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Evolution of microRNAs expression during human bronchial squamous carcinogenesis.

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Running head: MicroRNAs in lung carcinogenesis

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Abbreviations:
miRNA: microRNA; CIS: *in situ* carcinoma; SQCC: squamous cell carcinoma
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Abstract

MicroRNAs, negative posttranscriptional regulators of gene expression, are involved in cancer. Their role in early bronchial carcinogenesis was analysed in 60 biopsies obtained by fluorescence bronchoscopy (6 per stage: normal tissue of non-smokers, normal normofluorescent and hypofluorescent bronchial tissue of smokers, hyperplasia, metaplasia, mild, moderate and severe dysplasia, *in situ* carcinoma and invasive squamous cell carcinoma (SQCC)).

Seventy microRNAs were found differentially expressed in the course of bronchial carcinogenesis. Among them, some microRNAs show a linear evolution of their expression level like miR-32 and miR-34c, whose expression progressively decrease from normal bronchial tissues of non smokers to SQCC. Others behave differently at successive stages, like miR-142-3p or miR-9, or are only altered from a specific stage, like miR-199a or miR-139. MicroRNAs globally follow a two-steps evolution, first decreasing, in a reverse way of embryogenesis, during the earliest morphological modifications of bronchial epithelium and thereafter increasing at later stages of lung carcinogenesis. Moreover, microRNAs expression is very efficient to predict the histological classification between low and high grade lesions and between *in situ* and invasive carcinoma.

These data show, for the first time, that microRNAs are involved in bronchial carcinogenesis from the very early steps of this process and thus could provide tools for early detection of lung cancer.

Introduction.

Each year, lung cancer kills 1,300,000 persons worldwide and its incidence is steadily increasing. Improving its cure rate is thus a major public health objective. The bad prognosis of that cancer is mainly explained by the fact that the diagnosis is generally made only at advanced stages. Therefore, identification of early biomarkers, especially if they are detectable by non invasive methods (serum, sputum…), in high risk patients (mainly smokers) is mandatory. Lung squamous cell carcinoma (SQCC) is the endpoint of a whole range of morphological abnormalities (1), that are diffusely displayed in the bronchial airways of smokers (concept of field cancerisation) and could be used to identify markers of the ongoing cancer process.

Discovery of the microRNAs (miRNAs), negative posttranscriptional regulators of gene expression, is a landmark in molecular biology. Recent studies demonstrated that mutations in miRNAs or their aberrant expression are associated with diverse human diseases including cancers. Indeed, miRNAs might act as oncogenes or tumour suppressor genes. Many miRNAs are located on fragile sites and genomic regions involved in cancer (2). They play important roles in cell differentiation, cell growth and cell death (3). They have been found to be involved in known oncogenic pathways like those of p53 (4), Bcl2 (5) or K-Ras (6). Finally miRNAs seem to be very significant prognostic factors in patients with different tumours (7-10) and could be useful for treatment (11).

Interestingly, miRNAs maintain, even in cancer, the specificity of their developmental lineage (7). Expression of miRNAs is tissue and cell-type regulated (12). Moreover, it has been also shown that even if different tumour types display highly different patterns of miRNAs expression, tumours with a common embryonic precursor still express common miRNAs patterns (7;13). Therefore, their pattern of expression very efficiently classifies different tumour types in a more robust way than gene expression microarrays (7).

We studied the evolution of miRNAs level of expression on biopsies at the successive stages of human bronchial squamous carcinogenesis in order to determine their potential role in this pathological process and to identify potential tools for early detection of lung cancer.

Material and methods

1) Sample collection and RNA isolation.

All biopsies were obtained between 2003 and 2007 after informed consent from the patients and approval by the local ethical committee. Selected patients had a minimum smoking exposure of 30 pack-years and/or a history of lung or head and neck cancer. Fluorescence bronchoscopy was performed under local anaesthesia and all hypofluorescent areas were biopsied for histopathological diagnosis. Biopsies were classified using the 1999 histological WHO/IASLC classification of pre-invasive and invasive squamous lesions of the bronchus (14) into microscopically normal bronchial epithelium, hyperplasia, metaplasia, mild, moderate and severe dysplasia, carcinoma *in situ* (CIS), and invasive squamous cell carcinoma (SQCC). Normal bronchial epithelium was biopsied both in normo- and hypofluorescent areas in smokers and in 6 non-smokers with healthy lungs. A total of 60 biopsies were included with 6 samples of each category: normal bronchial epithelium of non smokers, normal normofluorescent bronchial epithelial tissue of smokers, histologically normal but hypofluorescent bronchial epithelium of smokers, hyperplasia, metaplasia, mild, moderate and severe dysplasia, CIS and SQCC. In each case, two biopsies were taken with a clean forceps in the same area. One biopsy was used for histopathology and the second biopsy was immediately dropped into Tripure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA), lysed by ultraturaxing and frozen in liquid nitrogen. All samples were kept at –80°C. RNA extraction was performed according to a classical phenol/chloroform protocol as

described by the Tripure provider. Twenty µg of glycogen (Roche Diagnostics) was added as carrier and the separation between the organic and the aqueous phase was performed on Phase Lock Gel (Eppendorf, Hamburg, Germany) to optimize the recovery of nucleic acids. Isolated RNAs were assessed for quantity and purity on the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and for quality on the Agilent 2100 bioanalyser with RNA 6000 NanoAssay (Agilent Technologies, Palo Alto, CA, USA). RNAs were stored at –80°C.

2. MiRNAs expression analyses.

The miRNAs were retrotranscribed and amplified (PCR) using the multiplex RT TaqMan MicroRNA Low Density Array (LDA) Assays (Applied Biosystem, Foster City, CA, USA). An amount of 160 ng of starting total RNA (20 ng for each of the eight RT-PCR) was used for each sample. The global miRNAs profiling for 365 human miRNAs was then performed by using the TaqMan LDA Human microRNA Panel v1.0 (Applied Biosystem). All the quality control tests were validated: blanks and reproducibility (standard deviation of cycle threshold (CT) < 1) of the two small nucleolar house-keeping RNAS RNU48 (SNORD48) and RNU44 (SNORD44). The amount of RNA from each sample was calibrated to the more stable (between the different arrays) small nucleolar house-keeping RNA, RNU48. This value gave a delta CT (ΔCT) value for each miRNA (miRNAs CT value – RNU48 CT value). The average ΔCTs were calculated for each group of six samples and the delta ΔCTs (ΔΔCTs), corresponding to the differences between two groups were obtained by subtracting the average ΔCT of the second group from those of the first one. Fold differences were calculated as $2^{(-\Delta\Delta CT)}$ as a decrease in 1 CT value was equivalent to a two-fold increase in the starting amount of cDNA. For the down-regulated miRNAs, it was calculated by $1/2^{(-\Delta\Delta CT)}$.

3. Statistical assessment.

First, unsupervised average linkage hierarchical clustering including all samples was performed to exclude any experimental bias. Thereafter, Student's *t* tests were used to assess the statistical significance of the difference of miRNAs expression between two groups. In addition, we performed a correction for multiple testing (CMT), according to Bonferroni's family-wise error rate. For both tests, we considered the threshold for statistical significance at 0.05. Finally, in order to assess the discriminating potential of miRNAs expression profiles, clustering using centred correlation and average linkage were performed.

Results

1. Differentially expressed miRNAs during squamous cell carcinogenesis.

Comparisons of miRNAs between successive stages did not show any statistically significant differences, probably because of the small number of patients included by stage. We grouped thus the nine histological stages in larger groups according to a molecular classification previously performed on the same biopsies. These groups are described in table 1.

We first compared miRNAs expression profiles between normal bronchial epithelium of non-smokers (group 0) and other groups of biopsies at different stages of squamous carcinogenesis in smokers (groups 1 to 3). MiRNAs expression profiles between successive groups and subgroups in smokers (groups 1, 2, 3, 3A and 3B) (table 1) were then successively compared, and also between CIS and SQCC. Eight comparisons were thus successively performed between groups 0 versus 1, 2 or 3; 1 versus 2; 2 versus 3 or 3A; 3A versus 3B and CIS versus 3B, respectively. Seventy miRNAs were found significantly differentially expressed in a least one of the eight comparisons. The name of these 70 miRNAs and the stage(s) at which their expression is significantly modified are described in table 2. In the eight successive comparisons of miRNAs expression profiles, 6, 19, 19, 16, 30, 15, 20 and 10 miRNAs were found significantly differentially expressed. In particular,

expression of miR-34c, miR-15a, miR-32 is progressively reduced from group 0 to groups 1, 2 or 3. Sixteen of the 70 miRNAs remained significantly differentially expressed in one or more comparison(s) after correction for multiple testing: 0, 3, 6, 4, 5, 4, 1 and 2 miRNAs, respectively at each of the 8 successive comparisons.

2. Evolution in two-steps of miRNAs expression

As illustrated in table 3, two successive waves were globally observed in miRNAs expression profiles during lung squamous carcinogenesis: firstly, a down-regulation and secondly an up-regulation of the majority of miRNAs. In the four first comparisons of miRNAs profiles between groups 0 versus 1, 0 versus 2, 0 versus 3, and 1 versus 2, the majority of miRNAs were down-regulated: 83, 84, 74 and 87.5 %, respectively. In the comparison between groups 2 and 3, the proportion of down-regulated miRNAs (57 %) decreased and that of up-regulated miRNAs increased (43 %). On the other side, in the 3 last comparisons between groups 2 versus 3A, 3A versus 3B and CIS versus SQCC, the majority of miRNAs were up-regulated: 80, 60 and 50 %, respectively.

While some miRNAs were modified at one specific step of lung squamous carcinogenesis only (e.g. miR-199a or miR139), others evolved across successive steps. Among them, some miRNAs showed a two-waves evolution, all being firstly down-regulated and then up-regulated (miR-142-3p, miR-142-5p, miR-9, miR-10b and miR-214).

4. Sample classification according to their groups by miRNAs profiles

The miRNAs that are differentially expressed between groups 0, 1 and 2 were not able to segregate samples *a posteriori* according their respective groups. However the miRNAs differentially expressed between group 2 versus 3 (figure 1), as well as of group 2 versus 3A (figure 2), or CIS versus invasive carcinomas (group 3B) (figure 3) were very efficient to separate and classify individual samples of these groups. The first and the clustering experiments, based on the differentially expressed miRNAs between groups 2 and 3

or 3A, show that these miRNAs segregate samples of groups 2 and 3 or 3A in 2 groups and that only 3 samples of group 3A (2 CIS and 1 SQCC) are misclassified in group 2 (figures 1 and 2). Finally, the list of miRNAs found being differentially expressed between CIS and invasive carcinoma perfectly discriminates the samples of these groups in two categories according to their histological classification (figure 3).

Discussion

Our results show that several miRNAs are differentially expressed in the course of bronchial carcinogenesis and are very efficient tools to discriminate the samples between low grade lesions (metaplasia, mild and moderate dysplasia) and high grade preneoplastic ones (severe dysplasia and CIS) as well as between *in situ* and invasive carcinomas. Moreover we found two successive steps in miRNAs expression evolution. Indeed, at earliest stages of bronchial carcinogenesis corresponding to the progression from normal epithelium of non-smokers, to phenotypically normal epithelium from smokers and hyperplasia and until the group of relatively benign morphological abnormal bronchial tissue of smokers (metaplasia and mild and moderate dysplasia), we observed a significant reduction of the expression of the vast majority of miRNAs. Whereas, during the later stages of carcinogenesis (severe dysplasia, CIS and SQCC), even if 74 % of the altered miRNAs remain also down-regulated as compared with their level of expression in normal bronchial tissue of non-smokers, the proportion of up-regulated miRNAs (43 %) increases as compared to their level of expression in previous group of lesions (metaplasia, mild and moderate dysplasia) and more so (80 %) from that latter group to the group of severe dysplasia and CIS.

Bronchial carcinogenesis thus appears as a process with a two-steps evolution. This is reflected by the global decrease of miRNAs expression observed at earliest stages of smoke-induced morphological modifications of bronchial epithelium followed by an increase

of up-regulated miRNAs at the latter stages of carcinogenesis. It has been postulated that miRNAs expression is highly regulated according to the cell's developmental lineage and to its differentiation stage (3;7;13). In agreement with the hypothesis that increased level of miRNAs expression induces cell and tissue differentiation, miRNAs down-regulation often occurs in cancer where tissue is loosing its normal differentiation (7;15). Interestingly, our data illustrate this concept. Globally, in evolution from groups 0 to 2, corresponding to morphological modifications following the loss of the normal differentiation of the normal ciliated and pseudostratified bronchial epithelium which is replaced by a keratinised and metaplastic one, the large majority of miRNAs are down-regulated. Among them, several miRNAs expressions presented an inverse evolution of their level of expression as compared with their evolution during lung embryogenesis. Particularly, miR-34c and miR-15a, which are up-regulated during lung development (16), are progressively down-regulated during lung squamous carcinogenesis. MiR-99a, miR-142-3p and miR-142-5p, also up-regulated in lung development (16), are down-regulated at earliest steps of carcinogenesis and secondly up-regulated at latest ones. MiR-214 and miR-301 (16), down-regulated in lung embryogenesis, are up-regulated at stages of severe dysplasia, CIS and SQCC.

Several miRNAs enlisted in our study have been previously reported in lung cancer miRNAs studies. However, none of the 5 miRNAs recently reported as prognostic factors by Yu, was detected in our study (10). Ethnic differences, Yu's study involving exclusively Chinese patients, perhaps explain these descrepancies. Two miRNAs up-regulated in lung carcinogenesis, miR-224 and miR-214, and one down-regulated, miR-32, were reported in Yanaihara's study, but inversely for 224, down-regulated at invasive stages (9), perhaps in accordance with the two-steps model of bronchial carcinogenesis. MiR-9, down-regulated in a series of lung cancers with a higher proportion of adenocarcinomas (9), is also down-regulated at early stages, from normal tissue of non-smokers to the group 2 and

thereafter up-regulated from the latter stages, to group 3A and again to group 3B. This pattern is another example of the two-steps evolution observed for miRNAs expression level during bronchial carcinogenesis. MiR-203, the most significantly up-regulated miRNAs in group 2 compared with both normal bronchial tissues of non-smokers or of smokers, was previously reported to be increased in lung cancer A549 cell lines (17). Finally, miR-17-5p, up-regulated at the level of group 3A, belongs to the miR-17-92 oncogenenic polycistron (18), overexpressed in cancer, and, in particular, in lung cancer (19) where it enhances cell proliferation and inhibits cell differentiation.

Some miRNAs identified in our study have been described as related to known oncogenes or tumour suppressor genes and their expression level evolves through all successive stages from normal bronchi of non smokers to invasive squamous cell carcinomas in smokers. Particularly, the expression level of miR-34c progressively decreases from normal epithelium of non smokers to invasive bronchial lesions of smokers. This miRNA was recently identified as a transcriptional target for the tumour suppressor p53 (4). The progressive down-regulation of miR-34c reflects thus the progressive loss of the protective activity of p53 and is in accordance with previous studies reporting p53 alterations starting in histologically normal tissue and hyperplasia of smokers (20). MiR-15a was also progressively down-regulated. Firstly described in chronic lymphocytic leukaemia (21), miR-15a has tumour suppressor properties through the inhibition of the anti-apoptotic Bcl2 protein, which is involved in lung carcinogenesis (5).

Other miRNAs enlisted in our study were not yet reported as related to lung cancer but were described in other cancers : miR-196a, in pancreatic carcinogenesis (22), miR-487b, in Burkitt lymphoma (23), mir-204, inhibitor of apoptosis and reported in acute lymphocytic leukaemias (24), miR-106b, in hepatocellular carcinomas (25) and miR-199a in ovarian cancer (26) and in acute myeloid leukaemia, where it is a signature for a bad prognosis (27).

On the other hand, several miRNAs identified in this study, like miR-452, miR-196b, miR-375, miR-193a, miR-139, have not been reported to be associated with any type of cancer until now and their function needs to be determined.

Finally, this study of the evolution of miRNAs expression in bronchial pre-invasive lesions aimed at identifying early biomarkers, especially detectable ones by non invasive methods (serum, sputum…) in high risk patients, for early detection of lung cancer. To our knowledge, no test or biomarkers are routinely available to solve this problem. The only attempt to identify a molecular pattern for preneoplastic bronchial lesions by using a high throughput technique was performed by Rahman et al (28) who used a proteomic mass spectrometry approach called MALDI MS. Invasive lung cancer was correctly classified but they could not validate their results for preneoplastic lesions. Potential explanations given by the authors were the lack of true normal epithelium for reference, heterogeneity of the sources of analysed tissues, technical problems related to MALDI MS method and a small number of available preneoplastic lesions. Most of these problems were avoided or solved in our study. We could not assess the prognostic value of miRNAs expression in our biopsies because lesions were resected and cured by endoscopic procedure when necessary. However, previously published data indicate that severe dysplasia and CIS are at higher risk of pejorative evolution than lower grade lesions (32 to 87 % versus 2 to 9 %). Analysis of the data revealed miRNAs signatures differentiating high grade lesions (severe dysplasia and more) from lower grade ones. If confirmed by internal and external validity studies and after assessing them in more adequate techniques for screening such as sputum examination, our observation could have potential important practical implications for the early detection of bronchial lesions requiring treatment. This miRNAs signature provides thus an original and promising tool for new lung cancer diagnosis researches.

In conclusion, this study is the first to assess miRNAs expression during the successive steps of bronchial carcinogenesis on human biopsies. Most miRNAs expression levels evolve in two steps during bronchial carcinogenesis. Initally decreasing during the earliest morphological modifications and the aberrant differentiation of normal bronchial epithelium in keratinised metaplastic one, they are thereafter found to increase when malignant transformation is engaged at the stage of severe dysplasia. Several miRNAs involved in human lung development show an inverse evolution of their level of expression during bronchial carcinogenesis, like miR-34c, transcriptional target of p53, which is up-regulated in lung formation, but inversely and progressively down-regulated during the whole process of bronchial carcinogenesis. However, the functional role of the majority of up-regulated or down-regulated miRNAs in this study remains unknown. These questions require further experiments. Finally, the miRNAs patterns of expression are able to remarkably segregate high grade preneoplastic lesions from the lower grade ones and also CIS from invasive carcinoma. The microRNAs differentially expressed between these stages are thus potential tools for early detection of lung cancer, at pre-invasive stage before the spreading of micrometastases.

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Table 1: composition of groups

<table>
<tr><td></td><td>Non smokers</td><td colspan="9">Smokers</td></tr>
<tr><td rowspan="2">Stages</td><td>Nl ns</td><td>Nl Nfl</td><td>Nl Hf</td><td>H</td><td>M</td><td>MiD</td><td>MoD</td><td>SD</td><td>CIS</td><td>SQCC</td></tr>
<tr><td>0</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td></tr>
<tr><td rowspan="2">Groups</td><td rowspan="2">Group 0</td><td colspan="3" rowspan="2">Group 1</td><td colspan="3" rowspan="2">Group 2</td><td colspan="2">Group 3A</td><td>Group 3B</td></tr>
<tr><td colspan="3">Group 3</td></tr>
</table>

Legend : Nl ns : normal of non smokers ; Nl Nfl : normal in normofluorescent area; Nl Hf : normal in hypofluorescent area; H: hyperplasia; M: metaplasia; MiD: mild dysplasia; MoD: moderate dysplasia; SD: severe dysplasia; CIS: *in situ* carcinoma ; SQCC : squamous cell carcinoma.

Stages 0 to 9 were assembled in larger groups of samples according to transcriptomic analyses performed on the same samples, thereby increasing the power of statistical tests as follows: normal normo- and hypofluorescent bronchial epithelium of smokers and hyperplasia (group 1), metaplasia, mild and moderate dysplasia (group 2) and severe dysplasia, CIS and SQCC (group 3). Normal bronchial epithelium of non smokers was considered a separate group (group 0). In addition, group 3 was subdivided in group 3A (severe dysplasia + CIS) and group 3B (invasive tumours).

Table 2: statistically significantly evolving miRNAs during lung carcinogenesis

Name of the microRNA	Comparison between groups (down = down-regulated ; up = up-regulated)							
	0 to			1 to	2 to		3A to	CIS to
	1	2	3	2	3	3A	3B	3B
hsa-miR-1				Down (5.88)			Down (10.00)	
hsa-miR-7					Up (4.11)	Up (3.96)		
hsa-miR-9		Down (11.11)			**Up (17.89)**	**Up (15.97)**	Up (3.05)	
hsa-miR-9- 4373272							Up (5.66)	
hsa-miR-9*					Up (2.49)			
hsa-miR-10b				Down (3.03)		Up (2.95)		
hsa-miR-15a	Down (5.00)	Down (6.67)	Down (3.45)					
hsa-miR-17-5p						Up (2.15)		
hsa-miR-32	Down (6.67)	Down (5.88)	Down (4.17)					
hsa-miR-34a		Down (5.00)						
hsa-miR-34c	Down (6.67)	**Down (14.29)**	**Down (25.00)**		Down (2.50)			
hsa-miR-99a				Down (1.96)	Down (1.89)			
hsa-miR-101		Down (5.56)	**Down (3.57)**					
hsa-miR-106b					Up (2.06)	Up (2.36)		
hsa-miR-132							Up (2.18)	
hsa-miR-133a				Down (5.88)				
hsa-miR-134					Down (2.70)			
hsa-miR-135a		Down (4.17)	**Down (4.00)**		Down (4.00)			
hsa-miR-139					Down (2.27)		Down (5.26)	**Down (5.88)**
hsa-miR-142-3p	Down (2.94)	Down (5.56)			Up (2.60)	Up (2.50)	Up (2.51)	Up (2.24)
hsa-miR-142-5p	Down (2.38)				Up (2.86)	Up (3.36)		
hsa-miR-143				Down (3.85)				
hsa-miR-145				Down (2.70)				
has-miR-183					Up (2.66)			
hsa-miR-190		Down (4.76)						
hsa-miR-193a				**Down (8.33)**				
hsa-miR-193b		Down (4.17)					Up (5.66)	

hsa-miR-196a			**Up (16.95)**		**Up (25.69)**	**Up (28.29)**		
hsa-miR-196b			Up (10.48)		**Up (10.80)**	**Up (10.78)**		
hsa-miR-199a							**Up (8.77)**	Up (20.87)
hsa-miR-203		**Up (5.26)**	Up (4.89)	**Up (5.79)**				
hsa-miR-204							Down (11.11)	Down (16.67)
hsa-miR-206		Up (3.86)			Down (2.33)			
hsa-miR-213					Down (3.57)			
hsa-miR-214				**Down (2.00)**			Up (2.35)	Up (2.67)
hsa-miR-216			Down (3.33)		Down (2.63)	Down (2.44)		
hsa-miR-218		Down (5.56)	Down (3.57)					
hsa-miR-224		Up (2.58)	**Up (1.63)**	**Up (3.79)**	Up (1.63)			
hsa-miR-301		down (3.57)			Up (2.47)			
hsa-miR-324-5p		Down (10.00)						
hsa-miR-338							Up (5.07)	
hsa-miR-361		Down (6.25)	Down (4.76)					
hsa-miR-362					Up (2.75)	Up (3.36)		
hsa-miR-374			Down (1.49)					
hsa-miR-375			Down (6.25)		**Down (7.14)**		Down (11.11)	
hsa-miR-382				Down (2.44)				
hsa-miR-410				Down (2.63)				
hsa-miR-411				Down (4.76)				
hsa-miR-422b			Down (4.76)		Down (4.76)	Down (4.17)		
hsa-miR-432							Up (3.46)	
hsa-miR-449		Down (3.33)	Down 5.56)		Down (5.56)			
hsa-miR-449b			Down (11.11)					
hsa-miR-451								Down (8.33)
hsa-miR-452			**Up (14.81)**		**Up (6.47)**	**Up (6.10)**		
hsa-miR-486							Down (10.00)	
hsa-miR-487b							Up (8.19)	**Up (19.03)**
hsa-miR-492					Down (2.5)			

hsa-miR-518d					Down (2.63)			
hsa-miR-521					Down (1.56)			
hsa-miR-545				Down (2.78)				
hsa-miR-565					Down (1.67)			Down (2.08)
hsa-miR-572					Down (3.45)			Down (3.85)
hsa-miR-597							Up (5.43)	
hsa-miR-615								Up (9.74)
hsa-miR-622							Down (6.25)	
hsa-miR-639						Down (2.86)		
has-miR-642		**Down (5.00)**	Down (12.50)	Down (2.17)	Down (3.33)		Down (6.67)	
hsa-miR-646							Down (5.00)	
hsa-miR-650						Up (4.97)		
hsa-miR-659	Up (4.83)			Down (3.70)			Up (6.81)	

Legend: this table shows all the miRNAs found differentially expressed in at least one of the 8 comparisons. The miRNAs statistically differentially expressed in a specific comparison after correction for multiple testing (CMT) (at Bonferroni corrected p-value < 0.05) are labelled in grey and in bold. The non labelled miRNAs are those where the *t* test p-value is < 0.05 and the corrected p-value is > 0.05, with indication for the type of regulation ("down" for down-regulated miRNAs or "up" for up-regulated miRNAs. In the first comparison (0 versus 1), no miRNAs expression remained statistically significant after CMT. Whereas, in the seven other comparisons, the number of miRNAs remaining statistically significant after CMT was: 3, 6, 4, 5, 4, 1 and 2, respectively (they are highlighted in bold and labelled in grey). The fold change of miRNAs transcriptional level of expression between the compared groups is expressed by numbers in brackets.

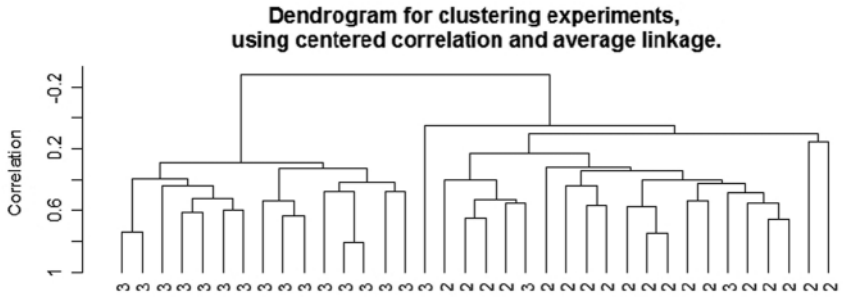
Table 3: two-steps evolution of miRNAs in lung squamous carcinogenesis

Differentially expressed miRNAs	Groups compared for miRNAs expression							
	0 versus			1 vs	2 versus		3A vs	CIS vs
	1	2	3	2	3	3A	3B	3B
Total number	6	19	19	16	30	15	20	10
Down-regulated	5 83 %	16 84 %	14 74 %	14 87,5%	17 57 %	3 20 %	8 40 %	5 50 %
Up-regulated	1 17 %	3 16 %	5 26 %	4 12,5%	13 43 %	12 80%	12 60%	5 50%

Legend: vs = versus.

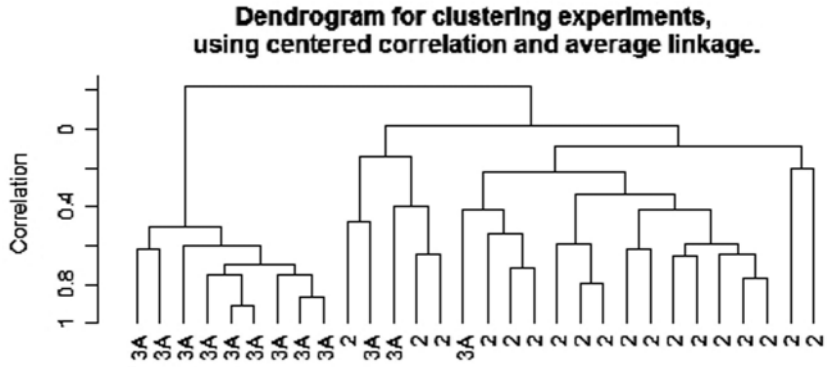
As shown by grey cases, two successive waves were observed in miRNAs expression profiles during lung squamous carcinogenesis: firstly, a down-regulation and secondly an up-regulation of the majority of miRNAs. In the 4 first comparisons of miRNAs profiles between groups 0 versus 1, 0 versus 2, 0 versus 3, and 1 versus 2, the majority of miRNAs are down-regulated. In the comparison between groups 2 and 3, the proportion of down-regulated miRNAs decreases and that of up-regulated miRNAs increases. In the 3 last comparisons between groups 2 versus 3A, 3A versus 3B and CIS versus SQCC, the majority of miRNAs are up-regulated.

Figure 1: classification of samples from groups 2 and 3 based on miRNAs differentially expressed between these groups



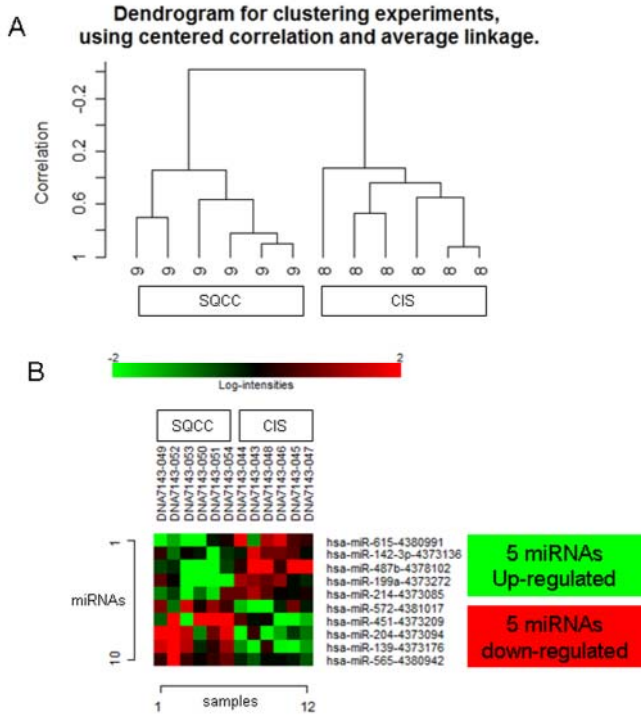
Legend: The dendrogram, based on the differentially expressed miRNAs between groups 2 (metaplasia , mild and moderate dysplasia) and 3 (severe dysplasia , *in situ* carcinoma (CIS) and invasive carcinoma, segregates samples of groups 2 and 3 in 2 groups. Only 3 samples of group 3 (2 severe dysplasias and 1 CIS) are misclassified in group 2.

Figure 2: classification of samples from group 2 and 3A based on miRNAs differentially expressed between these groups



Legend: The dendrogram, based on the differentially expressed miRNAs between groups 2 (metaplasia , mild and moderate dysplasia) and 3A (severe dysplasia and *in situ* carcinoma (CIS), segregates samples of groups 2 and 3A in 2 groups. Only 3 samples of group 3A (2 severe dysplasias and 1 CIS) are misclassified in group 2.

Figure 3: classification of samples between in situ and invasive carcinomas based on miRNAs differentially expressed between these groups



Legend: 8 = *in situ* carcinoma (CIS); 9 = invasive carcinoma.

These figures show that differentially expressed miRNAs between CIS and invasive carcinomas segregate the samples of these groups in two groups according to their histological classification: the six samples of group 8 and of group 9 are well separated in two different branches. In part B of the figure, the log intensities are directly proportional to the CT and the green (smaller CT) and the red (higher CT) colours represent up- and down-regulated miRNAs, respectively. The 5 first miRNAs are up-regulated in invasive carcinomas (the 6 first samples) as compared with CIS (the 6 last samples). The five last miRNAs are down-regulated from CIS to invasive carcinomas.

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