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Association of *HLA-B27* Gene and Rheumatoid Arthritis: Analysis of Potential Role as a Predictive Biomarker

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Abstract: Rheumatoid arthritis (RA) is a predominant inflammatory arthritis in human. The function of HLA-B27 gene in other types of arthritis has been well investigated; however, its function in RA is unclear. This study investigated the relative expression of HLA-B27 gene in RA patients compared to normal control and assesses its suitability as a biomarker for early detection of RA. Bioinformatics analysis was used to determine the profile of HLA-B27 gene in different human tumors to demonstrate the possible involvement of HLA-B27 in both RA and cancer; and to discover its functional association with other human genes. Samples of human blood from RA patients and healthy individuals were collected, and RNA extraction, cDNA synthesis and qPCR were carried out to detect expression of HLA-B27. ROC analysis was undertaken to investigate HLA-B27 diagnostic performance; GENT2 platform was used to compare HLA-B27 expression levels in different human tumors, and gene-gene interaction network was generated using GeneMANIA to identify correlation of HLA-B27 with other human genes. The qPCR analysis demonstrated an increase in the HLA-B27 expression by 1.65 fold in RA compared to normal control. ROC analysis indicated that HLA-B27 expression could efficiently differentiate RA from normal, supporting its potential use as diagnostic molecular biomarkers. The GENT2 revealed that HLA-B27 expression levels vary across different tumor types, most notably in heart tissue. The gene-gene interaction network revealed that KIR3DL1, KIR3DS1, LILRB1, B2M and LILRA1 were the leading genes showing the highest correlations with the HLA-B27. Our results indicate that HLA-B27 gene is involved in the RA pathogenesis and it can be used as a molecular biomarker for the diagnosis of RA. Our outcomes could lead to the discovery of novel diagnostic, preventive and therapeutic strategies.

1. Introduction

Rheumatoid arthritis (RA) is a prevalent inflammatory arthritis in human described by persistent inflammation of the joints with pain [1]. Analyses of RA epidemiology indicate a population incidence rate of 0.5- 1% [2]. RA symptoms usually appear at the age of 30 to 50 years, more frequently among females [3]. The exact origin of this disease is unknown; however, it has become obvious recently that genetic and epigenetic components, as well as environment significantly contribute to the onset and progression of RA [4]. Infectious incidents also play a crucial part in RA pathogenesis [5]. RA patho-

genesis involves a synthesis of auto-antibodies against proteins that have been altered post-translationally, erosion of joint lining tissue with inflammation amid emergence of intrusive effector T cells, and transition of synovial stromal cells into auto-aggressive effector cells [6]. COVID-19 infection was shown to amplify the risk of getting RA versus the healthy people due to harm inflicted on the immune system [7].

Inflammatory rheumatic diseases increase risk of cardiovascular diseases [8]. Genetic tendency take part in the RA progression, and its overall heritability was anticipated to be about 66% [9, 10]. So far, over 100 genetic loci were suggested to be linked with RA pathogenesis, but the exact mechanism of these loci's involvement in RA development is still unclear [11]. LA-DRB1, HLA-DPB1, HLA-DOA, PADI4, PTPN22, CTLA4, IL2RA, STAT4, TRAF1-C5, CD40, CCR6, IRF4, BACH2, RAD51B, DPP4, RBFOX1, PADI2, CDK4RAP2, LBH, COG6, TYK2, PADI4 and GATA3 are the most significant genes currently associated with RA susceptibility [12]. Genetic alterations in the human leukocyte antigen (HLA) are regarded as a key genetic predisposition to RA [13, 14]. HLA class I molecule B27 (HLA-B27), which is a surface antigen encoded by the B locus on chromosome 6, has been reported as strongly associated with arthritis such as reactive arthritis (ReA), and ankylosing spondylitis (AS); but its pathogenic role remains unknown [15-17]. HLA-B27 has been also linked with several other non-arthritis disorders including extra-ocular disorder [18, 19], and sensorineural hearing loss (SNHL) [20].

Precise and timely diagnosis of RA is crucial for its therapy, as early diagnosis was shown to slow down or stop RA progression [21]. The molecular mechanisms underlying RA development remain largely unclear. The function of the HLA-B27 gene in other types of arthritis has been studied, but not in RA. In this study, we investigated the relative expression of HLA-B27 gene in patients with RA versus normal control and assessed the possible diagnostic performance of HLA-B27 mRNA expression in order to find a reliable biomarker for the early detection of RA from blood samples. As RA can increase risk of cancer development, we used bioinformatics analysis to determine the profile of HLA-B27 gene in different human tumor types compared to normal control and to demonstrate the functional association of HLA-B27 with other human genes.

2. Materials and Methods

2.1. RNA Isolation and Reverse Transcription

For this study, 47 samples of blood were collected from RA patients and a matching number from healthy individuals who were all Iraqi Kurdish. Patients recruited were those who fulfilled at least four criteria of the American College of Rheumatology (ACR) [22]. Samples were obtained from Harem Private Hospital in Sulaymaniyah between March to October 2022, after obtaining appropriate ethical approval. Blood samples (2ml) were collected in a blood collection tube type EDTA-k2 (Ethylene diamine tetra acetic acid-di-potassium). In accordance with the guidelines provided by the manufacturer, Prime PrepTM Blood RNA Extraction Kit (Genet Bio, Daejeon, South Korea) was used to extract total RNA from the blood samples. The RNA concentration and purity were assessed using an Eppendorf Biophotometer (Eppendorf AG, Germany) at wavelengths of 260 nm and 280 nm, as well as through agarose gel electrophoresis. To synthesize the first-strand cDNA by reverse transcription, the 2X SuPrimeScript RT Premix (SR-3000) kit was used according to the manufacturer's instructions.

2.2. Quantitative Real-Time PCR

In order to assess the differential expression of *HLA-B27* between individuals with RA and those without the condition, a quantitative polymerase chain reaction (qPCR) analysis was conducted using QuantiTect SYBR Green PCR kit (Qiagen, Crawley, UK; Cat. No. 204143) and real-time cycler instrument (Applied Biosystems Fast 7500; CA, USA). All reactions were performed in triplicate in 96-well plates. *GAPDH* was used as a housekeeping gene. Each reaction mix was prepared in a 25 µl reaction volume containing 2x QuantiTect SYBR Green Master Mix (1x final concentration), forward and reverse primers (*GAPDH* forward: 5′-ATGGGGAAG-GTGAAGGTCG-3′, *GAPDH* reverse: 5′-GGGTCATTGAT-GGCAACAATATC-3′[23]; *HLA-B27* forward: 5′-GGGTC-TCACACCCTCCAGAGC-3′, *HLA-B27* reverse: 5′-CGGCGGTCCAGGAGCT-3′)[24],

QuantiTect RT Mix, cDNA (<500 ng/reaction). cDNA was replaced with nuclease-free water for the negative controls to ensure that no primer dimers or genomic nucleic acid contaminations were present. The PCR cycling conditions run according to (Table 1). The amplicon size of *GAPDH* and *HLA-B27* were 107 and 135 bp, respectively. After each PCR cycle, fluorescence data were collected to produce an amplification plot to determine the Ct value. The delta-delta-CT (ΔΔCT) method was used to find out the relative expression of each gene [25, 26]. The primers were synthesized by Qiagen (Crawley, UK).

Table1: qPCR cycling conditions for *HLA-B27*.

Steps	Temperature	Time
Denaturation (initial)	95°C	15 min
Denaturation	94°C	15 sec
Annealing	58°C	30 sec
Extension	72°C	30 sec

2.3. In Silico Analysis of HLA-B27 Gene

2.3.1. Expression of HLA-B27 Gene Across Different Human Tumor Tissues versus Control Using GENT2

Gene Expression database of Normal and Tumor tissues 2 (GENT2) was used to explore the expression level of *HLA-B27* transcript (mRNA) across a variety of healthy and tumorous human tissues, to relate them to the *HLA-B27* transcript level in RA. GENT2 provides an exploration platform for determining the patterns of gene expression throughout diverse control and cancer tissues [27].

2.3.2. Analysis of HLA-B27 Interaction with other Human Genes Using GeneMANIA

To further unveil the molecular mechanisms of RA development and determine the functional association of *HLA-B27* gene with other human genes, GeneMANIA prediction server was used. GeneMANIA predicts gene function and generates data including gene co-expression, co-localization, pathways involved and shared protein domains [28]. A gene-gene interaction network for *HLA-B27* was constructed using GeneMANIA and results were generated using human genome data.

2.4. Statistical Analysis

Normal distribution of the experimental results was confirmed using Kolmogorov-Smirnov test, and GraphPad Prism version 8 (California, USA). Relative quantity (RQ) has been usedto characterize the relative mRNA expression level of *HLA-B27* measured by qPCR. The RQ represents the expression amount of *HLA-B27*, relative to a *GAPDH* reference gene. The likely diagnostic value of *HLA-B27* expression was evaluated by ROC study. A ROC curve was created for *HLA-B27* and the optimal diagnostic cut-off point was determined via Youden's J Index [29]. The area under the ROC curve (AUC) was analyzed using Hanley and McNeil's methodology.

3. Results

3.1. Comparative Relative Expression of HLA-B27 Between RA and Healthy Individuals Using qPCR

The qPCR analysis was conducted to compare the relative expression level of *HLA-B27* between RA and healthy individuals. The qPCR results revealed a statistically significant change in the *HLA-B27* expression level in RA patients compared to the normal control (p<0.001). The *HLA-B27* gene expression was up-regulated significantly in RA versus normal control (p<0.001; Figure 1A), with a relative fold gene expression ($\log_2$) of 1.65 foldas mentioned in figure 1B.

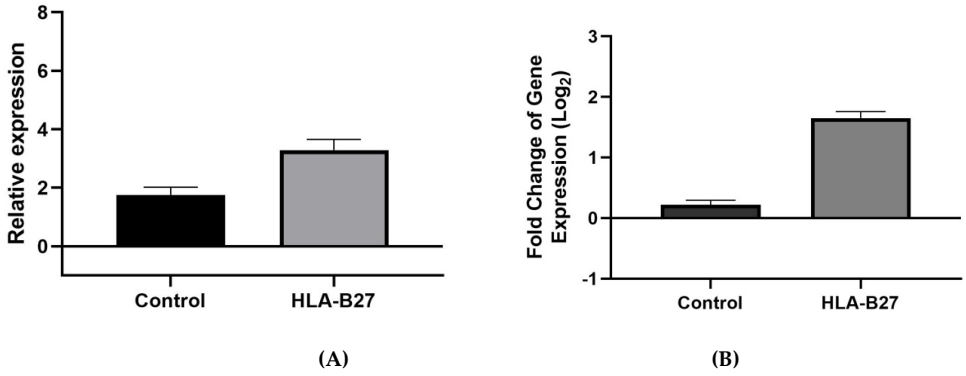

Figure 1: The relative quantity and fold gene expression of *HLA-B27* in human RA. The qPCR analysis results for (A) mean relative quantity (RQ) values and (B) relative fold gene expression (log_2) of *HLA-B27* in RA versus normal control (n=47). The relative expression of *HLA-B27* gene was significantly up-regulated in RA blood tissue versus normal control tissue (p<0.001), with a relative fold gene expression (log_2) of 1.65-fold. Data represent the mean ± SEM.

3.2. Diagnostic Effectiveness of HLA-B27 mRNA Expression in RA

The ROC study was used to evaluate the diagnostic potential of HLA-B27 mRNA expression for RA detection from blood samples. The ROC curve demonstrated that HLA-B27 mRNA expression can competently distinguish RA samples from the normal control (AUC = 0.92; p<0.001) (Figure 2). The optimal diagnostic cut-off value detected by the ROC analysis for HLA-B27 mRNA expression was 0.76 RQU; the associated sensitivity for the cut-off value was 83% and specificity was 79%. The optimal diagnostic cut-off value indicates that the HLA-B27 mRNA could be used as a molecular biomarker for the detection of RA.

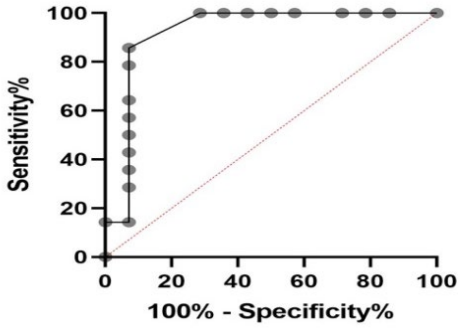

Figure 2: The analysis of the ROC curve for *HLA-B27* mRNA expression. ROC analysis for quantified *HLA-B27* mRNA expression indicates our target gene has a sensitive and specific optimal diagnostic cut-off value that can successfully distinguish RA from normal samples. The AUC for *HLA-B27* = 0.92 (p<0.001). The *HLA-B27* optimal diagnostic cut-off value = 0.76 RQU. The sensitivity and specificity attained with the cut-off value for *HLA-B27* were 83% and 79% respectively. AUC: Area Under Curve; RQU: relative quantification unit.

3.3. In Silico Analysis: Tissue-wide Expression Profile of HLA-B27 Gene Across Multiple Human Tumorous and Healthy Tissues

To demonstrate the expression profile of *HLA-B27* gene in diverse human tumors and healthy tissues and a possible shared genetic predisposition among RA and cancer, GENT2 platform was used. The GENT2 platform compared relative *HLA-B27* expression in different human normal and tumor tissues and revealed that *HLA-B27* mRNA expression levels vary greatly between different tumor types (Figure 3). The *HLA-B27* mRNA expression was significantly greater in tumor tissues of brain (p<0.001), breast (p<0.001), cervix (p=0.045), esophagus (p<0.001), heart (p<0.001), liver (p<0.001), ovary (p=0.019), pancreas (p<0.001), skin (p<0.001), testis (p<0.001), and tongue (p<0.001) compared to control non-tumor tissues. Meanwhile, the *HLA-B27* mRNA expression was significantly lesser in tumor tissues of adrenal gland (p=0.003), blood (p<0.001), bone marrow (p<0.001), colon (p=0.018), joint (p=0.015), kidney (p<0.001) and lung (p<0.001) compared to the control non-tumor tissues (Figure 3). Interest-

ingly, the most notable elevation in *HLA-B27* mRNA expression was detected in the heart tumor tissue compared to normal control.

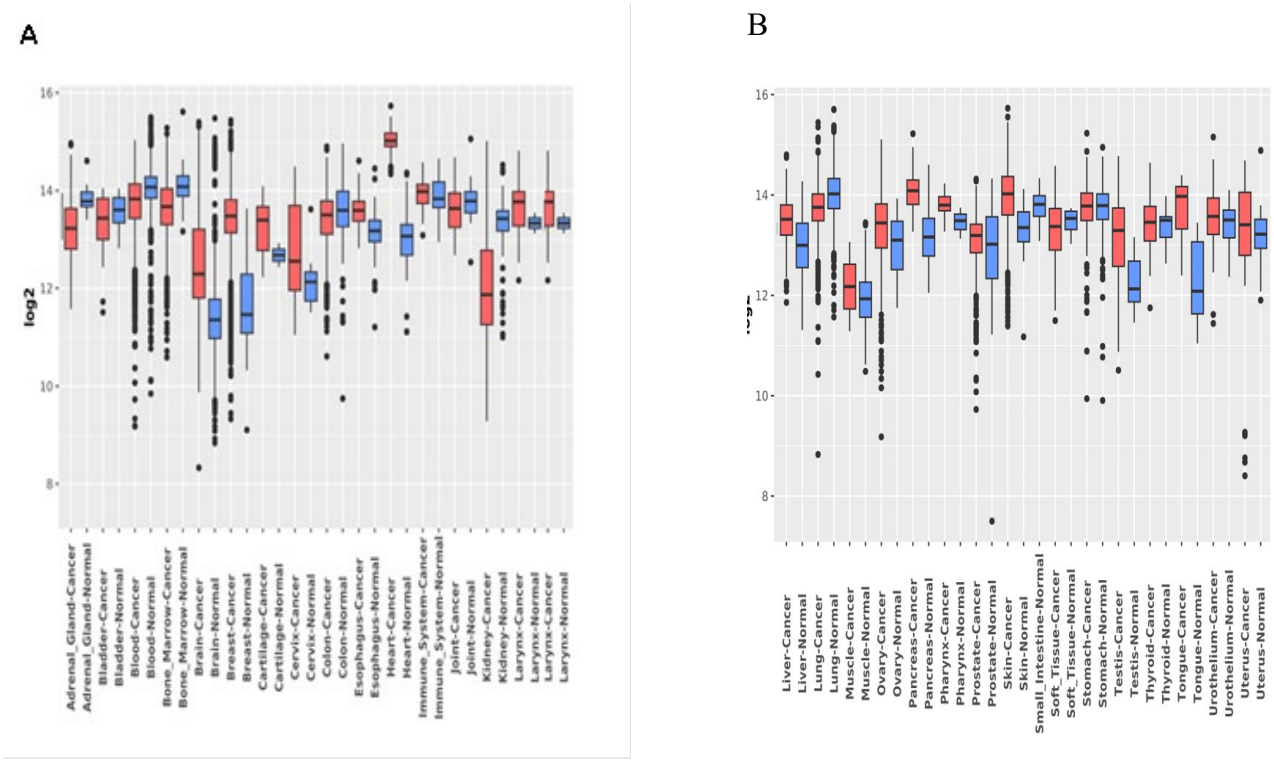

Figure 3: Tissue-wide expression pattern of HLA-B27 in multiple human tumors versus normal tissues. A: tumor and normal tissues of adrenal gland, bladder, blood, bone marrow, brain, breast, cartilage, cervix, colon, esophagus, heart, kidney and larynx. B: tumor and normal tissues of liver, lung, muscle, ovary, pancreas, pharynx, prostate, skin, small intestine, stomach, testis, thyroid, tongue, and uterus.

3.4. Functional Association of HLA-B27 with other Human Genes

To determine the functional link of *HLA-B27* gene with other human genes, GeneMANIA prediction server was used. Gene-gene interaction networks generated by GeneMANIA revealed that the highest functional association of *HLA-B27* gene with other human genes are co-expression and physical interactions (Figure 4). The key genes showing the highest correlations with *HLA-B27* were *KIR3DL1*, *KIR3DS1*, *LILRB1*, *B2M* and *LILRA1*. Concerning other human genes, *HLA-B27* share a protein domain with *B2M*, *TAPBP*, *HLA-C*, *HLA-G*, and *HLA-A*. The *HLA-B27* shared pathway with *KLRC4*, *KIR3DL3*, *TAPBP*, *KLRD1*, *KIR2DL5A*, *KIR2DS3*, *KIR2DS4*, *CD8A*, *KIR2DS1* and *KIR3DL1*. *HLA-B27* demonstrated genetic interactions with *KIR3DS1*. *HLA-B27* showed co-expression with *B2M*, *TAPBP*, *KLRD1*, *HLA-C*, *HLA-G*, and *HLA-A*. *HLA-B27* has physical interaction with *NDUFA9*, *CD8A*, *KIR3DL1*, *KIR3DS1*, *LILRB1*, *B2M*, *LILRA1*, *TAPBP*, *KLRD1*, *CALR*, *HLA-C*, *ARHGEF4*, *HLA-G* and *HLA-A*.

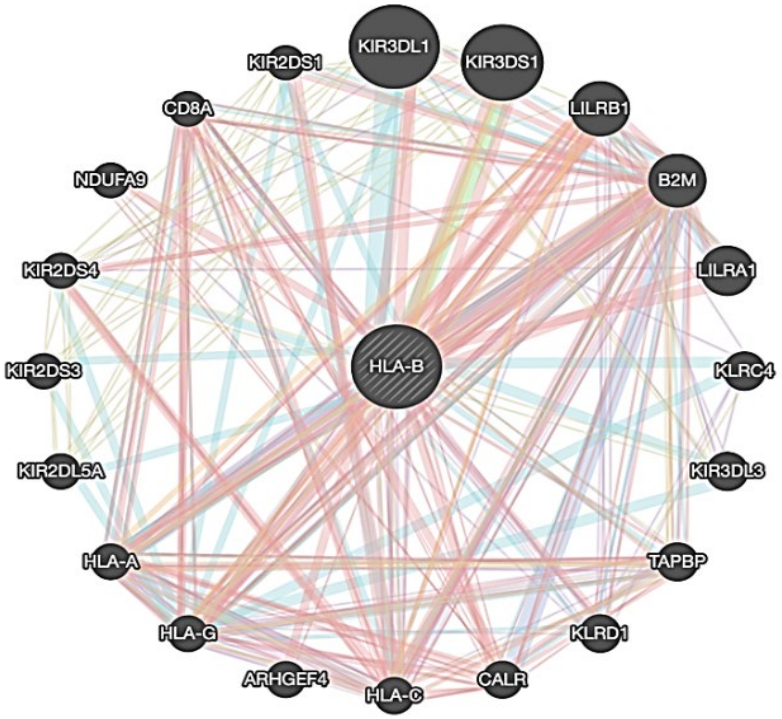

Figure 4: The functional association network of the *HLA-B27* gene using GeneMANIA. The central node represents the target gene bounded by 20 nodes representing human genes that are significantly correlated with the target gene in terms of the shared pathway (blue lines), physical interactions (pink lines), prediction (orange lines), co-expression (purple lines), genetic interactions (green lines), and shared protein domains (beige lines).

4. Discussion

RA is a chronic autoimmune disorder causing persistent inflammation in both small and large joints, resulting in joint pain, stiffness, and swelling with progressive damage [30]. The molecular mechanisms underlying the development of RA are unclear. While role of *HLA-B27* gene in other types of arthritis has been demonstrated, its function in RA remains largely unknown. As an early diagnosis of RA is key to prevent joint damage, this study aimed to explore the relative expression of *HLA-B27* gene in RA patients compared to normal control and assess the possible diagnostic performance of *HLA-B27* mRNA expression to find a reliable biomarker for the early detection of this disease.The qPCR analysis demonstrated an increase in the *HLA-B27* gene expression by 1.65 fold in RA compared to the normal control. ROC analysis indicated that the *HLA-B27* expression could efficiently differentiate RA patients from normal individuals, supporting its potential use as a diagnostic molecular biomarker.

A study of *HLA-B27* Subtypes in Iranian patients with Ankylosing Spondylitis detected two subtypes, B*2705 (63.4%) and B*2702 (36.6%) [31]. Meanwhile, B*2746, B*2749, B*2767, B*2702 and B*2705 subtypes were detected among Ankylosing Spondylitis patients in Turkey [32]. HLA-B27 was shown to have highest prevalence in ankylosing spondylitis and least prevalence in juvenile rheumatoid arthritis among Iranian population [33]. Studies reported an increased prevalence of HLA-B27 in rheumatoid arthritis patients in Turkey [34]. It was reported that prevalence of HLA-B27 antigen in the normal population is significantly lower in the middle eastern and Arab countries compared to the western countries [35]. The prevalence of HLA-B27 among normal Arab populations ranges between 2% and 5%, while the prevalence of HLA-B27 among patients with ankylosing spondylitis among various Arab populations was 64% [36]. In an early study on Japanese population, HLA-B27 has been noticed in 83% of the patients with ankylosing spondylitis which is a kind of arthritis producing inflammation in the ligaments and joints of the spine [37]. Numerous genes have been linked with the onset and progress of RA, and the HLA family is regarded as the most significant that subsidizes about %50 of genetic vulnerability for developing RA [38, 39]. An analysis proposed TBX3 as a predictive biomarker for RA and showed a reduced quantity of TBX3 levels in mice with induced arthritis [40].

Studies using genome-wide analysis connected potentially pathogenic genes with RA including *PADI4, TRAF1-C5, PTPN22, HLA-DRB1, STAT4, TNFAIP3,* and *CCR6*[39, 41, 42]. One of the first genes reported to be associated with RA pathogenesis is *HLA-DRB1*[14]. Over 150 loci with polymorphisms are currently associated with RA and the HLA associations are among the strongest ones [42], further supporting the results obtained in our current study. RA complications can lead to an increased risk of cancer development [43-46]. In this study, we used bioinformatics analysis to determine the profile of *HLA-B27* gene in different human tumor tissues versus normal control to demonstrate a possible shared genetic predisposition among RA and cancer; and also, to find out the functional link of *HLA-B27* with other human genes.

The GENT2 platform revealed that *HLA-B27* expression levels vary across different tumor types, most notably in heart tissue. The gene-gene interaction network generated by the GeneMANIA showed that *KIR3DL1, KIR3DS1, LILRB1, B2M* and *LILRA1* were the key genes showing the highest correlations with the *HLA-B27*. A higher incidence rate of developing cardiovascular disease was reported among RA patients [47] which may explain the high expression level of *HLA-B27* gene detected in both RA and heart tumor tissue. Patients with rheumatic disease show elevated rates of colorectal cancer recurrence [48]. A study showed that some cancer biomarkers such as *CEA, CA 15-3, CA 125,* and *CA 19-9* had been elevated in individuals suffering from RA [49]. An investigation demonstrated people with RA have an amplified risk of developing cancer by 10% compared to non-RA individuals [50]. It has been reported that RA patients display an elevated risk of developing nonmelanoma skin cancer [51], lymphoma and lung cancer in contrast to healthy individuals [50]. A study reported amplified level of *HLA* is linked with extended survival in most cancer types [52]. Absence of response to cancer immunotherapy was shown to rely on the molecular type of *HLA* class I abnormalities [53]. Nineteen *HLA-B* alleles including *HLA-B27* were detected among patients with breast cancer [54]. The findings of these reports are in order with the findings of our current investigation which reveals an elevated level of *HLA-B27* gene in RA and several cancer types.

5. Conclusions

Limited sample size, diversity of participants, lack of long-term follow-up of disease progression or treatment response, clinical relevance and implications of biomarker in real life, resource and time constraints were among imitations of the current study. The findings of this study suggest that the overexpression of the *HLA-B27* might be related to the RA in humans, and it has the potential to be used as a molecular biomarker in the diagnosing of the RA, and also a number of cancer conditions including colon and breast cancers.

Authors contributions: Karzan Ghafur Khidhir: Supervision, Project administration, Data analysis, Writing – original draft. **Baban Osman Ahmad**: Data curation, Methodology, Resources. **Dana Khdr Sabir**: Investigation, Formal analysis, Writing, Review and editing.

Data availability: Data will be made available on request.

Conflicts of interest: Theauthors declare that they haveno known competingfinancial interests or personal relationships that could have appeared to influence the work reported in this paper.

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