Salivary extracellular vesicles as non-invasive source of microRNAs in detection of malignant transformation of oral potentially malignant lesions

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ABSTRACT

Background: The study was designed to evaluate the sensitivity and specificity of using the salivary extracellular vesicles as a non -invasive source for miRNA-412, and miRNA-512 to detect the malignant transformation of the oral potentially malignant lesions (OPMLs).

Materials and Methods: The case-control study comprised 60 subjects; 20 participants in each of the 3 groups: patients having OPMLs, patients having oral squamous cell carcinoma (OSCC) and normal individuals as a control group. Saliva of all participants was analyzed using quantitative real-time polymerase chain reaction (qRT-PCR) to detect miRNA-412, and miRNA-512 in the extracellular vesicles.

Results: Upregulation of miRNA-412 and miRNA-512 was identified in OSCC and OPMLs groups in comparison to normal group, the highest values for miRNA-412 and miRNA-512 were seen in OSCC group, followed by the OPML group, followed by control group. The ROC curves for miR-512and miR-412 resulted to be both sensitive and specific, as shown by high AUC values (0.986 and 0.995 respectively) and maximum Youden's Index.

Conclusions: miR-412 and miR-512obtained from salivary extracellular vesicles are good predictors for malignant transformation in OPMLs.

Key words: Extracellular vesicles; liquid biopsy; malignancy; non-invasive diagnostic techniques; oral cancer; premalignant lesions.

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INTRODUCTION

Oral cancer (OC) ranks eighth among all other malignancies; with oral squamous cell carcinoma (OSCC) representing the most prevalent OC (1). Mortality represents a possible prognostic endpoint due to the disease itself or due to treatment-related complications; especially when detected at late stage. Therefore, the most effective therapeutic management of oral malignancy is achieved via early diagnosis and regular screening protocol (2)**.**

Currently, the gold standard diagnostic tool for detecting malignancies is the histological assessment of the tissues obtained by means of surgical biopsy. However, surgical biopsy has drawbacks including being medically invasive and risky; besides the need for sufficient time and a well-trained pathologist for its preparation and interpretation (3)**.** Thus, there is still an urgent clinical need for a safer, faster and a more standardized alternative for cancer early detection (3)**.**

Extracellular vesicles (EVs) are lipid bilayer-delimited components discharged by almost all body cells to communicate with their surrounding cells (4). During cancer development, tumor cells secrete EVs that carry micro-RNA (miRNA), RNA, DNA and/or proteins from their parent cells (5). Thus, their contents have been recently investigated as biomarkers for different malignancies (6), as well as head and neck cancer (7).

MiRNA contents of EVs of oral cancer cells were successfully extracted from saliva specimens (8). Obviously, malignancy-related miRNAs impact three main constituents of the tumor milieu: vasculature, extracellular matrix, and immune cells; causing the cells to acquire malignancy hallmark traits leading to genetic abnormalities (9)**.**

Among miRNAs within tumor-derived EVs that were proven important in oral cancer process is miRNA-412. It was found that detection of miRNA-412 can highly predict the percentage of malignancy-associated mortality and the degree of cancer progression. In addition, inhibition of miRNA-412 leads to reducing the vital processes of cancer stem cell development such as proliferation, migration, and invasion. Nevertheless, reduction of miRNA-412 can indicate cellular apoptosis(10).

Besides**,** evidence has been demonstrated that miRNA-512 can be considered as a tumor suppressor in different kinds of human malignancies like hepatocellular carcinoma (11) and lung adenocarcinoma (12). However, both miRNAs have not been tested in patients having oral premalignant lesions (OPMLs).

Based on the aforementioned information, the present study was thus conducted in an attempt to investigate the possible use of miR-412 and miR-512 in salivary extracellular vesicles as diagnostic markers for early detection of malignant transformation of OPMLs.

MATERIALS AND METHODS:

Study Design & Setting. This prospective, case-control study was performed during the period from October 2019 to August 2020. The protocol was registered at ClinicalTrials.gov with an ID NCT04913545. Patients were recruited at Oral Medicine clinic, Faculty of Dentistry and the outpatient clinics of National Cancer Institute.

Study groups. The study included 60 participants, recruited into 3 groups: Group I included 20 patients suffering from newly diagnosed untreated primary OSCC (13), Group II included 20 patients with clinically and histologically confirmed OPMLs with no signs of malignant transformation, and Group III included 20 healthy control subjects with no clinically detectable oral lesions who were matched for age, gender, and risk factors.

The study included subjects of both sexes, within age range of 30-70 years, who were systemically free. Conventional oral examination was performed first. Accordingly, if clinical features of oral tissues were altered and histological features confirmed OPMLs (innocuous, or non-suspicious lesions) (14), the participant was included in the OPML group; while if clinically evident suspicious lesions were detected during CVTE, the patient was included in the OSCC group (14).

On the other hand, pregnant and breast-feeding females, as well as, psychiatrically or mentally unstable patients were excluded.

Salivary sample collection. Whole unstimulated saliva (WUS) was harvested using standard technique (15). Subjects were instructed to refrain from eating, drinking, using chewing gum, etc., for at least one and a half hour before the evaluation. To obtain the samples, subjects were requested to swallow first, tilt their head forward, and expectorate all saliva in a tube for 5 minutes without swallowing (16)**.**

Tissue biopsy. specimens were harvested from patients included in group II. Biopsies were obtained from lesions that were clinically diagnosed as one of the OPMLs (oral lichen planus, Leukoplakia, lichenoid lesions) under local anesthesia. After being transformed into paraffin blocks, the diagnosis was confirmed by examining thin (5-μm-thick) sections of each tissue specimen stained with H&E. All tissue specimens were processed by the Oral Pathology department, Faculty of Dentistry. Analysis was performed on coded samples by 2 different oral pathologists who were masked regarding the subjects' clinical diagnoses until all analysis was finished.

Laboratory steps: MicroRNA Extraction:

EV isolation. For all saliva samples, dilution 1:1 with PBS (phosphate buffered saline) was applied and then samples were centrifuged at 3000 xg for 15 minutes at room temperature to remove cells, debris, and bacteria. The supernatant was, then, filtered using 0.2 μm filters and transferred to a sterile tube. Afterwards, a precipitation solution (65 μL per 250 μL of saliva) was added and the mixture incubated at 4°C overnight. The next day, samples were centrifuged at 3000 gx for 30 minutes to precipitate EVs. The resulting supernatant was removed and samples re-centrifuged at 1500 xg for 5 min to remove any

remaining supernatant. The pellet was resuspended in 600 µL of Lysis Buffer (miRNAeasy MA, USA) and stored at -80°C for subsequent RNA extraction.

RNA extraction was done using QIAzol lysis reagent and incubated for 5 minutes at room temperature. Afterwards, 100 uL chloroform was added, vortexed for 15 seconds, and incubated for 2–3 minutes at room temperature. Then, samples were centrifuged at 14000 xg at 4°C for 15 minutes. The upper watery phase was removed followed by addition of 100 % ethanol. Each 700 uL of this mixture was placed in RNeasy Mini spin column in 2 mL collection tube and centrifuged at 10000 xg at room temperature for 15 seconds.

After the mixture had completely passed the column, buffer RWT was added to each column, and again centrifuged at 10000 xg at room temperature for 15 seconds. 500 uL buffer RPE was then, added to the column and centrifuged at 10000 xg at room temperature for 15 seconds. After this, another 500 uL buffer RPE was added to the column and centrifuged at 10000 xg at room temperature for 2 minutes. The column -which had been placed in a new collection tube- was then centrifuged at full speed for 2 minutes. Lastly, the column was transferred to a new 1.5 mL collection tube and 50 uL RNase-free water was pipetted directly onto the column and centrifuged for 1 minute at 10000 xg to elute RNA. The extracted RNA was stored at −80 °C until use.

PCR Analysis for miRNA-412 and miRNA-512 Expression. Reverse transcription was carried out on 100 ng of total extracted RNA in a final volume of 20-µL (incubated for 60 min at 37°C, 5 min at 95°C, and then maintained at 4°C) using the miRNeasy Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

For real-time PCR, diluted reverse transcription products (cDNA template) were mixed with SYBR Green Master Mix (Qiagen, Valencia, CA, USA) in a volume of 25 uL. Quantitative real time PCR expression of mature miRNAs -412 and -512 were evaluated using an Applied Biosystems 7500 Real Time PCR System (Foster city, CA, USA) according to the manufacturer's instructions. The housekeeping miRNA SNORD68 was used as the endogenous control. Realtime PCR was performed with the following conditions: 95°C for 15 minutes, followed by 40 cycles at 94°C for 15 seconds, and 55°C for 30 seconds, and 70°C for 34 seconds.

After the PCR cycles, melting curve analyses were performed to validate the specific generation of the expected PCR product. The expression levels of miR-412 and -512 were evaluated using the delta cycle threshold (ΔCt) method. The cycle threshold value (CT) is defined as the number of cycles required for the fluorescent signal to cross the threshold in real-time PCR. ΔCt was calculated by subtracting the CT values of miRNA SNORD68 from the CT values of the target miRNAs.

As there is an inverse correlation between ΔCt and miRNA expression level, lower ΔCt values were associated with increased miRNA. The resulting normalized ΔCt values were used in calculating relative expression values using 2- ΔCt, and these values are directly related to the miRNA expression

levels. The 2- ΔΔCt calculation was then used to determine the relative quantitative levels of individual miRNAs

Statistical Analysis. The sample size was calculated using G* Power version 3.1.9.2 using ROC curve analysis according to an effect size of 1.497 (13), level of significance 0.05 and 80% power. The calculated sample size was 18 subjects per group, giving a total of 54 patients. However, we increased the number of the participants to 60 to make up for any defective samples.

Data was represented as mean and standard deviation when appropriate. One-way ANOVA was used to compare between tested groups for mean markers followed by Scheffe test for multiple comparison. A receiver operating characteristic (ROC) curve was created for each miRNA to estimate a preliminary cutoff point. Also, area under the ROC curve (AUC) was calculated. (α=0.05). Statistical Analysis was performed using IBM® SPSS® (version 26, Armonk, USA) and Med Calc version 19.4, taking in consideration the guide for classifying the accuracy of a diagnostic test by the traditional academic point system (17): 0.90–1.0 = excellent, 0.80–0.90 = good, 0.70–0.80 = fair , 0.60–0.70 = poor, and 0.50–0.60 = fail.

RESULTS

Baseline data. The participants in the 3 groups showed no statistically significant differences regarding age or gender (p = 0.1 and 0.627, respectively) (table 1).

Salivary levels of miRNA-412 and miRNA-512. The mean values for the 2 miRNAs in all groups studied seem comparable in the healthy controls, while great variation occurs in OPMLs and OSCC cases; where the mean values for the healthy control group are away from other tested groups. It is also evident that the highest mean values for miRNA-412 and miRNA-512 are seen in OSCC group, followed by the OPMLs group, followed by the control group (table 2).

Furthermore, the values of miRNA-512 and miRNA-412 were statistically significantly higher in OSCC grade III than grade II and grade I. Moreover, the values of miRNA-512 were statistically significantly different between mild to moderate dysplastic OPMLs and the non-dysplastic ones; however, miRNA-412 values showed more statistical significance with mild to moderate dysplastic lesions than miRNA512 values.

When the groups were compared, there was a highly significant difference ($P < 0.001$) (table 3).

Sensitivity and specificity for miRNA-412 and miRNA-512. The area under the ROC curve (AUC) of 0.98 for miRNA-512 and 0.995 for miRNA-412 indicate excellent diagnostic accuracy of each marker according to the academic point system (17). (Table 4)

For miRNA-512, the positive predictive value (PPV) of 100 means that 100% of subjects with a positive result have the disease (OSCC); while the negative predictive value (NPV) was 95.2, i.e., 95.2%, of

subjects with a negative result do not have the disease (OSCC). Similar interpretation fits for the 90.9 PPV and 100 NPV for miRNA-412.

The cutoff point was detected using Youden's index to be 0.9000 for miRNA-512 and 0.995 for miRNA-412.

Lastly, the ROC curve delineates the occurrence of the malignant changes in OPMLs with 90% sensitivity and 100% specificity for miRNA-512 (figure 1); and 100% sensitivity and 95% specificity for miRNA-412 (figure 2).

Comparison between miRNA-512 and miRNA-412. No significant difference between both markers (p=0.3785) was discovered (figure 3).

DISCUSSION

Early detection represents a key question that needs to be addressed in almost all diseases; especially early detection of malignant transformation in OPMLs where the lesions with dysplasia have the highest transformation rates (18). Certain miRNAs were revealed to be highly expressed in EVs from patients with OSCC. Differential miRNA expression profiles of OSCC-EVs have been reported from both in-vitro and clinical studies (19)**.**

Hence, as miRNA expression profiles existing for OSCC and normal tissues are distinct, miRNA expression analysis offers an opportunity for early-stage diagnosis of OSCC (9)**.** Consequently, the present study was designed to evaluate the sensitivity and specificity of using the salivary EVs as a non -invasive source of miRNA-412 and miRNA-512 to detect the malignant transformation of oral potentially malignant lesions using qRT-PCR analysis.

In order to achieve the aim of the study, we included patients diagnosed with oral lichen planus, oral lichenoid reaction and oral leukoplakia as they are the most common OPMLs, according to a study on cumulative meta-analysis of malignant transformation of OPMLs (18) and compared their results to patients diagnosed as having OSCC on one side and healthy individuals on the other.

The choice of miRNAs included in the present investigation (miRNA-412 and miRNA-512) was based on the fact that they were previously reported to be overexpressed in salivary EVs from OSCC patients (13). These authors were the first to investigate the usefulness of detecting deregulation of miRNAs in salivary EVs as potential biomarkers in OSCC. As the study was carried out in Italy, it was found of benefit to test such finding among Arabs with OSCC to elucidate the possible generalization of previous conclusions and prove the usefulness of their salivary levels analysis in detecting oral lesions with actual malignant transformation, specially that there has been controversial results presented in other researches, reporting anti-tumor activity of one of these miRNAs in non-small cell lung cancer (12) and hepatocellular carcinoma (11)**.** Furthermore, the present study adds another piece of information by adding a third group of patients with OPMLs in order to assess the ability of the mentioned biomarkers

to distinguish between OSCC and OPMLs, thus helping to point out the malignant transformation within the latter risky lesions.

In the present investigation, we isolated EVs from saliva utilizing the methodology described by (20) and which proved to be efficient for isolation of EVs from biological samples and may be exploited for the search of new biomarkers. In our study, this method had the merit of being simple and avoids the need for expensive equipment. Furthermore, isolated EVs retained their biological activity. In the present study, quantitative real time PCR was chosen because the technique is not influenced by non-specific amplification, while amplification and quantification can be monitored real-time. The technique of qRT-PCR was utilized by many previous works on salivary miRNA detection in OPMLs and OSCC patients (13)**.**

The results of the present investigation prove the findings of previous authors (19) who indicated that salivary EVs could harbor specific molecular biomarkers with potential for diagnosing oral cancer. Also, the present results, which showed an upregulation of miR-412and miR-512 in salivary EVs from OSCC patients, are in accordance with a previous study (13), where the level of miRNA-412 and miRNA-512 expression was significantly higher in OSCC group than the healthy group. The results showed that these miRNAs overexpression might play a pivotal role in OSCC tumorigenesis. Actually, miRNA-412 was considered as therapeutic target for colon cancer in a previous study (21), denoting its importance in disease initiation and progression.

Not only that, but the present results also showed that the higher the degree of dysplasia in OPMLs, the higher the over expression of the 2 markers, with higher significance for miRNA- 412, denoting the capability of the 2 markers to discriminate between OPMLs with different levels of dysplastic changes as equally as they could differentiate between OPMLs and controls on one hand and between OPMLs and OSCC on the other hand.

An additional finding in the present investigation was the increase in levels of both miRNA-412- and miRNA-512 with the increase in the grade of malignancy. This gives the two markers a prognostic value besides their diagnostic efficiency.

To better evaluate the discrimination power of the upregulated miRNAs between malignant lesions and OPMLs, we constructed ROC curves where MiR-512 and miR-412 proved to be both sensitive and specific, as shown by high AUC values (0.986 and 0.995, respectively) and maximum Youden's Index. This, again, was more or less in accordance with the results obtained by previous study (13)**.** Their AUC values were 0.847 and 0.871, respectively. This indicates that the two miRNAs are good predictors and can be suggested as new candidate biomarkers for malignant transformation in OPMLs which can be evaluated through further studies on a larger population. It has been previously stated that a major obstacle to the translation of salivary miRNAs results from laboratory studies into the clinic is the lack of consistency, and that one of the confounding factors is a high degree of inter-individual variability in

the levels of miRNAs, even when focusing only on healthy populations (3)**.** However, this does not appear to be the case with our present results.

Up to our knowledge, our study is the first research concerned with the clinical utility of both markers (miR-412 and miR-512) in detection of malignant transformation of oral potentially malignant lesions. Combining the present results with previous work, the possibility to use salivary EVs as a non-invasive source of miRNAs for OSCC diagnosis and early detection of malignant transformation in OPMLs is quite evident. The two studied miRNAs (miR-412 and miR-512) with their high sensitivity and specificity have the potential to be used as reliable biomarkers. Thus, cell-free miRNA profiles seem to represent a promising noninvasive approach for oral cancer diagnosis, prognosis, and therapeutic targets.

Conclusions. Salivary extracellular vesicle-associated miRNAs-412 and miRNAs-512 show high sensitivity and specificity in the discrimination between malignant lesions and OPMLs, thus they can furnish a reliable screening method for the early detection of malignant transformation in OPMLs.

Conflicts of interest: The authors have no conflicts of interest to disclose.

Ethics. The protocol received ethical approval from the institutional review board with ID number (18-9-5). After discussing the whole procedure with the subjects, an informed consent form was signed by the willing participants. The study steps were held following the Helsinki Declaration and its amendments.

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Authors' contribution. All the authors have substantial contribution in the work:

- Conception and design were performed by N.N.G., A.A.W. and F.M.Z
- Data analysis and interpretation by N.N.G, O.G.S. and F.M.Z.
- Manuscript drafting by N.N.G. and F.M.Z
- All authors have read and approved the final manuscript.

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TABLES.

Table 1. Baseline demographic data of the included participants

	Age		**Sex**	
	Mean	**SD**	**Male**	**Female**
OSCC (n=20)	55.4	10	11	9
OPMLs (n=20)	53.9	8.3	10	10
Control (n=20)	54	9.3	8	12

Table 2. Salivary levels of the 2 miRNAs in all groups studied.

	miRNA-412			**miRNA-512**		
	Mean	**SD**	**95% CI**	**Mean**	**SD**	**95% CI**
OSCC (n=20)	10.6	2.33	9.51-11.69	7.51	2.08	6.54-8.48
OPMLs (n=20)	4.13	1.73	3.32-4.94	2.65	0.77	2.29-3.01
Control (n=20)	1.09	0.14	1.02-1.16	1.07	0.08	1.03-1.1

Table 3. Comparison between salivary values of each marker in the 3 groups.

			Mean Difference*	95% Confidence Interval	
				Lower Bound	Upper Bound
miRNA-512	Control	OSCC	-6.44600	-7.4623	-5.4297
		OPML	-1.58100	-2.5973	-0.5647
	OSCC	Control	6.44600	5.4297	7.4623
		OPML	4.86500	3.8487	5.8813
	OPML	Control	1.58100	0.5647	2.5973
		OSCC	-4.86500	-5.8813	-3.8487
miRNA-412	Control	OSCC	-9.51150	-10.8453	-8.1777
		OPML	-3.03700	-4.3708	-1.7032
	OSCC	Control	9.51150	8.1777	10.8453
		OPML	6.47450	5.1407	7.8083
	OPML	Control	3.03700	1.7032	4.3708
		OSCC	-6.47450	-7.8083	-5.1407

*Scheffe test.

Table 4. Diagnostic accuracy parameters for miRNA-512 and miRNA-412 markers.

	Area under the ROC curve (AUC)*	**95% Confidence interval**	**PPV †**	**NPV‡**	**Sensitivity**	**Specificity**
miRNA-512	0.986	0.915 to 1.000	100	95.2	90%	100%
miRNA-412	0.995	0.931 to 1.000	90.9	100	100%	95%

* Binomial exact

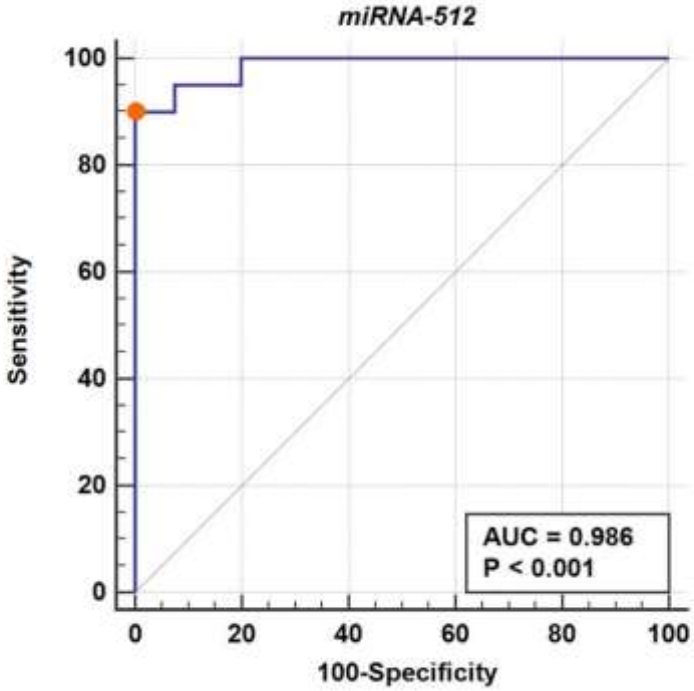
NPV‡, negative predictive value; PPV†, positive predictive value.

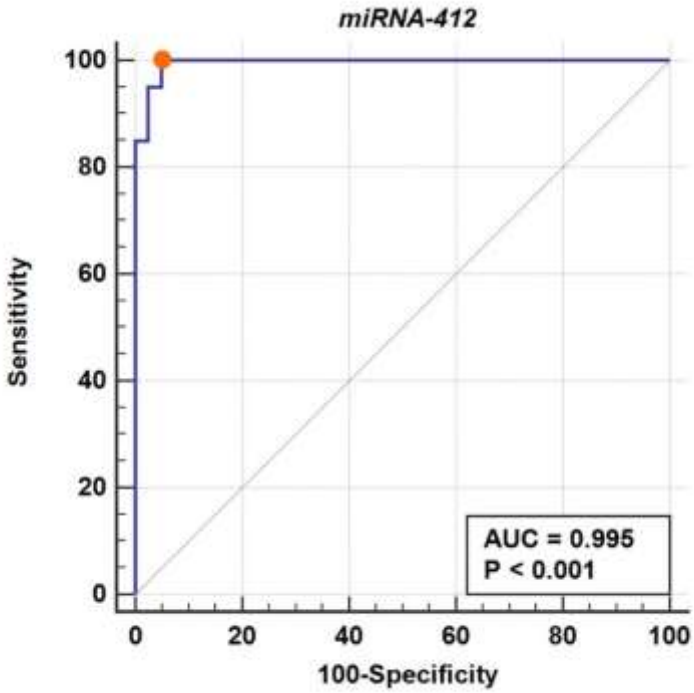
FIGURE LEGENDS

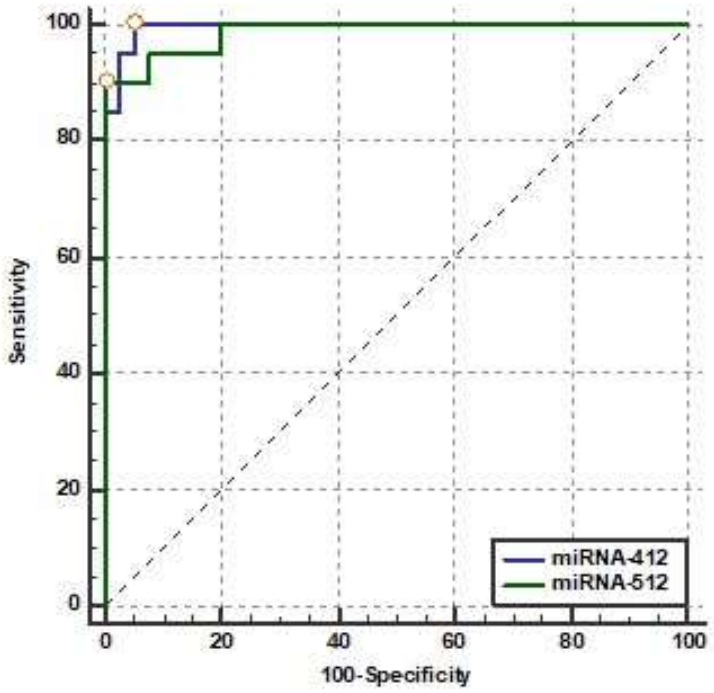
Figure 1. ROC curve for miRNA-512. Cut-off point marked Orange at 0.9 as identified by Youden index.

Figure 2. ROC curve for miRNA-412. Cut-off point marked Orange at 0.95 as identified by Youden index.

Figure 3. ROC curve for miRNA-512 and miRNA-412.






Sensitivity
100-Specificity
0
20
40
60
80
100
miRNA-412
miRNA-512