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RESEARCH ARTICLE

Novel Dysregulated MicroRNAs in Primary Laryngeal Squamous Cell Cancer

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ABSTRACT

Introduction: MicroRNAs (miRNAs) are endogenous, small, noncoding RNAs of 17 to 25 nucleotides that regulate approximately 30% of human genes. They are differentially expressed in various types of cancers compared with noncancerous tissues, suggesting that they may have crucial roles in tumorigenesis. The objective of this study was to identify laryngeal squamous cell cancer (LSCC)-specific miRNAs.

Materials and methods: A retrospective cohort of 10 LSCC and five normal laryngeal squamous epithelium samples were examined using a global miRNA profiling approach (HTG, Tucson, AZ, USA, 800 human miRNAs plus 10 endogenous control miRNAs). The expression status of selected dysregulated miRNAs that were significantly different from normal were verified by real-time quantitative PCR (qPCR).

Results: Twenty-three of the 800 human miRNAs had significantly different expression levels ($p < 0.05$) between LSCC and normal tissues. Fifteen of the 23 have not been previously reported in HNSCC and include: miR-663b, miR-663, miR-193b, miR-1291, miR-720, miR-191, miR-1224-3p, miR-214, miR-1285, miR-1207-5p, miR-483-5p, miR-1225-3p, miR-1228, miR-1280 and miR-638. Consistently upregulated miR-31 and miR-193b and differentially expressed miR-663b in LSCC were verified by qPCR.

Conclusion: The 15 novel miRNAs identified in this exploratory study, pending further confirmation and validation, may have clinical utility as LSCC-specific markers.

Keywords: MicroRNAs, Laryngeal squamous cell cancer, Global microRNA profiling, qPCR validation.

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INTRODUCTION

MicroRNAs (miRNAs) are endogenous, small, noncoding RNAs of 17 to 25 nucleotides that are thought to regulate approximately 30% of human genes.[1-3] They are involved in regulating target gene expression through imperfect base pairing with the 3'-untranslated region (3'-UTR) of target mRNAs of protein-coding genes, leading to the cleavage of homologous mRNA or translational inhibition. They are differentially expressed in various types of cancers compared with noncancerous tissues, suggesting that they may have crucial roles in tumorigenesis.[4-8]

Quite a few miRNAs have been linked to head and neck squamous cell carcinoma (HNSCC) based on their differential expression in tumors. miR-21, a commonly dysregulated miRNA in cancer, is frequently upregulated in HNSCC.[9-14] Progression of oral cancers has been significantly associated with miR-345, -21 and -181b.[12] Significantly low levels of miR-125a and miR-200a have been detected in the saliva of oral cancer patients[15] while high levels of miR-184 have been detected in the plasma of tongue SCC.[16] Altered expression of specific miRNAs are beginning to provide much needed insights into tumorigenesis mechanisms of abnormal cell-cycle regulation, evasion of apoptosis, reduced response to anti-growth signals and epithelial-mesenchymal transition (EMT).[17] These reports strongly support the potential utility of miRNAs as diagnostic biomarkers in HNSCC. The objective of this pilot study was to identify laryngeal squamous cell carcinoma (LSCC)-specific miRNAs using a global discovery approach.

MATERIALS AND METHODS

Cohort

The retrospective pilot cohort comprised 10 primary LSCC cases (cancer cohort) and five normal laryngeal squamous epithelium tissues (control cohort). This study was approved by the Henry Ford Health System Institutional Review Board Committee.

RNA Extraction

miRNA from tumor and normal tissue, present in separate formalin-fixed, paraffin-embedded (FFPE) tissue blocks was extracted using the High pure miRNA Isolation Kit (Roche). Final dilution of total RNA used for reverse transcription was 20 ng/ul.

Quantitative Nuclease Protection miRNA Microarray Assay

Global miRNA profiling using quantitative nuclear protection (qNPA) miRNA microarray assays was provided by High Throughput Genomics Inc. (HTG, Tucson, AZ, USA). This assay interrogates a panel of 800 human miRNAs plus 10 endogenous control miRNAs simultaneously.[18] We provided two curls of 5 µm tissue sections in tubes for each of the 15 cases.

HTG Data Analysis

Each microarray has two elements (replicates) for each transcript (A1.1 and A1.2, for example); therefore the total number of data points for a single transcript is 2. Data is normalized to the total signal for each microarray. A particular transcript is considered expressed, if the signal is more than three standard deviations above the background signal as determined by the *ANT* gene.

Significantly differently expressing miRNAs between normal and tumor samples were obtained using the Student's t-test. Significantly ($p < 0.05$) upregulated and downregulated miRNAs detected in the LSCC group (as compared to the normal group) by the qNPA assay are listed in Table 1.

Table 1: Significantly upregulated and downregulated miRNAs

miRNA	*Ratio*	*p-value*	*Chromosome location*
Upregulated			
hsa-miR-663b†	6.67	0.000	2
hsa-miR-320a	4.81	0.000	8p21.3
hsa-miR-320b	4.32	0.000	1
hsa-miR-663	3.38	0.000	20p11.1
hsa-miR-193b†	10.83	0.001	16p13.12
hsa-miR-92a	5.72	0.001	13q31.3/Xq26.2
hsa-miR-1291	3.98	0.001	12
hsa-miR-16	3.97	0.003	13q14.3/3q26.1
hsa-miR-720	2.07	0.004	3
hsa-miR-27a	5.11	0.005	19p13.13
hsa-let-7f	2.89	0.006	9q22.32/Xp11.22
hsa-miR-191	3.30	0.009	3p21.31
hsa-miR-1224-3p	3.19	0.009	3q27.1
hsa-miR-31†	6.16	0.010	9p21.3
hsa-miR-214	3.15	0.010	1q24.3
hsa-miR-1285	2.23	0.010	7/2
Downregulated			
hsa-miR-1207-5p	0.61	0.000	8
hsa-miR-483-5p	0.62	0.000	11p15.5
hsa-miR-1280	0.76	0.000	3
hsa-miR-1228	0.76	0.001	12
hsa-miR-1225-3p	0.76	0.002	16p13.3
hsa-miR-296-5p	0.75	0.008	20q13.32
hsa-miR-638	0.83	0.035	19p13.2

†Verified by qRT-PCR
Bolded: Previously unreported miRNAs

Five miRNAs with the lowest p-values (three upregulated: miR-663b, miR-193b, and miR-31; two downregulated: miR-923 and miR-1826) were selected for further verification by quantitative real-time PCR.

TaqMan MicroRNA Reverse Transcription

Reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (ABI) which was used in conjunction with the TaqMan MicroRNA Individual Assays (ABI) containing the individual reverse transcription primers. The manufacturer's prescribed protocol was followed for both kits. Final volume for 1 reaction is 7.5 ul (3.5 ul RT master mix, 2.5 ul total RNA, and 1.5 ul RT primer).

Real-Time Quantitative PCR (qPCR)

Real-time quantitative PCR (qPCR) approach was employed to verify expression status of selected miRNAs that were significantly different from normal controls as proof-of-principle. This was performed using TaqMan Universal Master Mix II (no UNG). Manufacturer's protocol was followed for microRNA individual assays. Each sample was run in duplicate.

qPCR Data Analysis

miRNA expression in the normal and tumor groups was measured using $^{\Delta\Delta}$Ct relative quantization. miRNAs with a value <0 were considered as downregulated and >0 were upregulated (Fig. 1).

RESULTS

All five normal samples and nine of 10 LSCC cases were part of the analyses (one tumor sample indicated outlier values and was excluded). Twenty-three of the 800 human miRNAs were differentially expressed ($p < 0.05$) between LSCC and normal tissues (Table 1), of which 16 were upregulated and seven were downregulated.

Fifteen of the 23 dysregulated miRNAs have not been previously reported in HNSCC and include: miR-663b,

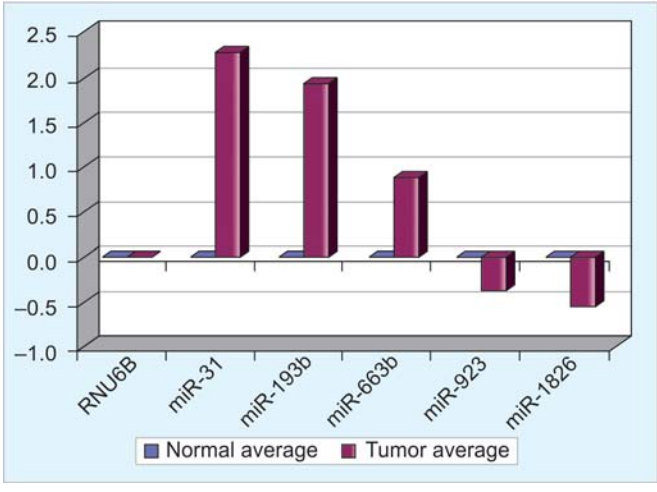

Fig. 1: qPCR data—miRNA expression levels in normal and tumor groups

RNU6B, the internal control miRNA, is not expressed in normal or tumor samples. miR-31, miR-193b and miR-663b are upregulated, whereas miR-923 and miR-1826 were downregulated in the tumor samples. miR-923 and miR-1826 are now listed as fragments in recent updates of the miRNA database and, therefore, not considered as miRNAs

miR-663, miR-193b, miR-1291, miR-720, miR-191, miR-1224-3p (3' arm of miR-1224 hairpin), miR-214, miR-1285, miR-1207-5p (5' arm of miR-1207 hairpin), miR-483-5p, miR-1225-3p, miR-1228, miR-1280 and miR-638 (highlighted in bold in Table 1).

Selected miRNAs, miR-923, miR-1826, miR-663b, miR-193b and miR-31, with p-values 0.000, 0.000, 0.000, 0.001 and 0.01 respectively, were verified by qPCR and were concordant with microarray data. miR-31 and miR-193b were consistently upregulated (9/9), and miR-663b was differentially regulated (7/9 upregulated and 2/9 down-regulated) in LSCC (Table 2). miR-923 and miR-1826 (not shown in Table 1) showed downregulation in 6/9 and upregulation in 3/9 samples; however, these are now listed as fragments in recent updates of the miRNA database and no longer regarded as legitimate miRNAs.

DISCUSSION

miRNAs, though lurking behind the scenes, are now being viewed less as molecular noise and more as exotic players with increasing prominence in theories about cancer.[19] By binding to mRNA, miRNA can silence or modulate mRNA function. Expression profiling of miRNAs has shown that some miRNAs are upregulated or down-regulated in cancer, suggesting that it is important to understand the specific roles miRNAs may have in cancer.

There are two types of cancer-related miRNAs: Oncogenic (i.e. miR-155, miR-21)[8] or tumor suppressor miRNAs (i.e. miR-15a and let-7 family).[20,21] In HNSCC, miRNA profiling has been performed in oral, oropharyngeal, tongue, laryngeal and thyroid cancers. A study by Hui et al[22] found no distinct differences in the global miRNA profiles between squamous cell cancers arising from the larynx, oropharynx or hypopharynx with the exception of miR-133b which was more highly expressed in laryngeal *vs* the other two subsites. They found about a third of the miRNAs examined in their HNSCC samples to be dysregulated. Because several of the differentially expressed miRNAs in their study were in frequently amplified or deleted regions, genomic amplifications or deletions were offered as one possible mechanism for abnormal miRNA expression in HNSCC.

We found 23 significantly differentially expressed miRNAs for many of which, information is limited to chromosomal locations (Table 1). Of the 23 significantly differentially expressed miRNAs, eight have been reported previously in HNSCC. These include miR-320a, miR-320b, miR-92a, miR-16, miR-27a, let-7f, miR-31 and miR-296-5p.

miR-27a is significantly upregulated in HNSCC cell lines and its expression is correspondingly reduced by using knock down anti sense approaches.[22] It is considered a non-causal HNSCC miRNA as its manipulation *in vitro* does not have an effect on phenotype.[17] miR-92a, previously named miR-92 in the miRNA database, has two precursor sequences: miR-92a-1 and miR-92a-2.[23] miR-92a-1 is located on the oncogenic miR-17-92 polycistron of chromosome 13q31.3, a locus that harbors quite a few upregulated miRNAs.[22] mir-92a-2, located on chromosome Xq26.2, belongs to the miR-106a-92 cluster and is also overexpressed in HNSCC.[22] miR-16 is commonly down-regulated due to deletion of the 13q14 region in chronic lymphocytic leukemia;[24,25] however, it is significantly upregulated in HNSCC.[22] The let-7 family of miRNA is known as a tumor suppressor miRNA that functions through inhibiting oncogenic mRNAs, such as *RASA1*, *MYC*, *HMGA2*.[21,26,27] The let-7 family members are commonly downregulated in HNSCC except for let-7i which is upregulated.[10,22,28] Low levels of both let-7d and miR-205 have been reported to be significantly related to poor survival in primary HNSCC.[11]

miR-31, dysregulated in many cancers,[29-32] appears to favor upregulation in HNSCC[14] and oral squamous cell carcinoma (OSCC)[6,16] cell lines. Its function in tumorigenesis is unclear; however, Liu et al[33] found miR-31 to be

Table 2: qPCR confirmation of qNPA assay

	miR-31	*miR-193b*	*miR-663b*	*miR-923*	*miR-1826*
LT-ID6	Up	Up	Up	Down	Down
LT-ID8	Up	Up	Down	Down	Up
LT-ID9	Up	Up	Up	Up	Up
LT-ID10	Up	Up	Up	Down	Down
LT-ID11	Up	Up	Up	Down	Down
LT-ID12	Up	Up	Up	Down	Down
LT-ID13	Up	Up	Up	Up	Up
LT-ID14	Up	Up	Up	Up	Down
LT-ID15	Up	Up	Down	Down	Down
	9/9 up	9/9 up	7/9 up 2/9 down	6/9 down 3/9 up	6/9 down 3/9 up

$^{\Delta\Delta}C_t$ relative quantization (tumor $^{\Delta}$Ct—normal $^{\Delta}$Ct) used to determine up or downregulation

significantly elevated in the plasma of OSCC patients, which was remarkably reduced following surgery. This suggests that miR-31 may be an oncogenic miRNA. Its detection in plasma could be clinically useful as a noninvasive diagnostic approach. miR-296 is downregulated in OSCC.[6]

Several studies have reported significant differential expression of miR-100, miR-125b and miR-375 in HNSCC,[5,13,14,16,34] none of which reached significance in this study.

Of the 23 significantly differentially expressed miRNAs, 15 have not been previously reported in HNSCC and include: miR-663b, miR-663, miR-193b, miR-1291, miR-720, miR-191, miR-1224-3p, miR-214, miR-1285, miR-1207-5p, miR-483-5p, miR-1225-3p, miR-1228, miR-1280 and miR-638.

Dysregulation of miR-193b is thought to influence melanoma development[35] via its role of cell proliferation repression and regulation of CCND1 expression.[35] In prostate cancer, miR-193b can present as an epigenetically silenced putative tumor suppressor.[36] It has also been detected in cervical cancer cell lines.[37] The role of miR-663b reported in the development of human leukemias[24] and of miR-663 in human colorectal cells[38] is not known.

miR-191 is upregulated in hepatocellular carcinoma (HCC) and affects the *TGF*-β and *MAPK* pathways, which play an important role in HCC tumorigenesis.[39,40] Inhibition of miR-191 by 2-*O*-metoxyethyl (MOE) anti-miR was found to decrease cell proliferation and induce apoptosis *in vitro* with significant reduction of tumor mass in an *in vivo* mouse model of HCC. Therefore, in HCC, miR-191 may signal a potential therapeutic target.

miR-1224-3p has been identified as a mammalian mirtron which is a short hairpin intron that is a precursor for miRNA biogenesis.[41] Mirtrons were originally found in invertebrates but are now known to exist in mammals also. miR-214 has been detected in melanomas, cervical and ovarian cancers.[42-44] In melanomas, miR-214 suppresses *TFAP2C* leading to tumor progression.[42] miR-1285 has been shown to inhibit expression of *TP53*.[45] miR-1207-5p is expressed abundantly in colon cancer cell lines along with miR-1207-3p, and Northern blot data suggested a common promoter and transcriptional regulatory unit making these likely complimentary overlapping miRNAs.[46] miR-483-5p is significantly upregulated in malignant adrenocortical carcinoma (ACC) as compared with benign tumors.[47] Its expression can accurately distinguish tumors as benign or malignant.

miR-638 has been detected in human colorectal cells[38] and in gastric cancer,[48] where it is significantly downregulated. It is present in a stable form in human plasma. Tanaka et al.[49] found that the ratio of miR-92a/miR-638 in plasma is useful for distinguishing leukemia patients from healthy individuals.

CONCLUSION

The human miRNA database continues to get fine tuned with respect to new additions as well as exclusions. This pilot study identified previously unreported miRNAs in LSCC that upon further investigation in larger studies may have clinical utility in LSCC. Our 23 aberrantly expressed miRNAs in LSCC, including the 15 unreported in HNSCC, require further examination with subsequent validation in larger cohorts for clinical relevance as LSCC-specific markers.

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