Gene 593 (2016) 58–63

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

MicroRNA and target gene expression based clustering of oral cancer, precancer and normal tissues☆,☆☆

Roshni Roy [a,1], Richa Singh [a], Esita Chattopadhyay [a], Anindita Ray [a], Navonil De Sarkar [a,2], Ritesh Aich [a,b], Ranjan Rashmi Paul [a,b], Mousumi Pal [a,b], Bidyut Roy [a,*]

[a] Human Genetics Unit, Indian Statistical Institute, 203 B. T. Road, Kolkata 700108, India
[b] Department of Oral Pathology, Guru Nanak Institute of Dental Science & Research, 157/F Nilganj Road, Kolkata 700114, India

ARTICLE INFO

Article history:
Received 11 April 2016
Received in revised form 2 August 2016
Accepted 5 August 2016
Available online 08 August 2016

Keywords:
MicroRNAs
Target genes
Expression
Oral cancer
Precancer

ABSTRACT

Purpose: Development of oral cancer is usually preceded by precancerous lesion. Despite histopathological diagnosis, development of disease specific biomarkers continues to be a promising field of study. Expression of miRNAs and their target genes was studied in oral cancer and two types of precancer lesions to look for disease specific gene expression patterns.
Methods: Expression of *miR-26a*, *miR-29a*, *miR-34b* and *miR-423* and their 11 target genes were determined in 20 oral leukoplakia, 20 lichen planus and 20 cancer tissues with respect to 20 normal tissues using qPCR assay. Expression data were, then, used for cluster analysis of normal as well as disease tissues.
Results: Expression of *miR-26a* and *miR-29a* was significantly down regulated in leukoplakia and cancer tissues but up regulated in lichen planus tissues. Expression of target genes such as, *ADAMTS7*, *ATP1B1*, *COL4A2*, *CPEB3*, *CDK6*, *DNMT3a* and *PI3KR1* was significantly down regulated in at least two of three disease types with respect to normal tissues. Negative correlations between expression levels of miRNAs and their targets were observed in normal tissues but not in disease tissues implying altered miRNA-target interaction in disease state. Specific expression profile of miRNAs and target genes formed separate clusters of normal, lichen planus and cancer tissues.
Conclusion: Our results suggest that alterations in expression of selected miRNAs and target genes may play important roles in development of precancer to cancer. Expression profiles of miRNA and target genes may be useful to differentiate cancer and lichen planus from normal tissues, thereby bolstering their role in diagnostics.

1. Introduction

India has one of the highest incidence of oral cancer in the world. Lifestyle-related habits such as smoking, drinking of alcohol, chewing of smokeless form of tobacco, which is highly prevalent in South Asia, including India, are the major causative agents (Gupta and Ray, 2003). Oral cancer is generally preceded by precancerous lesion/s but these lesions show varying degree of transformation potential. Oral leukoplakia is one such prominent precancerous lesion which is designated as white lesions that include hyperplasia and dysplasia of the oral mucosa with malignant transformation rates from 0.13 to 17.5% (Kondoh et al., 2007). Lichen planus is another precancerous lesion that has an autoimmune pathology (Warnakulasuriya et al., 2007). There is considerable difference in opinion in the transformation potential of lichen planus to cancer (van der Meij et al., 2003), though some have stated that lichen planus carries malignant potential with an unspecified risk (Silverman, 2000). Genetic variations and expression deregulation of miRNAs and their targets have long been implicated in cancer and precancer processes (Liu et al., 2010). We have previously shown that SNPs at *miR 26a*, *miR-29a*, *miR-34b* and *miR-423* can modulate risk of leukoplakia and cancer (Roy et al., 2014a; Roy et al., 2014b). Here, we investigated whether these microRNAs and their targets showed expression deregulation in lichen planus, leukoplakia and cancer tissues.

2. Materials and methods

2.1. Sample collection and processing

The study was approved by "Review committee for protection of research risk to humans, Indian Statistical Institute". Written consents from all patients and healthy individuals were taken prior to

Abbreviations: LP, lichen planus; LK, leukoplakia; NOR, normal; *miR*, microRNA.

☆ Financial support by: Indian Statistical Institute (ISI/HGU/9633/2015-16), Kolkata, India and Department of Biotechnology (BT/PR10987/MED/12/1407/2008), Government of India.

☆☆ Conflict of Interest: The Authors declare that they have no conflict of interest.

* Corresponding author.
E-mail address: broy@isical.ac.in (B. Roy).
[1] Present address: Baylor Scott and White Research Institute, Dallas, Texas, USA.
[2] Present address: Fred Hutchinson Cancer Research Center, University of Washington, Seattle, USA.

http://dx.doi.org/10.1016/j.gene.2016.08.011
0378-1119/

recruitment with the information that biological samples collected from them will be used for research. Lichen planus (N = 20), leukoplakia (N = 20) and oral cancer (N = 20) patients were recruited for this study. Patients were referred to this hospital for diagnosis by primary health centres. Apart from limited time span, our sample size was also restricted due to our intention of collecting disease tissues only from gingivo-buccal site. Normal healthy individuals (N = 20) who had visited same hospital for other dental ailments and had no familial history of cancer, were also recruited in the study. Demographic details and tobacco habit were noted with informed written consent. About 20 mg of diseased and normal oral tissues were collected from patients and healthy controls, respectively, and used for RNA isolation. Tissues were stored in *RNA Later* solution at −20 °C until isolation of RNA. Tissue RNA was extracted using *QiagenAllPrep DNA/RNA Mini Kit* as per the manufacturer's instructions.

2.2. Selection and expression of miRNAs and target genes

MicroRNAs were selected on basis of polymorphism study published by us (Roy et al., 2014a; Roy et al., 2014b). Initial list of validated miRNA targets was prepared using MirTarBase and MirWalk data bases but the final list of miRNA targets comprised of a subset of those targets whose expression was also deregulated in our RNASeq transcriptome data (unpublished result) obtained from same set of cancer samples that have been used in this study (Supplementary Fig. 1, Supplementary Table 1). In this way, 11 target genes and 4 miRNAs were taken for expression study. Along with the 11 target genes, *CTDSPL* and *CTDSP2*, 2 host genes of *miR-26a*, were also considered for expression study.

One microgram RNA from each sample was taken for *DNaseI* treatment and subsequently cDNA was prepared using random hexamer for target genes and miRNA specific primers for the 4 miRNAs (Invitrogen Inc., USA). Taqman Real Time PCR Assay, i.e. qPCR, was performed to study expression of miRNA, using *RNU44* as endogenous miRNA control, in duplicate tubes using Taqman probes. Quantification of target genes was performed using SYBR Green qPCR Assays with *RPPH1* (*RNase P RNA component H1*) as endogenous control (primer sequences in Supplementary Table 2).

2.3. Data analysis and statistical tests

Normalized expression was calculated as ΔCt (i.e. $Ct_{Gene} - Ct_{Endogenous\ control}$). Average ΔCt along with standard deviation was compared between control and different disease tissues, separately, using independent sample *t*-test after ensuring normal distribution by Kolmogorov Smirnov test and equality of variance by *Levene's* test. Hierarchical clustering was performed using R programming to see whether healthy individuals could be segregated from diseased individuals on the basis of normalized expression (i.e. ΔCt values) of miRNAs and target genes. All miRNAs along with those target genes, whose expressions were deregulated in at least two of three disease types with respect to control tissues, were taken for analysis. The final result was plotted using '*plot*' and *hclust* function. Spearman rank correlation analysis, between normalized gene expression of miRNAs and their target genes and between target genes was performed using PASW software.

3. Result

3.1. Demography and expression of miRNA and target genes

Normal healthy individuals and oral cancer group had similar distribution of males and females. On one hand, lichen planus had predominance of females while leukoplakia patients were all male, a preponderance seen in other studies as well (Nagao et al., 2005) (Table 1). Independent *t*-test analysis showed that the mean normalized expression (i.e. ΔCt values) of *miR-26a* and *miR-29a* was significantly different in all three disease tissues with respect to normal tissues (Fig. 1, Table 2 and Supplementary Table 3). Expression of all the four miRNAs were up regulated in lichen planus while those of *miR-29a* and *miR-26a* were significantly down regulated in leukoplakia and cancer tissues. Expression of *miR-34b* was up regulated while that of *mir-423* was down regulated in cancer tissues. It was observed that mean fold change (i.e. $2^{-\text{mean }\Delta\Delta Ct}$) in expression of *ATPB1*, *CPEB3* and *PIK3R1* was significantly downregulated in lichen planus, leukoplakia and cancer tissues. *ADAMTS7* had significantly reduced expression in lichen planus and cancer, *CDK6* had reduced expression in lichen planus and leukoplakia tissues while *DNMT3a* had reduced expression in leukoplakia and cancer tissues. Expression of *CTDSP2*, host genes of *miR-26a*, and *COL4A2* was significantly down-regulated in leukoplakia and *EZH2* was down regulated in lichen planus tissues only (Fig. 1, Table 2 and Supplementary Fig. 2).

3.2. Correlation among expression data

Spearman rank correlation analysis was carried out using expression data (i.e. ΔCt values) of four miRNAs and target genes from all four tissue types. Except *mir423*, expression of other 3 miRNAs and their target genes showed significant negative correlation in normal tissues (Table 3). All these negative correlations in normal tissues disappeared in disease tissues but significant positive correlation between expression of *ATP1B1* and *miR-423* and negative correlation between expression of *miR-26a* and *MYBL2* in lichen planus tissues was observed (Supplementary Table 4). Expression of *DNMT3a* was negatively correlated with that of *miR-26a* in leukoplakia tissues. Expression of *CTDSP2* was positively correlated with that of *mir-423* in cancer tissues. Some significant negative correlations were also observed between miRNAs and "*non-targets*". For example, significant negative correlation between *ADAM19* and *mir26a* and *ADAM19* and *mir34b* in normal tissue were observed. Several significant correlations between expressions of only target genes were also observed in normal and disease tissues (Supplementary Table 5). Some correlations between target genes were observed in all tissues while some are disease tissues specific.

3.3. Cluster analysis

Hierarchical clustering analysis using ΔCt or normalized gene expression showed disease or normal tissue specific expression profile, except for leukoplakia (Table 4). Dendrograms of the clustering show that expression profile comprising *miR-26a*, *miR-29a*, *ATP1B1*, *CDK6*, *COL4A2*, *CPEB3*, *PIK3R1* and *DNMT3A*, clearly clustered normal and lichen tissues, separately, from other two disease tissues (Supplementary Fig. 3). Gene expression profile comprising *miR-26a*, *miR-29a*, *miR-34b* and *ADAMTS7* clearly segregated cancer and lichen tissues, separately, from other two tissue types (Supplementary Fig. 4). Apart from above-mentioned clustering, lichen planus was also clustered by expression profile comprising *miR-26a*, *miR-34b* and *miR-29a* (Supplementary Fig. 5). We could not find any leukoplakia tissue specific gene expression profile for clustering.

4. Discussion

Down regulation of *mir-26a* has been consistently reported in oral carcinoma (Jia et al., 2014; Fukumoto et al., 2015). Investigators have studied miRNA profiles in saliva of patients with progressive and non-progressive leukoplakia and found that *mir-26a* was down regulated in progressive leukoplakia with respect to non-progressive samples (Yang et al., 2013). No report on the nature of expression deregulation of *miR-26a* in oral lichen planus tissues is available. This miRNA is reported to act as a tumor suppressor thus plays a significant role in tumor growth (Gao and Liu, 2011). In this study, expression of *mir-26a* was significantly upregulated in lichen planus but downregulated in leukoplakia and cancer tissues suggesting role of this miRNA as tumor suppressor in both leukoplakia and cancer. Study on differential