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THE ROLE OF microRNA-613 AND ITS RELATED GENES IN OVARIAN CANCER

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Ovarian cancer (OC) is the most lethal gynecological cancer. Multiple genetic and epigenetic abnormalities have been detected in ovarian cancers. As microRNAs (miRNAs) play important roles in carcinogenesis, numerous researchers aim to determine the molecular mechanism that regulates the cancer cells proliferation and metastasis. In the current study, the expression of microRNA-613 and related KRAS and Ezrin genes was assessed by Real-time PCR in ovarian cancer tissue and the adjacent apparently normal tissues. Our results revealed that the expression of miRNA-613 was downregulated in ovarian cancer while the expression of KRAS and Ezrin was higher in cancer tissues compared to apparently normal ovarian tissues. There was a negative correlation between the expression of miRNA-613 and both KRAS and Ezrin genes expression and a positive correlation between KRAS and Ezrin gene expression. The results obtained confirm that miRNA-613 acts as a tumor-suppressive gene in ovarian cancer and can realize such impact through the expression of KRAS and Ezrin genes. These data contribute to the identification of potential biomarkers and novel targets for OC early detection and treatment.

K e y w o r d s: ovarian cancer, miRNA-613, KRAS, Ezrin, gene expression.

Ovarian cancer is the leading cause of death in women diagnosed with gynecological cancers [1]. The ovarian cancer is divided histologically as serous, endometrioid (EC, endometrioid carcinoma), mucinous (MC, mucinous carcinoma), with clear cells and squamous cells (CCC for clear cell carcinoma and SCC for squamous cell carcinoma) [2].

Most of the cases are diagnosed at an advanced stage, which leads to poor outcomes of this disease [3]. So the early detection of the disorder is one of the most important steps that facilitate a good prognosis and a good response to the medical therapy for the patients [4].

Early diagnosis is better represented by non-invasive prognostic biomarkers, like non-coding RNAs (microRNAs) [4] as miRNAs have an important role in tumorigenesis, and the involvement of these transcripts in the etiology of cancer is a subject of study for researchers across the world [5].

MiRNAs are non-coding RNA structures, of 19–24 nucleotides, which are never translated into proteins, they are found in circulation, both in serum and plasma, either free or “encapsulated” within small vesicles named exosomes [6]. These short transcripts are occasionally excreted by tumors and thus are able to play diagnostic and prognostic roles regarding the development and progression of tumors [7].

MicroRNA-613 (miR-613) was firstly reported in 2011 that directly target the liver X receptor (LXR) gene and ensure tight regulation of LXR in many metabolic functions [8]. Its function and mechanism of action in biological processes and diseases are not fully understood [9], however growing numbers of evidences reveal that miR-613 may play a significant role in tumorigenesis and is identified as a tumor suppressor in multiple cancers by downregulating oncogenes expression and inhibiting the malignant potential of tumor [8]. For example, miR-613 inhibits KRAS expression by targeting its 3′UTR, indicating that KRAS is a direct target of miR-613. Therefore the overexpression of miR-613 could inhibit KRAS protein expression [9].

KRAS is a member of the RAS superfamily RAS-like GTPase. This protein is important in the RAS/MAPK pathway and regulates several signal transduction pathways [10]. Alterations in

KRAS have been identified in 25% of all cancers. KRAS mutations not only promote and maintain tumorigenesis, but they also increase the chance of resistance and poor prognosis [11] as this alteration increases the ability for GTP-loading and the GTP-binding pocket holds onto its substrate very tightly, making it difficult to displace [12].

On the other hand, overexpression of miR-613 was revealed to reduce tumor invasion, metastasis and angiogenesis through inactivating the AKT signaling pathway [13] as AKT regulates several cellular processes such as cell proliferation, apoptosis and tumorigenesis. AKT activity is increased by overexpression of a transit protein between membrane proteins and actin filaments called Ezrin [14].

Ezrin, a member of the ezrin–radixin–moesin (ERM) family, is not only a key membrane cytoskeletal cross linker, but also involved in signal transduction [15]. Ezrin controls signaling transduction by interacting with adhesion molecules and various growth factor receptors [16]. Ezrin has roles in tumor growth, metastasis, and morphogenesis in cancer biology, as increased Ezrin expression is correlated with poor prognosis in various cancers [17]. So there is considerable rationale for studying the relationship between Ezrin and KRAS and miR-613.

The aim of the current study was to investigate the expression of miRNA-613 and the related genes in ovarian cancer as this may ensure the importance of certain markers in the diagnosis of this tumor.

Materials and Methods

Patients and samples. The current study included 30 Egyptian women, with age range of 26-60 years old (mean 48.6 ± 7.91). Ovarian cancer tissues and apparently normal adjacent tissue specimens (~1 cm from tumor margins) were obtained from the archives of private pathology lab. Specimens of paraffin-embedded tissue were provided for total RNA extraction and miRNA extraction. The clinico-pathological data were obtained from pathology reports of the cases. Patients with other cancers or any targeted treatment were excluded. The study was approved by the Research Advisory Ethical Committee of the Faculty of Medicine, Minia University, Egypt.

The Histopathological study. Five µm sections were prepared and the sections were stained with hematoxylin and eosin (H&E) to be examined by light microscopy for general histopathological study [18].

RNA extraction from formalin-fixed paraffin-embedded tissues. Five to eight µm-thick sections were cut from each block of FFPE tissue, and the samples were deparaffinized with xylene and digested with proteinase K according to RNeasy FFPE kit, deoxyribonuclease (DNase) booster buffer and DNase I stock solution (Qiagen, USA). RNA samples were subjected to RNA quantitation and purity assessment using the NanoDrop® (ND)-1000 s spectrophotometer (Nano Drop Technologies, Inc. Wilmington, USA).

Real-time PCR for miRNA613 expression. The Reverse transcription kit (Qiagen, USA) was used for RNA reverse transcription into cDNA. Real-time PCR was done using (miScript SYBR® Green PCR Kit-code no BIO-218073) according to the manufacturer's protocol. The internal control was human Snord gene. The Primer sequence for amplification of human miRNA 613 gene; Catalog number: MS00005054 (Qiagen, USA). The Primer sequence for amplification of human Snord gene; Catalog number: MS00033712. The relative quantification (RQ) of target genes was determined using the $2^{-\Delta\Delta CT}$ method.

Real-time PCR for KRAS and Ezrin expression. Real-time PCR was performed according to manufacturer instructions (VERSO SYBR Green 1-step PCR ROX MiX KIT (Thermo Cat. # AB4105A). The Primer sequence sets for amplification were as follow: for KRAS gene; forward: 5'-CAAGACAGGTAAGTAACACTGA-3', reverse: 5'-GCAGTACCATGGACACTGGAT-3'. For Ezrin gene; forword: 5'-TGGACGAAGCTGAGACAACC-3', reverse: 5'-GAGTCTGAAGCACTCTCGCA-3'. For GAPDH gene as a housekeeping gene forward: 5'-TAGAGGGGTGATGTGGGGAG-3', reverse: 5'-TCTGCCCCAGACCCTAGAAT-3'.

Statistical analysis. The collected data were statistically analyzed using SPSS program (Statistical Package for Social Sciences) software version 25. Descriptive statistics were done for parametric quantitative data by mean, standard deviation (SD) and minimum and maximum of range and for non-parametric quantitative data by median and interquartile range (IQR), while for qualitative data by frequency and percentage. Correlations between different variables were done using Pearson's correlation coefficients. ROC curve analysis was done to determine AUC, optimal cutoff point, sensitivity,

Specificity of measured variables. The level of significance was set at (P value ≤ 0.05).

Results

Demographic and clinical characteristics of study subjects. The current study was done on 30 ovarian cancer tissues and the surrounding apparently normal tissues were considered as control. Demographic and clinical characteristics of the patients are shown in Table 1.

The histopathological results. The normal histology of the ovary and the pathological changes of ovarian carcinoma are shown in Fig. 1.

Relative change of expression of miRNA-613, KRAS and Ezrin genes by real-time PCR (qRT-PCR). Our results revealed that the expression of miRNA-613 gene was highly significantly decreased (P value < 0.001) in the cancer tissue as compared to non-cancerous tissue while KRAS and Ezrin was highly significant increased (P value < 0.001) in cancer tissue as compared to adjacent normal tissue (Table 2).

Correlation between miRNA-613, KRAS and Ezrin gene expression in ovarian cancer. Our results showed that there was a significant negative correlation between miRNA-613 and both *KRAS* and *Ezrin* gene expression and there was a significant positive correlation between KRAS and Ezrin expression in ovarian cancer (Table 3).

ROC curve analysis. In ovarian cancer, the best cutoff value of miRNA-613 was 0.98 with a sensitivity of 96.67 and 100% specificity, the best cutoff value of KRAS was 1.4 with a sensitivity of 94.34 and 95% specificity and the best cutoff value of Ezrin was 1 with sensitivity 93.33 and 96% specificity.

Discussion

Ovarian cancer is common gynecologic cancer that has a heterogeneous nature which complicates its early detection and primary prevention. The high mortality rate is mostly due to the detection of cancer in the late stages of its progression [19]. Most patients are diagnosed at an advanced stage due to the lack of early symptoms and available diagnostic techniques [15]. Therefore, understanding the molecular mechanism that regulates the proliferation and metastasis of ovarian cancer cells is crucial.

In recent years, the role of miRNAs in human cancer has become a hotspot in the research of oncology and many miRNAs have been identified as

Table 1. Demographic and clinical characteristics of all subjects in the study

Age	Range (mean ± SD)	(26-60) 48.6 ± 7.91
Side (%)	RT	7 (23.3%)
	LT	5 (16.7%)
	Bilateral	18 (60%)
Laterality (%)	Unilateral	12 (40%)
	Bilateral	18 (60%)
Grade (%)	G II	19 (63.3%)
	G III	11 (36.7%)
Stage (%)	IIA	14 (46.7%)
	IIC	5 (16.7%)
	IIIA	8 (26.7%)
	IIIC	3 (10%)
Total		30

biomarkers for tumor diagnosis, prognosis and potential target for novel genetic therapies [8].

In the current study, the expression of miRNA-613 is assessed in ovarian cancer tissue. In the human genome, miR-613 gene is located on chromosome 12 at 12p13.1 and increasing numbers of evidences reveal that miR-613 may play a significant role in tumorigenesis [8]. Our results revealed that the expression of miRNA-613 was downregulated in ovarian cancer compared to non-cancerous tissue confirming what was found previously as the level of miR-613 is decreased in colorectal cancer (CRC) [20], hepatocellular carcinoma (HCC) [21], gastric cancer (GC) [22], non-small cell lung cancer (NSCLC) [23], breast cancer (BC) [24].

Although miR-613 is known to be a tumor suppressor gene, it has distinct functions in specific cancers. For example; miR-613 may suppress hepatocellular carcinoma via targeting YWHAZ [25] and inhibit migration and invasion of esophageal carcinoma by targeting glucose 6 phosphate dehydrogenase [26]. Therefore, the underlying mechanism of miR-613 in different cancer requires further investigations.

Previously, it was demonstrated that miR-613 inhibited KRAS expression by targeting its 3′UTR, indicating that KRAS is a direct target of miR-613 [27] and KRAS overexpression was shown to reverse the effects of miR-613 on ovarian cancer cell

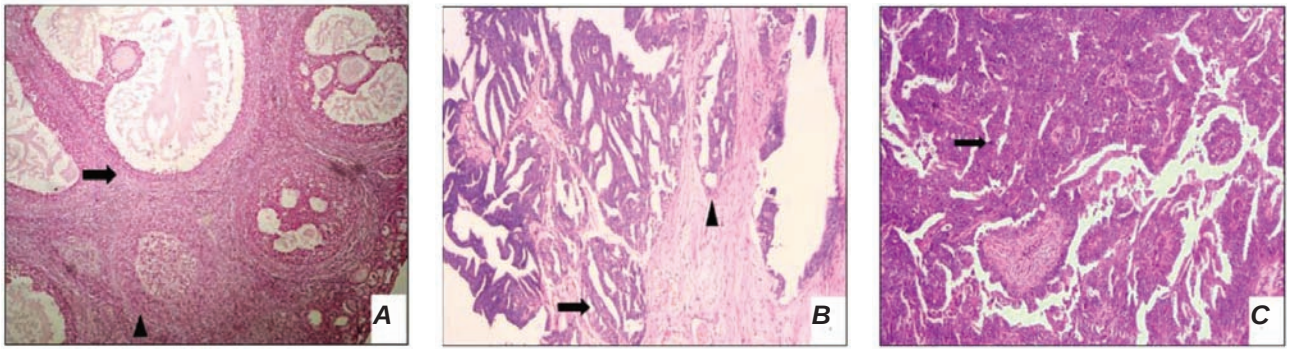

Fig. 1. ***A*** *– Sections in normal adjacent tissues showed ovarian cortex with multiple ovarian follicles in variable maturation stages. Mature ovarian follicle with bilayer lining (granulosa and theca cells) (arrow) surrounded by cellular stroma (arrow head).* ***B*** *– Section in serous ovarian carcinoma grade II showing solid nests admixed with both papillary and micro papillary invading the ovarian stroma (arrow). The neoplastic cells showing stratification, cellular and nuclear pleomorphism with moderate nuclear atypia. Surrounded by desmoplastic stroma (arrow head).* ***C*** *– Section in Serous ovarian carcinoma grade III showing solid masses of columnar to cuboidal cells with eosinophilic cytoplasm and slit-like spaces (fusion pf papillae) (arrow). Significant atypia, significant nuclear pleomorphism with large bizarre multinucleated forms and prominent nucleolus (H&E X100)*

Table 2. The median value of miRNA-613 KRAS and Ezrin gene in ovarian tumor tissue and adjacent normal tissue according to RQ value

		Control (adjacent normal tissue), $n = 30$	Cases (tumor tissue), $n = 30$
miRNA-613	Median IQR	1 (0.9-1.1)	0.4 (0.3-0.7)
KRAS	Median IQR	1 (0.85-1)	2.3 (1.6-5.2)
Ezrin	Median IQR	1 (0.9-1.2)	3.8 (1.7-5.8)

Mann Whitney test for non-parametric quantitative data between the two groups, $P < 0.001$

proliferation and invasion in human ovarian cancer cell lines.

In the assesment of KRAS gene expression, our results revealed that the expression of KRAS was up-regulated in ovarian cancer tissue compared to non-cancerous tissues. This was in consistent with the previous results which showed that KRAS mRNA was highly expressed in melanoma tissues compared to adjacent non-tumor tissues [28] and also KRAS overexpression is associated with lung cancer progression and with poor prognosis [29] confirming

Table 3. Pearson's correlation between miRNA-613, KRAS and Ezrin gene expression

	miRNA-613		KRAS	
	r	*P* value	*r*	*P* value
KRAS	-0.648	<0.001	-------	-------
Ezrin	-0.603	<0.001	0.819	<0.001

the hypothesis that KRAS amplification promotes cancer progression because it may be strongly associated with down-regulation of mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene (PI3K/AKT) pathways which further results in excessive cell proliferation and subsequently carcinogenesis [30].

A large number of evidence has demonstrated that a variety of human ovarian cancer cell lines have an abundant expression of *Ezrin* gene [31] so in this work, the expression of *Ezrin* gene in human ovarian carcinoma tissue was studied to enrich our understanding of this important molecular pathway.

Since both breast and ovarian carcinomas exhibit a similar ability to proliferate due to malignant effusion formation, the significant increase of Ezrin serves as a future therapeutic intervention target [17]. Ezrin promotes breast cancer progression and enhances metastasis through Akt signaling [14] and pharmacological inhibition of Ezrin has significant-

ly reduced cancer cell invasion and migration to the lymph nodes and lungs [32].

Our results revealed that the expression of *Ezrin* gene was highly significantly increased in ovarian cancer tissue compared to adjacent apparently-normal tissues. Köbel M. et al. reported that lacking of Ezrin may predict an improved prognosis of ovarian carcinoma as it may be necessary for tumor cell invasion [33]. This is consistent with the previous study which done on ovarian cancer cell lines SKOV3 and CaOV3 and demonstrated that the upregulated expression of Ezrin significantly promoted cell growth, EMT, metastasis, and invasiveness [15].

The Ezrin expression was associated with bad prognosis in a cancer type-specific manner [34]. Although Ezrin is known associated with poor prognosis in several cancers [35], however in few cases such as bladder cancer, higher Ezrin expression indicates better prognosis rather than worse [17].

Our results revealed that there is a significant negative correlation between miRNA-613 and *Ezrin* gene expression and a positive correlation between KRAS and Ezrin expression in ovarian cancer suggesting that miR-613 may regulate expression of both KRAS and Ezrin or they are contributing to the pathogenesis of ovarian carcinoma through a common pathway.

Although the pathogenesis of ovarian cancer is well recognized, however, some issues still hinder a full understanding of the complexity of this disease so better understanding of molecular alterations in ovarian carcinoma is important to identify novel targets for early detection and advanced treatment.

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Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

ЕКСПРЕСІЯ мікроРНК-613 ТА ВІДПОВІДНИХ ГЕНІВ ЗА РОЗВИТКУ РАКУ ЯЄЧНИКІВ

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Рак яєчників є одним із найбільш летальних гінекологічних онкологічних захворювань. За раку яєчників виявлено численні генетичні та епігенетичні аномалії. Оскільки мікроРНК відіграють важливу роль у канцерогенезі, багато дослідників націлені на визначення молекулярного механізму, який регулює проліферацію та метастазування ракових клітин. У даному дослідженні оцінювали експресію мікроРНК-613 та відповідних генів *KRAS* і *Ezrin* у тканині раку яєчників та прилеглих до неї, очевидно, нормальних тканинах методом ПЛР в реальному часі. Наші результати показали, що експресія мікроРНК-613 була знижена за раку яєчників, у той час, як експресія *KRAS* і *Ezrin* була вищою в ракових тканинах порівняно з нормальними тканинами яєчників. Виявлено негативну кореляцію між експресією мікроРНК-613 та експресією генів *KRAS* і *Ezrin*, а також позитивну кореляцію між експресією генів *KRAS* і *Ezrin*. Встановлено, що мікроРНК-613 діє як пухлино-супресивний ген за раку яєчників і може реалізовувати такий вплив через експресію генів *KRAS* та *Ezrin*. Отримані результати допоможуть визначити потенційні біомаркери та нові мішені для ранньої діагностики та лікування раку яєчників.

Ключові слова: рак яєчників, мікроРНК-613, KRAS, Ezrin, експресія генів.

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