Expression deregulation of mir31 and CXCL12 in two types of oral precancers and cancer: importance in progression of precancer and cancer

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Oral cancer generally progresses from precancerous lesions such as leukoplakia (LK), lichen planus (LP) and oral submucous fibrosis (OSMF). Since few of these precancers progress to cancers; it is worth to identify biological molecules that may play important roles in progression. Here, expression deregulation of 7 miRNAs (mir204, mir31, mir31*, mir133a, mir7, mir206 and mir1293) and their possible target genes in 23 cancers, 18 LK, 12 LP, 23 OSMF tissues compared to 20 healthy tissues was determined by qPCR method. Expression of mir7, mir31, mir31* and mir1293 was upregulated and that of mir133a, mir204 and mir206 was downregulated in cancer. Expression of most of these miRNAs was also upregulated in LK and LP tissues but not in OSMF. Expression deregulation of some of the target genes was also determined in cancer, LK and LP tissues. Significant upregulation of mir31 and downregulation of its target gene, CXCL12, in cancer, LK and LP tissues suggest their importance in progression of precancer to cancer. Expression upregulation of mir31 was also validated using GEO data sets. Although sample size is low, novelty of this work lies in studying expression deregulation of miRNAs and target genes in oral cancer and three types of precancerous lesions.

Among all the non-coding RNAs, the most studied category is microRNA (miRNA) which is 20–22 nucleotide single stranded RNAs known for gene silencing by mRNA degradation or inhibition of translation. Each miRNA can regulate expression of many target genes and expression of each target gene may also be regulated by multiple miRNAs. By this miRNA-mRNA interaction, miRNAs take part in fundamental cellular processes like cell proliferation, differentiation, apoptosis, stress response etc.

Primarily, miRNAs were frequently found to be located in fragile genomic sites and genomic regions which were found to be associated with cancer risk\textsuperscript{1}. This led to the hypothesis that miRNAs might have some role in carcinogenesis. More recent studies have proved the definite signature of miRNAs in initiation and progression of cancer. Based on their expressions and functions in cancer tissue, miRNAs are also classified as tumor suppressor and oncogenic miRNAs. Since expression profiles of miRNAs are very different from one cancer type to another, they bring the opportunity of using them as cancer specific biomarkers\textsuperscript{2}.

Some of the pre-cancerous lesions progress to cancer, in spite of report of tumorigenesis from normal tissues. In expression profiling study of miRNAs in oral cancer tissue, we reported that expression of 7 miRNAs was found to be significantly deregulated in cancer with respect to their adjacent normal tissues\textsuperscript{3}. The purpose of this study was to compare expression of those seven miRNAs and some of their target genes in oral cancer and pre-cancer compared to normal tissues from different set of healthy individuals.

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**Results**

**miRNA expression in cancer tissues.** Expressions of 7 miRNAs in cancer and precancer tissues were compared with respect to those in normal tissues from healthy individuals. At least 2 fold expression change in disease tissue (up or down-regulation) compared to the normal was considered to be the threshold value for calling it deregulation in expression since Ct value of duplicate of a sample vary by less than 1 Ct value i.e. less than 2 fold change. Expression of all 7 miRNAs were found to be significantly deregulated in cancer samples, 4 of them were up-regulated (hsa-miR-7, hsa-miR-31, hsa-miR-31* and hsa-miR-1293) while expression of remaining 3 miRNAs were down-regulated (hsa-miR-133a, hsa-miR-204 and hsa-miR-206) as was observed in a previous report in which adjacent control was used to compare the expression deregulation3 (Table 1). It is important to note that both the disease and normal tissues were collected from the same cancer patients in the previous report whereas cancer and normal tissues were taken from two different set of individuals in this study (Table 1 and Supplementary Figure S1). The direction of expression (up or down) deregulation was found to be perfectly matched for each miRNA, though the magnitude of expression (i.e. fold changes) showed differences between this study and our previous study (1).

**miRNA expression in three types of pre-cancer tissues.** Except hsa-miR-204, expression of remaining 6 miRNAs, were found to be significantly up-regulated in leukoplakia. Similarly, except hsa-miR-133a and hsa-miR-206, expression of remaining 5 miRNAs was significantly up-regulated in lichen planus tissues (Table 2 and Supplementary Figure S1). In OSMF samples, expression of hsa-miR-31 and hsa-miR-204 were significantly up- and down regulated, respectively. Expression of hsa-miR-7, hsa-miR-31, hsa-miR-31* and hsa-miR-1293 was significantly up-regulated in cancer, leukoplakia and lichen planus samples and that of hsa-miR-204 was significantly down-regulated in cancer and OSMF samples. Hsa-miR-31 is the only miRNA which was significantly up-regulated in cancer and all precancer tissues (Table 2).

**Expression of target genes in cancer and pre-cancer tissues.** For miR-204, five target genes (MMP9, PLAUR, SERPINE1, SNAI2 and COL5A3); for miR-31, three target genes (DMD, CXCL12 and WASF3); for miR-31*, one target gene (KIAA1737); for miR-7, one target gene (TGM2); for miR-133a, three target genes (H2AFX, RUNX3 and MTHFD1L) and for miR-206, one target gene (FYB) were selected for expression study (Table 3, Supplementary Figures S2–S5). Expression of miR-204 was significantly down-regulated in cancer and OSMF tissues, while expression of its three target genes (MMP9, PLAUR and SERPINE1) showed significant up-regulation (Table 4 and Supplementary Figure S2) in cancer only. In contrast, expression of miR-31 was significantly

### Table 1. miRNA expression in fold changes in paired and unpaired cancer samples. Paired samples: cancer and adjacent control tissue from same patients (published data)3. Unpaired samples: cancer and control tissues from cancer patients and healthy individuals, respectively. Significant with P value < 0.05 after Benjamini-Hochberg multiple testing correction for all miRNAs. ↑: upregulation; ↓: downregulation of expression.

| miRNA     | Paired cancer-normal tissues (n = 18)* | Paired cancer-normal tissues (can = 23, nor = 20)* |
|-----------|----------------------------------------|--------------------------------------------------|
| hsa-miR-7 | 4 ↑                                   | 2 ↑                                               |
| hsa-miR-31| 5 ↑                                   | 30 ↓                                              |
| hsa-miR-31*| 7 ↑                                  | 41 ↓                                              |
| hsa-miR-1293| 5 ↑                                | 5 ↓                                               |
| hsa-miR-133a | 97 ↑                               | 209 ↓                                             |
| hsa-miR-204| 27 ↑                                  | 23 ↓                                              |
| hsa-miR-206 | 32 ↓                               | 126 ↓                                             |

### Table 2. Expression of miRNAs in cancer and precancer. Expression in fold change of miRNAs in cancer and pre-cancer tissues were calculated with respect to the normal oral tissues from healthy individuals. *Significant after independent t-test with P value < 0.05 and Benjamini-Hochberg multiple testing correction. ↑: upregulation; ↓: downregulation of expression.

| miRNA     | Cancer (n = 23) | Leukoplakia (n = 18) | Lichen planus (n = 12) | Oral submucous fibrosis (n = 23) |
|-----------|----------------|----------------------|------------------------|----------------------------------|
| hsa-miR-7 | 2 ↑*           | 6 ↑*                 | 14 ↑*                  | 1                                |
| hsa-miR-31| 30 ↑*          | 68 ↑*                | 19 ↑*                  | 3 ↑*                             |
| hsa-miR-31*| 41 ↑*          | 65 ↑*                | 182 ↑*                 | 2 ↑*                             |
| hsa-miR-133a | 209 ↑*        | 20 ↑*                | 1                      | 3 ↑*                             |
| hsa-miR-204| 23 ↑*          | 4 ↑*                 | 3 ↑*                   | 8 ↑*                             |
| hsa-miR-206| 126 ↑*         | 22 ↑*                | 3 ↑*                   | 2 ↑*                             |
| hsa-miR-1293| 5 ↑*           | 33 ↑*                | 6 ↑*                   | 1                                |
Expression of seven miRNAs and fourteen target genes were studied in oral cancer, 3 types of pre-cancer and normal oral tissues from healthy individuals. MiRNAs and target genes selected for expression study were consistent with the result of the previous study on a different set of cancer and adjacent control tissues. It is noteworthy to mention that the control tissues were taken from a different set of healthy individuals in this study whereas it was adjacent normal tissue in previous study. But expression deregulation of miRNAs in cancer tissues were not taken for expression study.

Table 3. MiRNAs and their target genes selected for expression study. Experimentally validated target genes of the miRNAs were selected from miRTarBase (http://miRTarBase.mbc.nctu.edu.tw/) and our unpublished RNAseq data. Expression of other target genes were not deregulated in our unpublished RNA-Seq expression data, so, was not taken for expression study.

| miRNA       | Target genes |
|-------------|--------------|
| hsa-miR-7   | TGM2         |
| hsa-miR-31  | DMD, CXCL12, WASF3 |
| hsa-miR-31* | KIAA1737     |
| hsa-miR-204 | MMP9, PLAUR, SERPINE1, SNAI2, COL5A3 |
| hsa-miR-133a| H2AFX, RUNX3, MTHFD1L |
| hsa-miR-206 | FYB         |

Table 4. Fold change in expression of miRNAs and their target genes in cancer and precancer. Expression fold change of miRNA and target genes in cancer and pre-cancer tissues were calculated with respect to the normal oral tissues from healthy individuals. Significant, after independent t-test with P value <0.05 and Benjamini-Hochberg multiple testing correction, \( \uparrow \): upregulation; \( \downarrow \): downregulation of expression; LK-Leukoplakia, LP-Lichen planus, OSMF-oral sub-mucous fibrosis, ND: not determined due to non-availability of RNA samples.

| miRNA      | Cancer (n = 23) | LK (n = 18) | LP (n = 12) | OSMF (n = 12) | Targets |
|------------|----------------|-------------|-------------|---------------|---------|
| hsa-miR-204| 23 \( \downarrow \) | 4 \( \uparrow \) | 3 \( \uparrow \) | 8 \( \uparrow \) | MMP9, PLAUR, SERPINE1, SNAI2, COL5A3 |
| hsa-miR-31 | 30 \( \uparrow \) | 68 \( \uparrow \) | 19 \( \uparrow \) | 3 \( \uparrow \) | DMD, KIAA1737, CXCL12 |
| hsa-miR-31*| 41 \( \uparrow \) | 65 \( \uparrow \) | 182 \( \uparrow \) | 2 \( \uparrow \) | KIAA1737, TGM2 |
| hsa-miR-7  | 2 \( \uparrow \) | 6 \( \uparrow \) | 14 \( \uparrow \) | 1 \( \uparrow \) | DMD |
| hsa-miR-133a| 209 \( \uparrow \) | 20 \( \uparrow \) | 1 \( \uparrow \) | 3 \( \uparrow \) | RUNX3, H2AFX, MTHFD1L |
| hsa-miR-206| 126 \( \uparrow \) | 22 \( \uparrow \) | 3 \( \uparrow \) | 2 \( \uparrow \) | FYB |

Validation miRNA expression using GEO data sets. Upon analysis, GSE33299 data set showed upregulation of expression of miR-31 in both leukoplakia and leukoplakia-transformed cancer with respect to normal tissue but expression was more in leukoplakia-transformed cancer tissues. Similarly, GSE62809 revealed that miR-31 to be one of the highly expressed miRNAs in both progressive and non-progressive leukoplakia groups but its expression was higher in progressive leukoplakia group. Expression of 7 miRNAs (mir204, mir31, mir31*, mir133a, mir7, mir206 and mir1293) in our study showed similar patterns of expression deregulation like GSE33299 data set except miR-7 and miR-206 in leukoplakia and leukoplakia-transformed cancer tissues, respectively. Apart from mir31, GSE62809 data set also showed similar trend in expression deregulation of mir-31* and miR-204 but remaining miRNAs were not considered for expression analysis due to low read count.

Discussion

Expression of seven miRNAs and fourteen target genes were studied in oral cancer, 3 types of pre-cancer and normal tissues from 5 different sets of individuals. It is observed that miRNA expressions in oral cancer tissues were consistent with the result of the previous study on a different set of cancer and adjacent control tissues. But expression deregulation of miRNAs in cancer tissues
is in same direction (i.e. up- or down-regulation) when compared with control tissues collected from either adjacent site of cancer tissue or healthy individuals (Table 1). In case of leukoplakia, expression deregulation of only \( \text{miR-31} \) and \( \text{miR-31}^* \) was in same direction when compared with control tissues from either adjacent site or different healthy individuals (Table 2). In lichen planus tissues, expression of none of these miRNAs was deregulated when compared to adjacent controls but most of them have deregulated expression when compared to control tissues taken from different healthy individuals (Table 2). In this study, most of the seven miRNAs was found to be up-regulated in cancer and all precancer tissues (Table 4) and similar result was also reported in other studies on different cancers and oral precancer tissues5–7. GEO datasets (GSE33299, GSE62809) was used for expression validation and it revealed that \( \text{miR-31} \) expression is up-regulated in oral leukoplakia and leukoplakia-transformed cancer samples, thus, suggesting importance of \( \text{miR-31} \) upregulation in progression of leukoplakia to cancer9. \( \text{MiR-31} \) is capable to promote tumor growth and has oncogenic potential in head and neck and other cancers9–11. In contrast, it is also reported to be down-regulated in cancer and prevent metastasis in cancer cells12. Like \( \text{miR-31} \), expression of \( \text{miR-31}^* \) is also up-regulated in cancer and two pre-cancer tissues in this study like a report in another study13, but its expression was found to be significantly associated with progression free survival of metastatic colorectal cancer14. \( \text{MiR-7} \) is a known OncomiR and its expression was up-regulated in cancer, leukoplakia and lichen planus tissues in this study, like in different cancers from other report15. Expression of \( \text{miR-133a} \), \( \text{miR-204} \) and \( \text{miR-206} \) was down-regulated in cancer tissues but up-regulated in precancer tissues in this study. Their expressions are also found to be down-regulated in head and neck and other cancers and considered as tumor-suppressors16–18. Restoration of expression of these miRNAs causes significant inhibition on cell proliferation, migration, invasion, and tumorigenesis as well as significant increase in apoptosis16,18–22. Only few reports are available regarding expression of \( \text{miR-1293} \) in cancer but expression was found to be up-regulated in cancer, leukoplakia and lichen planus tissues in this study (Table 2).

In this study, expression of miRNAs and target genes was significantly deregulated in both cancer and precancer tissues (Table 4). Expression of three target genes (\( \text{H2AFX} \), \( \text{RUNX3} \) and \( \text{MTHFD1L} \)) of \( \text{miR-133a} \) and one target gene (\( \text{FYB} \)) of \( \text{miR-206} \) was upregulated while expression of these two miRNAs (\( \text{miR-133a} \) and \( \text{miR-206} \)) were significantly downregulated in cancer tissues (Table 4). \( \text{H2AFX} \) is one of the histone family member gene which are involved in DNA double strand break repair pathway23. Although methylation, copy number alterations, deletion and single nucleotide polymorphisms in \( \text{H2AFX} \) are well-linked with different types of cancer, expression deregulation of the gene is also reported in a few studies24–27. \( \text{RUNX3} \) is a transcription factor that functions as a tumor-suppressor in bladder, colorectal, hepatocellular, lung and gastric cancer28–29. But in head and neck cancer it plays an oncogenic role. Upregulation of \( \text{RUNX3} \) in head and neck cancer leads to increased cell proliferation and reduced apoptosis30. \( \text{MTHFD1L} \) is an enzyme that works in the mitochondrial pathway of glycine biosynthesis. Higher expression of glycine pathway genes have been reported to induce rapid proliferation in cancer cells as well as associated with greater mortality of cancer patients31. \( \text{FYB} \) (also known as \( \text{ADAP} \) and \( \text{SLAP-130} \)) participates in T-cell receptor mediated actin cytoskeletal rearrangement with activation of integrins function, T cell migration and production of cytokines32–34. Knockout of \( \text{FYB} \) gives protection against tumor formation and metastasis in mice35. So, expression deregulation of \( \text{FYB} \) might impair the immune system of cancer cells. Significant upregulation of expression of 3 target genes (\( \text{MMP9} \), \( \text{PLAUR} \) and \( \text{SERPINE1} \)) of \( \text{miR-204} \) had been observed in cancer tissues as expected since it is known that downregulated expression of miRNAs may upregulate expression of target genes. Expression of the target genes (\( \text{DMD} \) and \( \text{CXCL12} \)) was also downregulated in precancers and cancer tissues probably due to upregulation of \( \text{miR-31} \) in these tissues. These target genes might have significant roles in progression of diseases since they function in remodeling of extracellular matrix (\( \text{MMP9} \), \( \text{PLAUR} \) and \( \text{SERPINE1} \))36, muscle formation and maintenance and tumor suppressor (\( \text{DMD} \))37–39, tumor growth and metastasis (\( \text{CXCL12} \))37–38,40, cell shape and motility (\( \text{WASF3} \))41 and regulation of circadian clock (\( \text{KIAA1737} \)). Except OSMF, expression of only one target gene (i.e. \( \text{CXCL12} \)) of \( \text{miR-31} \) was significantly deregulated in both cancer and two other pre-cancer tissues (Table 4). Lower expression of \( \text{CXCL12} \) was also reported in head and neck cancer due to hypermethylation in promoter region37. It has been reported that high expression of \( \text{miR-31} \) can immortalize or transform oral keratinocytes4 and is also proposed that \( \text{miR-31} \) can disrupt DNA repair genes to favor genomic instability and epithelial-mesenchymal transition3. Thus, \( \text{miR-31} \) has the potential for malignant transformation of precancer to cancer since upregulated expression of this miRNA has also been observed in leukoplakia and leukoplakia-transformed cancer tissues42. So, expression deregulation of \( \text{miR-31} \) and its target gene (\( \text{CXCL12} \)) in pre-cancer and cancer tissues suggest for a common mechanism leading to these diseases. Due to lack of follow-up data in this study, validation of expression in non-progressive and progressive precancer samples could not be performed by us but it was validated by GEO data sets generated from non-progressive and progressive leukoplakia samples. So, expression deregulation of \( \text{miR-31} \) and its target gene (\( \text{CXCL12} \)) seems to have significance in the progression of pre-cancer to cancer, but it needs to be validated in a larger set of samples with follow-up data.

Methods

Collection of samples. All experimental protocols were approved by the ethical committee of Indian Statistical Institute, Kolkata. Methods were carried out in accordance with the relevant guidelines and regulations. Written informed consents were taken from all patients and controls. Oral tissue samples were collected with informed consent from five groups of unrelated individuals consisting of four disease groups (oral squamous cell carcinoma, OSCC; leukoplakia, LK; lichen planus, LP and oral submucous fibrosis, OSMF) and one normal/healthy group (Table 5 and Supplementary Table 1A–E) from Pathology Department, Guru Nanak Institute of Dental Science and Research, Kolkata, India. Follow-up of precancer patients could not be performed since they...
did not turn up to the same hospital for review. So, we do not have data to say how many of our precancers are non-progressive (i.e. not transformed to cancer) or progressive (i.e. transformed to cancer). Tissues were kept in RNA Later solution and stored at −80°C until use. Diseases were confirmed by histopathology from the same department. Though the cancer and pre-cancer patients had tobacco habit but none of the healthy individuals had tobacco habit.

### Isolation of RNA and preparation of cDNA.
Total RNA isolation was performed from the tissue samples using Qiagen AllPrep DNA/RNA Mini Kit and RNA yield was quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). From the RNA samples, random hexamer based cDNA for target genes and miRNA primer specific cDNAs were prepared using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) and Taqman miRNA Reverse Transcription Kit (Applied Biosystems Inc.) respectively.

### Expression assay.
**MiRNA.** Expression of 7 miRNAs (hsa-miR-7, hsa-miR-133a, hsa-miR-204, hsa-miR-206, hsa-miR-31, hsa-miR-31*; hsa-miR-1293) were determined from miRNA specific cDNA prepared from RNA samples isolated from oral cancer, three types of pre-cancers and normal tissues. Though 3 miRNAs such as RNU-44, RNU-46 and mmu-6, were primarily included as endogenous controls for normalization of the expression data, only RNU-44 was finally selected as endogenous control as the expression of the other two miRNAs vary a little from sample to sample. Taqman-based expression assays were performed using miRNA specific probe and primers in 7900HT Fast Real-Time PCR System (Applied Biosystems Inc.)

**Target genes.** Experimentally validated target genes of these 7 miRNAs were obtained from miRTarBase database (release 5.0 and 6.0) (http://mirtarbase.mbc.nctu.edu.tw/) and expression of the targets was, then, checked in another list of expressed genes (i.e. whole transcriptome) in cancer tissues determined by high-through-put RNA-Seq method (unpublished data). Expression of 14 target genes of six miRNAs (hsa-miR-206, hsa-miR-133a, hsa-miR-7, hsa-miR-31, hsa-miR-31* and hsa-miR-204) were found to be de-regulated in our RNA-Seq unpublished data. Among these 14 target genes, 10 were selected for expression study in cancer, precancer and normal tissues (Table 4) and expression values of remaining 4 target genes (i.e. H2AFX, RUNX3, MTHFD1L and FBY) were taken from whole transcriptome data on same cancer tissues (unpublished data). Expression of these 4 target genes could not be determined in precancer tissues due to non-availability of RNA samples. RT-PCR primers were designed for target genes and Sybr-Green expression assays were performed in 7900HT Fast Real-Time PCR System. Expression of RNaseP gene was used as endogenous control to calculate the expression deregulation of target genes.

Expression data, i.e. $C_t$ value which is the number of cycle at which quantity of PCR product reaches a certain threshold level in log phase of amplification, were converted to normalized expression data i.e. $AC_t = C_{target} - C_{endogenous}$ expression value of a gene in a tissue − $C_{endogenous}$ expression value of endogenous control gene in the same tissue. Expression deregulation of a gene in disease tissues compared to control tissues is calculated as mean of $ΔAC_t$ values of all samples of a disease group, where mean $ΔAC_t = mean of ΔCts$ of a gene in disease tissues − mean of $AC_t$s of the same gene in normal tissues. Fold change is calculated as $2^{ΔAC_t}$.

### Statistical analysis.
Expression data ($ΔAC_t$) for each of the five groups were tested for normality (Kolmogorov-Smirnov test). ANOVA and independent t-test were done to check whether the mean $ΔAC_t$ of any gene in a disease group is different from that in normal group. The OSMF patients were sub grouped on the basis of presence or absence of dysplasia and checked for any difference in mean $AC_t$. Benjamini-Hochberg multiple testing corrections were done on the p values to avoid false positive significance.

### Expression validation.
For miR-31, expression validation was performed using two data sets of Gene Expression Omnibus (GEO) database\textsuperscript{42}. GSE33299 data set had expression of miRNAs from 5 normal oral tissues, 20 leukoplakia and 5 leukoplakia-transformed cancer tissues and GSE62809 data set had miRNA expression profiling from 10 non-progressive and 10 progressive leukoplasias (i.e. turned into oral squamous cell carcinoma within 5yrs).

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| Sample groups          | Sample size for miRNA expression | Sample size for Target gene expression |
|------------------------|----------------------------------|---------------------------------------|
| Cancer                 | 23                               | 23                                    |
| Leukoplakia            | 18                               | 16                                    |
| Lichen planus          | 12                               | 14                                    |
| Oral submucous fibrosis* | 23                            | 15                                    |
| Normal/healthy         | 20                               | 15                                    |

**Table 5. Cancer, precancer and normal tissue samples used in this study.** *OSMF samples have 11 dysplasia and 12 without dysplasia tissues.*
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Author Contributions
E.C. experiment, data analysis and interpretation, manuscript writing; R.S., A.R., R.R. and N.D.S.: data analysis and critical interpretation of outcome; R.R.P., M.P. and R.A.: diagnosis of patients, collection of samples and disease confirmation by histology. B.R.: Supervised the entire work, contributed in design of experiment, critical review of data interpretation and manuscript writing.

Additional Information
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