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ORIGINAL ARTICLE

Genomic imbalances and microRNA transcriptional profiles in patients with mycosis fungoides

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Abstract Mycosis fungoides is the most common type of primary cutaneous T cell lymphoma. We have evaluated *CDKN2A* losses and *MYC* gains/amplifications by FISH analysis, as well as expression of *miR-155* and members of the oncogenic cluster *miR-17-92* (*miR17*, *miR18a*, *miR19b*, and *miR92a*) in MF patients with advanced disease. Formalin-fixed paraffin-embedded skin biopsies from 36 patients at diagnosis, 16 with tumoral MF (T-MF), 13 in histological transformation to a large T cell lymphoma (TR-MF), and 7 cases with folliculotropic variant (F-MF), were studied. Twenty cases showed genomic alterations (GAs): 8 (40 %) had *CDKN2A* deletion, 7 (35 %) showed *MYC* gain, and 5 (25 %) exhibited both alterations. GAs were more frequently observed in F-MF (p = 0.004) and TR-MF (p = 0.0001) than T-MF. GAs were significantly higher in cases presenting lesions in head, neck, and lower extremities compared to those observed in trunk and upper extremities (p = 0.03), when ≥25 % neoplastic cells were CD30 positive (p = 0.016) as well as in cases with higher Ki-67 proliferation index (p = 0.003). Patients with GAs showed bad response to treatment (p = 0.02) and short survival (p = 0.04). Furthermore, MF patients showed higher miRNA expression compared to controls ($p \leq 0.0223$). T-MF showed higher *miR17* and *miR-18a* expression compared to F-MF and TR-MF ($p \leq 0.0387$) while *miR19b*, *miR92a*, and *miR-155* showed increased levels in F-MF and TR-MF with respect to T-MF ($p \leq 0.0360$). Increased expression of *miR17* and *miR19b* in GA group compared to cases without alterations ($p \geq 0.0307$) was also detected. Our results add new information about genomic imbalances in MF patients, particularly in F-MF, and extend the present view of miRNA deregulation in this disease.

Keywords Mycosis fungoides · Genomic alterations · FISH · microRNA expression · miR-155 · miR-17-92 cluster

Fuad Huaman Garaicoa and Alejandro Roisman contributed equally to this study.

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Introduction

Cutaneous T cell lymphomas (CTCL) represent a heterogeneous group of extranodal malignant lymphomas. Among them, mycosis fungoides (MF) are the most common type of primary CTCL, representing more than 50 % of all lymphomas originated in the skin [1]. It is characterized by the infiltration and accumulation of malignant CD4-positive helper T lymphocytes in the skin (epidermal and dermal infiltration). Classic MF has a multistep process from patches to more infiltrated plaques and eventually, tumor development. The disease has an indolent clinical course at early stages with slow progression over years or decades, but in advanced stages (IIB–IVB), the 10-year survival decreases to less than 40 % [2]. A few MF cases may undergo large cell transformation which is characterized by an aggressive clinical course, refractoriness to treatment, presence of more than 25 % of large cells, and a median survival of less than 2 years [3]. In addition, three morphological variants have been described: folliculotropic MF (F-MF), granulomatous slack skin, and pagetoid reticulosis [1], from which F-MF is the only one associated to poor outcome. In contrast to MF, Sézary syndrome (SS) is a rare and aggressive variant of CTCL, characterized by the triad erythroderma, generalized lymphadenopathy, and presence of malignant T cells in the skin, lymph nodes, and peripheral blood. This entity is associated with a poor clinical outcome with an estimated 5-year survival rate of 24 %; about 10–15 % of MF patients may evolve to SS [4].

Up to date, different studies have provided insights in the understanding of the molecular pathogenesis of CTCL [5]. Among them, array-comparative genomic hybridization (CGH) studies allowed the identification of specific chromosome regions of probably prognostic value, particularly 9p21.3 losses and 8q24.21 gains/amplifications, involving *CDKN2A–CDKN2B* and *MYC* genes, respectively [6–8]. *CDKN2A–CDKN2B* genes are involved in cell cycle progression, particularly in G_1-S transition, and are able to induce cell cycle arrest in the G_1 phase by inhibiting the cyclin-dependent kinases CDK4 and CDK6, mediated by the phosphorylation of the retinoblastoma protein [9, 10]. Inactivation of *CDKN2A–CDKN2B* genes by homozygous deletions, mutations, and hypermethylation occurs in a wide range of human tumors and has been identified in lymphoid malignancies [11–15], indicating a role in disease progression [16]. As regards to *MYC*, it represents a global transcription factor that is thought to regulate 10–15 % of genes in the human genome [17]. Deregulation of *MYC* by rearrangements or gains/amplifications has been observed in different neoplasias, including malignant lymphomas, associated to genetic instability and increased tumor aggressiveness [7, 8, 13, 18]. *MYC* controls many aspects relevant to the survival and division of cells, such as DNA replication, protein synthesis, and the regulation of metabolism and energy. Activation of *MYC* enables the transition of cells from G_0/G_1 to the S phase, regulates the expression of microRNAs (miRNAs), and determines the upregulation of pro-apoptotic pathways [17, 19].

MicroRNAs (miRNAs) are short single-strand noncoding regulatory RNAs of 21–25 nucleotides that regulate gene expression by either mRNA degradation or inhibition of translation [20]. It has been well recognized that miRNAs fulfill and exert a wide spectrum of central regulatory functions in virtually all cellular processes such as apoptosis, cell proliferation, development, metabolism, survival, genome instability, and differentiation [21]. Many tumor-associated miRNAs are involved in tumorigenesis mainly by achieving transcriptional and post-transcriptional misregulation of both oncogene and tumor suppressor, and thus, these regulatory aspects may have prognostic significance as has been previously shown in certain lymphoid malignancies [22]. *miR-155* is one of these miRNAs with an oncogenic property being overexpressed in numerous solid tumors and in B cell lymphomas, where it was found to promote genomic instability, proliferation, and survival of malignant cells [23–25]. In addition, it has been suggested that *miR-155* plays a crucial role in development and activation of several types of immune cells, including B and T cells [26]. In line with this, Kopp et al. [27] showed that signal transducer and activator of transcription 5 (*STAT5*)-mediated upregulation of *miR-155* is an important step in CTCL carcinogenesis. Furthermore, some studies found *miR-155* upregulated in MF especially in tumoral MF (T-MF) [28–31], supporting its role in disease progression. Simultaneously, a large amount of experimental data demonstrated the oncogenic properties of *miR17–92* cluster in both hematological malignancies and solid tumors, being involved in cell cycle and cell death regulation [32–34]. Moreover, the oncogenicity of this cluster may involve several mechanisms including the cooperation with the *MYC* oncogene to decrease apoptosis [35–37]. Therefore, the aim of this study was to assess the importance of genomic imbalances on chromosomes 9p21.3 and 8q24.21 as well as miRNA expression, specifically *miR-155* and members of the oncogenic cluster *miR-17-92* in patients with advanced MF. Results were correlated with clinicopathological features of the disease.

Materials and methods

Patients

The study was performed on formalin-fixed paraffin-embedded (FFPE) skin biopsy samples with high tumor cell content from 36 patients with MF at diagnosis (26 males, 10 females; mean age: 62.5 years; range: 31–81 years): 29 cases showed clinically T-MF, 13 of them in histological transformation to large T cell lymphoma (TR-MF), and 7 cases had F-MF variant. Patients were diagnosed according to the World Health

Organization criteria [1]. MF staging according to the International Society of Cutaneous Lymphomas/European Organization for Research and Treatment of Cancer (ISCL/EORTC) [38] showed the following distribution: IIB (30; 83.3 %), IIIA (1; 2.7 %), IVA (2; 5.5 %), and IVB (3; 8.3 %). Seven patients died due to complications of the disease. In addition, 10 biopsy samples of benign inflammatory skin diseases were also evaluated and used as controls. The study was approved by the local Ethics Committee. All individuals provided their informed written consent.

Hematoxylin and eosin (H&E) stains and immunohistochemical (IHC) studies

Skin samples were formalin (buffered-formol 10 %) fixed for 6–48 h and paraffin embedded for tissue processing, cutting, and mounting sections (3–5 μm) in common and silanized slides. The analysis was performed using H&E stain (Biopur, Argentina). The IHC study was performed using the standard three-step technique (streptavidin-biotin-peroxidase) post-heat-induced antigen retrieval. Then, the slides were revealed with 3.3′-diaminobenzidine (DAB) and counterstained using hematoxylin. CD45RO, CD3, CD20, CD7, CD8, CD30, and Ki-67 (Dako, St Louis, USA), Granzyme B (BioSB, CA, USA), CD2, CD5, CD4, and TIA-1 (CellMarque, CA, USA) antibodies were used for this analysis.

Fluorescence in situ hybridization (FISH) analysis

A locus-specific FISH analysis was performed on histological sections (3–5 μm) on silanized slides. They were heating at 60 °C during 60 min and deparaffinized by two 100 % Xylene baths (15 min each at room temperature) and rehydrated in decreased ethanol baths (3 min each at room temperature). Then, slides were pre-treated in citrate buffer at 95 °C for 25 min, cooled at room temperature, and washed in 1× phosphate-buffered saline (PBS) for 5 min. Afterward, pepsin digestion for 10–40 min was performed (checking until nuclei were with well-defined perimeters), followed by 1× PBS wash for 5 min, dehydration in graded alcohol baths, and air-dried. FISH was carried out with OTS9P21.3 (CDKN2A) and OTS8Q24 (MYC) probes (LiVE-Lexel, Buenos Aires, Argentina), according to manufacturer's protocol. For each patient, at least 100 interphase nuclei were analyzed for each probe. In each sample, tumor and normal tissues were evaluated separately. The cut-offs for positive values (mean of normal control +3 standard deviations) were as follows: 3.5 and 12 %, for 8q24 gain and 9p21 loss, respectively (Supplementary Material Online Resource 1 Fig. S1).

RNA isolation

Total RNA was isolated using the RecoverAll® kit (Ambion, Austin, TX, USA), following manufacturer's guidelines with minor modifications. Briefly, samples were placed in a 1.5-ml microcentrifuge tube containing 1 ml of xylene, vortexed, and incubated at 50 °C for 3 min to melt the paraffin. The material was then centrifuged at 14,000 rpm for 10 min at room temperature to pellet the specimen, after which the xylene was carefully removed and the pellet was washed three times with 1 ml of 100 % room temperature ethanol and air-dried for 45 min. Following deparaffinization, tissue was protease digested by incubating the pellet in 200-ml digestion buffer and 4 ml protease at 80 °C for 15 min. For total RNA isolation, 240 ml of isolation additive was added to the sample, followed by vortexing and addition of 550 ml of 100 % ethanol. The mixture was then loaded onto a prepared filter and collection tube according to the manufacturer's procedure. The sample was centrifuged for 30 s at 10,000 rpm. Flow through was discarded and filter washed twice with wash buffer. Nuclease digestion was obtained by adding 60-ml DNase master mix, containing 6-ml DNase buffer, 4 ml DNase, 50 ml nuclease-free water, to the center of each filter and incubated for 30 min at room temperature. The filter was subsequently washed three times according to the manufacturer's protocol, and RNA was eluted with 60 ml preheated nuclease-free water. RNA quality and quantity were determined by spectrophotometry at 260 nm. All eluted RNAs were immediately aliquoted into 10 ml volumes each and stored at −80 °C until all extractions were accomplished.

Reverse transcription (RT) and quantitative real-time PCR (qRT-PCR)

miRNA reverse transcription was performed using TaqMan® MicroRNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA, cat. no: 4366597). TaqMan microRNA expression assays of *miR155* (002623), *miR17* (002308), *miR18a* (002422), *miR19b* (000396), and *miR92a* (000431) (Applied Biosystems, Foster City, CA, USA) were used to provide specific primers for reverse transcription and quantitation of mature miRNAs. *RNU6B* (001093, Applied Biosystems, Foster City, CA, USA) was used to normalize sample-to-sample differences in cDNA input, RNA quality, and RT efficiency. The relative expression fold change of *miR155* and *miR17–92* cluster was calculated using the $2^{-\Delta\Delta Ct}$ method [39]. All reactions were performed in triplicate.

Statistical analysis

All statistical evaluations were performed using the software package XLSTAT (Version 2015.1) and the GraphPad Prism

Version 6.0 (2012). The analysis of mRNA expression data was done using the Mann–Whitney test. Spearman correlation was used to analyze gene expression profiles. Group-wise comparison of the distribution of clinical and laboratory variables was performed with the Student's *t* test (for quantitative variables) and the χ^2 or Fisher's exact test (for categorical variables). Receiver operating characteristic (ROC) curve analysis was applied to calculate gene expression cut-off values, with the highest sensitivity and specificity. Overall survival (OS) was defined from the date of diagnosis to the death of the patient or last follow-up. Survival curves were estimated by the Kaplan–Meier method and compared with the log-rank test. For all tests, $p < 0.05$ was regarded as statistically significant.

Results

Genomic alteration analysis

FISH analysis was performed on 34 biopsies of MF patients: 14 T-MF, 7 F-MF, and 13 TR-MF. Twenty cases (59 %) showed genomic alterations (GAs): 8 patients (40 %) had deletion of *CDKN2A* (9p21), 7 (35 %) showed *MYC* gain (8q24), and 5 (25 %) exhibited both alterations. Considering the histopathological diagnosis, GAs were observed in 2/14 (14.3 %) T-MF cases compared to 6/7 (85.7 %) patients with F-MF and 12/13 (92.3 %) TR-MF cases, with significant differences in these last two subtypes with respect to T-MF ($p = 0.004$ and $p = 0.0001$, respectively) (Table 1). Specifically, *CDKN2A* deletion was found in 2/14 (14.3 %) T-MF, 4/7 (57 %) F-MF, and 7/13 (53.8 %) TR-MF, with statistical differences between T-MF and TR-MF ($p = 0.047$), while *C-MYC* gains were detected in 5/7 (71.4 %) F-MF and 7/13 (54 %) TR-MF patients, but it was not observed in T-MF cases ($p = 001$ and $p = 0.003$, respectively). No differences between F-MF and TR-MF patients for both GAs were detected (Table 2).

In order to evaluate the importance of our results, we analyzed the distribution of GAs taking into account the clinicopathological characteristics of patients. Table 3 lists the clinical features of both groups: GA and NA (no alterations), which not differ significantly with respect to age, extension and size of lesions, and the presence of ulcerative process. On the contrary, both groups of patients showed significant differences in their anatomical location. In this regard, GA was predominantly found in lesions located in head, neck, and lower extremities (14/18 cases; 77.8 %) compared to those observed in trunk and upper extremities (6/15; 40 %) ($p = 0.03$). Figure 1a showed the distribution of *CDKN2A* deletion and *MYC* gains as well as of total alterations according to the anatomical location. Interestingly, significant differences were found when we analyzed immunophenotypic characteristics and proliferation status. In this aspect, an association between the presence of GAs and the percentage of CD30+ cells using as cut-off ≥25 % was observed. This analysis showed that 50 % of GA cases had ≥25 % of CD30+ cells compared to 8.3 % of patients with NA ($p = 0.016$). Simultaneously, the proliferation index assessed with Ki-67 antibody also detected an increased rate in GA cases (50 %) compared to the NA group (5 %) ($p = 0.003$).

Table 1 Distribution of genomic aberrations according MF type

MF type (*n*)	GA (%)	NA (%)
T-MF (14)	2 (14.3)*	12 (85.7)
F-MF (7)	6 (85.7)	1 (14.3)
TR-MF (13)	12 (92.3)	1 (7.7)

GA genomic alterations, *NA* no alterations, *MF* mycosis fungoides, *T-MF* tumoral-stage MF, *F-MF* folliculotropic MF, *TR-MF* large T cell lymphoma transformation MF

*Significant differences with respect to TR-MF: $p = 0.0001$ and F-MF: $p = 0.004$

With regards to clinical evolution of the disease, the histological analysis showed that 33 % of T-MF and 41.6 % of F-MF underwent transformation to large T cell lymphoma. The evaluation taking into account the type of GA detected that 80 % TR-MF of folliculotropic origin had 9p21 deletion compared to 42.8 % of classical tumor origin, while in relation to 8q24 gain, the greater frequency was found in cases of T-MF origin group (71.4 %) compared with 40 % of F-MF derivation, suggesting a different role for these alterations in the progression of the pathology (Table 4). Three out of 13 TR-MF were constituted by large cells that did not express CD30 protein; all of them were originated from classical tumor stage MF. Simultaneously, our analysis did not show differences in the frequency of lymphadenopathies between groups as well as in laboratory parameters.

In regard to the relapse location although no differences were found, they were more frequently extracutaneous or systemic in the GA group (4; 20 %) compared to NA group (1;

Table 2 Genomic disbalances of *CDKN2A* and *C-MYC* genes in our MF patients

MF type (*n*)	Genomic disbalances (%)	
	CDKN2A	*C-MYC*
T-MF (14)	2 (14.3) #	0 ‡
F-MF (7)	4* (57.0)	5* (71.4)
TR-MF (13)	7** (53.8)	7** (53.8)

MF mycosis fungoides, *T-MF* tumoral stage MF, *F-MF* folliculotropic MF, *TR-MF* large T cell lymphoma transformation MF. Presence of both abnormalities in: *three cases, **two cases

‡Significant differences with respect to F-MF: $p = 0.001$ and TR-MF: $p = 0.003$; #significant differences with respect to TR-MF: $p = 0.047$

Table 3 Clinico-pathologic features in MF patients with and without genomic aberrations

Clinic characteristics	Groups		*P* value
	GA	NA	
Sex (M/F)	17/3	8/6	0.07
Median age in years (range)	62.7 (35–82)	62 (31–80)	0.88
Multifocality (%)	18 (90)	9 (64)	0.163
Localization (head, neck, and lower limbs) (%)	14/18 (77.8)	4/13 (30.8)	**0.013**
Size (cm); media (range)	7.5 (1–15)	5.3 (1–10)	0.2
Ulcerative lesion (%)	13 (65)	6 (42.8)	0.3
CD30 cells ≥25 % (%)	10 (50)	1 (8.3)*	**0.016**
Ki-67 (%); media (range)	50 (5–90)	5 (0–8)	**0.003**
β2M (μg/ml); media (range)	3.35 (1.5–6.7)	3 (1.7–3.6)	0.66
LDH (UI/l); media (range)	507 (200–1209)	439 (178–1060)	0.65
Refractoriness to treatment (%)	11 (55)	2 (14.3)	**0.02**
Extracutaneous relapse (%)	4 (20)	1 (7.1)	0.58
Adenopathies (%)	12 (60)	5 (35.7)	0.6
Mortality (%)	7 (100 %)	0	**0.049**

GA genomic abnormalities, *NA* no alterations, *M* male, *F* female, *B2M* beta-2 microglobulin, *LDH* lactate deshidrogenase

*12 cases evaluated

7.1 %). Even though this disease is chronic and recurrent, with partial remissions or no improvement, we observed that the response to treatment was completed in 2/14 (14.3 %) NA cases versus none of the GA group, partial in 9/20 (45 %)

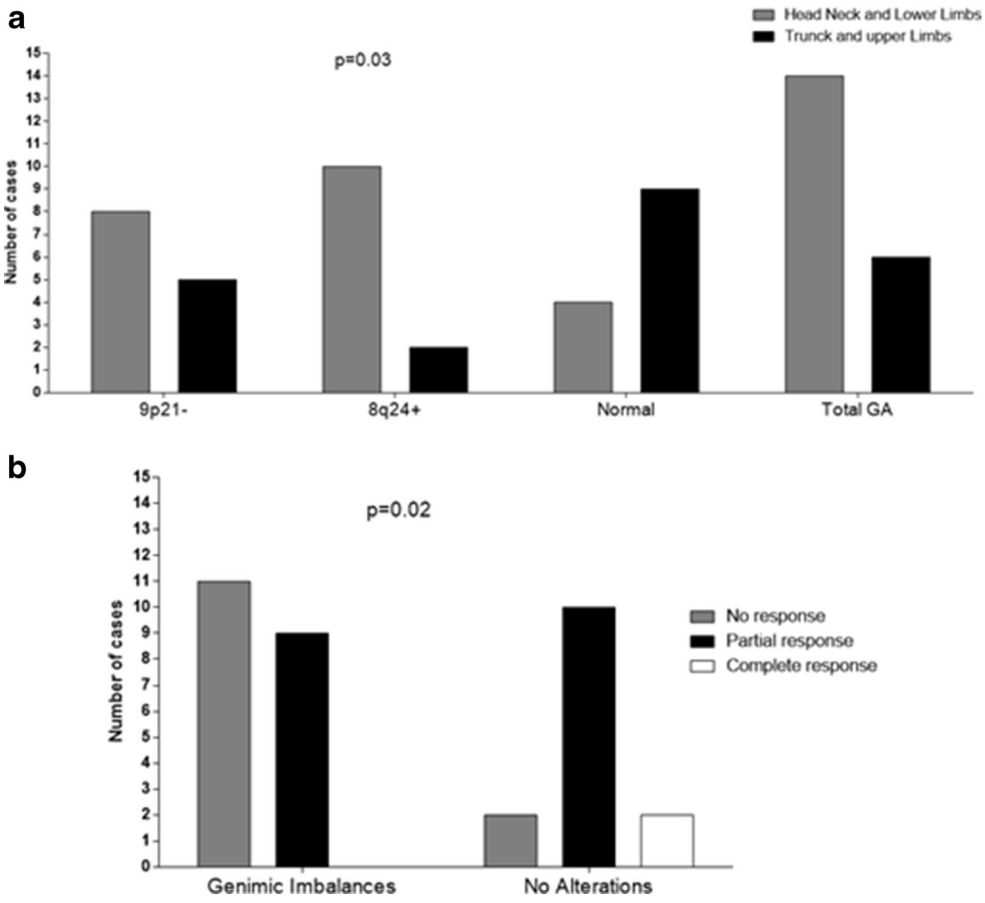

Fig. 1 Distribution of genomic imbalances in 9p21 (CDKN2A) and 8q24 (MYC) in patients with MF according to the following: **a** the anatomical location of lesions; **b** response to treatment

Table 4 Genomic abnormalities in TR-MF patients according the initial diagnosis

TR-MF origin	Genomic imbalances (%)	
	CDKN2A	*C-MYC*
T-MF	3* (42.8)	5 (71.4)
F-MF	4* (80)	2 (40)

MF mycosis fungoides, *T-MF* tumoral-stage MF, *F-MF* folliculotropic MF, *TR-MF* large T cell lymphoma transformation. *One case with both alterations

patients with GA compared to 10/14 (71.4 %) NA, and being non-response in 11/20 (55 %) cases belonging to the group with genomic imbalances regarding 2/14 (14.3 %) cases considered normal for the loci studied ($p = 0.02$) (Fig. 1b). Interestingly, patients with GA had a significantly poor prognosis with shorter OS (media: 92 months) than cases belonging to NA group that did not reach the median OS ($p = 0.04$) (Fig. 2), indicating an association with a worse clinical outcome for the first group.

Members of the *miR17–92* cluster and *miR-155* are aberrantly expressed and display similar gene expression profiles in MF

Another aspect of this study was to evaluate the expression of *miR-155* as well as of members of the *miR-17-92* cluster: *miR17*, *miR18a*, *miR19b*, and *miR92a*, in our MF cohort. This analysis was performed in 32 cases and 10 samples of benign inflammatory skin lesions, considered as our control group.

The comparison of *miR-155* expression between patients and controls showed higher expression levels in the whole cohort of MF patients ($p = 0.0044$) (Fig. 3e) as well as in each morphology subtype compared to controls (T-MF: $p = 0.0231$; F-MF; $p = 0.0033$ and TR-MF: $p = 0.0002$) (Supplementary Material Online Resource Fig. S2). In addition, increased levels in F-MF ($p = 0.004$) and TR-MF ($p = 0.03$) with respect to T-MF were also observed, without statistical differences between F-MF and TR-MF patients (Fig. 4e). In addition, the relationship between genomic imbalances detected by FISH and miRNA expression was also explored showing an increased expression in MF patients with GA compared to NA cases but without significant differences.

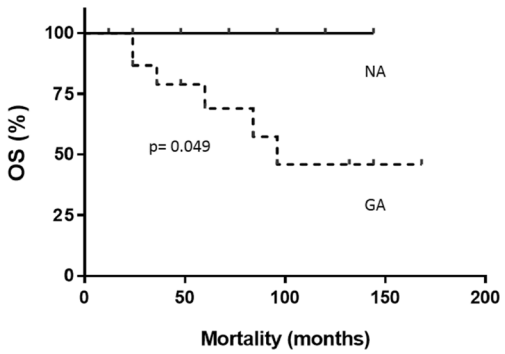

Fig. 2 Kaplan–Meier analysis of MF patients showing a short overall survival (OS) in cases with genomic alterations (GA) compared to those with no alterations (NA) ($p = 0.04$)

Furthermore, we aimed this study in order to identify if miRNAs from the *miR17–92* cluster were aberrantly expressed in MF; so we compared the expression of these miRNAs in our series of patients and controls. This assessment resulted in a striking finding, revealing that gene expression analysis from *miR17*, *miR18a*, *miR19b*, and *miR92a* members of the *miR17–92* cluster showed higher levels in comparison with controls ($p \leq 0.0223$) (Fig. 3a–d). In addition, we explored gene expression profiles between the transcription patterns exhibited according to the morphology previously described. All of them, except *miR-18a* in F-MF, had significant differences with respect to controls ($p \leq 0.033$) (Fig. S2). Because of the striking deregulation seen in the gene expression levels evaluated in the present study and in order to obtain a more detailed outlook of the potential tumorigenic roles of the oncomiR *17–92* in MF, we next evaluated if there was any inter-morphological differences in our cohort. Interestingly, *miR17* and *miR-18a* transcriptional profiles were higher in T-MF compared to F-MF and TR-MF patients ($p \leq 0.0387$), and no statistical significance was found when we analyzed F-MF vs TR-MF. In contrast with these findings, *miR19b* and *mir92a* showed increased levels in F-MF and TR-MF with respect to T-MF ($p \leq 0.0360$), but similarly to those previously observed, no statistical differences regarding F-MF and TR-MF were found (Fig. 4a–d). In line with this, miRNA gene expression profiles taking into account genomic alteration showed that *miR17* and *miR19b* were upregulated in GA group compared to NA cases ($p = 0.0307$ and $p = 0.0107$, respectively) (Fig. 5). No significant differences in the remaining miRNAs evaluated here were found.

Finally, we assessed the potential association between miRNA gene expression profile and clinical features from our cohort. In this regard, data analysis showed no statistically differences regarding clinical parameters nor therapeutic response (Supplementary Material Online Resource Table S1).

Discussion

In this study, we report the analysis of a series of patients with clinical and histopathological diagnosis of MF in advanced stages and variants, using FISH technique for detecting genomic imbalances in 9p21 and 8q24, and qRT-PCR analysis to evaluate gene expression profiles of *miR-155* and members of the *miR17–92* cluster. Our results showed that these genomic abnormalities are common in MF, reaching about 60 % of our cohort, with similar frequencies for 9p21 deletions (65 %; 13/

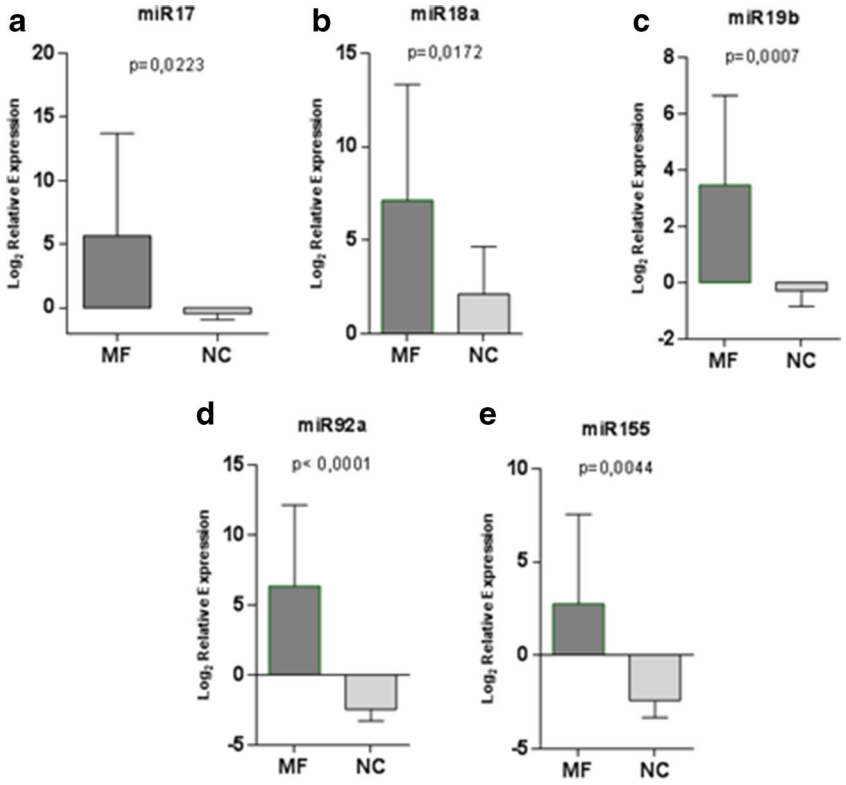

Fig. 3 miRNA expression analysis in MF patients and controls: **a**–**d** members of the *miR-17–92* cluster ($p \leq 0.0223$); **e** *miR-155* ($p = 0.0044$). *NC* normal controls

20 cases with GA) and 8q24 gains (60 %; 12/20 cases with GA). Interestingly, we have observed five cases with both GAs, indicating the presence of clonal evolution. The literature shows discordant data in relation to the frequency of GA in MF, with authors that observed similar distribution of both alterations like our study [6] while others found higher frequency of 9p21 losses [8, 40] or increased levels of *MYC* gains [41]. These controversial results may be explained by the clinical and pathological heterogeneity of patients studied in different cohorts.

Regarding the histological diagnosis, all subtypes showed some degree of genomic aberrations, being less frequent in

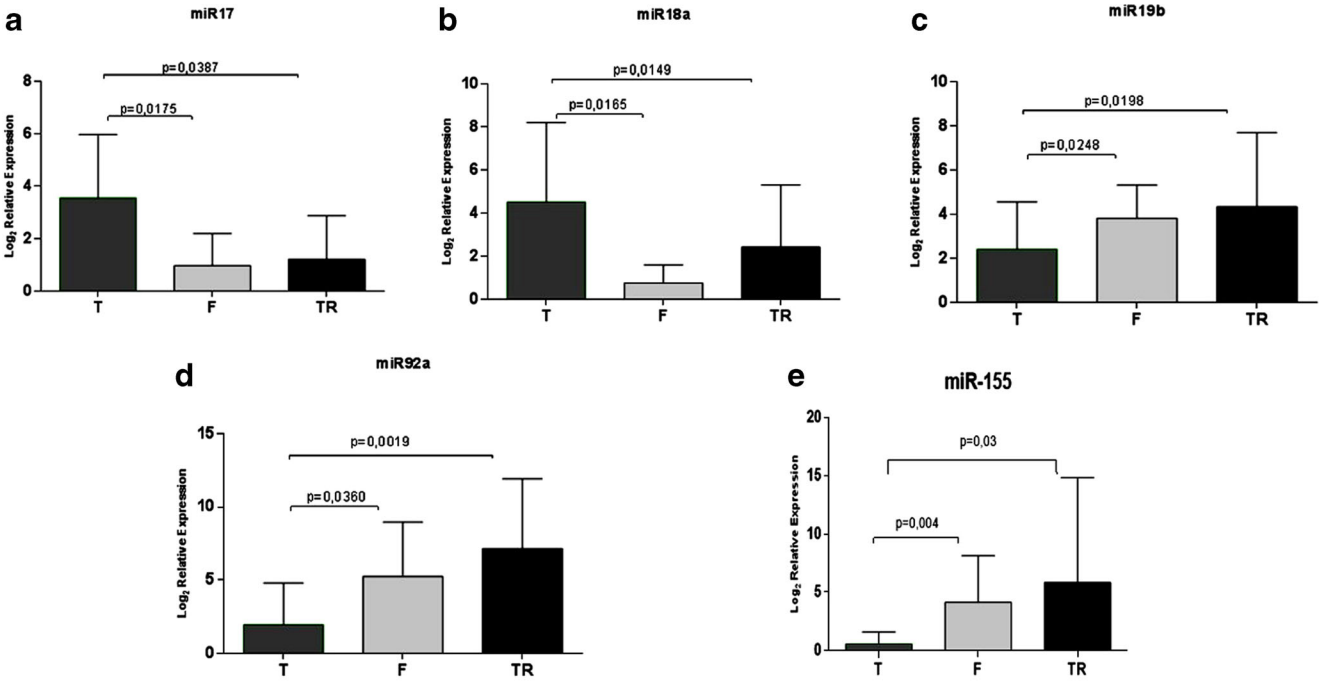

Fig. 4 miRNA expression taking into account morphological subtypes: **a**–**d** members of miR-17–92 cluster; **e** miR-155 ($p \leq 0.03$)

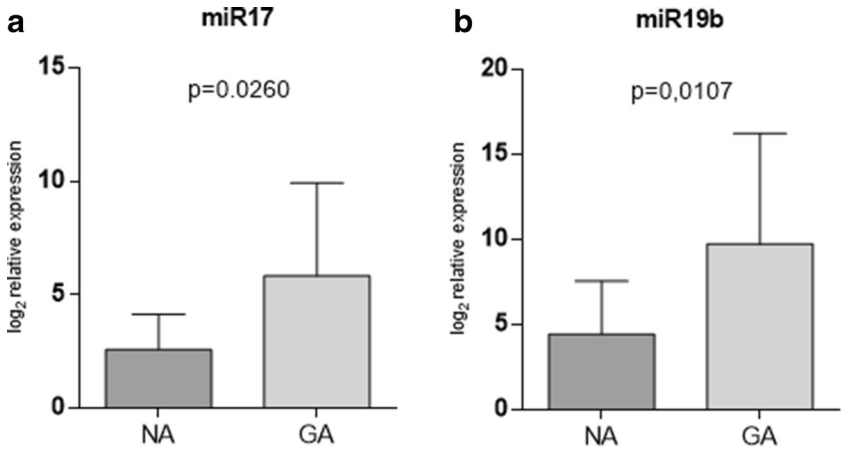

Fig. 5 miRNA expression in MF patients with genomic alterations (GA) and with no alterations (NA); significant differences for *miR17* ($p = 0.0307$) and *miR19b* ($p = 0.0107$)

the tumor stage of classical MF, when compared with the high frequency in cases of TR-MF and F-MF. In particular, the *CDKN2A* deletion was detected in just over half of our patients with TR-MF and F-MF but showed low frequency in T-MF. In addition, *MYC* gains were common in F-MF than in TR-MF, and they were not observed in any patient with T-MF. Our findings are consistent with data that associate these alterations to worse clinical outcome and support the importance of these genomic rearrangements in tumor progression [42]. Also, the presence of 9p21 deletions in the tumor stage and the absence of 8q24 gains would support that the loss of *CDKN2A* would be an earlier event in the development and progression of MF.

With respect to clinical and morphological characteristics, the frequency of GA was significantly higher in patients presenting lesions located in head, neck, and lower extremities, when neoplastic cells expressing CD30 protein were ≥25 % of total tumor volume and with higher Ki-67 proliferation index. The association between GA and the frequency of CD30+ cells is interesting because of its relation with poor prognosis especially in the TR-MF [43, 44]. Edinger et al. [45] found shorter OS in MF cases in which cells located in the dermis were strongly CD30 positive. However, it is noteworthy that some small published series have reported that patients with CD30-positive transformation tend to have a better prognosis than CD30-negative ones [46, 47]. Comparing within the group of TR-MF cases with and without CD30+ cells, we found no significant differences in OS, being necessary further studies to clarify these results. Also, a higher rate of Ki-67 in cases with GA indicates its association with increased replicative capacity of the disease and supports the importance of this parameter in clinical evolution. Interestingly, we found higher genomic instability in cases involving the head, neck, and lower extremities with respect to trunk and upper extremities. To our knowledge, this is the first association between GA and the location of lesions, suggesting that it is a new prognostic factor in this pathology.

In reference to clinical and therapeutic features, the absence of a favorable response to treatment and the frequency of histological transformation to large cell lymphoma were significantly more frequent in patients with GA, associated with lower survival. Indeed, the seven patients that died due to complication of the disease belonged to the GA group. These findings indicate an important role of genes located on 9p21 and 8q24 in MF transformation and support the limited data reported in the literature [6–8, 42] suggesting these genetic imbalances as tumor markers of neoplastic progression in this pathology. Particularly, the study of Salgado et al. [7] using oligonucleotide-based array-CGH allowed to distinguish two groups of T-MF patients: stable and unstable, taking into account the number of genetic imbalances and chromosome abnormalities, being the last one associated to a more aggressive clinical outcome. As known, losses and/or mutations of *CDKN2A/CDKN2B* locus and silencing by promoter methylation have been described in various solid tumors and hematological malignancies as well as skin tumors associated with poor prognosis [11, 13]. Such inactivation promotes tumorigenesis and generates resistance to treatment that could be explained by defects in the induction of apoptosis and senescence in response to therapy [6]. Conversely, *MYC* gene translates an oncoprotein that functions as a transcription factor with multiple target genes and is generally recognized as an important regulator of proliferation, growth, differentiation, and apoptosis [18]; all of them are critical process in tumor development and progression.

Moreover, we conducted the evaluation of *miR-155* expression. Our results showed overexpression of this miRNA in MF patients in relation to that observed in samples of benign inflammatory skin diseases used as controls. Taking into account the histological type, such overexpression was significantly higher in F-MF and TR-MF compared to T-MF, indicating a relationship between aggressive forms of MF and the presence of an increased expression of this oncomiR. The literature is even contradictory; where some authors like Maj et al. [30] have reported overexpression of this gene in early stages and advanced classical MF compared to controls, whereas others like Moyal et al. [31] showed only advanced stages with increased expression of this miRNA. Different reports [30, 48] suggest

that prolonged or sustained overexpression of this gene can lead to uncontrolled proliferation, inhibition of apoptosis, and malignant transformation, so that *miR-155* could be a good candidate in the possible link between inflammation and skin carcinogenesis. Among the T cell lymphomas, overexpression of *miR-155* has been detected in anaplastic large cell lymphoma ALK negative, and recently in TR-MF and T-MF [28, 49]. Thus, MF variants have been relegated probably due to their lower incidence. Only two studies [50, 51] have evaluated *miR-155* in F-MF, and both have detected overexpression of this miRNA compared to the control group. However, we also found increased expression of *miR-155* in lesions of TR-MF, a particular finding that would suggest a role for this miRNA in the process of transformation to large T cell lymphoma.

In line with this, miRNAs encoded by the *miR17–92* cluster have been proposed to play both tumor suppressor and oncogenic roles depending upon the cellular context [52–54]. Moreover, it has been well established that miRNAs from this cluster are deregulated in a wide range of B cell lymphomas and solid tumors [55–57]. Furthermore, miRNAs from the *17–92 cluster* have been linked with advance MF or SS compared to benign control including *miR17* and *miR92a* [28, 58]. Here, we show an enhanced *miR17–92* expression in MF in comparison with controls as well as *miR19b* and *miR92a* transcriptional activity in advanced disease compared with tumor stage MF, suggesting that these members of *miR17–92* cluster may be involved in disease progression. Additionally, it has been shown that the two members of the *miR19* seed family (*miR-19a* and *miR-19b*) are responsible for promoting cell proliferation [59] and they are also essential to achieve the oncogenic activity of the complete cluster [60, 61]. Furthermore, two different experimental studies [62, 63] found that *miR92a* plays a central role in cancer development by enhancing cell proliferation, metastasis, and preventing cell to undergo apoptosis. Limited results are offered about these miRNA functions in CTCL. Narducci et al. [58] and Cristofoletti et al. [64] found that *miR106b* is upregulated in SS and *PTEN* targets indicating for this *miR17–92* paralog a potential oncogenic function. Moreover and in contrast to Narducci et al. [58] and Cristofoletti et al. [64] findings, Ballabio et al. **[68]** showed that *miR17*, *miR19a*, *miR92*, and *miR106a* are downregulated, diminishing apoptosis rates and enhancing proliferation in SS.

Simultaneously, we have analyzed the association between the expression levels of the different miRNAs evaluated in this study and the presence of GA (loss of 9p21 and 8q24 gain). This analysis showed an increased expression in patients with these abnormalities compared to the group without them, with significant differences for *miR17* and *miR19b*. To the best of our knowledge, this is the first report that studies the relationship between miRNAs and GA in CTCL, supporting a probable relationship of them with progression and transformation of MF [29, 30, 65].

Finally, our study did not find associations between miRNA deregulation and clinical, therapeutic, and laboratory parameters. The literature shows few reports studying the association of miRNAs with clinicopathologic features; one of them is Maj et al. [30] who detected *miR-155* overexpression in MF but no association with overall survival. They showed that high expression of other miRNAs different *to miR-155* was associated with better prognosis but neither reached statistical significance. However, other series as van Kester et al. [28] and Ralfkiær et al. [29] suggest *miR-155* upregulation as an adverse prognostic marker in MF. In reference to members of the miR-17-92 cluster, some reports have associated the expression of *miR-92a* to advanced disease stages [28, 50], suggesting to be involved in disease progression. Simultaneously, and to the best of our knowledge, no association between other members of the *miR-17-92* cluster and clinical characteristics of MF patients has been reported.

In summary, our results add new information about genomic imbalances in patients with MF, particularly F-MF, establishing the first study of GA in this MF variant of recognized poor prognosis, and also extend the present view of miRNAs deregulation in MF patients providing new insights in the comprehension of MF pathobiology that may be helpful to improve risk prediction in this disease.

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Compliance with ethical standards

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments.

Conflicts of interest None.

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