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RESEARCH

MicroRNA Expression Profiles in Kaposi's Sarcoma

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Abstract Kaposi's sarcoma (KS) is a mesenchymal tumor, caused by Human herpesvirus 8 (HHV8) with molecular and cytogenetic changes poorly understood. To gain further insight on the underlying molecular changes in KS, we performed microRNA (miRNA) microarray analysis of 17 Kaposi's sarcoma specimens. Three normal skin specimens were used as controls. The most significant differentially expressed miRNA were confirmed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). We detected in KS versus normal skin 185 differentially expressed miRNAs, 76 were upregulated and 109 were downregulated. The most significantly downregulated miRNAs were *miR-99a*, *miR-200* family, *miR-199b-5p*, *miR-100* and *miR-335*, whereas *kshv-miR-K12-4-3p*, *kshv-miR-K12-1, kshv-miR-K12-2, kshv-miR-K12-4-5p* and *kshv-miR-K12-8* were significantly upregulated. High expression levels of *kshv-miR-K12-1* ($p=0.004$) and *kshv-miR-K12-4-3p* ($p=0.001$) was confirmed by RT-PCR. The predicted target genes for differentially expressed miRNAs included genes which are involved in a variety of cellular processes such as angiogenesis (i.e. THBS1) and apoptosis (i.e. CASP3, MCL1), suggesting a role for these miRNAs in Kaposi's sarcoma pathogenesis.

Keywords Array profiling · FFPE · Kaposi sarcoma · miRNA · Quantitative reverse transcriptase polymerase chain reaction

Introduction

Kaposi's sarcoma (KS) is a mesenchymal tumor characterized by the presence of proliferating spindle-shaped tumor cells of endothelial origin, usually in a directional streaming pattern as well as by neoangiogenesis, inflammation with fibrosis and hyperemia (extravasated erythrocytes and hemosiderin storage) [1]; and it is the most common tumor associated with HIV-infection. Human herpesvirus 8 (HHV8) (also known as Kaposi's sarcoma-associated herpesvirus, KSHV), an oncogenic gamma-2 herpesvirus, is considered the etiological agent of KS [2].

Clinically Kaposi's sarcoma is classified into four groups: classical Kaposi's sarcoma (specific for elderly men of Mediterranean or Eastern European origin), African-endemic Kaposi's sarcoma, AIDS-associated Kaposi's sarcoma and iatrogenic Kaposi's sarcoma related to patients undergoing immunosuppressive treatment [3, 4].

Molecular and cytogenetic changes are poorly known in KS [5]. However, it has been suggested that many factors play an important role in the neoangiogenesis and proliferation of KS cells, including cytokines, angiogenic and growth factors (*OSM, IL-1β, TNF-α, IFN-γ, TGF-β, IL-6, VEGF, bFGF, PDGF,* etc.) [6]. *RDC1* (Chemokine orphan receptor 1), *LMO2* (Lim-domain only 2), *SPP1* (Osteopontin), *MERTK* (c-mer proto-oncogene tyrosine kinase), *NRN1* (Neuritin1), *KIT* as well as *MFAP* represent novel cellular genes essential for transformation of endothelial cells by Kaposi's sarcoma-associated herpesvirus [7]. In addition, other genes, known to be involved in KS pathology,

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such as *THBS1* (Thrombospondin 1), *S100A2* (S100 calcium binding protein A2), *PRG1* (plasticity related gene 1, now named *SRGN*), and *ITM2A* (integral membrane protein 2A), have roles in such processes as proliferation, immunomodulation, angiogenesis and apoptosis [8].

MicroRNAs (miRNAs) are a recently discovered class of endogenous, small non-coding RNAs of about 22 nucleotides in length. They have an important role in post-transcriptional gene regulation and their role in tumorigenesis is well acknowledged [9–11]. MiRNA expression patterns are correlated with the histological type of cancer and have diagnostic and prognostic value. Since the target genes regulated by miRNAs are involved in various biological processes including development, differentiation and proliferation, miRNAs may also have an important therapeutic role [12]. Recent studies showed that formalin-fixed paraffin-embedded tissue (FFPE) sample are reliable source of miRNA and are suitable for miRNA expression profiling [13, 14] and this was the premise to use FFPE samples for miRNA analyses in our study.

In the current study we aimed to identify differentially expressed miRNAs in KS samples compared to normal skin samples. Also miRNA expression differences between different histological stages of KS were investigated. Potentially target genes of the differentially expressed miRNAs were identified using prediction tools of four miRNA databases (miRBase, TargetScan, miRanda and mirTarget2).

Materials and Methods

Patients and Tissue Samples

In the present study we investigated 17 Kaposi's sarcoma FFPE (formalin-fixed paraffin-embedded) samples and used 3, KSHV-negative, normal skin FFPE samples as controls. All tissue samples were selected from the archives of Histopathological Department of Dermatology, Clinic University Hospital, Bucharest. All diagnoses were verified by reviewing the H&E-stained sections of each case and confirmed by a dermatopathologist. Informed consent was obtained from individual subjects for all procedures. All the patients were HIV (Human Immunodeficiency Virus) negative. No association with lymphoproliferative diseases was found at the moment of surgical excision. The following data collected from the patients' medical files were recorded: gender, origin, age at diagnosis, anatomic distribution (size, location, multicentricity, immunocompromised conditions, second malignancies, clinical course). The KS lesions mostly occurred in men aged 60 or older and were, predominantly, located on the distal parts of lower limbs.

The classification of KS samples into patch/plaque or tumor stage was based on three criteria: clinical feature (macroscopic appearance), histopathological and immunohistochemical profiles. Specific endothelial markers, such as CD34, CD31, and vimentin (mesenchymal marker) and actin were assessed to differentiate between incipient and tumor stages of KS. HHV8 immunostaining was performed also. Specific staining such as Pearls stain was used to evidence the hemosiderin storage.

HHV8 Immunohistochemistry

Fixed, paraffin-embedded tissue sections were examined immunohistochemically using the mouse monoclonal antibody to HHV8 LNA-1, ORF-73 (clone 13B10, Cell Marque, USA). Tissue sections were stained with an automated immunostainer (Ventana BenchMark XT) using heat-induced epitope retrieval and standard DAB detection kit (Ventana). Primary antibody was ready to use. Strong, diffuse, nuclear staining was scored as +++, diffuse but moderate staining was scored as ++, diffuse but weak or equivocal staining was scored as +, and no staining above background was scored as −. A case of nodular basal-cell carcinoma was used as negative control and a case of Kaposi sarcoma was used as positive control.

RNA Isolation and miRNA Array Experiments

Total RNA was extracted from formalin-fixed, paraffin-embedded tissue sections (5 sections of 10 μm each) using the RNeasy FFPE Kit (Qiagen, Valencia, CA, USA). The concentration of total RNA was measured with a NanoDrop Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA). The samples were then analyzed running the Small RNA and Nano 6000 RNA chip kit assays with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to verify the integrity and to quantify miRNA. The microarray experiments were performed using the miRNA Microarray Kit V2 platform, containing 723 human and 76 human viral miRNAs from the Sanger database v.10.1, (Agilent Technologies) according to the manufacturer's instructions (protocol version 2.1). In brief, after phosphatase treatment, total RNA (100 ng for each sample) was denaturated by incubation with DMSO (100 %) at 100 ° C for 7.5 min, and then the samples were immediately transferred to an ice-water bath to prevent the RNA from reannealing. They were labeled with Cyanine3-pCp using T4 RNA Ligase and incubated at 16 °C for 2 h. After labeling reaction, samples were vacuum-dried and resuspended in nuclease-free water. Microarray slides were assembled into the Agilent SureHyb Chamber and hybridized at 55 °C for 20 h.

Data Analysis

The microarray slides were washed and immediately scanned using the Agilent's high-resolution Microarray Scanner. Data was extracted with the Agilent Feature Extraction Software (v.9.5), using data extraction default protocol for miRNA arrays, and analyzed using default settings for advance miRNA analysis of the Gene Spring GX10 software (Agilent Technologies). Data was log2 transformed before processing. Differentially expressed miRNAs between tumor and control were calculated with the *p*-value cut off ≤0.05, and using the Benjamini-Hochberg procedure for multiple testing corrections, FC≥2.

Validation of Microarray Data by Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Microarray expression profiles of three selected miRNAs, *kshv-miR-K12-1, kshv-miR-K12-4-3p* and *kshv-miR-K12-7* were verified by quantitative RT-PCR using miScript SYBR Green PCR kit (Qiagen, Valencia, USA) on a Lightcycler (Roche, software v.3.5). With miScript Reverse Transcription Kit was performed reverse transcription of RNA samples according to manufacturer's protocol (Qiagen, Valencia, CA, USA). For each reaction were used 5 ng of cDNA template in a total volume of 20 µl as described Mosakhani et al. [15]. For data normalization was used U6 gene (Qiagen, Valencia, CA, USA). For each miRNA the relative quantification (RQ) was calculated using $2^{-\Delta\Delta Ct}$ method, and fold change as a log2 RQ [16]. To see the statistical significance of the miRNA expression between KS patient and control cases *p*-value was calculated and $p<0.05$ was considered statistical significant. Due to limited amount of RNA, two sample could not been study with RT-PCR.

Target Prediction

The predicted target genes and the miRNAs chromosomal location were retrieved from the miRBase database (http://microrna.sanger.ac.uk) [17], TargetScan (http://www.targetscan.org) [18], miRanda (www.microRNA.org) [19] and mirTarget2 (http://mirdb.org/miRDB) [20, 21]. We considered only those miRNA targets which were present in more than 2 databases, in order to reduce the false positive results.

Results

HHV8 Status in Patients with Kaposi's Sarcoma In all 17 study cases, immunohistochemistry for HHV8 was performed to confirm the diagnosis. Staining was assessed semiquantitatively on a scale from weak (+) to strong (+++). The HHV8 staining was localized in the nuclei and usually was diffuse with a superimposed granular pattern (Fig. 1). Of the 17 KS cases, 11 cases showed strong nuclear staining for HHV8. Three nodular Kaposi's sarcoma samples showed moderately nuclear, granular expression for HHV8, but also an occasional light cytoplasmic background staining. HHV8 staining was absent in patch/plaque stage KS lesions (3 cases). Considering the histology of these KS stages with a stronger vascular component than cellular one, HHV8 PCR could be more sensitive. Normal vascular endothelial cells were negative in all cases of Kaposi's sarcoma.

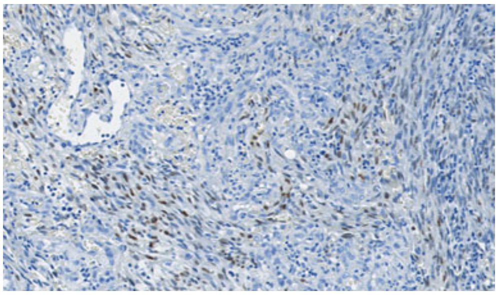

Fig. 1 Most of the tumor cells show positive staining for human herpesvirus 8 latent nuclear antigen-1 (HHV-8 LNA-1): diffuse nuclear staining with superimposed granular pattern. Normal vascular endothelial cells were HHV8 negative (HHV-8 LNA-1 immunostaining, ×20)

Distinct miRNA Signatures in Kaposi's Sarcoma, Compared with Normal Tissue Initially, the miRNA profiles of Kaposi's sarcoma samples and normal skin samples were compared, whereby differentially expressed miRNAs between the two groups were identified by using the volcano plot analysis (Fig. 2). The analysis showed 76 miRNAs upregulated in KS versus normal, while 109 miRNAs were downregulated. Among the most significantly downregulated miRNAs were *hsa-miR-99a, miR-200 family, hsa-miR-199b-5p, hsa-miR-*

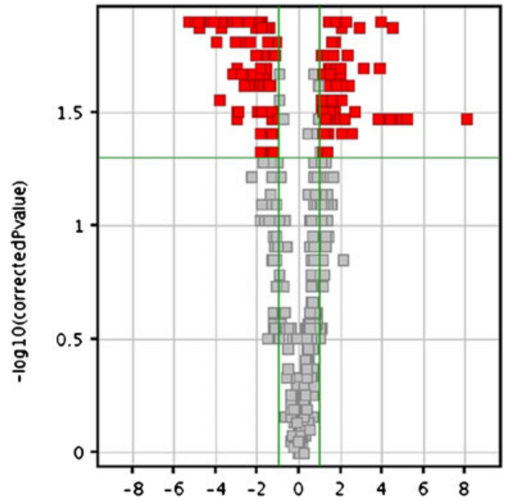

Fig. 2 Volcano plot of differential expressed miRNAs in KS versus normal tissue. Each *point* represents a miRNA. The *vertical green lines* represent FC = 2, and the *horizontal green line* represents *p*=0.05. The *red points* lying to the right of the right *vertical green line* are upregulated in KS versus normal samples (*p*≤0.05). The *red points* situated to the left of the left *vertical green line* are down regulated in KS versus normal samples (*p*≤0.05)

100, hsa-miR-335, hsa-miR-125b, hsa-miR-14, while the most upregulated ones were 12 KSHV-related miRNAs.

Validation of Microarray Results Three miRNAs, *kshv-miR-K12-1*, *kshv-miR-K12-4-3p* and *kshv-miR-K12-7,* with the highest fold change in KS versus normal cases were selected for RT-PCR validation. In agreement with the microarray results, higher expression of *kshv-miR-K12-1* (p=0.004), *kshv-miR-K12-4-3p* (p=0.001), and *kshv-miR-K12-7* (p=0.22) was seen in KS specimens.

MicroRNA Signatures and Histological Stages of KS Next, the same analysis was applied to see the differences between tumor and incipient stages (patch and plaque) of Kaposi's sarcoma. The expression of miR-486-5p and kshv-miR-K12-8 was observed only in the tumor stage of Kaposi's sarcoma (in 12 out of 14 samples). Although the two subtypes clustered separately, the analysis did not show any other significantly differentially expressed miRNAs between these two groups (Fig. 3).

MicroRNAs Predicted Targets There is strong evidence that the miRNA expression is negatively correlated with the expression of their predicted target genes in different types of cancer [22]. Each miRNA can regulate the expression of multiple mRNA targets [23]. Using four different databases (see materials and methods), we identified several predicted target genes for the miRNAs found to be differentially expressed in our KS samples, including oncogenes or tumor suppressors previously described as essential for KS pathogenesis. These include target genes such as *MYC, FGF3 (INT2), LMO2, SPP1, MERTK, NRN1, S100A2, ITM2A, RAB27A, PRG1* (also known as *SRGN*), *TGF-βR2, MCL1, CASP3* and *THBS1* (Table 1).

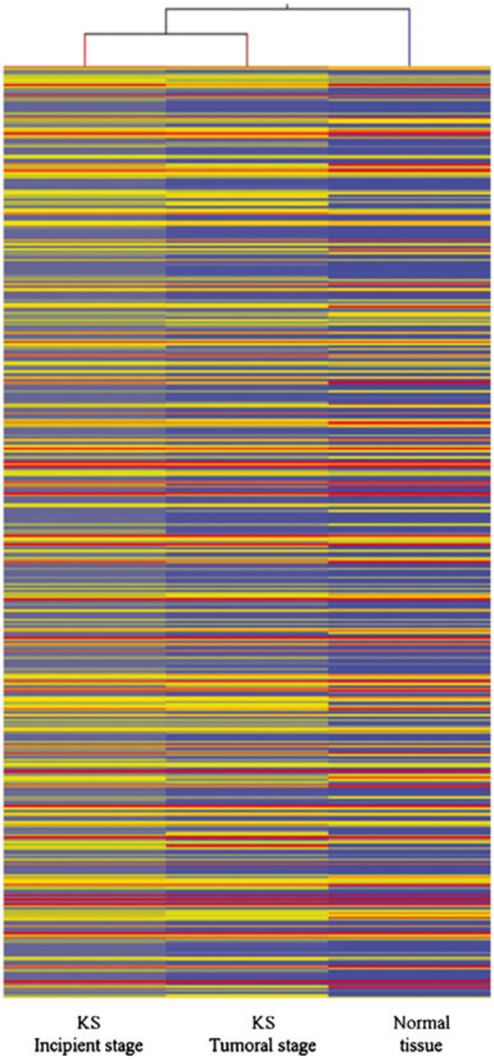

Fig. 3 Hierarchical clustering of the KS and normal tissue samples based on miRNA expression values. The KS stages (tumoral and incipient) have a distinct expression profile that separates them from the normal tissue samples

Discussion

Currently, little is known about the underlying molecular mechanisms of KS. In this study we examined differences in miRNA expression between 17 classic KS samples and three KSHV-negative normal skin samples, and also the differences between KS incipient and tumor stages.

The results clearly distinguished the KS and normal skin sample groups and identified 185 differentially expressed miRNAs, where 76 were upregulated and 109 were downregulated in KS samples. Among the most significantly upregulated miRNAs were 12 KS associated herpesvirus miRNAs while the most significantly downregulated miRNAs included *miR-99a* and *miR-200* family.

MiR-99a, the most downregulated in our KS cases, targets oncogenic mammalian target of rapamycin (*mTOR*) and fibroblast growth factor receptor 3 (*FGFR3*). It is known that mTOR have a central role in cell proliferation and is upregulated in cancers. The importance of mTOR pathway for KS has been previously reported by Merimsky et al. [24]. They showed that activation of PI3K/Akt pathway followed by mTOR activation may be important in the tumorigenesis of KS. Rapamycin inhibits the function of mTOR, resulting in cell cycle arrest in G1 phase (antiproliferative activity). Rapamycin also displays antiangiogenic activities linked to a decrease in production of vascular endothelial growth factor (VEGF) [24].

The *miR-200* family (including *miR-200a, b, c, miR-141* and *miR-429*) as well as *miR-205* were downregulated in KS samples (in both incipient and tumor stages) compared to controls. MiRNAs have been previously implicated in the epithelial-to-mesenchymal transition (EMT) occurring during

Table 1 Differentially expressed miRNAs in Kaposi's sarcoma and their putative target genes, predicted by at least two of the four analyzed (miRBase, TargetScan, miRanda and mirTarget2)

MicroRNAs	Predicted target genes
let-7a, let-7b, let-7c, **miR-494**	*MYC*
miR-99a	*FGFR3*
miR-25; miR-92a	*LMO2*
miR-513a-3p	*SPP1*
kshv-miR-K12-4-3p; kshv-miR-K12-6-3p; kshv-miR-K12-1 miR-203, **miR-298**	*MERTK*
miR-194	*NRN1*
kshv-miR-6-5p; kshv-miR-6-3p	*S100A2*
kshv-miR-K12-6-5p; kshv-miR-K12-1; kshv-miR-K12-4-3p	*ITM2A*
miR-96	*RAB27A*
miR-10a	*SRGN*
kshv-miR-K12-1; kshv-miR-K12-3; kshv-miR-K12-11 miR-20a; miR-17	*TGFβR2*
miR-29b; miR-101	*MCL1*
let-7a,b,c,d,e,f,g,i; miR-98	*CASP3*
kshv-miR-K12-1; kshv-miR-K12-3; kshv-miR-K12-4-3p miR-1; miR194; **miR-206**	*THBS1*
kshv-miR-K12-1; kshv-miR-K12-6-3p; kshv-miR-K12-11	

Upregulated miRNAs are showed in bold and downregulated miRNAs are shown in normal font

carcinogenesis [25]. In the pathogenesis of KS the role of the EMT has been described both in vivo and in vitro studies [26]. As for the target genes of *miR-200* family, a recent study reported these to be *ZEB1* and *ZEB2,* the E-cadherin transcriptional repressors. According to the study, inhibition of miR-200 reduced E-cadherin expression, increased the expression of Vimentin and induced EMT [23, 25, 27]. Thus, *miR-200* is a powerful regulator of the complex EMT differentiation process involved in tumor progression [25].

Let-7 family members (*let-7a* to *let-7i*) were, similarly, among the downregulated miRNAs in KS observed in the current study. These miRNAs are often downregulated in a variety of human cancers and are known to exhibit tumor suppressor function. Downregulation of *let-7* family was reported previously [28] in KSHV-associated cancers, including KS. Several reports have shown that *let-7* is unlikely to regulate EMT process but may contribute to tumor progression by regulating some embryonic genes re-expressed during cancer development (e.g., *HMGA2*) [29]. The target genes of *let-7* family members include *RAS* and *MYC* [29–31].

One of the target genes of *miR-20a*, upregulated in our KS samples, was reported *TGF-ßR2* (TGF-beta type II receptor). *TGF-ß* pathway is an important element in regulation of cell growth, differentiation, matrix production and apoptosis [32]. Inhibition of *TGF-ß* signaling pathway confers cells the ability to proliferate. Furthermore, KSHV-mediated *TGF-ß* signaling cascade has been demonstrated to be centrally involved in KSHV-associated cancers [33–35]. For instance, TGF-β signaling is blocked in KSHV-infected primary effusion lymphoma (PEL) through epigenetic silencing of the TβRII gene by KSHV-encoded LANA [33]. In addition, lytic protein K-bZIP [35] and vIRF1 (viral IFN regulatory factor 1) [34] have been reported to interfere and inhibit TGF-β pathway. KSHV microRNAs were recently reported to inhibit TGF-β activity. In a recent study KSHV-infection was noted to induce lower *TGF-ß2* expression in KS [33]. *THBS1* is shown to act as a strong tumor suppressor and critical regulator of cell adhesion, migration and angiogenesis [8]. It has been reported that in the presence of KSHV miRNAs, reduced *THBS1* expression translates into decreased *TGF-β* activity, suggesting that

Table 2 Deregulated miRNAs at regions with recurrent chromosome aberration in Kaposi sarcoma (Pyakurel et al. [49])

miRNAs	Chromosomal location
miR-193b	16p13.12
miR-662	16p13.3
*miR-21**	17q
miR-10a	17q21.32
miR-152	17q21.32
miR-195	17p13.1
miR-22	17p13.3
miR-497	17p13.1
miR-155	21q21.3
miR-99a	21q21.1
let-7c	21q21.1
miR-513b, miR-513c, miR-513a-5p	Xq27.3
miR-424	Xq26.3
miR-224, miR-452	Xq28

KSHV-encoded miRNAs may play an essential role in pathogenesis by downregulation of *THBS1*[8].

MiRNAs are shown to be involved in different biological processes, such as differentiation, apoptosis, initiation and progression of human cancers [9]. The current study revealed differentially regulated miRNAs whose target genes have been implicated in these vital processes. *miR-1*, downregulated in KS samples and described as a regulator of apoptosis [23], targets *HSP60* and *HSP70* [36, 37]. Recently *MCL1*, a member of *BCL-2* family of anti-apoptotic proteins, is found to be regulated by *miR-29a, b* and *c*, all downregulated in KS samples. It is commonly known that *MCL1* is upregulated while *miR-29b* is downregulated in cancer and in addition, *miR-29b*/*MCL-1* interaction is considered to be potential therapeutic possibility in cancer [38, 39].

Twelve KSHV miRNAs were upregulated in the current study. Similar expression tendency were also reported in two recent studies [40, 41]. *Kshv-miR-K12-4-3p* and *kshv-miR-K12-1* were the most significantly differentially expressed in KS samples also in the study by Hansen et al. [41].

K12 is a latent gene that is activated during KSHV lytic replication [42]. *K12* gene has been reported to be a target for cellular immune response. Recent findings have indicated *kshv-miR-K12-3* and *kshv-miR-K12-7* in the induction of IL-6 and IL-10 secretion by macrophages and monocytes, suggesting its involvement in immune response to KSHV tumor progression [43]. Also, KSHV open reading frame 50 (called RTA) has been reported to be a target for *kshv-miR-K12-9**, which functions as the switch between latency and lytic viral replication [44].

Using four different databases, we predicted *SPP1, PRG1, ITM2A, S100A2, RAB27A* and *THBS1* to have binding sites for KSHV miRNAs within their 3′UTRs. In particular, *THBS1* is targeted by multiple KSHV miRNAs, including *kshv-miR-K12-1, kshv miR-K12-3-3p, kshv-miR-K12-6-3p* and *kshv-miR-K12-11*. Samols et al. [8] confirmed KSHV miRNAs dependent inhibition of the 3′UTRs of the *SPP1, PRG1* and *THBS1*, suggesting that the observed gene expression changes are due to the viral miRNAs expression [8]. Suffert et al. [45] showed that KSHV miRNAs decrease endogenous Casp3 levels (a critical factor for the control of apoptosis), supporting the hypothesis that Casp3 3′ UTR is regulated via three binding sites (from 5′ to 3′) for *kshv-miR-K12-1, kshv-miR-K12-3 and kshv-miR-K12-4-3p* [45]. Other studies provide supporting evidence that not only regulation of Casp3 is responsible for KSHV-mediated inhibition of apoptosis. For example, Abend et al. [46] reported that *kshv-miR-K12-10* targets and downregulate TNF-like weak inducer of apoptosis (TWEAK) receptor and protects cells from TWEAK-induced apoptosis, suggesting an antitumor effect for TWEAK signaling in KS lesions [46]. In addition, Bcl-2-associated factor BCLAF1 is another identified target for several KSHV miRNAs [47].

The involvement of miRNAs in cancer has been described in many studies demonstrating that genomic loci containing miRNA genes show DNA copy number alterations [48]. Comparative genomic hybridization analysis of Kaposi's sarcoma lesions revealed recurrent changes in chromosomes 16, 17, 21q and X, [49] which were also the locations for many of the deregulated miRNAs identified in the current analysis (Table 2). Hence, *miR-224* and *miR-452*, which were downregulated in our study, located at Xq28, the chromosomal area known to be deleted in KS [49]. Similarly, *miR-25*, also downregulated in our KS samples, resides in the common deleted chromosomal area in KS, namely at 7q22.1 [49]. Also a recurrent gain at 11q13, with *FGF4* and *INT2* oncogenes amplified and expressed, has been reported in KS [5], whereas in our study, none of the miRNAs was found to be located at 11q13 region.

In conclusion, the current study revealed differentially regulated miRNAs that are involved in KS pathogenesis, and many of them were found to be located in the common altered chromosomal locations of KS. Their target genes included frequently affected genes in KS and important genes in signaling pathways, such as TGF-Beta pathway. Although, the current results revealed the miRNA profile of KS, further validations are required.

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Disclosure/Conflict of Interest The authors declare no conflict of interest.

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