included in this study without stratification of the cancer types making the study generalized rather than cancer specific. To our knowledge, this is the first study in Saudi Arabia to investigate genetic predictive risk factors related to VTE risk in cancer patients.

5. Conclusion

Venous thromboembolism showed a major complication of cancer in our study population. The molecular and clinical predication biomarkers for VTE risk in Saudi Arabian population are limited and need further research. This step is beneficial to establish the appropriate thromboprophylaxis plans maximizing the efficacy of anticoagulation treatments and minimizing the risk of bleeding. We recommend running this study in a prospective design to monitor the cancer patients closely and collect the required data precisely as needed. Larger study population with a case: control ratio with 1:1 or 1:2 for an accurate statistical comparison is recommended too. Also, it is recommended to conduct this study on a specific cancer type starting from the common type in the region. High throughput molecular technologies are recommended to explore the VTE molecular prediction markers. Finally, exploring the clinical VTE risk factors in our population is highly recommended.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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