Review

Potential Predictive Role of MicroRNAs in the Neoadjuvant Treatment of Esophageal Cancer

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Abstract. *Esophageal cancer is a disease with disappointing prognosis. Currently, there are no predictive factors that can identify patients who on the one hand would likely benefit from tri-modality management and, on the other hand, would not be significantly affected by the morbidity accompanying the treatment. MicroRNAs are short non-coding RNAs responsible for post-transcriptional modification of gene expression by binding to 3'-UTR of messenger RNA and represent emerging potential predictive biomarkers of treatment (chemotherapy and radiotherapy) efficacy and toxicity. We reviewed the current literature, addressing the potential predictive role of microRNAs for efficacy of chemotherapy (specifically cisplatin, 5-fluorouracil, doxorubicin and paclitaxel) and radiotherapy, including predicted targets in the cell. Altogether 82 articles were identified and included in this review. This may be the first review on this topic specifically focusing on neoadjuvant treatment of esophageal cancer.*

Esophageal cancer (OMIM: 133239; C15; histologically squamous cell carcinoma or adenocarcinoma) (EC) remains a disease with poor prognosis. In 2012, 456,000 new cases were diagnosed worldwide (1). The treatment strategy for EC has remained the same over the past 30 years despite intensive research. Tri-modality treatment, consisting of neoadjuvant chemoradiation followed by surgical resection

(esophagectomy), is currently the gold standard in the management of non-metastatic EC (2).

About 30% of patients will achieve pathological complete response (pCR), and these patients may not necessarily need esophagectomy which exposes them to considerable surgical morbidity. The selection of patients who can achieve pCR after neoadjuvant treatment remains a challenge and currently there are no predictive biomarkers for identifying this subgroup of patients. Positron-emission tomography combined with computed tomography (PET/CT) was investigated, but the negative predictive value of this examination is not sufficient for pCR of the primary tumor, although it remains an option for excluding disease progression after neoadjuvant treatment or distant metastasis (3). On the other hand, in patients whose disease progresses on inactive neoadjuvant treatment, the delay of surgery may represent a significant risk for patient management. At the same time, the toxicity of chemoradiation can be significant, especially considering frequent high prevalence of patients with poor performance status. Considering these facts, it is evident that there are numerous unresolved issues in EC management. Biomarkers represent a promising option in how to respond to at least some of these challenges and better personalize the treatment.

MicroRNAs (miRNAs, miR) have attracted much research interest with a large number of articles published on this topic. miRNAs are short non-coding DNA fragments, 18-22 nucleotide long, transcribed into single-stranded RNA. miRNAs are to be distinguished from short-interfering RNAs (siRNAs), which are double-stranded and interfere with only one target gene, compared with miRNA, which can regulate expression of multiple messenger RNAs (mRNAs). Similarly, multiple miRNAs can regulate expression of the same mRNA, making the situation even more complicated. miRNAs influence cell proliferation and apoptosis, as well

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as differentiation, on different cellular levels. Furthermore, miRNAs can regulate other cells by being transported into the extracellular space as exosomes (4, 5).

miRNAs are transcribed from non-coding DNA to pri-miRNA, then cleaved in the nucleus by type III RNase (DROSHA) to pre-miRNA, transported through the nuclear membrane (utilizing exportin-5) to the cytoplasm and further cleaved to produce mature miRNAs by another type III RNase (DICER). Mature miRNA associates with argonaut (AGO) protein to form RNA-induced silencing complex (RISC) which binds to the 3'-untranslated region (UTR) of mRNA, modifying its expression (6, 7).

Determination of biomarkers represents an integral part of the management of patients with cancer (8). In EC, miRNAs represent potential biomarkers for early detection in primary diagnosis, diagnosis of disease progression and metastasis, and prediction of disease outcome. miRNAs can act as tumor suppressors as well as promoting tumor growth, depending on the gene regulated, and may influence the response to chemotherapy as well as radiation by different mechanisms (9, 10).

The aim of this review is to summarize published data regarding miRNAs and their potential predictive value in the neoadjuvant treatment of EC. To our knowledge, this is the first review summarizing the data regarding the role of the miRNAs in prediction of chemotherapy and radiation efficacy in EC.

MicroRNAs in Esophageal Cancer

There are currently no effective screening methods for EC in clinical practice, largely accounting for why EC is mostly diagnosed in a locally advanced or metastatic stage. Several authors have proposed use of circulating miRNAs including *miRNA-367*, *miRNA-25*, *miRNA-100*, *miRNA-193-3p*, *miRNA-194*, *miRNA-223*, *miRNA-337-5p*, *miRNA-483-5p*, *miRNA-1246*, *miRNA-21*, *miRNA-18a*, *miRNA-155*, and *miRNA-107* as potential tools for early detection of EC (11-17). The principal advantage of circulating miRNA evaluation from peripheral blood compared to upper gastrointestinal endoscopy is patient comfort as well as higher cost-effectiveness. This approach could be useful specifically among high-risk patients, such as patients with Barrett's esophagus or patients with a family history of EC, in order to potentially avoid repeated endoscopy and biopsy.

Dysregulation of multiple miRNAs in EC carcinogenesis have been identified including of *miRNA-101*, *miRNA-655*, *miRNA-124*, *miRNA-21*, *miRNA-29c*, *miRNA-145*, *miRNA-133a*, *miRNA-133b*, *miRNA-183*, *miRNA-130b*, *miRNA-100*, *miRNA-27a*, *miRNA-195*. Potential targets in EC development are oncogenes [e.g. signal transducer and activator of transcription 3 (*STAT3*), extracellular signal-regulated kinase/mitogen-activated protein kinase (*ERK1/2/MAPK*), mechanistic target of

rapamycin (*mTOR*), and kirsten rat sarcoma oncogene (*KRAS*)], tumor-suppressor genes [e.g. enhancer of zeste homolog 2 (*EZH2*), and phosphatase and tensin homolog (*PTEN*)], genes regulating the cell cycle [e.g. cyclin E, and cell division control protein 42 homolog (*CDC42*)], apoptosis [e.g. programmed cell death protein 4 (*PDCD4*)] and cell motility [e.g. fascin homolog 1 (*FSCN1*)] (18-28).

The determination of miRNAs also seems to be a viable option for the estimation of patient prognosis and at the same time for predicting the extent of the disease and its metastatic potential. For example, *miR-92a* was reported as a biomarker predicting lymph node involvement, as well as shorter patient survival in EC (29). One possible mechanism for this is the regulation of the membrane glycoprotein cadherin 1 which participates in cell adhesion and motility. Similarly, *miRNA-150*, *miRNA-574-3p*, *miRNA-142-3p*, *miRNA-16*, *miRNA-21*, *miRNA-335* were reported as prognostic biomarkers in EC (30-34).

MicroRNAs as Biomarkers Predictive of the Efficacy of Neoadjuvant Chemoradiation

In EC, the most widely utilized chemotherapeutic agents in the neoadjuvant, adjuvant and palliative settings are cisplatin, oxaliplatin, 5-fluorouracil (5-FU) and paclitaxel. External-beam radiation (usually to a total dose of 45-60 Gy) is administered along with chemotherapy in neoadjuvant treatment over a 4-6 weeks period, with daily fractions of 1.8-2.0 Gy. Published data including putative mechanisms of action of miRNAs in chemotherapy resistance in EC are summarized in Table I.

Sensitivity to cisplatin. Cisplatin is transported into the cell through solute carrier membrane transporters (SLC), specifically SLC31A1 (CTR1, OMIM: 603085). At the same time, cisplatin molecules are pumped out of the cell by the ATP-binding cassette (ABC) proteins, mostly ABCC2 (OMIM:601107), and ABCG2 (OMIM: 603756). After entering the cell nucleus, cisplatin acts at the DNA level, producing DNA-cisplatin adducts which repress DNA replication and cell division. The cellular response to DNA damage is the activation of repair mechanisms. If the cell cannot repair this damage, the internal pathway of apoptosis is activated. All these pathways are regulated by miRNAs and can influence sensitivity to chemotherapy (35).

Ma *et al.* have reported that the expression of *miRNA-196a* is related to ABCG2. Overexpression of *miRNA-196a* increases the cell expression of the membrane transporter ABCG2 which participates in cisplatin efflux and possible resistance (36). DNA polymerase beta also plays an important role in DNA repair. Wang *et al.* reported that *miRNA-499* can affect the activity of DNA polymerase-beta, thus increasing the efficacy of cisplatin treatment (37). Poly ADP ribose polymerase (*PARP*) gene, responsible for repair of single-

Table I. MicroRNA and sensitivity of esophageal cancer to chemotherapy.

Chemotherapeutic	miRNA	Specimen	Predicted target	Dysregulation	Dysregulation effect	Reference
Cisplatin	<i>miRNA-483</i>	cancer tissue, surrounding non-cancer tissue, cell lines (ECA109)	Unknown	Overexpression	Cisplatin resistance	46
	<i>miRNA-214</i>	Cancer tissue, surrounding non-cancer tissue, cell lines (ECA109)	Unknown	Overexpression	Cisplatin resistance	46
	<i>miRNA-499</i>	Cell lines (EC9706, KYSE30)	DNA polymerase β	Overexpression	Cisplatin sensitivity	37
	<i>miRNA-223</i>	Cancer tissue, cell lines (OE33, JHesoAD1)	PARP	Overexpression	Cisplatin sensitivity	38
	<i>miRNA-214-3p</i>	Cell lines (hESO, TE7, TE10)	CUG-BP1, survivin	Underexpression	Cisplatin resistance	39
	<i>miRNA-196a</i>	Cell lines (TE1, EC109)	ABCG2	Underexpression	Cisplatin sensitivity	36
	<i>miRNA-141</i>	Cell lines (KYSE series)	YAP1	Overexpression	Cisplatin resistance	40
	<i>miRNA-27a</i>	Cell lines (ECA109, TE-13)	P-Glycoprotein, BCL2	Underexpression	Cisplatin sensitivity	70
	<i>Let-7c</i>	Cancer tissue, cell lines (TE1, TE5, TE8, TE9, TE10, TE11, TE13)	IL-6/STAT3	Underexpression	Cisplatin resistance	41
5-FU	<i>miRNA-192-5p</i>	Cell lines (OE19)	ABCC3	Overexpression	5-FU resistance	45
	<i>miRNA-378a-3p</i>	Cell lines (OE19)	CBL-B	Underexpression	5-FU resistance	45
	<i>miRNA-192-5p</i>	Cell lines (OE19)	TYMS	Underexpression	5-FU resistance	45
	<i>miRNA-27b-3p</i>	Cell lines (OE19)	RAS	Overexpression	5-FU resistance	45
	<i>miRNA-193b-3p</i>	Cell lines (OE19)	RAS	Overexpression	5-FU resistance	45
	<i>miRNA-18a-3p</i>	Cell lines (KYSE410)	RAS	Underexpression	5-FU resistance	45
	<i>miRNA-125a-5p</i>	Cell lines (KYSE410)	ERBB2	Underexpression	5-FU resistance	45
	<i>miRNA-141-3p</i>	Cancer tissue, cell lines (EC109, EC9706, TE-1, KYSE-150)	PTEN	Overexpression	5-FU resistance	47
	<i>miRNA-221</i>	Cell lines (OE19, OE33, PT1590, LN1590)	DKK2	Overexpression	5-FU resistance	48
	<i>miRNA-193b</i>	Cell lines (OE19, OE21, OE33, KYSE450)	STATHMIN1	Overexpression	5-FU sensitivity	49
	<i>miRNA-483</i>	Cancer tissue, surrounding non-cancer tissue, cell lines (ECA109)	Unknown	Overexpression	5-FU resistance	46
	<i>miRNA-214</i>	Cancer tissue, surrounding non-cancer tissue, cell lines (ECA109)	Unknown	Overexpression	5-FU resistance	46
	<i>miRNA-27a</i>	Cell lines (ECA109, TE-13)	P-Glycoprotein, BCL2	Underexpression	5-FU sensitivity	70
Paclitaxel	<i>miRNA-133a/b</i>	Cancer tissues, adjacent non-cancer tissues	Unknown	Underexpression	Paclitaxel sensitivity	51
Doxorubicin	<i>miRNA-483</i>	Cancer tissue, surrounding non-cancer tissue, cell lines (ECA109)	P-Glycoprotein	Overexpression	Doxorubicin resistance	46
	<i>miRNA-214</i>	Cancer tissue, surrounding non-cancer tissue, cell lines (ECA109)	P-Glycoprotein	Overexpression	Doxorubicin resistance	46
	<i>miRNA-223</i>	Cancer tissue, cell lines (OE33, JHesoAD1)	PARP	Overexpression	Doxorubicin sensitivity	38
	<i>miRNA-27a</i>	Cell lines (ECA109, TE-13)	P-Glycoprotein, BCL2	Underexpression	Doxorubicin sensitivity	70
Chemotherapy combination	<i>miRNA-200c</i>	Cancer patient, serum	Unknown	Overexpression	Chemoresistance	42
	<i>miRNA-634</i>	Cancer tissue, non-cancer tissue, cell lines (HeLa, U2OS, WI38, KYSE850, KYSE170, KYSE150, LCL)	OPA1, TFAM, LAMP2	Overexpression	Chemosensitivity	43

5-FU, 5-Fluorouracil.

strand DNA breaks, has been well studied in different tumor types and also represents a therapeutic target for PARP inhibitors, which are currently being introduced into clinical practice. It was also suggested that miRNA-223 regulation of

PARP1 expression plays a role in increasing sensitivity to cisplatin. Overexpression of *miRNA-223* impedes the function of PARP1, thus reducing the opportunity for DNA repair and directing the cell to apoptosis (38).

Similarly Phatak *et al.* investigated the potential influence of the inhibitor of the apoptosis protein survivin, which also acts as a regulator of the post-transcriptional modifications of the mRNA CUG triplet repeat-RNA binding protein 1 (CUG-BP1). Expression is regulated by *miRNA-214-3p* which if overexpressed it increases the response to cisplatin (39). Imanaka *et al.* have studied *miRNA-141* and its potential role in cisplatin resistance in EC and have observed *miRNA-141* overexpression in cisplatin resistant Cell lines (40). *miRNA Let-7* discovered in 2000 by Reinhart *et al.* was demonstrated to have a significant role in chemotherapy sensitivity in EC. *Let-7* regulates the STAT pathway, which then regulates cell proliferation and apoptosis. In particular, apoptotic potential may be a crucial factor in increasing sensitivity to cisplatin. Sugimura *et al.* showed that the transfection of *Let-7c* into EC Cell lines increased their sensitivity to cisplatin (41). Similarly, low expression of *miRNA-200c* predicted chemosensitivity to neoadjuvant cisplatin-based chemotherapy in EC (42). A possible explanation for this finding may come from the tumor-suppressor gene protein phosphatase 2, regulatory subunit A, beta (*PPP2R1B*) associated with lower *miRNA-200c* expression. *PPP2R1B* protein is serine/threonine phosphatase that has a negative effect on intracellular signal transduction by dephosphorylating activated (phosphorylated) protein kinases, more specifically those of the AKT pathway. *miRNA-634* is another microRNA regulating cell apoptosis and also potentially involved in sensitivity to chemotherapy (43). *miRNA-634* expression leads to underexpression of mitochondrial dynamin-like GTPase (*OPA1*), mitochondrial transcription factor A (*TFAM*) and lysosomal-associated membrane protein 2 (*LAMP2*) genes participating in cell homeostasis. *OPA1* is crucial for mitochondrial formation and mitochondrial metabolism, *TFAM* is involved in mitochondrial genome expression and *LAMP2* membrane glycoprotein plays a role in lysosome integrity and function (lysosomes mediate degradation of damaged mitochondria preventing the launch of intrinsic apoptosis).

Sensitivity to 5-FU. The antimetabolite 5-FU is transported through the cell membrane by SLC22A7 (OMIM: 604995) and at the same time expelled by ABCG2 (OMIM: 603756) and ABCC4 (OMIM:605250). 5-FU is further metabolized by thymidylate phosphorylase to the active metabolite fluorodeoxyuridine monophosphate, which is incorporated into DNA while inhibiting cell replication by inhibition of thymidylate synthase. The principal mechanism of 5-FU elimination is its metabolism by dihydropyrimidine dehydrogenase, dihydropyrimidinease and beta-ureidopropionase to the inactive product fluoro-beta-alanine (44).

Hummel *et al.* in a study investigating chemosensitivity of EC Cell lines identified several potential intracellular targets for miRNAs (45). Transmembrane transporter

ABCC3 (OMIM:604323) was overexpressed in 5-FU-resistant esophageal adenocarcinoma, which correlated with underexpression of *miRNA-192-5p*. At the same time, it was noted that ABCB1 (OMIM:171050) may be deregulated by *miRNA-378a-3p* through inhibition of the trafficking of ABCB1 into the cell membrane mediated by Casitas B-lineage lymphoma protein B (CBL-B). A decrease in the level of *miRNA-378a-3p* correlated with overexpression of CBL-B and resistance to 5-FU. Inhibition of thymidylate synthase represents the principal target for 5-FU action, and overexpression of this enzyme can increase cell resistance to 5-FU. This hypothesis was confirmed by Hummel *et al.* who found low expression of *miRNA-192-5p* being linked to overexpression of thymidylate synthase in 5-FU-resistant EC Cell lines. The overexpression of *miRNA-483*, *miRNA-214* and resistance to cisplatin and 5-FU was described by Zhou *et al.* (46). Jin *et al.* investigated the potential role of *miRNA-141-3p* in chemotherapy sensitivity and noted that *miRNA-141-3p* was up-regulated in EC Cell lines resistant to 5-FU. The proposed role of *miRNA-141-3p* was confirmed by its inhibition, which sensitized the cell to chemotherapy (47). The suggested target was PTEN, the expression of which was inversely correlated with *miRNA-141-3p* level. PTEN negatively regulates the phosphatidylinositol 3-kinase (PI3K) pathway, one of the crucial cell pathways regulating cell proliferation. Similarly, RAS GTPase is involved in the MAPK pathway. The RAS family of genes are the most frequently mutated proto-oncogenes in involved in carcinogenesis. Hummel *et al.* found three miRNAs associated with the RAS pathway, *miRNA-27b-3p*, *miRNA-193b-3p*, and *miRNA-18a-3p*, to be dysregulated in EC (45). One of the cell membrane receptors utilizing the MAPK pathway is human epidermal growth factor receptor 2 (ERBB2 or HER2/neu). Underexpression of *miRNA-125a-5p* has been demonstrated to be inversely correlated with *HER2/neu* expression leading to 5-FU resistance in EC (45). The third important pathway in the cell mediation of cell proliferation is the WNT/ β -catenin signaling pathway. Similarly to MAPK and PI3K/AKT pathways, this pathway transduces the proliferation signals from the cell membrane to the nucleus in response to growth factors. One of potent inhibitor of the WNT/ β -catenin pathway is the Dickkopf WNT signaling pathway inhibitor 2 (DKK2). Wang *et al.* reported that *miRNA-221* is a regulator of DKK2 (48). Overexpression of *miRNA-221* increases the level of DKK2, inducing cell proliferation and inhibiting apoptosis, resulting in 5-FU resistance. Proper functioning of the cytoskeleton that forms the mitotic spindle responsible for cell division is essential for the physiological cell-cycle course. It was suggested that an important regulator of the cytoskeleton stathmin 1 (STMN1) is regulated by *miRNA-193b* in EC (49). Overexpression of *miRNA-193b* seems to correlate with sensitivity to 5-FU. At the same time, it was suggested that

Table II. *MicroRNA and sensitivity to radiotherapy of esophageal cancer.*

miRNA	Specimen	Predicted target	miRNA dysregulation	Role in EC therapy	Reference
<i>miRNA-21</i>	Cell lines (TE-1)	PTEN	Overexpression	Increases radioresistance	53
<i>miRNA-31</i>	Cell lines (OE33 P, OE33 R)	DNA repair genes (<i>PARP1</i> , <i>SMUG1</i> , <i>MLH1</i> , <i>RAD51L3</i> , <i>MMS19</i> , <i>LIG3</i> , <i>ERCC1</i> , <i>NEIL1</i> , <i>CDK7</i> , <i>DDB1</i> , <i>TOP3A</i> , <i>XPC</i> , <i>RPA3</i>)	Underexpression	Increases radioresistance	54
<i>miRNA-22</i>	Cancer tissues	Rad51	Overexpression	Increases radiosensitivity	55
<i>miRNA-98</i>	Cell lines (TE1, ECA109, EC9706, KYSE30, KYSE150 and KYSE450)	BCL2	Overexpression	Increases radiosensitivity	56
<i>miRNA-196a</i>	Cell lines (TE1, EC109)	Cyclin B1	Underexpression	Increases radiosensitivity	57
<i>miRNA-16</i>	Cancer patient, plasma	Unknown	Overexpression	Increases radiosensitivity	71
<i>miRNA-381</i>	Cell lines (TE1, ECA109, EC9706, KYSE30, KYSE150, KYSE450)	CTNNB1, LEF1, CDK1, XIAP, CXCR4	Underexpression	Increases radioresistance	72
<i>miRNA-96</i>	Cell lines (TE-1, ECA-109, EC-9706 HEEC), cancer tissue, adjacent non cancer tissue	RECK	Overexpression	Increases radioresistance	73
<i>miRNA-124</i>	Cancer tissues, cell lines (TE-1)	CDK4	Overexpression	Increases radiosensitivity	74

miRNA-193b can influence cell autophagy, thus participating in regulation of cell death.

Sensitivity to paclitaxel. Paclitaxel is transported through the cell membrane into the cell utilizing SLCO1B3 (OMIM:605495) membrane transporter. Cell efflux is mediated mostly through ABCB1 (OMIM:171050), ABCC2 (OMIM:601107), ABCC1 (OMIM:158343), and ABCG2 (OMIM: 603756). Paclitaxel binds to microtubules (alpha-tubulin), thereby inhibiting depolymerization in the M phase of the cell cycle. Paclitaxel is further metabolized by cytochrome *P450 3A4* (CYP3A4) (OMIM: 124010) and CYP2C8 (OMIM:01129) to three main metabolites: 3'-*p*-hydroxypaclitaxel, 6 α -hydroxypaclitaxel and 6 α ,3'-*p*-dihydroxypaclitaxel (50).

Chen *et al.* found *miRNA-133a* and *miRNA-133b* underexpression in patients with EC with increased sensitivity to paclitaxel (51). Nevertheless, to our knowledge, this is the only study describing the role of miRNAs in sensitivity to paclitaxel in EC.

Sensitivity to doxorubicin. Doxorubicin is transported into the cell by SLC22A16 (OMIM:608276) and the efflux is mediated by ABCC1 (OMIM:158343), ABCB1 (OMIM:171050), ABCC2 (OMIM:601107), ABCG2 (OMIM: 603756). Antitumor activity of doxorubicin is mediated partly by reactive oxygen radicals produced during redox cycling of doxorubicin and also by DNA intercalation and topoisomerase inhibition. The main cause of doxorubicin resistance seems to

be either increased efflux from the cell or increased DNA repair capacity (52).

The effect of miRNAs on membrane transport of cytotoxic drugs was studied by Zhou *et al.* (46). Antagomirs (miRNA inhibitors) of *miRNA-483* and *miRNA-214* were shown to increase the intracellular concentration of doxorubicin, possibly by modifying cell membrane transporters. Increased cellular concentration of cytotoxic drugs can increase the chance of DNA damage and tumor cell apoptosis. As already described above, PARP has an important role in DNA damage repair. *miRNA-223* overexpression seems to increase sensitivity to doxorubicin in cell lines by reducing PARP function (38). Again, there are no other studies specifically investigating miRNA role in sensitivity of patients with EC to doxorubicin.

Radiosensitivity

As outlined above, the data regarding the role of miRNAs in sensitivity to chemotherapy are sparse and there are even fewer articles describing the potential role of miRNA in the efficacy of radiotherapy for EC. miRNAs known to play a role in sensitivity of EC to radiation are summarized in Table II. The molecular basis of radiotherapy efficacy lies at the DNA level, with some data even suggesting changes of the function of cell membrane or intracellular enzymes. Radiation (photons, electrons or protons) causes DNA damage by direct and indirect mechanisms of action. A direct effect is DNA damage caused by the energy transferred by

Table III. MicroRNA single nucleotide polymorphisms in esophageal cancer.

MicroRNA SNP position	miRNA	SNP	Allele	Histology	Risk	p-Value	Predicted target	Role in EC	Reference
Pri-miRNA	<i>miRNA-34b/c</i>	rs4938723	T>C	SCC	OR=0.65	0.036	GATA-X transcription factor	Reduces risk of EC	82
Pri-miRNA	<i>miRNA-100</i>	rs1834306	T>C	SCC	OR=0.50	8.05×10 ⁻⁵	Unknown	Reduces risk of EC	81
Pri-miRNA	<i>miRNA-219-1</i>	rs107822	G>A	SCC	OR=0.57	2.837×10 ⁻⁵	Unknown	Reduces risk of EC	75
Pri-miRNA	<i>miRNA-124</i>	rs531564	C>G	SCC	OR=0.61	0.005	PTBP1	Reduces risk of EC	62
Pri-miRNA	<i>miRNA-125a</i>	rs12976445	C>T	SCC	HR=2.17	0.014	ERBB2	Worsens survival	65
Pri-miRNA	<i>miRNA-218</i>	rs11134527	A>G	SCC	OR=0.68	0.016	BMI1, LAMB3, LASP1	Reduces risk of EC	76
Pri-miRNA	<i>miRNA-34b/c</i>	rs4938723	T>C	SCC	OR=0.82	0.007	Unknown	Reduces risk of EC	62
Pre-miRNA	<i>miRNA-423</i>	rs6505162	C>A	85.5% AC	OR=0.64	<1×10 ⁻⁴	PABPC1, FGFR2	Reduces risk of EC	63
Pre-miRNA	<i>miRNA-196a-2</i>		C>T	SCC	OR=1.43	0.037	Multiple	Increases risk of EC	64
	<i>miRNA-146a</i>		G>C						
	<i>miRNA-499</i>		T>C						
	<i>miRNA-423</i>		C>A						
	(combined risk)								
Pre-miRNA	<i>miRNA-146a</i>	rs2910164	G>C	SCC	OR=0.37	0.014	NF-κB	Hematological toxicity	65
Pre-miRNA	<i>miRNA-196a2</i>	rs11614913	T>C	SCC	HR=1.55	0.010	Unknown	Worsens survival	65
Pre-miRNA	<i>miRNA-196a-2</i>	rs11614913	T>C	SCC	OR=0.62	0.04	Unknown	Reduces risk of EC	77
Pre-miRNA	<i>miRNA-146a</i>	rs2910164	G>C	SCC	OR=2.39	<0.01	TRAF6, IRAK1		78
Pre-miRNA	<i>miRNA-196a2</i>	rs11614913	T > C	SCC	OR =0.72	0.070	Unknown	Reduces risk of EC	79
Pre-miRNA	<i>miRNA-423</i>	rs6505162	C>A	SCC	OR=4.94	0.012	Unknown	Increases risk of EC	82
Mature-miRNA	<i>miRNA-608</i>	rs4919510	C>G	SCC	HR=0.47	0.008	Unknown	Improved survival	69
Mature-miRNA	<i>miRNA-449b</i>	rs10061133	A>G	SCC	OR=0.77		Unknown	Reduces risk of EC	80
Mature-miRNA	<i>miRNA-4293</i>	rs12220909	G>C	SCC	OR=0.77		Unknown	Reduces risk of EC	80
Binding site	<i>miRNA-663b</i>	rs3088440	G>A	AC	HR=0.34	0.037	CDKN2A	Reduces risk of disease progression	66
Binding site	<i>miRNA-196a</i>	rs6573	C>A	SCC	OR=0.43	0.020	RAP1A	Increases risk of EC	67
Binding site	<i>miRNA-191</i>	rs4245739	A>C	SCC	OR=0.54	0.004	MDM4	Reduces risk of EC	68
miRNA processing genes	<i>RAN</i>	rs14035	C>T	SCC	HR=1.16	0.586	mRNA stability	Worsens survival	69
miRNA processing genes	<i>GEMIN4</i>	rs7813	C>T	SCC	HR=1.77	0.235	RISC	Worsens survival	69

HR: Hazard ratio; OR: odds ratio; AC: adenocarcinoma; SCC: squamous cell carcinoma.

the beam directly to the DNA chain, resulting in single- or double-strand breaks. Indirect effects of radiotherapy are mediated by the reactive particles induced by the energy transfer to water molecules, causing the formation of reactive oxygen species. Considering these facts, radioresistance of the cancer cells is caused mainly by the increased capacity to repair this damage, or reduced susceptibility to apoptosis.

PTEN has been associated with the pathogenesis of several tumor types. Recently, PTEN relation to miRNA-21 was also characterized (53). Overexpression of miRNA-21 was found in radioresistant cell lines. The exact mechanism is Unknown, but PTEN negatively regulates cell oncogenes and slows cell proliferation, directing the cell towards apoptosis.

Efficacy of radiotherapy-induced DNA damage is closely related to the efficacy of the DNA repair mechanisms. *miRNA-31* seems to increase radiosensitivity by inhibition

of several DNA repair enzymes, as reported by Lyman-Lennon *et al.* (54). Another study presented by Wang *et al.* investigated the potential role of miRNA-22 in EC radioresistance. The efficacy of radiotherapy in this study seems to be related to the expression of *miRNA-22* (55). The explanation for this effect may be its relation to the RAD51 pathway. RAD51 is a repair enzyme responsible for homologous recombination [similarly to breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2)]. Overexpression of *miRNA-22* reduces the level of RAD51 leading to decreased capability for DNA repair after radiotherapy, which results in the increased radiosensitivity observed. Another potential miRNA which may play a role in EC radiosensitivity is *miRNA-98*. Underexpression of *miRNA-98* was proven to increase radioresistance of EC Cell lines (56). One potential explanation for this observation may be the interaction

between miRNA-98 and the B-cell lymphoma 2 (*BCL2*) gene family. Members of this gene family are crucial regulators of apoptosis, with mostly pro-apoptotic, but also anti-apoptotic potential mediated through alteration in the permeability of the mitochondrial membrane (intrinsic pathway of apoptosis).

In the clinic, it is difficult to evaluate radiotherapy sensitivity or resistance because almost all patients are treated with concomitant chemoradiation and the individual contribution of chemotherapy or radiation to the final outcome cannot be precisely discerned. Most of the data discussed above were obtained from *in vitro* experiments on cell lines, and it is important to note that situation *in vivo* may be different.

An important target for miRNAs is the cell cycle, the first step on the path of a cancer cell towards apoptosis is cell-cycle arrest. There are several time-points in the cell cycle where this can occur, the most significant being the transitions from G₁ to S phase and G₂ to M phase. On detection of DNA damage, the cell interrupts the cell cycle in attempt to repair the damage, and if this does not happen, the cell will be directed towards apoptosis (necrosis is not the desirable mechanism of cancer cell death during radiotherapy or chemotherapy treatment). The potential role of miRNA-196a was studied on the EC Cell lines TE1 and EC109 (57). It was observed that underexpression of miRNA-196a increased the sensitivity of these cells to radiotherapy. A potential explanation might be cell-cycle arrest caused by the underexpression of *miRNA-196a* mediated through its target cyclin B.

miRNA Single Nucleotide Polymorphisms and EC

Single-nucleotide polymorphisms (SNPs) may play a critical role in increasing the risk of carcinogenesis through different mechanisms, including their effects on the level of signal transduction pathways, apoptosis, xenobiotic biotransformation or cell membrane transport. Several SNPs participating in these processes have been studied (58-61). In general, SNPs are located in three different regions of the DNA connected with RNA function, including the coding sequences of the DNA which is transcribed into pri-miRNA, the DNA sequences containing binding sites for the mRNA-miRNA interaction, and the DNA sequences coding enzymes participating in miRNA expression (DICER, DROSHA). SNPs reported to be associated with EC are summarized in Table III.

SNPs in DNA-coding miRNA. SNPs in pri-miRNA, pre-miRNA or mature miRNA that affect the function of miRNAs have been described. Zhang *et al.* have reported potential association of SNPs (rs531564, rs4938723) in pri-miRNA with EC risk (62). Possible targets could be polypyrimidine

tract-binding protein 1 (PTBP1) which regulates miRNA processing for miRNA-124, and Wnt pathway for *miRNA34/b/c*. A case-control study presented by Ye *et al.* reported the lower risk of EC with the polymorphism rs6505162 (C>A) in pre-miRNA-423 (63). Similarly, Umar *et al.* found that the combination of four pre-miRNA (miRNA-196a-2, miRNA-146a, miRNA-499, miRNA-423) SNPs increased the EC risk (OR=1.43) (64). The relation of SNPs in miRNA to survival of patients with EC was studied by Wu *et al.*, who reported that patients with SNPs rs11614913 (T>C) in pre-miRNA-196a2 and rs12976445 (C>T) in pri-miRNA-125a have inferior survival (65).

SNPs in the binding sites of mRNA. The binding site for miRNA on mRNA is the 3'-UTR of the mRNA. Several SNPs in 3'-UTR regions of mRNA encoding crucial proteins participating in carcinogenesis have been identified. SNP rs3088440 (G>A) in the binding site for miRNA-663b in cyclin-dependent kinase inhibitor 2A (*CDKN2A*) has been connected with decreased risk of EC (HR=0.34, *p*=0.0368), while rs6573 (A>C) in *miRNA-196a*-binding site of RAS related protein 1A (*RAP1A*) (OR=0.43, *p*=0.02) and rs4245739 (A>C) in miRNA-191-binding site of mouse double minute 4 (*MDM4*) gene (OR=0.54, *p*=0.004) have been reported to reduce the risk of EC (66-68).

SNPs in enzymes regulating and processing miRNA. The steps in the processing of pri-miRNA to pre-miRNA, double-stranded RNA duplex and finally mature miRNA have been described above. A crucial step in miRNA function is the formation of the RISC complex. Key proteins in the RISC complex are GEM-associated proteins 3 and 4 (GEMIN3, GEMIN4). Yang *et al.* reported that rs7813 SNP in *GEMIN4* worsens patient survival. Similarly, SNP rs14035 of RAS-related nuclear protein (*RAN*), participating in transportation of pre-miRNA through the nuclear membrane into the cytoplasm, was reported to be possibly associated with inferior patient survival (69).

There are currently no studies specifically describing miRNA SNPs in relation to sensitivity of patients with EC to chemotherapy or radiotherapy.

Conclusion

The investigation of miRNAs represents a promising direction in the development of novel approaches for screening, early diagnosis, staging, prognostic assessment and treatment of EC. Further research is necessary to move the data from the laboratory through translational research to the bedside.

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