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A search for epigenetic biomarkers predicting sensitivity to therapy in cervical cancer

Bachelor essay

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Abstract

Cervical cancer is the fourth most common cancer in women, with an overall mortality ratio of 52%. Treatment with chemoradiation improves progression-free and overall survival, when compared with radiation treatment alone. Unfortunately, resistance to radiotherapy and/or chemotherapy is limiting further improvement of survival rates for cervical cancer patients. Aberrant gene expression is playing a big role in either resistance or sensitivity to (chemo)radiation therapy. Methylation of genes as well as microRNAs (miRNA) can cause changes in gene expression and in this way either enhance or decrease sensitivity to therapy. We could possibly use this epigenetic information as a biomarker to predict outcome of treatment in cervical cancer patients. The aim of this study was to provide an overview of the latest studies in this field concerning alterations in methylation patterns or miRNAs expression levels associated with either better or worse response to therapy. Although relatively not many studies have been performed in this field focussing on cervical cancer, research is still ongoing and in the last years researchers have shown correlations between methylation and miRNAs patterns and therapy outcome. These studies could contribute to an ideal situation where patients will be screened for aberrant methylation or miRNAs patterns to predict if standard (chemo)radiation therapy will be effective. If patients are predicted to respond bad to therapy due to certain methylation or miRNA patterns, this information could provide us where to intervene with other therapeutic drugs to enhance sensitivity to therapy. Further research in this area will be of great value, taking us a step closer to further improvement of survival rates for cervical cancer patients.

Introduction

Cervical cancer is the fourth most common cancer in woman, with an overall mortality ratio of 52% [1]. The human papillomavirus (HPV) is detected in 99.7% of cervical cancers, making it central to the development of cervical cancer [2]. The two most common histological types of cervical cancer are squamous cell- and adeno carcinoma, with a ratio of 69 % and 25 % respectively [3]. Based on clinical examination, cervical cancer is classified in different stages, according to the International Federation of Gynecology and Obstetrics (FIGO) [4]. There are four main stages, which are also subdivided in an A- or B group. The first two stages, except for stage IIB, are the early stages and the last two stages (including IIB) are the locally advanced stages. Used treatment is based on this staging, in which early stages are treated by surgery and locally advanced stages are treated with chemoradiation therapy [5]. Adjuvant concurrent chemoradiation improves progression-free and overall survival, when compared with radiation treatment alone [4,6]. Unfortunately, resistance to radiotherapy and/or chemotherapy limits further improvement of survival rates. Tumor biological factors such as aberrant gene expression patterns can play a big role in the response to chemoradiation therapy. It would be of great value if we could use biomarkers that could predict this resistance or sensitivity to therapy [6].

Epigenetic changes are suggested as another hallmark of cancer, because of its ability to induce pro-cancer characteristics, even in mutation-free cells [8]. Epigenetic changes enable alterations in gene expression without changing the DNA sequence. This can be done in various ways, including DNA methylation, histone methylation or acetylation and changes in microRNAs [7].

Methylation is a process in which a methyl group is added to the base cytosine. In humans and other mammals, only cytocines that precede a guanosine in the DNA sequence are methylated (called a CpG dinucleotide). These dinucleotides can be clustered in small stretches of DNA, which are called 'CpG islands'. Promoter regions of genes often contain these CpG islands. About 80% of the CpG dinucleotides distributed in the genome, which are not associated with CpG islands, are strongly methylated. CpG dinucleotides in CpG islands are in contrast usually unmethylated, whether or not the gene is being transcribed [9]. DNA methylation plays a role in gene expression regulation. It is thought that DNA methylation can target the formation of chromatin, since specific proteins are able to bind to methyl-cytosines, which on their turn are involved in protein complexes which are able to recruit chromatin remodelling proteins. Strong correlation between DNA methylation and gene inactivition is found [10].

This epigenetic mechanism is often deregulated during carcinogenesis [6]. Neoplastic cells often harbour an imbalance in methylation patterns, including hypermethylation of normally unmethylated CpG islands located in gene promoter regions. In this way it is, for example, possible to silence tumor suppressor genes via abberant hypermethylation [11]. As said before, factors such as aberrant gene expression patterns, which can be established by changes in DNA methylation patterns, can play a big role in either resistance or sensitivity to chemorediation therapy. When DNA demethylating agent decitabine was administered prior to, or in combination with chemotherapy, increases in partial as well as complete responses were reported in clinical trials, in multiple malignancies. [13,14]. Important is that not all patients respond well to demethylating agents, sometimes even showing indications of adverse effects. [15] This reverse effect could be explained by the fact that decitabine is a global DNA demethylator, which could for that reason, also cause re-expression of genes involved in resistance to anti-cancer therapy, or re-expression of oncogenes, which were initially silenced via methylation. We are in need for guidelines, which could help us predict outcome of treatment in cervical cancer patients. Roossink et al (2012) showed a summary of reports where hypermethylated genes were related to good as well as poor response to therapy in different malignancies [6]. Only patients that will respond beneficial to demethylating agents should be additionally treated. Patients who are expected to respond negatively to demethylating agents would possibly benefit from more specific demethylating agents.

Regulation of gene expression is very complex, and many other factors may contribute to it, including microRNAs. MicroRNAs (miRNAs) are non-coding RNAs with a length of around 21-23 nucleotides, functioning as posttranscriptional regulators. Several studies have highlighted the association between expression levels of miRNAs and carcinogenesis [7]. Recently, several miRNAs have been documented to be deregulated and in this way can contribute in tumor radioresistance [12].

Since aberrant methylation as well as changes in miRNA levels can lead to enhanced or decreased sensitivity to therapy, we could possibly use this information as a biomarker to predict outcome of treatment in cervical cancer patients and we could try to counter or reinforce these mechanisms. Following will be an overview of different studies that showed alterations in methylation patterns and miRNAs expression associated with better or worse response to treatment.

Methylation patterns related to therapy response in cervical cancer

As said before, altered methylation patterns have been associated with changes in development and progression of cancer. Different studies have shown a link between certain methylation patterns and therapy response in different types of cancer [44,45,46,47]. Unfortunately only few studies have been exploring this relation in cervical cancer [6]. Next will follow a few of the latest studies where methylation in promoter regions of certain genes were associated with therapy outcome and response in cervical cancer. Table 1 shows an overview of the discussed genes.

TP73, *BRCA*, *FANCF* and *RARB* are possible biomarkers for predicting therapy outcome in cervical cancer patients

A previous review study from Roossink et al. (2012) [6] suggested *TP73*, *BRCA1* and *FANCF* to be possible biomarkers for predicting therapy outcome in cervical cancer patients. Promoter methylation of *TP73* was related to poor response to radiotherapy in cervical cancer tissue specimens. *TP73* methylation was indicated to result in loss of expression, since methylation was inversely correlated with *TP73* protein expression. Also, high levels of *TP73* protein expression was associated with increased radiotherapy sensitivity. These findings indicate that methylation op *TP73* could lead to worse response to radiotherapy [16].

Narayan et al. (2003) [17] showed that cervical cancer patients who showed methylation of *BRCA1* and also *RARB* failed or responded bad to treatment (radiation and/or chemotherapy), suggesting that promoter methylation of these genes may be a bad prognostic indicator. However, two independent studies concerning epithelial ovarian cancer patients, showed that promoter hypermethylation of *BRCA1* was related to better response to cisplatin [18,19].

In a later paper of Narayan et al. (2004) [20] results showed that promoter hypermethylation of *FANCF* is also common in cervical cancer. Cervical cancer cells with *FANCF* promoter hypermethylation are hypersensitive to DNA-damaging drugs [6]. An earlier study showed that promoter demethylation resulted in cisplatin-resistance in ovarian cancer cells [21]. However, there are doubts about the relationship between *FANCF* methylation and therapy response. Lim et al. (2007) [43] performed a study where they tried to confirm the hypothesis that inactivation of *FANCF* is a mechanism of sensitisation to platinum chemotherapy in ovarian cancer cell lines and ovarian cancer samples. Results did not support methylation-dependent silencing of *FANCF* as a mechanism of sensitisation to platinum-based chemotherapy in ovarian cancer, since they could not find an association between methylation of *FANCF* and overall survival.

TP73

TP73 encodes a member of the p53 tumor suppressor gene family, the tumor protein p73. It is a gene, which is involved in the activation or execution of the apoptosis process. P73 can activate transcription of p53-responsive genes and in this way induce apoptosis in a p53-like manner [22]. Deregulation in *TP73* may lead to radioresistance in cells when this deregulation leads to distortion in the apoptotic process in cancer cells.

FANCF&BRCA1

FANCF is one of the Fanconi anemia complementation group (*FANC*). *FANC* genes are essential in DNA repair pathways, responding to cisplatin and other DNA cross-linking agents.
FANC proteins can interact with *BRCA* genes. Results of Narayan et al. (2004) [20] showing promoter hypermethylation of *FANCF* is common in cervical cancer as well as results of Taniguchi et al (2003) [21] showing promoter hypermethylation of *FANC* can disrupt the *FA-BRCA* pathway resulting in cisplatin sensitivity in ovarian tumors, are in line with the knowledge of the function of the *FA-BRCA* pathway. More studies exploring *BRCA1* however are showing different results [18,19.] Also, results of Lim et al. (2007) [43] are contradictory to the previous studies. These different outcomes show that more research concerning the role of *BRCA* and *FANCF* in the development of therapy resistance is needed.

RARB

The *RARB* gene encodes for retinoic acid receptor beta, a member of the thyroid-steroid hormone receptor superfamily. It is a nuclear transcriptional regulator. It is thought that this protein limits growth of many cell types. In this way, *RARB* can function as a tumor suppressor gene. Methylation, assuming this would lead to gene silencing, would for this reason be unfavourable.

Unmethylated *MYOD1*, unmethylated *ESR1* and methylated *hTERT* promoter prediction for chemoradiation resistance.

Sood et al. (2015) [5] evaluated methylation status of gene promoters of six genes, which have previously shown to be methylated in invasive cervical cancer. This evaluation was done in cervical cancer patients with locally advanced stage FIGO IIB or III, treated with chemoradiation. Patients were followed for several years to be able to analyse outcome and response to chemoradiation therapy. They found that gene promoter methylation of *MYOD1* and *ESR1* predicted a favourable outcome in patients. In contrast, they found that gene promoter methylation of *hTERT* predicted a worse outcome.

MYOD1

Gene *MYOD1* encodes a transcription factor that is able to regulate muscle cell differentiation. Epigenetic dysregulation in cancer is seen more often [23].

Dey et al. (2013) proposed *MYOD* to be a tumor suppressor gene in medulloblastoma [24]. Widschwendter et al. (2004) [25] showed that cervical cancer patients with unmethylated *MYOD1* serum DNA had a significantly better overall survival compared to patients with methylated *MYOD1,* which is in contrast with results of Sood et al. (2015) [5]. Unmethylated *MYOD1* and response to therapy is poorly understood [5]. Altered functioning of *MYOD1* could be related to cellular de-differentiation or activation of the epithelial-mesenchymal transition pathways, both associated with chemoresistance and radiation resistance. Epigenetic changes of *MYOD1* and the outcome of functioning of *MYOD1* are not fully clear, based on the contradictory results of these studies.

ESR1

Gene *ESR1* encodes for estrogen receptor alfa, important for hormone binding. It is a transcription factor and is involved in control of cell differentiation. Kirn et al. (2014) [26] found that *ESR1* methylation was associated with higher grade tumors, although it was not of prognostic relevance in cervical cancer.

Interestingly, Sood et al. (2015) [5] also showed that lower *ESR1* transcript levels were correlated with chemoradiation resistance. This would be contradictory if we assume that methylation of gene promoter *ESR1* would lead to gene silencing of *ESR1*, since methylation of *ESR1* predicted for a favourable outcome in patients. However, none of the genes analysed showed a statistical significant association between methylation and gene expression, assuming that methylation of *ESR1* found in this study may not cause silencing of the gene.

hTERT

In contrast to *MYOD1* and *ESR1*, *hTERT* gene promoter methylation predicted a worse outcome. *hTERT* is a potential oncogene and part of the telomerase complex. Overexpression of *hTERT* is thought to be able to induce immortalization of many human cell types, via prevention of progressive loss of telomere length during DNA replication [27].

Zinn et al. (2007) [28] showed that although methylation of *hTERT* in its promoter region in many cancer is seen, this methylation does not directly mean that the gene is silenced. They showed that different cancer cell lines retained alleles with little or no methylation around the transcription start site, despite being densely methylated in the surrounding area. In addition, Renaud et al. (2006) [53] hypothesised that methylation in certain areas in the promotor CpG island of *hTERT* could prevent binding of CTCF inhibitors, which are able to repress *hTERT* expression. If these areas where hypermethylated, but a small part of the core promotor region was not methylated, *hTERT* could still be transcribed and the transcriptional repressor CTCF could not bind anymore, resulting in expression of *hTERT.* Apparently it does also matter where in the promoter CpG island methylation occurs. This

could also be an explanation for the fact that Sood et al. did not find an association between methylation and expression of the analysed genes.

Methylation of CpG unit in *MDR1* predictive for chemotherapy efficiency

Huang et al. (2016) [29] looked at methylation status of 16 CpG units in the gene promoter region of *MDR1*, a gene which codes for P-glycoprotein (P-gp). This evaluation was done in cervical cancer patients with different stages, ranging from Ib to IIIb, receiving chemotherapy before the main treatment. They found that before therapy, methylation rate of one CpG unit (Loci 2, 3, and 4) was significantly higher following effective chemotherapy than when patients experienced ineffective chemotherapy.

MDR1 and P-glycoprotein

P-gp is one of the protein members in the superfamily of ATP-binding cassette transporters. It has a membrane efflux pumping function, which makes it able to pump a variety of antitumor drugs out of cells and in this way producing drug resistance.

Findings of Huang et al. (2016) showed that positive expression rate of P-gp before chemotherapy reduced efficacy of chemotherapy. In addition, they showed that the methylation rate of six CpG units was higher in cervical cancer tissue before chemotherapy
than after chemotherapy. So, it seems that during chemotherapy, certain methylation patterns can change.

Altered methylation of *Casp8AP2* possible predictor for oxaliplatin resistance

Chen et al. (2015) [30] performed a study with sensitive cervical cancer cells (SiHa cells) and cervical cancer cell lines which were resistant for oxaliplatin (S3 cells). They found methylation changes in S3 cells compared to SiHa cells, both genome-wide and within individual loci. Cell line S3 showed an increase in methylation within the promoter region of different genes including *Casp8AP2* . Treatment with demethylation agent 5-Aza-2′-deoxycytidine (5-aza) was able to reverse these findings. They hypothesized that if DNA methylation in drug-resistant cancers cells is necessary for maintenance of drug resistance, reversal of DNA methylation in S3 should be able to restore their sensitivity to drug treatment. Their findings showed that after S3 cells were treated with 5-aza, the sensitivity of S3 cells to oxaliplatin was restored. These data show that methylation of promoter regions of different genes can play a role in drug resistance in cervical cancer.

Casp8AP2

Caspase 8 associated protein 2 is a protein which is most likely involved in Fas-mediated apoptosis. This protein is suggested to be a component of the death-inducing signalling complex that includes Fas receptor, Fas-binding adapter FADD and caspase 8. Methylation of *Casp8AP2* could possibly cause defects in the apoptosis mechanisms where anti-cancer drugs are most often dependent on.

***SOCS1* methylation possible predictor for favourable therapy outcome**

Kim et al. (2015) [31] performed a study with radiosensitive human cervical cancer cell lines: CaSki, HeLa, ME-180 and SiHa and normal human fibroblast cell lines CCD-18Lu, CCD-18Co and WI-38. Also normal cervix tissue was obtained from one patient.

First of all this study showed that *SOCS* 1, 2, and 5 where all downregulated in cervical cancer cells compared to normal cervix tissue and the fibroblast cell lines. DNA methylation analysis of the *SOCS* gene promoter regions showed that *SOCS1* expression was correlated with differential DNA methylation in CaSki, Hela and ME-180 cells. When they treated their cell lines with 5-azacytidine (5-AzaC), *SOCS1* expression was prominently increased in CaSki and HeLa cells, and slightly increased in ME-180 cells. Lastly, they also showed that overexpression of *SOCS1* made HeLa cells more resistant to radiation. Combining the results that DNA gene promoter methylation of *SOCS1* is correlated with *SOCS1* expression and that overexpression of *SOCS1* showed a more resistant phenotype in HeLa cells, methylation of the *SOCS1* gene could be favourable for patients

when predicting outcome of therapy.

One of the first results from Kim's study was that *SOCS1* was downregulated in cervical cancer cells. This could possibly be due to methylation of the promoter region of *SOCS1* and could also be a positive predictor for therapy outcome. A test where methylation status of *SOCS1* is compared with (chemo)radiation therapy sensitivity would be of great value to confirm this suggestion.

SOCS1

SOCS1 codes for a suppressor of cytokine signalling protein, which plays a key role in the negative regulation of cytokine signalling transduction. It can inhibit the JAK/STAT pathway to modulate cellular responses. *SOCS1* appears to have tumor suppressor activity [25]. Kim et al. (2015) [31] showed that overexpression of *SOCS1* was related to a more resistant phenotype in HeLa cells. These observations are unexpected considering the function of *SOCS1* and further research exploring the role of *SOCS1* in therapy resistance is needed.

Table 1 Methylation patterns in genes associated with favourable or unfavourable outcome to therapy.

Gene	Name	Gene ID	Status	Therapy	Variant	Outcome	Ref.
TP73	Tumor Protein p73	7161	Methylated	radiotherapy	Cervical cancer	unfavourable	16
FANCF	Fanconi anemia complementation group F	2188	Methylated	Chemotherapy	Cervical cancer	favourable	20,21
FANCF	Fanconi anemia complementation group F	2188	Methylated	Chemotherapy	Ovarian cancer	unfavourable	43
BRCA1	BRCA1, DNA repair associated	672	Methylated	Radiation and or Chemotherapy	Cervical cancer	unfavourable	17
BRCA1	BRCA1, DNA repair associated	672	Methylated	Cisplatin	Ovarian cancer	favourable	18,19
RARB	retinoic acid receptor beta	5915	Methylated	radiation and/or chemotherapy	Cervical cancer	unfavourable	17
MYOD1	myogenic differentiation 1	4654	Methylated	chemotherapy	Cervical cancer	favourable	5
ESR1	estrogen receptor 1	2099	Methylated	chemotherapy	Cervical cancer	favourable	5
hTERT	human telomerase reverse transcriptase	7015	Methylated	chemotherapy	Cervical cancer	unfavourable	5
MDR1 (ABCB1)	ATP binding cassette subfamily B member 1	5243	Methylated	chemotherapy	Cervical cancer	favourable	29
Casp8AP2	caspase 8 associated protein 2	9994	Methylated	Oxaliplatin	Cervical cancer	unfavourable	30
SOCS1	suppressor of cytokine signaling 1	8651	Methylated	(Chemo)radiation	Cervical cancer	favourable	31

MiRNA expression patterns related to therapy response in cervical cancer

Most miRNA genes are located in DNA sequences between genes, although they can also be found in exonic or intronic regions. They can be independently transcribed from their own promoters. The biosynthesis of miRNAs involve different steps, including generation of hairpin-shaped long transcripts by RNA polymerase II. The primary long transcript undergoes processing in both the nucleus and the cytoplasm by type III ribonucleases calles Drosha (in nuclei) and Dicer (in cytoplasm). Mature miRNAs can, after formation with different proteins, form a miRNA-induced silencing complex (miRISC), which can act as a guide to deliver the complex to target mRNA. Here it will negatively regulate gene expression. Gene silencing is achieved by either mRNA degradation or inhibiting the translation of mRNA. Figure 1 shows an overview of the synthesis of miRNA and miRISC.

MiRNAs are considered to play an important role in regulating cellular processes such as apoptosis, cell cycle progression, metastasis and radioresistance. This knowledge makes it easy to understand that changed expression of miRNAs could lead to alterations in progression of cancer and sensitivity or resistance to different therapies [32].

Many studies have been performed, exploring the association between miRNAs and development and progression of cancer [48,49,50]. Here will follow two studies that explored the link between miRNA expression patterns and outcome to therapy in cervical cancer. Table 2 shows an overview of the discussed miRNAs.

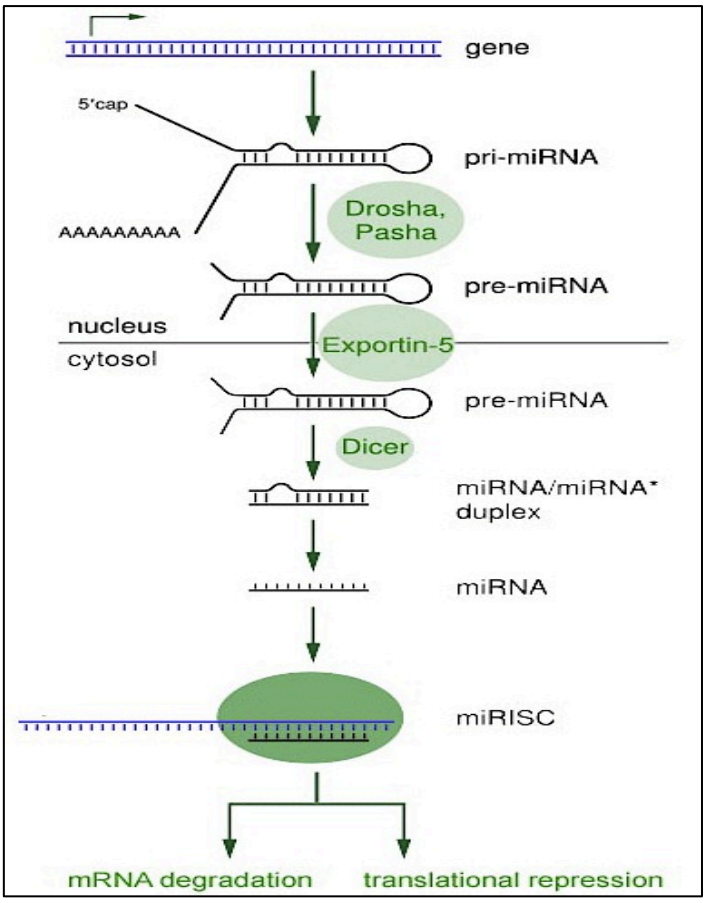

Fig 1. Synthesis of miRNA and miRISC [54]
Polymerase II generates pri-miRNA by transcribing the miRNA gene. Drosha cleaves pri-miRNA, forming pre-miRNA. Pre-miRNA is transported to the cytosol and cleaved by Dicer, leaving a double stranded miRNA duplex. One miRNA strand is loaded into the RISC complex and is transported to the mRNA, where it will cause mRNA degradation or translational repression.

MiRNA expression including miR-630, miR-1246, miR-1290 and miR-3138 could promote radioresistance of cervical cancer cells

Zhang et al. (2013) [12] studied miRNA profiles of three cervical cancer cell lines and their radioresistant variant: Hela/Hela-R11, Siha/ Siha-R15 and Hela-C/ Hela-NDRG2 cells. They showed that 20 miRNAs (14 upregulated and 6 downregulated) had similar alteration in all three radioresistant cervical cancer cell variants compared to their control.

Four of these microRNAs appeared to be up-regulated 5 fold in the radioresistant cells (miR-630, miR-1246, miR-1290 and miR-3138). Expression of all these 4 miRNAs was increased upon exposure to radiation. They transfected Hela cells with miRNA mimics (small, chemically modified double-stranded RNAs that mimic endogenous miRNAs) which where specific for these 4 miRNAs. These Hela cells expressed higher miR-630, miR-1246, miR-1290 and miR-3138 after transfection compared with the control cells. Overexpression of each 4 miRNAs could dramatically increase the survival fraction of irradiated Hela cells. MiR-630 mimics were the most effective in attenuating radiosensitivity of Hela and Siha cells. Suppressed miR-630 by specific miRNA inhibitors significantly lowered survival fraction in all three resistant cervical cancer cell lines. According to these data Zhang et al. (2013) [12] indicated that this specific miRNA signature could promote radioresistance of human cervical cancer cells.

MiR-630

MicroRNA 630 (miR-630) is thought to play a role in modulation steps in the intrinsic apoptosis pathway. It is hypothesized that miR-630 can block the upstream signalling pathways that are activated after DNA damage and converge on p53 activation [33]. MiR-630 also appeared to acts as a regulator downstream of phospho- ΔNp63α in autophagy [34]. It is suggested that miR-630 can regulate cisplatin-induced cancer cell death. Findings of Zhang et al. (2013) [12] indicated that suppressed MiR-630 had a sensitizing effect on resistant cervical cancer cells. These findings are plausible, when the function of miR-630 is indeed blocking signaling pathways which could normally lead to cell apoptosis after DNA damage has occurred.

MiR-18a overexpression promotes radiosensitivity in cervical cancer patients

Liu et al (2015) [35] were also interested to find biomarkers to predict radiosensitivity in cervical cancer patients. Their aim was to examine the role of miR-18a in regulation of radiosensitivity, since Ke et al. (2013) [52] showed that miR-18a was significantly decreased in radio-resistant cervical cancer samples compared to radio-sensitive tumor samples. Liu showed that cervical cancer patients with high expression levels of miR-18a showed hypersensitisation to radiation compared to patients showing low expression levels. To test if expression levels of miR-18a could serve as a diagnostic tool to predict radiosensitivity, they used a receiver operating characteristic (ROC) curve. Using miR-18a as a classifier, the ROC curve showed clear separation of the radioresistant and the radiosensitive patients groups. Liu et al concluded that these results indicated that miR-18a could be a potential molecular marker for detection of sensitivity to radiotherapy.

MiR-18a

MicroRNA 18a (miR-18a) was in the same report of Liu et al (2015) indicated to be playing a role in the DNA damage response, by targeting the ATM gene, which is responsible for double strands break (DSB) repair. When ATM has a reduced ability to repair DSBs caused by radiation, this will lead to increased genomic instability, resulting cells to go into apoptosis. MiR-18a could in this way contribute to enhanced sensitivity to radiation in cervical cancer patients.

Table 2 MicroRNAs genes associated with favourable or unfavourable outcome to therapy.

MiRNAs	Name	Gene ID	Status	Therapy	Variant	Outcome	Ref
MiR-630	microRNA 630	693215	upregulated	radiotherapy	Cervical cancer	unfavourable	12
MiR-18a	microRNA 18a	406953	upregulated	radiotherapy	Cervical cancer	favourable	35

Conclusions and future perspectives

This review shows a recap of the present studies exploring possible epigenetic markers related to prediction of therapy outcome in cervical cancer. Although relatively not many studies have been done in this field concerning cervical cancer, research is still ongoing and the importance of epigenetic control in regulation of expression of genes is explicitly acknowledged.

This review shows that in cervical cancer many genes show altered methylation patterns in their promoter regions, which appeared to be related to either better or worse outcome to therapy. This given, mapping of methylation status could become a usable biomarker for predicting therapy response.

Since microRNAs are also able to change gene expression without changing DNA sequence, microRNAs can be seen as epigenetic modulators as well. Besides acting as epigenetic modulators, other epigenetic mechanisms are also able to influence microRNA expressions. It has been proposed that alterations in the methylation status of miRNA promoters could be the driving mechanism behind their aberrant expression in cervical cancer [36].

Human papilloma virus (HPV), which is almost always present in cervical cancer patients, seems to be able to influence alterations in the methylation status of protein-coding genes and various miRNA genes, through the E6 and E7 oncoproteins [36]. These oncoproteins are transcribed from viral genes that are integrated into cellular DNA of host cells. They can target transcription factors such as p53. P53 plays a key role in regulation of methyltransferase DNMT1 transcription as well as in regulating miRNA expression. HPV infection can contribute to altered methylation patterns and miRNA expression via targeting p53 and in this way also alter response to different therapies [32].

In our mission to reduce the number of cervical cancer cases, vaccination against the HPV virus has shown to be very effective [37]. Treatment of chemoradiation instead of only treating with radiotherapy has been of importance for improving survival of cervical cancer patients [6]. Still, the problem involving development of resistance to radiotherapy and/or chemotherapy remains a big problem in further improvement of survival rates of cervical cancer patients and this problem is seen in many types of cancer. Using epigenetic markers as a biomarker could potentially help us. Ideally, patients will be screened for altered epigenetic patterns before therapy, and based on this information, we will be able to predict if standard therapy will be either effective or not.

When patients are prognosed to respond negatively to chemoradiation therapy, the next step is to intervene in the mechanisms that are related to bad response. This can be done at different levels. DNA demethylators such as 5-aza-2′-deoxycytidine (decitabine, dacogen) can be used to reverse methylation. The downside of using decitabine is the fact that this is a global DNA methylator, which will cause demethylation of desired genes but also demethylation of genes which are undesired to be demethylated, such as oncogenes. As seen in this study, there are multiple genes which are demethylated and related to favourable therapy outcome. The problem with lack of specificity could be tackled when we are able to specifically target genes for demethylation.

Synthetic epigenome engineering tools have been produced, which typically consist of a protein-based programmable DNA-binding domain (DBD) fused to an enzymatic scaffolding effector domain. Commonly used DBDs are Zinc Finger Proteins (ZFPs), transcription activator-like effectors (TALEs) and type II CRISPR-Cas9 system. ZFPs and TALEs can specifically bind to a certain DNA sequence. Recently, the CRISPR-Cas9 system is used to create a new DBD. Deactivated Cas9 (no longer able to cleave DNA) is guided to a target sequence by guide RNA. When these different DBDs are paired with effector enzymes which can demethylate CpGs at target promoters, like Thymidine DNA glycosylase (TDG) or Ten-eleven Translocation (TET), specific demethylation will be possible [38]. Huisman et al. (2016) [39] explored effectiveness of locus-targeted demethylation using ZFP-TET2. They showed that by targeting TET2 to hypermethylated TSGs, efficient DNA demethylation could be achieved.

Since miRNAs are involved in various human diseases, development of inhibitors for specific miRNAs is ongoing an expected to be useful in providing tools for basic research as well as generating promising new therapeutic drugs [40]. In the future, these inhibitors could be given to patients who show upregulated levels of miRNAs which correlate with unfavourable therapy outcome.

Besides looking at either methylation or miRNA patterns, it is also possible to directly look at gene expression or protein expression. Wong et al. (2003) [41] showed that expression profiles of cervical cancer were distinguishable from those of normal cervix and they were able to predict response to radiotherapy based on the signature expression patterns of cervical cancer. Coherent, protein expression can also correlate with therapy outcome. Noordhuis et al. (2009) [51] showed that positive immunostainings of EGFR and pEGFR predict poor response to (chemo)radiation in cervical cancer.

Although methylation of gene promoter regions is often correlated with silencing of the gene, this does not always have to be the case. Sood et al. (2015) [5] did not find a statistical significant association between methylation and gene expression. Also, Renaud et al. (2006) [53] showed that CTCF inhibitors (which can repress gene expression) can bind to certain areas in the promotor CpG island. If these areas were methylated but a small core promotor region was not, this resulted in expression of *hTERT*.

If we want to be able to intervene in downstream pathways, understanding how methylation of the gene of interest influences the gene expression and therefore also the protein expression is necessary. Another example showing the necessity to understand consequences of methylation, follows from a study by Paulíková et al. (2013) [42] who showed that hypermethylation of the *XRCC2* and the *RAD51L3* genes was related to late complications after chemoradiotherapy in radiosensitive cervical cancer patients. This hypermethylation was also assumed to be a possible cause of increased radio sensitivity. This shows that we have to be careful in linking methylation status to sensitivity to therapy, since methylation of genes could also be responsible for late grade toxicity or other side effects in patients. Also, various studies find contradictory results of relation between methylation patterns and therapy outcome, for example Narayan et al. (2003) [17] who found that cervical cancer patients with methylation of *BRCA1* responded bad to treatment, whereas to other independent studies showed that promoter hypermethylation of BRCA1 related to better response to cisplatin in ovarian cancer patients [18,19]. Studies exploring the relationship between *FANCF* or *MYOD1* and chemotherapy response also showed contradictory results [18,19,43 and 5,24,25]. More research in this field is needed, especially when functions of genes are not completely understood.

This study and many others show that epigenetic changes can serve as possible biomarkers for predicting outcome of therapy. In the future, this information could hopefully help us to provide better therapy for cervical cancer patients, especially those who are expected have an unfavourable outcome to standard therapy.

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