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MicroRNA-9 expression is a prognostic biomarker for benign angiofibroma and malignant nasopharyngeal tumors

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Abstract
In recent years, microRNAs have been shown to be involved in tumor pathogenesis and regulation of gene expression. The objective of the present study was to examine the expression level of microRNA-9 (miR-9) in 17 angiofibroma, 57 nasopharyngeal carcinoma and 60 control blood samples, using a real-time quantitative assay, and to investigate the relationships between miR-9 expression, clinic pathological features and the prognosis of these patients
Results: A significant decrease in miR-9 expression (P=0.03) was observed in angiofibromas compared to controls and that decrease was significantly associated with clinical staging and complications ($P < 0.05$).Compared to angiofibroma and healthy samples, the expression levels of miR-9 in nasopharyngeal carcinoma were significantly decreased ($P < 0.01$) and were significantly associated with clinical stage and distant metastasis ($P < 0.01$). The Kaplan–Meier curve showed that patients with high miR-9 expression survived significantly longer than patients with low miR-9 expression ($P <0.001$). Furthermore, these levels were significantly higher in post-treatment samples than in pre-treatment samples ($P < 0.01$) in all patients.
Conclusions: The findings of the present study suggested that decreased miR-9 expression has a strong correlation with the clinical staging and progression of nasopharyngeal angiofibroma and carcinoma and its low expression is a significant risk factor affecting cancer patients' survival, suggesting that it could be a valuable marker for prognosis of nasopharyngeal tumors.

Keywords: nasopharyngeal fibroma-carcinoma - MicroRNA-9 prognosis

Introduction
Nasopharyngeal angiofibroma is a fibrovascular benign but aggressive and invasive tumor of the nasopharynx that affects males mainly and shows a destructive growth pattern with bone erosion [1]. Nasopharyngeal carcinoma is a squamous cell carcinoma that tend to present at an advanced stage and 60% of patients develop distant metastasis, hence the need for early screening, diagnostic and prognostic markers. During tumorgenesis, multiple genetic abnormalities disrupt normal cell function thus contributing to nasopharyngeal tumor pathogenesis [2].
MicroRNAs (miRNAs) are small non-coding RNA involved in various types of cancer [3, 6] and could be potential prognostic markers for tumor progression [7, 9].
It was reported that miR-9 is downregulated in colon, ovarian, renal cancers but upregulated in breast, biliary and brain cancers suggesting that it may exert different effects in different types of cancer [10, 17]. However, the clinical significance of miR-9 in angiofibroma as a novel biomarker and nasopharyngeal carcinoma was investigated in the current study.

Methods
This study was approved by the Ethics Committee of Assiut University and informed consent was obtained from all patients. Before and 2 weeks after treatment, 10 ml blood was taken from 17 angiofibroma male patients (mean age: 15 years as it affect mainly teenage males[18], 7 patients were grade I i.e. limited to nasopharynx and 10 were grade III i.e. extending into the orbit) [18], 57 patients with nasopharyngeal carcinoma (staged according to the TNM classification [19],the patients were mainly male (88.2%), with age ranging from 44 to 70 years (mean 60.3 years, in the Middle East,the highest incidence rate is between 41-60 years as reported by a similar study [20], 94.1% of the patients were current or former smokers.) and 60 healthy individuals (volunteers for blood donation)that served as control. The blood samples were taken in EDTA-containing tubes and separated into cellular fractions and plasma by centrifugation at 1500 g for 5 min. 500 μl plasma was mixed with 1.5 ml TRIzol reagent (Invitrogen, USA), incubated for 5 min at room temperature and mixed with 400 μl chloroform. The aqueous phase containing RNA was taken, and RNA was precipitated by addition of 100% ethanol, the mixture was taken to RNeasy Mini spin columns (Qiagen, USA) and washed several times [21].

The expression levels of miR-9 in angiofibroma, nasopharyngeal carcinoma and control samples were detected using a quantitative real-time PCR assay, total RNA from samples was subjected to reverse transcription using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. U6 snRNA was used as an endogenous control, and then PCR was carried out using TaqMan PCR Master Mix kit (Applied Biosystems). The specific primers were as follows: U6 F, 5′-CTCGCTTCGGCAGCACA-3′; U6 R, 5′-

AACGCTTCACGAATTTGCGT-3′ miR-9F, 5′-GTGCAGGGTCCGAGGT, miR-9R 5′-GCGCTCTTTGGTTATCTAGC-3′ [22].

Statistical analysis

SPSS 11.0 statistical software (SPSS Inc, Chicago, IL, USA) was used and the variables were expressed as mean ± standard deviation (SD). To evaluate the differences in miR-9 expression levels before and after treatment, the paired *t*-test was used, patient survival were investigated by the Kaplan–Meier method. $P < 0.05$ was considered statistically significant.

Results

The expression levels of miR-9 in control and angiofibroma (mean ± SD: 7.9 ± 3.73, 5.37 ± 2.3 respectively,*P*=0.03) were significantly elevated compared to nasopharyngeal carcinoma (mean ± SD: 3.33 ± 2.13,P < 0.01, Figure 1), but significantly increased in benign and cancer patients after treatment (mean ± SD: 7.31 ± 1.3,4.33 ± 2.31 respectively, P < 0.01, Figure 2, 3). Statistically significant lower expression was observed in Grade III angiofibroma compared to grade I (Figure 3). The nasopharyngeal fibroma patients were further divided into two groups according to miR-9 expression levels using the mean as a cutoff: low miR-9 expression group (n = 8, mean ± SD: 4.3 ± 1.13) and high miR-9 expression group (n = 9, mean ± SD: 5.3 ± 0.32). Table 1 summarizes the correlation of miR-9 expression levels with clinic pathological features of patients with nasopharyngeal fibroma. It was significantly associated with clinical staging and complications (P <0.05) only.

The nasopharyngeal carcinoma patients were further divided into two groups according to miR-9 expression levels using the mean as a cutoff: low miR-9 expression group (n = 27, mean ± SD: 2.31 ± 1.03) and high miR-9 expression group (n = 30, mean ± SD: 4.32 ± 1.3). Table 2 summarizes the correlation of miR-9 expression levels with clinic pathological features of patients with nasopharyngeal carcinoma. It was significantly associated with clinical staging and distant metastasis (P < 0.01) only. The correlation between miR-9 expression level and survival time of the nasopharyngeal carcinoma patients was evaluated using Kaplan–Meier survival analysis (Figure 4). The curve shows that the patients with high miR-9 expression survived significantly longer than patients with low miR-9 expression (P = 0.001). The analysis of miRNA-9 in nasopharyngeal carcinoma presented a sensitivity of 0.733 (95% CI: 0.67-0.79, Figure 5).

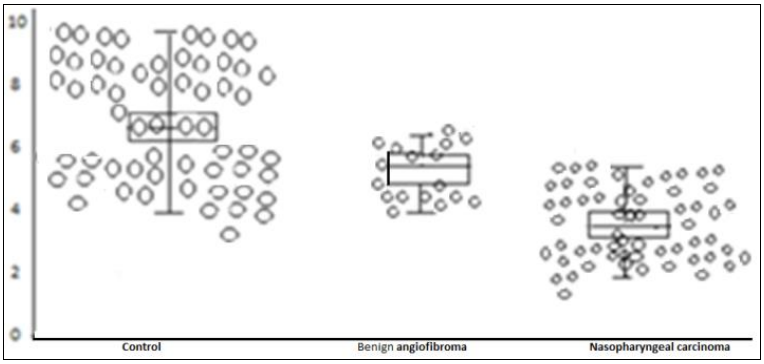

Fig 1: The expression levels of mir -9 in noncancerous sample, benign angiofibroma and nasopharyngeal carcinoma

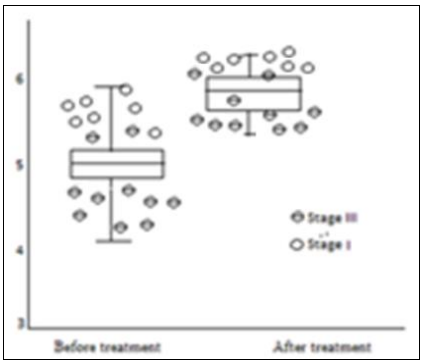

Fig 2: The expression levels of mir -9 in angiofibroma before and after treatment

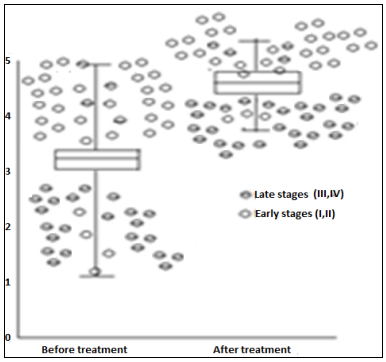

Fig 3: The expression levels of mir -9 in nasopharyngeal carcinoma before and after treatment

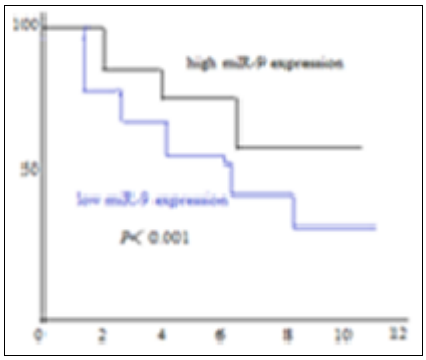

Fig 4: Kaplan –Meier survival curves for nasopharyngeal carcinoma patients with high and low miR -9 expression

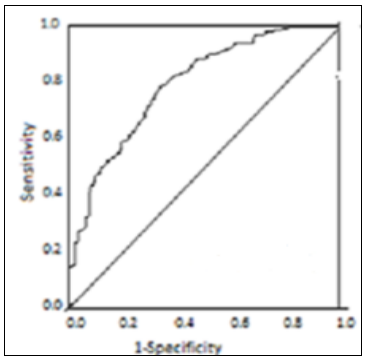

Fig 5: ROC curve of miR-9 in preoperative samples.

Table 1: Correlation of miR-9 expression levels with clinicopathological features of patients with nasopharyngeal fibroma.

		Number of cases (%)	miR-9 expression	
			Low (n, %)	High (n, %)
Age at diagnosis & Gender (mean ± SD)	males only	13.9±3.1 17(100)	8(47)	9(53)
Presenting symptom	Nasal obstruction epistaxis both	5(29.4) 5(29.4) 7(41.2)	3 (60) 4(80) 3(42.9)	2(40) 1(20) 4(57.1)
Staging	Stage I Stage III	7(28.6) 10(71.4)*	-- 8(80)	7(100) 2(20)
Complications	Trismus Palatal perforation Proptosis Transient hemiperesis	-- 2(28.6) 3(42.9) --	-- 1(50) 3(100)* --	-- 1(50) -- --

Table 2: Correlation of miR-9 expression levels with clinicopathological features of patients with nasopharyngeal carcinoma.

		Number of cases (%)	miR-9 expression	
			Low (n, %)	High (n, %)
Gender	Male	54(94.7)**	27(50)	27(50)
	Female	3 (5.3)	--	3 (50.0)
Age	≤60 years	28 (49.1)	13 (46.4)	15 (53.6)
	>60 years	29 (50.9)	14 (48.28)	15(51.7)
Smoking habits	Smoker	55(96.5)**	26(47.3)	29(52.7)
	Nonsmoker	2 (3.5)	1 (50.0)	1 (50.0)
Survival	< 12months	18 (31.5)	18(100)**	--
	>12months	39 (68.5)	9 (23)	30(77)**
Tumor staging	stage I,II(4.1±.2.4)	33 (57.9)	7 (12.1)	26 (87.8)**
	stage III,IV(2. 9±1.49)	24 (42.1)	20 (83.3)**	4(16.7)
Distant metastasis	Present (1.51±1.03)	21 (36.8)	20 (95.2)**	1(4.7)
	Absent (5.2±2.7)	36 (63.1)	7 (19.4)	29(80.5)**

*P<0.05, ** P<0.01

Discussion

To our knowledge, this is the first study to analyze miRNA -9 in angiofibroma, and compares it with angiocarcinoma. miRNAs are involved in various types of cancer by regulating gene expression and play important role in apoptosis and cell differentiation [23, 24]. Many biomarkers showed prognostic value for nasopharyngeal masses and predict the response to treatment. MiR-9 is downregulated in colon, ovarian, gastric, renal and esophageal cancers [25, 30] but overexpressed in brain, biliary tract, and breast and lung cancers [30, 34]. These results suggest that it may play important roles in tumor progression with different effects according to the type of cancer. In the current study, miR-9 expression was decreased in nasopharyngeal fibroma compared with controls; marked downregulation was observed in nasopharyngeal carcinoma, it was significantly correlated with the disease stage. Furthermore, patients with low miR-9 expression had higher incidence of complications as proptosis and palatal perforation in fibroma, a shorter survival rate, distant metastasis in carcinoma than those with higher miR-9 expression suggesting that the level of miR-9 expression may be an independent factor for predicting the prognosis of those patients. Furthermore, these levels were significantly higher in post-treatment samples than in pre-treatment samples similar to previous studies [35, 36].

There are several possible causes for the downregulation of miRNA in benign and malignant nasopharyngeal masses including DNA methylation as in colorectal cancer [12]. Zheng *et al*. identified cyclin D1 as a target of miR-9 in gastric cancer suppressing metastasis, proliferation and invasion [25]. Guo *et al*. reported that miR-9 inhibited ovarian cancer through regulation of NF-κB1 [28].

Conclusions

In conclusion, decreased miR-9 expression has a strong correlation with the aggressive prognosis of both benign and malignant nasopharyngeal tumors and is a statistically significant risk factor affecting overall survival in patients with nasopharyngeal carcinoma; it could be a valuable marker for screening, diagnosis and follow up of these patients. However, the precise mechanism is still not understood.

Acknowledgments

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Conflict of interest: None

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