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Deciphering the unique microRNA signature in human esophageal adenocarcinoma: dissecting esophageal adenocarcinoma progressive profile and its distinctions from gastric cancer

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A Thesis submitted to the

Biotechnology Graduate Program

in partial fulfillment of the requirements for

the degree of Master of Science

By Rama Ahmed Saad Ahmed Ali

(under the supervision of Dr. Wael El-Rifai and Dr. Ari J. S. Ferreira)

May 2013

The American University in Cairo

School of Sciences and Engineering (SSE)

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Has been approved by

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Thesis Supervisor

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Date _____

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Thesis first Reader

Affiliation:

Date _____

[Name of second reader] _____

Thesis Second Reader

Affiliation: _____   Date _____

Department Chair _____   Date _____

Dean _____   Date _____

DEDICATION

THIS WORK IS DEDICATED TO MY BELOVED FATHER AND BROTHER. WITHOUT THER SUPPORT, WISDOM, AND UNDERSTANDING, I WOULDN'T HAVE BEEN ABLE TO ACHIEVE MY AIM. I ALSO DEDICATE THIS WORK TO MY BELOVED MOTHER AND ALL OTHER CANCER VICTIMS WHO LOST THEIR PRECIOUS SOULS WHILE FIGHTING THIS DEVASTATING DISEASE.

ACKNOWLEDMENTS

The American University in Cairo

Deciphering the Unique MicroRNA Signature in Human Esophageal Adenocarcinoma: Dissecting EAC Progressive Profile and its Distinctions from Gastric Cancer

By Rama Ahmed Saad Ahmed Ali

Under the Supervision of Dr. Wael El-Rifai and Dr. Ari Ferreira

ABSTRACT

Background and Methods: Esophageal adenocarcinoma (EAC) is characterized by its dramatic increase in incidence rates in the US and European countries. The specific miRNA signature that would stratify EAC from other upper gastrointestinal cancers remains not elucidated. In an attempt to elucidate the unique miRNA signature in EAC, I performed a comprehensive microarray profiling for the specific miRNA signature present in EAC. This signature was subjected to independent validation by qRT-PCR. **Results:** 21 miRNAs overlapped between 2 different microarray platforms to be dysregulated in EAC. 9 miRNAs were selected for validation in 46 normal squamous (NS), 23 Barrett's esophagus (BE), 17 Barrett's high grade dysplasia (HGD), 34 EAC, 33 gastric adenocarcinoma (GC), and 45 normal gastric (NG) tissues in order to identify the miRNAs that are specifically unique for EAC. Upon validation by qRT-PCR, 2 miRNAs (miR-21 and miR-133b) were similarly dysregulated in EAC and GC. On the other hand, 6 miRNAs (up-regulated: miR-194, miR-31, miR-192, and miR-200a; down-regulated: miR-203 and miR-205) displayed a dysregulation pattern specific to EAC, as compared to BE rather than GC. This suggests their underpinning specific role in EAC. Results have indicated the over-expression of miR-194, miR-192, miR-21, and miR-31 BE adjacent to HGD lesions as compared to isolated BE samples. Clinicopathological features were found to be associated with differential expression of miR-203, miR-194, miR-200a, and miR-192. The expression levels of miR-203 have exhibited further dysregulation with EAC progression. On the other hand, the expression levels of miR-194, miR-200a, and miR-192 have dropped significantly in late EAC stages, suggesting that these miRNAs may be involved in EAC tumor development rather than progression. **Conclusion:** These data highlight the EAC specific miRNA signature. This indicates evidence for the unique molecular perturbations underpinning EAC that can be employed as disease biomarkers. Further studies using larger cohorts of patients are required for further validation[1].

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[1]This abstract is modified from (Saad et al., 2013). The data in this thesis was published in PloSOne. The student (Rama Ahmed Saad Ahmed Ali) is the first author, and thereby retains the copyright of the figures in the paper. The copyright license is at Appendix G.

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LIST OF ABBREVIATIONS

UGC: Upper gastrointestinal cancers

BE: Barrett's esophagus

NS: Normal squamous epithelia

CLE: Columnar lined esophagus

GERD: Gastroesophageal reflux disease

HGD: High grade dysplasia

GC: Gastric adenocarcinoma

NG: Normal gastric tissue

miRNA: MicroRNA

qRT-PCR: Quantitative Real Time- Polymerase Chain Reaction

EGJ: Esophageal gastric junction

BMI: Body mass index

H. Pylori: *Helicobacter pylori*

OR: Odds ratio

LGD: Low grade dysplasia

H & E: Hematoxylin & Eosin staining

THE: Transhiatal esophagectomy

5-FU: 5-Fluorouracil

C. elegans: Caenorhabditis elegans

DGCR8: DiGeorge syndrome critical region 8

ADARs: adenosine deaminases

RISC: RNA induced silencing complex

miRISC: miRNA-RISC complex

UTR: Un translated region

ORF: open Reading Frame

CLL: Chronic lymphoblastic leukemia

FFPE: Formalin-Fixed Parrafin-Embedded

ER: Estrogen receptor

HER2: Human epidermal growth factor receptor 2

PR: Progesterone receptor

EMT: Epithelial mesenchymal transition

HCC: Hepatocellular carcinoma

TKI: Tyrosine kinase inhibitors

PTEN: Phosphatase and tensin analogue

PDCD4: Programmed cell death 4

ANXA1: annexin A 1

IGF1R: Insulin-like growth factor 1 receptor

SNP: Single nucleotide polymorphism

DNMT1: DNA methyl transferase 1

CTs: threshold cycles

RFU: Relative florescence unit

FSCN1: Fascin homologue 1

AFE: Agilent Feature Extraction:

FDR: False discovery rate

FC: Fold change

CGH: Comparative genomic hybridization

TNF: Tumor necrosis factor

TRAIL: TNF-related apoptosis-inducing ligand

MSI-H: Microsatellite instability-high

APC: Adenomatus polyposis coli

ERBB2: Erythroblastic leukemia viral oncogene homologue 2

CHAPTER 1:-LITERATURE REVIEW

1.1 : Esophageal Adenocarcinoma

1.1.1:- General Description and Etiology

Esophageal adenocarcinoma is a type of upper gastrointestinal cancers (UGCs) that involves the malignancy initiated in a glandular tissue that is not an original part of the esophagus (Chen et al., 2011; DeMeester, 2006). Barrett's esophagus (BE) is the premalignant status that precedes EAC. BE is associated with the substitution of the normal squamous (NS) epithelium of the esophagus with columnar epithelium, and thus it is also known as columnar-lined esophagus (CLE). BE is predominantly caused by chronic acid reflux from the stomach which in turn triggers the transformation of the cells located in the distal part of the esophagus (Chen & Yang, 2001; DeMeester, 2006). Typically BE is recognized to harbor columnar tissue. Accordingly, BE can be technically described with the presence of a columnar tissue of any size (Flejou, 2005). BE is classified into three histological types: gastric-fundic type epithelium, gastric-junctional type epithelium, specialized or intestinal type epithelium. The intestinal type BE is the histological type associated with the risk of development of EAC. BE develop from the exposure of the NS epithelium to gastric and bile acids refluxates, a condition known as gastroesophageal reflux disease (GERD), which results in the replacement of the NS epithelium with columnar tissue which in turn able to resist the reflux (Flejou, 2005).

1.1.2:- Epidemiology and Risk Factors

EAC exhibit high incidence rate in Western populations and industrial countries. The incidence rate of EAC has escalated by 8 times from 1973 to 2006 (Pohl, Sirovich, & Welch, 2010) (Figure 1). In US, the incidence rate of EAC has escalated throughout the past three decades to affect approximately 10000 people per year and constitute > 50 % of total esophageal cancer cases. Similarly, the United Kingdom, France, Switzerland, Scandinavia, New Zealand, and Australia reported increased incidence rates of EAC (Melhado, Alderson, & Tucker, 2010).
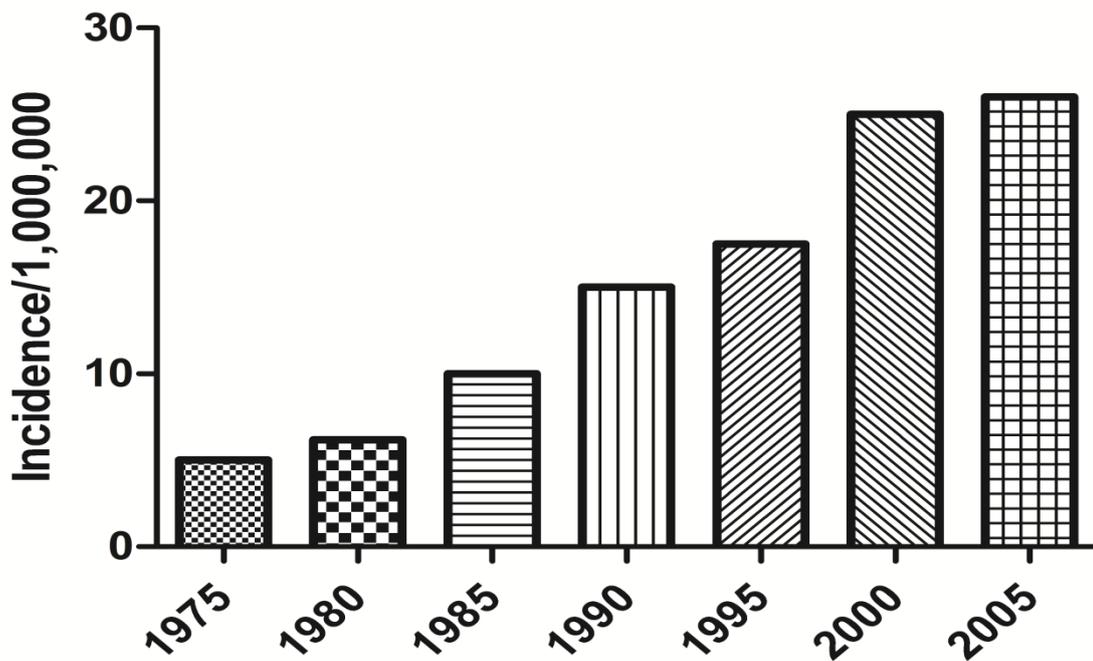
Figure 1: The overall escalation of EAC incidence rates. The figure depicts the escalating rates of EAC in western population. Overall esophageal adenocarcinoma incidence increased from 3.6 per million in 1973 to 25.6 per million in 2006.

In US, the incidence rate of EAC has witnessed an increase among white males by 350 % as compared to the mid-1970s of adenocarcinoma of the esophagus, and thereby squamous cell carcinoma in 1990 (Pera, Manterola, Vidal, & Grande, 2005). Additionally, black males had an increased incidence rate of EAC but in general their incidence is much less than others. White people have higher incidence of developing EAC than black people where it was reported that the incidence in black people constitute 30% of the total incidence in white people (Blot, Devesa, Kneller, & Fraumeni, 1991). Older men have displayed higher incidence of EAC than younger men, where most EAC cases are above 50 years old. Furthermore, Devesa et al demonstrated the doubled incidence of EAC in males below 65 years, and 3 to 4 times increase of EAC cases in people above the age 65 years (Devesa, Blot, & Fraumeni, 1998). Despite the increase in the incidence rates in females, the total number of incidence among females is 7 times less than that in males (Pera et al., 2005).

The incidence rate of EAC has increased in several European countries. For instance, in Denmark the age-standardized incidence of EAC per 100,000 (persons − year) have increase from 4.7 in 1978 to 6.6 in 1992 (Botterweck, Schouten, Volovics, Dorant, & van Den Brandt, 2000). Similarly in Italy, age-standardized incidence has increased from 2.3 in 1975 to 5.2 in 1992 (Melhado et al., 2010). Slovakia has witnessed a sharp increase in the incidence of EAC where the incidence has escalated from 0.7 in 1969 to 3.3 in 1992. In England and Wales, the age-standardized incidence has increased from 2.6 in 1969 to 7.7 in 1992. In Scotland, the incidence has almost tripled in 1995 as compared to 1975 (Melhado et al., 2010).

Although several risk factors are well known to be associated with EAC such as alcohol, some dietary factors, tobacco, some medications, obesity and infection with *Helicobacter pylori,* GERD remains the major factor predisposing to EAC (Chen & Yang, 2001; DeMeester, 2006; Holmes & Vaughan, 2007). The firm association between GERD and EAC has been laid down. At least twofold risk for adenocarcinoma was associated with a preceding history of GERD, with a directly proportional relationship between GERD and EAC development (DeMeester, 2006; Holmes & Vaughan, 2007; Pera et al., 2005). Furthermore, a population-based, case-control study that was conducted in Sweden pointed out to a firm association between GERD and EAC (Green, Amaro, & Barkin,

2000). The odds ratio in EAC patients with GERD history was 7.7 whereas as for patients with no preceding GERD, the odds ratio was 2 (Green et al., 2000). Additionally, the severity, duration, and frequency of the symptoms are associated with higher EAC risk. Severe and long-term symptoms are associated with odds ratio of 45.5 for EAC. GERD is well-recognized as a widespread condition in the general population. 15-20% of the adults suffer GERD every week. More than 10% of the population is expected to develop reflux symptoms with EAC incidence of 2.3/100,000 per year (Holmes & Vaughan, 2007).

BE develops secondary to the long-term continuous exposure of NS esophageal tissue to gastric refluxate. BE is defined as the metaplastic substitution of NS tissue with columnar intestinal-type epithelium. BE represents a central risk factor for developing EAC (Flejou, 2005). Almost all EAC cases were preceded by BE. 3-7 % of GERD patients subject to endoscopic examination were diagnosed with BE. The trend of BE has escalated by 28 folds throughout the past 30 years. In Scotland, 1.4 BE cases were reported per 1000 endoscopic procedures in the years of 1980 and 1981 (Prach, MacDonald, Hopwood, & Johnston, 1997; Botterweck et al., 2000). These incidence increased up to 42.7 per 1000 endoscopic procedures in 1992 (Pera et al., 2005). BE is more prevalent in males than females with ratio 2:1, and thereby the incidence of Barrett's adenocarcinoma has increased in males with ratio 3:1 as compared to females. The same pattern is implied in the mean age of BE diagnosis in males, where males are diagnosed with BE at a mean age of 62 years while females are diagnosed at an average age of 67.5 years. Additionally, mean age of EAC diagnosis in males is 64.7 years whereas that in females is 74 years (Zheng et al., 1993). This 20 years gap between BE and EAC diagnosis in females might underlie the lower incidence of EAC in females. Most BE conditions are not diagnosed.

Approximately 1 million of the population in the USA is expected to get BE (Cameron, 1993). Usually BE conditions are not detected unless the patient was subject to endoscopic diagnosis or the case have progressed to EAC. Among all EAC cases, GERD symptoms is of similar frequency in cases with or without BE. 62% of EAC suffered BE, and it constituted <1% of asymptomatic individuals and 3-7% of population with GERD that didn't progress to EAC. BE patients are expected to develop EAC with a risk of 1% per year (Cameron & Romero, 2000).

Smoking is well known to be a risk factor for EAC. Upon conducting a population-based, multicenter, case-control study, smoking was reported to double the risk of EAC development (Pera et al., 2005; Gammon et al., 1997). Furthermore, a dose-dependent effect was found to be associated with the risk among smokers. However, smoking cessation didn't result in a significant decrease in EAC risk before 30 years of quitting (Gammon et al., 1997). On the other hand, a case-control study conducted in Sweden indicated that a strong dose-dependent association between smoking and EGJ carcinoma with OR 4.2 and incidence rates 2.5-7 between heavy smokers and never-smokers (Lagergren, Bergstrom, Lindgren, & Nyren, 2000). Furthermore, it was inferred from this study that smoking is not associated with EAC risk.

Western population display high levels of obesity which in turn renders them predisposed to several cancers including EAC, gastric, postmenopausal breast and colorectal cancer. Several studies have documented the association between obesity and EAC. There are several theories for mechanism by which obesity results in EAC. The most well-recognized mechanism is that obesity attributes to EAC through increasing the intra-abdominal pressure, and thereby promoting GERD which in turn results in BE and EAC (Kuczmarski, Flegal, Campbell, & Johnson, 1994). However, 2 studies have reported that role of obesity in the development of EAC, and EGJ with no GERD association. A population based multicenter, case-control study has shown that the body mass index (BMI) display a directly proportional relationship with the risk for EAC. This relationship was more apparent with young age groups (<50 years) presenting a legitimate potential that BMI might be associated with the early onset tumor development whereas other risk factors are central to tumor developing in relatively older ages (Engel et al., 2003). Furthermore, a BMI above the lowest quartile is reported to be associated with an EAC risk of 41.1%. On the other hand, overweight was associated with increased risk for EGJ carcinoma rather than EAC. The combination of GERD and increased BMI results in multiplicative increase of the risk for EAC and EGJ (Brown et al., 1995). Obese individuals (BMI >30 kg/m^2) suffering reflux have OR of 179.2 for EAC, and 12.2 for EGJ as compared to non-obese persons not suffering reflux (Engel et al., 2003).

Dietary components are well-recognized to be associated with esophageal squamous cancer rather than EAC. Few studies have addressed the association between dietary factors and EAC. Case-control studies reported fatty foods as strong risk factors for EAC and EGJ. In addition, vegetables, fruits, niacin, b-carotene, lutein, folate, iron, vitamins B12, B6, and C, and Zinc are protective against EAC and EGJ (Zhang et al., 1997). Low intake of fruits is associated with EAC. High intake of antioxidants such as vitamin C, alpha-tocopherol, and beta-carotene scavenge the free radicals and thereby prevent their DNA damaging effects (Brown et al., 1995; Zhang et al., 1997). High intakes of antioxidants resulted in decreased risk to EAC and EGJ. Cereal fiber is known to be a scavenger for nitrites. Low serum selenium is associated with a risk for EAC and EGJ. Selenium is known to have anti-carcinogenic actions and reduced risk of EAC (Rudolph et al., 2003).

Long-term administration of medications that relax the lower esophageal sphincter, such as anticholinergic drugs, is associated increased risk for GERD and eventually, EAC. The chronic administration of these medications was associated with an OR of 3.8 and 95% incidence as compared with individuals who never took these drugs (Eisen, 2000). Medications-mediated EAC was reported to weigh 10% of all EAC cases (Pera et al., 2005). Some drugs such as aspirin and anti-inflammatory drugs are associated with a 50-90% decrease in the EAC risk (Corley, Kerlikowske, Verma, & Buffler, 2003; Hur, Nishioka, & Gazelle, 2004).

Cag A$^+$ strains *of Helicobacter pylori* are associated with reduced risk for EAC and EGJ as compared to Cag A$^-$ strains. It is generally speculated that the increased rates of EAC and EGJ in western population is partially attributed to the lower incidence of *H. pylori* infection (Boulton-Jones & Logan, 1999).

1.1.3:- Pathogenesis

The development of EAC is a complicated multi-step process that requires the interaction of several genetic and epigenetic perturbations. A typical scenario for EAC development is that prolonged exposure to gastric acid or bile acid through gastric or duodenogastric reflux respectively results in inflammation-stimulated hyperplasia, followed by multifocal dysplasia, carcinoma in-situ and eventually invasive adenocarcinoma (Chen & Yang, 2001; Flejou, 2005; Holmes & Vaughan, 2007). Upon prolonged exposure to gastric &/or bile acid, the highly sensitive normal squamous epithelium is substituted by columnar epithelium (i.e. transformed to BE). There are several theories proposing the mechanism of BE development. The most acceptable one is that the metaplastic transformation of the pluripotent stem cells in the basal layer upon prolonged exposure to gastric or bile acid (Flejou, 2005). Another theory is that columnar cells from the gastric cardia substitute the NS epithelia. Furthermore, the propagation of the columnar cells from the esophageal duct has been proposed as an underlying mechanism for BE transformation. The first mechanism is regarded as the most predominant among BE patients (Chen & Yang, 2001).

One of the major characteristics of BE is that being histologically indistinguishable from gastric intestinal metaplasia type II or III. BE encompasses several types of columnar-cell types such as gastric-type small intestinal-like, endocrine, Paneth and goblet cells (Spechler, 2002). Goblet cells contain acidic mucins such as sulfomucins and sialomucins, and thereby are stained with Alcian blue. In addition, goblet cells also harbor colonic-like mucins that can be visualized upon high-iron diamine staining (Kim & Ho, 2010). Dysplasia, the pre-neoplastic stage preceding EAC, results from a series of genetic aberrations and molecular perturbations. These alterations result in uncontrolled growth

and morphological abnormalities that characterize dysplasia. Thereby, in a broad sense, dysplasia can be defined as the accumulation of histological defects caused by several interacting molecular alterations. The histological aberrations are classified as cytological changes and architectural changes (Spechler, 2002). Cytological changes involve pleomorphism, stratification, nuclear enlargement, loss of cytoplasmic maturation, atypical mitosis, and hyperchromatism. Architectural changes comprise the crowding of tubules and villiform surfaces. The presence of these changes indicates the persistent genetic damage that causes cells to undergo clonal proliferation associated with abnormal differentiation. According to the severity of both cytological and architectural changes, dysplasia can be graded as low or high grade (Warnakulasuriya, Reibel, Bouquot, & Dabelsteen, 2008).

Metaplastic cells undergo malignant transformation upon either the deactivation of tumor suppressor genes &/or the activation of proto-oncogenes. Molecular aberrations in the tumor suppressor genes p53 and p16, as well as, the proto-oncogene cyclin D1 result in the malignant progression of BE to EAC (Flejou, 2005). Diploid progenitor cells develop mutations in these genes, which in turn result in clonal expansion and proliferation. The proliferation of these cells produces daughter cells with further mutations including the loss of heterozygosity at chromosomes 13q, 5q, and 18q (Dolan et al., 1998). Following the neoplastic division of these autonomous cells that are subject to continuous acquiring of mutations, a clone of cells evolve with the ability to invade nearby tissues, and later on metastasize (Flejou, 2005).

The bile acid reflux promote the transformation of BE to carcinogenesis. Continuous exposure of BE to bile acid triggers DNA damage as well as the activation of the NF-κB pathway. While DNA damage may embark apoptosis, the activation of NF-κB inhibits apoptosis, and thereby promotes the survival of mutated progenitor cells (Chen & Yang, 2001; Spechler, 2002).
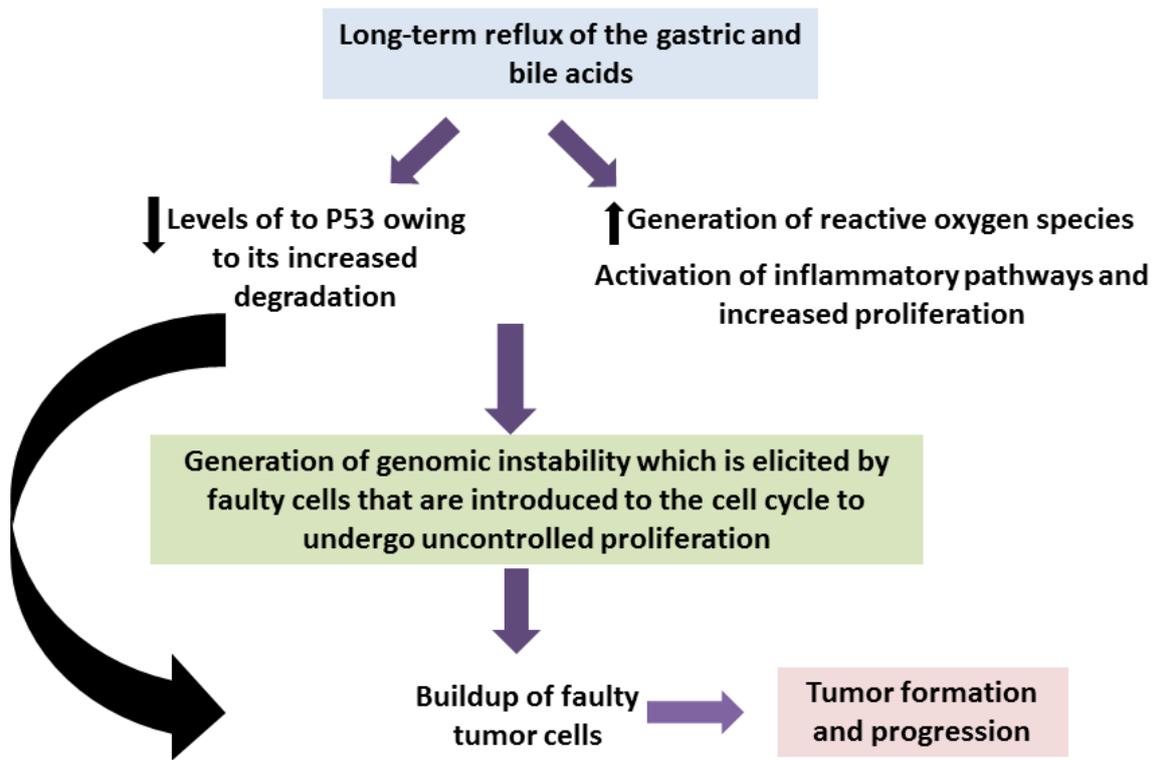
Figure 2: A flowchart demonstrating the pathogenic progression from BE to EAC.
Chronic exposure to gastric and bile acids results in the activation of inflammatory pathways, and the failure of cell cycle arrest. Both events result in the massive buildup of tumor cells, and tumor formation.
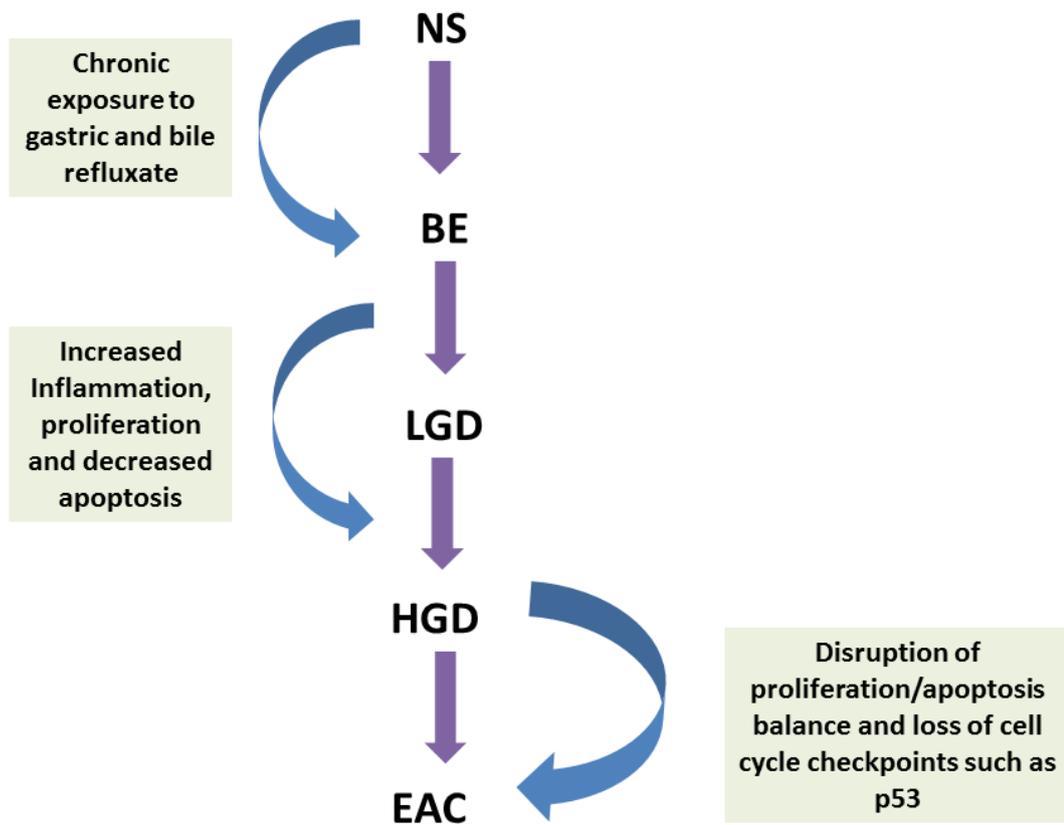
Figure 3: The pathogenesis of EAC. Continuous exposure of normal squamous epithelium results in its transformation to BE, and later on to dysplasia. Further exposure to refluxate will allow for the progression from dysplasia to EAC.

1.1.4:- Diagnosis, Staging, and Prognosis

The asymptomatic nature of EAC development presents a big challenge for early diagnosis of EAC. Usually EAC is diagnosed by means of endoscopic and histological examination (Bergman, 2006)). Endoscopic examination usually detects EAC easily since the tumor is diagnosed at late stage where the tumor mass and lumps are obvious (Spechler, 2002). Upon hematoxylin and eosin (H&E) staining, the cancerous tissue appears as dark blue tissue with cellular clusters that are assembled as cohesive glands. In addition, cells will display the typical morphology of malignant cells such as altered nuclear size, and shape. Usually NS epithelium appears as a pink color cells lining cancerous tissue (Waterman et al., 2004).
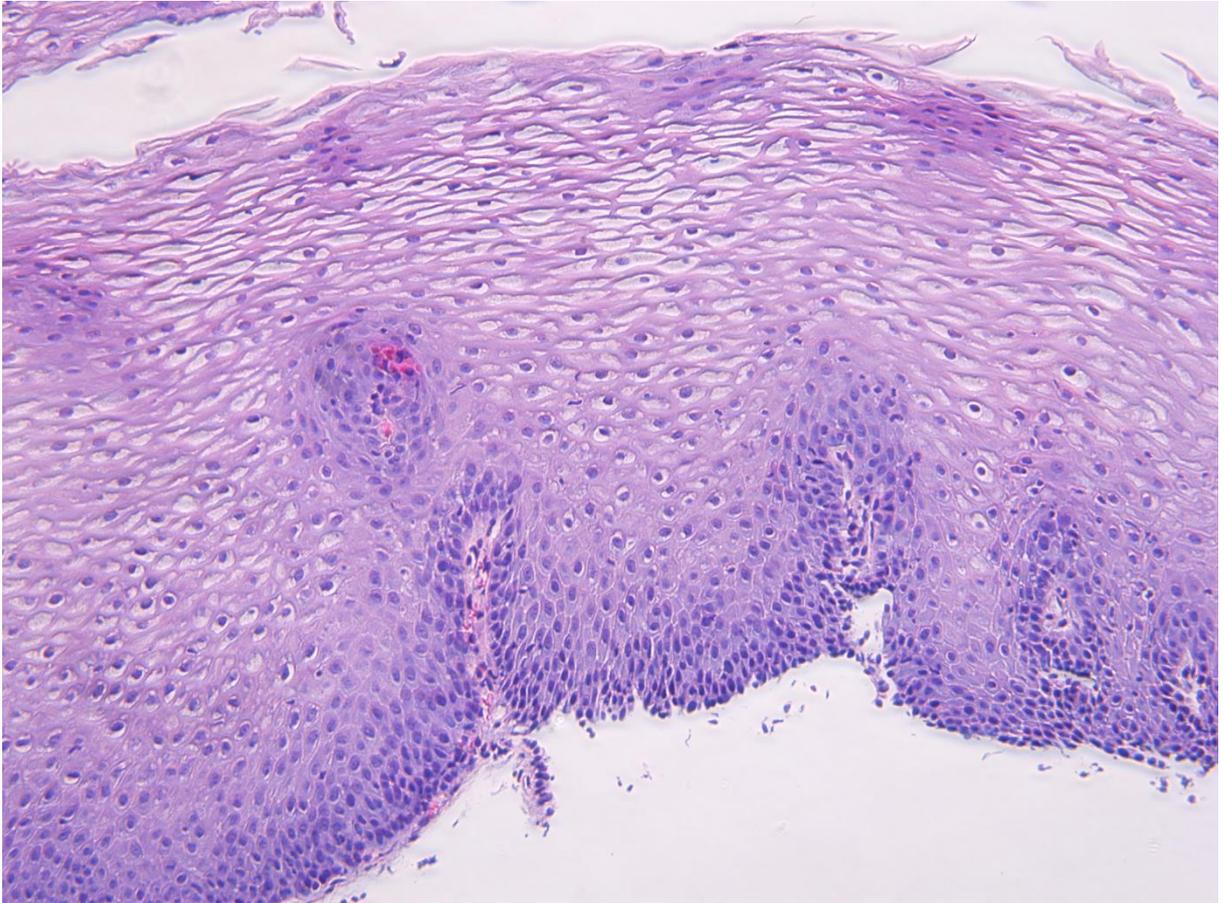
Figure 4: H & E staining of NS tissue section. The NS appears in violet color with and cells have consistent shape with regular sized nuclei. H&E stained slides were obtained from Mary Kay Washington's lab.
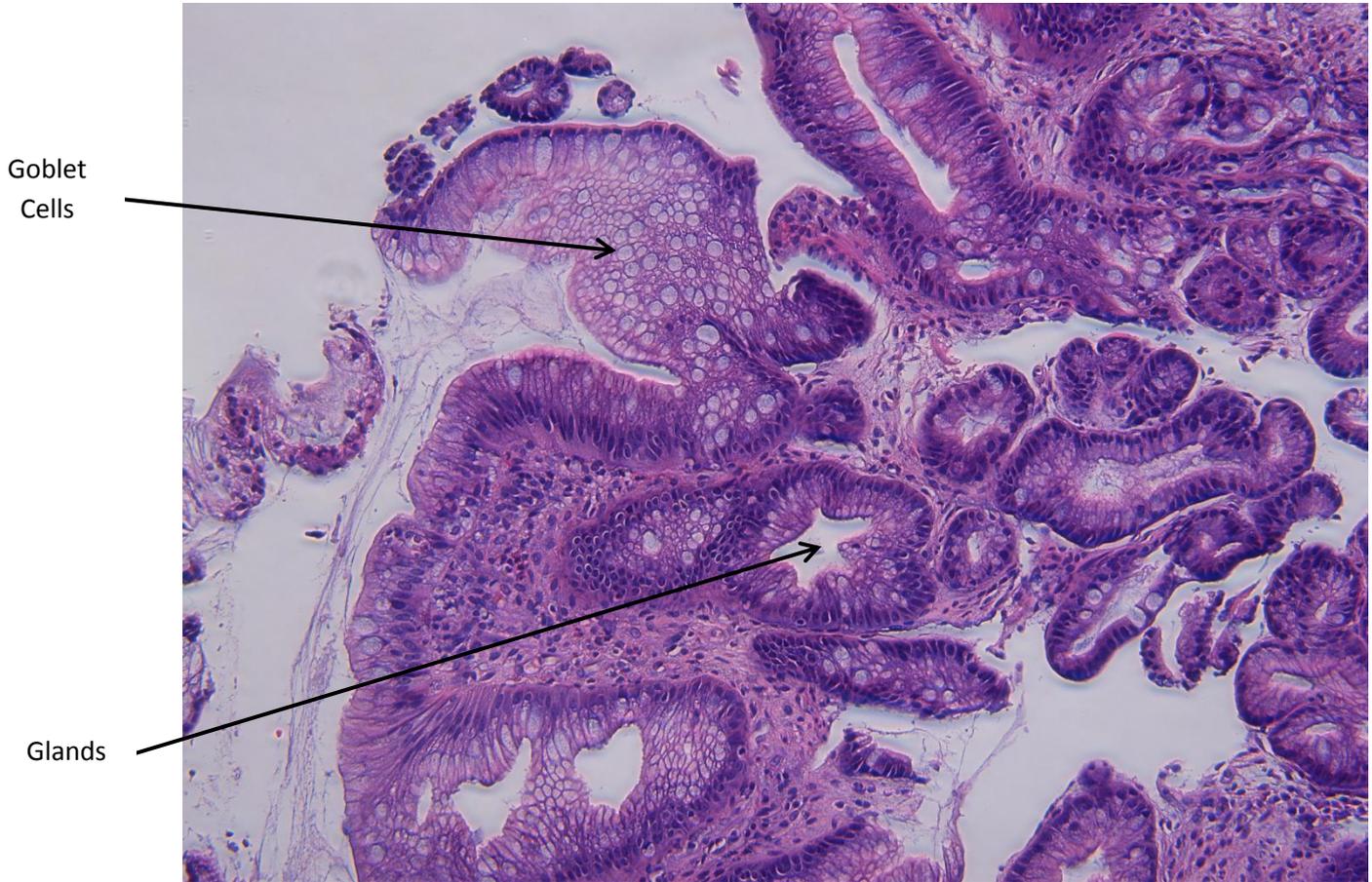
Figure 5: H & E staining of BE tissue section. The BE tissue is characterized by the presence of goblet cells. The light pink surrounding tissue is stroma. H&E stained slides were obtained from Mary Kay Washington's lab.
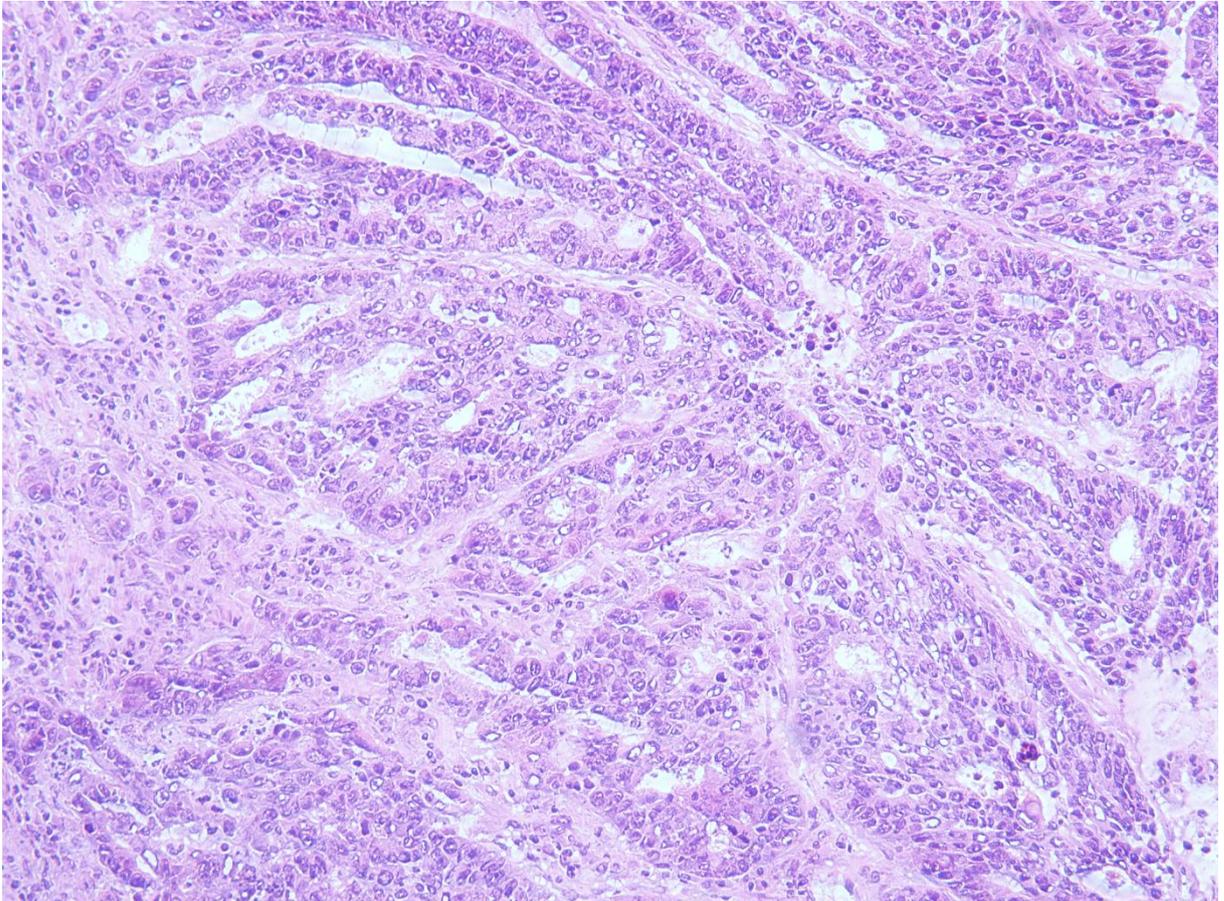
Figure 6: H & E staining of EAC tissue. Cells are arranged in glandular cohesive clusters cohesive clusters of cells that also display excessive mitosis. They exhibit irregular morphology and variable cellular and nuclear sizes. The light pink color surrounding the EAC cells is stroma. H&E stained slides were obtained from Mary Kay Washington's lab.

Based on the TNM classification, EAC can be staged from I to IV. The TNM classification describes tumors as a function of the tumor size (T), regional lymph nodes (N), and metastasis (M) (National Cancer Institute, 2013). An EAC is diagnosed to be at stage 0, if carcinoma is in-situ, with no regional lymph node metastasis or distant metastasis. This stage is denoted as (Tis, N0, M0). In stage I (T1N0M0), the tumor invades the lamina propria or submucosa, and there is no regional or distant metastasis (National Cancer Institute, 2013). Depending on the presence of distant metastasis, stage II is classified to IIA and IIB. Stage IIA (T2N0M0 or T3N0M0) is characterized by the presence of a tumor mass invading muscularis propria or adventitia with no regional or distant metastasis. Stage IIB (T1N2M0, or T2N1M0) involves the presence of a tumor that invades lamina propria, submucosa or muscularis propria, in addition to regional lymph node metastasis but no distant metastasis (National Cancer Institute, 2013). Stage III (T3N1M0, or T4NXM0) is associated with either a tumor that has invaded the advetitia, together with lymph node metastasis, and no distant metastasis, or a tumor that invaded adjacent structures regardless the presence of regional or distant metastasis (National Cancer Institute, 2013). Based on the degree of metastasis, stage IV is further classified into two different stages: IVA, and IVB. Stage IVA is mainly characterized by the cervical lymph nodes metastasis in case of tumors located in the upper thoracic esophagus (M1A), whereas, in case of stage IVB, a distant metastasis in non-regional lymph nodes or other structures is present. Table (1) summarizes TNM classification of EAC tumors.

Table 1: TNM classification of EAC. The table illustrated the details of EAC TNM-based tumor staging based on primary tumor status of invasion, regional lymph nodes metastasis, and distant metastasis.

Primary Tumor (T)		**Regional Lymph Nodes (N)**		**Distant Metastasis (M)**	
T1	Tumor in Mucosa/submucosa	N0	No metastasis in regional lymph nodes	M1a	Cervical nodes metastasis
T2	Tumor in muscularis propria	N1	Regional lymph nodes metastasis	M1b	Distant metastasis
T3	Tumor is in the wall and the serosa or pre-luminal fat	*EAC TNM-Based Staging*			
T4	Tumor is in the surrounding organs: pleura, aorta, trachea, and pericardium				

Stage	**T**	**N**	**M**
I	Tumor-in-situ (Tis)	N0	M0
II	T1-T3	N0, N1	M0
III	T3/T4	N1/Any N	M0
IV	Any T	Any N	M1a or M1b

The late diagnosis of EAC contributes significantly to the bad prognosis associated with this type of cancer. EAC are known to be associated with less response to chemotherapy and lower survival rates. Long-term survival becomes challenging upon distant metastasis, especially if the tumor invades the muscularis propria. Usually, long-term survival in EAC does not exceed 30% (Chen et al., 2011).

1.1.5:- Therapeutic Measures

Surgery is the mainstay treatment for small-sized tumors that neither invaded regional lymph nodes nor metastasized to distant organs (Stahl, Budach, Meyer, Cervantes, & ESMO Guidelines Working Group, 2010). Upon invasion of muscularis propria or other tissues, dysphagia appears as a major symptom. In case of invasion without metastasis, surgical removal of the tumor becomes a mainstay option (Boshier, Anderson, & Hanna, 2011; Bonavina, Bona, Luporini, Navoni, & Zucali, 2003). There are several surgical approaches for EAC. Transhiatal esophagectomy involves incision through the abdomen with anastomosis of the stomach to the esophagus (University of Michigan, Thoracic Surgery, 2012). This approach includes the abdominal mobilization of the stomach together with, transthoracic excision of the esophagus and anastomosis of the stomach to the upper part of the esophagus (University of Michigan, Thoracic Surgery, 2012) (Figure 7).
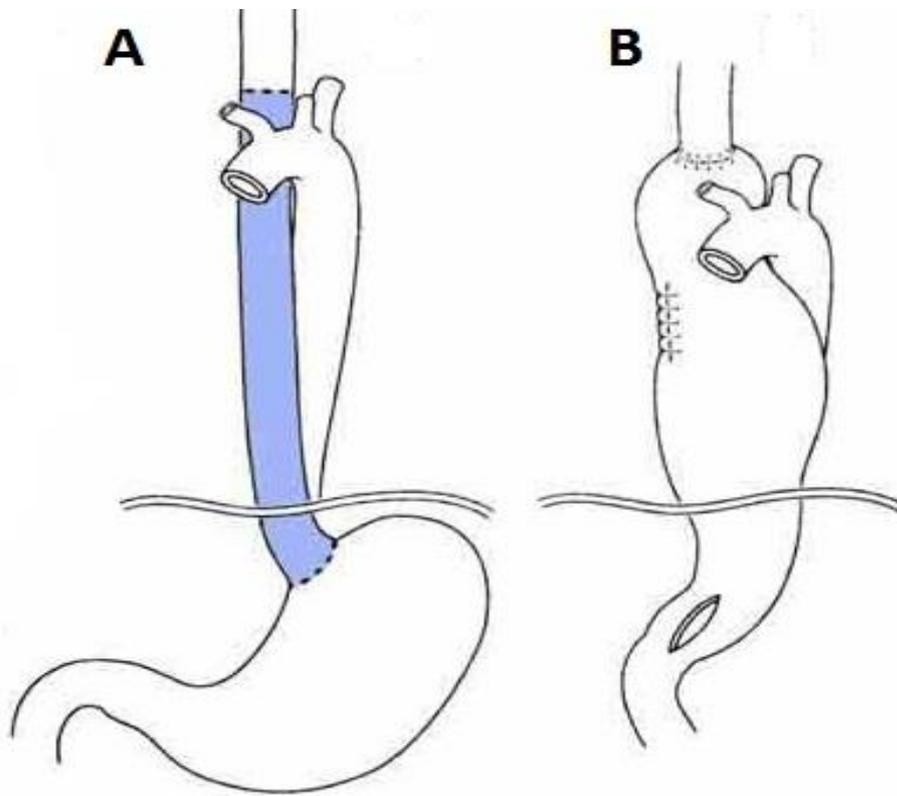
Figure 7: The figure demonstrates the steps of THE. A) Transhiatal esophagectomy involves incision through the abdomen with anastomosis of the stomach to the esophagus. B) Following the removal of the esophagus, abdominal mobilization of the stomach together with, transthoracic excision of the esophagus and anastomosis of the stomach to the upper part of the esophagus is performed. The figure is modified from (University of Michigan, Thoracic Surgery, 2012).

To relief dysphagia, radiation or an expandable metallic stent is advised. These two options are indispensable especially if the patient has developed metastasis and thereby, not eligible to surgery. Laser therapy and electrocoagulation are also means of relief of dysphagia. Surgical intervention has a 5-year survival rates that range from 5% to 30% (Stahl et al., 2010). Patients diagnosed at early stage have even higher chances of survival. Chemotherapy combined with radiations had been also described to decrease tumor size and relief dysphagia (Shridhar, Imani-Shikhabadi, Davis, Streeter, & Thomas, 2013). For patients with tumors diagnosed at stages IB, II, III, and IVA, chemoradiation prior to surgery is a first line treatment strategy (Ronellenfitsch et al., 2013). 5-fluorouracil (5-FU), and cisplatin are commonly used in chemotherapeutic regimens (Shridhar et al., 2013).

1.1.6:- Challenges in Current Diagnostic and Treatment Measures

EAC are known to have a bad prognosis. This is attributed to two facts: the late diagnosis, and poor response to chemotherapy. The asymptomatic nature of BE and early stages of EAC renders the tumor diagnosed at late stages, upon the invasion of muscularis propria which is manifested as dysphagia (National Cancer Institute, 2013). Most patients are diagnosed with EAC at advanced stages, and therefore, a 5-year relative survival rate is 14% (Polednak, 2003). Furthermore, the relatively low survival rates following surgical resection and the poor response to chemotherapy renders EAC treatment more challenging than other UGCs (Hong, Peng, Chen, Sehdev, & Belkhiri, 2013). Although, surgical removal of the tumor represents a mainstay radical treatment, long-term survival following surgery are relatively low (Stahl et al., 2010).

EAC is associated with poor clinical outcome and more resistance to chemotherapy than gastric cancers. EAC are known to be associated with resistance to DNA-damaging drugs (Hong et al., 2013). These challenges recall for the identification of biomarkers for early detection of EAC.

The elucidation of specific molecular mechanisms underpinning EAC is central to early diagnosis and effective treatment. A major requirement for understanding tumorigensis is the detection of the driving molecular events that take place in early stages of tumor formation. Accordingly, elucidating the molecular biomarkers associated with all EAC pre-tumorigenic and tumorigenic stages will provide a comprehensive understanding for driving genetic alterations.

1.2: MicroRNA: Discovery, Molecular Genetics and Biogenesis

MicroRNAs (miRNAs) are 20-25 nucleotide sequences that negatively regulate gene expression through targeting the complementary mRNA or blocking its translation (Chen & Rajewsky, 2007). In 1993, Victor Ambros, Rhonda Feinbaum, and Rosalind Lee discovered miRNAs upon studying *lin-14* in the development of *C. elegans* (Lee, Feinbaum, & Ambros, 1993). They observed that the protein expression of LIN-14 is regulated by a short nucleotide sequence encoded by *lin-4* gene. This nucleotide sequence gets transcribed to a 61 nucleotide sequence precursor, which later matures to a 22 nucleotide product (Lee et al., 1993).

Although *lin-4* was identified as a miRNA regulating LIN-14 expression, it was not regarded as universal gene regulation mechanism. For almost a decade, this phenomenon was considered as an idiosyncratic process occurring in nematodes. In 2000, let-7 was discovered in *C. elegans* as the second miRNA, which was later found to be present in several other species, which caused miRNA-mediated targeting to be encrypted in the molecular biology dogma as a gene regulation mechanism (Pasquinelli et al., 2000; Reinhart et al., 2000).

Experimentally discovered miRNAs are given names prior to their discovery (Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006). The nomenclature system is based on two parts: the word miR, followed by a dash, and a number that refers to the order of discovery (Ambros et al., 2003). For instance, miR-31 was discovered before miR-205. The un-capitalized r as in mir indicated the pre-miRNA that is transcribed directly from the gene, whereas, the capitalized R as in miR, indicated the mature form (Griffiths-Jones et al., 2006). Some miRNAs are almost identical but are encoded by different regions in the genome, and thereby they are denoted by the same number but with a different lower case letter following this number such as miR-148a, and miR-148b. If 2 miRNA genes located at different spots in

the genome and have identical mature forms, they are denoted by the same number, followed by a dash and a different unit digit (Ambros et al., 2003). This can be exemplified as the 2 pre-miRNAs mir-194-1, and mir-194-2 which produce a similar mature form (miR-194). The miRNA species is indicated by 3 letters preceding the name (Griffiths-Jones et al., 2006). For example, a *Homo sapiens* miRNA is denoted by has-miR-148, a *Mus musculus* miRNA is indicated by mmu-miR-194, and an *Ovis aries* miRNA is indicated as oar-miR-194. If two mature miRNAs are produced from the same arm or a pre-miRNA, they are indicated in the suffix as -5p or -3p (Ambros et al., 2003; Griffiths-Jones, 2004). If the expression levels of two miRNA coming from opposite arms in the same hairpin are known, the less abundant would be followed by an asterisk. For instance, miR-123 and miR-123* are originated from same hairpin, but miR-123 is more abundant (Griffiths-Jones, 2004).

miRNAs are transcribed from either intronic regions of host genes or non-coding intergenic areas. Most miRNA genes are intergenic, and thereby their transcription is regulated by their own promoters, where they have an antisense orientation (Lee, Kim, Han, Yeom, Lee, Baek, & Kim, 2004). However, the transcription intronic miRNAs is regulated by their host gene promoter. Additionally, some miRNAs are located in the exons of non-coding regions. 40% of miRNA genes are located in host genes' introns and exons, and exhibit sense orientation (Rodriguez, Griffiths-Jones, Ashurst, & Bradley, 2004). Several miRNA genes are present in polycistronic units, and thereby are commonly regulated by same promoter (Altuvia et al., 2005). However, this doesn't essentially imply that these miRNAs have similar sequence or biological function. Adding to the complexity of scenario, the DNA sequence of miRNA gene doesn't necessarily reflect the final structure of the mature miRNA. This is attributed to 2 facts: 6% of miRNAs are subject to RNA editing, and the site-specific modification of RNA (Lee et al., 2004).

The biogenesis of miRNA takes place on 4 major stages: transcription, nuclear procession, nuclear export, and cytoplasmic processing. miRNA genes are transcribed by means of RNA polymerase II (Pol II) (Zhou, Ruan, Wang, & Zhang, 2007). Pol II binds to the promoter regions and transcribes the gene to produce the pri-miRNA which is a hairpin loop structure consisting of two opposite arms and a loop (Cai, Hagedorn, & Cullen, 2004). A pri-miRNA contains up to 6 pre-miRNAs. Each hairpin loop contains approximately 70 nucleotides, and flanked by other sequences required for processing. The pri-miRNA transcript is subject to 3 modifications: 5' end cap, 3' polyadenylation, and splicing (Cai et al., 2004). In the nucleus, the processing of pri-miRNA starts with the binding of the DiGeorge Syndrome Critical Region 8 (DGCR8), also known as Pasha, to the double-stranded structure of the RNA. DGCR8 also binds to the Drosha, an enzyme that excise the RNA, resulting in a Mircoprocessor complex (Gregory, Chendrimada, & Shiekhattar, 2006). The RNase III domain of the Drosha gets directed by DGCR8 to separate the hairpins at the flanking sequences. The resulting product is called the pre-miRNA, and it has a 3' hydroxyl and a 5' phosphate groups (Gregory et al., 2006). In some cases, some pre-miRNAs are biosynthesized from the introns without the catalysis of the Microprocessor complex, and they are known as Mirtrons (Berezikov, Chung, Willis, Cuppen, & Lai, 2007). A small percentage of the pri-miRNAs are modified in the nucleus. This process is catalyzed by RNA acting denosine deaminases (ADARs). These enzymes catalyze the transition of adenosine to inosine (A to I) (Kawahara et al., 2008). Following the nuclear processing, pre-miRNAs are subject to nuclear export by means of a nucleocytoplasmic shuttle known as Exportin-5. Exportin-5 recognizes and bind to the 2 nucleotides at the 3' end of the pre-miRNA, and exports the pre-miRNA out of the nucleus by means of energy-requiring mechanism that utilizes GTP (Murchison & Hannon, 2004).

Following exportation to the cytoplasm, the pre-miRNA undergoes cytoplasmic processing by means of an RNase III enzyme known as Dicer, and an RNA-induced silencing complex (RISC) (Iorio & Croce, 2012). The Dicer recognizes and binds to the 3' end of the hairpin and cleaves the loop that ligates the 5' and 3' ends. This results in the production of a duplex of 2 opposite miRNA strands exhibiting incomplete complementarity. Usually the hairpin loop length is about 22 nucleotides (Lund & Dahlberg, 2006). Then the RISC recognizes and binds one strand forming a miRNA-RISC (miRISC) complex which later on binds the target (Iorio & Croce, 2012) (Figure 8).

1.3: miRNA Mechanism of Action

The miRISC complex regulates gene expression at the post-transcriptional level. miRNA acts by binding to the partially or almost complementary mRNA target resulting in degradation of mRNA or inhibition of translation. miRNA-mediated gene regulation is classified to 3 mechanisms: 1) mRNA degradation, 2) blocking of translations, and 3) site-specific cleavage (Figure 8). Based on the degree of the complementarity between the miRNA and its mRNA target, the mechanism of the regulation will vary. If there is a complete or almost complete match between the miRNA and mRNA target, site-specific cleavage will prevail (Iorio & Croce, 2012). However, this mechanism is rare in mammals . The two other mechanisms are predominant in mammals, where incomplete complementarity between the miRNA and target mRNA is the usual case. These two processes are usually referred to as non-cleavage repression (Su, Trombly, Chen, & Wang, 2009). The complete mechanisms by which miRNA blocks translation is still not fully understood. It is widely accepted that miRNA binds to the 3' UTR of the complementary sequence. However, some miRNAs were reported to bind to the 5' UTR or even the ORF. The 5' end of the miRNA is known as the seed site and it is the essential sequence for mRNA binding (Lytle, Yario, & Steitz, 2007). On the other hand, based on the degree and the location of complementarity, the target sites are classified into three main classes: the 5' seed only targets (dominant seed site targets), 5' dominant (5' canonical seed site targets), and the 3' canonical (3' complementary seed site targets) (Iorio & Croce, 2012). miRNA can target several genes and thereby can elicit several functions. 60% of the mRNAs have conserved sequences that interact with miRNAs. Furthermore, based on the results of computational analysis, the 3'UTR of genes were found to be targeted by several miRNAs (Lewis, Burge, & Bartel, 2005). Another mechanism of

RISC-independent mechanism miRNA-gene regulation is that miRNA binds to the ribonucleoproteins, and thereby inhibits its RNA binding functions. This mechanism is known as decoy activity (Beitzinger & Meister, 2010). Additionally, several studies pointed out to the ability of miRNA to regulate gene expression at the transcriptional levels via binding to the DNA (Gonzalez, Pisano, & Serrano, 2008) (Figure 8).
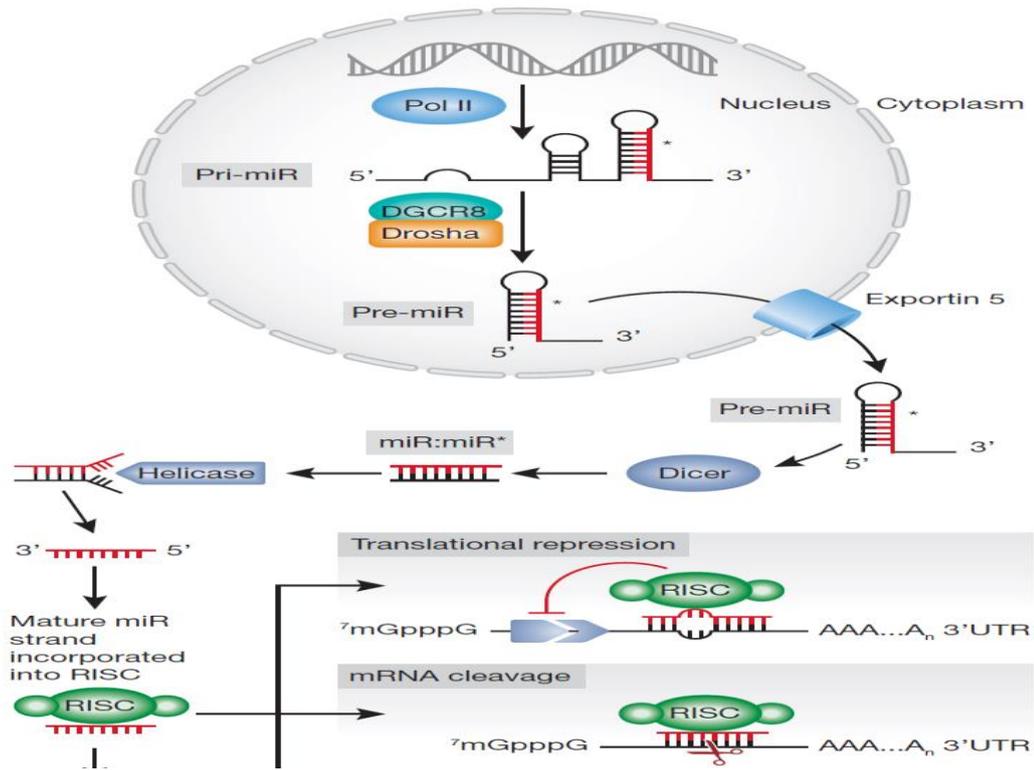
Figure 8: miRNA pathogenesis and mechanism of action. The diagram depicts the biogenesis of miRNA and its mechanism of action. miRNA undergoes nuclear processing by means of Drosha-DGCR8 complex to produce pre-miRNA that is later processed in cytoplasm by Dicer and gets incorporated in RISC. The RISC-miRNA complex represses expression by either blocking translation or mediating mRNA degradation. The figure is modified from (Iorio & Croce, 2012).

1.4: The Role of miRNA in Cancer

The genomic mapping of miRNA genes revealed that they are located in chromosomal loci that are predisposed to amplifications or deletions. It was reported that genomic regions harboring miRNAs that negatively regulated an oncogenic target are prone to deletions or mutations which in turn results in the down-regulation of the miRNA and the over-expression of the oncogenic target (Calin et al., 2004). For instance, miR-15a and miR-16-1 are tumor suppressor miRNA located in the same polycistron (13q14) (Raveche et al., 2007). This region was found to be frequently deleted in CLL. On the contrary, chromosomal loci having miRNAs that down-regulates tumor suppressors are liable to amplifications. Accordingly, this results in the over-expression of the oncogenic miRNA and the down-regulation of the tumor suppressor target (Calin et al., 2004).

Given the oncogenic or tumor suppressor role of miRNAs in cancer, miRNA profiling in cancer can be useful for distinctions between tumor and normal tissues. miRNA profiling in tumors provides insights for diagnosis, prognosis, tumor type, and stage (Calin & Croce, 2006; Calin et al., 2004). Genome-wide miRNA expression profiling revealed the miRNA signatures associated with different types of cancer, as well as the fingerprint of the poorly differentiated tissue of origin (Calin & Croce, 2006). On the contrary, mRNA profiles didn't stratify the cancer or tissue types. Furthermore, miRNA expression patterns allow for the identification of the primary tissue of origin, especially in case of metastatic tumors (Iorio & Croce, 2012). For instance, 48 miRNAs were reported to identify the primary tissue of origin upon the blind measuring their expression in 336 primary and metastatic tumors (Rosenfeld et al., 2008). This is very beneficial since metastatic tumors of unknown origin are associated with poor prognosis. miRNA profiling can also serve in the elucidation of biomarkers for early diagnosis, prognosis, and response to chemotherapy. For instance, miR-21, and miR-205

were found to be associated with ductal adenocarcinoma where the phenotypic alterations in the ducts follow their aberrant expression. Accordingly, miR-21 and miR-205 are potential biomarkers for early detection of ductal adenocarcinoma (du Rieu et al., 2010). Furthermore, from a technical point of view, the fact that the size of mature miRNA sequence ranges from 20 to 22 nucleotides, they are more stable than mRNA. Accordingly, it is feasible to detect miRNAs in challenging biological specimens such as formalin-fixed paraffin-embedded (FFPE), and blood. Detection of miRNAs in FFPE will allow the retrieval of expression data associated with long term clinical outcome (Iorio & Croce, 2012). However, there are several challenges associated with the analysis of FFPE. The integrity of the nucleic acid material is one of the challenges encountered by the analysis of FFPE (Turner, Heath, & Kurn, 2011). The fact that these samples have undergone formalin fixation and embedded in paraffin results in the degradation of RNA. However, the short nucleotide sequence of miRNAs (21-25) renders them intact for analysis (Liu & Xu, 2011). The minute amount of tissue in FFPE, its embedding in excessive paraffin, and the presence of paraffin artifact represent major limitation for retrieving valid and quantifiable data from FFPE (Liu & Xu, 2011). Overcoming this technical limitation essentially necessitates the tweaking of the analytical procedures including de-parrafinization. Firstly, to render FFPE tissues accessible for extraction buffers and lysis, effective de-paraffinization is a requirement (Kotorashvili et al., 2012). Several de-paraffinizing reagents are known such as xylene, n-octane and d-limonene (Ribeiro-Silva, Zhang, & Jeffrey, 2007). Incubating the tissue with the de-paraffinizing reagents at room temperature or 50 ℃ allows for the dissolution of the paraffin (Kotorashvili et al., 2012). On the other hand, detection of miRNA in blood represents a high potential for non-invasive diagnostic tests (Mitchell et al., 2008). Additionally, miRNAs can be detected from circulating exosomes, urine, saliva, and sputum (Iorio & Croce, 2012). Furthermore, the miRNA expression profile retrieved from blood was similar to that obtained from frozen tissues indicating the high potential of blood miRNAs as non-invasive diagnostics that are accurately reflecting the miRNA profile associated with tumors (Taylor & Gercel-Taylor, 2008). For instance, the plasma samples of lung cancer patients revealed lung cancer-specific

miRNA profile 1-2 years before the development of the disease (Boeri et al., 2011). Given the ability of miRNA signature to discriminate between the different cancer subtypes, miRNA profiling can serve as an efficient diagnostic tool (Calin & Croce, 2006). Although gene expression profiling can also distinguish between different tumor subtypes, miRNA profiling provides further information to the cancer-associated network between miRNA-target (Iorio & Croce, 2012). For instance, the differential miRNA expression between basal and luminal breast cancer subtypes can also relate to the expression of estrogen receptors (ER), human epidermal growth factor receptor 2 (HER2), and (PR) progesterone receptors, as well as the epithelial and myoepithelial origins (Iorio et al., 2005; Mattie et al., 2006). This can be exemplified by the association of the dys-regulation of miR-200 family with the luminal subtype where it targets the epithelial-mesenchymal transition (EMT) regulators such as ZEB1 and ZEB2 (Gregory, Bracken, Bert, & Goodall, 2008). On the other hand, miR-145, and miR-205 which are normally over-expressed in myoepithelial cells, exhibit down-regulation in triple negative breast cancers (ER-/PR-/HER2-) (Sempere et al., 2007). This indicates that the alteration in expression of these miRNAs is associated with the tumor progression in this particular subtype. Additionally, the miRNA signature discriminates between the various histological subtypes of ovarian cancer (Iorio et al., 2007), and miR-205 differentiates between non-squamous and squamous non-small cell lung cancer (Lebanony et al., 2009).

Given the accumulating evidence highlighting the role of miRNA to identify tumors, differentiate between tumor subtypes, and associate with cancer predisposition, miRNAs can serve as diagnostic and prognostic markers to dictate therapy (Iorio & Croce, 2012). This can be exemplified by the association of miR-155 up-regulation and let-7a down-regulation with poor outcome in CLL and lung cancer (Yanaihara et al., 2006). Furthermore, the expression profile of 7 miRNAs in gastric cancer is able to determine the overall survival and relapse-free events (Li et al., 2010). In hepatocellular carcinoma (HCC), the down-regulation of miR-26 is associated with poor survival, but a good response to interferon-α therapy (Ji et al., 2009). On the contrary, miR-21 is associated with poor response to treatment in adenocarcinoma (Schetter et al., 2008) and pancreatic cancer (Giovannetti et al., 2010). Over-expression of miR-125b in breast cancer correlates with a poor response to taxol-based therapy (Zhou et al., 2010). Owing to its influence on response to treatment, it is hypothesized that the over-expression or knock-down of miRNAs can potentially enhance response to treatment. This concept has been applied extensively in in-vitro cell models (Iorio & Croce, 2012). The knock-down of miR-21, and miR-200b in cholangiocarcinoma cell lines resulted in enhanced response to gemcitabine (Meng et al., 2006). Furthermore, adjuvant miRNAs can improve the sensitivity to targeted therapies and avoid drug resistance. The reconstitution of miR-205 improves the response to tyrosine kinase inhibitors (TKI) via targeting HER3 (Iorio et al., 2009).

Several gain-of-function studies have elucidated the tumor suppressor role of miRNAs. Tumor suppressor miRNAs essentially target oncoproteins. miR-15a, and miR-16-1 targets BCL2 (Cimmino et al., 2005), and let-7 targets RAS and MYC (Johnson et al., 2005). miR-205 and the family of miR-200 are able to target ZEB transcription factors which are EMT activator and thereby, decrease migration and invasion (Gregory et al., 2008).

Furthermore, miR-221 and miR-222 are able to inhibit proliferation and metastasis through targeting c-Kit (Poliseno et al., 2006). On the other hand, loss-of-function studies have demonstrated the role of oncomiRs which target tumor suppressors. The knock-down of miR-21 in glioblastoma resulted in the activation of caspases and thereby apoptosis (Chan, Krichevsky, & Kosik, 2005). Other reports indicated the tumor suppressors phosphatase and tensin homologue (PTEN), and programmed cell death (PDCD4) as targets for miR-21 (Meng et al., 2007). miR-17-92 cluster and miR-155 are oncogenes that are up-regulated in lymphoproliferative disorders that enhanced the lymphomas development, and increased the proliferation rate (Garzon et al., 2008; He et al., 2005).

Given the implications of miRNA dysregulation in proliferation, invasion, migration, metastasis, and angiogenesis, together with, its several targets, miRNAs elicit their action through several interactions with many targets. Therefore, understanding the miRNA dysregulation associated with each cancer type gives deeper insight about the cancer-associated network. Indeed, this is the foundation for proposing that miRNA can be promising in the context of understanding and tackling of the multifactorial complicated nature of cancer.

miRNA is known to be highly dysregulated in cancers. There are several genetic and epigenetic mechanisms resulting in aberrant miRNA expression. Chromosomal aberrations: deletions, translocations and extra copies, single nucleotide polymorphisms (SNPs), CpG islands, altered DNA methylation, and defects in miRNA biosynthesis mechanisms, underlie the dysregulation of miRNAs (Iorio & Croce, 2012).

One of the causes of miRNA down-regulation is the structural variations such as SNPs which were reported to abolish the expression of miR-15a and miR-16-1 in CLL (Raveche et al., 2007), and lung cancer (Hu et al., 2008). Defective Dicer or Drosha is another cause for miRNA down-regulation (Merritt et al., 2008; Nakamura, Canaani, & Croce, 2007; Thomson et al., 2006). Furthermore, the dysregulation of some genes might in turn result in miRNA silencing. For instance, Lin-28 inhibits Let-7 biosynthesis (Viswanathan, Daley, & Gregory, 2008). Among the common reasons for miRNAs deregulation is the aberrant expression of a master gene that regulates the miRNA (Iorio & Croce, 2012). For instance, p53 induces miR-34a, miR-34b, and miR-34c, and thereby these miRNAs are suggested to one of the underlying mechanisms for the tumor-suppressor effect of p53 (Chang et al., 2007; He et al., 2007).

One prominent reason for miRNA silencing is epigenetic alterations. Several miRNA genes have highly dense CpG islands in their promoter regions. In fact, analyses have indicated that half of the miRNA genes harbor CpG islands (Iorio & Croce, 2012). Extensive methylation causes miRNA silencing. For instance, the treatment of T24 bladder cancer cells with a de-methylating agent known as 5'-Aza-2'deoxycytidine restores the expression of miR-127 which harbors a CpG island in its promoter region. The methylation-mediated silencing of miR-127 results in the over-expression of its oncogenic target BCL-6 (Saito et al., 2006). Similarly, the expression of miR-9-1 (Lehmann et al., 2008), miR-34b, and miR-34c get restored upon treatment with 5'-Aza-2'deoxycytidine (Toyota et al., 2008). Another epigenetic perturbation that is results in miRNA silencing is histone deacetylation (Iorio & Croce, 2012). Upon treatment of SKBR3 breast cancer cells with histone deacetylase inhibitor, the expression of several miRNAs significantly escalate, pointing out to the histone deacetylation as a silencing mechanism (Scott, Mattie, Berger, Benz, & Benz, 2006). Another intriguing mechanism for miRNA silencing is that some of the silenced miRNAs are able to target the DNA methylation machinery resulting in a feed-back loop that controls the expression of both the miRNA and the target. For instance, miR-148a/miR-152 were found to be silenced by methylation in breast cancer and target DNA methyl transferase 1 (DNMT1) levels (Xu et al., 2012). Similarly, miR-29s are also silenced miRNAs that target DNMT-3A

and DNMT-3B (Iorio & Croce, 2012). The reconstitution of miR-29s in lung cancers and acute myeloid leukemias (AMLs) caused the activation of the methylated tumor suppressors and thereby repressed tumorigensis (Fabbri et al., 2007; Garzon et al., 2008).

Hypomethylation underlies the up-regulation of several oncogenic miRNAs. For instance, let-7a-3 is up-regulated in lung cancer (Brueckner et al., 2007), and miR-21 is up-regulated in ovarian cancer owing to hypomethylation (Iorio et al., 2007). Upon profiling the miRNA expression profile in DNMT1 and DNMT3b deficient colorectal cancer cell lines, the expression of miR-124a, which is located within a CpG island and known to target cyclin D kinase 6, was restored (Lujambio et al., 2007). Other mechanisms for upregulation of oncogenic miRNAs is the regulation by master cancer-associated genes. For instance, c-MYC enhances the expression of the oncogenic miR-17-92 cluster (Chang et al., 2009).
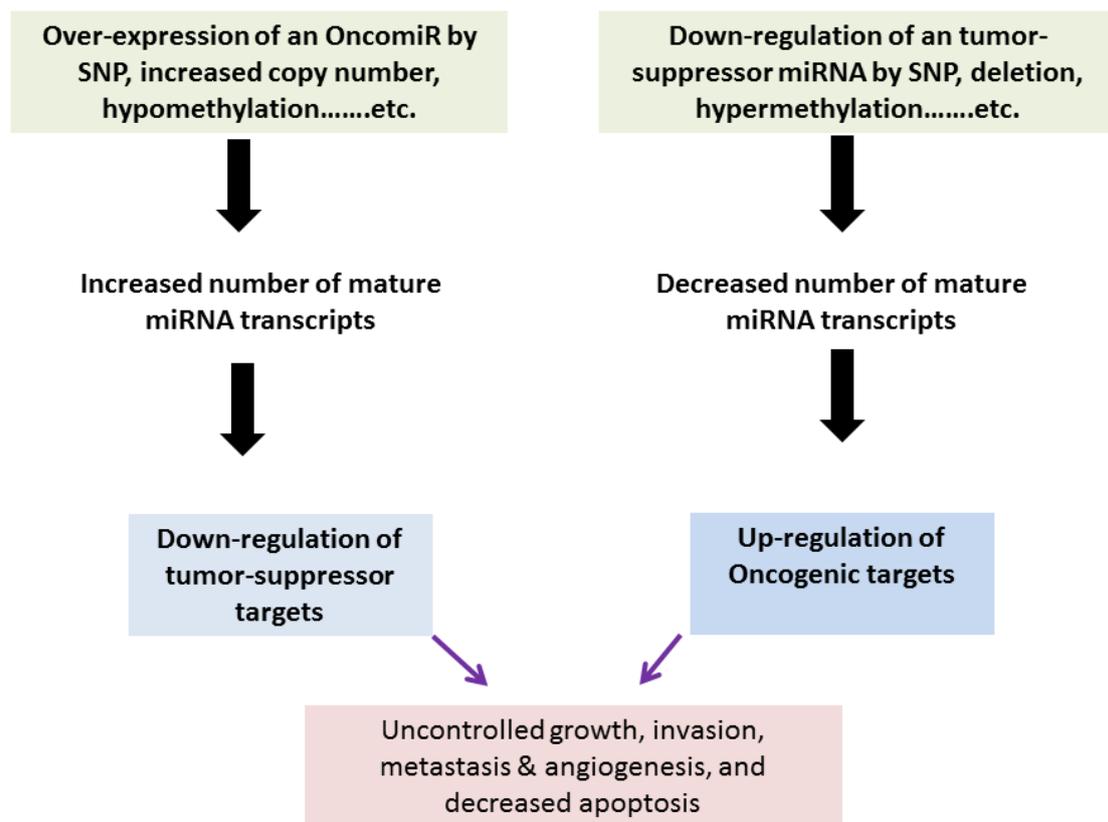
Figure 9: The role of miRNAs in cancer. The diagram demonstrates the diverse roles of miRNA in cancer. From the perspective of cancer, miRNAs that are down-regulated are regarded as tumor-suppressor miRNAs whereas up-regulated ones are oncomiRs. The dysregulation of miRNA in cancer results in tumorigenic functions such as increased proliferation, invasion and angiogenesis, and decreased apoptosis.

The Effects of miRNA Dys-regulation in Cancer

Increased Proliferation

Enhanced Invasion and Metastasis

Increased Angiogenesis

Inhibition of Apoptosis
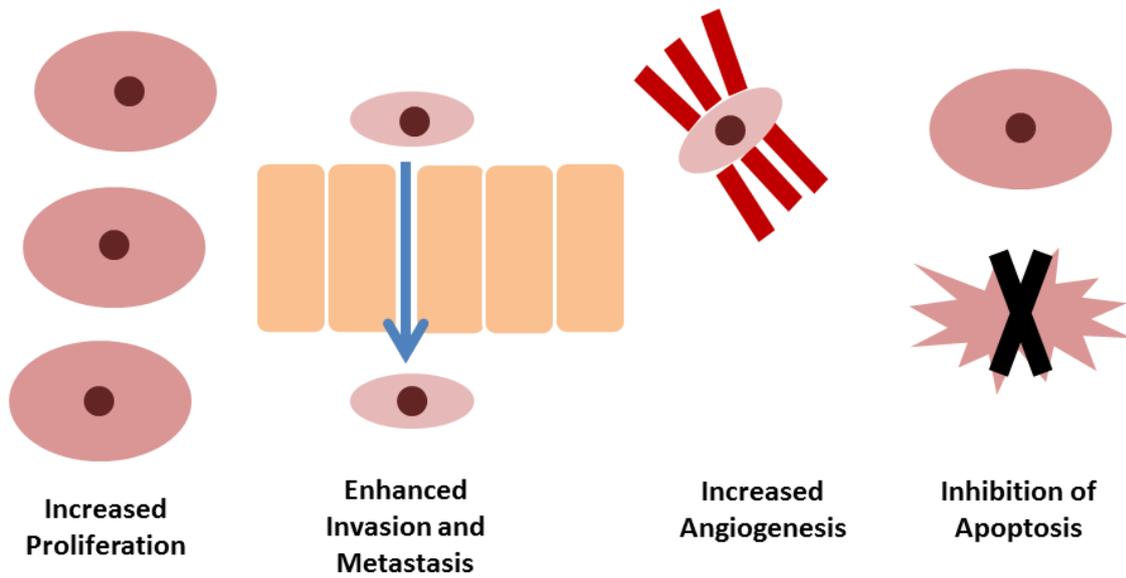
Figure 10: Biological functions elicited by miRNAs. miRNAs are known to play a variety of tumor suppressor or oncogenic roles. miRNAs can block apoptosis, potentiate angiogenesis, increase proliferation, invasion, and metastasis.
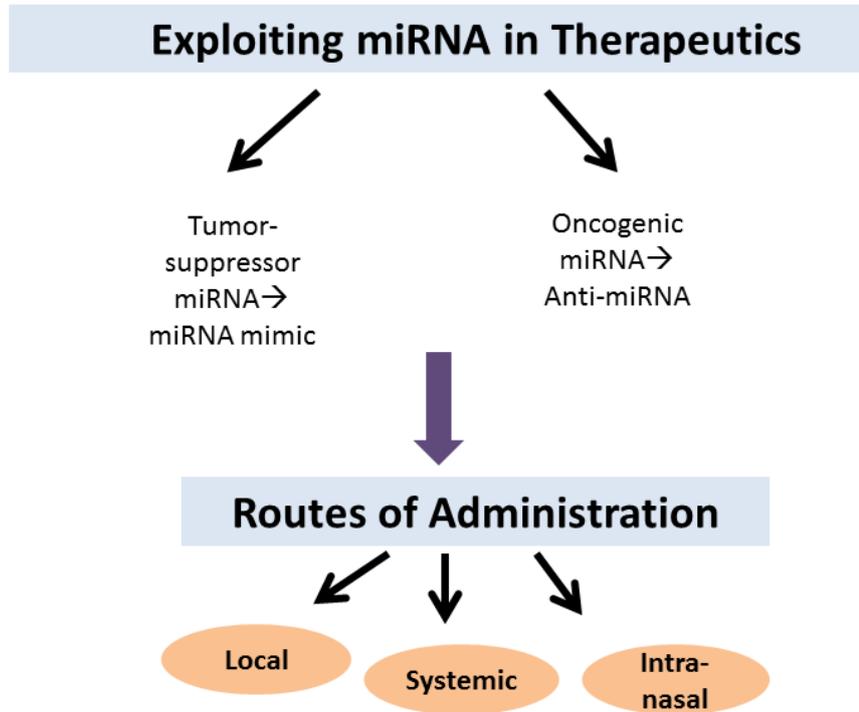
Figure 11: Routes of Delivery and Therapeutic potentials of miRNAs. Based on their role in cancer, miRNA can be exploited in therapy as miRNA mimics or antagomiRs. Several administration routes can be employed such as local, intranasal or systemic. Conjugation of miRNA mimic or antagomiRs to vehicles that facilitate delivery such as nanoparticles or viruses are possible ways for delivery.

1.5 Methods of miRNA Expression Profiling: A Comparison

Several approaches have been adopted for miRNA expression profiling such as microarray, quantitative real-time polymerase chain reaction (qRT-PCR), in-situ hybridization and high throughput sequencing. miRNA microarray is employed for preliminary assessment of miRNA expression where the samples are hybridized to probes that include thousands of miRNA targets (Sato, Tsuchiya, Terasawa, & Tsujimoto, 2009). Although microRNA microarray is well recognized as a highly throughput method, its inaccurate quantification poses a risk for false discovery as well as data reproducibility. Thus the requirement for independent validation by qRT-PCR is rendered crucial (Iorio & Croce, 2012).

qRT-PCR is very subtle and precise but it is very costly and not a high-throughput method. Quantitative determination of miRNA expression levels by means of qRT-PCR essentially requires a 2-step reaction in which the miRNA is firstly converted to the corresponding cDNA by means of reverse transcription followed by its quantitative measurement by real-time PCR (Chen et al., 2005). Although miRNA reverse transcription is similar to that of mRNA, where it includes flanking by poly-A-tail or using random hexamer primers, miRNA cDNA synthesis is characterized by some specific features. For instance, upon using poly-A-tail for miRNA 3' flanking, a second step is required where an oligo-dT primer that is flanked with a primer sequence complementary to a universal miRNA primer used in qRT-PCR. The third step of cDNA synthesis is the reverse-transcription step that includes the synthesis of the cDNA by means of reverse-transcriptase in the presence of dNTPs, and poly-A buffer. During qRT-PCR, the 3' flanked end (reverse) of the cDNA will be hybridized to the complementary universal miRNA primer, whereas the 5' end (forward) will be hybridized with the miRNA

specific primer (Chen et al., 2005). miRNA specific primers can be obtained from the online database miRbase (http://www.mirbase.org/). The qRT-PCR is performed usually in a specific thermal cycler that is able to illuminate each sample with a light bean at a specific wavelength (λ_{max} = 497 nm) followed by the detection of the fluorescence emitted by each sample (Rychlik, Spencer, & Rhoads, 1990). The reaction involves the amplification of the template by means of polymerase, dNTPs, and SYBR-Green. SYBR-Green is a cyanine dye that binds to the nucleic acids. This dye-nucleic acid complex absorbs the light at λ_{max} = 497, and releases green light at λ_{max} = 520 (Zipper, Brunner, Bernhagen, & Vitzthum, 2004). The amplification reaction usually involves 2 major steps. The first step is an initial denaturation at 95ºC for 10 minutes. The second step usually involves 40 cycles. Each cycle comprises 3 steps, the first step is denaturation which is performed at 95ºC for 30 seconds, followed by annealing step at 60ºC for 45 seconds, and finally the camera capture step where the machine detects the green light emitted that corresponds to the amount of the amplified product. Following the completion of the reaction, the cycle at which each sample has exceeded the background noise, known as threshold cycle (Ct) is being read and analyzed in comparison of other samples (Rychlik et al., 1990). The sensitivity and accuracy of the qRT-PCR is driven by several factors including the reaction kinetics, experimental design, and analysis method. The precision of qRT-PCR quantification relies on the high sensitivity of the detection of the SYBR-nucleic acid complex. Experimentally, a proper qRT-PCR experiment essentially requires that the same sample is run twice in the same reaction, and the variation between the Cts of the duplicates should be less than half a cycle. Furthermore, a proper analysis of the qRT-PCR reaction requires the adjusting of the threshold line to be within the midway of the logarithmic amplification curve where the quantification is within the exponential plateau (Figure 12).
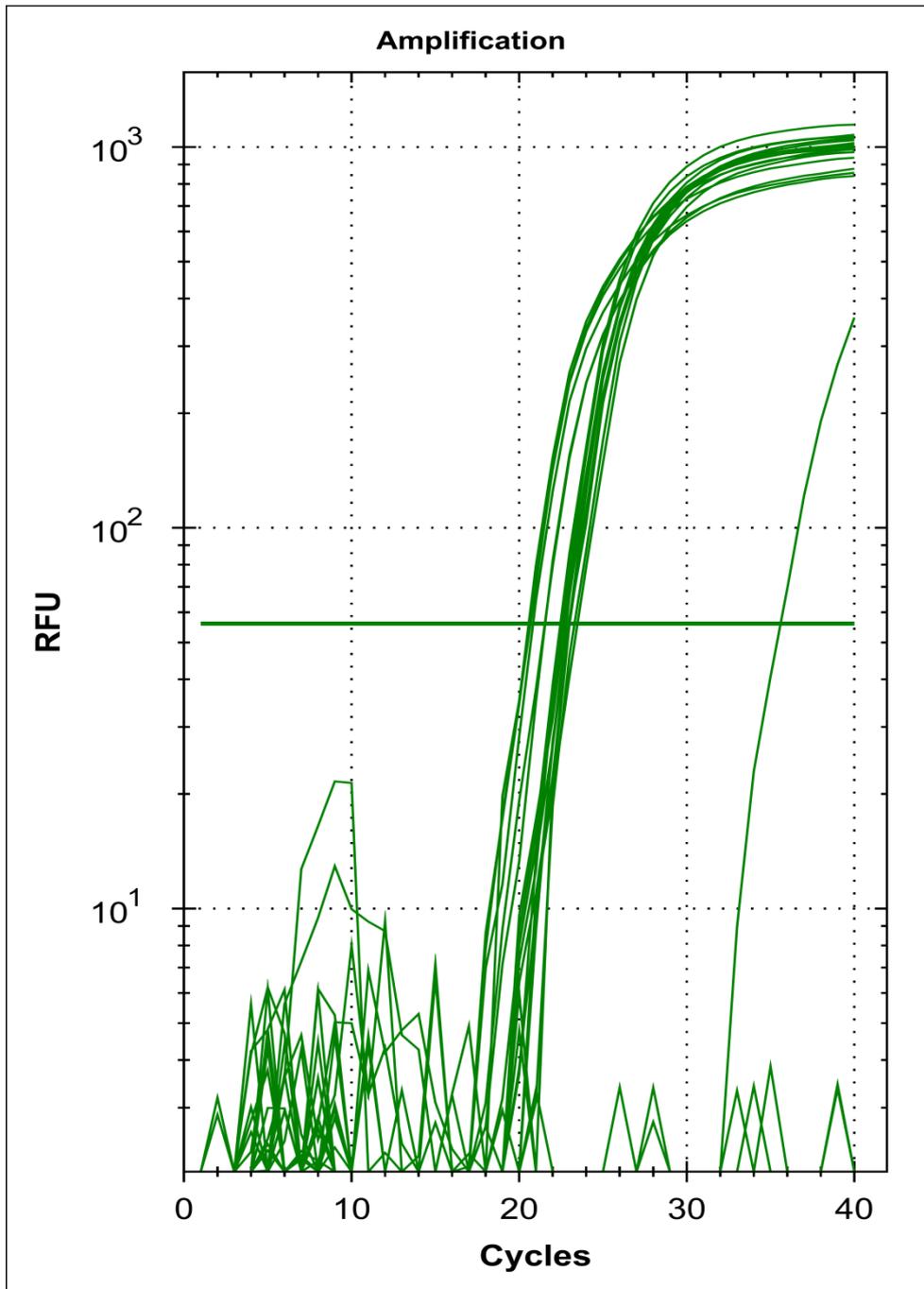
Figure 12: qRT-PCR quantification curve. The graph depicts a typical qRT-PCR quantification curve. The X-axis shows the Ct, whereas the y-axis shows the number of copies of the amplicon. Accurate quantification relies on the setting of the proper threshold line which should exceed the background noise. Usually the correct threshold line is in the middle of the logarithmic phase of amplification. The intersection of the threshold line with the amplification curve represents the Ct.

One of the drawbacks associated with florescent detection by means of SYBR-Green is the sensitivity to nonspecific by-products such as primer dimers, or PCR amplicon from a misannealed primer. This in turn recalls for the necessity of running a melting/dissociation curve following the completion of the qRT-PCR reaction (Ririe, Rasmussen, & Wittwer, 1997). The melting curve analysis will allow for the measurement of the dissociation of primer-template complexes, and thereby providing an accurate assessment of the specific melting point (Tm) of each primer. The Tm of a primer is the temperature at which half the primer-template amount dissociates into single strands. Accordingly, melting curve analysis serves as a quality control procedure that checks whether the amplicon corresponds to the required region or not (Figure 13). This measure is conducted by plotting the rate of change of SYBR green florescence, (i.e. relative florescence unit, RFU) upon subjecting the sample to various temperatures. At low temperatures, the sample-primer duplex will not dissociate, thereby the SYBR Green florescence will be low, and thereby the dRFU/dT will be low. Upon increasing the temperature, the duplex dissociates and thereby SYBR Green florescence increases, and thereby yielding a high dRFU/dT. The Tm of the amplicon is obtained from the peak of the melting curve where inflection from positive florescence rate to a negative one takes place (Ririe et al., 1997).
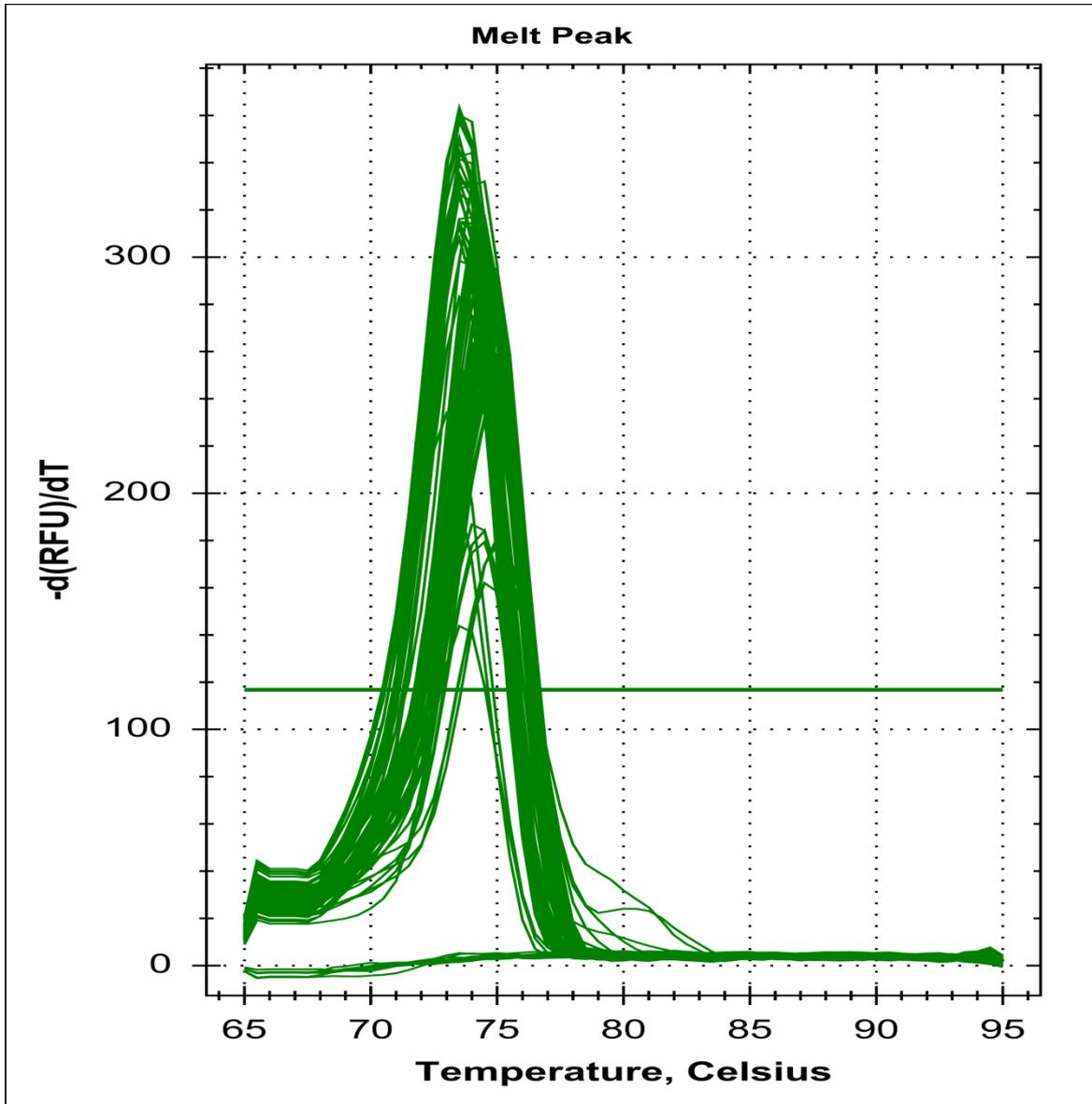
Figure 13: Melting curve for qRT-PCR. The graph depicts the melting curve of qRT-PCR. The X-axis represents the temperature in Celsius, and the Y-axis shows the rate of change of relative florescence unit as a function of temperature. At low temperatures, the nucleic acid-primer duplex don't dissociate and thus, the SYBR-Green dye don't quench, and the quantification is low. At high temperatures, the duplex dissociates and thereby the SYBR-Green emits florescence and the dRFU/dT increases. The peak of the curve is the Tm.

In-situ hybridization relies on the assessment of miRNAs via hybridizing them with a specific probe which is complementary to their sequence (Nielsen, 2012). However, it is disadvantaged by being a low-throughput and not a highly quantitative method. High-throughput sequencing of miRNA by means of RNA-Seq is specifically characterized by being able to discover new miRNAs. Furthermore, it is fast, and productive. On the other hand, it is disadvantaged by being expensive, demanding setup, and interfering artifacts (Baker, 2010).

1.5: The Role of miRNA in EAC: Current Understanding and Pending Questions

EAC is a multifactorial complicated disease that occurs on several stages, and underpinned by several molecular changes. Given the fact that miRNA can elicit one or more functions through several targets, the importance of studying miRNA in EAC has been underscored. Accordingly, the role of miRNA in EAC has been studied extensively. Several studies have tackled the role of miRNA dysregulation in EAC proliferation, migration, invasion, and metastasis.

Dysregulation of miRNA in esophageal cancer plays a central role in increasing cell proliferation, and thereby disturbing the homeostatic balance between proliferation and apoptosis. Disturbing the proliferation and/or apoptosis results in increased tumor growth. For instance, miR-375, a proliferation inhibitor that acts through targeting insulin-like growth factor 1 receptor (IGF1R), was reported to be downregulated in EAC (Kong et al., 2012). miR-31 which is upregulated in esophageal cancer enhances proliferation (He et al., 2012). Furthermore, miR-34, which is down-regulated, is controlled by p53, and it functions as an inhibitor for proliferation (Hunten, Siemens, Kaller, & Hermeking, 2013). The over-expression of miR-196a resulted in enhanced proliferation and blocking apoptosis in EAC cells through targeting the tumor suppressor annexin A 1 (ANXA1) (Luthra et al., 2008). miR-21 which is an oncogene that is highly expressed in EAC targets programmed cell death (PDCD4) pathway, and thereby increased proliferation (Fassan et al., 2010). miR-133b, which is down-regulated in esophageal cancer, was shown to block proliferation via targeting fascin homologue 1 (FSCN1) (Kano et al., 2010).

miRNA dysregulation is also responsible for invasion and metastasis in esophageal cancer. miRNA dysregulation enhances invasion and metastasis through the disruption of cell-cell adhesion matrix, dissociation of the extracellular matrix, and induction and sustainability of cell growth (He et al., 2012). These effects are achieved through the subsequent alteration of the signaling mechanisms central to invasion and metastasis. One

approach to study the effect of miRNA invasion and metastasis is to elucidate the association between the dysregulation of miRNA expression and cell motility and invasiveness. For instance, the gain-of-function approach elucidated the role of miR-143, miR-145, and miR-133b in blocking proliferation and invasion via targeting FSCN1 gene (Kano et al., 2010).

Owing to the role of miRNA dysregulation in EAC, several studies addressed the miRNA expression profile associated with EAC in an aim to retrieve miRNA biomarkers for early diagnosis. Feber et al reported the dysregulation of miR-21, miR-192, miR-194, miR-200c, miR-205, miR-203, and miR-93 in EAC (Feber et al., 2008). Furthermore, Mathe et al indicated the dysregulation of miR-21, miR-223, miR-192, mR-194, miR-203, and miR-375 in EAC primary tissues, and correlated the expression of these miRNAs with prognosis and response to treatment (Mathe et al., 2009). However, to date, the miRNA expression profile associated specifically with EAC rather than other types of UGCs, and EAC progression is not understood.

Few studies reported the role of miRNA in EAC therapy. miR-148a was shown to improve the response to cisplatin/5-fluorouracil (5-FU) in 7 out of 10 EAC cell lines as demonstrated by the reduction in cell viability by approximately 50% (Hummel et al., 2011). miR-31 was also reported to enhance the response of EAC tumors to radiation. However, the therapeutic role of miRNA in EAC is not studied extensively and not fully understood.

Given the substantial role played by miRNA dysregulation in EAC, a comprehensive understanding of the functional and mechanistic implications of miRNA is a necessity. However, several challenging are still ahead for the study of miRNA biology in tumors in general. For instance, miRNAs are known to be tissue specific (Calin & Croce, 2006). In other words, the effect that the miRNA may elicit in one tissue is not universal for other tissues. Furthermore, the off-target effects that might hinder or confound the effect of a

miRNA. In addition, the other biological mediators that might interfere with the role of miRNAs such as the other non-coding RNAs that compete with miRNA binding to the target. The lack of mouse models for miRNA dysregulation represents a major inadequacy in the field of miRNA biology (Iorio & Croce, 2012).

From a diagnostic perspective, the utilization of miRNAs as markers essentially implies the presence of highly accurate and reproducible methods. However, the observed variations between studies introduce further challenges for the miRNA translational research. Moreover, the role of miRNA as therapeutic targets or drugs is still pended by several challenges. One of the major challenges ahead miRNA therapies is the liability of degradation of miRNA, and miRNA antisense (Iorio & Croce, 2012). Furthermore, the bioavailability of miRNA sequence in the target organ represents a huge challenge. However, some chemical modifications for the miRNA sequence protect from degradation and enhance the bioavailability in the target organ. For instance, the integrity and bioavailability of miR-221 was significantly enhanced upon adding a cholesterol derivative to the 5' end of the miRNA (Park et al., 2011). This has been demonstrated by the increased cholesterol form of miR-221 levels in the liver and significantly enhanced function as compared to the unmodified molecule of miR-221 (Park et al., 2011). The ability of miRNA to predict response to treatment as an aspect of personalized medicine still needs to be explored and validated in large patient cohorts. This is essentially true given the fact that most miRNAs involved in proliferation or apoptotic networks are only addressed in in vitro models (Iorio & Croce, 2012).

1.6: Stratifications between EAC and Gastric Adenocarcinoma: Histological, Pathological and Molecular Differences

Upper gastrointestinal adenocarcinomas (UGCs) have resemblance in histology, and molecular characteristics; however, they possess differential pathophysiological characteristics. From a histological point of view, gastric adenocarcinoma (GC), and EAC display similarities and differences. The Lauren classification stratifies GC into 2 groups: intestinal and diffuse. This stratification is based on the growth pattern and microscopic morphology. Diffuse tumors are characterized by the presence of non-cohesive tumor cells that infiltrate the gastric stroma as well as the stomach wall without the formation of gland (Dicken et al., 2005). In addition, diffuse tumors also display the growth of fibrous or connective tissue (i.e. desmoplasia). Unlike the intestinal-type gastric cancers, diffuse cancers are not caused by environmental factors, and thus can occur in young patients. Diffuse-type gastric cancers originate from de-novo cells, and not resulting from chronic inflammation. These tumors are initiated from a single-cell mutations within normal gastric glands (El-Rifai & Powell, 2002). On the other hand, the intestinal-type GC is characterized by gland formation, and thereby is more similar to EAC. Both EAC and intestinal-type GC are associated with gland formation that resembles colonic mucosa under the microscope. The glandular morphology ranges from poor-to well-differentiated. Intestinal GC is preceded by chronic atrophic gastritis, and is characterized by the adherence of tumor cells to form tubular or glandular like structures (Dicken et al., 2005).

Although EAC, diffuse- and intestinal-type GC share some common molecular characteristics, there are several distinct molecular perturbations, and at least a main characterizing event associated with each of them. For instance, the genetic or epigenetic silencing of E-cadherin, a cell surface protein required for connection between cells, and cellular architect, is a major cancer-driving event underlying diffuse GC (Yakirevich & Resnick, 2013). However, in case of intestinal GC, genetic and epigenetic perturbations in MSI-H, p53, APC, and β-catenin are the major events (El-Rifai & Powell, 2002). On the other hand, as for EAC, perturbations in ERBB2, p53, and TGF-β are among the underlying causes.

Thus, some molecular lesions are shared among EAC and GC, whereas others are tumor-specific.

The incidence of esophageal adenocarcinoma (EAC) has increased dramatically throughout last 3 decades (Blot, Devesa, & Fraumeni, 1993; DeMeester, 2006; Hesketh, Clapp, Doos, & Spechler, 1989). One of the challenges associated with EAC is its late diagnosis where tumor is diagnosed at progressive stages which render the prognosis very poor (Shah & Kurtz, 2010). The elucidation of specific molecular mechanisms underpinning EAC is central to early diagnosis and effective treatment. A major requirement for understanding tumorigensis is the detection of the driving molecular events that take place in early stages of tumor formation. Accordingly, elucidating the molecular biomarkers associated with all EAC pre-tumorigenic and tumorigenic stages will provide a comprehensive understanding for driving genetic alterations (Kumar, Mohan, & Guleria, 2006; Shah & Kurtz, 2010).

Although some reports indicated the miRNA expression profile associated with EAC, the specific miRNA signature that can discriminates EAC from gastric adenocarcinoma is not elucidated. Furthermore, the miRNA expression across different EAC stages is not well understood. In this study, a comprehensive miRNA profiling and validation were attempted to examine the miRNAs that are specific to EAC, and not dysregulated in gastric cancer. Furthermore, miRNA expression across the different stages of EAC was investigated.

CHAPTER 2:-MATERIALS and METHODS[2]

Ethics Statement

The repositories of pathology at Vanderbilt University (Nashville, TN, USA) and the National Cancer Institute Cooperative Human Tissue Network (CHTN) have supplied human tissue samples. Both Institutional Review Boards at the American University in Cairo (AUC), and Vanderbilt University Medical Center (VUMC) provided approvals for the utilization of the specimens. Written consents were obtained from subjects. Samples included in the study were leftover tissues that were extra after diagnosis was that usually are trashed (Saad et al., 2013).

Sample collection and total miRNA extraction

46 NS, 13 isolated BE, 10 BE adjacent to HGD, 17 HGD, and 34 EAC tissues were used in this study. The esophageal tissue samples were either frozen or formalin-fixed paraffin-embedded (FFPE) (Saad et al., 2013). In addition, 78 (45 NG and 33 GC) frozen gastric tissue samples were utilized for comparison with esophageal tissues. All the NS tissues represent different individuals. 38 NS samples were adjacent to EAC (n=23), isolated BE (n=6), BE adjacent to HGD (n=3) or HGD lesions (n=6). From the BE tissues adjacent to HGD, 8 were matched with HGD tissues. All NG samples are from different patients except for 2 samples are obtained from the same patient. Similarly, all 33 GC tissue samples are from different patients except for 2 samples. 20 GC tissue samples were matched with NG. Out of the 33 GC samples, 25 are antral samples, 4 were from the gastro-esophageal junction (GEJ), 2 from the cardia, 1 from the pylorus, and 1 from the body. Histological diagnosis was performed according to H&E-stained sections. The samples had a wide spectrum that included well-differentiated adenocarcinomas and poorly-differentiated, in addition to both intestinal- and diffuse-type tumors (Saad et al., 2013). The total RNA/miRNA was isolated by miRNeasy kit (Qiagen, Maryland, USA).

Briefly, frozen tissue samples were minced using homogenizer and placed in 700 ul trizol for 10 minutes at room temperature, followed by adding 140 ul chloroform. This was followed by incubation at room temperature for 2 minutes then centrifugation at 13,400 RPM, 4°C for 15 minutes to ensure effective segregation between aqueous and organic phases. The upper aqueous phase was then separated in a separated microcentrifuge tube and treated with 3 times its volume 100% ethanol. The solution was then transferred to miRNAeasy columns where the total RNA/miRNA was bound to the column. Upon centrifugation, eluent was discarded and columns were subject to low-salt and high salt binding buffers in order to scavenge traces of thiocyanate and nuclease respectively.

Eventually, the total RNA/including miRNA was eluted by mean of nuclease free water. FFPE tissues were subject to similar extraction procedures in addition to an initial de-paraffinization step were carried out using D-limonene (Sigma-Aldrich, Saint Louis, MO) prior to extraction. Quality control analysis demonstrated no difference in the miRNA expression for samples purified from frozen or FFPE tissues.

Spectrophotometric Measurement of Total RNA/miRNA Yield:

Absorbance of RNA/miRNA was assessed by means of spectrophotometry at wave length (λ) of 260 nm. Total RNA/miRNA was diluted to suitable dilution using nuclease free water and the yield was calculated based on the formula:

RNA/miRNA Yield (ng/µl) = Absorbance at λ260 × 40 × Dilution Factor.

Measurements at λ 280 were performed to assess the purity of RNA/miRNA. The ratio between the absorbance at λ260 and λ 280 (A260/A280) ranged from 1.75 to 2 in all samples.

miRNA microarray analysis

Two different microarray platforms were utilized for the preliminary detection of dysregulated miRNAs: Agilent Human microRNA Microarray V2 (Agilent Technology, Santa Clara, CA) and Exiqon miRCURY LNA™ microRNA Array, v.11.0 – human (Exiqon Life Science, Woburn, MA). This approach served to identify the major changes and reduce the false discovery rate (FDR). For the preliminary detection, 3 EAC samples as compared to a pool of 3 adjacent normal tissue samples which were diagnosed histologically. This pooling was employed in order to further reduce the variations (Saad et al., 2013).

Microarray data was analyzed by Agilent Feature Extraction (AFE) program, as well as the R language. The following steps were executed for the analysis: 1) data transformation, 2) quantile normalization was carried out to achieve inter-array normalization, 3) log2 transformation of data, 4) miRNA log2 ratios calculation through abstracting log2 value of the pooled control sample from the average log2 value of 3 EAC. Exiqon miRNA data was analyzed by means of *limma* R package. The following steps were executed for the anaylsis (1) correction for background; (2) loess method for intra-array normalization; (3) median value calculation to reprint order to indicate the miRNA intensity value for replicate probes, and (4) In order to allow comparison among arrays, quantile normalization was carried out (Saad et al., 2013).

The two-class unpaired student's t-test was employed for the detection of miRNAs that exhibit differential expression between NS and EAC (Saad et al., 2013). For quality assurance purposes, the p values were subject to the false discovery rate (FDR) method (Benjamini & Hochberg, 1995). The set of miRNAs that overlapped between the two platforms to display at least a 2-fold change difference between both normal and tumor tissues were selected for validation to ensure that the difference is biological (Saad et al., 2013).

Quantitative real-time PCR (qRT-PCR)

Reverse transcription was carried out on 3 steps. The first step is the poly (A) tail synthesis that comprises: 2 μg RNA, 1.5 units of poly(A) polymerase, 10X poly(A) buffer, and 10X ATP. The reaction of 15 μl was incubated at 37°C for 30 minutes to allow for the annealing of Poly A tail. The second step that involves the annealing of the poly (dT)-adaptor upon incubation at 60°C for 5 minutes. The third step which is the reverse transcription was performed by iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, USA), where the manufacturer's instructions were followed (Saad et al., 2013).

CFX Connect Real-Time System (Bio-Rad, Hercules, USA) and iQ SYBR green supermix (Bio-Rad, Hercules, USA) were used for quantitative real-time PCR. Matue miRNA and primer sequences were identified using the miRbase (http://www.mirbase.org/). Table 2 illustrates the primers' sequences (Saad et al., 2013).

Table 2: List of qRT-PCR primers for miRNAs. Table is modified from (Saad et al., 2013).

miRNA	Primer Sequence
Universal primer	GCGAGCACAGAATTAATACGAC
miR-191	CAACGGAATCCCAAAAGCAGCTG
miR-21	TAGCTTATCAGACTGATGTTGA
miR-133b	TTTGGTCCCCTTCAACCAGCTA
miR-205	TCCTTCATTCCACCGGAGTCTG
miR-203	GTGAAATGTTTAGGACCACTAG
miR-215	ATGACCTATGAATTGACAGAC
miR-200a	TAACACTGTCTGGTAACGATGT
mir-194	TGTAACAGCAACTCCATGTGGA
mir-192	CTGACCTATGAATTGACAGCC
mir-31	AGGCAAGATGCTGGCATAGCT

miR-191 was used as a reference gene for normalization. The formula $2^{(Rt-Et)}$ was exploited for fold change calculation (Saad et al., 2013). Rt is the threshold cycle number of the reference gene in the sample. Et is the threshold cycle number of the experimental gene in the sample. The Student's t-test was set up for the assessment of statistical significance using a cutoff at ≤0.05. A heatmap demonstrating the relative fold changes esophageal tissue samples was created by means of Treeview® software. All values were log transformed and centered to the median (Saad et al., 2013).
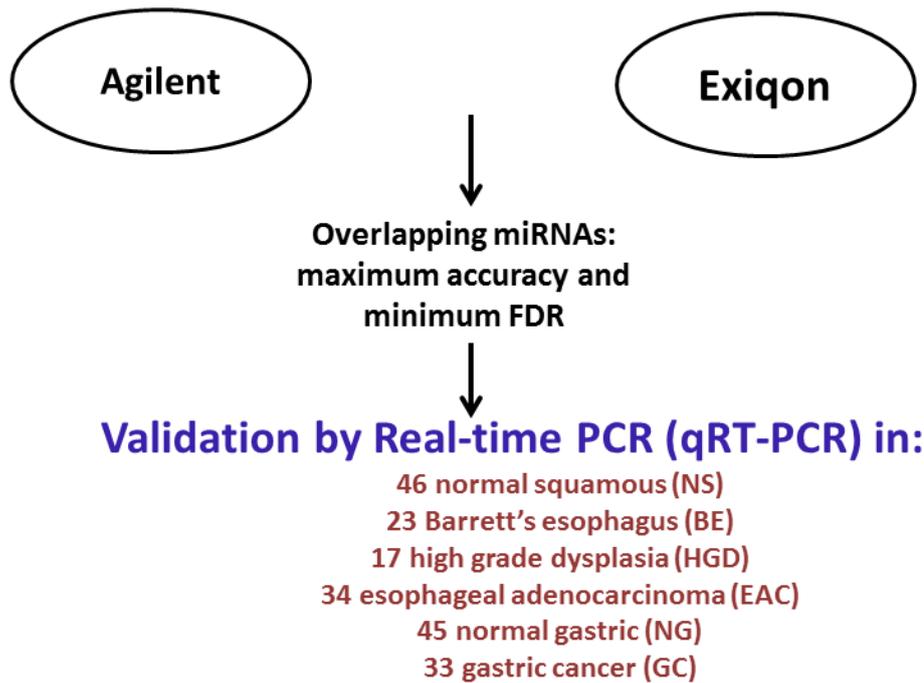
Figure 14: Flowchart for the expeimental methodology employed for profiling the unique miRNA signature associated with EAC. 2 microarray platforms were employed for the preliminary expression profiling: Agilent and Exiqon. The miRNAs that overlapped between both platforms to display differential expression between NS and EAC by at least 2 FC were subject to independent validation by qRT-PCR in esophageal and gastric tissues to identify those miRNAs whose dysregulation is specifically unique for EAC.

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[2]The Materials and Methods is paraphrased from (Saad et al., 2013). The copyright license is present in Appendix G.

I- Identification of deregulated miRNAs in EAC

The results of microarray indicated that 21 miRNAs overlapped between the 2 microarray platforms to be dysregulated in EAC as compared to NS (FC≥2.0 or ≤-2.0, p<0.05). Out of the 21 miRNAs, 11 miRNAs were down-regulated, whereas 10 miRNAs displayed overexpression (Figure 15, Table 3) (Saad et al., 2013). Among the 21 overlapping miRNAs, 8 miRNAs that were validated independently by for qRT-PCR in isolated BE, NS, BE lesions adjacent to HGD, HGD, and EAC tissue samples (Saad et al., 2013).
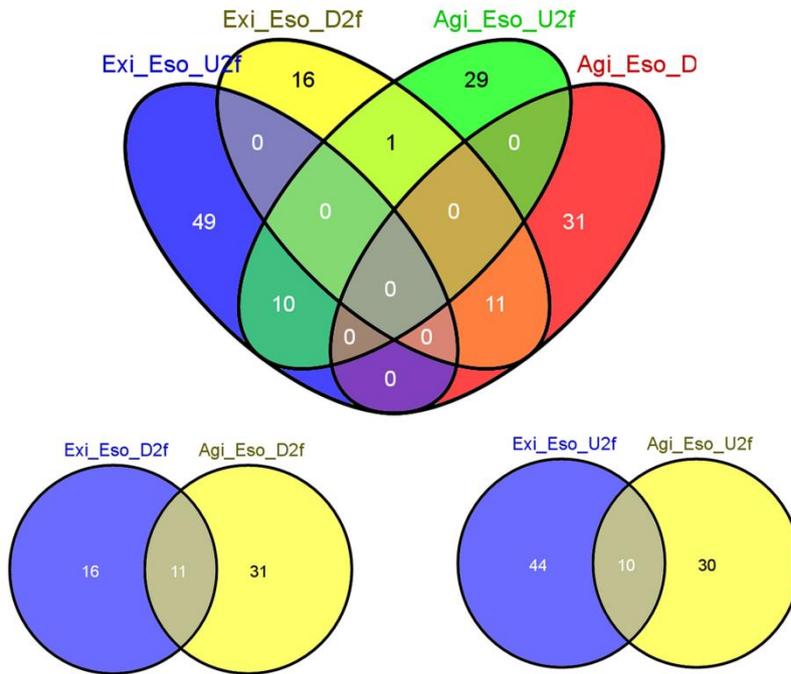
Figure 15: Venn diagram of miRNA analysis in EAC. Venn diagram depicting the miRNAs overlapping between Exiqon and Agilent platforms. The set of miRNAs that overlapped between the 2 platforms to display ≥2 FC of up-regulation or ≤-2 FC of down-regulation were considered significant. 11 miRNAs were consistently down-regulated across the 2 platforms whereas, 10 miRNAs were consistently up-regulated in both platforms. Exi, Exiqon; Agi, Agilent; D, down-regulated; U, up-regulated. Figure is modified from (Saad et al., 2013). Copyright license is present in Appendix G.

Table 3: miRNAs that displayed at least 2-fold change in Agilent and Exiqon. 21 miRNAs that overlapped between the 2 platforms to display ≥ 2 or ≤-2 FC of dysregulation in EAC. Table is modified from (Saad et al., 2013). Copyright license is present in Appendix G.

miRNA	Agilent	Exiqon
Down-regulated		
hsa-miR-1274a	-4.19	-3.63
hsa-miR-1274b	-2.22	-2.52
hsa-miR-133b	-6.70	-3.01
hsa-miR-143*	-5.87	-2.25
hsa-miR-144	-7.83	-8.80
hsa-miR-145	-5.63	-4.19
hsa-miR-145*	-3.18	-4.12
hsa-miR-203	-71.85	-30.44
hsa-miR-205	-149.71	-178.03
hsa-miR-31	-8.77	-8.87
hsa-miR-365	-2.78	-2.07
Up-regulated		
hsa-miR-192	145.21	58.40
hsa-miR-194	105.57	52.86
hsa-miR-196a	2.62	2.86
hsa-miR-200a	4.61	4.79
hsa-miR-200b	2.69	3.80
hsa-miR-21	2.25	2.68
hsa-miR-215	131.78	33.06
hsa-miR-429	3.93	3.11
hsa-miR-574-5p	2.19	2.06
hsa-miR-7	2.98	3.98

II-Identification of dysregulated miRNAs in BE

In order to identify miRNA deregulations in BE, miRNA expression by qRT-PCR in NS and BE frozen and FFPE tissue samples. 3 miRNAs (miR-192, miR-215 and miR-194) were found to be over-expressed in BE relative to NS (FC $\geq$2, P $\leq$ 0.05) (Figure 16, Table 4). On the other hand, 3 miRNAs (miR-205, miR-203, and miR-31) were of low abundance (FC $\leq$ -2, P $\leq$ 0.05) in BE as compared to NS (Figure 16, Table 4) (Saad et al., 2013). This data suggests the potential role of these miRNAs in BE development. On the contrary, miR-200a, and miR-133b didn't exhibit any statistically significant differential expression between NS and BE (Figure 17, Table 4) (Saad et al., 2013).
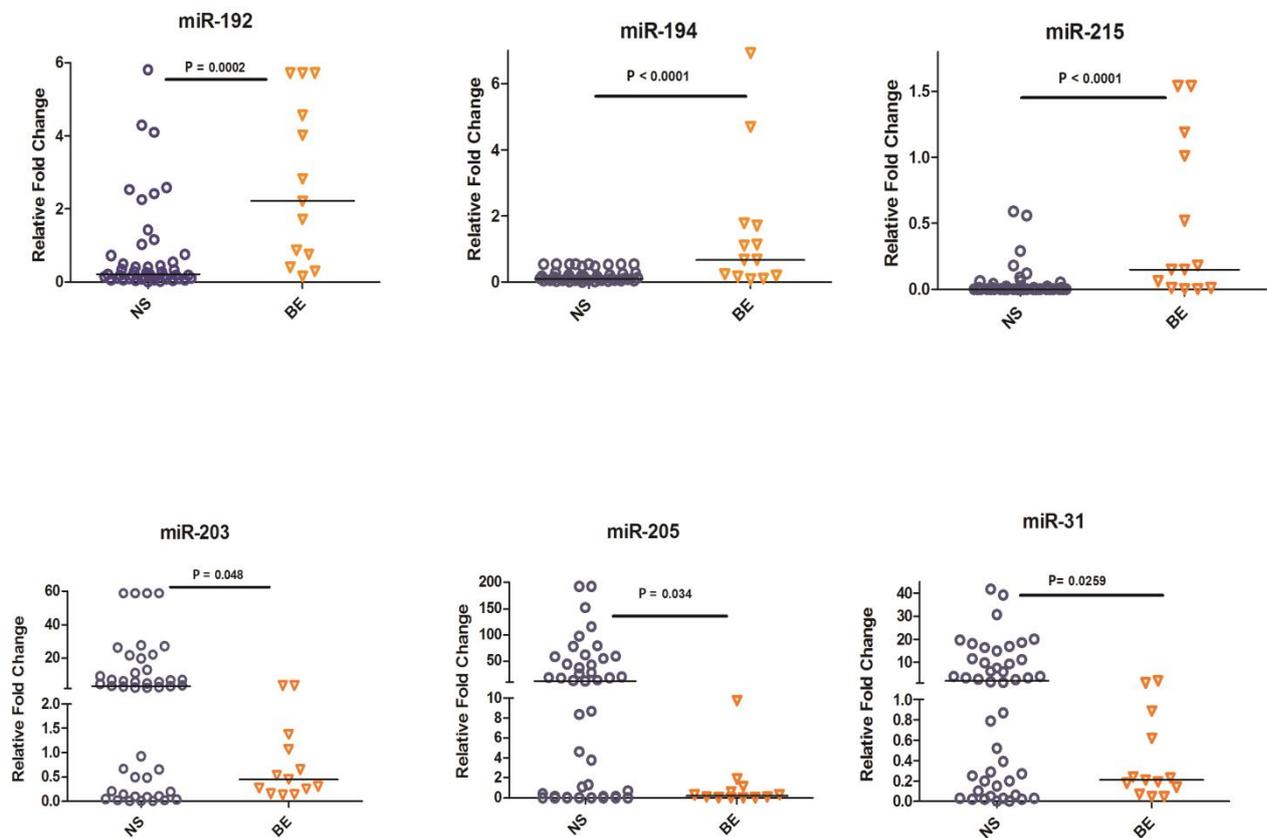
Figure 16: Differentially expressed miRNAs between NS and BE. miRNA expression was measured by qRT-PCR in NS and BE tissues. 6 miRNAs displayed aberrant expression in BE as compared to NS. The housekeeping miRNA miR-191 was used as a reference miRNA for normalization. Fold Change was calculated based on this formula: $2^{(Rt-Et)}$. Statistical significance was evaluated by means of Student's t-test. FC cutoff was set at $\geq$2

or ≤-2, and p value cutoff is ≤0.05. Figure is modified from (Saad et al., 2013). Copyright license is present in Appendix G.
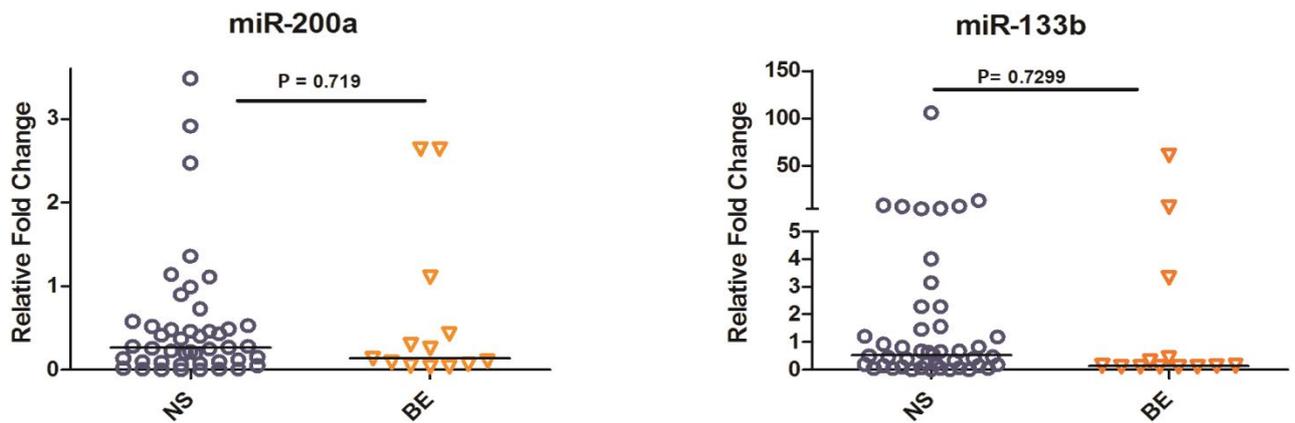
Figure 17: miRNAs that have similar expression trends in NS and BE. miR-200a and miR-133b didn't exhibit any statistically significant differential expression between NS and BE. The housekeeping miRNA miR-191 was used as a reference miRNA for normalization. Fold Change was calculated based on this formula: $2^{(Rt-Et)}$. Statistical significance was evaluated by means of Student's t-test. FC cutoff was set at ≥2 or ≤-2, and P value cutoff is ≤0.05. Figure is modified from (Saad et al., 2013). Copyright license is present in Appendix G.

Table 4: Differential expression of miRNAs between NS and BE. Summary of miRNA expression in BE. miR-194, miR-192, and miR-215 were up-regulated (FC ≥2, P ≤0.05). miR-203, miR-205, and miR-31 were down-regulated (FC ≤-2, P ≤0.05). miR-200a and miR-133b displayed similar expression in both miR-133b and miR-200a. Table is modified from (Saad et al., 2013). Copyright license is present in Appendix G.

miRNA	Median value of fold change in NS tissue	BE	
		Median value of fold change	**P value as compared to NS**
miR-194	0.1	0.67	< 0.0001
miR-192	0.2	2.21	0.0002
miR-215	0.01	0.1	< 0.0001
miR-205	11.87	0.20	0.03
miR-203	3.33	0.45	0.05
miR-31	2.63	0.21	0.024
miR-200a	0.2	0.14	0.719
miR-133b	0.51	0.14	0.45

III- *Differential Expression of miRNA between Isolated BE and BE Lesions Adjacent to HGD*

Using qRT-PCR, the expression of the selected 8 miRNAs was measured in 13 isolated BE, 10 BE adjacent to HGD, and 17 HGD samples. miR-192, miR-194, miR-31, miR-215, and miR-21 displayed as statistically significant over-expression in BE tissues adjacent to HGD, as compared to the isolated BE samples (P<0.05) (Figure 18, Table 5) (Saad et al., 2013). Furthermore, both BE adjacent to HGD and HGD samples didn't exhibit a statistically significant variation in expression levels. This indicates that BE adjacent to HGD has a similar miRNA expression signature to that of HGD. Furthermore, these data suggest that these miRNAs might be implicated in the early stages of Barrett's tumorigensis. On the other hand, miR-200a, miR-205, miR-133b, and miR-203 showed no difference in their expression levels among BE and BE adjacent to HGD (Saad et al., 2013).
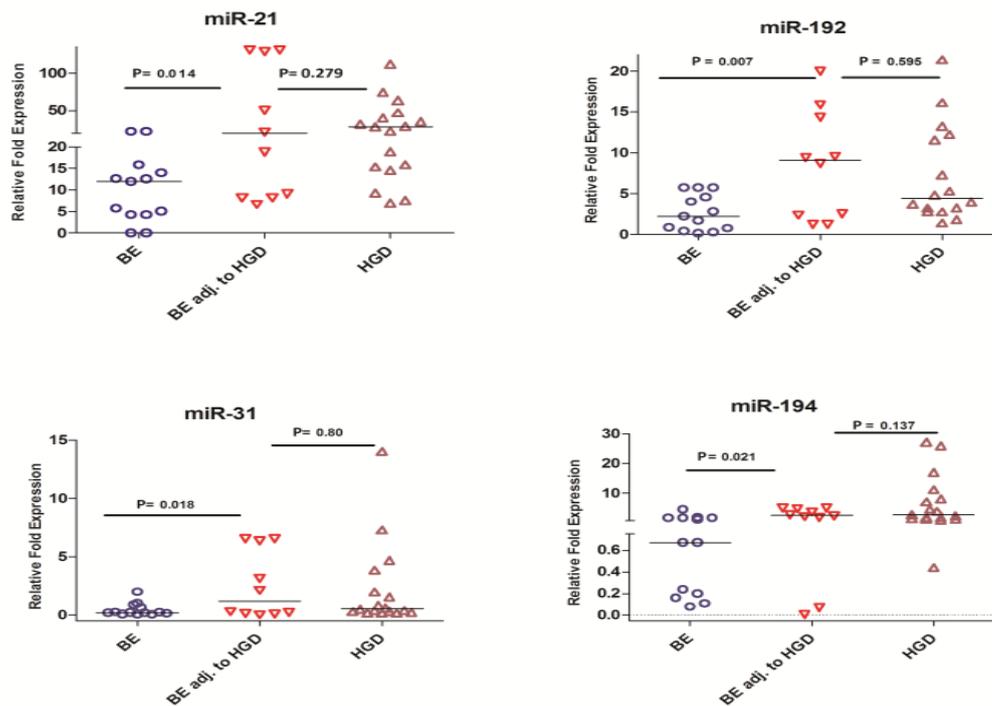
Figure 18: The differential miRNA expression between BE and BE adjacent to HGD.
qRT-PCR measurement of miRNA expression in isolated BE, BE adjacent to HGD and HGD. 4 miRNAs were differentially expressed between BE and BE adj. to HGD (FC ≥ 2 or ≤ -2, p ≤ 0.05). The housekeeping miRNA miR-191 was used as a reference miRNA for normalization. Fold Change was calculated based on this formula: $2^{(Rt-Et)}$. Statistical significance was evaluated by means of Student's t-test. FC cutoff was set at ≥ 2 or ≤ -2, and P value cutoff is ≤ 0.05. Figure is modified from (Saad et al., 2013). Copyright license is present in Appendix G.

Table 5: Median levels of miRNA expression in isolated BE and BE adjacent to HGD

miRNA	Isolated BE	BE adjacent to HGD		HGD	
	[#]Median value	[#]Median value	*P value	[#]Median value	**P value
miR-194	0.67	2.47	0.021	2.73	0.137
miR-192	2.21	9	0.007	4.37	0.595
miR-21	11.95	20.08	0.014	28.3	0.279
miR-31	0.21	1.2	0.018	0.455	0.8
miR-200a	0.14	0.93	0.287	1.22	0.465
miR-205	0.2	0.3	0.324	0.4	0.351
miR-203	0.45	1.12	0.565	0.93	0.327
miR-133b	0.14	0.21	0.597	0.08	0.120

[#]**The median values of FC are shown relative to reference miR-191**

*P value is shown for the comparison to BE

**P value is shown for the comparison to BE adjacent to HGD

Table adapted from (Saad et al., 2013). **Copyright license is present in Appendix G.**

IV- Identification of HGD-Associated miRNA Signature

Upon measuring miRNA expression levels in HGD tissue samples, miR-192, miR-194, and miR-200a were found to display statistically significant dys-regulation in HGD as compared to BE and NS tissues (FC≥2, or ≤-2, p≤0.05) (Figure 5, Table 3) (Saad et al., 2013). Furthermore, miR-215 and miR-31 were dys-regulated in HGD as compared to NS (FC≥2, or ≤-2, p≤0.05) (Figure 19, Table 3), whereas miR-21 was found to be up-regulated in HGD as compared to BE (Saad et al., 2013).

Table 6: HGD-associated miRNA signature. Table modified from (Saad et al., 2013). **Copyright license is present in Appendix G.**

MiRNA	Median value of FC in NS	Median value of FC in BE	Median value of FC in HGD	P values	
				P value upon comparing HGD to NS	P value upon comparing HGD to BE
miR-194	0.1	0.67	2.73	<0.0001	0.032
miR-192	0.2	2.21	4.37	<0.0001	0.01
miR-21	16.77	11.95	28.3	0.076	0.005
miR-215	0.01	0.1	0.99	<0.0001	0.19
miR-200a	0.2	0.14	1.22	0.0003	0.050
miR-31	2.63	0.21	0.455	0.02	0.07
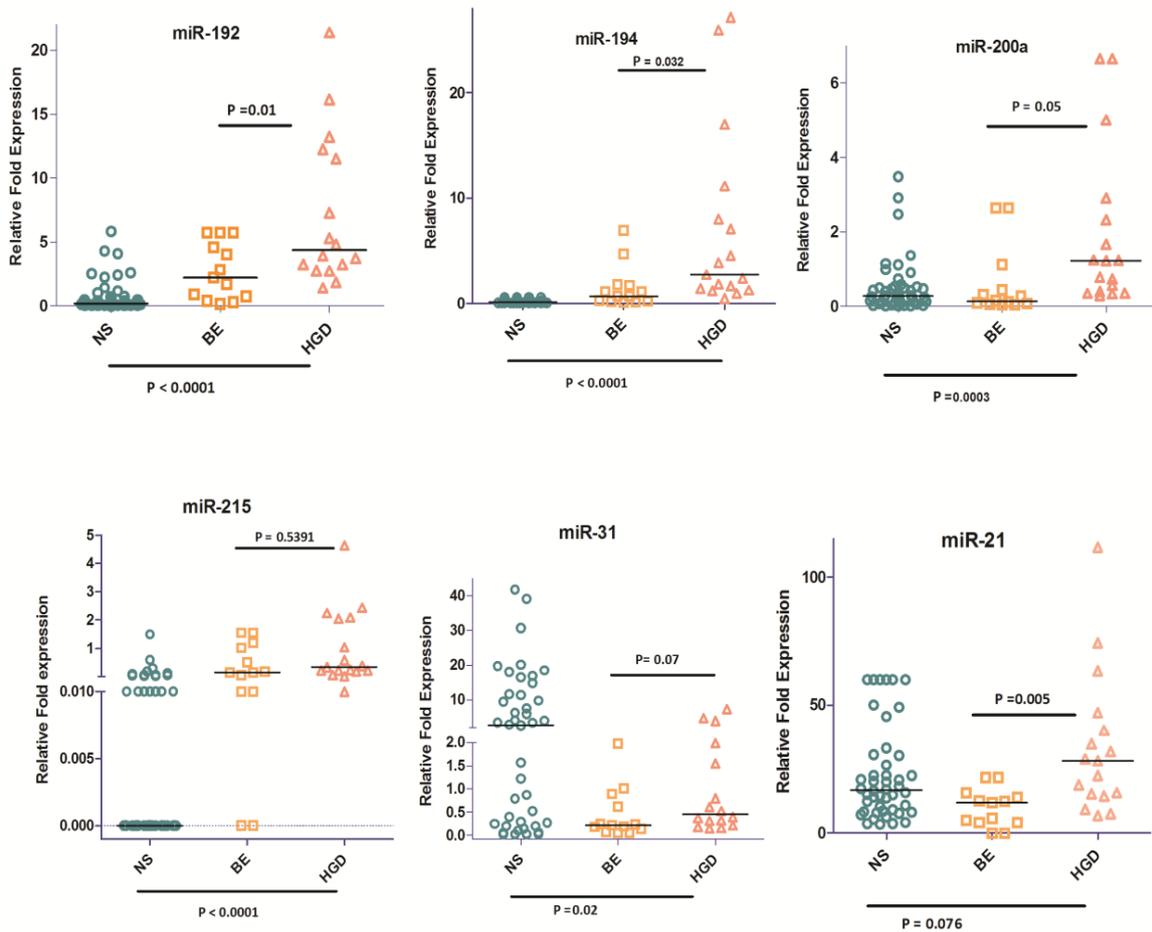
Figure 19: miRNAs that are dysregulated in HGD. The miRNA expression was evaluated in NS, BE, and HGD tissues by qRT-PCR. The housekeeping miRNA miR-191 was used as a reference miRNA for normalization. Fold Change was calculated based on this formula: $2^{(Rt-Et)}$. Statistical significance was evaluated by means of Student's t-test. FC cutoff was set at ≥ 2 or ≤ -2, and P value cutoff is ≤ 0.05. Figure is modified from (Saad et al., 2013). Copyright license is present in Appendix G.

V- Validation of the specific miRNA signature associated with EAC

Independent measurement of miRNA expression in a large set of esophageal tissue samples by qRT-PCR has validated the microarray data. miR-194, miR-192, miR-21, miR-215, and miR-200a were confirmed to be overexpressed, whereas miR-203, miR-205, miR-133b, and miR-31 were validated as down-expressed in EAC as compared to NS (Saad et al., 2013). On the other hand, miR-194, miR-192, miR-21, miR-31 and miR-200a were up-regulated (Figure 20, Table 3), and, miR-133b, miR-203, and miR-205 were down-regulated in EAC as compared to isolated BE (Figure 21, Table 3) (Saad et al., 2013).

Upon profiling the miRNA expression throughout the esophageal tissues: NS, BE, HGD, and EAC, we found that some miRNAs such as miR-194, miR-192, miR-21, miR-133b, and miR-200a displayed progressive dysregulation from NS to EAC (Saad et al., 2013). However, miR-203, miR-205, and miR-31 didn't exhibit this trend where the miRNA dysregulation in HGD was not concordant to EAC (Saad et al., 2013).
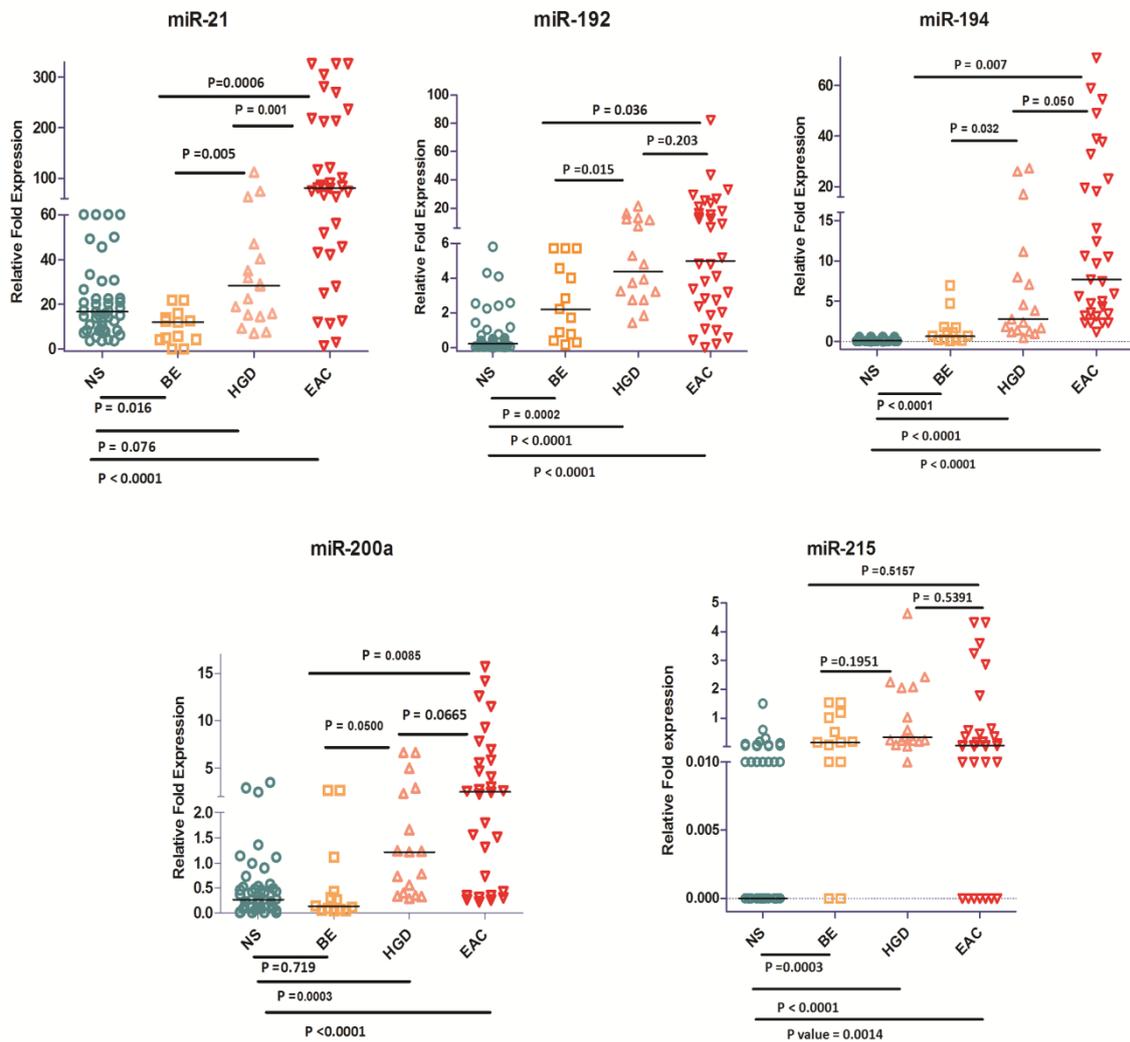
Figure 20: EAC up-regulated miRNAs. miRNA expression was evaluated in EAC by means of qRT-PCR. The graphs demonstrate the comparison between the expression levels across all esophageal tissues. miR-21, miR-192, miR-194, and miR-200a were up-regulated in EAC as compared to BE. miR-215 was up-regulated in EAC as compared to NS. The cutoffs were FC ≥2 or ≤-2, and p≤0.05. The housekeeping miRNA miR-191 was used as a reference miRNA for normalization. Fold Change was calculated based on this formula: $2^{(Rt-Et)}$. Statistical significance was evaluated by means of Student's t-test. FC cutoff was set at ≥2 or ≤-2, and P value cutoff is ≤0.05. The figure is adapted from (Saad et al., 2013). Copyright license is present in Appendix G.
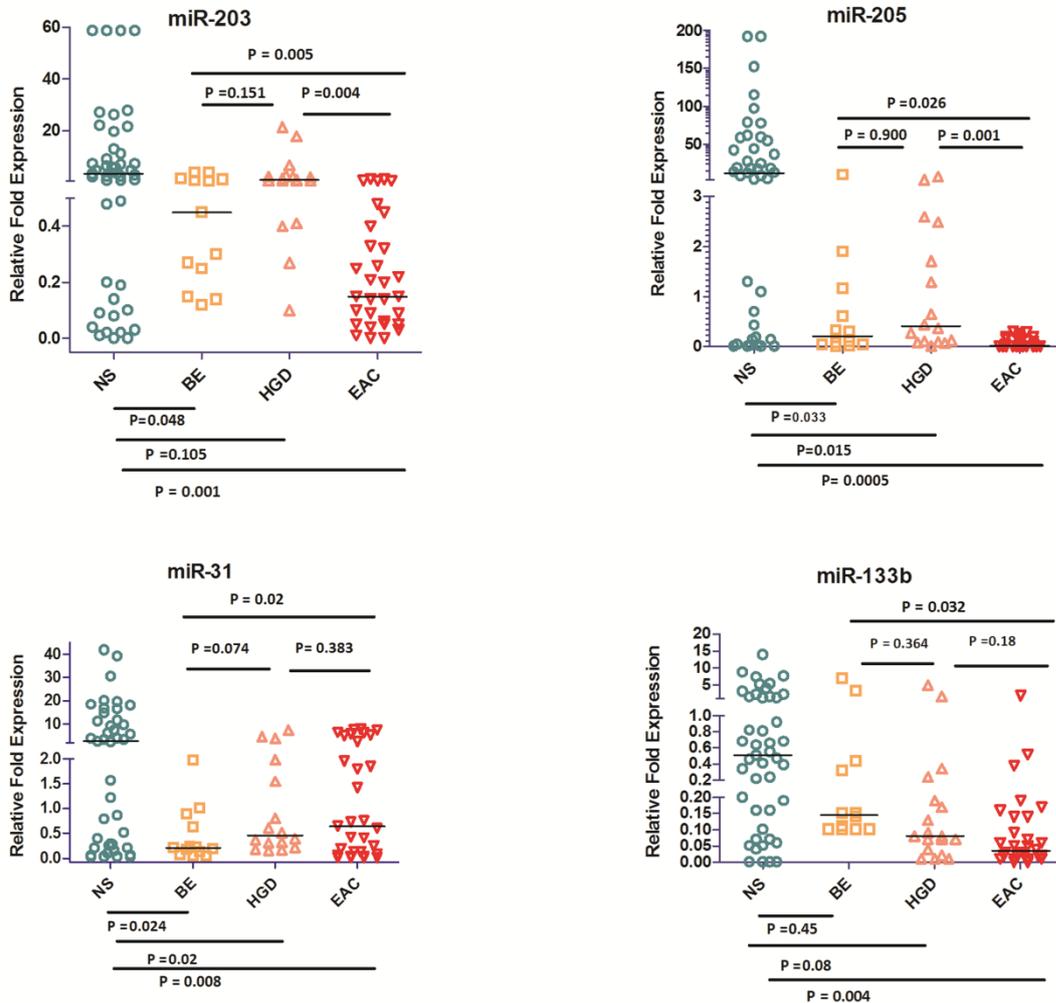
Figure 21: EAC down-regulated miRNAs. miRNA expression was evaluated in EAC by means of qRT-PCR. The graphs demonstrate the comparison between the expression levels across all esophageal tissues. miR-203, miR-295, and miR-133b were down-regulated in EAC as compared to BE and NS. miR-31 was paradoxically found to be up-regulated in EAC as compared to BE. However, it was down-regulated in EAC as compared to NS which is consistent with our microarray data. The cutoffs were FC ≥ 2 or ≤ -2, and $p \leq 0.05$. The housekeeping miRNA miR-191 was used as a reference miRNA for normalization. Fold Change was calculated based on this formula: $2^{(Rt-Et)}$. Statistical significance was evaluated by means of Student's t-test. FC cutoff was set at ≥ 2 or ≤ -2, and P value cutoff is ≤ 0.05. Figure is adapted from (Saad et al., 2013). Copyright license is present in Appendix G.

Table 7: Median levels of miRNA expression in NS, BE, HGD, and EAC. Table is adapted from (Saad et al., 2013). Copyright license is present in Appendix G.

miRNA	NS	BE		HGD		EAC	
	#Median value	#Median value	*P value	#Median value	**P value	#Median value	**P value
miR-194	0.1	0.67	<0.0001	2.73	0.032	7.68	0.007
miR-192	0.2	2.21	0.0002	4.37	0.015	5.00	0.036
miR-21	16.77	11.95	0.01	28.3	0.005	80.30	0.0006
miR-200a	0.2	0.14	0.719	1.22	0.050	2.5	0.008
miR-205	11.87	0.20	0.03	0.4	0.9	0.03	0.026
miR-203	3.33	0.45	0.05	0.93	0.15	0.15	0.005
miR-133b	0.51	0.14	0.45	0.08	0.36	0.03	0.032
miR-31	2.63	0.21	0.024	0.455	0.074	0.64	0.02

#**The median values of FC are shown relative to reference miR-191**

*__P value is shown for the comparison to NS__

**__P value is shown for the comparison to BE__

In an attempt to comprehensively visualize the signature of the validated miRNAs across all esophageal tissues, hierarchical clustering was carried out by means of TreeView® Software. The relative fold change expression values were converted to their corresponding log10 values, and hierarchical clustering was achieved as complete linkage. Figure 22 demonstrates the miRNA signature across the 4 histological groups of NS, BE (isolated BE, and BE adjacent to HGD), HGD and EAC (Saad et al., 2013). EAC can be stratified from NS and BE by the over-expression levels of miR-21, miR-194, miR-192, and miR-200a, and the down-regulation of miR-203, miR-205, and miR-133b. In contrast, the over expression of miR-203, miR-205, miR-31, and miR-133b in NS have stratified it from BE, HGD and EAC. HGD follows a miRNA expression trend similar to EAC in miR-192, miR-194, miR-21, and miR-200a. It is noteworthy to mention that the miRNA expression profile of BE lesions adjacent to HGD is similar to HGD rather than BE. The down-regulation of miR-203, miR-205, miR-31, and the up-regulation of miR-21, miR-192, and miR-194 in BE renders it clustered from NS.
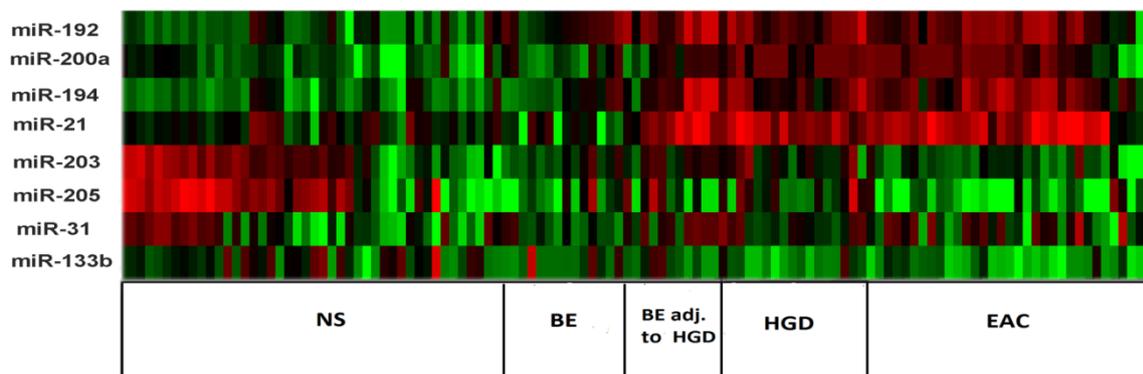
Figure 22: Heat map depicting the miRNA expression signature across esophageal tissues. Hierarchical biclustering of miRNAs and tissue samples by means of tree-view software demonstrates the stratification between the different esophageal tissues based on their miRNA expression trend. Expression levels range from low (Light Green) till high (Bright Red). EAC tissues are characterized by the over-expression signature of miR-192, miR-200a, miR-194, and miR-21, and the down-regulation of miR-203, miR-205, and miR-133b. HGD tissues shows miRNA expression trend that is similar to EAC. BE lesions that are adjacent to HGD are similar to HGD rather than isolated BE. BE is stratified from NS by the down-regulation of miR-205, miR-203 and miR-31, and the up-regulation of miR-192 and miR-194. Figure obtained from (Saad et al., 2013). Copyright license is present in Appendix G.

To determine the miRNA signature specific to EAC, the expression of the 8miRNAs was measured in gastric cancer tissue samples. miR-194, miR-192, miR-31, and miR-200a displayed over-expression in EAC rather than GC (Figure 23) (Saad et al., 2013). Moreover, miR-203 and miR-205 exhibited down-regulation in EAC and not in GC (Figure 23). These differences underscore the biological and pathogenic perturbations between the 2 tumors (Saad et al., 2013). On the other hand, miR-21 and miR-133b showed a similar pattern of dysregulation in EAC and GC (Figure 24) (Saad et al., 2013). This may indicate that these miRNAs are implicated in mutual pathways among both UGCs.
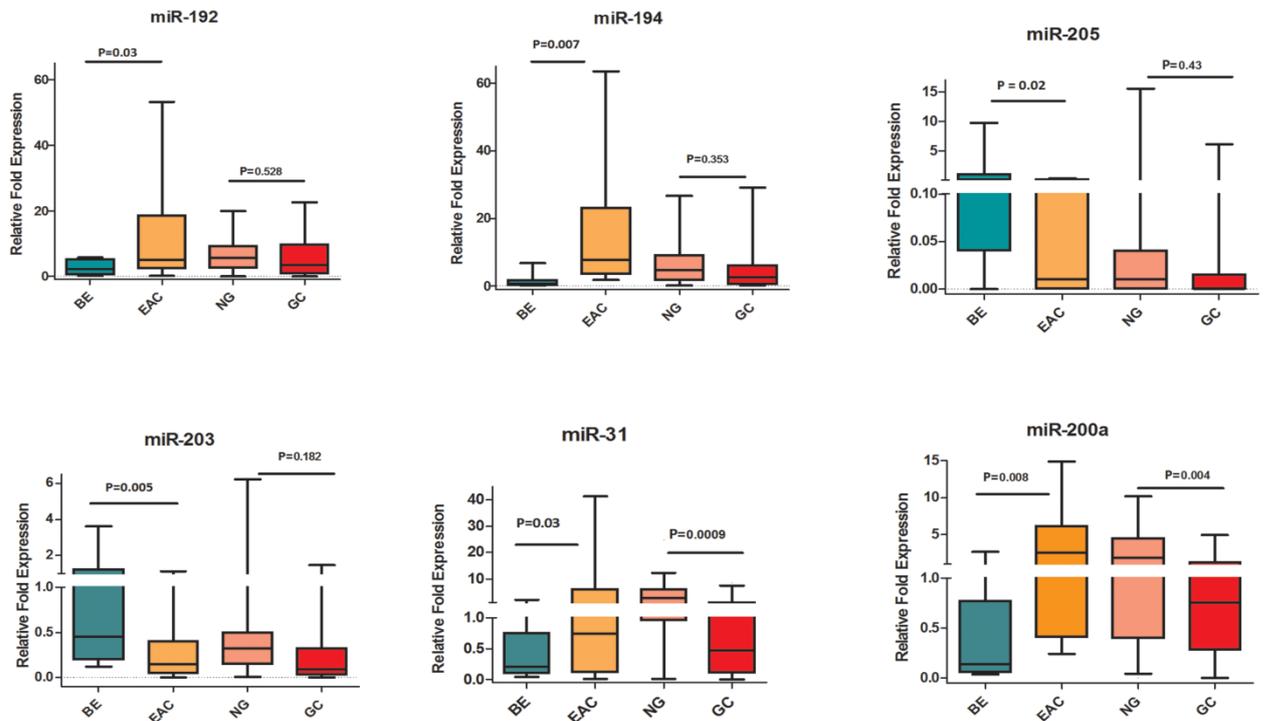
Figure 23: EAC unique miRNA signature. Box-and-Whisker plot depicting the qRT-PCR measurement of miRNA expression levels in BE, EAC, NG, and GC tissues. All expression levels were normalized to miR-191. BE was used as a tissue comparator for EAC, and NG was used as a tissue comparator for GC. miR-192, miR-194, miR-205, miR-203, miR-31, and miR-200a displayed a dys-regulation signature that is specific for EAC rather than GC. miR-192, miR-194, miR-205, and miR-203 didn't exhibit any differential expression among gastric tissues. miR-31 and miR-200a displayed opposite expression trends between EAC and GC. The cutoffs were FC ≥ 2 or ≤ -2, and $p \leq 0.05$ (Saad et al., 2013). Copyright license is present in Appendix G.

Table 8: Profile of differentially expressed miRNAs in EAC as opposed to GC. Table obtained from (Saad et al., 2013)**. Copyright license is present in Appendix G.**

miRNA	Status in EAC using BE as a comparator	Status in GC using NG as a comparator
miR-194	Up-regulated	Not significant
miR-192	Up-regulated	Not significant
miR-200a	Up-regulated	Down-regulated
miR-31	Up-regulated	Down-regulated
miR-205	Down-regulated	Not significant
miR-203	Down-regulated	Not significant
miR-133b	Down-regulated	Down-regulated
miR-21	Up-regulated	Up-regulated
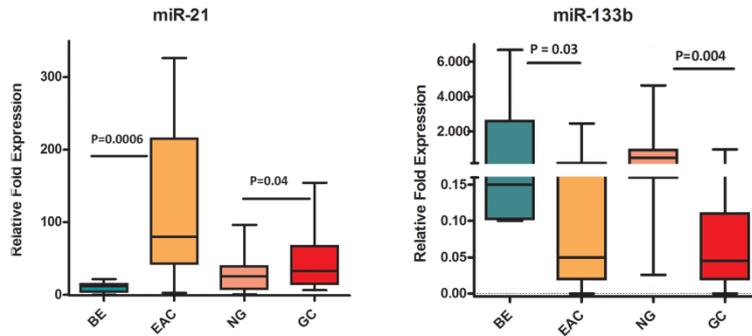
Figure 24: miRNAs that are common in EAC and GC. Box-and-Whisker plot depicting the qRT-PCR measurement of miRNA expression levels in BE, EAC, NG, and GC tissues. All expression levels were normalized to miR-191. BE was used as a tissue comparator for EAC, and NG was used as a tissue comparator for GC. miR-21 and miR-133b displayed similar expression trends among both EAC and GC. The cutoffs were FC ≥2 or ≤-2, and p≤0.05 (Saad et al., 2013)**. Copyright license is present in Appendix G.**

VI- Association of the miRNA Expression Levels with Different EAC Stages

To assess the miRNA expression across different EAC stages, the miRNA expression levels in different EAC stages were measured by qRT-PCR. According to the TNM classification system, samples were clustered from stage I to stage III. miR-203 was significantly down-regulated in stage III as compared as stage I (P_{ANOVA}=0.0006, $P_{I\&II}$=0.054, $P_{II\&III}$=0.002, and $P_{I\&III}$=0.01) (Saad et al., 2013). This suggests that the dysregulation of this miRNA may be involved in the progression of EAC. On the other hand, the miR-194, miR-200a and miR-192 exhibited a significantly higher expression in stage I as compared to later stages (P_{ANOVA} ≤0.0009, $P_{II\&III}$ ≤0.003, and $P_{I\&III}$ ≤0.006) (Saad et al., 2013). This may indicate that role of these miRNAs in early stages of Barrett's tumorigensis (Figure 25).
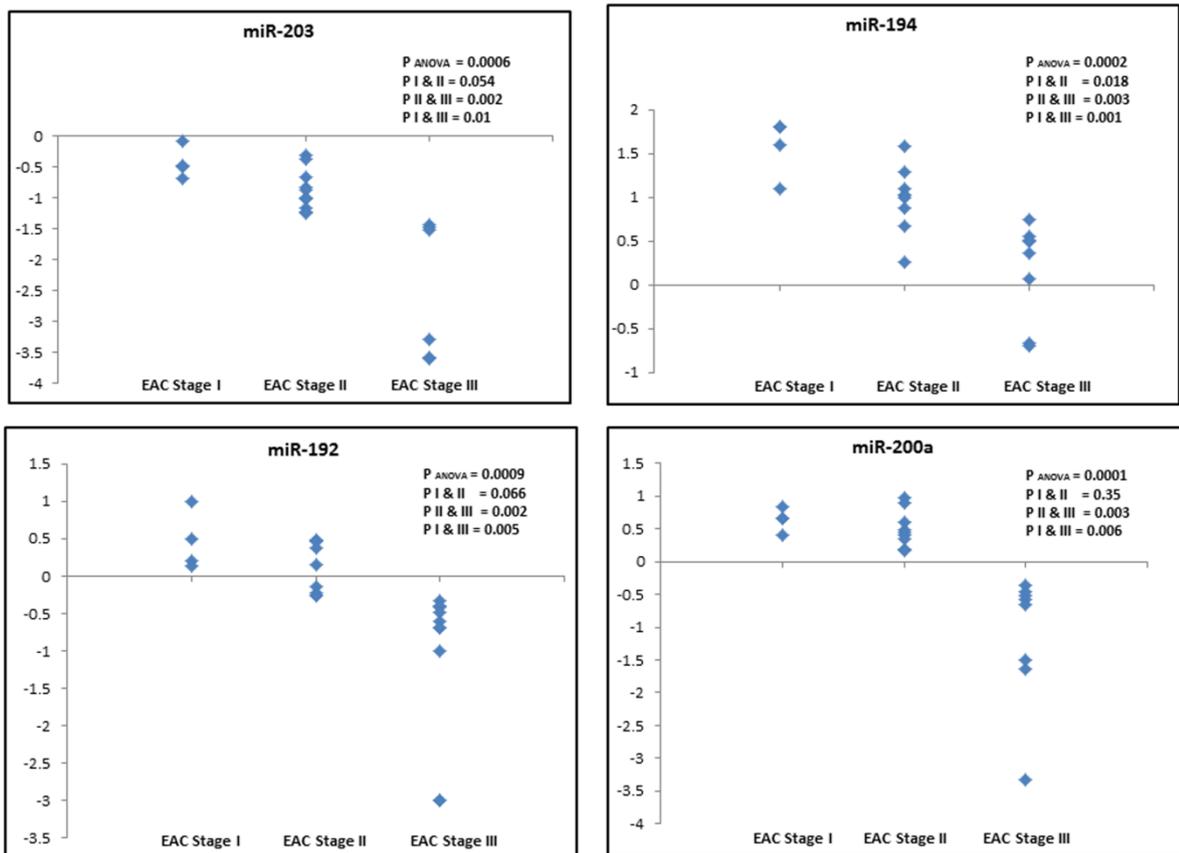
Figure 25: miRNA expression across different EAC stages. miRNA expression across EAC stages I, II, and III was compared upon measurement by qRT-PCR. All expression levels were normalized to miR-191. ANOVA was used for the assessment of statistical significance between the 3 EAC stages. Student's t-test was used for analyses of significance between each 2 stages. Modified from (Saad et al., 2013)**. Copyright license is present in Appendix G.**

Table 9: Median levels of miRNA expression in different EAC stages.

miRNA	Stage I	Stage II		Stage III			
	#Median	#Median	*P	#Median	**P	***P	P_AVOVA
miR-203	-0.1	-0.9	0.054	-1.5	0.002	0.01	0.0006
miR-194	1.7	1.0	0.018	0.4	0.003	0.001	0.0002
miR-192	0.4	-0.2	0.066	-0.7	0.002	0.005	0.0009
miR-200a	0.7	0.4	0.35	-0.6	0.003	0.006	0.0001

#The median values of FC are shown relative to reference miR-191

*P value is shown for stage II as compared to stage I

** P value is shown for stage III as compared to stage II

***P value is shown for stage III as compared to stage I

---

[3]The data of this thesis was accepted for publication in PloSOne. The citation is (Saad et al., 2013). The copyright license is present in Appendix G.

CHAPTER 4: DISCUSSION

Several evidence points out to the central role of the aberrant miRNA expression in the development and cancer progression in UGCs (Ding et al., 2010; Fassan et al., 2011; Hamfjord et al., 2012). miRNAs have been considered significantly owing to their high potential to modulate several signaling pathways that pose major oncogenic or tumor suppressor functions. According to the function of their targeted moiety, miRNAs are considered to embark oncogenic or tumor suppressor actions (Asangani et al., 2008; Babak, Zhang, Morris, Blencowe, & Hughes, 2004; Wang et al., 2010). This has rendered miRNA in the focus of the tumor-associated networks which modulate main tumorigenic features such as proliferation, invasion, metastasis, and apoptosis (Becker Buscaglia & Li, 2011; Crawford et al., 2009; Ding et al., 2010; Matsushima et al., 2011).

Two different microarray platforms were utilized in this study in order to determine the most substantial variations in expression. Analysis of the microarray data has indicated the down-regulation of 11 miRNAs and the up-regulation of 10 miRNAs in both platforms. It is noteworthy to mention that there were several variations in the detected miRNAs between the 2 platforms. This could be attributed to alteration in sensitivity of both platforms in detecting minor alterations of low abundance. This has driven us to set a FC cutoff of ≥ 2 for up-regulation or ≤ -2 for down-regulation. This rigorous analytical strategy may have resulted in disregarding some dysregulated miRNAs. However, it is expected that the excluded miRNAs will not tolerate the standard validation methods, and will not underpin a biological function associated with EAC. As a result of this rigorous approach, all the miRNAs that were chosen for subsequent measurement by qRT-PCR were validated.

It was observed that the expression of some miRNAs didn't exhibit difference between NS and BE. On the other hand, another set of miRNAs showed a differential expression between NS and BE suggesting that these miRNAs can serve as markers for BE development since they are associated with the anomaly of the lineage from NS to BE.

The choice of proper tissue comparator for expression profiling studies in EAC is a big controversy. Based on the tissue comparator used, the miRNA dysregulation profile will vary in terms of miRNAs, and their pattern of dysregulation. The controversial nature of the choice of proper tissue comparator in EAC expression profiling studies has been evident in the different tissue controls used among several studies. While BE is considered as the pre-malignant condition that precedes EAC, and thereby could be regarded as a proper tissue control, NS should not be ignored. The fact that miRNA can elicit one or more biological functions in several pre-tumor or tumor stages via targeting one or more proteins makes the choice of a proper control far more complicated. For instance, several miRNAs were previously reported to be dys-regulated in BE as well as EAC such as miR-203, and miR-205. Furthermore, miR-194 as reported to be up-regulated in BE and EAC and is suggested to be involved in intestinal epithelial differentiation. Additionally, miR-196a was reported to be up-regulated in BE and EAC, and is thought to play a role in enhancing growth and suppressing apoptosis (Smith, Watson, Michael, & Hussey, 2010). Accordingly, a comprehensive understanding of miRNA expression profile in EAC requires the inclusion of both NS and BE.

The over-expression of miR-21, miR-192, and miR-194 is in frame with preceding studies that addressed miRNA expression in EAC (Feber et al., 2008; Mathe et al., 2009). It has been well-recognized that miRNA dysregulation pattern expression and biological roles are specific to each tissue type. Accordingly, the data from one disease cannot be simply applied to other diseases (Babak et al., 2004; Liu & Kohane, 2009). Although some studies have indicated that EAC and GC have shared molecular characteristics employing comparative genomic hybridization (CGH) or gene expression microarrays (van Dekken et al., 1999), the alteration in their miRNA expression profiles has not been investigated. To approach this query, the expression of EAC validated miRNAs was measured in gastric tissues.

Remarkably, miR-21 and miR-133b exhibited a likewise pattern of dysregulation among both EAC and GC. This indicates that these 2 miRNAs are potentially linked to regulating common molecular pathways that are shared between EAC and GC regardless the location or pathology. miR-21 is a known to be an oncogenic miRNA that is over-expressed in all epithelial solid tumors. It is also known to target many apoptotic pathways. miR-21 was reported to target several tumor suppressor genes such as programmed cell death 4 (PCDC4) and TAp63 (Buscaglia & Li, 2011). This is the first study, to the best of our knowledge, to show the down-regulation of miR-133b in EAC as compared to BE. However, miR-133b was also found to be down-regulated in lung cancers. miR-133b is reported to target MCL1 and BCL2L2, and thereby improve the response gemcitabine (Matsushima et al., 2011). Furthermore, resistant HeLa and PC3 cells were more sensitive to tumor necrosis factor alpha (TNFα) and to TNF-related apoptosis-inducing ligand (TRAIL) in the presence of miR-133b (Patron et al., 2012).

In this study, 6 miRNAs were found to be EAC specific rather than gastric cancer in the presence of BE as a tissue comparator. This observation is highly relevant especially with the fact that both diseases display significant alterations in terms of etiology, therapeutic response, and clinical outcome. The identification of EAC unique miRNA signature points out that this specific miRNA marking may be involved in targeting major molecules that are central to EAC associated signaling. One of the main specific features associated with EAC is its striking resistance to chemotherapy as well as poor clinical outcome than that observed in gastric cancers (Ilson, 2002). In this study, miR-203 expression levels were found to be inversely correlated with EAC progression. This indicates that miR-203 is potentially regulating major pathways involved in EAC tumor progression. The function and the targets of miR-203 have not been studied previously in EAC, however, it was reported to be of low abundance in metastatic breast cancer cell lines which was correlated by the enhanced motility and invasion via targeting SNA12 (Zhang et al., 2011). Although miR-194, miR-192, and miR-200a were significantly up-regulated in EAC, they exhibited an opposite pattern

upon EAC progression. Their up-regulation levels were higher in early EAC stages than later ones. This observation may indicate that these miRNAs are involved in EAC development not progression. In concert with this observation, the data indicated that miR-192, miR-194, miR-21, and miR-31 displayed a statistically significant up-regulation in BE adjacent to HGD as compared to isolated BE tissue samples. These data point out to the differential miRNA expression in cancer stages and the possible functions elicited by these miRNAs. These alterations in the miRNA levels across the stages highlights the complexity in cancer-associated signaling in which several molecular knockouts are being implicated which in turn reduce the requirement for over-expression levels of these miRNA in late stages. This recalls for further studies to examine the mechanistic and functional implications associated with EAC progression.

Using NS as a comparator, miR-192, miR-194, miR-200a, miR-215, and miR-21 were found to be up-regulated, whereas miR-203, miR-205, miR-133b, and miR-31 were found to be down-regulated. This confirms the validity of the microarray data. However, there are some discrepancies between the data obtained upon utilizing NS and BE as comparators. Using NS as comparator, miR-215 was found to be up-regulated while miR-31 showed down-regulation in EAC. However, using BE as comparator, miR-31 was found to be up-regulated whereas miR-215 didn't show any differential expression between BE and EAC. This discrepancy reflects the differential expression between NS and BE, and the dys-regulation of these 2 miRNAs in BE as compared to NS. This data underscores the importance of including both NS and BE upon miRNA expression profiling in EAC. Furthermore, the fact that miR-31 is down-regulated in BE as compared to NS, and up-regulated in EAC as compared to BE and NS indicates that the dysregulation of the same miRNA whether by its up-regulation or down-

regulation may elicit a role in BE transformation or tumorigensis. Further functional and mechanistic studies are required to decipher the role of miRNAs across different pre-tumorigenic and tumorigenic stages.

miR-194 is thought to elicit two different actions in the development of BE and EAC. Its expression was found to be regulated by HNF-1a which is a transcription factor that exhibits up-regulation in BE (Smith et al., 2010). The expression of miR-194 was shown before to be triggered during intestinal epithelial differentiation. This indicates that miR-194 is associated with intestinal transformation required for BE, as well as other tumorigenic functions. miR-192 was found to be up-regulated in lung cancer and poses proliferative functions. In addition, it was found to be over-expressed in colon cancer enhancing cell cycle progression. Thus, miR-192 is known to be an oncomiR. miR-215 is known to be induced by p53 and assists miR-192 to regulate cell cycle, and thereby the reduction in its expression results in the abrogation in the cell cycle regulation. We here report the down-regulation of miR-215 in EAC as compared to BE. It was also reported to be down-regulated in gastric, and colon cancers. miR-200 family members are known to be involved in epithelial mesenchymal transition (EMT) through targeting ZEB1 and ZEB2 which are transcription factors involved in EMT (Xiong et al., 2012). Moreover, miR-200a is regulated by ZEB1 and ZEB2, which are targeted by miR-200a and miR-205, and thereby creating a miRNA regulatory loop (Smith et al., 2010). Reconstitution of miR-205 resulted in increased expression of the tumor suppressors IL24 (Kim et al., 2013).

Upon comparing EAC to GC, we found that miR-200a and miR-31 displayed opposite trend of expression where both miRNAs were up-regulated in EAC as compared to BE, and on the contrary they were down-regulated in GC. This data highlights the concept supports miRNA has different functional and mechanistic implications that are vary among different tissues. On contrary to the studies reporting the up-regulation of miR-200a in EAC, miR-200a was reported to be down-regulated in EBV-associated GC (Shinozaki et al., 2010). miR-31 has been characterized as a tumor-suppressor miRNA. It's down-regulation in GC has been reported by several studies. Here we report the down-regulation of miR-31 in GC as

compared to NG, and in BE as compared to NS. On the other hand, we found that miR-31 is up-regulated in EAC as compared to BE. To date, the effect of the over-expression of miR-31 in EAC has not been investigated. However, our findings are consistent with several reports.

One of the interesting observations is that miR-192, miR-194, and miR-200a displayed progressive up-regulation in esophageal tissues starting from NS till EAC. Furthermore, miR-133b displayed progressive down-regulation from NS till EAC. This suggests that these 4 miRNAs are implicated in columnar intestinal transformation and tumorigensis. On the other hand, miR-31, miR-203, and miR-205 didn't show any statistical difference between BE and HGD suggesting that these miRNAs are not involved in early stages of tumorigensis. miR-21 showed up-regulation in both HGD and EAC as compared to BE and NS indicating its role as an oncomiR. miR-215 did not exhibit any statistical significant differential expression between BE and EAC. However, it was significantly up-regulated in BE suggesting that it plays a role in its development. This data are consistent with previous reports showing the up-regulation of miR-215 in BE as compared to NS and EAC.

To the best of our knowledge, there are 2 limitations for this study. The first one is the limited sample size. Future studies that include larger cohorts are required for independent validation. Another limitation is that the microRNA microarray, although it is a high throughput method, it doesn't allow the discovery of novel miRNAs. RNA-seq is a high throughput method that allows for the identification and discovery of novel microRNAs.

CONCLUSION:

In summary, herein the difference between EAC and GC miRNA expression profiles is addressed and elucidated. These data highlight the fact that EAC and GC have variable molecular mechanisms involved in their development and progression. Further studies are required to specifically analyze the role of specific miRNAs in terms of their targets, mechanisms and functions in EAC. Moreover, larger cohorts are required for independent validation. This will serve to provide new perceptions for the EAC development and progression.

APPENDIX A

IRB APPROVAL LETTER 1:

THE AMERICAN UNIVERSITY IN CAIRO
OFFICE OF THE ASSOCIATE PROVOST FOR RESEARCH ADMINISTRATION

To:	Wael El-Rifai (Vanderbilt University Medical Center)
Advising:	Rama Ali (AUC Biotechnology graduate student)
From:	Graham Harman, Chair of the IRB
Date:	June 22, 2012
Re:	approval of study

This is to inform you that I reviewed your research proposal entitled "Validation of novel microRNA (miRNA) biomarkers in esophageal adenocarcinomas (EAC)," and determined that approval by the Vanderbilt University IRB allows me to approve this study as "exempt" under AUC IRB guidelines.

Please note that IRB approval is valid only for one year from the date of this letter. For projects taking longer than one year to complete, IRB approval must be sought again *before* the expiration date of the current approval.

Thank you and good luck.

graham harman

APPENDIX B

IRB LETTER 2:

THE AMERICAN UNIVERSITY IN CAIRO
INSTITUTIONAL REVIEW BOARD

To: Dr. Rama Ali

cc: Ragya Sorour

From: Atta Gebril, Chair of the IRB

Date: February 12 , 2013

Re: Approval of study

This is to inform you that I reviewed your research proposal entitled **"Validation of novel microRNA (miRNA) biomarkers in gastric cancer,"** and determined that it used appropriate procedures to minimize risks to human subjects and that adequate provision was made for confidentiality and data anonymity of participants in any published record. I believe you will also make adequate provision for obtaining informed consent of the subjects. Thus, the proposal qualifies for exempt review, meaning that I have approved it without convening the full Institutional Review Board.

Please note that IRB approval does not automatically ensure approval by CAPMAS, an Egyptian government agency responsible for approving much off-campus research involving surveys and interviews. CAPMAS issues are handled at AUC by the office of the University Counsellor, Dr. Amr Salama. The IRB is not in a position to offer any opinion on CAPMAS issues, and takes no responsibility for obtaining CAPMAS approval.

This approval is valid for only one year. In case you have not finished data collection within a year, you need to apply for an extension.

Thank you and good luck.

Atta Gebril

IRB chair, The American University in Cairo
2046 HUSS Building
T: 02-26151919
Email: agebril@aucegypt.edu

Institutional Review Board
The American University in Cairo
AUC Avenue, P.O. Box 74
New Cairo 11835, Egypt.
tel 20.2.2615.1000
fax 20.2.27957565
Email: aucirb@aucegypt.edu.

APPENDIX C

IRB LETTER 3:

Vanderbilt University
Institutional Review Board

504 Oxford House Nashville, Tennessee 37232-4315
(615) 322-2918   Fax: (615) 343-2648
www.mc.vanderbilt.edu/irb

08/12/2011

Wa'el El-Rifai, M.D.,Ph.D.
Surgery - Oncology
1255 Light Hall   37122-8720

RE: IRB# 111096 "Molecular Pathobiology of Barrett’s Tumorigenesis"

Dear Wa'el El-Rifai, M.D.,Ph.D.:

A designee of the Institutional Review Board reviewed the research study identified above. The designee determined the study does not qualify as "human subject" research per §46.102(f)(2).

(f) Human subject means a living individual about whom an investigator (whether professional or student) conducting research obtains:
(1) data through intervention or interaction with the individual, or
(2) identifiable private information.

Intervention includes both physical procedures by which data are gathered (for example, venipuncture) and manipulations of the subject or the subject's environment that are performed for research purposes. Interaction includes communication or interpersonal contact between investigator and subject. Private information includes information about behavior that occurs in a context in which an individual can reasonably expect that no observation or recording is taking place, and information which has been provided for specific purposes by an individual and which the individual can reasonably expect will not be made public (for example, a medical record). Private information must be individually identifiable (i.e., the identity of the subject is or may be ascertained by the investigator or associated with the information) in order for obtaining the information to constitute research involving human subjects.

[The study will include tissue samples received de-identified of esophageal and gastroesophageal adenocarcinoma. This study does not involve interaction with human subjects or collection of private information.]

Please note:
Any changes to this proposal that may alter its "non-human"/"non-research" status should be presented to the IRB for approval prior to implementation of the changes. In accordance with IRB Policy III.J, amendments will be accepted up to one year from the date of approval. If such changes are requested beyond this time frame, submission of a new proposal is required.

Sincerely,

Shannon Simmons

Shannon Simmons
Regulatory Compliance Analyst IV
Institutional Review Board
Health Sciences Committee #1

Electronic Signature: Shannon D Simmons/VUMC/Vanderbilt : (F9146BB52DE7323E8B60279C1C26B9C8)
Signed On: 08/12/2011 11:43:13 AM CDT

El-Rifai, Wa'el   IRB # 111096   1   08/12/2011

APPENDIX D

IRB APPROVAL LETTER 4:

Vanderbilt University
Institutional Review Board

06/11/2008

Wa'el El-Rifai, M.D.,Ph.D.
Surgery - Oncology
1255 Light Hall   37122-8720

Wa'el El-Rifai, M.D.,Ph.D.
Surgery - Oncology
1255 Light Hall   37122-8720

RE: IRB# 080587 "Targets of gene amplification and overexpression at 17q in gastric cancer"

Dear Wa'el El-Rifai, M.D., Ph.D.:

A designee of the Institutional Review Board reviewed the research study identified above. The designee determined the study does not qualify as "human subject" research per §46.102(f)(2).

   (f) Human subject means a living individual about whom an investigator (whether professional or
      student) conducting research obtains:
         (1) data through intervention or interaction with the individual, or
         (2) identifiable private information.

   Intervention includes both physical procedures by which data are gathered (for example,
   venipuncture) and manipulations of the subject or the subject's environment that are performed for
   research purposes. Interaction includes communication or interpersonal contact between
   investigator and subject. Private information includes information about behavior that occurs in a
   context in which an individual can reasonably expect that no observation or recording is taking place,
   and information which has been provided for specific purposes by an individual and which the
   individual can reasonably expect will not be made public (for example, a medical record). Private
   information must be individually identifiable (i.e., the identity of the subject is or may be ascertained
   by the investigator or associated with the information) in order for obtaining the information to
   constitute research involving human subjects.

This study PI will receive the samples that are coded without a link to patient identifiers. Approval is
extended for the Request for Determination of Non-Human Subject Application submitted on May 27,
2008.

Please note: Any changes to the protocol that could conceivably alter the "non-human" status of this
research should be presented to the IRB for approval prior to implementation of the changes.

Sincerely,

Lu Ellen Davie

Lu Ellen Davie, RN, PA IV
Institutional Review Board
Health Sciences Committee #3

Electronic Signature: Lu Hoekstra/VUMC/Vanderbilt :
Signed On: 06/11/2008 04:54:20 PM CDT

El-Rifai, Wa'el   IRB # 080587   1   06/11/2008

APPENDIX E

CERTIFICATE OF COMPLETION OF NIH TRAINING FOR PROTECTION OF HUMAN
RESEARCH PARTICIPANTS

Certificate of Completion

The National Institutes of Health (NIH) Office of Extramural Research
certifies that **Rama Ali** successfully completed the NIH Web-based
training course "Protecting Human Research Participants".

Date of completion: 01/25/2013

Certification Number: 1069424

APPENDIX F

PUBLICATIONS:

Saad, R., Zheng, C., Shoumin, Z., Peilin, J., Zhao, Z., Washington, M. K., et al. (2013).

Deciphering the unique microRNA signature in human esophageal adenocarcinoma.

PLoS One, 8(5): e64463. doi:10.1371/journal.pone.0064463.

Chen, Z., Saad, R., Jia, P., Peng, D., Zhu, S., Washington, M. K., et al. (2013). Gastric

adenocarcinoma has a unique microRNA signature not present in esophageal

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10.1002/cncr.28002

APPENDIX G

Copyright Information:

The data in the thesis is accepted for publication in PlosOne journal and will appear online in June 2013. The student (Rama Ahmed Saad Ahmed Ali) is the first author on this paper. Since some of the figures and the data in the paper were reproduced in the thesis, it is essential to attach the copyright permissions and guidelines of the journal. PlosOne is an open access journal that allows the authors to own and retain the copyright of their articles. The data in the thesis were cited and referenced to this journal article. The link and the screen shot below show the copyright information for PlosOne Articles.

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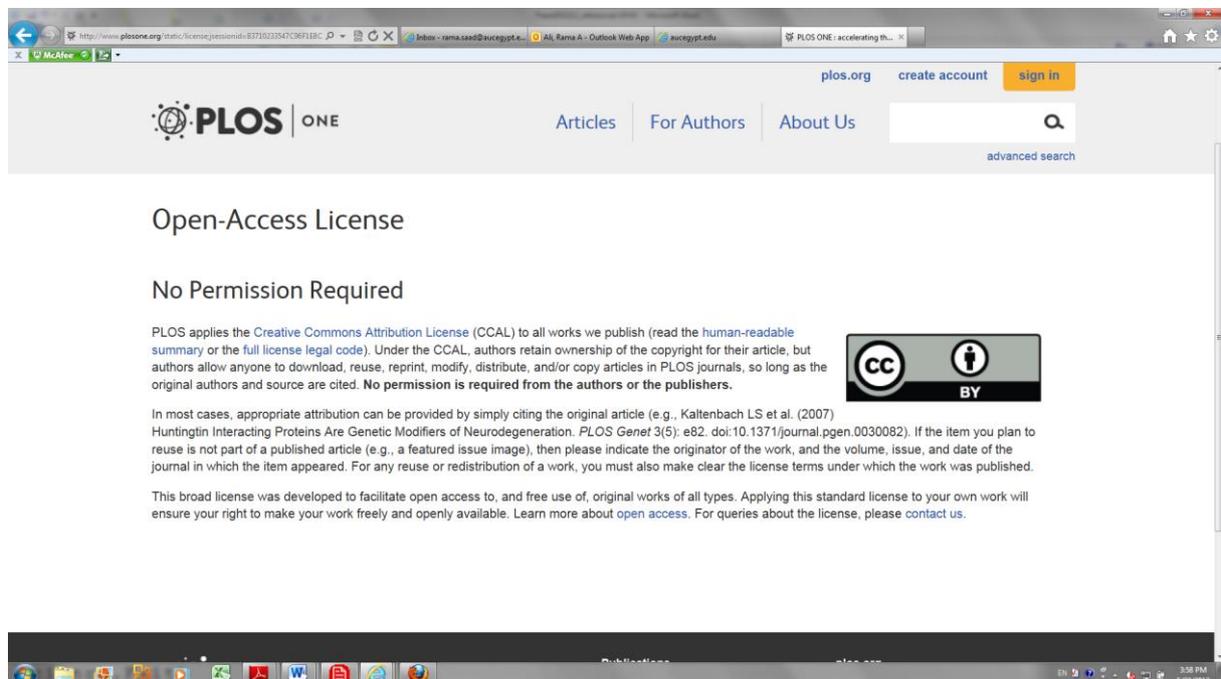
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06/11/2008

Wa'el El-Rifai, M.D.,Ph.D.
Surgery - Oncology
1255 Light Hall   37122-8720

Wa'el El-Rifai, M.D.,Ph.D.
Surgery - Oncology
1255 Light Hall   37122-8720

RE: IRB# 080587 "Targets of gene amplification and overexpression at 17q in gastric cancer"

Dear Wa'el El-Rifai, M.D., Ph.D.:

A designee of the Institutional Review Board reviewed the research study identified above. The designee determined the study does not qualify as "human subject" research per §46.102(f)(2).

(f) Human subject means a living individual about whom an investigator (whether professional or student) conducting research obtains:
(1) data through intervention or interaction with the individual, or
(2) identifiable private information.

Intervention includes both physical procedures by which data are gathered (for example, venipuncture) and manipulations of the subject or the subject's environment that are performed for research purposes. Interaction includes communication or interpersonal contact between investigator and subject. Private information includes information about behavior that occurs in a context in which an individual can reasonably expect that no observation or recording is taking place, and information which has been provided for specific purposes by an individual and which the individual can reasonably expect will not be made public (for example, a medical record). Private information must be individually identifiable (i.e., the identity of the subject is or may be ascertained by the investigator or associated with the information) in order for obtaining the information to constitute research involving human subjects.

This study PI will receive the samples that are coded without a link to patient identifiers. Approval is extended for the Request for Determination of Non-Human Subject Application submitted on May 27, 2008.

Please note: Any changes to the protocol that could conceivably alter the "non-human" status of this research should be presented to the IRB for approval prior to implementation of the changes.

Sincerely,

Lu Ellen Davie

Lu Ellen Davie, RN, PA IV
Institutional Review Board
Health Sciences Committee #3

Electronic Signature: Lu Hoekstra/VUMC/Vanderbilt :
Signed On: 06/11/2008 04:54:20 PM CDT

THE AMERICAN UNIVERSITY IN CAIRO
INSTITUTIONAL REVIEW BOARD

To: Dr. Rama Ali

cc: Ragya Sorour

From: Atta Gebril, Chair of the IRB

Date: February 12 , 2013

Re: Approval of study

This is to inform you that I reviewed your research proposal entitled **"Validation of novel microRNA (miRNA) biomarkers in gastric cancer,"** and determined that it used appropriate procedures to minimize risks to human subjects and that adequate provision was made for confidentiality and data anonymity of participants in any published record. I believe you will also make adequate provision for obtaining informed consent of the subjects. Thus, the proposal qualifies for exempt review, meaning that I have approved it without convening the full Institutional Review Board.

Please note that IRB approval does not automatically ensure approval by CAPMAS, an Egyptian government agency responsible for approving much off-campus research involving surveys and interviews. CAPMAS issues are handled at AUC by the office of the University Counsellor, Dr. Amr Salama. The IRB is not in a position to offer any opinion on CAPMAS issues, and takes no responsibility for obtaining CAPMAS approval.

This approval is valid for only one year. In case you have not finished data collection within a year, you need to apply for an extension.

Thank you and good luck.

Atta Gebril

IRB chair, The American University in Cairo
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THE AMERICAN UNIVERSITY IN CAIRO
OFFICE OF THE ASSOCIATE PROVOST FOR RESEARCH ADMINISTRATION

To: Wael El-Rifai (Vanderbilt University Medical Center)

Advising: Rama Ali (AUC Biotechnology graduate student)

From: Graham Harman, Chair of the IRB

Date: June 22, 2012

Re: approval of study

This is to inform you that I reviewed your research proposal entitled "Validation of novel microRNA (miRNA) biomarkers in esophageal adenocarcinomas (EAC)," and determined that approval by the Vanderbilt University IRB allows me to approve this study as "exempt" under AUC IRB guidelines.

Please note that IRB approval is valid only for one year from the date of this letter. For projects taking longer than one year to complete, IRB approval must be sought again *before* the expiration date of the current approval.

Thank you and good luck.

Graham Harman