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Research article

Candidate gene analysis in Israeli soldiers with stress fractures

Ran Yanovich [1], Eitan Friedman [2,4]✉, Roni Milgrom [2], Bernice Oberman [3], Laurence Freedman [3] and Daniel S. Moran [1, 5]

[1] Heller Institute of Medical Research, [2] Susanne Levy Gertner Oncogenetics Unit, and [3] Biostatistics Unit Gertner Institute of Epidemiology and Health Policy Research, Sheba Medical Center, Tel-Hashomer, Israel; [4] Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel, [5] Ariel University Center of Samaria, Israel

Abstract

To investigate the association of polymorphisms within candidate genes which we hypothesized may contribute to stress fracture predisposition, a case-control, cross-sectional study design was employed. Genotyping 268 Single Nucleotide Polymorphisms- SNPs within 17 genes in 385 Israeli young male and female recruits (182 with and 203 without stress fractures). Twenty-five polymorphisms within 9 genes (*NR3C1, ANKH, VDR, ROR2, CALCR, IL6, COL1A2, CBG*, and *LRP4*) showed statistically significant differences ($p < 0.05$) in the distribution between stress fracture cases and non stress fracture controls. Seventeen genetic variants were associated with an increased stress fracture risk, and eight variants with a decreased stress fracture risk. None of the SNP associations remained significant after correcting for multiple comparisons (false discovery rate- FDR). Our findings suggest that genes may be involved in stress fracture pathogenesis. Specifically, the CALCR and the VDR genes are intriguing candidates. The putative involvement of these genes in stress fracture predisposition requires analysis of more cases and controls and sequencing the relevant genomic regions, in order to define the specific gene mutations.

Key words: Stress fractures; Bone remodeling; genetic variance; SNPs; inherited predisposition.

Introduction

Stress fracture (SF) is a prevalent overuse injury that usually affects bones of the lower extremities in physically active individuals, especially amongst athletes and military recruits of combat units during basic training (Hod et al., 2006; Murray et al., 2006). While the precise underlying mechanisms operative in SF pathogenesis remain elusive, the current paradigm stipulates that repeated, cyclical, weight-bearing activity leads to a change in the bone's remodeling processes- osteoclast mediated bone resorption and osteoblast driven bone formation (Romani et al., 2002). When this process is unbalanced, microscopic, and subsequent macroscopic bone fissures are encountered that are collectively referred to as SF (Orava and Hulkko, 1984).

Numerous variables have been reported as SF risk factors: mechanical [(e.g., training intensity and errors (Jones et al., 1993), hip joint (Giladi et al., 1991), tibia width (Giladi et al., 1987)], environmental [(e.g., shoes (Schwellnus et al., 1990) training surface (Albisetti et al., 2010)], and behavioral [(e.g., nutritional habits (Frusztajer et al., 1990), smoking (Altarac et al., 2000), motivation (Hallel et al., 1976)]. SF represents a common disorder that, in all likelihood results from the combined effects of environmental factors in genetically susceptible individuals- akin to other common multifactorial multigenic disorders.

Indirect lines of evidence do support the existence of genetic factors in SF pathogenesis: the existence of SF in monozygotic twins (Singer et al., 1990), SF in the pediatric age group (Bachmann et al., 2011, Lee et al., 2005), the high rate (10.6%) of SF recurrence within one year at different anatomical sites among recruits who suffered from SF during their basic training (Giladi et al., 1986). Moreover, anthropometric differences such as height, weight, narrow tibia, that have been reported to be associated with SF risk are also at least partially genetically determined (Milgrom et al., 1989).

Lastly, family history of SF and/or other bone associated pathologies have been reported to occur at significantly higher rates among American female soldiers with SF (Friedl et al., 1992). Notably, a family history of osteoporosis was reportedly associated with a 10-fold increased risk for developing SF in that population (Friedl et al., 1992). In the Israeli Defense Forces (IDF), family history of SF of first degree relatives was elicited in 8.5% of soldiers who developed high grade SF (Givon et al., 2000). Taken together, these data are consistent with the existence of a genetic susceptibility to SF.

To date, there are only two published reports on genetic analysis of SF not originating from our group. Välimäki and his coworkers (Valimaki et al., 2005) genotyped 15 Finnish soldiers with SF and compared the rates of two polymorphisms in the Estrogen receptor alpha gene- *ESR1* (MIM 133430)- *Xba*I (351A→G) and *Pvu*II (397T→C), and to CAG repeats in the Androgen receptor gene- *AR* (MIM 313700) in these cases with that of 164 non SF controls. No differences in polymorphisms rates were noted between cases and controls. Another group of researchers (Chatzipapas et al., 2009) assessed the rates of four polymorphisms (FokI, BsmI, ApaI, and TaqI) in the Vitamin D Receptor- *VDR* (MIM 601769) in 32 Greek individuals with SF and 32 controls, and reported a significant 2.7-fold and a non significant 2.0-fold increased SF risk associated with the FokI and BsmI alleles, respectively.

The aim of this study was to assess the putative association of sequence variants within several candidate

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genes with predisposition to SF in a group of IDF combat male and female soldiers using a case control study design.

Methods

This study was approved by the IDF IRB and the Ministry of Health's IRB for genetic studies ethic committees for human studies. All participants were active duty soldiers and were recruited from among combat soldiers referred to the Institute of Nuclear Medicine at the Medical Services and Supply Center of the IDF's Medical Corps with clinical symptoms compatible with SF from January 2007 to December 2009. Each participant, after signing a written informed consent, filled a questionnaire that detailed demographic data, personal and family history of SF and bone diseases, engagement in sports and smoking and alcohol history, and additional data relevant to SF risk.

All participants were clinically evaluated by an orthopedic surgeon whose physical examination focused on the lower limbs. Imaging included Technetium-99m methylene diphosphonate (Tc^{99}) bone scan. This is the "gold standard" method for diagnosing stress fractures with estimated sensitivity and specificity rates of close to 100% (Moran et al., 2008). Thus, it is routinely practiced and is the current protocol in the Medical corps of the IDF for all soldiers in the IDF who have symptoms compatible with stress fractures (Hod et al., 2006) in the Institute of Nuclear Medicine at the Medical Services and Supply Center for IDF soldiers. The results of the bone scan were interpreted by two different expert radiologists, and based on the results, the soldiers were classified as either having no evidence of SF, or having grade 1-4 SF, according to practiced criteria and protocols (Zwas et al., 1987). Individuals with grade 1 or a single grade 2 SF by the stated criteria for SF diagnosis and grading (Zwas et al., 1987) as well as individuals with metatarsal SF were excluded from the study, as a clearer phenotypic distinction between cases and controls was deemed appropriate for the current study design.

Blood (20ml) was drawn into EDTA containing tubes by certified phlebotomists and the DNA extraction was completed within 4 hours from the blood sample collection. DNA was extracted from peripheral blood leukocytes using the PUREGENE kit by Gentra Systems Inc (Minneapolis, MN), following the manufacturer's recommended protocol.

The genetic profile of the study participants was determined by characterizing 268 SNPs from the 17 candidate genes listed in Supplementary Table 1. The selected genes were deemed candidate genes to be involved in stress fracture pathogenesis as they are involved in a variety of bone pathologies: osteogenesis imperfecta, osteoporosis, osteopenia, (see Supplementary Table 1). All SNPs were selected from known SNPs international internet databases (www.genecards.org, www.ncbi.nlm.nih.gov/SNP/ index.html, and http://hapmap.ncbi.nlm.nih.gov) using the following criteria: SNPs which were previously reported as polymorphic in more than one population, minor allele frequency (MAF) greater than 10%, intra-gene location with preference for tagging SNPs, selected from the HapMap project (www.hapmap.org), polymorphisms with a known or predicted effect on protein's structure and/or function. All SNPs within and flanking the genes were eligible for inclusion as long as they fulfilled the above mentioned criteria.

The genotyping platform was the SEQUENOM™ MassARRAY system spectrometry (La Jolla, CA, USA), using the SEQUENOM Homogeneous MassEXTEND (hME) Assay SNPs analysis, as previously described (Storm et al., 2003).

Allele frequencies for each SNP between all cases and controls were calculated. SNPs with a minor allele frequency of less than 10% were excluded from further analysis. A Hardy-Weinberg equilibrium (HWE) test for each SNP within the entire control population was performed, and those SNPs not in HWE were also excluded from subsequent analysis. The association of each SNP with SF was evaluated using 5 different genetic models (inheritance patterns); co dominant, dominant, recessive, over dominant and log-additive, with adjustment for body mass index and gender as possible confounders. Results from individual SNP association analyses, were given as odds ratios with accompanying confidence intervals (CI). A FDR (false discovery rate) procedure was used to adjust for multiple comparisons for all SNPs and for all possible inheritance models using a recently developed multi-stage extension of the previously described method (Benjamini and Hochberg, 1995).

A pairwise allelic linkage disequilibrium (LD) test for each adjacent pair of SNPs within each gene was performed, and for those SNPs in significant LD with each other (using the normalized D' measure), haplotypes were constructed using the R haplo.stats software (http://cran.r-project.org/web/packages/haplo.stats/. Haplotypes and haplotype frequencies between the different groups (cases and controls) were estimated. The associations of these haplotypes with SF were then evaluated.

An association test (haplo.glm) was used that applies a haplotype-trait association test based on a general linear model using maximum likelihood estimates for haplotypes, allowing for ambiguity of haplotype phase. Specific risks were estimated as odds ratios (Tress et al., 2007) with associated 95% confidence intervals (CI), and were adjusted for putative confounders. As with the SNP association models different genetic models were examined. The most common haplotype was consistently used as the reference haplotype, whilst haplotypes with very low frequencies and counts were grouped together. The haplotype analysis was unadjusted for multiple comparisons.

Results

Two hundred and three soldiers (162 males and 41 females) ranging between 18-32 years, mean age: 20.2±1.3 years, were classified as having no evidence of SF and were assigned "control" status (C group), and 182 soldiers

(165 males and 17 females) ranging between 18-30 years, mean age: 20.1 ± 1.7 years, with grade 3-4 SF or multiple grade 2 SF were assigned "case" status (SF group). Both cases and controls were for the most part soldiers in basic training with 79% among SF cases and 73% among controls within 4 months of recruitment and initiation of their basic training. The compliance rate was low: the overall number of seemingly eligible individuals for both cases and controls over the time of recruitment was 3208 IDF soldiers, most of these being controls. There was a 12% compliance rate overall.

There were no statistically significant differences in anthropometric measures, physical activity habits prior to enrollment, smoking habits, and alcohol consumption between the two study groups (Table 1). The ethnic origin of the study population consisted of 49.5% (196 / 396) Ashkenazi, 38.1% (151/396) Non-Ashkenazi, and in 12.4% (49/ 396) ethnicity could not be ascertained as a result from refusal to answer this specific question.

Table 1. Selected characteristics of the study groups. Data are averages (± SD).

Characteristics	SF *(N=182)*	Controls *(N=203)*
Anthropometrics		
Age (years)	20.1 (1.7)	20.2 (1.3)
BMI ($kg \cdot m^{-2}$)	23.0 (.3)	23.2 (.2)
Height (m)	1.77 (.07)	1.77 (.07)
Weight (kg)	71.5 (9.5)	73.1 (11.1)
Ethnicity		
Ashkenazi	52.4%	46.3%
Non-Ashkenazi	41.6%	35.9%
Unknown	3.9%	17.8%
Lifestyle		
Physically active 6 months before recruitment	81.2%	78.9%
Running (km/wk)	8.0 (4.7)	7.6 (3.8)
Practice (h/wk)	5.2 (2.5)	5.1 (2.1)
Smoking habits		
Never (%)	63.6%	64.5%
Ever (%)	31.8%	33.1%
Current (%)	22.7%	27.7%
Past (%)	9.1%	5.4%
Didn't answer (%)	4.5%	2.5%
Alcohol consumption		
Yes (%)	72.1%	67.8%
No (%)	24.0%	29.7%
Didn't answer (%)	3.9%	2.5%

With respect to the bone scan results, 110 participants from the SF group (71.4%) were diagnosed with multiple SFs, while 53.4% (59 /110) suffered from bilateral tibia fractures, 17.3% (19/110) had bilateral femoral fractures. Among the 28.6% (44/154) of participants who sustained single SF, 48.2% had tibial SF, 39.3% had femoral SF, and 12.5% had SF in the fibula.

Ten participants from the SF group (6.5%) reported a family history of bone disorders or SF, as follows: five participants (3.2%) reported a previous SF in their father during military service (three a tibia grade 2 SF and two a femoral SF of unknown grade), four participants (2.6%) reported maternal osteopenia, and a single male participant (0.7%) reported on maternal osteoporosis. All five participants who reported that their mothers have osteopenia and/or osteoporosis were diagnosed with multiple grade 3 tibial SF. There were no reports on known family history of bone disorders and/or SF among controls that filled in the same questionnaires at the same time as the cases.

A total of 268 SNPs were selected from 17 genes spanning 12 chromosomes, such that the selected genes had maximum coverage (Supplementary Table 1). Overall, 220/268 (82.1%) SNPs within the 17 genes that were genotyped were used in the subsequent analysis. Forty eight genotyped SNPs (17.9%) were excluded from subsequent statistical analyses for the following reasons: 5.6% (15/268) were not polymorphic, 1.5% (4/268) had MAFs less than 10%, and 10.8% (29 out of 268) were not in HWE, as presented in Supplement 1.

A total of 11.4% polymorphisms (25/220 SNPs) within the below listed 9 genes were found to have a statistically different distribution between cases and controls: *NRC31, ANKH, VDR, ROR2, CALCR, IL6, COL1A2, CBG, and LRP4*. Seventeen genetic variants were associated with an increased SF risk (9 SNPs and 8 haplotypes) and eight variants- were associated with a decreased SF risk (7 SNP and 1 haplotype) as detailed in Table 2.

Sequence variants in a total of 8 genes were associated with an increased risk for SF development: *NR3C1*, *ANKH*, *VDR*, *ROR2*, *CALCR*, *IL6*, *CBG*, and *COL1A2* (Table 2). Among the sequence variants which were significantly associated with an increase in SF risk, the CCAGGCAC haplotype of the *VDR* gene (composed of the following 8 SNPs: rs2853564, rs4760648, rs11168287, rs4328262, rs4334089, rs7136534, rs10782318, and rs7299460) was found to most prominently increase SF risk (12.22-fold, 95%CI 1.45-102.7, p = 0.022). In addition, the CGTTCTCCGA haplotype of the *CALCR* gene (composed of 10 SNPs: rs18011972, rs2283004, rs7790825, rs12666831, rs757033, rs1326124, rs972978, rs2214213, rs7783961, and rs2188805) was found to significantly increase SF risk by 1.93-fold (95%CI 1.11-3.50), p = 0.00255).

Sequence variants in six genes were associated with decreased SF risk: *NR3C1*, *AR*, *VDR*, *CALCR*, *COL1A2*, and *LRP4* (Table 2). While the ACCTATAAG haplotype of the *VDR* gene (composed of the following 9 SNPs: rs7975232, rs987849, rs2107301, rs2239182, rs2525044, rs2239179, rs2248098, rs1540339, and rs40734735) was associated with the lowest (84%) ORs for SF development (OR = 0.16 (95%CI 0.04-0.62, p = 0.00876)), the rs2306033 polymorphism of the *LRP4* was found to be strongly associated (p = 0.001722) with a 61% decreased probability for SF (OR = 0.39 (95%CI 0.21-0.72)). None of the single SNP associations retained the significance level of 0.05 after adjusting for multiple comparisons using the multi stage FDR methodology.

Results remained unchanged when the 49 individuals whose ethnicity was unknown were excluded from the analysis (data not shown).

Discussion

In this pilot study the association of SF with 25 sequence

Table 2. List of the 25 genetic markers found to be significant different between the SF and the C groups, hence associated with SF pathogenesis: both SNPs and haplotypes analyses within the following 9 genes: *NR3C1, ANKH, VDR, ROR2, CALCR, IL6, COL1A2, CBG, and LRP*

Gen	Genetic marker		†Data	OR (95% CI, p-value)	#Model analysis
NR3C1	SNP	*rs4244032*	*G/A (A/A)*	.94 (.39-.61, p=.04)	*CD*
			G/A (A/A-G/G)	.58 (.38-.89, p=.01)	*OD*
		rs12656106	*G/C (G/G)*	.57 (.36-.91, p=.04)	*CD*
			C/C (G/G)	.54 (.29-.98, p=.04)	*CD*
			G/C-C/C (G/G)	.56 (.36-.87, p=.01)	*D*
	Hap.	GAAAA	13.74 (6.66)	2.11 (1.01-4.39, p=.05)	CD
		GT	44.12 (36.87)	1.49 (1.07-2.07, p=.02)	CD
ANKH	SNP	rs4701616	C/T/-C/C (C/T)	1.67 (1.11-2.53, p = .01)	D
VDR	SNP	*rs4328262*	*G/T (T/T)*	.57 (.33-.99, p = .04)	*D*
			G/T (T/T-G/G)	.53 (.32-.88, p=.01)	*OD*
	Hap.	*ACCTATAAG*	*2.65 (3.3)*	.16 (.04-.62, p = .01)	*CD (1st block)*
		CCAGGCAC	4.2 (NA)	12.22 (1.45-102.7, p = .02)	CD (2nd block)
ROR2	SNP	rs10992075	G/A (A/A)	1.68 (1.04-2.69, p = .02) ‡	CD
	Hap.	CCCTACC	6.97 (4.45)	2.71 (1.03-7.12, p = .04)	CD (block 5a)
CALCR	SNP	*rs12154667*	*T/T (C/C)*	.48 (.27-.85, p =.01)	*CD*
			C/T (C/C)	.47 (.29-.77, p =.01)	*CD*
			C/T-T/T (C/C)	.47 (.30-.75, p =.001)	*D*
		rs1548456	T/T (C/C)	2.23 (1.2-4.15, p = .01)	CD
			T/T (C/C-C/T)	2.39 (1.35-4.25, p = .002)	R
	Hap.	CGTTCTCCGA	12.91 (8.43)	1.93 (1.09-3.43, p = .003)	CD
		CC	16.05 (13.75)	1.68 (1.05-2.71, p = .02) ‡	CD
		CT	41.25 (33.96)	1.47 (1.08-2.02, p = .02)	CD
IL6	SNP	rs1554606	T/T (G/G-G/T)	3.48 (1.35-8.94, p = .005)	R
COL1A2	SNP	rs420257	C/C (T/T-T/C)	2.29 (1.10-4.80, p = .02) ‡	R
		rs42517	G/G (A/A-A/G)	11.03 (1.36-89.20, p = .003) ‡	R
		rs42522	G/G (A/A-A/G)	2.21 (1.02-4.77, p = .04) ‡	R
		rs24531	*A/G (G/G)*	.54 (.34-.88, p = .01) ‡	*CD*
		rs413826	*C/G-G/G (C/C)*	.53 (.32-.88, p = . 01) ‡	*D*
CBG	SNP	rs11629171	C/T-T/T (C/C)	1.65 (1.09-2.50, p = .02)	D
		rs2281518	T/C (T/T-C/C)	1.52 (1.00-2.32, p = .05)	OD
	Hap.	CATCCT	14.5 (9.75)	1.82 (1.03-3.22, p = .04)	CD
LRP4	SNP	*rs2306033*	*A/G-A/A (G/G)*	.39 (.21-0.72, p =.002)	*D*

Inducer marker / *Protective marker (Italic style)*. ‡ Adjusted for BMI

† **SNP analysis**- SNPs genotyping using the relevant model: CD- Co dominant model, whereas rare genotype (in comparison to the frequent genotype), e.g., A/T (T/T) = the frequent genotype is T/T; D- Dominant model, whereas both heterozygous and rare homozygous (in comparison to the frequent genotype, the frequent homozygous)side; OD- Over dominant model, whereas heterozygous genotype (in comparison to both homozygous genotypes), e.g., A/C (A/A-C/C); R- Recessive model, whereas rare homozygous (in comparison to both: frequent homozygous and heterozygous genotypes). **Hap. (haplotype analysis)**- Distribution (%) among SF group (in brackets the haplotype frequency among the C group); e.g., 2.65 (3.3) = 2.65% distribution among the SF group in comparison to 3.3% distribution among the C group

Model analysis: CD=Co dominant SNP analysis; D=Dominant SNP analysis; OD=Over dominant SNP analysis.

variants within the following 9 genes: *NRC31, ANKH, VDR, ROR2, CALCR, IL6, COL1A2, CBG*, and *LRP4* were assessed. Specifically sequence variants within two genes *CALCR* and *VDR* emerged from the current study as seemingly associated with stress fracture. Recently, six *CALCR* polymorphisms were reportedly associated ($p < 0.005$) with lower lumbar spine BMD (rs10488551, rs2283002, and rs6976450) and lower hip BMD (rs2214213, rs7790825, and rs972978) among 709 pre menopausal Canadian females (average age: 44.5±7.2 years) (Giroux et al., 2010). In the present study, one of these 6 SNP's (rs12154667) was associated with lower SF risk, another (rs1548456) with an increased SF risk and two haplotypes- with an increased stress fracture risk. Since Calcitonin is one of the hormones involved in regulating osteoclast activity and calcium secretion (Bijvoet et al., 1971; Bussolati and Pearse, 1967), it seems plausible that these intronic polymorphisms affect intracellular molecular pathways eventually manifesting as SF.

VDR polymorphisms have been associated with os teoporosis risk in some (Eisman, 1999) but not all (Arden et al., 1996; Willing et al., 1998) women of diverse ethnicity. These contradicting findings are in part attributed to the notion that the effects of polymorphisms in this receptor are dependent on interactions with the environment, particularly calcium intake (Ferrari et al., 1998). Notably, *VDR* binds the active form of vitamin D (1,25-dihydroxyvitamin D) with high affinity to mediate its effects on calcium homeostasis, skeletal development and bone mineralization (Achermann and Jameson, 2003).

Moreover, some of these VDR polymorphisms have been associated with SF risk in other military recruits (Chatzipapas et al., 2009). The results of the present study emphasize that the association between VDR and SF risk may be complex and should be further studied both genetically and functionally. At the time of writing only two published studies, not originating from our group, reported the involvement of seven polymorphisms

within 3 genes (*ESR1*, *AR*, and *VDR*) in SF pathogenesis (Chatzipapas et al., 2009; Valimaki et al., 2005). The data presented herein strengthen the notion that *VDR* sequence alterations and specific haplotypes are affecting SF risk. Yet the fact that SNPs and haplotypes within *VDR* were found to have divergent and in fact opposing effects on SF risk in our study population may be indicative of the complex interaction between *VDR* and SF pathogenesis.

Noteworthy, all SNPs genotyped in the present study and shown to be associated with altering SF risk are localized in intronic, non-coding regions within candidate genes. Although they are not functional variants, these polymorphisms may be directly involved in protein function via several regulatory elements, such as intron splice enhancers and silencers that regulate alternative splicing (Tress et al., 2007). Alternatively, these SNPs may simply be in linkage disequilibrium with other functional SNPs or mutations in the gene (Cooper, 2010).

The fact that none of these associations remained significant after correcting for multiple comparisons needs to be emphasized. The reasons for the lack of significance between the SNPs and stress fracture phenotype may stem from the limited number of patients that were analyzed and the fact that for the most part the SNPs were not actually causative but rather indicative of a presumed existence of a causative mutation in the vicinity of the SNP or residing on a specific haplotype. Moreover, in some genes (e.g., VDR, COL1A2) SNPs or haplotypes were shown to increase the risk whereas other SNPs from the same gene were associated with lowering the risk. Such a distinct effect on phenotype may be accounted for by different mutation types in linkage disequilibrium with the specific SNP or haplotype: the effect of an activating mutation compared with the effect of an inactivating mutation in the same gene that result in two opposing phenotypes. This is a well known phenomenon in a variety of human disorders (Lerner et al., 2010; Vaclavikova et al., 2009).

The lack of significance following FDR may also indicate that these "associations" between the SNPs and stress fracture phenotype were obtained spuriously and represent a chance occurrence only and not a real biological plausible pathway or mechanism underlying stress fracture. In this case, the negative results of any real association with genes and SNPs that were previously associated with osteoporosis may be indicative that the mechanism that underlies stress fractures is distinct from that of osteoporosis and the additional rare inherited bone diseases that were queried in the current study.

The limitations of this study should be pointed out. Although it is the largest study published that addresses the issue of genetic predisposition to SF, it is still possibly underpowered to detect genetic differences tagged by SNPs. It also focused on a specific population of young recruits in the IDF and the mechanisms operative in non military service SF (e.g., elite athletes) may differ. The low compliance rate (12%) may have led to selection bias affecting the current study results. Moreover, the retrospective case-control study design precludes any conclusions as to the predictive value of our findings. This study was based on the candidate gene approach that inherently is limited to the selected candidate genes. Lastly, the p-values for the haplotypes were not adjusted for any possible multiplicity problem, which could mean that some of the p-values found to be significant would become non-significant if an appropriate adjustment method were applied.

Conclusion

This study suggests that there may be genetic factors that contribute to SF pathogenesis and has indicated some of the genes that may underlie SF predisposition in young military recruits. Specifically the CALCR and the VDR genes are intriguing candidates to be involved in stress fracture pathogenesis. Obviously, these results need to be replicated, confirmed and extended by analyzing more cases and controls, before any conclusions can be drawn.

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Key points

- Understanding the possible contribution of genetic variants to stress fracture pathogenesis.
- There is a paucity of data on the involvement of polymorphisms in specific genes in active military personnel/athletes which may contribute to stress fractures development.
- The results from the current study should facilitate a more comprehensive look at the genetic component of stress fractures.

AUTHORS BIOGRAPHY

Ran YANOVICH

Employment

The deputy director of the military physiology unit, Heller Institute of Research, Sheba Medical Center in Israel.

Degree

PhD

E-mail: ran.yanovich@hseba.health.gov.il

Eitan FRIEDMAN

Employment

Head of the Oncogenetics unit, Sheba Medical Center, Tel Hashomer, A full professor at the Department of Internal Medicine, Sackler Medical School, Tel-Aviv University.

Degree

MD, PhD

Research interests

The genetic basis of common disorders and inherited predisposition to cancer

E-mail: eitan.friedman@sheba.health.gov.il

Roni MILGROM

Employment

Doctoral candidate at the Weitzman Institute of Science, Rec Israel.

Degree

MSc

E-mail: ronimilgrom@gmail.com

Bernice OBERMAN

Employment

The biostatistics unit, at the Gertner Institute of Epidemiology and Health Policy Research teaches at the Tel-Aviv University

Degree
MSc
E-mail: BerniceO@gertner.health.gov.il

Laurence FREEDMAN
Employment
Professor, Bar Ilan University, Israel and the head of the Biostatistics Unit, Gertner Institute of Epidemiology and Health Policy Research, Sheba Medical Center.
Degree
PhD
E-mail: lsf@actcom.co.il

Daniel S. MORAN
Employment
Professor, Ariel University Center of Samaria, and Senior researcher at the Heller Institute of Research, Sheba Medical Center.
Degree
PhD
Research interests
Adaptation to stress and extreme conditions encountered during military service, including stress fractures.
E-mail: Dani.Moran@sheba.health.gov.il

✉ **Eitan Friedman, MD, PhD**
Head, the Susanne Levy Gertner Oncogenetics Unit, The Danek Gertner Institute of Human Genetics, Chaim Sheba Medical Center, Tel-Hashomer, 52621, Israel

Supplementary Table 1. List of the SNPs within the candidate genes which were analyzed for SF, their chromosomal loci, and their bone associated pathologies or other disorders.

Gene symbol	Gene	SNPs	SNPs analyzed / (total SNPs)	Chrom. loci	Bone pathology/other disorders
COL1A1 (MIM 120150)	Procollagen type I alpha 1	rs1061237, rs2277632, rs2586488, rs2075555, rs2075559	5 / (5)	17q21.3-q22.1	[d]OI, [e]OST, [f]EDS
COL1A2 (MIM 120160)	Procollagen type I alpha 2	rs420257, rs42518[a], rs2521206, rs2621212, rs411717, rs6465412, rs369982, rs388625, rs11764718, rs42522, rs42531, rs2521205, rs1858822, rs42526, rs2621215, rs42517, rs413826	16 / (17)	7q22.1	[d]OI, [e]OST, [f]EDS
ESR1 (MIM 133430)	Estrogen receptor alpha	rs3020434, rs4583998[a], rs9340799, rs3003924[a], rs1643821, rs12199722, rs4870062, rs3778099, rs7755185, rs11155819, rs827420, rs2982712[a], rs9322331, rs3020369, rs2077647, rs4870061, rs827421, rs3020404, rs9340933[c], rs3020382, rs3020376[a], rs6911230[a], rs12662655[c], rs3020370, rs3020394, rs6902771, rs3853248[a], rs1709184, rs532010, rs3798577, rs9479130, rs1884052, rs2982896[a], rs1709183, rs3020407, rs3844508[a], rs4870056, rs1884051, rs2347867, rs9340835[a], rs3020414[c], rs2474148, rs1709182, rs3138774, rs2234693, rs9340954, rs1999805[a]	34 / (47)	6q25.1	[e]OST, [g]OSA
VDR (MIM 601769)	Vitamin D receptor	rs40734735, rs4334089, rs4237855[a], rs1540339, rs2853564, rs3890734[a], rs4760648, rs2239179, rs2248098, rs2525044, rs2239182, rs2107301, rs7136534, rs7299460, rs731236[a], rs2254210, rs987849, rs4328262, rs11168287, rs7975232, rs10783219, rs38195845	19 / (22)	12q13.11	[e]OST
IGF1 (MIM 147440)	Insulin like growth factor	rs2195240, rs17796225, rs5009837, rs6214, rs7136446, rs5742629, rs10735380, rs4764697, rs11111272, rs9308315, rs12821878, rs2373721, rs10778176	13 / (13)	12q22-q23	[e]OST
IL6 (MIM 147620)	Interleukin 6	rs1554606, rs2069832, rs13306435[c], rs11544633[c], rs2069840, rs2069860[b]	3 / (6)	7q21	[h]BMD
TGF-β1 (MIM 190180)	Transforming growth factor, beta-1	rs8179181	1 / (1)	19q13.2	[e]OST
NR3C1 (MIM 138040)	Glucocorticoid receptor	rs12655166, rs4244032, rs852980[c], rs9324911[c], rs9324916, rs2963155[c], rs6877893, rs258751[c], rs2918417, rs7701443, rs852979, rs10482682, rs17100289, rs9324924, rs12656106, rs4607376, rs33388	13 / (17)	5q31.3	[e]OST, [i]OSP
CALCR (MIM 114131)	Calcitonin receptor	rs13226124, rs12154667, rs7783961, rs12666751[c], rs2301680[a], rs2214213, rs10237272, rs1548457, rs9656015, rs6465381, rs2299249, rs1548456, rs1801197, rs17788132, rs10282132[b], rs10488551, rs2051748, rs12666831, rs2188805, rs757033, rs7790825, rs6970701[b], rs2283002[c], rs972978, rs2283004, rs9641123	21 / (26)	7q21.3	[e]OST

Lower line- SNPs excluded from the statistical analysis, from the following reasons: [a]- Not in HWE (Hardy-Weinberg Equilibrium); [b]- MAF<10%; [c]- Not polymorphic

Bone pathology and other disorders- [d]OI- Osteogenesis imperfecta, [e]OST- Osteoporosis, [f]EDS- Ehlers-Danlos syndrome, [g]OSA- Osteoarthritis, [h]BMD- Bone mineral density, [i]OSP- Osteopenia, [j]CL2- Chondrocalcinosis 2, [k]CRD- Craniometaphyseal Dysplasia, [l]BTB- Brachydactyly type B, [m]RRS- Recessive Robinow Syndrome, [n]OPS- Osteoporosis-Pseudoglioma syndrome, [o]CWP- widespread pain disorder

Supplementary Table 1. Continued.

Gene symbol	Gene	SNPs	SNPs analyzed / (total SNPs)	Chrom. loci	Bone pathology/other disorders
ANKH (MIM 605145)	Progressive ankylosis protein homolog	rs706293, rs17251763, rs258219, rs879253, rs1061813, rs31916, rs826188[a], rs153930, rs1004673, rs258226, rs2453327, rs4701616, rs826352, rs10513186, rs1374080, rs2921600, rs875525, rs31912, rs1353258, rs31934, rs28005, rs379016, rs258231, rs1620976, rs10052744, rs40970, rs27353, rs31989[a], rs17317655, rs3006069, rs1550826, rs697565, rs697571, rs744165, rs2454873, rs1697124, rs13170282[c], rs4702049[c], rs412056, rs258360[a], rs398961, rs31911	37 / (42)	5p15.1	[h]BMD, [j]CL2, [k]CRD
TNFRSF11A (MIM 603499)	Activator of Nuclear Factor κ B	rs1805034	1 / (1)	18q22.1	[e]OST
ROR2 (MIM 602337)	Receptor tyrosine kinase-like orphan receptor 2	rs10125466, rs7031729[a], rs10992075, rs7869182, rs10761134, rs2312735[a], rs4073735, rs10992149, rs7855522, rs6479385[a], rs10761130[a], rs7029814, rs9409461, rs10820921, rs10992145, rs7038397, rs11789973, rs7874148, rs4639579[a], rs7037255, rs7039620, rs4467996, rs1534533, rs10992124, rs4744105[a], rs4744107, rs7858435, rs10992123[a], rs4744113, rs16907725, rs12683181, rs4743855, rs9774945, rs12685556[c], rs9409652, rs10992072, rs10761129[a], rs6479386, rs3905385, rs7021744, rs10992065, rs12376130, rs1881389[a], rs4237215, rs7871522[c], rs7863557, rs10992063, rs12554679, rs4595189, rs2141368, rs10992158	40 / (51)	9q22	[l]BTB, [m]RRS
CBG (MIM 122500)	Corticosteroid binding globulin	rs2281518, rs11160168, rs2144834, rs11629171, rs941599, rs11622970[a], rs1042394, rs2144835, rs2281519, rs1998056	9 / (10)	14q32.1	[o]CWP
LRP4 (MIM 604270)	Low density lipoprotein receptor-related protein 4	rs2306033	1 / (1)	11p11	[e]OST
LRP5 (MIM 603506)	Low density lipoprotein receptor-related protein 5	rs3736228, rs4988300[a], rs4988321[b]	1 / (Jones, Bovee)	11q13.4	[e]OST, [n]OPS
BMP2 (MIM 112261)	Bone morphogenetic protein 2	s1980499, rs2273073, rs235764, rs15705	4 / (4)	20p12.3	[e]OST
ARHGEF3 (MIM 612115)	Rho guanine nucleotide exchange factor GEF 3	rs7646054, rs983739, rs1110866	3 / (3)	3p21-p13	[e]OST
Total SNPs	**220 / (268)**				

Lower line- SNPs excluded from the statistical analysis, from the following reasons: [a]- Not in HWE (Hardy-Weinberg Equilibrium); [b]- MAF<10%; [c]- Not polymorphic

Bone pathology and other disorders- [d]OI- Osteogenesis imperfecta, [e]OST- Osteoporosis, [f]EDS- Ehlers-Danlos syndrome, [g]OSA- Osteoarthritis, [h]BMD- Bone mineral density, [i]OSP- Osteopenia, [j]CL2- Chondrocalcinosis 2, [k]CRD- Craniometaphyseal Dysplasia, [l]BTB- Brachydactyly type B, [m]RRS- Recessive Robinow Syndrome, [n]OPS- Osteoporosis-Pseudoglioma syndrome, [o]CWP- widespread pain disorder