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# The Differential Estimation of Let-7b and miRNA-96 in the Serum and the Tissue Samples Obtained from Chemically Induced Oral Carcinogenesis in Rats

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**Abstract**

Let-7b and miRNA-96 are among the dysregulated microRNAs in various diseases. Selected seventy-five male rats were divided into five equal treatment groups and were given 4-nitroquinoline 1-oxide (4-NQO) by drinking water (50 ppm) for five different drenching periods. To each treatment group, a control group of five males was also assigned. The oral pathological changes were microscopically evaluated thereafter. Histopathological changes indicating dysplasia or frank carcinoma were observed in the fifth treatment group only. Let-7b and miRNA-96 expressions in the tongue tissue and serum were quantified using quantitative reverse transcription PCR (qRT-PCR). The fifth treatment group only showed signs of severe and well differentiated squamous cell carcinoma in percentages of 40% and 26.7%, respectively. Let-7b expression appeared to be statistically non-significantly different when up and downregulation were compared in tissue and serum samples ($p$=.3; $p$=.08). For miRNA-96, it seemed that no statistically significant difference ($p$=.08) was attained when tissue samples were studied, while the difference was statistically significant ($p$=.04) when serum samples were considered. Let-7b can be considered a good biomarker for oral squamous cell carcinoma (OSCC) in tissue and body fluid as well, while miRNA-96 demonstrated a controversial result as far as the tissue type was considered and, thus, can play the role of an oncogene (OG) or a tumor suppressor gene (TS).

**Keywords**: Let-7b, miRNA-96, Oral carcinogenesis, Squamous cell carcinoma, Rats

# التقدير المقارن لل Let−7b و miRNA−96 في مصل وانسجة الجرذان المصابة بسرطان الفم المستحث كيمياويا

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**الخلاصة**

الرنا الميكروي هو فئة من من احماض الرنا غير المشفرة والموزعة موزعة على نطاق واسع في الأنسجة المتغيرة وسوائل الجسم ، ووجد أنها تلعب دورًا مهمًا في التسرطن الفموي .يعتبر Let−7b و

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miRNA−96 من بين الرنا الميكروي غير المنتظم في أمراض مختلفة .تم تقسيم خمسة وسبعون من ذكور الجرذان إلى خمس مجموعات متساوية المعاملة وتم إعطاؤهم 4−نيتروكينولين 1− أكسيد (4−NQO) عن طريق شرب الماء بجرعة 50 جزء في المليون لخمس فترات غمر مختلفة .كما أستخدامت مجموعة سيطرة مكونة من خمس ذكور من الجرذان مقابل كل مجموعة معاملة .بعد نهاية كل فترة نقع ، تم تقييم التغيرات المرضية عن طريق الفم مجهريًا .حيث قيست كمية تعبير Let−7b و miRNA−96 في أنسجة اللسان وعينات المصل وقورنت في المجاميع المعاملة وكذلك مجموعة السيطرة باستخدام النسخ العكسي الكمي PCR (RT−qPCR) النتائج: أظهرت المجموعة العلاجية الخامسة فقط العلامات النسيجية المرضية لسرطان الخلايا الحرشفية الحاد والمتباين بشكل جيد بنسب 40٪ و 26.7٪ للحيوانات المعالجة على التوالي .تراوحت الاستجابة في مجموعات العلاج الأخرى ولفترات التعرض الأقصر من خلال التنسج الطبيعي إلى المعتدل .على المستوى الجزيئي ، يبدو أن تعبير Let−7b يختلف من الناحية الإحصائية بشكل غير معنوي عند مقارنة التنظيم العالي و الواطئ في عينات الأنسجة والمصل (p = .3) ؛ في حالةmiRNA−96 ، بدا أنه لم يتم تحقيق أي فرق ذي دلالة إحصائية(p = 0.08) عند دراسة عينات الأنسجة بينما كان الاختلاف ذا دلالة إحصائية (p = 0.04) عند النظر في زيادة وتناقص ا miRNA في عينات المصل ااستنتجت الدراسة انخفاض تنظيم Let−7b بشكل ملحوظ في عينات المصل والأنسجة للحيوانات المعالجة ، ويمكن اعتبارذلك علامة حيوية جيدة لسرطان الخلايا الحرشفية في الفم OSCC و الأنسجة بالإضافة الى سوائل الجسم ، بينما أظهرmiRNA−96 نتيجة مثيرة للجدل فيما يتعلق بنوع الأنسجة وبالتالي يمكن أن يلعب دور الجين المسرطن اوالجين الكابت للورم.

## Introduction

Oral cancer (OC), mainly squamous cell carcinoma, is a common malignancy, categorized in the top 20 most deleterious human cancers [1]. Oral carcinogenesis is a complex multifactorial process regulated by genetic, environmental, social and other exogenous factors [2]. In the last decade, an appreciably interest has been applied in analyzing the series of molecular events involved in oral squamous cell carcinoma (OSCC). Accordingly, microRNAs (miRNAs) have attracted increasing attention due to their role in the development and progression of several pathologies, including OC. Mature miRNAs bind to the 3' untranslated regions of the messenger RNA (mRNA) and represses the translation. In this context, they can be linked to cellular proliferation, apoptosis, invasion and metastasis [3]. Mounting evidences showed an up or downregulation of miRNAs expression in oral neoplastic tissues when compared to the normal tissue. In OSCC, many miRNAs with a distinct expression profile and a potential biological significance have been discovered [4, 5]. As per their stability in tissues as well as body fluids, including plasma and serum, miRNAs are considered as potential biomarkers for the detection of OSCC [6]. Let-7b and miRNA-96 are found to be downregulated and can act as a tumor suppressor in many cancers [7, 8]. However, their functions and mechanisms in OC have rarely been explored previously.

The chemically induced tumors rat model, in spite one of the facts that it is earliest model developed, is still the best to study all stages of the OC due to a close similarity to the clinical form of the disease. The 4-nitroquinoline 1-oxide (4-NQO) is a powerful carcinogen inducing cancer in many organs. It can specifically induce dysplastic changes and OSCC when applied in different concentrations via drinking water [9].

There are many methods for quantifying miRNAs, one of the most common is quantitative real-time PCR (qRT-PCR) which is a good and dependable method to monitor expression profiles of miRNAs in different tissues and organisms. This technique has been utilized to estimate the overexpression or the under expression of miRNAs in cancerous tissues and body fluids [10]. The present study was set up to investigate the pathological changes of the oral mucosa after the carcinogen exposure as well as to explore the expression of two miRNAs, Let-7b and miRNA-96, extracted from the oral tissues and serums.

## Materials and Methods

*Experimental Design*

The current study included an animal model for oral carcinogenesis that was induced chemically. Animal drenching of a carcinogen was practiced at the animal house of the College of Science, Duhok University. Whereas the tissue samples processing and examination were performed at the Central Public Health Laboratory of Duhok. Finally, the molecular investigation was accomplished at Nabu corporation for scientific researches, Baghdad, Iraq.

*Experimental Animals*

One hundred albino Wistar male rats, of 4 weeks age, were selected for the study. Seventy-five rats were given 4-NQO (Santa Cruz, USA) solution in their drinking water at a concentration of 50 ppm. The remaining twenty-five animals were also divided into five control groups, each of five animals assigned for a particular treatment group. The experimental animals were divided into five equal groups each included fifteen animals. The first group was euthanized after 4 weeks of the carcinogen drenching, the second group after 8 weeks of drenching and so forth up to the fifth group which was exposed to the carcinogen for 20 weeks. In each treatment group, rats' tongues were dissected immediately after the cervical dislocation. The autopsies and the serum samples were taken, and kept with TRIzol™ (Santa Cruz, USA) for later use.

*Histopathological Examination*

Tissue samples were prepared for histopathological investigation according to the literature [11]. Grading of the tissue samples depended on the currently accepted World Health Organization classification [12, 13].

*Molecular Investigation*

The total RNA was extracted from tissue and serum samples by using the TRIzol® LS Reagent, according to the instruction provided by the manufacturing company. Complementary DNA (cDNA) belonging to the particular miRNA was synthesized by the aid of the primers listed in Table 1. ProtoScript® First Strand cDNA Synthesis Kit (NEB, UK) was used for this purpose. The program used for the synthesis of the cDNAs is elucidated in Table 2.

**Table 1:** The primers and their sequences

| Primer | Sequence | Reference |
|---|---|---|
| Let-7b | CTCAACTGGAGCTAGTTTCGTCGTAGGGCAGTTG AGAACCACAC RT | Wang *et al.,* 2018 [14] |
| miRNA-96 | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCAC TGGATACGACAGCAAA RT | Song *et al.,* 2015[15] |
| U6 | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTG AGAAAAATATG | Wang *et al.,* 2018[14] |

**Table 2:** Program of cDNA synthesis

| Step | Time (Minute) | Temperature (ºC) |
|---|---|---|
| Hold | 5 | 25 |
| Hold | 60 | 42 |
| Hold | 20 | 65 |
| Hold | ∞ | 4 |

The expression level of Let-7b and miRNA-96 was estimated by the quantitative-real time PCR (qRT-PCR) using the SYBER green to recognize double-stranded DNAs. Table 3 shows the forward and reverse primers utilized for the amplification of the cDNAs.

**Table 3:** Primers for cDNA amplification

| Primers | Sequence | Reference |
|---|---|---|
| Let-7b | F 3´ CCAGCTGGGTGAGGTAGTAGGTTGT 5´<br>R 3´ CTGGAGCTAGTTTCGTCGTAGGG 5´ | (Wang *et al.*, 2018) [14] |
| miRNA-96 | F 3´ CGGCGGTTTGGCAATGGTAGAACT 5´<br>R 3´ CCAGTGCAGGGTCCGAGGTAT 5´ | (Song *et al.*, 2015) [15] |
| U6 | F 3´ CTCGCTTCGGCAGCACA 5´<br>R 3´ AACGCTTCACGAATTTGCGT 5 | (Wang *et al.*, 2018) [14] |

The reaction components and the amplification conditions of the RT-PCR used in this study are shown in Tables 4 and 5 respectively.

**Table 4:** RT-PCR reaction components

| The Component | 20 µl Reaction |
|---|---|
| Template DNA | 5µl |
| Luna Universal qPCR Master Mix | 10µl |
| Forward primer (10 µM) | 1µl |
| Reverse primer (10 µM) | 1µl |
| Nuclease-free Water | 3µl |

**Table 5:** The amplification conditions of the qRT-PCR

| Steps | Temperature (ºC) | Duration | Cycles |
|---|---|---|---|
| Initial Denaturation | 95 | 60 Sec | 1 |
| Denaturation | 95 | 15 Sec | |
| Anneal/Extension | 60 | 30 Sec | 40-45 |
| Dissociation | 60-95 | 40 Min | 1 |

The analysis and the interpretation of the results of the gene expression study were based on Livak formula [16]. The relative expression of Let-7b and miRNA-96 was normalized with U6 [17, 18] and calculated based on $\mathbf{2^{-\Delta\Delta Ct}}$ method. The statistical analysis software SPSS (version 25.0) (IBM Corporation, New York, NY, USA) was applied for data analysis. Data was presented as mean ± standard deviation. Chi-square and Fisher's exact tests were performed to find the statistical differences. Probability level $p \leq 0.05$ was considered statistically significant.

## Results

*Morphologic and Histologic Evaluation*

No apparent abnormal clinical and histological changes were observed in rats within the control group. In the experimental groups, white and rough appearance of the tongue mucosa was noticed only after the 20 weeks intake of the carcinogen. Twelve (80%) rats showed normal histology and three (20%) rats from the first treatment group exhibited mild dysplasia in their tongue samples, where the changes confined within basal and parabasal layers only. In the second treatment group, normal, mild and moderate dysplastic changes were noticed in 2 (13.3%), 10 (66.7%) and 3 (20%) rats respectively. Six (40%) and nine (9%) animals showed mild and moderate dysplasia respectively in the third treatment group. All fifteen animals (100%) showed moderate levels of dysplastic changes in the fourth treatment group. While 5 (33.3%) rats had the same manifestation in the fifth treatment group, 6 (40%) animals experienced severe dysplasia and 4 (26.7%) rats had the signs of OSCC (Table 6). Figures 1 and 2 show the normal histological appearance and SCC of the rat tongue respectively.

**Table 6:** Histopathological changes of the rats' tongue autopsies. Data is presented by frequency and percentages.

| Experimental groups | Normal | Mild Dysplasia | Moderate Dysplasia | Severe Dysplasia | Carcinoma (SCC) |
|---|---|---|---|---|---|
| **First Group (n=15)** | 12 (80%) | 3 (20%) | 0 (0%) | 0 (0%) | 0 (0%) |
| **Second Group (n=15)** | 2 (13.3%) | 10 (66.7%) | 3 (20%) | 0 (0%) | 0 (0%) |
| **Third Group (n=15)** | 0 (0%) | 6 (40%) | 9 (60%) | 0 (0%) | 0 (0%) |
| **Fourth Group (n=15)** | 0 (0%) | 0 (0%) | 15 (100%) | 0 (0%) | 0 (0%) |
| **Fifth Group (n=15)** | 0 (0%) | 0 (0%) | 5 | 6 (40%) | 4 (26.7%) |

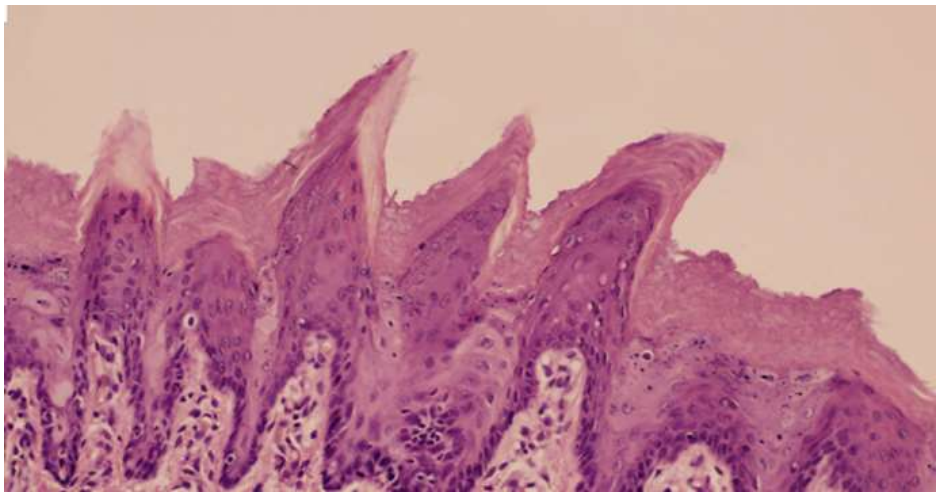

**Figure 1**: Microphotograph of normal tongue mucosa in the control group (H & E at 40x).

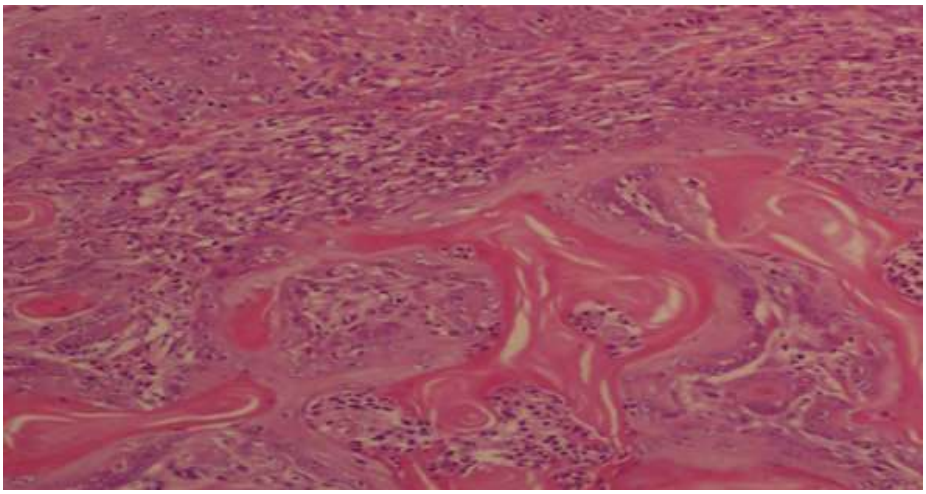

**Figure 2:** Microphotograph of a well differentiated squamous cell carcinoma (H & E at 40x).

*Molecular Findings*

When oral tissue samples were examined, the results of the present investigation revealed that Let-7b was down-regulated in 62 (82.6 %) animals, while up-regulation appeared in 13 (17.4 %) animals. The comparison of both showed a statistically insignificant difference ($p$=0.3). miRNA-96 expression levels comparison indicated insignificant statistical difference ($p$=0.08) with a down-regulation and up-regulation frequencies of 34 (45.4 %) and 41 (54.6 %) respectively (Table 7).

**Table 7**: Up and down regulation of miRNAs in tissue samples. Data is presented by frequency and percentages.

| MicroRNAs | | Group One | Group Two | Group Three | Group Four | Group Five | Total | *P*-value |
|---|---|---|---|---|---|---|---|---|
| **Let-7b** | Up regulated | 1 (6.7%) | 3 (20%) | 4 (26.7%) | 4 (26.7%) | 1 (6.7%) | 13 (17.4%) | 0.3 |
| | Down regulated | 14 (93.3%) | 12 (80%) | 11 (73.3%) | 11 (73.3%) | 14 (93.3%) | 62 (82.6%) | |
| **miRNA 96** | Up regulated | 7 (46.7%) | 13 (86.7%) | 8 (53.3%) | 6 (40%) | 7 (46.7%) | 41 (54.6%) | 0.08 |
| | Down regulated | 8 (53.3%) | 2 (13.3%) | 7 (46.7%) | 9 (60%) | 8 (53.3%) | 34 (45.4%) | |

The results of the comparisons of the miRNAs expression levels within one tissue types showed variable miRNAs up and down-regulated responses when they were isolated from serum samples. Let-7b was downregulated in 54 (72 %) animals, while they up-regulated in 21 (28 %) cases with a statistically non-significant difference ($p$=0.08). The difference of the miRNA-96 expression levels was statistically significant ($p$=0.04) when the down-regulation frequency of 41 (54.6 %) animals was compared with that of the up-regulation of 34 (45.4 %) (Table 8).

**Table 8**: Up and down regulation of miRNAs in serum samples. Data is presented by frequency and percentages.

| MicroRNAs | | Group One | Group Two | Group Three | Group Four | Group Five | Total | *P*-value |
|---|---|---|---|---|---|---|---|---|
| **Let-7b** | Up regulated | 4 (26.7%) | 8 (53.3%) | 2 (13.3%) | 5 (33.3%) | 2 (13.3%) | 21 (28%) | .08 |
| | Down regulated | 11 (73.4%) | 7 (46.7%) | 13 (86.7%) | 10 (66.7%) | 13 (86.7%) | 54 (72%) | |
| **miRNA 96** | Up regulated | 12 (80%) | 4 (26.7%) | 6 (40%) | 6 (40%) | 6 (40%) | 34 (45.4%) | .04* |
| | Down regulated | 3 (20%) | 11 (73.4%) | 9 (60%) | 9 (60%) | 9 (60%) | 41 (54.6%) | |

**(*)** Significant at $p \leq 0.05$, $X^2=9.9$

## Discussion

Animal models can be considered an essential part in studying how cancer develops and progresses. The use of a carcinogenic material to induce OSCC in experimental animals has gained paramount interest [9, 19, 20].

The qualitative assessment of our samples revealed dysplastic and neoplastic changes at the mucosal epithelia of the tongue, especially sections near the posterior part. Histopathological outcomes of this study were in accordance with earlier observations in which OSCC was detected between 18 and 22 weeks, following the administration of 50 ppm 4-NQO [21, 22].
For better understanding of the cancer development and progression two miRNAs, Let-7 and miRNA-96, were used in our study to assess their dysregulation pattern of expression in both serum and tissue samples. Accumulated evidences demonstrated that this dysregulation in cancer is through different mechanisms, deletion or amplification of genes, dysregulation of epigenetic events, and abnormal transcriptional control of certain miRNA genes [23]. Extensive profiling investigations have identified miRNAs to act as oncogenes (OGs) or as tumor suppressor genes (TSs) [24]. The downregulation of Let-7b expression in the current study is similar to what has already been achieved by others [25, 26]. These authors proved the link between Let-7b loss of expression and the development of poorly differentiated carcinoma, the

fact that can explain the presence of downregulation findings of Let-7b even in the first group where the histological changes included only mild dysplasia. With few exceptions, absence or downregulation of the Let-7b is closely correlated with aggressive or high-grade tumors, and poor prognosis, while higher levels of expression can be associated with good prognosis and probably prolonged survival, suggesting the Let-7b to be a good diagnostic and prognostic tool for oral malignancies [27]. According to our results, Let-7b can be classified as a TS. However, Chirshev and his team reported that Let-7, in rare cases, can act as an OG [28]. Similarly, Let-7b downregulation was observed in serum samples as well. This aberrant expression was observed formerly by many authors [29, 30].

The current investigation also aimed to assess the oncogenic mechanism of miRNA-96 in tissue and serum samples. Up until now, miRNA-96 has been categorized as an OG in ovarian cancer, breast cancers, head and neck squamous cell carcinoma, bladder cancer and hepatocellular carcinoma [31-35]. Interestingly, it may act as a TS in colorectal cancer [36]. However, the downregulation results of miRNA-96 within the tissue and serum samples of our study were inconsistent with the oncogenic role proposed by these studies. According to Piotrowski and colleagues, a non- significant expression of the miRNA-96 in oral cancer tissues, with an upregulation in laryngeal tumor tissues of the same patient observed in their study. [37]. The current results agree with a fact that in certain cases the same miRNA, like miRNA-96, can act as an OG in a particular cell type and as a TS in another. The dual activity of some miRNAs is probably related to the tissue-specific nature of that miRNA expression which suggests that different cancers can express certain miRNA signatures [38].

## Conclusion

The results indicated that histological and molecular findings can give a good evaluation for cancer progression rather than being utilized individually. On the other hand, Let-7b was markedly downregulated in both serum and tissue samples of the treated animals, and, therefore, can be considered a good biomarker for OSCC in tissue and body fluid as well. Whereas miRNA-96 demonstrated a controversial result as far as the tissue type is considered and, thus, can play the role of an OG or a TS.

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## Ethics Approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 12092018-7 in 12/09/2018.

## Competing Interests

The authors declare that they have no competing interests.

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