miR375-3p Distinguishes Low-Grade Neuroendocrine From Non-neuroendocrine Lung Tumors in FFPE Samples

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Lung cancer is still one of the leading cause of death worldwide. The clinical variability of lung cancer is high and drives treatment decision. In this context, correct discrimination of pulmonary neuroendocrine tumors is still of critical relevance. The spectrum of neuroendocrine tumors is various, and each type has molecular and phenotypical differences. In order to advance in the discrimination of neuroendocrine from non-neuroendocrine lung tumors, we tested a series of 95 surgically resected and formalin-fixed paraffin embedded lung cancer tissues, and we analyzed the expression of miR205-5p and miR375-3p via TaqMan RT-qPCR. Via a robust mathematical approach, we excluded technical outliers increasing the data reproducibility. We found that miR375-3p levels are higher in low-grade neuroendocrine lung tumor samples compared to non-neuroendocrine lung tumors. However, miR375-3p is not able to distinguish among different types of neuroendocrine lung tumors. In this work, we provide a new molecular marker for distinguishing non-neuroendocrine from low-grade neuroendocrine lung tumors samples establishing an easy miRNA score to be used in clinical settings, enabling the pathologist to classify more accurately lung tumors biopsies, which may be ambiguously cataloged in routine examination.

Keywords: neuroendocrine, microRNA, biomarker, lung cancer, miR-375

INTRODUCTION

Pulmonary neuroendocrine (NE) tumors form a distinct group of neoplasms that share morphological, immunohistochemical, ultrastructural, and molecular features. The clinical spectrum is various, from low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AT) to high-grade large cell NE carcinoma (LCNEC) and small cell lung carcinomas (SCLC). Currently, the 2015 World Health Organization classification of pulmonary NE tumors is based on combined architectural patterns with the two most relevant parameters, the mitotic index and presence of necrosis, observed by hematoxylin and eosin (H&E) staining, for the purpose of recognizing the four different subtypes (Travis et al., 2015). However, lung NE tumors represent
The samples had been stored at and were retrieved from 2011 by the Units of Surgical Pathology of the hospitals of embedded (FFPE) samples were collected between 1981 and 2012) and directly transactivates miR375-3p in cell lines and tissues. This procedure involves DNase treatment, purification, and RNA elution. All samples were stored at −80°C until used for analysis. The concentration of each sample (ng/µl) along with the purity ratio (O.D.: 260/280) was determined using a NanoDrop Spectrophotometer ND-3300 (Thermo Scientific).

**RNA Extraction**

Four 10-µm sections were cut from the selected paraffin tissue blocks, placed in xylene and heated at 50°C for 13 min. The tube was centrifuged at 12,000 × g for 2 min, and the xylene was decanted. Residual xylene was extracted by the addition of 100% ethanol to the dewaxed tissue sections and centrifugation at 12,000 × g for 5 min was performed. The ethanol was removed and the process was repeated once. The samples were then air-dried for 30 min at room temperature. The Recoverall kit (Applied Biosystems) was used to extract total RNA from dried sections. This procedure involves DNase treatment, purification, and RNA elution. All samples were stored at −80°C until used for analysis. The concentration of each sample (ng/µl) along with the purity ratio (O.D.: 260/280) was determined using a NanoDrop Spectrophotometer ND-3300 (Thermo Scientific).

**RT-qPCR**

Quantification of miRNAs expression was carried out using TaqMan MicroRNA Assay kits according to the manufacturer’s protocol (Applied Biosystems). Prefabricated TaqMan MicroRNA Assays (containing miRNA-specific forward and reverse PCR primers and miRNA-specific Taqman MGB probe) were used for the investigation of miR21-5p (ID 000397), miR205-5p (ID 000509), miR375-3p (ID 000564), and RNU6B (ID 001093). RNU6B was used as an endogenous control to normalize miRNAs expression. Complementary DNA was generated using the TaqMan MicroRNA Reverse Transcription (RT) Kit (ABI P/N 4366596) according to the manufacturer's instructions. Reverse transcriptase reactions contained 10 ng of total RNA as the template, 5 µL of gene-specific stem-loop RT primer, 1.5 µL of 10 × RT buffer, 0.15 µL of 100mM dNTPs, 1 µL of MultiScribe reverse transcriptase, and 4.16 µL of nuclease-free water. The 15-µL reactions were incubated on a Labcycler (SensoQuest GmbH) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C. Quantitative PCR was carried out using the CFX384 Touch™ Real-Time PCR Detection System (BIORAD). The 20-µL PCR reactions contained 1.33 µL of RT product, 10 µL of FastStart TaqManProbe Master Mix (Applied Biosystems), 0.9 µL of Assay mix, and 7.17 µL of nuclease-free water. The 96-well plates were run in triplicate. A no-template control was included with each plate to rule out contamination. The relative expression of each miRNA was calculated using the ∆∆Cq method relative to the endogenous control RNU6B.
Master (ROCHE, P/N 04673417001), 7.67 µL of nuclease-free water, and 1 µL of TaqMan MicroRNA Assay (Applied Biosystem). Reactions were incubated at 95°C for 10 min, followed by 40 cycles of incubation at 95°C for 15 s and at 60°C for 1 min. The threshold cycle data (Ct) and baselines were determined using auto settings. All measures were done in technical triplicates and negative controls were included in each assay. Statistical analysis and technical outlier identification were performed as described elsewhere (Ricci et al., 2015). Briefly, technical outlier identification and statistical analysis were performed according to a pipeline developed within our research group: first, outliers are identified by checking their variability via chi-square test as well as their deviation by the respective population mean via Student's t-test; second, on the basis of a training set of data, a Bayesian classifier is implemented which, by relying on the fact that triplicate expression averages are proven to be normally distributed, can be optimized and characterized also in terms of prediction uncertainty. For more details, the R package is available here: https://github.com/LeonardoRicci/MiRNA-QC-and-Diagnosis.

RESULTS

Patients’ Cohort

At the time of this analysis, not all the patients the clinical data of sex, age and cancer stage were available (23 out of 95 missing partial clinical data), due to the age of the stored samples and difference in the procedure of managing and saving clinical data among different hospitals. The patients cohort showed no statistical difference in the median age between TC, AT, AD, SQC, LCNEC and SCLC patients (All = 67.2 ± 5.9; TC = 54 ± 10; AT = 67.4 ± 13.9; AD = 69 ± 9.2; SQC = 70 ± 11.1; LCNEC = 64 ± 11.2; SCLC = 67 ± 9.7). The cohort was more represented by males than females (61M VS 17F). Clinical stage data among different hospitals. The patients cohort in a training and validation set and we measured U6 and miR375-3p expression by RT-qPCR TaqMan based technology. The training set included 27 non-NE (14 AD and 13 SQC) and 31 low-grade NE (8 AT and 23 TC). Each measurement was done in technical triplicates, and all the samples passed through a statistical reliability check (see section “Materials and Methods”) (Ricci et al., 2015). Using the average Ct we calculated, for each sample, ΔCt375 as:

\[ \Delta C_{375} = Ct_{U6} - Ct_{375} \]

Supplementary Figure S2 shows how U6 is stable in both groups analyzed bearing optimal characteristics as normalizer. The scatter plot in Figure 1A depicts in x-axis the samples ordered with a decreasing ΔCt375 (y axis), as well as the three thresholds \( \chi:10:90 \) (where 10:90 represents the odds of the sample of being non-NE or NE), \( \chi:90:10 \). The two colors represent the two different classes assigned via immunohistochemical analysis and gene profiling by the pathologist: the non-NE lung tumors (blue) and low-grade NE lung tumors (red). The thresholds divide the plot into four boxes which reflect the different outcomes of the classifier based on the likelihoods:

- orange: AT-TC class with odds larger than 90:10 (\( \Delta C_{375} > 4,9 \))
- yellow: AT-TC class with odds between 50:50 and 90:10 (4,9 > \( \Delta C_{375} > 3 \))
- light blue: AD-SQC class with odds between 50:50 an 90:10 (3 > \( \Delta C_{375} > 1,4 \))
- green: AD-SQC class with odds larger than 90:10 (\( \Delta C_{375} < 1,4 \))

The accuracy is equal to 91.4%. However, if only high-reliable responses are considered, namely with odds at least 90:10 the accuracy is 72.4%. Considering statistical analysis reported in Materials and Methods, a maximum accuracy of 90.3% can be predicted for miR375-3p as Bayesian classifier. Figure 1B shows the probability density histograms regarding the two assigned classes: the non-NE lung tumors (blue) and low-grade NE lung tumors (red), with the Gaussian curves that fit the data representing the probability density functions. The two groups are well-separated with a Student's t-test \( p\) value = 5.5e10. The reliability of miR375-3p as classifier can be inferred by considering the confusion matrix (Table 1) which reports the numbers of each tumors class samples per threshold-defined box. The quantity \( \Delta C_{375} \) discriminates between the two groups with 92.6% of sensitivity and 90.4% of specificity.

Test of the Improved Classifier on an Independent Data Set

Once we set the thresholds with the training set, we tested \( \Delta C_{375} \) reliability on the validation set of samples (22): 11 non-NE (8 AD, 3 SQC) and 11 low-grade NE (3 AT and
We performed RT-qPCR measures of miR375-3p and U6 and then calculated the $\Delta Ct_{375}$. We plotted the results using the classifier thresholds calculated previously with the training set (Figure 1C). With the exception of 4 misclassified cases, the classification provided by the classifier coincides with the immunohistochemical diagnosis (Supplementary Table S2). There are also three technical outliers, based on statistical analysis (Ricci et al., 2015), which, however, are correctly classified by $\Delta Ct_{375}$. The resulting ROC (Figure 1D) displayed an AUC equal to 0.88. Moreover, the results of a one-way analysis of variance (ANOVA) on the data allow to state that $\Delta Ct_{375}$ is not correlated with the clinical stage (Supplementary Figure S3), both for NE ($p > 0.2$) and non-NE ($p > 0.05$).

### Classifier Test for High-Grade Neuroendocrine Lung Tumors

We speculated whether the $\Delta Ct_{375}$ classifier was able also to discriminate LCNEC (Figure 2A) and SCLC (Figure 2B) in either NE or non-NE lung tumors groups. We measured $\Delta Ct_{375}$ in 11 LCNEC and 4 SCLC tissue samples and plotted the results
FIGURE 2 | Scatter plots for the ΔCt375 analysis of LCNEC and SCLC samples. Neither LCNEC (A) nor SCLC (B) samples may be sharply discriminated by the ΔCt375 (ΔCtU6–ΔCt375) since they do not cluster in any of the ΔCt375 defined boxes (green and light blue: non-NE; yellow and orange: low-grade NE). Empty-crossed circles and full squares represent technical outliers (see Ricci et al., 2015).

according to the thresholds calculated previously. In both cases, the built classifier cannot divide either LCNEC nor SCLC in NE or non-NE type. However, considering the small cohort size, due to the rarity of such samples, the results are not conclusive.

DISCUSSION

Lung cancer is still a worldwide leading cause of death (Siegel et al., 2018). The treatments for this disease are different and vary depending on the type of lung cancer. In the era of personalized medicine, there is an increasing need for biomarkers and devices to classify the disease allowing proper treatment for each class of patients. miRNAs have been widely accepted as good biomarkers for several diseases, among which cancer. miRNAs are stable molecules well preserved in FFPE as well as in fresh snap-frozen specimens unlike larger RNA molecules as messenger RNAs. Being nucleic acids, they are easy to measure by RT-qPCR (Xi et al., 2007). It is well accepted that the expression of a set of miRNAs is a more reliable indicator of physiological or pathological changes, compared to one single miRNA. However, technological limitations, costs, and ease of use push toward the development of fast and immediate assays. In this sense, few miRNAs would be desirable for a diagnostic test compared to a whole set. For this reason, building an innovative collection of biomarkers requires a precise idea of its use and influence in clinical practice. miR375 has been shown to be involved in NE cellular development in several tumors (Abraham et al., 2016; Miller et al., 2016; Arvidsson et al., 2018). Post-transcriptional regulation of Notch signaling pathway and ASH1 make miR375 molecularly involved in NE differentiation (Nishikawa et al., 2011; Abraham et al., 2016). Interestingly, miR375 has also been demonstrated to be a good biomarker of diabetes, however, it is not clear whether the increase of the levels of miR375 is due to autoimmune mechanisms or pancreatic beta-cells destruction. A recent work, showed that the major part of the miR375 in circulation from NE cells in adrenal gland, the thyroid, the lungs and the gastrointestinal tract (Latreille et al., 2015; Eliasson, 2017). Our present study shows that using a mathematical approach based on a Kolmogorov-Smirnov statistic for the outlier classification and a Bayesian index it is possible to distinguish low-grade NE from non-NE lung tumors, based on the levels of miR375-3p (ΔCt375), with an 88% accuracy. This marker improves the differential diagnosis between non-NE and low-grade NE lung tumors which may be particularly challenging in small biopsies. Another level of complexity is given by the existence of different types of NE subsets. Reproducible and objective pathologic criteria with clinical and prognostic value must be established when comparing the various grades of pulmonary NE tumors (Travis et al., 1998). Additionally, there are no specific immunohistochemical or molecular markers that allow for separation of these tumors in clear groups. So far, some markers like Ki-67 are used to separate the high-grade SCLC and LCNEC and TTF-1 for LCNEC and basaloid squamous carcinoma, otherwise easily confused by morphology. However, Ki-67 is not very efficient in classifying AT vs TC tumors (Travis et al., 2015). Hence, in our work, we also investigated the possibility to discriminate high-grade NE lung malignancies by the measurement of ΔCt375. However, due to the biological differences of these tumors compared to the low-grade cases, the analysis was not relevantly proficient. The molecular complexity of these tumors (Rekhtman et al., 2016) dampens the capability of a clear classification. Thus, the pathologist pre-classification may not be able to cluster in clear groups the different tumor samples. The quantity ΔCt375 was tested on such pre-classification of high-grade NE lung tumors and its failure in subsetting the LCNEC and SCLC samples may also be due to the high molecular heterogeneity of these tumors. In this study we used FFPE samples being part of a standard procedure in clinical settings, however, liquid biopsies are becoming relevant for ease of use and patients' compliance. Thus, in a future study of ΔCt375 ability...
in classifying NE from non-NE lung tumors, may be of critical value the use of plasma or serum. Moreover, a higher number of LCNEC and SCLC samples may increase the robustness of our findings in which miR375-3p is not able to distinguish among NE lung tumors. Concluding, here we report a miRNA marker which may fulfill the unmet need of the pathologists in clinical settings for the discrimination of NE from non-NE lung tumors also from small and challenging biopsies, making our findings of practical relevance.

DATA AVAILABILITY STATEMENT
The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT
The studies involving human participants were reviewed and approved by Ethical committee of Santa Chiara Hospital. The patients/participants provided their written informed consent to participate in this study.

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AUTHOR CONTRIBUTIONS

MD, VD, MG, MB, GR, PG, and AC designed the experiments. VD, MG, SM, SD, CC, and LR performed the experiments. MB, GR, PG, AC, and LC collected the samples. SD, VD, MG, and MD wrote the manuscript. SD and MD revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2020.00086/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.