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Altered peritumoral microRNA expression predicts head and neck cancer patients with a high risk of recurrence

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Head and neck squamous cell carcinoma is typically characterized by a high incidence of local recurrences. It has been extensively shown that mucosa from head and neck squamous cell carcinoma patients carries both genetic and gene expression alterations, which are mostly attributable to major etiologic agents of head and neck squamous cell carcinoma. We previously identified a signature of microRNAs (miRNAs) whose high expression in tumors is predictive of recurrence. Here, we investigated whether the deregulation of miRNA expression in the tumor-surrounding mucosa is correlated to disease recurrence. Specifically, comparing the miRNA expression in matched tumoral, peritumoral, and normal tissues collected from head and neck squamous cell carcinoma patients, we identified 35 miRNAs that are deregulated in both tumoral and peritumoral tissues as compared with normal matched samples. Four of these composed a miRNA signature that predicts head and neck squamous cell carcinoma local recurrence independently from prognostic clinical variables. The predictive power of the miRNA signature increased when using the expression levels derived from both the peritumoral and the tumoral tissues. The expression signal of the miRNAs composing the predictive signature correlated with the transcriptional levels of genes mostly associated with proliferation. Our results show that expression of miRNAs in tumor-surrounding mucosa may strongly contribute to the identification of head and neck squamous cell carcinoma patients at high risk of local recurrence.
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Head and neck squamous cell carcinoma comprises 5.5% of all incidence cancers and is the sixth leading cancer worldwide.[1,2] It is typically characterized by locoregional development with 60% of patients affected by relapse. Unfortunately, advances in the surgical and medical treatments for head and neck squamous cell carcinoma over the past two decades have not improved overall disease outcomes. As a consequence, locoregional failure is the most common cause of death in head and neck squamous cell carcinoma patients.[3] Typically, the treatment consists of surgical resection followed by ionizing radiation or chemoradiation. The goal of surgical treatment is complete eradication of the primary tumor with a 'safe margin'. However, despite apparent complete macroscopic and microscopic excisions of primary head and neck squamous cell carcinoma, there is a significant rate of local recurrence in patients with histologically negative margins.[4] There are two biological explanations for the mechanism of local recurrence. First, it may be due to the existence of preneoplastic processes at multiple sites in the mucosa ('field cancerization' hypothesis).[5,6] These preneoplastic tissues, from which the primary carcinoma may have developed, are apparently tumor free when analyzed at a

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histological level but present several genetic alterations when analyzed at a molecular level.[5] According to this hypothesis, it is currently accepted that the development of cancer is driven by the accumulation of genetic and epigenetic changes within a clonal population of cells.[4] These molecular alterations confer to the adjacent tissue a premalignant nature and the potentiality to evolve into an invasive cancer. The evidence that often there are clonal relationships between the primary tumor and the tumor-adjacent premalignant epithelium supports this hypothesis.[4,5,7]

A second possibility is that recurrence may be due to minimal residual disease, according to which small clusters of histopathologically undetectable tumor cells, which survive to the treatment, proliferate leading to local recurrence.[4,5] These evidences indicate that standard histopathology has limitations in the examination of the surgical margins.

One of the current major questions in the management of head and neck squamous cell carcinoma addresses the prediction and treatment of local recurrence by the identification of molecular alterations occurring in surgical margins. This ultimately is likely to provide a more rational therapeutic approach, potentially relevant to diagnosis and prognosis of head and neck squamous cell carcinoma.

Currently, potential molecular markers for head and neck squamous cell carcinoma include proteins and epigenetic markers, DNA copy number changes, and loss of heterozygosity.[4] *TP53* is one of the best-characterized molecular markers in tumor resection margins. Several studies have shown that a correlation exists between positive p53 expression by immunohistochemistry, or the presence of *TP53* mutations assessed by DNA sequencing in surgical margins and the risk of recurrence.[8,9] However, the limitation of these studies is due to the difficulty to discriminate whether the observed changes represented early malignant changes or simply a reaction to cellular stress. In addition, not all *TP53* mutations give p53 protein stabilization and overexpression detectable by immunohistochemistry.

More recently, also miRNAs expression has been introduced in the identification of minimal residual disease in head and neck squamous cell carcinoma.[10] miRNAs are 22 nucleotides long double-stranded small RNAs, able to modulate gene expression at post-transcriptional level. Deregulation of miRNAs leads to an altered expression of genes involved in cell fate regulation. Therefore, by modulating oncogenes and tumor suppressor pathways, miRNAs could contribute to tumorigenesis.[11] According to several studies miRNAs expression profile is also emerging among the best markers for diagnosis, staging, and treatment of cancer, including head and neck squamous cell carcinoma.[12–14] In particular, our group together with others has observed a correlation between the expression of specific miRNAs and outcome of head and neck squamous cell carcinoma patients.[12,14–16]

Here, we aimed at identifying key alterations of miRNAs expression occurring in tumor-surrounding mucosa (referred to as 'peritumoral' mucosa hereafter) from head and neck squamous cell carcinoma patients, which predict local recurrence. In particular, the identification of a predictive miRNA signature in peritumoral tissues may have relevance for early detection of minimal residual disease and of pre-cancer molecular alterations implicated in malignant transformation. Ultimately, this information may be important for the patient follow-up and treatment strategy for the head and neck squamous cell carcinoma patients.

In the present report, we compared miRNAs expression in matched tumoral, peritumoral, and normal tissues from 66 head and neck squamous cell carcinoma patients. We identified 35 miRNAs which were deregulated in both tumor and peritumoral tissues, compared with normal matched samples. In peritumoral tissues, out of the 35 deregulated miRNAs, 4 predicted local recurrence independently from prognostic clinical variables, either when considered individually or as a group. There was no evidence of a similar predictive effect when we analyzed the matched normal tissue samples from the surgical resection margins.

Furthermore, the predictive power of the miRNA signature increased when considering the expression levels of both the peritumoral and the tumoral tissues.

Materials and methods

Study Population and Clinical Samples

We enrolled 132 serially and prospectively selected patients with histologically confirmed primary head and neck squamous cell carcinoma who underwent curative treatment at the Otolaryngology Head and Neck Surgery Department (Figure 1). The study was approved by the scientific ethic committee of the Italian National Cancer Institute 'Regina Elena' (Rome) (protocol CE/379/08).

From each patient, three biopsies, from tumoral, peritumoral, and normal tissues, were collected at surgery and preserved in RNA later (Ambion, Austin, TX, USA). The tumoral samples were divided into two pieces: the first one assigned to the molecular characterization and the second one for formalin-fixed paraffin-embedded inclusion followed by histological characterization. Only tissues presenting $>80\%$ of tumor cells at the histological characterization were molecularly analyzed. We followed the same procedure for both the peritumor (sampled at the distance of at least 1 cm from the external margin of the tumor) and the normal tissue (sampled on disease-free surgical resection margins, at a distance of at least 2 cm from

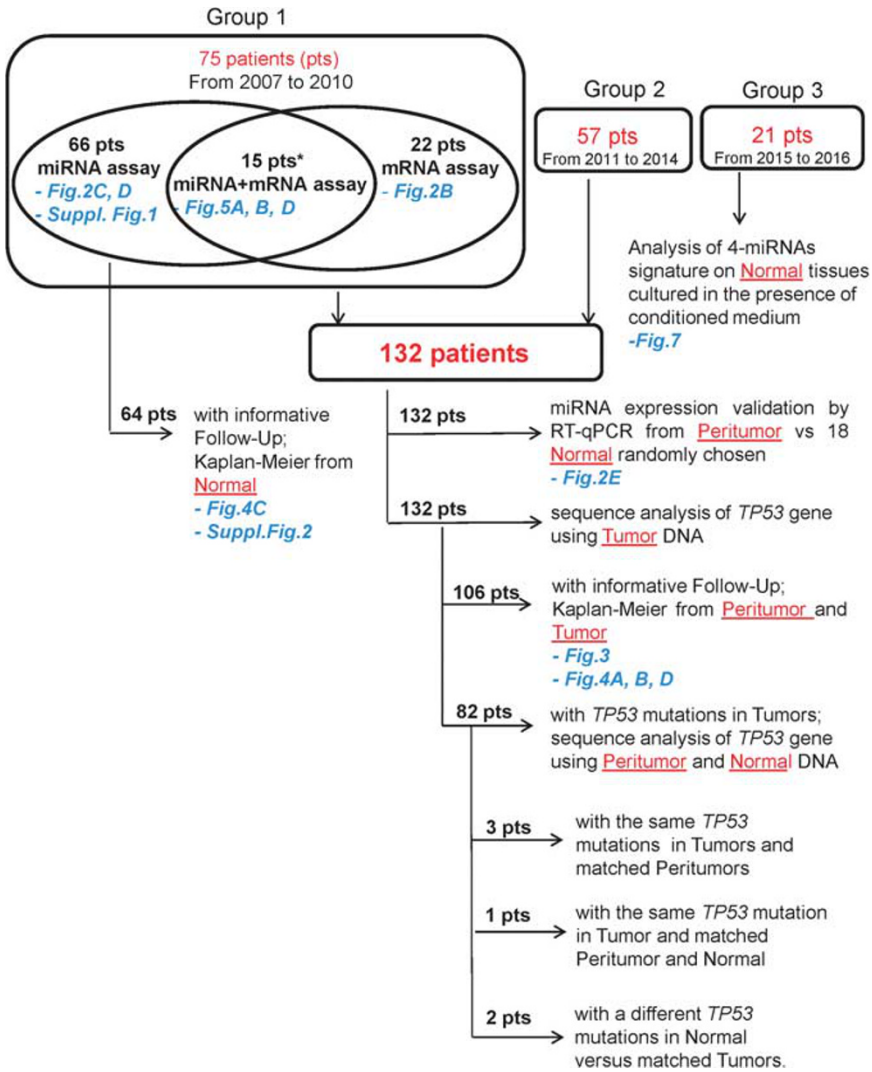


Figure 1 Flow chart of head and neck squamous cell carcinoma patients enrolled in the study. FU = follow-up; * = these 15 patients are part of the 66 and 22 head and neck squamous cell carcinoma patients. The three groups of head and neck squamous cell carcinoma patients all belong to the Italian National Cancer Institute 'Regina Elena' cohort and were consecutively enrolled from 2007 to 2016.

the external margin of the tumor). Histological analysis by hematoxylin & eosin staining has been performed on all normal tissues and on 20 randomly selected peritumoral tissues in order to evaluate the presence of cancer or pre-cancer cells. Both tissues are histologically mainly represented by mucosa.

To validate the obtained results, we also included in the analyses expression data from an additional subset of 42 matched head and neck squamous cell carcinoma patients (tumor and normal samples) of The Cancer Genome Atlas (TCGA) project. mRNA and miRNA sequencing data were downloaded from Firehose Broad GDAC (https://gdac.broadinstitute.org) of Broad Institute, whereas Reverse Phase Protein Array (RPPA) data were downloaded from The Cancer Proteome Atlas (http://tcpaportal.org/tcpa/) of MD Anderson Cancer Center.

Inclusion and Exclusion Criteria

We included only patients with histologically proven squamous cell carcinoma with primary tumor site from oral cavity, oropharynx, hypopharynx, and larynx, and who did not receive any anticancer therapy before surgery. As a consequence of these inclusion/exclusion criteria, our cohort is mainly represented by HPV-negative cases. In the follow-up study, to evaluate the prognostic value of the identified miRNA signature, we included head and neck squamous cell carcinoma patients who developed disease persistence or local recurrence after more than one month from surgery and with a follow-up equal to or more than twelve months. Local recurrence was the main study outcome and it was defined by both local lymph node metastases as well as local recurrences at the exact site of resection. Recurrence-free survival was considered

as the length of time interval from the time of surgery to the time of loco-regional recurrence.

TP53 Mutational Analysis

The mutational analysis of the exons 2–11 of *TP53* gene was performed as described in refs 15,17 using genomic DNA as template.

HPV Detection and Genotyping

HPV detection and genotyping assessment has been previously described in the study by Ganci *et al.*[15]

RNA Extraction, Labeling, and Microarray Hybridization

RNA was extracted using the miRNAeasy kit (Qiagen, Chatsworth, CA, USA) following the manufacturer's instructions. The concentration purity and quality of total RNA were assessed using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent). Total RNA (100 ng) for each specimen was labeled and used for microarray analysis of miRNAs and mRNAs expression, respectively, on the Agilent and the Affymetrix platforms. Specifically, 66 paired tumoral, peritumoral, and normal samples were hybridized on 'Human miRNA Microarray (V2) 8x15K' slides, containing probes for 723 miRNAs, while 55 additional tumoral samples were hybridized on 'SurePrint G3 Human v16 miRNA 8x60K' slides, containing probes for 1205 miRNAs. The 716 common human miRNAs were considered for further bioinformatics analyses. For mRNA expression, 22 paired normal, peritumoral, and tumoral samples were hybridized on Affymetrix Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA), following the manufacturer's instructions.

microRNAs Expression Analysis

Reverse Transcription of miRNAs and qPCR quantification of miRNA expression were performed, respectively, by TaqMan MicroRNA RT assay and TaqMan MiRNA Assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. RNU44 and RNU48 were used as endogenous controls to standardize miRNA expression. All reactions were performed in duplicate.

Microarray Data Analyses

The nomenclature of miRNAs analyzed is referred to the Rel.16 of miRNABase (http://microrna.sanger.ac.uk/).

Normalization. Expression signals were verified for quality control and extracted by Agilent Feature Extraction 10.7.3.1 software. All values lower than 1 were considered below detection and set to 1. The arrays were normalized dividing by the mean intensity calculated on data comprised in the interquartile range, thus preventing outliers from skewing the normalization. Affymetrix gene expression arrays were background adjusted and quantile normalized. The gene expression values were obtained using Robust Multiple-array Average procedure. All data were log2-transformed and entirely processed by MATLAB (The MathWorks) with in house scripts.

miRNAs and clinical features. Paired or unpaired Student's *t*-test and Wilcoxon sign-rank test were used to identify differentially expressed miRNAs and genes. A false discovery procedure[18] was included for multiple comparisons. Hierarchical clustering was performed to highlight specific pattern of expression. Pairwise distance between rows and columns were computed by using Euclidean metric. Samples were sorted by the intensity level of the miRNAs and divided into three subgroups (high intensity, medium intensity, low intensity) approximately counting the same number of samples. Differentially expressed miRNAs have been identified comparing their expression levels in the two groups where the difference in the signal levels was wider (ie, high intensity *vs* low intensity samples). Selected miRNAs were evaluated for local recurrence-free survival using Kaplan–Meier analysis and univariate and multivariate Cox proportional hazard regression models. The log-rank test was used to assess differences between curves. Significance was defined at $P < 0.05$ for univariate analysis and at $P < 0.1$ for multivariate models. The hazard risk and the 95% confidence intervals were estimated for each variable. All analyses were performed by MATLAB.

Definition of patient signature score. To analyze the prognostic value of the miRNA signature, we performed Cox and Kaplan–Meier analyses on a signal score obtained merging signal information of selected miRNAs.[15] Briefly, we assigned a score to each patient according to the expression level of the miRNAs composing the signature. We defined a signature score $SCORE_j$ obtained, for every patient *j*, summarizing the binary representation $s_i(j)$ of the expression level for each miRNA *i* composing the miRNA signature. Specifically, we set, for each miRNA *i* in each patient *j*, the binary representation of its expression level $s_i(j)$ equal to 1 if the miRNA had a high intensity level, to 1 if the miRNA had a low intensity level, and to 0 if the miRNA had a medium intensity level. The patient signature $SCORE_j$ was then quantified averaging these values over the number of miRNAs composing the signature in each patient:

$$SCORE_j = \sum_{i=1}^{n} s_i(j)/n \quad j = 1 \ldots \mathrm{N}$$

where *n* represents the number of miRNAs composing the signature and *N* is the total number of patients.

The binary value $s_i(j)$ for the expression level of each miRNA was obtained first sorting the expression levels of the given miRNA *i* and then partitioning all samples in three groups with equal number of elements and high, medium, and low expression level of miRNA *i*. If, for miRNA *i*, sample *j* belongs to the group of samples with the highest intensity level then $s_i(j) = 1$; conversely $s_i(j) = -1$ or $s_i(j) = 0$ if sample *j* belongs to the group of samples with the lowest or with a medium expression level of miRNA *i*. Finally, samples were stratified into two groups with positive (miRNA signature *high*) or negative (miRNA signature *low*) values of the patient signature score.

Expression and Correlation Analyses from TCGA Data

RNA-Seq by Expectation Maximization (RSEM) normalized count, log2 Reads Per Million of mapped reads (RPM), and replicates-based normalized data were retrieved for mRNA, miRNA, and protein expression, respectively. RSEM normalized count were then log2 transformed. Patients were selected based on the availability of mRNA and miRNA expression data for both normal and tumoral tissues. A total of 42 patients were used for transcriptome expression profiles analysis; 35 out of these 42 patients also presented information about protein expression and were included in the analysis of miRNA–proteins correlation. Four prognostic miRNAs were tested for differential expression by means of a two-sided paired Wilcoxon test using *wilcox.test* function included in *stats* R package. The miRNA/mRNA and miRNA/protein correlation analyses were performed by two-sided Pearson's correlation test using the *cor.test* function of *stats* R package. All analyses were conducted in R (version 3.0.1).

Functional Classification Analysis

In *silico* prediction pathways analysis on four prognostic miRNAs deregulated in tumoral *vs* normal samples was performed using ConsensusPathDB program (consensuspathdb.org/).

Cell Lines and Preparation/Use of Conditioned Medium

Cal27 line was obtained from ATCC (Rockville, MD, USA) and was maintained in RPMI-1640 medium (Invitrogen-GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (200 U/ml), streptomycin (200 mg/ml) (Invitrogen-GIBCO), and Amphotericin B antifungal (2.5 μg/ml) (Lonza). For the production of conditioned medium, 2×10^6/ml Cal27 cells were plated in Corning 150 mm Culture Dish and cultured for 24 h in RPMI-1640 complete medium (Invitrogen-GIBCO). Afterwards, the resulting conditioned medium was collected, centrifuged at 1200 r.p.m. for 5 min, and the supernatant was aliquoted and stored at −80 °C until use.

Biopsy from surgical resection margin (normal tissue) of 21 head and neck squamous cell carcinoma patients has been collected in 15 ml sterile canonical tubes in RPMI-1640 complete media. The biopsy was processed into 2 h from the collection; the tissue was cut into small equal pieces and cultured into six-well culture plate (BD Falcon) for 48 h. Afterwards, two out four pieces of biopsy were moved into a new six-well culture plate and cultured in the presence of conditioned medium from Cal27 cells. Finally, cultured tissues were collected and homogenized by a gentleMACS dissociator (Miltenyi Biotec) in 700 μl of Qiazol (Qiagen, Chatsworth, CA, USA). RNA was extracted using the miRNAeasy kit (Qiagen) following the manufacturer's instructions and analyzed for the expression of four prognostic miRNAs by RT-qPCR as indicated above.

Expression Signatures from the LINCS Data Set

The LINCS data set (www.lincsproject.org) includes an extensive catalog of gene-expression profiles generated by the Library of Integrated Network-based Cellular Signatures (LINCS) project from several human cancer cells in response to ~20 000 chemical perturbations.[19] The interrogation of LINCS project using Broad Institute LINCS Cloud web tool resulted in a list of several small-molecule perturbations, which are able to significantly decrease or increase the expression level of the positively and negatively miRNA-correlated genes. In particular, LINCS Cloud web tool generates a list of perturbations rank ordered by the strength of the match to the query, from highest to lowest, based on consistent connectivity across cell types. Perturbations are characterized by a score that ranges from −100 to 100, based on the overlap, with up- or downregulated genes after the perturbation, and on the strength of the enrichment. Here, we selected positive and negative perturbations with a minimal score of 90 and −90, respectively, when considering the mean connectivity score across the four cell lines in which the pertubagen connected most strongly to the query.

Immunohistochemistry

Immunohistochemical staining was performed on 3 μm formalin-fixed paraffin-embedded sections in an automated autostainer (BOND-III; Leica, Milan, Italy) by a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system (Leica), using the monoclonal antibody anti-Human Ki-67 Antigen clone MIB-1 (1:100 dilution; Dako, Ely, UK), after antigen retrieval by heating in citrate buffer (pH 6.0).

Results

Histologically Tumor-Free Peritumor Tissue from Head and Neck Squamous Cell Carcinoma Patients Shows Altered MicroRNA Expression

It has been shown that continuous tracts of mucosa surrounding the tumor may contain not only genetic alterations but also gene expression alterations without presenting any histopathological evidence of dysplasia in head and neck squamous cell carcinoma patients. On this basis, we investigated whether molecular biomarkers that present altered expression in the peritumoral tissue were also able to predict recurrence development in head and neck squamous cell carcinoma patients.

We first evaluated the histopathology of the peritumoral tissue. In a group of 20 randomly selected peritumoral tissues, corresponding to 20 patients, with information on histopathological characteristics, we did not identify any sign of dysplasia. Representative images are shown in Figure 2a.

Secondly, we evaluated whether cancer-derived cells were present in peritumoral and normal tissues from the 20 subjects. This was done by checking for the presence of mutations in the *TP53* gene in these tissues, being *TP53* the most frequently mutated gene in HPV(−) head and neck squamous cell carcinoma (86% from the TCGA study); specifically, we analyzed whether the *TP53* mutations previously identified in the tumor tissues of 82 patients from this cohort (reported in refs 15,17) were also detectable in the matched peritumoral and normal tissues from the same patients. We identified one case presenting the same *TP53* mutation in the three tissues, three cases presenting the same *TP53* mutation in tumor and peritumor, and two cases presenting in the normal tissue a *TP53* mutation that was different from the one found in the matched tumors (Figure 1). These results suggest that, despite histologically tumor-free, a number of peritumoral and normal tissues from this cohort may present infiltration of cancer-derived cells and/or presence of genetically damaged cell clones.

Thus, we evaluated mRNA and miRNA expression profiles on matched tumoral, peritumoral, and normal tissues from the cohort of head and neck squamous cell carcinoma patients (Figure 1). Demographic and clinical characteristics of all head and neck squamous cell carcinoma patients included in this study are reported in Supplementary Table S1. The analyzed patients carried almost exclusively HPV(−) tumors (as described in Materials and methods).

Expression profiling of mRNAs and miRNAs from tumoral, peritumoral, and normal tissues showed 1827 mRNAs (1002 down and 825 up) and 150 miRNAs (88 up and 62 down) whose expression is altered between tumor and normal tissues (Supplementary File 1). Supervised clustering of these mRNAs and miRNAs showed that global expression in peritumors is quite similar to that of normal tissues (Figures 2b and c).

However, when we compared the expression of miRNAs individually, comparing peritumoral *vs* normal tissues, we found that 35 out of 150 tumor-associated miRNAs (up = 23, down = 12) were also deregulated in peritumoral mucosa (Supplementary Table S2). These miRNAs showed in the peritumor an expression level that was intermediate to that of normal and tumoral tissues (Figure 2d). Interestingly, high expression of four of these miRNAs (miR-96-5p, miR-21-3p, miR-21-5p, and miR-429) was previously shown to be predictive of recurrence in head and neck squamous cell carcinoma.[15,16] Expression of these four miRNAs in peritumors was intermediate to that of tumoral and normal tissues, except for miR-429, which showed similar expression levels in tumoral and peritumoral tissues (Supplementary Figure S1). Validation of altered expression level of these miRNAs between peritumoral and normal tissues was performed by RT-qPCR on a series of 132 peritumors and 18 normal tissues (Figure 2e).

microRNA Signature Expression in Peritumors is Predictive of Recurrence Development in Head and Neck Squamous Cell Carcinoma Patients

As above mentioned, 4 of the 35 miRNAs presenting altered expression in the peritumor were previously reported by our group as predictive of recurrence when highly expressed in head and neck squamous cell carcinoma tumors.[15,16] We here evaluated whether their elevated expression in peritumoral tissue is able to predict disease recurrence in head and neck squamous cell carcinoma patients. Expression of these four miRNAs was analyzed by RT-qPCR on peritumors from the group of 106 patients for which we had complete follow-up (average = 31 months, standard deviation = 19 months) information. As shown in Figures 3a–d, the survival analysis showed that high expression of all four miRNAs was predictive of shorter recurrence-free survival in this cohort (Supplementary Table S3). On the contrary, high expression of these miRNAs in the normal tissues is not predictive of recurrence (Supplementary Figure S2).

In the multivariable Cox proportional hazards regression model, adjusting for other significant prognostic factors identified for the same patients cohort in our previous work (postoperatory adjuvant therapy, pharynx as primary site, and *TP53* mutation, as described in ref. 15), we determined that a high expression level of each of these four miRNAs in the peritumoral tissue predicts disease recurrence independently from the other variables (Figures 3a–d).

We next investigated whether these four miRNAs predict local recurrence-free survival when they are considered as a signature (miRNA signature). We assigned a score to each patient based on the expression levels of the four miRNAs (see Materials

and methods), and Kaplan–Meier analysis was carried out comparing patients with positive and negative scores. As shown in Figures 4a and b, patients with positive score for the miRNA signature (miRNA signature *high*) in, respectively, peritumoral and tumoral tissues showed reduced local

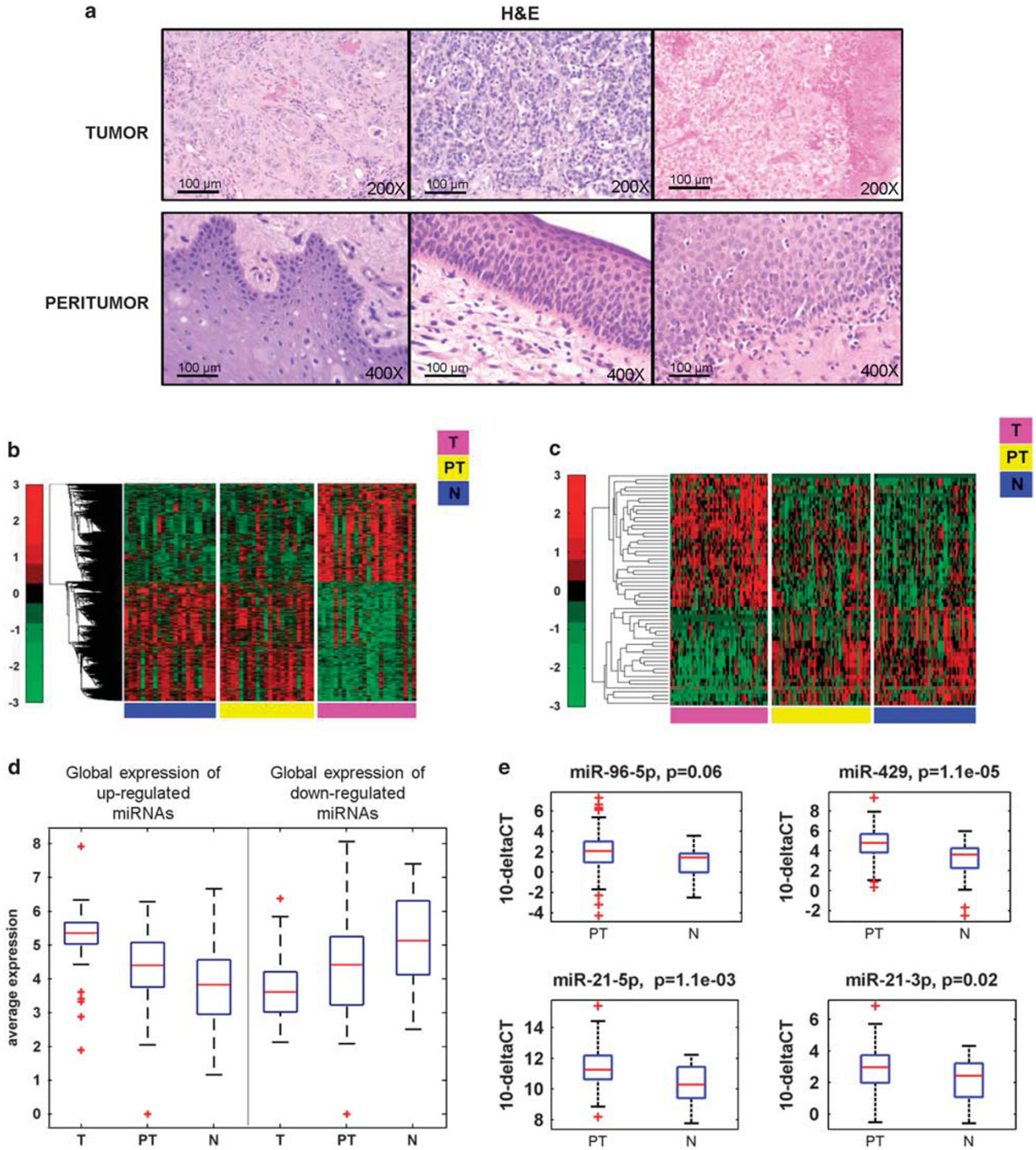


Figure 2 Molecular alteration in peritumoral *vs* normal tissue of head and neck squamous cell carcinoma patients. (**a**) H&E staining performed on formalin-fixed paraffin-embedded tissue slices showing the histological analysis of peritumoral and tumoral areas from three representative head and neck squamous cell carcinoma patients. (**b**) Supervised clustering analysis representing the genes differentially expressed between 22 tumor tissues *vs* their normal counterparts ($P < 0.05$). Expression of these genes in peritumoral tissues is also shown. (**c**) Supervised clustering analysis representing the miRNAs differentially expressed between 66 tumor tissues *vs* their normal counterparts ($P < 0.05$, fold change > 2). The expression matrix shows the behavior of these miRNAs also in peritumoral tissues. (**d**) Box plot showing the overall expression of the miRNAs differentially expressed between 66 peritumoral tissues and their normal counterparts ($P < 0.05$). (**e**) Box plots showing the expression of four selected miRNAs (miR-429, miR96-5p, miR-21-5p, and miR-21-3p) evaluated by RT-qPCR in 132 peritumors *vs* 18 normal samples from head and neck squamous cell carcinoma patients.

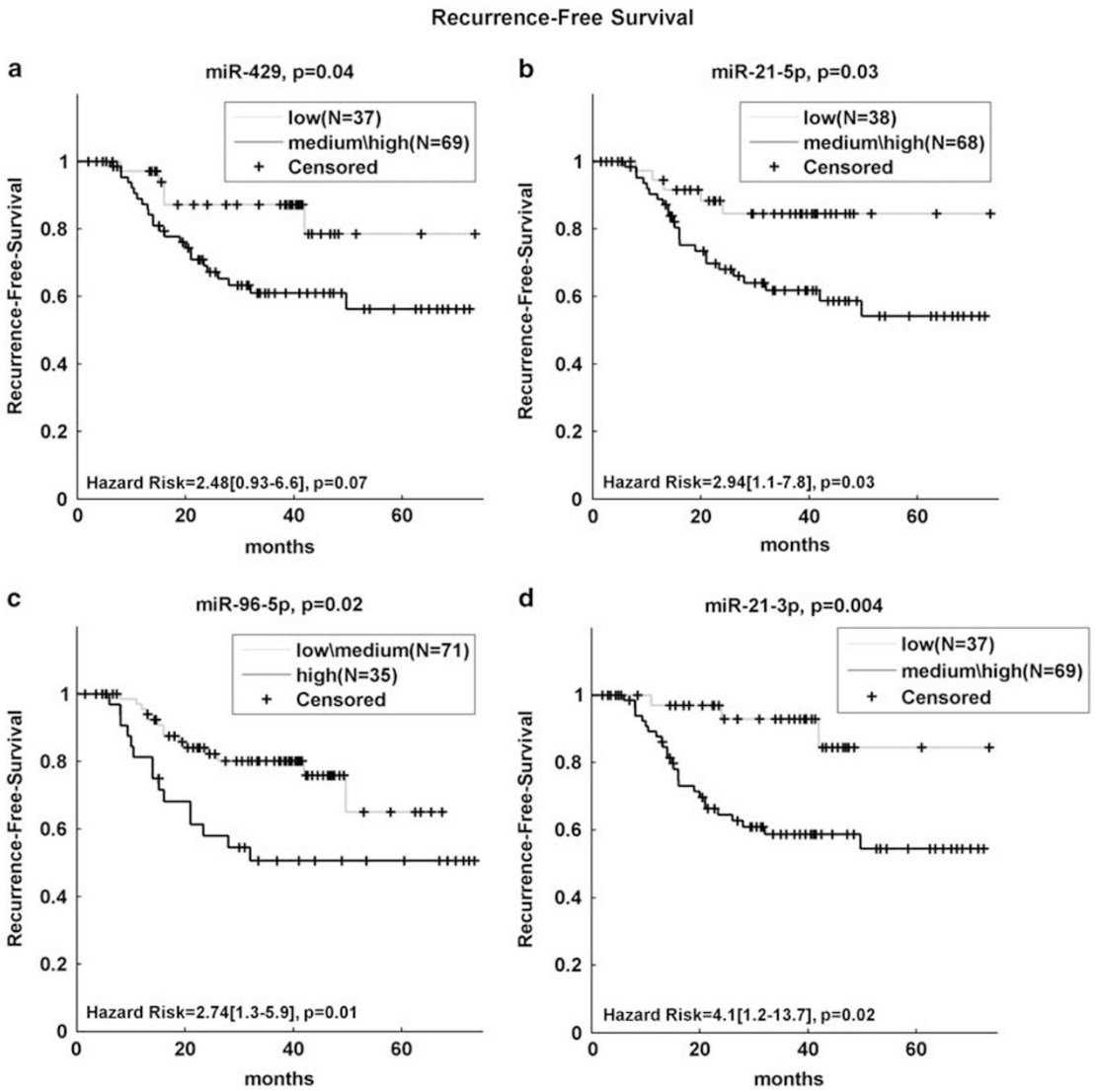


Figure 3 Expression of four miRNAs in peritumoral tissues predicts development of local recurrence in head and neck squamous cell carcinoma. Kaplan–Meier (KM) analyses representing the correlation between the expression of four miRNAs (miR-429, miR96-5p, miR-21-5p, and miR-21-3p) and local recurrence development in peritumoral tissues of head and neck squamous cell carcinoma patients (relative *P*-value is indicated above each KM). The analyses were performed on patients ($N=106$) with an informative follow-up (≥12 months), considering two subgroups according to the expression level (high/low) of each prognostic miRNA (see Materials and methods). For each KM the hazard risk, the confidence interval, and the relative *P*-value (*P*) of the multivariate Cox analysis are also indicated.

recurrence-free survival compared with patients with negative score (miRNA signature *low*). By multivariable analysis we assessed that the miRNA signature predicts local recurrence-free survival independently from the other prognostic clinical variables. On the contrary, expression of the miRNA signature in normal tissues was not predictive of recurrence (Figure 4c).

We next combined information about the miRNAs signature scores in peritumor and tumor, obtaining four groups (high in T/high in PT, low in T/low in PT, high in T/low in PT, low in T/high in PT) and we compared local recurrence-free survival of these groups by Kaplan–Meier analysis. As shown in Figure 4d, patients with high expression of miRNA signature in both tumor and peritumor show a shorter local recurrence-free survival compared with the other groups, also independently from the other clinical variables (T-high/PT-high: *vs* T-low/ PT-low, $P=0.0002$; *vs* T-high/PT-low, $P=0.02$; *vs* T-low/PT-high, $P=0.04$). Of note, a high score of miRNA signature in both tumoral and peritumoral tissues shows a stronger predictive power for local recurrence-free survival (hazard risk = 7.3 (2.4–22.1), $P=0.0004$, Figure 4d), compared with these tissues considered individually (hazard risk = 2.59 (1.16–5.8), $P=0.02$, Figure 4a; hazard risk = 2.69 (1.25–5.8), $P=0.01$, Figure 4b).

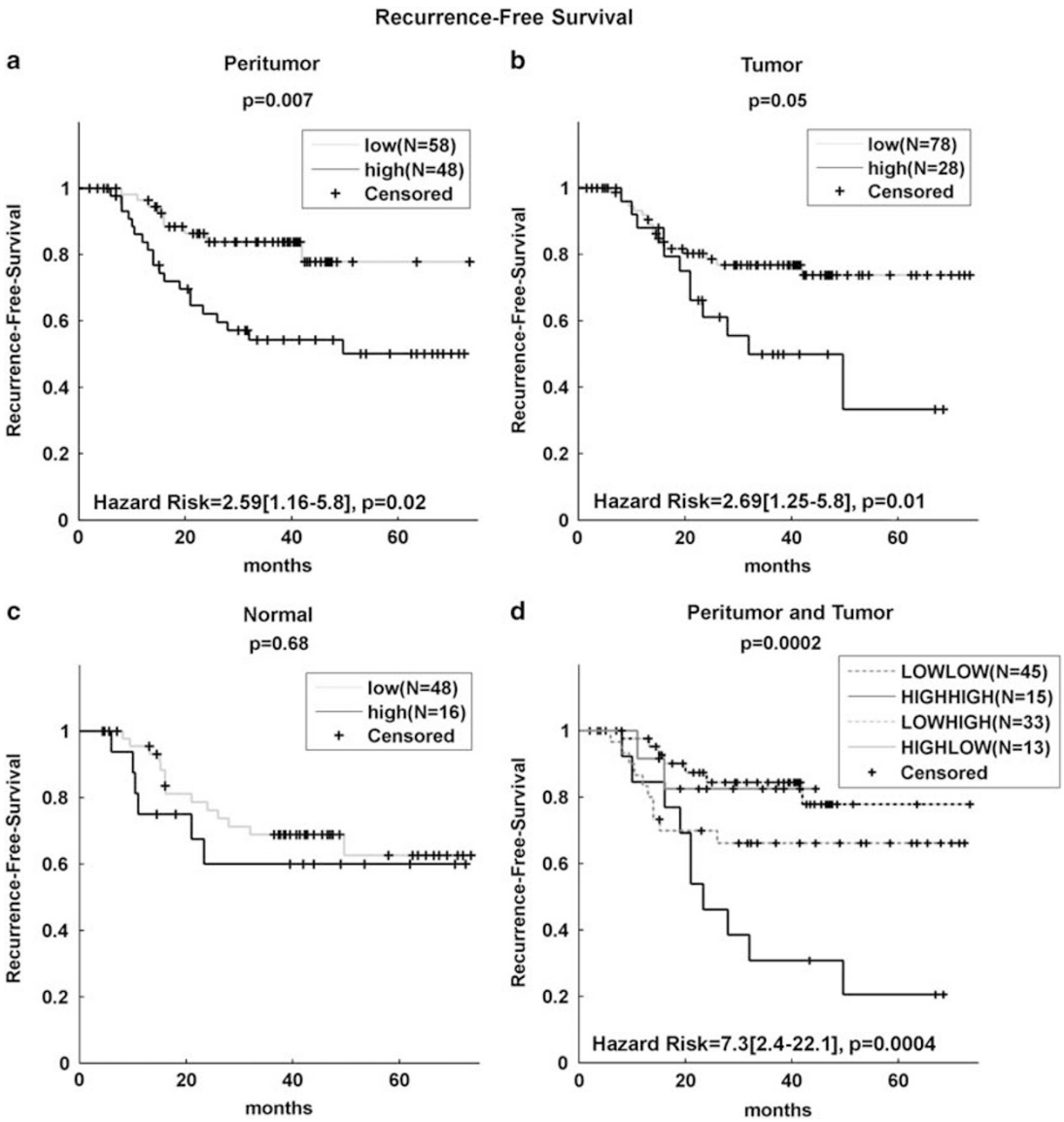


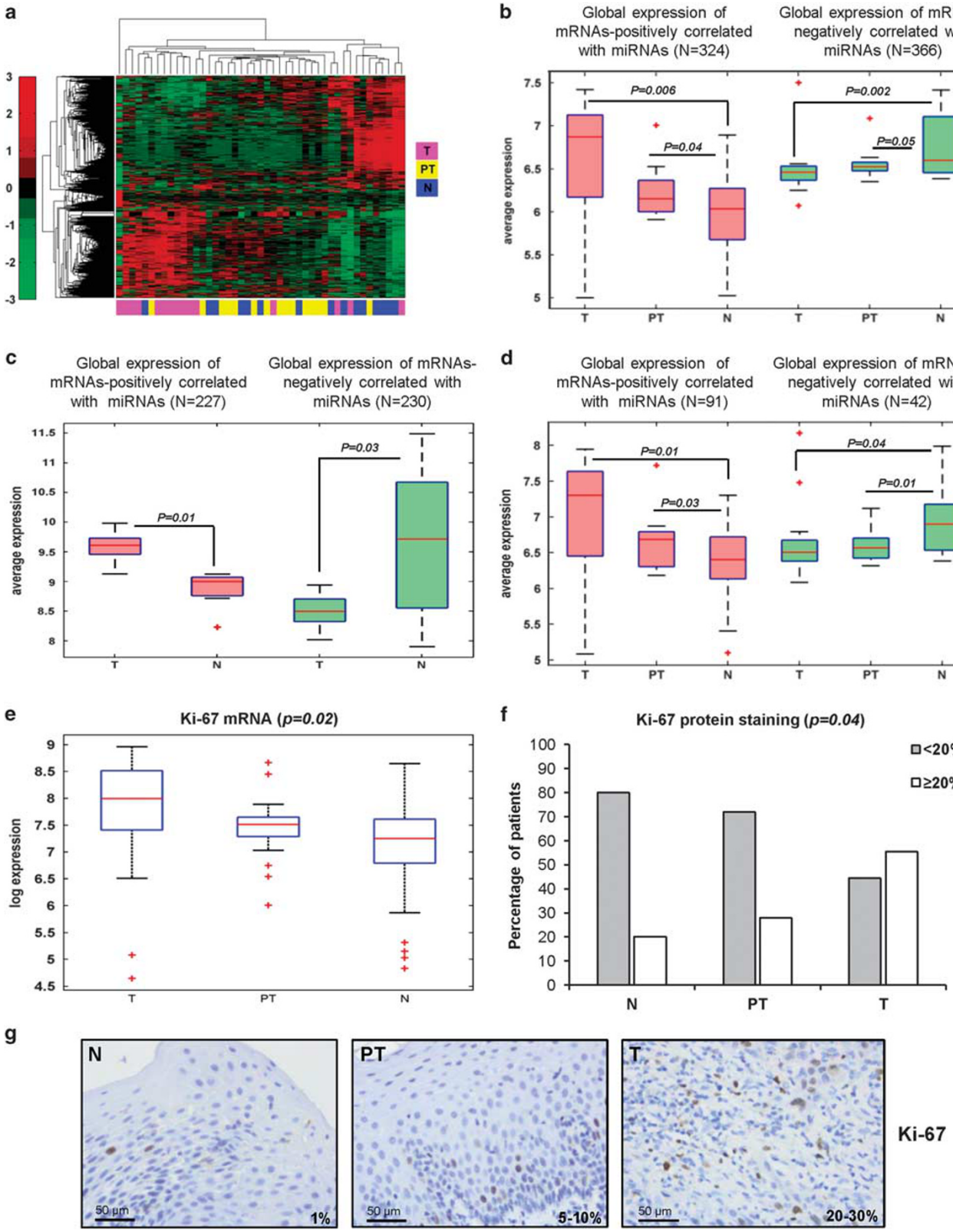
Figure 4 Expression of a 4-miRNAs signature predicts development of local recurrence in head and neck squamous cell carcinoma. Kaplan–Meier analysis representing the correlation between the expressions of four miRNAs considered as a group and recurrence-free survival in peritumoral (**a**) and tumoral (**b**) tissues of head and neck squamous cell carcinoma patients (see Materials and methods for score calculation). Kaplan–Meier analyses were performed comparing patients with high expression of the signature and patients with low expression of the signature, according to the expression level of each prognostic miRNA (see Materials and methods). (**c**) Kaplan–Meier analysis representing the correlation between the expression levels of four miRNAs considered as a group and recurrence-free survival in normal tissues. (**d**) Kaplan–Meier analysis representing the correlation between the expression levels of four miRNAs considered as a group and recurrence-free survival by the combination of their expression in tumor and peritumoral tissues of head and neck squamous cell carcinoma patients. Kaplan–Meier analyses were performed dividing the patients in four subsets: patients with high expression of the miRNA signature for both tissues (tumor and peritumor), patients with high expression in tumor and low expression in peritumor and vice versa, and finally patients with low expression of the signature in both tissues. The *P*-value was calculated on the two subgroups expressing high and low level of miRNAs signature in both tissues (tumor and peritumor). The multivatiate Cox analysis relative to recurrence-free-survival is shown at the bottom of each KM.

mRNAs Correlated to the Peritumor-Associated Pognostic miRNAs Belong to the Proliferation Pathway

To explore the functional significance of the deregulation of these four prognostic miRNAs in peritumoral tissue, we combined the information of miRNAs expression levels with the gene expression profiles obtained by microarrays. First, we compared gene expression of peritumoral tissues with that of normal tissues, thus identifying genes whose expression is significantly altered in peritumoral *vs* normal matched samples ($P < 0.05$). Then, we analyzed whether the expression of a subset of these genes was positively or negatively correlated with the expression of the four miRNAs, considered separately (Supplementary File S2). We obtained 690 correlated mRNAs, of which 324 positively and 366 negatively correlated to the expression of at least 1 of

the 4 prognostic miRNAs according to a Pearson's correlation coefficient higher than 0.3 in absolute value (Figures 5a and b and Supplementary File 3).

Similarly, when considering miRNA and mRNA expression levels obtained from matched tumor and normal head and neck squamous cell carcinoma



samples of the TCGA study cohort, three of the four miRNAs composing the signature were also significantly upregulated in the TCGA data set (miR-96-5p: FC = 1.91, P = 8.00e-04; miR-21-5p: FC = 2.39, P = 5.20e-07; miR-21-3p: FC = 1.67, P = 1.03e-03 and miR-429: FC = 0.95, P = 8.72e-01). The analysis of miRNA–mRNA correlations in the TCGA samples highlighted that 88 and 93% of the mRNAs had a, respectively, positive and negative correlation with the four miRNAs (Figure 5c, Supplementary File S3).

Functional classification of correlated mRNAs showed enrichment for genes belonging to various cancer-related pathways, as the cell cycle, DNA replication, and replication stress (Supplementary Table S4). The cell cycle pathway was the most relevant in terms of number of genes and significance. A box plot representing the overall expression of the cell cycle-related genes altered in tumoral and peritumoral tissues (listed in Supplementary File 3), compared with normal counterparts, is shown in Figure 5d. One of the best established proliferation markers is Ki-67, which is included in the list of mRNAs correlated to the four miRNAs that we found deregulated in peritumoral tissues (in particular, with miR-429, miR-21-3p, and miR-21-5p) (Figure 5e). To assess if also Ki-67 protein expression correlates with the deregulated miRNAs, we performed immunohistochemistry (IHC) to detect Ki-67 in a subset of tumoral, peritumoral, and normal samples for which formalin-fixed paraffin-embedded material was available (Figures 5f and g). IHC highlighted that a greater number of tissues characterized by high Ki-67-positivity (≥20% of positive cells) was present in the peritumoral and tumoral groups, as compared with normal samples (N) (P = 0.04) (Figures 5f and g and Supplementary File 5).

Finally, we evaluated the four miRNAs and their correlated mRNAs/proteins on the TCGA data set focusing our analysis on the subset of 35 matched tumor and normal samples for which data on mRNA, miRNA, and protein expression were available. Of 690 correlated genes, only 9 (ARAF, BAK1, BRCA1, CDK1, CHk1, CHk2, EZH2, FOXM1, and RAD51) were represented in the proteomic profile from the TCGA study. Analysis of the correlation between the expression of the four miRNAs and these proteins highlighted that miR-96-5p correlates with the level of CHK2 (R = 0.36, P = 0.03) and EZH2 (R = 0.42, P = 0.01) proteins, both involved in cell cycle pathway.

Prediction of Novel Drug Compounds for Locally Advanced Recurrent Head and Neck Squamous Cell Carcinoma

To identify novel putative therapeutic combinations for head and neck squamous cell carcinoma patients, especially for those with high risk of local recurrence development, we used the list of 690 miRNA-correlated genes to interrogate the Library of Integrated Network-based Cellular Signatures (LINCS) database (http://lincs.hms.harvard.edu/). In the LINCS database, 439 of these 690 miRNAs-correlated mRNAs were represented (mRNAs accepted by the LINCS database are listed in Supplementary File 4) and then considered for subsequent analyses. The database returned more than 300 statistically significant perturbations for negatively miRNA-correlated genes and more than 400 statistically significant perturbations for positively miRNA-correlated genes. As summarized in Figure 6, we observed that the expression of peritumoral miRNA-correlated genes is perturbed upon silencing or overexpression of various well-known cell cycle regulators, such as p21waf1 (CDKN1A), MYC, and Aurora Kinase A. In addition, we identified a subgroup of 22 small molecules leading to the upregulation of genes negatively correlated and to the downregulation of genes positively correlated with the four prognostic miRNAs (Table 1). Interestingly, among the 22 candidate drugs, 10 are inhibitors for PI3K–mTOR pathway, which is one of the most frequently activated pathways in head and neck squamous cell carcinoma and which also plays a critical role in mediating radiation resistance.[3]

Histologically Tumor-Free Mucosa from Head and Neck Squamous Cell Carcinoma Patients is Prone to the Modulation of miRNAs Expression

As shown in Figure 2 the identified prognostic miRNAs present increased expression level in the peritumors compared with normal matched

Figure 5 Functional impact of the four peritumor-associated prognostic miRNAs. (**a**,**b**) Unsupervised clustering analysis (**a**) and box plot (**b**) representing the expression of 690 genes positively (mRNAs-PCm) and negatively (mRNAs-NCm) correlated with the four prognostic miRNAs and differentially expressed between peritumoral tissues and their normal counterparts ($P < 0.05$) in the Italian National Cancer Institute 'Regina Elena' head and neck squamous cell carcinoma cohort. (**c**) Box-plot representing the expression of 457 genes positively (mRNAs-PCm) and negatively (mRNAs-NCm) correlated with the four prognostic miRNAs and differentially expressed between tumor tissues and their normal counterparts ($P < 0.05$) in the TCGA head and neck squamous cell carcinoma cohort. (**d**) Box plot representing the overall expression of the subset of cell cycle-related genes negatively (N = 42) and positively (N = 91) correlated with the expression of prognostic miRNAs ($P < 0.05$) from the analysis of the Italian National Cancer Institute 'Regina Elena' data set. (**e**) Box plots showing the differential expression of Ki-67 mRNA among tumor, peritumoral, and normal tissues (ANOVA test, $P < 0.05$). (**f**) Graph representing the percentages of patients showing < 20% or ≥ 20% of Ki-67-positive cells in tumoral, peritumoral, and normal tissues (statistical significance calculated by 3 × 2 chi squared test ($P < 0.05$), ANOVA test P = 0.06) (see also Supplementary File 5). (**g**) Representative immunostaining of Ki-67 protein expression in formalin-fixed paraffin-embedded tissues from head and neck squamous cell carcinoma. The percentage of Ki-67-positive cells is indicated in each panel. N, normal tissue; PT, peritumoral tissue; T, tumor tissue.

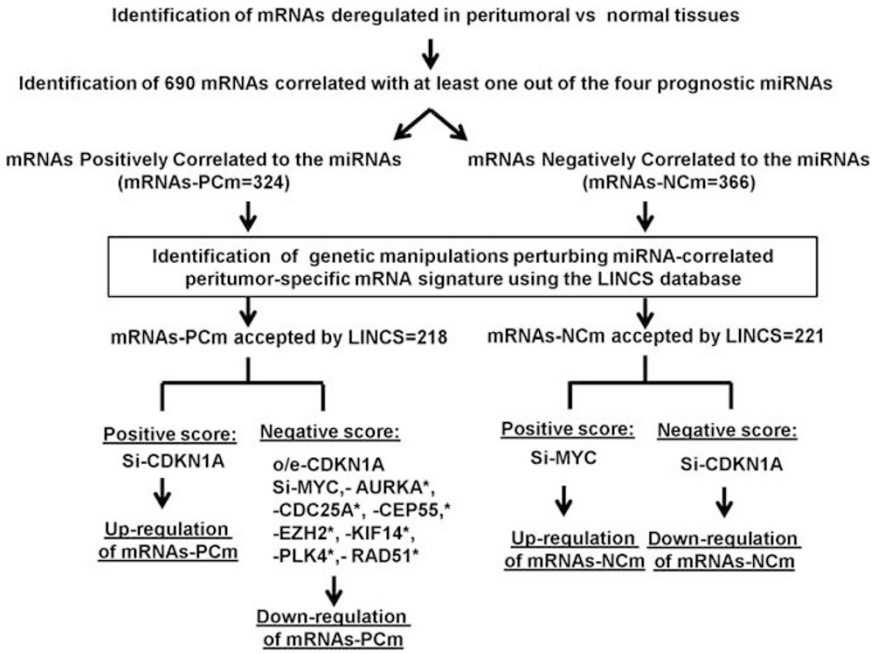


Figure 6 Workflow to predict novel drug compounds for locally advanced recurrent head and neck squamous cell carcinoma. Scheme representing the workflow and the results relative to the identification by LINCS database of the genetic manipulations perturbing mRNAs positively (mRNAs-PCm) and negatively (mRNAs-NCm) correlated to the miRNAs deregulated in peritumoral tissue. *Genes deregulated in peritumoral *vs* normal tissues and in tumoral *vs* normal tissues. Mean rank score (best 4 cell lines) >90 for positive score and <-90 for negative score. Si, silencing.

Table 1 Drugs perturbation analysis by LINCS database of mRNAs correlated to at least one of four prognostic miRs

Drug family	*Name of drug*
Statins	Simvastatin
mTOR and mTORC inhibitors	Sirolimus, AZD8055, KU-0063794, NVP-BEZ235, Wortmannin
Actin filament polymerization inhibitors	Cytochalasin b
PI3Ks inhibitors	Wortmannin, AS605240, KU-0060648, PI-103, PI-828, PP-110, TGX-115
EGFR inhibitor	WZ-3146
Cox-2 inhibitor	Valdecoxib
IGF1R inhibitor	BMSF754807, BMS536924 BMSF536924
MEK1, MEK2	PD-198306, selumetineb
Inhibitors of sphingolipid metabolism	DL-PDMP

Mean rank score (best four cell lines) >90 for positive score and <-90 for negative score. We selected only the drugs able to negatively perturb mRNAs-PC as well as positively perturb mRNAs-NC.

counterparts. An increasing distance from the site where the tumor is localized characterizes peritumoral and normal tissues.

We hypothesized that tumor might affect the expression of miRNAs in the surrounding mucosa through the release of soluble factors. This would agree with the intermediate level of expression that was observed for a group of miRNAs in peritumors, compared with tumor and normal tissues (Figure 2e and Supplementary Figure S1).

To test our hypothesis we cultured normal tissue specimens from 21 newly enrolled head and neck squamous cell carcinoma patients (Figure 1) with conditioned medium from the head and neck squamous cell carcinoma cell line Cal27, or with complete RPMI medium as control, and then evaluated the expression of the four prognostic miRNAs in the two conditions (Figure 7a). Normal tissues cultured in the presence of conditioned medium (in Figures 7a and b) presented a significant increase of miR-429 and miR-21-3p levels, compared with the same tissues cultured in control RPMI medium ($P=0.007$ and $P=0.029$, respectively) (Figure 7b). miR-21-5p and miR-96-5p showed upregulation only in a small subset of tissues and then resulted not significant (Figure 7a). These results indicate that a subset of normal tissues is prone to the deregulation of miRNAs relevant for the clinical outcome of the disease.

Discussion

According with our study hypothesis, the study indicated that a signature of miRNAs in peritumoral tissue in head and neck squamous cell carcinoma patients is predictive of disease re-occurrence.

Alterations occurring at a genetic level and also at genes/miRNAs expression level have been previously reported in histologically tumor-free mucosa from head and neck squamous cell carcinoma patients.[10,20,25–29] However, these alterations have not been previously associated with patients'

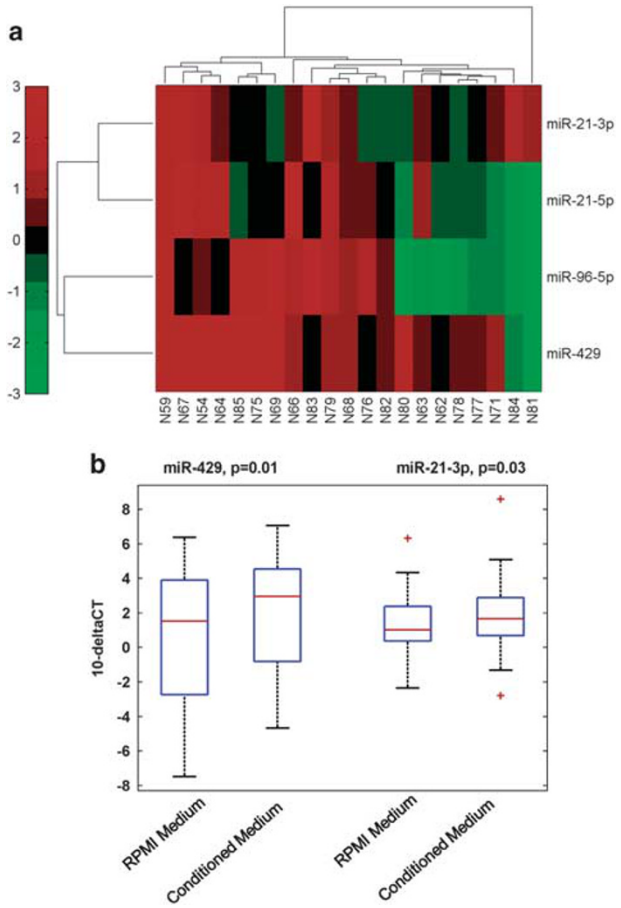


Figure 7 Histologically tumor-free mucosa from head and neck squamous cell carcinoma patients is prone to the modulation of miRNAs expression. (**a**) Unsupervised clustering analysis representing the expression of the four prognostic miRNAs of normal tissue specimens from 21 head and neck squamous cell carcinoma patients cultured with conditioned medium from Cal27 cells or with control RPMI 10% fetal bovine serum medium (RPMI). (**b**) Box plot representing the expression of the two prognostic miRNAs (miR-21-3p and miR-429) significantly upregulated in normal tissue specimens from 21 head and neck squamous cell carcinoma patients, cultured with conditioned medium from Cal27 cells compared with the same tissues cultured in control RPMI medium ($P=0.007$ and $P=0.029$, respectively).

survival. In our study, the upregulation of a signature composed of four miRNAs (eg miR-21-5p, miR-21-5p, miR-96-5p, miR-429) in the peritumoral tissue of head and neck squamous cell carcinoma is associated with shorter local recurrence-free survival, independently from the other prognostic clinical variables; of note, combination of the information about these 4-miRNA signature expression level in peritumor with that of the tumor outperforms the predictive power observed in each tissue.

As already mentioned, genetic and mRNA/miRNA expression alterations observed in histologically tumor-free mucosa of head and neck squamous cell carcinoma patients may rely on: (1) the presence of tumor cells spreading in the tumor-surrounding mucosa; (2) the presence at multiple sites in the mucosa of preneoplastic clonal cell populations which may potentially become malignant. Various evidences present in the literature indicate that both hypotheses are probably true. Relatively to the first hypothesis, the infiltration of neoplastic cells in tumor-surrounding areas of head and neck squamous cell carcinoma patients has been previously indicated by genetic and molecular evidences;[4] also in our study, the identification of peritumoral tissues containing the same *TP53* mutation present in the matched tumor tissue suggests that spreading tumor cells may be present in the tumor-surrounding mucosa. However, there are also evidences that support the second hypothesis. An example is represented by the fact that miR-96-5p and miR-21-5p, which we found upregulated in peritumor, has been also found increased in preneoplastic lesions, such as the oral leukoplakia. Interestingly, these two miRNAs were identified among a pool of miRNAs, which is able to predict malignant transformation in oral leukoplakia.[10,28]

Head and neck squamous cell carcinoma adjacent tissue has been also shown to carry altered levels of some of the proliferation-associated factors that we found altered in peritumor. In particular, Ki-67, a very well-established proliferation marker, was shown to be upregulated in head and neck squamous cell carcinoma adjacent tissue compared with tonsillectomy specimens of non-cancer individuals and was also shown to correlate with pathological features and disease-free-survival.[20–24] Additionally, antibodies detecting Ki-67 proliferation marker are already commonly used as diagnostic tools for several cancer types.[25] These evidences suggest that Ki-67 may present prognostic and diagnostic usefulness also in head and neck cancer. Other genes differentially expressed in peritumor *vs* normal counterpart in our data set, and in tumoral *vs* normal samples in the TCGA cohort, have been previously shown to be altered in tumor adjacent tissue (eg MMP1, MMP14).[4,20,26,27] The interrogation of the LINCS database with the miRNA-related gene signature altered in the peritumoral tissues led to the identification of novel putative therapeutic options for head and neck squamous cell carcinoma patients. Interestingly, for some of the identified molecules an antitumor activity and the capability to radiosensitize tumor cells have been reported specifically in head and neck squamous cell carcinoma.[30–33] One such example is represented by inhibitors targeting proteins in the PI3K/mTOR pathway (Table 1); the activation of this axis is one the main mechanisms of acquired radioresistance in head and neck squamous cell carcinoma patients and their inhibitors have emerged as a promising therapeutic approach for head and neck squamous cell carcinoma.[3,31,32] In agreement with these data, some of the identified genetic perturbations leading to the deregulation of the miRNA-related gene signature are proteins (eg KDR, FGFR1, K-RAS, STAT6, CDK4, and SRC) involved in the activation of the PI3K–mTOR

pathways and associated with an aggressive, invasive phenotype, and radioresistance in head and neck squamous cell carcinoma.[31,34–38] Finally, among the genetic perturbations affecting the miRNA-related gene signature, we found crucial cell cycle controllers, as MYC, Aurora kinase A, and CDKN1A, supporting the association of the four prognostic miRNAs with the deregulation of proliferation-associated factors in head and neck squamous cell carcinoma adjacent normal tissue.

Increased expression of the four prognostic miRNAs is strongly connected to the activation of a proliferative program in tumor-surrounding tissues, while miRNAs expression is not predictive of recurrence development in the normal tissue.

However, despite not prognostic, in normal tissue miRNAs are positively related to the proliferation program (data not shown). It is then possible that the peritumor, being anatomically closer to the tumor, maybe more subjected to the tumor secretome-derived factors, which could ultimately participate to the activation of the proliferation program. This hypothesis is sustained by the observation obtained on normal tissue specimens cultivated in the presence of conditioned medium from head and neck squamous cell carcinoma cells; these tissues indeed present a rapid response to the conditioned medium from Cal27 cells, characterized by the upregulation of two of the prognostic miRNAs.

Finally, the identification of miRNA-related peritumor-specific gene signature provides preliminary insight into the molecular mechanisms of action of these prognostic miRNAs in head and neck squamous cell carcinoma adjacent tissues that could be relevant for recurrence development and for the identification of novel promising therapeutic approaches for head and neck squamous cell carcinoma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)