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MicroRNA (miR)-203 and miR-205 expression patterns identify subgroups of prognosis in cutaneous squamous cell carcinoma*

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Summary

Background Cutaneous squamous cell carcinoma (CSCC) is the second most widespread cancer in humans and its incidence is rising. These tumours can evolve as diseases of poor prognosis, and therefore it is important to identify new markers to better predict its clinical evolution.

Objectives We aimed to identify the expression pattern of microRNAs (miRNAs or miRs) at different stages of skin cancer progression in a panel of murine skin cancer cell lines. Owing to the increasing importance of miRNAs in the pathogenesis of cancer, we considered the possibility that miRNAs could help to define the prognosis of CSCC and aimed to evaluate the potential use of miR-203 and miR-205 as biomarkers of prognosis in human tumours.

Methods Seventy-nine human primary CSCCs were collected at the University Hospital of Salamanca in Spain. We identified differential miRNA expression patterns at different stages of CSCC progression in a well-established panel of murine skin cancer cell lines, and then selected miR-205 and miR-203 to evaluate their association with the clinical prognosis and evolution of human CSCC.

Results miR-205 was expressed in tumours with pathological features recognized as indicators of poor prognosis such as desmoplasia, perineural invasion and infiltrative growth pattern. miR-205 was mainly expressed in undifferentiated areas and in the invasion front, and was associated with both local recurrence and the development of general clinical events of poor evolution. miR-205 expression was an independent variable selected to predict events of poor clinical evolution using the multinomial logistic regression model described in this study. In contrast, miR-203 was mainly expressed in tumours exhibiting the characteristics associated with a good prognosis, was mainly present in well-differentiated zones, and rarely expressed in the invasion front. Therefore, the expression and associations of miR-205 and miR-203 were mostly mutually exclusive. Finally, using a logistic biplot we identified three clusters of patients with differential prognosis based on miR-203 and miR-205 expression, and pathological tumour features.

Conclusions miR-205 and miR-203 tended to exhibit mutually exclusive expression patterns in human CSCC. This work highlights the utility of miR-205 and miR-203 as prognostic markers in CSCC.
Cutaneous squamous cell carcinoma (CSCC) is the second most common cancer in humans after basal cell carcinoma (BCC). The incidence of CSCC is rising dramatically. It is estimated that more than 700,000 new cases of CSCC are diagnosed in the U.S. per year, leading to high healthcare costs. The risk of developing CSCC in a lifetime is between 7% and 11%. Owing to the high frequency of CSCC, it is the nonmelanoma skin cancer that causes most metastases and deaths.

CSCC can evolve as a disease of poor prognosis. Although significant advances have been made in our understanding of the development of CSCC, there are many unknown aspects concerning its pathogenesis and prognosis. Thus, it is necessary to seek molecular markers that can help physicians to predict the biological behaviour and clinical evolution of CSCC. Recently, microRNAs (miRNAs or miRs) have emerged as a new class of molecule closely related to the pathogenesis of cancer. miRNAs are small molecules of noncoding RNA of 17–25 nucleotides that act as post-transcriptional regulators of mRNA expression. miRNAs have been implicated in the pathogenesis of several forms of cancer, and their expression patterns may have prognostic value.

Despite the fact that important work has been done regarding the role of miRNAs in the biology of CSCC, and other skin cancers such as BCCs, the utility of miRNAs in defining the prognosis of the disease still requires further study. Although some authors have evaluated the expression patterns of miRNAs in skin cancer cell lines and in CSCC, their potential prognostic value has not been explored in detail. In the present work, we identified differential miRNA expression patterns at different stages of CSCC progression in a well-established panel of murine skin cancer cell lines. Later, based on our findings and previous data in the literature, we selected miR-205 and miR-203 to evaluate their association with the clinical prognosis and evolution of human CSCC. Based on the expression of miR-203 and miR-205 and pathological tumour features, we predicted the prognosis of CSCC using multinomial logistic regression models. We also identified three clusters of CSCC with a logistic biplot, which highlights the utility of miR-203 and miR-205 expression to predict CSCC prognosis.

Patients, material and methods

Patients and tumour variables

Seventy-nine human primary CSCCs were collected at the University Hospital of Salamanca in Spain. The collection and use of tumour samples was approved by the institutional Ethics Review Board of the University Hospital of Salamanca. Written informed consent for research using these tumour samples was obtained from all patients. We evaluated different pathological and clinical variables of evolution whose distribution is described in Table S1 (see Supporting Information).

Cell lines

We used a panel of murine cell lines that represent different stages of CSCC, a generous gift from Dr Balmain (University of California at San Francisco). The panel includes (i) non-tumoural cell lines: C50, C5N, NK; (ii) papilloma cell lines: MSCP1 and P6; (iii) malignant, well-differentiated cell lines with squamous morphology: PDV, PDVCS7, B9 and E4;
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and, (iv) poorly differentiated cell lines, with the epithelial to mesenchymal transition (EMT) phenotype: H11, D3, A5, CarB and CarC. \(^{17-20}\) Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and 4 mmol L\(^{-1}\) L-glutamine at 37 °C and 5% of CO\(_2\).

**Total RNA isolation**

Cells were collected and centrifuged at 200 \(\times\) g. Total RNAs were isolated using the Qiagen™ kit (miRNA easy; Qiagen, Courtaboeuf, France) following the manufacturer’s instructions. Briefly, RNAs were extracted with a mix of phenol–chloroform followed by precipitation in ethanol, and were purified through RNase-free columns. RNA concentrations were determined with a spectrophotometer (Nanodrop; Wilmington, DE, U.S.A.) and microfluidic chips (Agilent; Santa Clara, CA, U.S.A.).

**miRNA expression analysis**

miRNA expression was analysed with specific arrays miR-CURY LNA microRNA Array, v.10.0 (Exiqon; Vedbaek, Denmark), following the manufacturer’s recommendations. Then, fluorescent reagents were reconstituted and cDNAs were synthesized and labelled. Following this, samples were heated at 95 °C in darkness, hybridized, and washed with the robotic HS 4800 Pro system (Tecan™; Männedorf, Switzerland). Fluorescence was scanned with a GenePix 4000B (Axon Instruments™, Sunnyvale, CA, U.S.A.) microarray scanner. Image processing was accomplished with the Gene Pix Pro (v 6.0). The data for the analysis were generated from a single channel following the manufacturer’s instructions. Raw data processing was performed with the ExiMiR package from R.\(^{24,29}\) Quantile normalization was performed using the Robust Microarray Analysis (RMA) algorithm\(^{30}\) from the BioConductor (http://www.bioconductor.org/) tools suite. All expression data were deposited in the GEO database (GEO: GSE71923).

**Tissue-array and in situ hybridization**

Tissue samples embedded in paraffin were used to prepare tissue microarrays with a tissue arrayer device (Beecher Instruments; Sun Prairie, WI, U.S.A.). Three 1-mm-diameter tissue cylinders from each tumour were included. Different areas of each tumour were selected to analyse the heterogeneity of tumours. miR-203 and miR-205 expression was detected in the tissue sections by in situ hybridization (ISH) using the miRCURY LNA™ microRNA ISH kit (Exiqon) and following the manufacturer’s recommendations.

For the quantification of miRNA expression, we evaluated the percentage of positive cells, the intensity of the staining and the location as follows: the intensity of staining was considered negative when there were no stained cells or fewer than 5% of the cells were stained; weakly positive (+) when the percentage of stained cells was more than 5% and less than 25%; moderately positive (++) when more than 25% and less than 75%; intensely positive (+++) when more than 75% of the cells were stained. Analysis of miRNA expression by ISH was performed by three independent observers (J.C., C.R.-C. and E.C.-A.).

**Immunohistochemistry**

P63 and E-cadherin expression were evaluated by immunohistochemistry with specific antibodies against P63 (Biocare, Clon BC4A4) and E-cadherin (Vitro, Clone EP700Y). We evaluated P63 and E-cadherin expression with the same semiquantitative method as that used to assess miRNA expression, described in the Supplementary Materials and Methods (see Supporting Information).

**Statistical analyses**

To compare dichotomous variables we used the Chi-square or Fisher exact tests, and to evaluate two independent samples, the Mann–Whitney U-test. To assess more than two independent groups we used the Kruskal–Wallis test. To compare temporal intervals we used the Kaplan–Meier estimator, followed by the Mantel–Cox log-rank test. To build graphical representations of the statistical associations among variables, we used the Cytoscape (v.3.1.0) software, freely available at www.cytoscape.org (accessed 11 December 2014). To evaluate which variables predicted events of poor clinical evolution, we developed logistic regression models and used the Wald test. We considered \(P\)-values < 0.05 as significant, and confidence intervals at 95%. To generate clusters of prognosis we used the logistic biplot.\(^{31}\)

**Results**

Tumours with poor clinical evolution are associated with specific histopathological traits

We defined CSCCs with poor clinical evolution as those tumours that presented local recurrence, lymph nodal progression or metastases to distant organs. In the literature, a number of other types of histopathological tumour traits associated with poor clinical evolution of CSCC have been established, such as poor degree of differentiation, perineural infiltration, infiltrative growth pattern, desmoplasia and so on.\(^{13-14}\) Therefore, we first evaluated to what extent the tumours in our cohort, known to have a poor clinical outcome, exhibited these histopathological features, all associated with a poor prognosis (Table 1). Undoubtedly, perineural infiltration was statistically more frequent in tumours with local recurrence (\(P = 0.002\)) and with lymph nodal progression (\(P = 0.048\)). Thicker tumours also showed more local recurrence (\(P = 0.009\)) and lymph nodal progression (\(P = 0.035\)). Nodal progression was also associated with a poor grade of differentiation (\(P = 0.006\)) (Table 1). We did not find statistical association with dissemination to distant organs because of the low
number of tumours with this clinical event (n = 1) in our series (see Table S1; see Supporting Information). As expected, we observed several associations among the different histopathological tumour traits, which in turn were also associated with poor or good prognosis based on the literature (Table S2; see Supporting Information). In conclusion, in our cohort of CSCC we observed a number of associations among several pathological tumour traits with events of poor clinical evolution.

Identification of miRNA differentially expressed in skin cancer cell lines with different grades of aggressiveness

Owing to the increasing importance of miRNAs in the pathogenesis of cancer,9–11 we considered the possibility that miRNAs could help to define the prognosis of CSCC. To identify miRNAs that could be associated with human CSCC with different grades of aggressiveness and prognosis, we identified miRNAs differentially expressed between groups of CSCC cell lines. Later, we chose some of these miRNAs to be evaluated in human CSCC prognosis, because of their importance in skin homeostasis.

As a result, we identified a number of miRNAs overexpressed in squamous nonmalignant cell lines (C5N, NK, MSCP1, P6) when compared with a group of malignant cell lines with a squamous morphology (PCVC57, B9, E4) (Fig. 1A and Table S3A; see Supporting Information). Among the miRNAs most differentially expressed in the immortalized nonmalignant group, we identified some miRNAs already known as tumour suppressors within different contexts, such as let-7, miR-34b, miR-200c, others with protumoural effects, such as miR-19a,35,36 and others related to skin homeostasis, such as miR-203 and miR-205 (Fig. 1A and Table S3A; see Supporting Information).14,21,23,37,38 We also identified miRNAs downregulated in the EMT stage, within squamous CSCC cell lines (PCVC57, B9, E4) compared with spindle CSCC cell lines (D3, H11, CarB, CarC, A5). In our study, the miRNA most differentially expressed was miR-205, which is in agreement with the fact that it can inhibit EMT in CSCC (Fig. 1B and Table S3B; see Supporting Information).39

Table 1  Associations of poor clinical evolution of CSCCs with histopathological tumour features, and miR-205 and miR-203 expression

<table>
<thead>
<tr>
<th>Poor prognosis criteria</th>
<th>Local recurrence</th>
<th>Nodal progression</th>
<th>Events of poor clinical evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>P-value</td>
</tr>
<tr>
<td><strong>Pathological tumour traits</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth pattern</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expansive</td>
<td>0</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>Mixed</td>
<td>1</td>
<td>16</td>
<td>NS</td>
</tr>
<tr>
<td>Infiltrative</td>
<td>3</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>Good</td>
<td>1</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
<td>35</td>
<td>NS</td>
</tr>
<tr>
<td>Poor</td>
<td>1</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td><strong>Perineural invasion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td>64</td>
<td>6</td>
</tr>
<tr>
<td><strong>Desmoplasia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td>19</td>
<td>NS</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Turnour thickness, mm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (SD)</td>
<td>11.5 (5.5)</td>
<td>5.28 (4.5)</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>Turnour size, mm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (SD)</td>
<td>21.5 (44.5)</td>
<td>18 (9)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>miRNA expression</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-203</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expression</td>
<td>0</td>
<td>29</td>
<td>2</td>
</tr>
<tr>
<td>No expression</td>
<td>4</td>
<td>46</td>
<td>27</td>
</tr>
<tr>
<td><strong>miR-205</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expression</td>
<td>4</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>No expression</td>
<td>35</td>
<td>40</td>
<td>3</td>
</tr>
</tbody>
</table>

CSCC, cutaneous skin cell carcinoma; miRNA, microRNA; NS, not significant; **bold**, significant P-values; **bold/italic**, statistical trend.
Because of the importance of miR-203 and miR-205 in skin homeostasis, we initially focused on these two miRNAs. It was observed that miR-205 was more expressed in nonmalignant and squamous CSCC cell lines than in spindle CSCC cells ($P = 0.041$). In contrast, miR-203 was more expressed in squamous nonmalignant cell lines than in
the malignant groups with a statistical trend \( (p = 0.086) \) (Fig. 1C).

**Mutually exclusive expression patterns of miR-203 and miR-205 in cutaneous squamous cell carcinoma**

Next, we evaluated the expression of miR-203 and miR-205 in human CSCCs. Firstly, we confirmed the expression patterns of both miRNAs in the normal adjacent skin, as described within the literature. Thus, miR-203 was predominantly expressed in the upper layers of the skin,\(^4^0\) and miR-205 was mostly expressed in the basal and immediate suprabasal layers (Fig. 2A).\(^4^1\)

We then evaluated the expression patterns of miR-205 and miR-203 in CSCC and found them to be reciprocally exclusive (Fig. 2B and Table S4; see Supporting Information). miR-203 was more frequently expressed, to a moderate or intense degree, in differentiated rather than in undifferentiated areas \( (p = 0.014) \), whereas miR-205 was more frequently expressed at high levels in undifferentiated areas \( (p < 0.0001) \) in the invasion front \( (p = 0.0008) \) and in zones with perineural invasion (Fig. 2C–F and Table S4; see Supporting Information). Interestingly, we observed that tumour cells, inside a blood vessel and forming a thrombus, sometimes overexpressed miR-205 (Fig. 2F).

In addition, miR-205 was, in general, more frequently expressed in tumours with an infiltrative growth pattern \( (p = 0.003) \), perineural invasion \( (p = 0.016) \), and in thicker tumours \( (p = 0.023) \) – all pathological tumour traits associated with a poor prognosis.\(^3^2,3^3,4^2\) By contrast, miR-203 expression was not associated with these same tumour traits (Table S5A; see Supporting Information).

Regarding the expression of these miRNAs in the invasion front, it was determined that miR-205 was more frequently expressed in tumours with aggressive traits, such as infiltrative growth pattern and perineural invasion. However, miR-203 was significantly less expressed in the invasion front of such aggressive tumours (Table S5B; see Supporting Information). In conclusion, miR-205 and miR-203 tended to exhibit mutually exclusive expression patterns in human CSCC.

**Reciprocally exclusive associations between miR-205 and miR-203 and P63**

It has been reported that miR-203 inhibits P63, leading to cell differentiation and the repression of stemness;\(^1^1,2^3\) whereas miR-205 represses E-cadherin and expands into the stem cell compartment.\(^4^1\) Thus, we evaluated the associations of miR-203 and miR-205 with P63 and E-cadherin as grading markers in epithelial differentiation. As expected, P63 was more frequently expressed in poorly differentiated tumours, and it was significantly more common in undifferentiated areas, whereas E-cadherin expression was more intense in well-differentiated tumours, and less common in undifferentiated areas (Fig. 2C,D,G).

P63 was inversely correlated to miR-203 expression, and most of the tumours with a remarkable P63 expression did not show miR-203 \( (p = 0.009) \). Conversely, the majority of tumours with P63 expression showed expression of miR-205 \( (p = 0.0001) \). We did not observe a statistically significant association between the expressions of miR-205 and E-cadherin in the tumours of our cohort (Fig. 2H and Table S5C; see Supporting Information). In conclusion, miR-203 and miR-205 showed a mutually exclusive P63 protein distribution in human CSCC.

**Prediction of cutaneous skin cell carcinoma prognosis based on miRNA expression and tumour traits**

We later evaluated the association between events of poor prognosis and miR-205 and miR-203 expression. miR-205 expression was associated with local CSCC recurrence \( (p = 0.038) \), and with the general events related to a poor clinical evolution \( (p = 0.011) \). We could not find an association between the expression of miR-203 with specific events of poor prognosis (Table 1).

To predict the prognosis of CSCC, we built a logistic regression model in order to predict local recurrence, lymph nodal progression, and the existence of any event associated with a poor clinical evolution. As predictive variables, we used miR-203 and miR-205 expression, perineural invasion, grade of differentiation (well-differentiated vs. poorly or moderately differentiated), growth pattern of invasion (infiltrative vs. noninfiltrative), tumour thickness (> 2 mm vs. < 2 mm) and tumour surface size (> 20 mm vs. < 20 mm in the largest diameter).

Perineural invasion was the only independent variable associated with local recurrence \( (p = 0.017) \); and a low grade of differentiation was the only variable related to lymph nodal progression \( (p = 0.004) \). Interestingly, miR-205 expression was the only independent variable associated with the occurrence of any general outcome of poor clinical evolution \( (p = 0.021) \) (Table 2). In summary, we report here that the logistic regression models are capable of predicting the clinical evolution of CSCC, based on miRNA expression and the pathological features of tumours.

**Identification of clusters of human cutaneous squamous cell carcinoma with different prognoses**

We then attempted to sort the 79 CSCCs from our study into clusters of different prognoses based on the multiple associations among the tumour traits and expression of miR-203 and miR-205 using a logistic biplot.\(^3^1\) Thus, we included the pathological variables involved in the prognosis of CSCC such as: presence/absence of perineural invasion and desmoplasia, degree of differentiation, growth pattern of invasion, tumour size and thickness;\(^3^2,3^4,4^3\) together with the expression of miR-203 and miR-205. We were able to discriminate three clusters of tumours, with different prognoses and with statistically significant differences, using all of the tumour characteristics considered (Table 2 and Fig. S1; see Supporting Information).
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(A) H&E staining of normal skin samples with miR-203 and miR-205 expression.

(B) Bar graph showing the number of tumors with miR-203 expression, with P = 0.019.

(C) H&E and miR-203 expression staining of tumors.

(D) H&E staining of tumors with miR-203 expression.

(E) Bar graph showing the number of tumors with different expression levels of miR-203, with P < 0.0001.

(F) H&E staining showing P63 expression in tumors.

(G) E-Cadherin staining in tumors with miR-203 and miR-205 expression.

(H) Bar graph showing the number of tumors with P63 expression, with P < 0.0001.
Fig 2. Expression of miR-203 and miR-205 in human CSCC. (A) Expression of miR-203 and miR-205 in normal skin as determined by in situ hybridization (ISH). Markedly, miR-203 is expressed in the epidermal upper layers of the epidermis [a, b (100×) and c (400×), black arrowheads], whereas miR-205 is expressed in the basal and immediately suprabasal layers of the epithelia [d, e (100×) and f (400×), red arrowheads]. (B) Mutually exclusive expression of miR-205 and miR-203 in CSCC. Pearson’s Chi-square test. (C) Example of a well-differentiated CSCC. The haematoxylin and eosin (H&E) section shows intense keratinization in the central zones of the tumoural lobules (100× and 400×). The pictures show miR-203 and miR-205 expression by ISH and P63 expression by immunohistochemistry from the same tumour, as indicated in the pictures. MiR-203 is highly expressed, whereas miR-205 and P63 were poorly expressed. (D) Example of a poorly differentiated CSCC. The H&E section shows strands of epithelial cells without keratinization (100×). MiR-205 and P63 were highly expressed, whereas miR-203 is poorly expressed. In (C) and (D), the images on the right are details (400×) of those on the left (100×). (E) Quantification of miR-203 and miR-205 in tumors that correspond to zones without expression of E-cadherin (f, lower portion of the spot) and tumoral spindle cells in the lower one (d) and corresponding to zones with strong staining to E-cadherin (e, upper portion of the spot). There is strong E-cadherin expression in differentiated and undifferentiated areas of CSCC. Pearson’s Chi-square test. (F) Upper figure: Strong miR-205 expression in CSCC cells that are forming an intravascular tumoural thrombus (a, and white square) (400×). Lower figure: High miR-205 expression present in tumoral spindle cells in the invasion front with perineural infiltration (b) (400×). Nerves are indicated by an asterisk (*). (G) H&E staining (a–d) and E-cadherin expression (e, f) (100×) done by immunohistochemistry in well-differentiated (a, c, e) and poorly differentiated (b, d, f) CSCCs. There is strong E-cadherin expression in the epithelial cells of the well-differentiated CSCC (e) (100×). In (d) (400×), there are typical epithelial cells in the upper portion of the picture (*) that correspond to zones with strong staining to E-cadherin (f, upper portion of the spot) and tumoral spindle cells in the lower one (***) that correspond to zones without expression of E-cadherin (f, lower portion of the spot). This zone illustrates the tumour heterogeneity and the epithelial to mesenchymal transition process. (H) Upper figure: miR-203 expression is significantly absent when P63 is present. Lower figure: miR-205 and P63 coexpressed in the same tumours in a statistically significant manner. P-values were obtained by the Pearson’s Chi-square test. CSCC, cutaneous squamous cell carcinoma.

<table>
<thead>
<tr>
<th>Logistic regression models of prognosis</th>
<th>Variables</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td>Local recurrence</td>
<td>Perineural infiltration</td>
<td>17:445</td>
<td>1-662–1383-359</td>
<td>0.017</td>
</tr>
<tr>
<td>Nodal progression</td>
<td>Grade of differentiation</td>
<td>7:909</td>
<td>1-912–32-717</td>
<td>0.004</td>
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<td>Events of poor clinical evolution</td>
<td>miR-205 expression</td>
<td>6:552</td>
<td>1-332–32-232</td>
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<table>
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<tr>
<th>Clusters of prognosis identified by logistic biplot</th>
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<tr>
<td>n</td>
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<tr>
<td>Events of poor clinical evolution</td>
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<td>Pathological tumour traits</td>
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<tr>
<td>Poor grade of differentiation</td>
</tr>
<tr>
<td>Perineural infiltration</td>
</tr>
<tr>
<td>Desmoplasia</td>
</tr>
</tbody>
</table>

Clustering analysis

Cluster 1 comprised 25 CSCCs (31.6%), but only one developed a clinical outcome of poor prognosis. In this cluster, only three tumours (12%) showed miR-205 expression, but 15 (60%) expressed miR-203. In addition, this cluster contained several tumours with pathological traits of a good prognosis. In addition, there was only one tumour with perineural infiltration, one tumour with desmoplasia and two tumours with a poor grade of differentiation.

Farthest from cluster 1, cluster 3 comprised 23 CSCCs (29.1%), and eight tumours (35%) developed outcomes of poor clinical evolution. Interestingly, 21 (91%) tumours expressed miR-205 and only three (13%) expressed miR-203. This cluster contained several tumours with histopathological traits of poor prognosis; in addition, 22 (96%) showed an infiltrative growth pattern, 13 (56.5%), a poor grade of differentiation, and the same percentage of tumours with perineural infiltration and desmoplasia (Table 2 and Fig S1; see Supporting Information).

In total, all clusters were well characterized by the percentage of tumours that expressed miR-205 and miR-203, such that a progressively increasing expression of miR-205 went from 12% of tumours in cluster 1 to 91% of tumours in cluster 3, whereas the trend of miR-203 expression was the opposite. This correlated perfectly with the percentage of cases with poor clinical evolution in each cluster, although the pathological tumour traits, including infiltrative growth pattern and poor grade of differentiation, showed a worse correlation among all three clusters.

Table 2 Definition of CSCC prognosis by multivariate analyses: pathological features of CSCC associated with a poor clinical evolution obtained by logistic regression; characteristics of the three clusters of CSCCs identified by the logistic biplot. The table shows the number and percentage of tumours within each cluster that showed the characteristics indicated.

<table>
<thead>
<tr>
<th>Variables OR 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logistic regression models of prognosis</td>
<td></td>
</tr>
<tr>
<td>Local recurrence</td>
<td>Perineural infiltration</td>
</tr>
<tr>
<td>Nodal progression</td>
<td>Grade of differentiation</td>
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<tr>
<td>Events of poor clinical evolution</td>
<td>miR-205 expression</td>
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<tr>
<td>Clusters of prognosis identified by logistic biplot</td>
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<tr>
<td>n</td>
<td>Cluster 1 (25 (31%))</td>
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miRNAs in CSCC prognosis, J. Canueto et al.

(Table 2 and Fig. S1; see Supporting Information). Thus, although this study requires further validation using a new cohort of patients, in our cohort, the expression of these two miRNAs more accurately sorted tumours in terms of prognosis rather than pathological features. In conclusion, this study illustrates the usefulness of miR-203 and miR-205 in predicting the prognosis of human CSCC.

Discussion

We have evaluated miRNA expression and histopathological tumour features of human CSCC, and its association with local recurrence, lymph node dissemination and metastasis to predict clinical prognosis. miR-205 expression in the primary tumour was associated with local recurrence (Tables 1 and 2). We have also demonstrated the differential localization of miR-203 and miR-205 in human CSCC (Fig. 2B–E), and have evaluated the associations of the expression pattern of miR-203 and miR-205 with the pathological traits of CSCC. Overall, miR-203 and miR-205 were associated with different tumour traits in a mutually exclusive manner (Tables S4 and S5; see Supporting Information). Thus, miR-205 was associated with clinical and histopathological variables of poor outcome.

Not much is known about the role of miR-205 in the pathogenesis of CSCC. It has been pointed out that miR-205 expression is higher in normal skin, in which it is restricted to basal layer progenitors. miR-205 maintains epithelial proliferation during the development of skin, and the lack of expression of miR-205 inhibits the proliferation of cells of the basal layer. Although it has been described that miR-205 inhibits EMT through the inhibition of ZEB factors, we observed the expression of miR-205 in undifferentiated areas, zones of perineural invasion and along the invasion front in CSCC (Fig. 2C–F and Tables S4 and S5; see Supporting Information). This could be in agreement with the fact that a pure EMT is rarely observed in the invasion front of epithelial tumours of human origin. In fact, while a complete EMT is accepted as a critical step during embryogenensis, its participation in carcinoma metastasis is debated by several authors.

Here we observed a statistical association between miR-205 expression and local recurrence. Moreover, miR-205 expression was the only independent variable selected by a logistic regression model to define general events of poor outcome of CSCC (Table 2). These facts, together with the expression of miR-205 in undifferentiated zones and in the invasion front, suggest a protumoural role for miR-205 in the pathogenesis of CSCC. miR-205 would probably help to maintain a poorly differentiated and more aggressive epithelial phenotype in the tumours, but at the same time would be an epithelial marker and would inhibit the whole mesenchymal transformation. It has been described that miR-205 could activate AKT in normal skin and in carcinomas of other origins. This is also consistent with the inverse correlation between miR-205 and the positive correlation with P63, found in this study, and in agreement with previous works. Recently it was demonstrated that EMT occurs in parallel with AKT activation during CSCC invasion.

Despite the important role of miR-203 in keratinocyte homeostasis, its role in CSCC has not been previously studied. Here, we did not find statistical association with events of poor clinical evolution, but according to our data, miR-203 helps to identify a subgroup of CSCC with a better prognosis (Table 2 and Fig. S1; see Supporting Information). This could be in agreement with the fact that miR-203, which has an antitumoural effect in BCCs, has been observed as being repressed in CSCC in mice, and also inhibits EMT. In conclusion, miR-203 could behave as a tumour suppressor in human CSCC, but additional studies are needed to clarify this possibility. An interesting aspect was the mutually exclusive pattern of expression between miR-203 and miR-205 in tumours, depending on the degree of differentiation, and in the invasion front (Fig. 2 and Tables S4 and S5; see Supporting Information).

Finally, we identified three clusters of tumours with different prognoses by integrating the expression pattern of these miRNAs with clinical and pathological parameters. Although these clusters encompassed different pathological variables, they were well defined by the percentage of cases that expressed miR-203 and miR-205, and indeed, miR-203 and miR-205 sorted tumours in terms of prognosis more accurately than histopathological variables alone. Thus, these miRNAs could be used as markers of prognosis in CSCC. Finally, taking into account that miRNAs are master molecules that affect different processes in cellular homeostasis through the regulation of multiple proteins, miRNA-based targeted therapies might be more effective than those directed towards a single protein. Accordingly, they may become interesting potential therapeutic targets in CSCC.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Supplementary Materials and Methods. Patients, materials and methods (supplementary information): clinico-epidemiological, pathological and clinical evolution variables of cutaneous squamous cell carcinoma.

Table S1. Descriptive data in the cohort of patients with cutaneous squamous cell carcinoma. The table shows the clinico-epidemiological, pathological and clinical evolution characteristics of the study cohort.

Table S2. Associations between different tumour features of cutaneous squamous cell carcinoma. The table shows associations among pathological traits that were statistically significant or showed statistical trend.

Table S3. List of miRNAs differentially expressed in skin cancer cell lines with different grade of aggressiveness. (A) List of 45 miRNAs most differentially expressed in nonmalignant vs. squamous CSCC cell lines. (B) List of 43 miRNAs most differentially expressed between squamous CSCC and spindle CSCC cell lines. These lists of miRNAs are represented in the heatmaps in Figure 1A and B, respectively. CSCC, cutaneous squamous cell carcinoma.

Table S4. Quantification of miR-203 and miR-205 expression in human cutaneous squamous cell carcinoma. The table shows the degree of expression in differentiated and undifferentiated areas, and in the invasion front.

Table S5. Associations of miR-205 and miR-203 general expression with different characteristics of cutaneous squamous cell carcinoma. (A) Associations of clinico-pathological tumour traits and miR-205 and miR-203 expression. (B) Associations of expression of miR-205 and miR-203 in the invasion front with the presence of different tumour traits. (C) Associations of miR-205 and miR-203 and epithelial markers, E-cadherin and P63 expression.

Figure S1. Clusters of cutaneous squamous cell carcinoma prognosis identified by the logistic biplot.