microRNA Portraits in Human Vulvar Carcinoma

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Abstract

Unregulated expression of microRNAs is well known and has already been demonstrated in many tumor types. However, in vulvar carcinoma this field has been unknown territory. Our study characterizes microRNA in vulvar tumors through an expression profile of 754 miRNAs, relating this with clinical and anatomopathologic data, and presence of HPV infection. Twenty HPV-negative and 20 HPV-positive samples, genotyped for high-risk HPVs (HPV16, 18, 31, 33) and a pool of seven normal vulvar skin samples were used for the identification of differentially expressed miRNAs by TLDA Quantitative Real Time PCR (qRT-PCR). Twenty-five differentially expressed microRNAs between HPV-positive and HPV-negative groups and 79 differentially expressed on the tumor compared with normal samples were obtained. A network between microRNA expression profiles and putative target miRNAs predicted by target prediction algorithms and previously demonstrated as relevant in vulvar carcinomas, such as TP53, RB, PTEN, and EGFR was constructed. Downregulation of both miR-223-5p and miR-19-b1-5p were correlated with the presence of lymph node metastasis; downregulation of miR-100-3p and miR-19-b1-5p were correlated with presence of vascular invasion; overexpression of miR-519b and miR-133a were associated with advanced FIGO staging. In conclusion, our study demonstrates that microRNAs may be clinically important in vulvar carcinomas and our findings may help for further studies on functional implications of miRNA deregulation in this type of cancer. Cancer Prev Res; 6(11); 1231–41. ©2013 AACR.

Introduction

Unregulated expression of microRNAs is well known and well described in a variety of human tumors (1). However, in vulvar carcinomas this field has been an uncharted territory.

Studies based on epidemiologic, clinical, histopathologic, and molecular data indicate the existence of two categories of vulvar carcinomas: one group in which the HPV (high risk, such as 16, 18, and 33) appears as the main causing factor and women are aged between 35 and 65 years old; and a second group, not related to HPV, in which women are aged from 55 to 85 years old and vulvar intraepithelial neoplasia (VIN) and differentiated p53 mutation are usually observed (2, 3).

Some years ago, the central role of miRNAs in susceptibility to viral infection was described: miRNA-122 expression and hepatitis C virus infection (4), and miRNA-125b and HPV infection in cervical cancer (5).

High-risk HPV infection causes oncogenic and tumor suppressive aberrant expression of miRNAs. Various miRNA genes are downstream targets of transcription factors such as c-Myc, p53, and E2F and their expression can be modulated by oncogenic HPV E6 and E7 (6). HPV infection in tumor cells can negatively regulate the levels of a specific miRNA (miR-34a) through the inhibition of p53 by the E6 viral protein (7). E6 and E7 HPV viral proteins cause decreased miR-218, a tumor suppressor microRNA (8); miR-125b can directly reduce proliferation of HPV in vitro models. The inactivation of this miRNA without the presence of the virus is able to induce cellular changes that mimic koilocytes (5).

Our group has shown several molecules having potential prognostic role for cancer of the vulva, such as c-KIT (9), p14ARF (10), and β-catenin (11). However, to date, the preferred treatment for this condition is surgical and the most important clinical prognostic factor in vulvar cancer is the lymph node status (12, 13). Little is known about prognostic factors that may indeed provide a better psychosocial quality of life for the women diagnosed with vulvar cancer.

Once microRNAs have never been studied in vulvar cancer, we performed expression analysis of 754 microRNAs to
describe a microRNA signature in vulvar carcinomas, aiming to provide a global microRNA profile in this type of cancer, in which both of the two etiologic groups were assessed, taking into account the previously demonstrated importance of HPV in this type of tumor and their capability of modulating microRNAs; we also evaluated the correlation between microRNA expression in HPV negative samples with clinical data in order to avoid viral interference in microRNA pathways.

Materials and Methods

Patient sample selection
Forty formalin-fixed paraffin-embedded (FFPE) vulvar squamous cell carcinomas were retrospectively and randomly selected from the Department of Anatomic Pathology, A.C. Camargo Hospital (São Paulo, Brazil) from patients who had surgical intervention between 1980 to 2008, being 20 HPV-negative and 20 HPV-positive samples (positive for high-risk HPVs - HPV16, 18, 31 and 33). HPV genotyping was performed using the Linear Array HPV Genotyping Test (Roche). All cases had their histologic diagnosis confirmed by experienced pathologist. Retrospective clinical data were collected from patient's medical records. A pool of seven normal vulvar skin samples (distal edge of the tumor, without the presence of histologically confirmed malignancy) was used as overall nontumor control for comparisons between microRNA expressions. Fresh frozen samples (5 surgically removed tumors, 5 adjacent noncancerous skin, and 11 samples constituents of a normal pool) were provided by Hospital A.C. Camargo Biobank for the microRNAs validation step. For validation of the sequences predicted by target prediction algorithms, 16 fresh frozen samples were also obtained from AC Camargo Biobank, being 10 HPV-positive and 6 HPV-negative samples.

Ethics statement
This work has been approved by the Ethics Committee of our institution (A.C. Camargo Research Ethics Committee—Number 1622/11). All experiments were conducted according to the principles expressed in the Declaration of Helsinki.

RNA isolation, microRNA profiling by qRT-PCR TLDA method
For TLDA analysis, total RNA from 40 FFPE tumor samples and 7 normal samples were extracted using RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion), according to the manufacturer’s instructions.

cDNA synthesis was performed from total RNA (300 ng) using TaqMan miRNA kit Reverse Transcription Kit (Applied Biosystems) according to manufacturer’s protocol. qRT-PCR using TaqMan human microRNA array A+2.0B cards was performed on the 7900HT Fast Real-Time PCR System (Both from Applied Biosystems). Fold change values were obtained from the ratio between $2^{-\Delta C_{t}}$, HPV-negative tumors/$2^{-\Delta C_{t}}$, HPV-positive tumors. Levels of expression of each miRNA were also compared with normal tissue sample pool. Samples with $C_{t}$ values $>35$ were excluded from the analysis to enable more reliable detection.

miRNA levels of expression were determined for each sample using microRNA hsa-miR-210 as the normalizing factor, which was chosen using the RefFinder online tool—Coton EST Database (14).

For validation of microRNAs obtained in the comparison of expression with clinical and anatomicopathologic data, specific cDNA for each miRNA were synthesized from 2 µg of total RNA using TaqMan Reverse Transcription Kits MicroRNA (Applied Biosystems) following manufacturer’s specifications. qRT-PCR reactions were performed in 7900HT Fast Real-Time PCR System using Taqman Universal PCR Master Mix System (Applied Biosystems) following the manufacturer’s specifications. As initiators of the polymerization reaction, the following Assays were obtained from Applied Biosystems: hsa-miR-223-5p (AssayID: 000512), hsa-miR-19b-1-5p (AssayID: 002425), hsa-miR-100-3p (AssayID: 002142); hsa-miR-519b-3p (AssayID: 002384); hsa-miR-133a (AssayID: 002246). Hsa-miR-210 (Assay ID: 000512) was used as the reaction control, as previously described.

Validation of five putative miRNA-targets was performed by qRT-PCR using the protocol mentioned above for the following assays: PTEN (Assay Hs00829813); CDKN1A (Assay Hs01121172); TP53 (Assay Hs01034249); RB1 (Assay Hs00153108); and CDKNA2 (p16 locus, Assay Hs00924091). HPRT was used as endogenous control (Assay Hs99999909_m1). Literature-based validation of the other targets was performed through PubMed search (15).

Bioinformatics and statistical analysis
For microRNA differential expression analysis, TMeV program version 4.4.1 (16) was used, and the parametric statistical test Significance Analysis of Microarrays (SAM) was applied with 1000 permutations FDR 0.05 in the identification of differentially expressed microRNAs between HPV-positive versus HPV-negative samples. For the identification of differentially expressed microRNAs between tumoral samples (HPV positive and negative) versus normal pool sample, the One Class t test was used. It was verified if the mean expression of each microRNA was different of 1 (one). Standard Bonferroni Correction to multiple tests was applied and the level of significance was 0.01. After SAM and t test, the expression Fold was calculated with $|2 |$ cutoff.

Differentially expressed microRNAs were further examined using hierarchical grouping in which clusters were performed using Euclidean distance metric parameter and average linkage as a measure of similarity (17, 18).

A microRNA-microRNA network was created using a list of genes previously described in the literature as related to vulvar cancer and the differentially expressed microRNAs, with MAGIA (miRNA And Genes Integrated Analysis) website (19) that uses three predictors—PITA (19), miRanda (20), and Targetscan (21) – in order to determine putative miRNAs which can be targets of these
differentially expressed miRNAs (22). Predictions found in at least two algorithms were selected for the study. Importantly, these predictions were not experimentally validated and therefore these target-mRNAs are called putative target mRNAs in this paper.

For the comparative analysis between the differential miRNAs expression fold change from the HPV negative group and the clinical and anatomopathological characteristics of patients, Student t test was used to access differences between two groups of numerical variables with normal distribution and ANOVA test for more than two groups. Prism 5 for Windows was used for this analysis.

Results

Patients and samples characteristics

Epidemiological, anatomopathological and clinical characteristics of the studied population are shown in Table 1. Median age of onset of the disease was similar to both HPV positive and negative groups (69 and 70 years, respectively). However, HPV positive women’s age ranged from 15 to 90 years old while HPV negative ranged from 48 to 85. The majority of patients were white in both groups (95% in HPV positive and 85% in HPV negative groups).

Approximately half of the tumors were classified as moderately-differentiated squamous cell carcinomas in both groups and the majority of cases did not present nodal metastasis, vascular or perineural invasion. The HPV negative group demonstrated a higher invasion grade - deep dermis/subcutaneous invasion - than HPV positive tumors (78.95% in the first group versus 55% in the second).

Clinical characteristics were also assessed. Seventy percent (70%) of HPV positive tumors belonged to FIGO stage IB, while in HPV negative, tumors were distributed between FIGO IB stage (47.4%), IIC (21.1%) and the remainder of the cases between other FIGO staging groups. Tumor recurrence was observed in 47.36% of HPV positive and 35.29% of HPV negative samples. In both groups the majority of patients were alive without disease or had died from cancer. HPV genotyping results are shown in Supplementary Table S1. Among the 20 genotyped samples, 16 (80%) were infected by one HPV type and 15% by 2 types of HPV virus.

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MicroRNA expression patterns: HPV positive versus HPV negative tumors

A genome-wide profiling of 754 microRNAs was assessed in 20 HPV positive and 20 HPV negative samples. HPV negative samples microRNA expression profiles (N) were distinctly different from HPV positive profiles (P), and 25 independent microRNAs were found to be differentially expressed in this comparison (P < 0.001). Fold change values obtained from the ratio between 2-ΔCT HPV negative tumors/2-ΔCT HPV positive tumors are demonstrated on Table 2. Ten microRNAs were expressed at higher levels in HPV positive tumors, while 15 were expressed at lower levels (Fig. 1). Importantly, RNU44 was found differentially expressed between HPV positive and HPV negative samples (Fold = −2.89). The number of potential mRNA targets corresponding to each differentially expressed microRNA ranged from 5 (miR-29c-5p) to 1313 (miR-186-5p), as shown in Table 2.

MicroRNAs selected in the previous analysis were analyzed by hierarchical grouping. No clear separation of major clusters between HPV positive and negative samples was observed. However, a subset of HPV negative samples were effectively separated from the other samples (Fig. 2).

MicroRNA expression patterns: tumor samples versus normal tissue

The comparison between the 754 microRNAs by TLDA was also performed between the normal pool and 40 tumoral samples (both with presence or absence of HPV). Seventy-nine differentially expressed microRNAs were obtained, being all of them downregulated on the tumor when compared to normal samples.

Comparative analysis between microRNAs expression, clinical, and anatomopathological data in vulvar carcinomas in the absence of HPV infection

The comparative analysis between microRNAs expression and important clinical and anatomopathological data was performed in samples with absence of HPV infection, in order to avoid viral interference in microRNA pathways. Five microRNAs were found to be correlated with important features: decreased expression of miR-223-5p and miR-19b-1-5p and presence of lymph node metastases; decreased expression of miR-100-3p and again of miR-19b-5p-1 and presence of vascular invasion; increased expression of microRNAs miR-519b-1-5p and miR-133a and more advanced tumor staging (FIGO IIIA, IIIB, IIIC).

In order to verify important characteristics of the 5 microRNAs correlated with clinicopathological features, miRBase and miRDB databases were used. Mature miRNA name, location, 5’-3’ sequence, gene family and number of potential mRNA targets corresponding to each differentially expressed microRNA are shown in Table 3. Putative target mRNAs predicted by algorithms ranged from 214 (miR-100-3p) to 594 (miR-519b-3p).

In the validation step, specific microRNA expression agreed with TLDA expression fold results for miR-19b-1-5p, miR-133a, and miR-223-5p expression.

Putative target mRNA-microRNA interactions network

An interaction network between 17 microRNAs expression profiles and their putative target sequences was created, demonstrating a possible interaction with genes previously shown to be relevant in vulvar carcinogenesis, such as TP53, RB, PTEN and EGFR. The network contains 64 connections obtained between 10 lower expressed and 7 higher expressed microRNAs (Fig. 3). Five of these computational algorithms predicted target mRNAs – RB1, PTEN, CDKN1A, TP53 and CDKN2A (p14 locus) - were analyzed by qRT-PCR and two were then validated: PTEN and CDKN2A (p14 locus), as shown in Supplementary Table.
Table S2. Literature-based validation was also performed and the validated target mRNAs are shown in Fig. 3.

Discussion

In the present study, we described a microRNA signature for vulvar carcinomas by qRT-PCR both in the presence and absence of HPV infection. Twenty five differentially expressed microRNAs between HPV positive and HPV negative vulvar cancer groups were identified. MicroRNA interaction networks along with their differential expression between groups suggest that changes in the expression patterns of these microRNAs can be key regulators of viral infection and tumor progression in this neoplasm.

MicroRNAs levels of expression were determined for each sample using microRNA hsa-miR-210 as the normalizing factor, which was chosen through RefFinder online tool.
Table 2. Differentially expressed microRNAs obtained from HPV-negative versus HPV-positive tumors comparison by TLDA qRT-PCR

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<th>Mature miR</th>
<th>Fold change</th>
<th>Average Ct HPV negative</th>
<th>Average Ct HPV positive</th>
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<th>5’-3’ sequence</th>
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aData obtained from the microRNA database miRBase (http://www.mirbase.org/).
bChromosomal location: genomic regions locations from which differentially expressed microRNAs are transcribed into corresponding microRNAs.
c5’-3’ sequence: sequence correspond to the mature microRNA.
dData obtained from miRDB MicroRNA Target Prediction And Functional Study Database (http://mirdb.org/miRDB/index.html).
eData obtained from miRMaid database (http://140.mirmaid.org/matures/hsa-miR-1274b).
xData not found.
developed for evaluation and screening of reference genes in extensive sets of experimental data through the integration of computer programs such as geNorm, Normfinder, BestKeeper, and the Comparative ΔCt method. It is well-known that normalization is one of the most critical steps in qRT-PCR and commonly used genes for this purpose, such as U6 and 5S (23), have already been described as being differentially expressed in cancer, which makes these genes not suitable as internal controls. In fact, RNU44, a microRNA recommended as a suitable reference gene, was found as differentially expressed among our cases. Therefore, in the present study, normalization was performed using the most stable among our data set, in order to avoid inaccurate expression results.

Some studies in other HPV related tumors such as cervical cancer have demonstrated that HPVs have oncogenic properties, mediated at least in part by reshaping the milieu of cellular miRNAs (24). HPV oncogenic proteins E6, E7 and E5 have been shown to directly or indirectly lead to deregulation of multiple miRNAs, including miR-29a in cervical cancer, contributing to the initiation and progression of this cancer (25). MiR-29c, found to be deregulated among our samples, is a member of miR-29 family which has already been shown to restrain cell cycle progression and induce apoptosis while involved in HPV infection – also in cervical cancer (24).

Other miRNAs such as miR-125b, also found differentially expressed in our groups of samples, plays important role in the regulation of HPV DNA duplication (25). Nuovo et al. 2010 (5) have shown a strong inverse correlation between this microRNA and HPV DNA in productive infection in cervical cancer. Other microRNAs observed in our...
study, such as microRNA-21, have also been identified as significantly deregulated in a range of cancers, including cervical cancer (26). miR-16 seems to be important within HPV pathogenesis in head and neck squamous cell carcinomas (27).

It is well known that vulvar carcinoma is a tumor with involvement of an HPV viral agent. In order to prevent viral interference in miRNA expression, comparative analyses between microRNAs expression and important clinical and pathological features were performed on HPV negative samples. Decreased expression of miR-223-5p is related to the presence of metastasis in our cases, which corroborates some previously described findings in esophageal tumors: miR-223 targets the artemin (ARTN), a neurotrophic factor which belongs to the family of neurotrophic factors and known to be related to metastasis. Moreover, overexpression of miR-223 in primary gastric tumors has been associated with worse metastasis-free survival and found only in metastatic cells (24).

In this study, decreased expression of miR-19b-1-5p was associated with the presence of vascular invasion and metastasis. Xu et al (2012) (28) demonstrated increasing expression of miR-19a and miR-19b in cervical tumor cells; this increase was related to cell growth and invasion.

Our data for miR-133a, showed that the increased expression of this microRNA was correlated with more advanced clinical stages, contradicting some published studies: In renal cell carcinomas, the expression of miR-133a was significantly suppressed in tumor cells, and restoration of

![Hierarchical clustering obtained from the comparison of HPV-negative versus HPV-positive samples.](image)
this molecule generated ectopic reduction of cell proliferation and invasion, as well as induction of apoptosis (29).

Regarding target mRNA validation by qRT-PCR in vulvar carcinoma clinical samples, our data showed a possible regulation of PTEN gene expression by miR-22 and CDKN2A regulation by miR-193a-5p, miR-1254, and miR-1291.

PTEN has already been shown as being repressed by several microRNAs and to be regulated by more than one microRNA. Among them, miR-22 has been shown to downregulate PTEN expression through its binding to a PTEN 3’UTR-specific site and therefore, is being considered a new-found regulatory molecule in the PTEN/AKT pathway. Among our HPV-negative samples, both miR-22 and PTEN were downregulated (data not shown), indicating that PTEN downregulation may be achieved by other targeting microRNAs rather than by miR-22. Moreover, both PTEN and miR-22 have common important sequence elements for promoter activity. This indicates that their activity can be reduced by deletions in the promoter regions (30). Looking from another perspective, both microRNA and target were upregulated on HPV-positive samples. This indicates that miR-22 may have a role on PTEN expression, post transcriptionally regulating this gene.

CDKN2A has also been previously demonstrated as a target for many microRNAs (31). Among the microRNAs found by qRT-PCR as regulators of CDKN2A—miR-193a-5p, miR-1254 and miR-1291—all were upregulated in our HPV-positive samples (data not shown). CDKN2A, on the other hand, was downregulated in these samples, which corroborates the reverse pattern, observed for microRNA-mRNA expression and can indicate a possible action of these microRNAs in CDKN2A gene transcription.

Among the literature-based validated targets, miR-21 a microRNA largely discussed in the literature has been found upregulated, similarly to the majority of previous studies (32, 33). miR-21 has been shown as an oncogene capable to regulate genes as Bcl-2 (34). Moreover, activation of miR-21 by Nanog, in turn activated by CD44, has been demonstrated as having importance in multidrug resistance (35, 36).

Of paramount importance, miR-21 and miR-106b are located near HPV integration sites, which indicate that these microRNAs may have implications in vulvar cancer, a tumor in which HPV has already been shown as having relevant clinical importance (33, 34). miR-106 was found upregulated among our HPV-positive samples. In fact, this microRNA has been shown as a cell proliferation promoter through the increase of cyclinD1 (CCND1) and E2F intracellular activity (37).

Downregulated expression of miR-365 has been found among our HPV-negative samples and also in human colon carcinoma samples. Nie and colleagues (38) showed that downregulation of miR-365 was associated with tumor progression and poor survival proportions and that this microRNA may have an antitumor effect, due to its target to CCND1 and Bcl2, which leads to cell-cycle arrest and apoptosis activation (38).

Still addressing cell-cycle interfering microRNAs, miR-186 has been shown as a proliferation inhibitor in lung...
adenocarcinomas, by targeting CCND1, CDK2, and CDK6 (39) and CCND1 has also been demonstrated as a predicted target of miR-26b (40). Among our HPV-positive samples, the expression of these two microRNAs was downregulated, which may be an important factor for the high proliferative status of the HPV-positive tumors.

Finally, miR-16, found upregulated among HPV-negative samples, was showed as having antitumor action through inhibition of CCND1, CCND2, CCND3, bcl-2, and other genes (41–43). When targeting CCND1 and CCND2, miR-16 seems to induce G1-phase arrest (38). In ovarian epithelial carcinoma cells, miR-26 upregulation contributed to the inhibition of proliferation and invasion and enhanced sensitivity to apoptotic stimuli (43).

This study presents for the first time a microRNA profile overview in vulvar cancer. Whether the added value of the generated data will be clinically meaningful remains to be tested and will be considered in future studies. As the main focus of this article was to describe a microRNA portrait in vulvar cancer, we did not perform any biologic or functional analysis of the microRNAs evaluated nor of their target genes. The number of clinical events evaluated on the HPV negative subset was low and, thus, we were not able to find robust associations between microRNAs expression and these features.

On the other hand, considering vulvar cancer is a rare disease, a relevant number of samples was used—40 FFPE samples for TLDA analysis and 21 independent fresh frozen samples for validation step. Also, our studies were performed using qRT-PCR–based techniques, which have already been demonstrated as powerful tools for microRNA quantification as they allow good sensitivity and specificity (28). Moreover, the reliability of TLDA method is based on the presence of specific forward PCR primer and TaqMan probes and it relies on qRT-PCR, the gold standard method for expression analysis (44, 45).

In summary, our descriptive analysis of microRNA expression in vulvar cancer showed 25 differentially expressed microRNAs between HPV-positive and HPV-negative tumors, and 79 altered microRNAs in cancer compared with normal vulvar tissue, all of them downregulated on the tumor compared with normal samples. This data adds new knowledge for the understanding of molecular pathways of vulvar carcinogenesis and tumor progression. Interactions between these microRNAs and algorithm-based predicted mRNAs previously described may have importance in vulvar cancer and, thus we believe that the microRNAs evaluated can be involved in cancer progression and support their function in HPV infection. Three microRNAs (miR-223-5p, miR-19b-1-5p and miR-133a) were correlated with important clinical and anatomopathologic features in vulvar cancer. Our findings may assist future analysis to reveal the actual functions of miRNAs in this disease, providing biomarkers for the establishment of prognostic and predictive values of response to novel targeting therapies in vulvar cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

![Figure 3. Interaction networks between differentially expressed microRNAs and their algorithm-based predicted target mRNAs, with relevance in vulvar carcinogenesis. The network contains 64 connections obtained between 10 lower expressed microRNAs (shown in green) and 7 higher expressed microRNAs (shown in red). Putative target mRNAs are shown in white circles. qRT-PCR validated targets are shown in thick lines; literature-based validated target mRNAs are shown in solid lines; nonvalidated targets (qRT-PCR or literature-based analysis) are shown in dashed lines.](image-url)
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.M. Maia, A.M. Lavorato-Rocha, I.S. Rodrigues, G. Baiocchi, R.M. Rocha


Writing, review, and/or revision of the manuscript: B.M. Maia, A.M. Lavorato-Rocha, F.A. Soares, R.M. Rocha

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.M. Lavorato-Rocha, G. Baiocchi

Study supervision: C.M. Coutinho, G. Baiocchi

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