Haplotypes of (–794(CATT)$_{5–8}$/–173G>C) *MIF* gene polymorphisms and its soluble levels in basal cell carcinoma in western Mexican population

Elizabeth Guevara-Gutiérrez,[1] María José Castro-Jonguitud,[1] Susana Elizabeth De la Torre-Flores,[1] José Francisco Muñoz-Valle,[2] Alberto Tlacuilo-Parra,[3] Francisco Javier Salazar-Torres ,[1] Yeminia Valle,[2] Jorge Ramón Padilla-Gutiérrez,[2] Diana Emilia Martínez-Fernández,[1] Emmanuel Valdés-Alvarado [2]

[1]Instituto Dermatológico de Jalisco "Dr. José Barba Rubio", Secretaría de Salud Jalisco, Zapopan, Jalisco, México
[2]Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Mexico
[3]División de Investigación, Unidad Médica de Alta Especialidad (UMAE), Hospital de Pediatría, Centro Médico Nacional de Occidente, IMSS, Guadalajara Jalisco, México

Correspondence to
Dr Emmanuel Valdés-Alvarado, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara 44340, Mexico; emmanuel634@hotmail.com

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ABSTRACT

Basal cell carcinoma (BCC) is the most common dermatological neoplasms in Caucasian populations. In Mexico, a prevalence of 3.9 per 1000 habitants is estimated. Recently, the macrophage migration inhibitory factor (MIF) has been related to different types of cancer. Therefore, this study aimed to investigate the genetic association of haplotypes of [-794(CATT)5-8/-173G>C]MIF gene polymorphisms and its soluble levels in BCC. A total of 360 individuals were recruited for the study, that is, 180 of the total amounts were patients with BCC histologically confirmed and the remaining 180 individuals were identified as control subjects (CS). Both polymorphisms were genotyped by PCR and PCR-RFLP (restriction fragment length polymorphism), and MIF serum levels were measured by ELISA kit. A borderline difference was found between the 55 genotype and the susceptibility to BCC (5.6% vs 1.7% in BCC and CS, respectively, OR=3.7 and p=0.04). Furthermore, the haplotype 7G showed a significant association with BCC (p=0.02, OR=1.99). Concerning MIF soluble levels, patients with BCC showed a media of 2.1 ng/mL and CS showed 4.4 ng/mL, the comparison between groups was significant (p<0.01). Our findings suggest that the 55 genotype and the haplotype 7G are associated with the susceptibility to BCC; furthermore, a significant difference was found between MIF soluble levels in both study groups.

Significance of this study

What is already known about this subject?

- Migration inhibitory factor (MIF) may contribute to carcinogenesis by preventing cellular apoptosis or by promoting chronic inflammation through the influx of inflammatory cells.
- MIF directly promotes tumor cell proliferation and invasion in various cancer cell types.
- The proinflammatory and proangiogenic activities of MIF position it as a potentially important player in the development and progression of non-melanoma skin cancer.
- *MIF* promoter polymorphisms affect MIF expression and soluble levels.

What are the new findings?

- For the first time influence of promoter polymorphism and soluble levels of MIF are analyzed in Mexican Mestizo population.
- MIF soluble levels are increased in control subjects with respect to patients with basal cell carcinoma (BCC); nevertheless, both groups are under the reference parameters.
- Haplotypes of MIF promoter polymorphism could increase the risk of development of BCC.
- MIF could have protection or risk roles in BCC, depending on the cellular sources of production (analyzed previously in mice, now in humans).

How might these results change the focus of research or clinical practice?

INTRODUCTION

Basal cell carcinoma (BCC) is the most common dermatological neoplasms in Caucasian populations. In Mexico, a prevalence of 3.9 per 1000 habitants is estimated.[1] Nevertheless, in recent years there has been an increase in the number of cases becoming a health problem.[2] BCC arises from the basal cell layer of the epidermis, rarely metastasize, nevertheless, are locally destructive. BCC is divided clinically into broad morphological subtypes including nodular, micronodular, superficial, and morphoeic/infiltrative.[3] The most common cause of BCC is exposure to ultraviolet (UV) light, particularly ultraviolet B (UVB),[2–4] other

Significance of this study

- The research of new biomarkers that could be predictive of cancer (genetic variants or soluble markers), as well as its relationship with environmental risk factors, falls on the challenges posed to close the gap between the evidence and medical practice and the further development and diversification of targeted therapies since they improve understanding the etiology, carcinogenic mechanisms, and prognosis of cancer. Results on which measures can be taken to guarantee the best care and treatment are available to the patient.

causes include ionizing radiation, immunosuppression, and toxin exposures. Inherited familial cancer syndromes, such as Gorlin, Bazex-Dupré-Christol, and Rombo syndromes, are associated with a predisposition to BCC.[2–5] However, non-syndromic genetic susceptibility to BCC is poorly investigated. Recently, some studies have shown the relation of macrophage migration inhibitory factor (MIF) and different types of cancer.[6–8] Recently, MIF expression is demonstrated to be elevated in various solid tumors. Furthermore, the level of MIF is correlated to the metastatic potential of tumors including hepatoma, gastric cancer, lung cancer, and breast cancer.[6] MIF is a pleiotropic proinflammatory mediator with chemokine-like functions that is secreted in both constitutive and inducible fashion, and MIF is an important immunoregulator of the inflammatory response in multiple organ systems and to be overexpressed in multiple human tumors, including melanoma and no melanoma skin cancer.[9] MIF's proinflammatory and proangiogenic actions make this cytokine a possible preventive and therapeutic target for non-melanoma skin cancer (NMSC).[8 9] MIF is a small homotrimeric (3×12.5 kDa) code by *MIF* gene, which is located at 22q11.2, and this protein was originally found to inhibit spontaneous random migration of macrophages out of capillary tubes in vitro.[10] Two functional polymorphisms are located in the promoter region of *MIF* gene, the first polymorphism is a CATT short tandem repeat (STR) at position −794, with five to eight-length variants (alleles 5–8), in which the number of repeats of CATT is associated with varying amounts of serum-circulating levels of MIF.[11] The second promoter polymorphism is a single-nucleotide polymorphism (SNP) in the position −173 and consists of a transversion of G>C.[12] It is important to highlight that a strong linkage disequilibrium among 7-CATT and −173*C alleles in western Mexican population was detected by Llamas-Covarrubias *et al*.[13] Moreover, the presence of the polymorphic alleles −794(CATT7) and −173*C MIF has been associated with higher expression of protein serum levels in the Caucasian population.[11 13 14] Based on this knowledge, this study aimed to investigate the association between haplotypes of ($-794(CATT)_{5-8}$/−173G>C) *MIF* gene polymorphisms and its soluble levels in BCC in western Mexican population.

MATERIALS AND METHODS

Subjects

The study group included a total of 360 Mexican Mestizo subjects: 180 of the total amounts were histologically confirmed as patients with BCC. All these patients were unrelated and were recruited from the "Instituto Dermatológico de Jalisco José Barba Rubio" in Guadalajara city, Mexico. Furthermore, we recruited 180 unrelated individuals identified as control subjects (CS) age–sex matched with patients with BCC. We considered Mexican Mestizo subjects, only those individuals who for three generations, including their own, had been born in western Mexico.

Ethics statement

The study was performed according to the ethical principles for experiments involving humans stated on the Declaration of Helsinki and ethical approval was obtained by Dirección General de Salud Publica. Informed consent was obtained from all patients for being included in the study. Furthermore, submitting authors are responsible for coauthors declaring their interests.

Genotyping of MIF $-794(CATT)_{5-8}$ and −173G>C polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using Miller's technique.[15] Conditions were performed as in previous studies in our research group,.[13] Genotyping of the STR $-794(CATT)_{5-8}$ polymorphism was achieved by conventional PCR, and cycling conditions were: initial denaturing 95°C for 4 min followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, then a final extension of 2 min at 72°C. Amplification products were further electrophoresed on a 19:1 7% polyacrylamide gel at 150 V for 15 hours and stained with $AgNO_3$. On the other hand, the −173G>C MIF polymorphism was genotyped by PCR-RFLP (restriction fragment length polymorphism), and cycling conditions were 40 cycles and an annealing temperature of 60°C were used. The 366 bp fragment obtained was further digested with Alu I restriction endonuclease (New England Biolabs, Ipswich, Massachusetts, USA) by overnight incubation at 37°C. Finally, the digest was resolved on a 29:1 6% polyacrylamide gel stained with 0.02% $AgNO_3$. The G allele resulted in a 268 bp and a 98 bp fragments, while the C allele was represented by a 206 bp, 98 bp, and a 62 bp fragments. Results were confirmed by the automatized sequencing of one random sample of each genotype of both polymorphisms (Applied Biosystems, USA).

MIF serum levels

The determination of MIF serum levels was performed by ELISA and the commercial Human MIF ELISA Kit (RayBio, USA), according to the manufacturer's instructions. MIF assay sensitivity was 6 pg/mL.

Statistical analysis

The Hardy-Weinberg equilibrium test and genotype and allele frequencies were calculated by the χ^2 test or Fisher's exact test, when applicable. ORs and 95% CIs were calculated to test the probability that the genotype and allele frequencies were associated with BCC. Haplotype inference was performed using EMHAPFREQ software.[16] Linkage disequilibrium was estimated through the Lewontin D' measure (LD). Haplotypic frequencies were also compared through χ^2 and Fisher's exact tests. MIF serum levels were compared among groups by Student's t-test and analysis of

Table 1 Clinical and demographic characteristics

Variable	BCC (N=180), n (%)	CS (N=180), n (%)
Demographics		
Age (years)*	66 (36–85)	62 (40–75)
Gender†		
Male	68 (38)	63 (35)
Female	112 (62)	117 (65)
Clinical characteristics		
Tumor size		
<5 mm	56 (31)	–
>5 mm	124 (69)	–
Tumor localization		
Head–neck	165 (92)	–
Body trunk	11 (6)	–
Others	4 (2)	
BCC grade		–
Low	131 (73)	–
High	49 (27)	

*Data show minimum and maximum.
†Data show median.
BCC, basal cell carcinoma; CS, control subjects.

variance tests for MIF and Mann-Whitney U test. A p value <0.05 was considered to be statistically significant. All the statistical analyses were done with the SPSS V.20.0 software.

RESULTS

Clinical and demographic characteristics

All clinical characteristics are shown in table 1. The median age of CS and BCC groups was 66 and 62 years, respectively. The gender distribution among BCC individuals was 62% males and 38% females. About the skin lesions identified in the patients with BCC, a total of 31% of the tumors were <5 mm of diameter and 69% were higher than 5 mm. Moreover, we also detected the localization of the tumor. 92% of our patients presented the lesion in the head–neck, 6% in the body trunk, and 2% in other body parts, such as genitals, lower and upper extremities. To find out the grade of BCC, the pathologist looks at a tissue sample from the skin under a microscope. The grade is a description of how the cancer cells look and act compared with normal cells. Low grade means that the cancer cells are well differentiated, in this sense, 73% of patients with BCC showed a low grade and the rest (27%) showed a high grade. High grade means that the cancer cells are poorly differentiated or undifferentiated.

Analysis of MIF –794(CATT)$_{5–8}$ and –173G>C polymorphisms

There was no deviation from the Hardy-Weinberg equilibrium for any of the polymorphisms in both groups (p>0.05). The genotype and allele frequencies of *MIF* −794(CATT)$_{5–8}$ polymorphism in both study groups are summarized in table 2. The most common genotypes in BCC and CS subjects were 67 and 66, respectively. Allele 6 was the most frequent in both groups (BCC 53% and CS 56%). A borderline difference was found between the 55 genotype and the susceptibility to BCC (5.6% vs 1.7% in BCC and CS, respectively, OR=3.7 and p=0.04). Concerning the genotype and allele frequencies of *MIF* −173G>C polymorphism, these are summarized in table 3. The most common genotype in both study groups was GG (BCC 56.7% and CS 52.2%). Allele G was also the most frequent in both groups (BCC 76.7% and CS 72.5%); however, the comparisons of genotype and allele frequencies for the −173G>C*MIF* polymorphism between both study groups did not show significant differences.

Table 2 Allele and genotype frequencies of the –794(CATT)$_{5–8}$ *MIF* polymorphisms

Polymorphisms	BCC (N=180), n (%)	CS (N=180), n (%)	OR (95% CI); p value*
–794(CATT)$_{5–8}$ MIF			
Genotype			
55	10 (5.6)	3 (1.7)	3.7(0.9 to 14.3); 0.04
56	49 (27.2)	56 (31.1)	0.9 (0.6 to 1.7); 0.92
57	14 (7.8)	8 (4.4)	1.9 (0.7 to 5.1); 0.16
66*	44 (24.4)	49 (27.2)	1
67	54 (30)	48 (26.7)	1.2 (0.7 to 2.2); p=0.43
77	9 (5)	16 (8.9)	0.6 (0.2 to 1.6); p=0.31
Allelle			
5	83 (23.1)	69 (19.2)	1.3 (0.9 to 1.9); p= 0.21
6*	191 (53.1)	202 (56.1)	1
7	86 (23.8)	89 (24.7)	1.1 (0.7 to 1.5); p=0.9

*p<0.05.
BCC, basal cell carcinoma; CS, control subjects; MIF, migration inhibitory factor.

Haplotypes of (–794(CATT)$_{5–8}$/–173G>C) MIF gene polymorphisms

The linkage disequilibrium analysis was tested and showed that the alleles of both polymorphisms do not segregate independently (LD=0.63). Subsequently, we compared the haplotype distribution between groups as shown in table 4. Haplotype 6G was the most frequent in BCC (47.9%) and CS (50.6%). Haplotype 7G showed a significant association with BCC (p=0.02, OR=1.99).

MIF soluble levels

MIF soluble levels were measured in patients with BCC and CS and are presented in figure 1. Patients with BCC showed

Table 3 Allele and genotype frequencies of the –173G>C*MIF* polymorphisms

Polymorphisms	BCC (N=180), n (%)	CS (N=180), n (%)	OR (95% CI); p value*
–173G>C *MIF*			
Genotype			
GG*	102 (56.7)	94 (52.2)	1
GC	72 (40)	73 (40.6)	0.9 (0.6 to 1.2); 0.66
CC	6 (3.3)	13 (7.2)	0.42 (0.15 to 1.2); 0.08
Allele			
G	276 (76.7)	261 (72.5)	1
C*	84 (23.3)	99 (27.5)	0.8 (0.6 to 1.1); 0.19

*p<0.05.
BCC, basal cell carcinoma; CS, control subjects; ; MIF, migration inhibitory factor.

Table 4 Haplotype frequencies of the (−794(CATT)$_{5–8}$ y −173G>C) *MIF* gene polymorphisms

	Frequency			
	BCC (N=360)	CS (N=360)		
Haplotypes	n (%)	n (%)	P value	OR (95% CI)
5G	70 (19.4)	61 (16.9)	0.34	1.21 (0.81 to 1.81)
5C	13 (3.6)	8 (2.2)	0.23	1.71 (0.69 to 4.25)
6G*	172 (47.9)	182 (50.6)		1
6C	19 (5.3)	21 (5.9)	0.9	0.9 (0.5 to 1.84)
7G	34 (9.4)	18 (5)	0.02	1.99 (1.1 to 3.67)
7C	52 (14.4)	70 (19.4)	0.25	0.78 (0.52 to 1.19)

*p<0.05.
BCC, basal cell carcinoma; CS, control subjects; ; MIF, migration inhibitory factor.

a media of 2.1 ng/mL and CS showed a media of 4.4 ng/mL. The comparison between groups was significant (p<0.01); nevertheless, media levels of both groups are under the reference parameters.

DISCUSSION

BCC is the most frequent malignant tumor in Caucasians in Mexico, that is, a prevalence of 3.9 per 1000 population is estimated; furthermore, the risk of recurrence varies from 2% to 10% and the risk for developing new tumors ranges from 10.4% to 21.2%.[1 3 17 18] Distinct etiological factors have been related to BCC; exposure to the UV radiation from sunlight is one of the principal factors, nevertheless, the immune system and genetic factors are closely related to the development and progression of the tumor.[5 19] In this sense, cytokines are strongly implicated with cancer in many different ways; certain cytokines promote the development of a protumorigenic microenvironment while others generate effective antitumor effects.[8 20] Recent studies have suggested that MIF, a pleiotropic cytokine with chemokine-like functions, is overexpressed in multiple human tumors and may serve as an important link between inflammation and the development of NMSC,[21] on the other hand, MIF

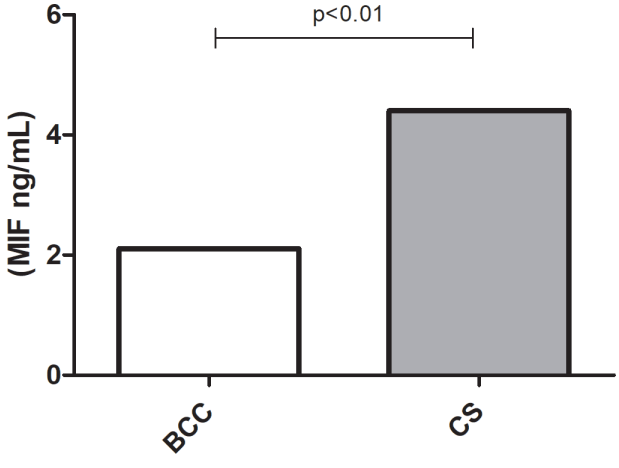

Figure 1 Migration inhibitory factor (MIF) soluble levels in patients with basal cell carcinoma (BCC) and control subjects (CS). Patients with BCC showed significant lower MIF soluble levels (p<0.01) compared with CS (2.1 and 4.4 ng/dL, respectively). Data are provided in medians, p values calculated by Mann-Whitney U test.

has been related with the recruitment and maintenance of antigen-presenting cells in the dermis/epidermis developing an unexpected tumor-suppressive activity.[8] In the present study, we analyzed the haplotypes of (−794(CATT)$_{5–8}$/−173G>C) *MIF* gene polymorphisms and its soluble levels in patients with BCC, which is important to highlight that to the best of our knowledge it has not been done yet in patients from western Mexico. About the clinical characteristics of our patients, we found that the average age was 66 years and that 62% of patients with BCC were males. The lesions in these patients were found principally in head and neck (92%). Lousquadro describes also similar information about gender and localization[2]; however, something to highlight is that we found one patient with a genital lesion, suggesting that the risk of light exposure could not be a factor of this tumor, in this manner we tried to associate the gender, tumor size, tumor localization, and tumor grade with the haplotypes of *MIF* polymorphisms, but no association was found (data not shown). Regarding our research, we tried to associate both *MIF* polymorphisms with BCC, but no association was found with −173G>C *MIF* polymorphism and this result is according to other research in western Mexican population, but in different diseases, such as multiple sclerosis and lepromatous leprosy.[22 23] Nevertheless, our results are different concerning Razzaghi *et al*, who report the association of *MIF*-173 C allele with clinical stages of patients with prostate cancer,[24] but prostate cancer and BCC have different etiology and also *MIF* gene could respond to different transcription factors—also Razzaghi *et al* recruited patients from Tehran, Iran. Both populations differ in their ancestry and these differences could be an explanation about why they found an association, and we do not. Concerning the −794(CATT)$_{5–8}$ *MIF* gene polymorphisms, a borderline association was found between the 55 genotype and BCC (OR=3.7, p=0.04). Various studies in western Mexican population have found an association of the higher repetition alleles and genotypes with different diseases, such as acute coronary syndrome (associated with 67 genotype),[25] lower risk to psoriatic arthritis (related to 58 genotype),[26] expanded disability status scale and multiple sclerosis severity score in males with multiple sclerosis (linked with the allele 7),[23] and early-onset rheumatic arthritis (connected to allele 7).[13] Reporter assays and human clinical studies indicate that repeat number in the −794(CATT)$_{5–8}$ *MIF* gene polymorphisms is associated with higher *MIF* expression and protein levels, as we already describe we found an unexpected borderline association of the 55 genotype, for that reason we inferred the haplotypes of both polymorphisms that are in a linkage disequilibrium and to analyze if they are responsible for influencing or modifying the risk of the disease by being in a nearby position. Haplotype 7G showed a significant difference, and the 7G carriers present 1.99 more susceptibility to develop BCC. These findings are consistent with a research by Llamas-Covarrubias *et al*, who found a tendency toward association (p=0.054) between the haplotype 7G and rheumatic arthritis,[13] otherwise haplotype analysis accord with the linkage disequilibrium reported among *MIF* variants is present in our population. For that reason, this haplotype significance association is more important than the analysis of each polymorphism. MIF soluble levels were also measured in patients with BCC and CS. The comparison

between groups was significant ($p<0.01$), notwithstanding, it is important to highlight that the media levels of both groups are under the reference parameters (2–6 ng/mL).[27] Moreover, is important to describe that MIF soluble levels as shown in the results are higher in CS, this could be principally for the age (media of 62) of our CS, as we mention these subjects were age–sex matched, is one of the possible causes. On the other hand, MIF has been related to BCC, but the functions described and its association with the pathology is contrasting. Martin *et al* describes that MIF plays an important role in UVB-induced NMSC development and progression in a murine model. They mention that the acute inflammatory phase occurs in the skin in response to UVB exposure, in this sense, MIF could be the link between inflammation and oncogenic mutations in an inflammatory environment, though and upregulation of inflammatory pathways and suppression of p53 and diminishing of apoptosis.[9] In a similar research Heise *et al* indicate a moderate expression of MIF in normal human skin samples but an enhanced expression of this cytokine in lesioned skin of patients with cutaneous squamous cell carcinoma (SCC)[21] is important to describe that the pathophysiology of BCC and SCC is different, and SCC is closely related to UVB exposure. In this sense, MIF could be activated by UVB, but its function depends also on the receptors that trigger. On the other hand, Brocks *et al* mention that MIF is essential for maintaining innate immunity in the skin and could protect from non-melanoma epidermal tumors by regulating the number of antigen-presenting cells in skin in a murine model.[8] Concerning our results, we can hypothesize that MIF could be performing an organ-specific and not a system function, nevertheless, it is important to note that a significant difference was found, but an immunohistochemistry test could help to elucidate this hypothesis. On the other hand, the studies cited previously are focused mainly on the activation of MIF trough UVB exposure in SCC. However, MIF could not be activated mainly by UVB in BCC, so from this information we could gather that MIF acts as a pleiotropic and immunoregulatory mediator with chemokine-like functions and is secreted in both a constitutive and inducible fashion. Additionally, the function that MIF performs depends on the kind of activation. For that reason, we could hypothesize that during chronic inflammation, MIF could be performing tumorigenic activities, and during acute inflammation, MIF is related with tumor development protection as Heise et al describes. All these functions are regulated by the genetic mutations associated with its expression. Taken together our results and previous studies, further investigations are mandatory to elucidate the exact mechanism of MIF in BCC, taking into consideration the constitutive or inducible expression, the pathway of activation, and which receptors are involved in its function.

CONCLUSION

In conclusion, our findings suggest a borderline association of the 55 genotype with BCC and a strong association of the haplotype 7G with the susceptibility to BCC. Furthermore, CS showed higher MIF soluble than patients with BCC, but MIF levels of both study groups are under reference parameters. Is important to highlight that to the best of our knowledge is the first time that *MIF* promoter polymorphisms and its soluble levels are analyzed in Mexican Mestizo population.

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ORCID iDs
Francisco Javier Salazar-Torres http://orcid.org/0000-0003-1960-2055
Emmanuel Valdés-Alvarado http://orcid.org/0000-0001-6810-9203

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