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GENETIC AND EPIGENETIC ALTERATIONS AS SERUM MARKERS FOR CANCER DETECTION

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II. **Benati M**, Montagnana M, Danese E, Giudici S, Ruzzenente O, Guidi GC, et al. MiR-199a and miR-125b expression levels in serum of patients affected by epithelial ovarian cancer. Biochim Clin 2016;40:328-33.

III. Montagnana M, **Benati M**, Danese E, Giudici S, Perfranceschi M, Ruzzenente O, et al. Aberrant miRNAs expression in patients with endometrial cancer. Int J Gynec Cancer (in press).

ABBREVIATIONS

C, Cytosine

MBD, methyl-CpG-binding domain proteins

Tfs, Trascription Factors

ACF, ATP-dependent chromatin assembly factor

5mC, methyl-modified cytosine

PCNA, proliferating cell nuclear antigen

RFTS, replication foci targeting sequence

HPLC, high performance liquid chromatography

PCR, Polymerase Chain Reaction

LUMA, luminometric methylation assay

WGBS, Whole genome bisulfite sequencing-based

HAT, histone acetyltransferase

HDAC, histone deacetylases

NO, Nitric Oxide

HL, Hodgkin's Lymphoma

KAT, lysine acetyltransferases

KDAC, lysine deacetylases

CBP, CREB-binding protein

PCAF, P300/CBP

PTM, histone post-translational modifications

MS, Mass spectrometry

ChiP, chromatin immunoprecipitation

RNA pol II, RNA polymerase II

RISC, RNA-induced silencing complex

ARGO, Argonaute

CRC, colorectal cancer

cfDNA, cell free DNA

WHO, World Health Organization

AJCC, American Joint Committee on Cancer

MSP, Methylation specific PCR

EGFR, epidermal growth factor receptor

WT, wild type

SE, sensitivity

SP, specificity

PPV, positive predictive value

NPV, negative predictive value

SEP9, septin 9

EOC, Epithelial Ovarian Cancer

CA125, Cancer Antigen 125

HE4, Human Epididymis Protein 4

WAP, Whey Acid Protein

miR, microRNA

VEGF, Vascular Endothelial Growth Factor

nf-kb, Nuclear Factor kappa-light-chain-enhancer of activated B cells

FIGO, International Federation of Gynecology and Obstetrics

CV, Coefficient of Variation

qPCR, Quantitative Polymerase Chain Reaction

cDNA, complementary DNA

Ct, Cycle threshold

AUC, Area Under the Curve

ROC, Receiver Operating Characteristic

IKKb, 3-Phosphoinositide-dependent Protein Kinase-1-mediated IκB Kinase β

EC, endometrial cancer

ABSTRACT

Several studies have shown that epigenetic mechanisms, as microRNAs (miRs) expression and gene methylation are involved in cancer, by regulating cell proliferation, apoptosis and angiogenesis. Accordingly, epigenetic changes occur from early stages and accumulate during cancer progression, by contributing to cancer development and progression.

Since tumour specific genetic and epigenetic alterations can be detected not only in cancer tissues but also in circulating serum or plasma cell-free DNA (cfDNA), this method is considered promising for improving non-invasive cancer detection and monitoring.

The present work includes three papers presenting genetic and epigenetic changes that could contribute to the identification of new non-invasive cancer biomarkers.

The aim of first work was to compare the status of *KRAS* mutation and *SEPT9* methylation between the primary tumors and matched plasma samples in patients affected by colorectal cancer (CRC).

KRAS mutations and *SEPT9* promoter methylation resulted present in 34.1% (29/85) and in 95.3% (81/85) of the primary tumour tissue samples. Patients with both genetic and epigenetic alterations in tissue specimens (31.8%, 27/85) were considered for further analyses on cfDNA. In 4 primary tumours with *KRAS* mutations, identical mutations were not observed in the corresponding plasma samples. The median methylation rate in tumour tissues and plasma samples was 64.5% (12.2-99.8%) and 14.5% (0-45.5%), respectively. The median *KRAS* mutation load (for matched mutations) was 33.6% (1.2-86%) in tissues and 4% (0-17%) in plasma samples. A statistically significant correlation was found between tissue and plasma *SEPT9* methylation rate ($r=0.41$, $p=0.035$), whereas no association was found between tissue and plasma *KRAS* mutation load ($r=0.09$, $p=0.65$).

These data show a discrepancy in epigenetic versus genetic alterations detectable in cfDNA as markers for tumour detection. Many factors could affect the mutant cfDNA analysis including the sensitivity of the detection method and the presence of tumour clonal heterogeneity.

The second line of research has focused on patients affected by epithelial ovarian cancer (EOC). Serum levels of miR-199a and miR-125b were found to be significantly higher in EOC patients compared to healthy controls ($p=0.007$ and $p=0.002$, respectively). A statistically significant correlation was found between miR-199a and miR-125b expression levels ($r=0.38$, $p=0.03$). The ROC curve analysis of the diagnostic performance on healthy controls and EOC patients revealed that HE4 had a significantly higher area under the curve (AUC: 0.90) when compared to CA125 (AUC: 0.85), miR-199a (AUC: 0.70) and miR-125b (AUC: 0.67). Despite the low specificity, mainly in pre-menopausal women, CA125 and HE4 seem to have better diagnostic performance compared to miRs investigated.

The third line of research has focused on investigate in human endometrial cancer (EC) the expression of miR-186, miR-222, miR-223 and miR-204.

Serum levels of miR-186, miR-222 and miR-223 resulted significantly up-regulated in patients compared to healthy controls ($p=0.004$, $p=0.002$ and $p<0.0001$). Contrarily, miR-204 resulted significantly down-regulated in EC patients compared to healthy controls ($p<0.0001$). A positive significant correlation was observed between miR-186 and both miR-222 ($r=0.71$, $p<0.0001$) and miR-223 ($r=0.64$, $p<0.0001$) as well also between miR-222 and miR-223 ($r=0.57$, $p<0.0001$). The AUCs for the selected miRs ranged from 0.70 to 0.87, significantly higher than for CA125 (0.59).

Our results confirm that these miRs are implicated in EC and hold promise as a novel blood-based biomarker for the diagnosis.

In conclusion, our results indicate that circulating nucleic acids are a potentially promising source of tumor-specific biomarkers in patients affected by different solid cancer. Accordingly, we have demonstrated that some circulating tumour-specific biomarkers can be detected at any time during the course of the disease and once detected indicate that a tumour is probably present.

The biggest challenge remains to standardize the methodologies including sample storage and DNA or miRs extraction to translate the quantitation of circulating epigenetic biomarkers into a clinical routine for cancer diagnosis and prognosis prediction.

RIASSUNTO

Recenti studi hanno dimostrato che cambiamenti epigenetici, quali ad esempio alterata espressione di microRNA (miR) o deregolazione nella metilazione di promotori, sono coinvolti nei processi di cancerogenesi in quanto eventi chiave nella regolazione del ciclo cellulare, nell'apoptosi o nell'angiogenesi.

Le alterazioni epigenetiche sono eventi precoci nello sviluppo del tumore e accompagnano l'intero processo di formazione neoplastica.

È possibile studiare le alterazioni epigenetiche non solo a livello tissutale, ma anche nei liquidi biologici quali ad esempio il sangue.

Lo studio di marcatori tumorali nel sangue può essere un potente strumento per studiare la dinamicità tumorale, tuttavia, ad oggi, poche molecole hanno una sensibilità e specificità tali da essere usate nella pratica clinica.

Il presente lavoro di tesi include tre studi volti ad analizzare i cambiamenti genetici ed epigenetici circolanti in pazienti affetti da tumori solidi, al fine di identificare nuovi biomarcatori diagnostici, misurabili in modo non invasivo.

Nel primo studio abbiamo analizzato lo stato di mutazione del gene *KRAS* e lo stato di metilazione del promotore del gene *SEPT9* in tessuto tumorale primario e in campioni di plasma ottenuti da pazienti affetti da cancro al colon-retto (CRC).

Le mutazioni di *KRAS* sono risultate presenti nel 34.1% (29/85) dei campioni tissutali, mentre l'alterato stato di metilazione del promotore del gene *SEPT9* nel 95.3% (81/85).

I pazienti che presentavano entrambe le alterazioni (31.8%, 27/85) nel tessuto, sono stati selezionati per analizzarne il plasma.

La mediana del tasso di metilazione nei tessuti tumorali e nei campioni di plasma è stata 64,5% (12,2-99,8%) e 14,5% (0-45,5%), rispettivamente.

La mediana del carico di mutazione di *KRAS* è stata del 33,6% (1,2-86%) nei tessuti e del 4% (0-17%) nei campioni di plasma. Una correlazione statisticamente significativa è stata osservata tra il tasso di metilazione di *SEPT9* nel tessuto ed il tasso di metilazione misurato nel plasma ($r=0.41$, $p=0.035$), mentre non è stata trovata alcuna associazione tra il carico mutazionale del gene *KRAS* in tessuto e plasma ($r=0.09$, $p=0.65$).

Questi dati mostrano una discrepanza nella rilevabilità delle alterazioni genetiche ed epigenetiche nel plasma. Molti fattori possono influenzare l'analisi del DNA circolante inclusa la sensibilità del metodo di rilevazione e la presenza di eterogeneità clonale della massa tumorale.

La seconda linea di ricerca ha riguardato il tumore epiteliale all'ovaio (EOC).

I livelli sierici di miR-199a e miR-125b sono risultati significativamente più elevati nelle pazienti con tumore rispetto ai controlli sani ($p=0.007$ e $p=0.002$, rispettivamente). Una correlazione statisticamente significativa, anche se marginalmente, è stata trovata tra i livelli di espressione del miR-199a e miR-125b ($r=0.38$, $p=0.03$). L'analisi della curva ROC, volta ad analizzare le performance diagnostiche dei miR studiati, ha rivelato che HE4 presenta un'area sotto la curva significativamente più alta (AUC: 0.90) rispetto a quella del CA125 (AUC: 0.85), del miR-199a (AUC: 0.70) e del miR-125b (AUC: 0.67).

Nonostante la bassa specificità, soprattutto nelle donne in pre-menopausa, CA125 e HE4 sembrano quindi avere una migliore performance diagnostica rispetto ai miR esaminati nel nostro studio.

La terza linea di ricerca ha riguardato lo studio dell'espressione di 4 miR (miR-186, miR-222, miR-223 e miR-204) nel siero di pazienti affette da tumore all'endometrio, al fine di analizzarne la performance diagnostica.

I livelli sierici di miR-186, miR-222 e miR-223 sono risultati significativamente più alti ($p=0.004$, $p=0.002$ e $p<0.0001$) e l'espressione di miR-204 è risultata significativamente più bassa nelle donne affette da neoplasia rispetto ai controlli sani ($p<0.0001$). È stata inoltre osservata una correlazione statisticamente significativa tra miR-186 e miR-222 ($r=0.71$, $p<0.0001$) e tra miR-186 e miR-223 ($r=0.64$, $p<0.0001$), così come tra miR-222 e miR-223 ($r=0.57$, $p<0.0001$). Le AUC per i miR selezionati sono risultate significativamente superiori a quella del CA125.

I nostri risultati confermano che questi miR sono implicati nella patogenesi del cancro all'endometrio e potrebbero essere utilizzati come marcatori di diagnosi precoce.

I lavori condotti dal nostro gruppo di ricerca in quest'ambito, indicano che gli acidi nucleici circolanti sono una fonte potenzialmente promettente di biomarcatori tumore-specifici in pazienti affetti da diverse forme di cancro.

Senza dubbio, il valore diagnostico di tali marcatori epigenetici, in pannelli multipli o in combinazione con biomarcatori tradizionali, potrebbe essere superiore a quello osservato per marcatori utilizzati singolarmente.

1. INTRODUCTION

1.1 Epigenetic and cancer

Cancer is a major public health problem in the United States and many other parts of the world. The overall estimate of 1,658,370 new cases is the equivalent of more than 4,500 new cancer diagnoses each day in 2015 (1).

Cancer is not a single disease, but a diverse group of conditions that all share in common an increase in cell numbers within particular tissues.

The development of colorectal cancer (CRC), for example, is a multistep process that involves an accumulation of mutations in tumor suppressor genes and oncogenes. It has provided a useful model for the understanding of the multistep process of carcinogenesis, characterized by the disruption of various cellular processes through the damage of their control mechanisms (2).

These are mainly faulty DNA repair system, dysfunctional cell cycle checkpoints leading to excessive cell proliferation, the failure of apoptosis, loss of contact inhibition, and cellular migration into other tissues to form distant metastases.

Hanahan et al. in 2000 (3) proposed that six hallmarks of cancer together constitute an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases. These six hallmarks were: sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, angiogenesis and resisting cell death.

Implicit in their discussion was the notion that as normal cells evolve progressively to a neoplastic state, they acquire a succession of these hallmark capabilities, and that the multistep process of human tumor pathogenesis could be rationalized by the need of incipient cancer cells to acquire the traits that enable them to become tumorigenic and ultimately malignant.

It has long been accepted that genetic alterations can cause cancer, however, throughout the last decades the importance of epigenetic changes in initiation and progression of cancer has been widely acknowledged. The genetic and epigenetic processes seem to be interconnected in driving the development of tumours.

The cancer stem cell hypothesis was first proposed 150 years ago. Cell surface marker expression analysis indicates that cells of tumours can be sorted into a major and a minor population, where the latter constitutes less than 1% of the cells in the tumour (4). The cells of the minor population display several abilities which resemble those of stem cells, for example self-renewal and differentiation, both crucial properties in driving malignancy. Self-renewal drives tumorigenesis, whereas differentiation contributes to the heterogeneity phenotype of the tumours. Because stem cells have an unlimited ability to proliferate, it is likely that the tumorigenic cancer stem cells are the drivers of multistep tumorigenesis (5).
However, accumulating evidence in the recent years indicate that tumor cell heterogeneity is in part due to significant contribution of 'epigenetic' alterations in cancer cells (6-8). Consequently, it is now becoming apparent that epigenetic plasticity together with genetic lesions drives tumor progression, and that cancer is the manifestation of both genetic and epigenetic modifications.

Genetic information of an organism is encoded in the DNA sequence. "Epigenetics" refers to the regulation of gene expression through certain chemical changes such as DNA methylation or histone modifications or the function of noncoding RNAs, without involving mutational changes in DNA sequence (9).
The term "epigenetics" was coined by Conrad Waddington in 1942 (10) and initially taken to describe the discipline in biology that studies "the interactions of genes with their environment that bring the phenotype into being" (11).
In 2009, a more complete definition proposed that "an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (12). In fact, all cells of a complex multicellular organism contain the same genetic information but during development, each single cell differentiates into a specific phenotype without any changes in DNA sequence. This feature of epigenetics implies that the accuracy of epigenetic modifications is vital for maintaining the genome integrity and the cell phenotype. Aberrant epigenetic modifications are associated with different heritable (for example imprinting disorders and some cancers) and non-heritable diseases (for example most cancer types). Indeed, epigenetics contributes to the understanding of mechanisms underlying different diseases for which genetic mutations are not the

only cause. Among the possible epigenetic modifications, DNA methylation, histone modifications and microRNAs (miRs) expression are the most intensively studied by epigenetic researchers for unravelling their role in gene expression regulation and their involvement in diseases.

Historically, the epigenetics was used to describe all biological phenomena that do not follow normal genetic rules. Nowadays, the field of epigenetics is considered as one of the most rapidly expanding fields of modern biology that has enormous influences on the understanding of biological phenomena and diseases, including cancer.

Recent work suggests that the global epigenetic changes in cancer may involve the dysregulation of hundreds of genes during tumorigenesis (13).

The mechanism by which a tumor cell accumulates such widespread epigenetic abnormalities during cancer development is still not fully understood. The selective advantage of these epimutations during tumor progression is possible, but it is unlikely that the multitude of epigenetic alterations that reside in a cancer epigenome occur in a random fashion and then accumulate inside the tumor due to clonal selection. A more plausible explanation would be that the accumulation of such global epigenomic abnormalities arises from initial alterations in the central epigenetic control machinery, which occur at a very early stage of neoplastic evolution. Such initiating events can predispose tumor cells to gain further epimutations during tumor progression in a fashion similar to accumulation of the genetic alterations that occurs following defects in DNA repair machinery in cancer. The “cancer stem cell” model suggests that the epigenetic changes, which occur in normal stem or progenitor cells, are the earliest events in cancer initiation (14).

Since epigenetic mechanisms are central to maintenance of stem cell identity (15), it is reasonable to speculate that their disruption may give rise to a high-risk aberrant progenitor cell population that can undergo transformation with gain of subsequent genetic mutations. Such epigenetic disruptions can lead to an overall increase in number of progenitor cells along with an increase in their ability to maintain their stem cell state, forming a high-risk substrate population that can readily become neoplastic on gain of additional genetic mutations.

Moreover, genetic and epigenetic mechanisms are not separate events in cancer; they can intertwine and take advantage of each other during tumorigenesis. Alteration in epigenetic mechanisms can lead to genetic mutations, and genetic mutations in epigenetic regulators lead to an altered epigenome (16).

1.2 DNA methylation

DNA methylation is a widely used epigenetic control mechanism in cells (17) and it is associated with the silencing of repetitive and centromeric sequences and transposable elements throughout the genome, as well as in genomic imprinting and X-chromosome inactivation (dosage compensation in human females) (18).

DNA methylation is involved in the control of genomic imprinting, which is an epigenetic form of gene regulation whereby a gene or genomic domain can be biochemically marked with information about its potential origin. Methylation of cytosine (C) in CpG repeat-rich elements is considered to be the one of the most important epigenetic traits in the regulation of transcriptional repression in mammals (19).

In normal tissue the methylation of particular subgroups of CpG island promoters can be detected. Impacts on gene transcription due to DNA methylation may occur in two different ways:

1. The binding of transcriptional proteins to the gene may be physically impeded by DNA methylation (20)
2. More importantly, methyl-CpG-binding domain proteins (*MBDs*) may bind to methylated DNA (21)

Jin et al. proposed a direct and simple mechanism for transcriptional regulation by DNA methylation. They showed in their work that altered affinity/stability between *Tfs* (Trascription Factors) and DNA elements caused by DNA methylation (particularly by non-CpG methylation) can serve as a direct source for fine tuning of gene expression (22).

DNA methylation is also influenced by histone modifications (23).

After *MDBs* binding, they are recruited more proteins to the locus such as histone deacetylases and other chromatin remodelling proteins. The ATP-dependent

chromatin assembly factor (*ACF*), for example, is a dimeric motor that spaces nucleosomes to promote formation of silent chromatin (24).

These proteins can also recruit histone modifying enzymes that alter nearby chromatin.

Aberrant patterns of DNA methylation may cause "incorrect" gene expression of certain genes, and in cancer, aberrant methylation, as well as both hypomethylation and hypermethylation, have been observed.

1.2.1 Molecular mechanism of methylation genes

The existence of methyl-modified cytosine (5mC) in nature was first discovered in 1925 by Johnson and Coghill, as a structural unit of nucleic acids isolated from tubercle bacillus, as anticipated by Wheeler and Johnson, the first to synthesize 5-methyl-cytosine in 1904. More than two decades after Johnson and Coghill's discovery, Wyatt showed that 5mC occurred in the nucleic acids of higher animals and plants (25). Cytosine methylation in vertebrates occurs predominantly at CG dinucleotide sequences (26), termed CpG sites. A family of proteins, known as the DNA methyltransferases (*DNMTs*), catalyzes the transfer of a methyl group from S-adenosylmethionine to a cytosine residue (27).

Four known *DNMT* proteins exist in mammals: *Dnmt1* (28), *Dnmt3A/B* (29), and *Dnmt3l* (30).

Work by Bessman et al. in 1958 characterizing the function of DNA polymerase showed that the enzyme cannot distinguish between the methylated and unmethylated cytosine nucleotide (31) prompting the possibility for the existence of a methyltransferase responsible for propagating 5mC through DNA replication.

Dnmt1 was originally reported by Bestor et al. (32). *Dnmt1* contains proliferating cell nuclear antigen (*PCNA*)-binding and replication foci targeting sequence (*RFTS*) domains, which are thought to contribute to maintenance methylation during replication. (33).

Dnmt1 also shows a preference for hemi-methylated DNA in which one strand is methylated, although it has de novo DNA methyltransferase activity (34). This set of features is the reason why Dnmt1 is often referred to as the 'maintenance methyltransferase'.

Dntm3a and *Dnmt3b* are responsible for the de novo methylation of unmethylated DNA (35) and have both overlapping and disparate DNA sequence affinities. Although somatic tissues show very little expression of *Dnmt3a* or *Dnmt3b*, *Dnmt3a* is ubiquitously expressed throughout the early embryo while Dnmt3b expression is specific to the forebrain and eyes. *Dnmt3b* isoforms can act as accessory proteins that interact with catalytically active enzymes to re-establish DNA methylation and could be one of many key factors for initiation of de novo DNA methylation during tumorigenesis (36), while Dnmt3a has been shown to methylate all CpGs regardless of genomic context (37).

Dnmt3a and *Dnmt3b* seem also to play a role in methylation maintenance. Early knockout studies showed that embryonic stem cells lacking *Dnmt3a* and *Dnmt3b* enzymes lose nearly all 5mC over progressive cell divisions, indicating *Dnmt1* is insufficient to fully maintain 5mC (38).

Dnmt3 has no active methylase domain but seems to play a role in ensuring proper methylation of imprinted loci and transposable elements through the interaction with *Dnmt3a* and *Dnmt3b*.

More recently, *Dnmt3b* together with *Dnmt3a* have been shown to be responsible for CpG and non-CpG methylation in oocytes (37)**.**

1.2.2 DNA methylation and cancer

The function of tumor suppressors is often lost in cancers, enabling uncontrolled cell proliferation, division and growth. In addition to alterations in the genome tumor suppressor genes can also be silenced by promoter DNA hypermethylation (39).

The majority of primary tumors show tissue-specific gains of methylation as compared to their normal counterparts (40).

However, the mechanism of cancer-associated hypermethylation remains unclear. One possibility is that aberrant CpG island hypermethylation in cancer occurs through the interaction of increased *DNMTs* and oncogenic transcription factors (41-42). An analysis comparing normal and cancer cell lines also showed that the presence of stalled or active RNA polymerase in normal cells predicts resistance to aberrant hypermethylation in cancer cells (43).

Thirty years ago, the first epigenetic change observed in cancer was loss of pattern methylation.

Hypermethylation in cancer has been suggested to occur in hight frequency at the CPG islands in the promotors regions (44).

Another epigenetic process was global DNA hypomethylation.

Hypomethylation is a process that reduces the methylation level of proto-oncogenes in the cell. Some studies have found that, in a large variety of hypomethylated tumour samples, the changes in the cell are not only correlated with altered methylation patterns but also with increased tumour progression (45).

Global DNA hypomethylation has been detected in various types of cancer such as breast (46), colorectal (47), ovarian (48).

Hypomethylation has been located to the intergenic region associated with higher expression of repetitive DNA sequences (49).

Consequently, particular DNA hypomethylations are linked to cancer initiation. Tumour cells also exhibit hypomethylation of CpG dinucleotides in various DNA regions that are responsible for increased gene expression, invasion and metastasis of cancer cells.

1.2.3 Methods for DNA methylation analysis

The appropriate approach for analysis of DNA methylation depends upon the goals of the study.

The earliest studies on DNA methylation were aimed at determining the overall levels of 5-methylcytosines in the genome by hydrolysing DNA chemically and quantifying the hydrolysed products by high performance liquid chromatography (HPLC) (50).

DNA hydrolysis can be carried out by incubation with formic acid at high temperature.

However, Catania et al. (51) suggested the use of hydrofluoric acid for chemical hydrolysis of DNA to prevent deamination of cytosine and methylcytosine, which often occurs with formic acid.

In any case, enzymatic hydrolysis of the DNA is reported to be a better alternative for quantifying the degree of DNA methylation (52).

Resulting deoxyribonucleosides are subsequently separated by HPLC, and the methylcytosine levels are quantified by comparing the relative absorbance of cytosine and methylcytosine at 254 nm in the sample with external standards of known bases.

A major advance in DNA methylation analysis was the development of a method for sodium bisulfite modification of DNA to convert unmethylated cytosines to uracil, leaving methylated cytosines unchanged.

This allows one to distinguish methylated from unmethylated DNA via PCR amplification and analysis of the PCR products. During PCR amplification, unmethylated cytosines amplify as thymine and methylated cytosines amplify as cytosine.

Methylation-specific PCR (53), is one of the most effective choices for investigating the methylation profile of these regions.

It is an application of bisulfite sequencing method. For a sequence in a gene containing CpGs, the allele on which those CpGs are methylated and another on which those CpGs are unmethylated should give different sequences after bisulfite modification.

When a primers set that are complementary to the sequence with methylated CpGs, but are not complementary to the originally same sequence with unmethylated CpGs, is used for PCR, only the sequence (allele) with methylated CpGs should be amplified. The same is true for the primer pair specific for sequence with unmethylated CpGs.

An other assay is the LUMA (luminometric methylation assay) technique, published by Karimi and colleagues in 2006 (54). It utilizes a combination of two DNA restriction digest reactions performed in parallel and subsequent pyrosequencing reactions to fill-in the protruding ends of the digested DNA strands. One digestion reaction is performed with the CpG methylation-sensitive enzyme HpaII; while the parallel reaction uses the methylation-insensitive enzyme MspI, which will cut at all CCGG sites.

The enzyme EcoRI is included in both reactions as an internal control. Both MspI and HpaII generate 5'-CG overhangs after DNA cleavage, whereas EcoRI produces 5'-AATT overhangs, which are then filled in with the subsequent

pyrosequencing-based extension assay. Essentially, the measured light signal calculated as the HpaII/MspI ratio is proportional to the amount of unmethylated DNA present in the sample. As the sequence of nucleotides that are added in pyrosequencing reaction is known, the specificity of the method is very high and the variability is low, which is essential for the detection of small changes in global methylation. LUMA requires only a relatively small amount of DNA (250–500 ng), demonstrates little variability and has the benefit of an internal control to account for variability in the amount of DNA input. However, high quality DNA is essential to ensure that complete enzymatic digestion occurs, and the polymerase extension assay requires a pyrosequencing machine and reagents.

WGBS (BS-seq; MethylC-seq) theoretically covers all the C information (55). In this method, genomic DNA is purified and sheared into fragments. The fragmented DNAs are end-repaired; adenine bases are added to the 3 end (A-tailing) of the DNA fragments, and methylated adapters are ligated to the DNA fragments (56). The DNA fragments are size-selected before sodium bisulfite treatment and PCR amplification, and the resulting library is sequenced. It should be noted that a high number of PCR cycles and inappropriate selection of a uracil-insensitive DNA polymerase may result in an over-representation in the methylated DNA data (57). Starting with sufficient genomic DNA may avoid a loss of information from regions of interest and over-amplification.

The major advantage of WGBS is its ability to assess the methylation state of nearly every CpG site, including low- CpG-density regions, such as intergenic ‘gene deserts’, partially methylated domains and distal regulatory elements. It can also determine absolute DNA methylation level and reveal methylation sequence context.

1.3 Histone modifications

Chromatin is the higher order of organization of genomic information. Nucleosome constitutes its basic unit, which is composed by a histone octamic protein core.

The structure of the nucleosome core is relatively invariant from yeast to metazoans (58) and includes a 147 bp segment of DNA and two copies each of four core histone proteins.

Chromatin proteins function as building blocks to package eukaryotic DNA into higher order chromatin fibers. Each nucleosome encompasses a tetramer of 2 histone 2A (H2A) and 2 histone 2B (H2B) molecules, flanked by H3 and H4 dimers. The histone proteins coordinate the changes between tightly packed DNA (heterochromatin) that is inaccessible to transcription and exposed DNA (euchromatin) that is available for binding to transcription and regulation of transcription factors (59).

Histone proteins contain a globular C-terminal domain and an unstructured N-terminal tail.

The N-terminal tails can undergo a variety of post-translational covalent modifications including acetylation, methylation, phosphorylation, ubiquitination and ADP ribosylation on specific residues, the most studied of which are acetylation and methylation of specific lysine residues on histones H3 and H4 (60).

About 30 short chains of amino acids protrude from the histones. These ‘histone tails` are subjected to various post□translational modifications, which form a ‘histone code`. By defining the accessibility of the transcription machinery to genes and gating the accessibility of the genome to other machineries, such as repair and DNA replication, the ‘histone code` regulates chromatin function and thus determines gene expression patterns. Within the histone tails, lysine (K) and arginine (R) residues are the major sites of modifications. Reversible acetylation and methylation of the basic side chains of these amino acids are common (61).

Histone acetylation was the first histone modification described and hyperacetylated histones were early associated with open chromatin formation and transcriptional activation (62).

Histone tails have a large number of lysine residues, which act as target for histone acetyltransferase (*HATs*) and histone deacetylases (*HDACs*). Both hyper and hypo histone acetylation of individual lysine, are associated with

trascriptional state. Histone acetylation can effected transcription by causing a confirmation change of nucleosome core, allowing a greater accessibility of DNA. The attachment of acetyl groups to lysine residues goes along with two functional consequences. First, the positive charge of physiologically protonated ε-amino groups is abolished, resulting in altered electrostatic as well as steric properties of the affected protein region. Second, acetylation serves as a mark for distinct "reader" domains, which comprise specialized tertiary structures (e.g., bromodomains) in proteins that undergo a selective interaction with acetylated lysines (63).

Upon acetylation, local affinity of the modified histone protein to negatively charged DNA is decreased, resulting in a less condensed chromatin structure and in exposure of promoter sites. As a consequence of the increased accessibility, the DNA globally becomes more prone to access of the transcriptional machinery (64).

On the other hand, acetylation of histones can attract proteins to elongated chromatin that has been marked by acetyl groups histone modifications serving as a signalling platform to recruit or occlude effector proteins. (65). These factors specifically recognize modifications via unique domains. They possess enzymatic activities such as remodelling ATPases and following their binding they can further modify chromatin.

1.3.1 Histone acetylation and diseases

Acetylated histones represent a type of epigenetic marker within chromatin. Modifications of histones cannot only cause secondary structural changes at their specific points but can also cause structural changes in distant locations which can also affect function.

Aberrant acetylation levels have been connected with a diversity of disease phenotypes including cancer, neurological disorders, and cardiovascular and metabolic malignancies (66-68).

Dysfunction of *HDAC* enzymes has been linked with a variety of human diseases, because the reversal of acetylation by *HDACs* correlates with transcriptional repression. *HDACs* can regulate diverse cellular functions, including cell cycle

progression, survival, proliferation (69-70) and also changes in histone acetylation have been reported to associate with inflammation (71).

For example Nitric Oxide (*NO*) plays an important role in a variety of physiologic and pathophysiologic processes in multiple tissues, as inflammation process. NO is a dual regulator of inflammation, contributing to vasodilation and cell activation as well as to processes involved in the resolution of inflammation.

The interaction of *HDAC* isoforms and *NF-kB* proteins to alter acetylation provides the potential to fine-tune the expression of iNOS and other downstream target genes contributing to inflammatory responses (72).

Because these are ubiquitously expressed and involved in cell proliferation and survival, aberrations in their gene expression have been implicated in a wide range of cancers. *HDAC1–HDAC3* genes are over-expressed in ovarian cancer tissues and probably have a significant role in ovarian carcinogenesis (73); these *HDAC* isoforms are also highly expressed in Hodgkin's Lymphoma (HL). However, decreased *HDAC1* expression is accompanied by worse outcome in HL (74). Over-expression of *HDAC1* has also been reported in prostate and gastric cancers (75), while contrastingly, under-expression was reported in colorectal cancer (76).

Because of the broad acceptance of substrates, histone acetyltransferases and deacetylases are hence often referred to as lysine acetyltransferases (*KATs*) and lysine deacetylases (*KDACs*), respectively (77).

Lysine acetylation of histone and non-histone proteins are generally linked to activation of transcriptional activity and therefore affects pivotal physiological processes within an organism. As a consequence of misregulated acetylase activity, the manifestation and progression of certain malignancy phenotypes correlates with pathological aberrations of the acetylation equilibrium. This could be either due to altered activity of the responsible enzymes or because of changes in their expression levels. The role of distinct *KAT* subtypes in diseases like cancer, neurodegenerative disorders, viral and parasitic infections, inflammation, and metabolic and cardiovascular malignancies have been extensively investigated (78-79).

CREB-binding protein (CBP) and *p300* are highly conserved and functionally related transcription coactivators and histone/protein acetyltransferases and involved in multiple cellular processes.

In human cancer, spontaneously occurring mutations in the P300 gene, that acts as a crucial element in the eukaryotic gene regulation network was shown to be regulated by phosphorylation, which greatly reduces its *HAT* activity (80-81).

This reinforce the idea that indicate *P300/CBP (PCAF)* activity can be under abnormal control in human disease**,** particularly in cancer, which may inactivate a p300/CBP tumor-suppressor-like activity (82).

p300 and *CBP* seem to have a dual role in oncogenesis; they can be either friends or foes. On the one hand, genetic studies show that they can act as tumor suppressors, as *CBP* and *p300*-null chimeric mice develop hematological malignancies. (83-84).

In addition to histone proteins, *HDACs* have many non-histone protein substrates, including *p53* and *STAT*, which are important transcription factors regulating the expression of a large number of genes (85).

P53 acts as a tumor suppressor protein by inhibiting cell cycle progression and S-phase entry. Several reports have been shown that acetylation of the C-terminal regulatory domain is involved in regulating activity of *p53* (86).

Acetylation of this site is observed after DNA damage *in vivo*, induced *p53* and caused cell cycle arrest or apoptosis; therefore, over expression of *PCAF* can cause growth arrest (87).

1.3.2 Methods for histone acetylation analysis

Antibody-based techniques such as western blotting have been extensively adopted to characterize histones. However, antibody-based approaches are limited for the following reasons: I) they can only confirm the presence of a modification and cannot identify unknown histone post-translational modifications (*PTMs*); II) they are biased due to the presence of co-existing marks, which can influence binding affinity; III) they cannot identify combinatorial marks, as only very few antibodies are available for such purpose and IV) they cross-react between highly similar histone variants or similar *PTMs* (*e.g.*, di- and trimethylation of lysine

residues). Egelhofer et al. described that more than 25% of commercial antibodies fail specificity tests by dot blot or western blot, and among specific antibodies more than 20% fail in chromatin immunoprecipitation experiments (88).

Mass spectrometry (MS) is currently the most suitable analytical tool to study novel and/or combinatorial *PTMs*, and it has been extensively implemented for histone proteins (89).

This is mostly due to high sensitivity and mass accuracy of MS, and the possibility to perform large-scale analyses.

A different method to analyze the chromatin is the chromatin immunoprecipitation (ChIP). This involves crosslinking of the protein–DNA complex within an intact cell using crosslinking agents, such as formaldehyde. The DNA is then sheared to smaller pieces (□500 bp) by sonication or nuclease digestion. The sheared protein-bound DNA is then immunoprecipitated using a highly specific Ab against the protein. An aliquot of the sheared DNA before immunoprecipitation is used as a reference sample. The protein–DNA complexes from reference and ChIP samples are then reverse crosslinked. The DNA is purified and enrichment of ChIP-ed DNA over the reference sample can be analyzed using a number of techniques, such as quantitative PCR, sequencing or microarray (90). Although ChIP has been widely used in other model systems, there are only a few labs that have successfully used ChIP in Caenorhabditis elegans (91).

1.4 MicroRNAs

MicroRNAs (miRNAs) are endogenous, non-coding RNAs that are ~22 nucleotides in length. MiRNAs control gene expression by binding to target mRNAs leading to their degradation or repression of translation (92).

MiRNA biogenesis begins in the nucleus where they are transcribed by *RNA polymerase II* (*RNA Pol II*) as long transcripts known as primary miRNA (pri-miRNA) (93). Like mRNAs, pri-miRNAs are poly-adenylated at the 3′ end and carry a 7-methyl-guanosine cap at the 5′ end (94).

Pri-miRNA is cleaved by the *RNase III enzyme Drosha* into a shorter (<100 bp) transcript known as the precursor miRNA (pre-miRNA) composed of a stem-loop structure which encodes the mature miRNA sequence in the stem (95).

The export receptor Exportin-5 (Exp5) binds to pre-miRNA and exports it from the nucleus into the cytoplasm (96). Once in the cytoplasm, the terminal base pairs and loop of the pre-miRNA are cleaved off by another *RNase III enzyme, Dicer*. This cleavage leaves a small double stranded RNA referred to as the miRNA:miRNA* duplex (97).

One strand of this duplex forms the mature miRNA and is incorporated into the RNA-induced silencing complex (*RISC*). The *RISC* is a ribonucleoprotein that contains the mature miRNA and an Argonaute (*Argo*) protein which is believed to be responsible for translational repression (98).

The resultant complex between mature miRNA and RISC is denominated miRSC. In mammals, selection of the guide strand is dictated by thermodynamic stability, the less stable strand at the 5´ end has more probability of being incorporated into the *RISC*; the remaining strand (miRNA*-passenger strand) is excluded and generally degraded (99).

However, miRNA sequencing data, demonstrate that a large number of miRNA* are not degraded and are expressed in similar concentrations to their corresponding guide strand (100).

Once the mature miRNA is incorporated into the *RISC* this complex inhibits the translation of target mRNA through 1) mRNA cleavage 2) mRNA destabilization or 3) translational repression (101).

Identification of miRNA targets has been difficult because only the seed sequence (about 6–8 bases) of the approximately 22 nucleotides aligns perfectly with the target mRNA's 3 untranslated region (102).

Bioinformatics approaches can identified putative targets for particular miRNAs through analysis of the miRNA seed sequences, (103) however, these miRNAs need to be assayed in vitro or in vivo to determine if they truly affect the proposed mRNA. Once a sequence has been determined to be a unique miRNA, the miRBase registry assigns a name according to existing guidelines (104).

In the database, a sequence of 3 or 4 letters designates the species (e.g., “hsa” for Homo sapiens); however, this prefix is usually dropped in the literature. The core of the miRNA name is the designation “miR” (denoting a mature sequence) followed by a sequentially assigned unique identifying number. Lettered suffixes are added to miRs that differ by only 1 or 2 bases (e.g., miR-10b), and numbered suffixes are assigned to miRs that have the same sequence but are derived from different primary transcripts. A suffix of 5p or 3p is given when mature miRNAs are derived from the 5 arm or the 3 arm, respectively, of the precursor miRNA.

1.4.1 MicroRNAs and cancer

Epigenetic profiling of miRNAs has revealed new insights into the altered epigenetic regulation of these molecules in diseases, including cancer.

Therefore, it is not surprising that studies have found miRNAs to be involved in many cellular processes including development, cell proliferation, apoptosis, fat metabolism, and cell differentiation (105).

Cancer in particular has been a major focus of microRNA research over the past decade, and many studies have demonstrated the importance of microRNAs in cancer biology through controlling expression of their target mRNAs to facilitate tumor growth, invasion, angiogenesis, and immune evasion (106).

For almost three decades, carcinogenesis has been primarily attributed to abnormalities in oncogenes and tumor-suppressing genes. It is now recognized that miRNA also have a primary role in cancer onset and progression. Oncomir is the term used to describe an miRNA involved in cancer.

With such widespread regulatory functions in gene expression and key roles in cancer associated cellular processes, the roles of miRNAs in cancer are now being extensively explored.

Abnormal miRNA expression in malignant cells compared with normal cells are often attributed to alterations in genomic miRNA copy numbers and gene locations (amplification, deletion or translocation).

The earliest discovery of miRNA gene location change is the loss of *miR-15a/16-1* cluster gene at chromosome 13q14, which is frequently observed in B-cell chronic lymphocytic leukemia patients (107).

This microRNA deletion was shown to act at least in part through allowing higher expression of the *miR-15/16* anti-apoptotic target B-cell lymphoma 2 (*BCL2*). Since then it has been documented that microRNAs have roles in all of the cancer hall-marks defined by Hanahan and Weinberg (108), and are implicated in the clinical management of cancers at every stage.

Although many specific examples have been reported, microRNA functions fall into two broad major functional categories: (i) homeostatic regulation of gene expression, through 'fine-tuning' of translation according to cellular requirements; and (ii) robustness in cellular responses, which is important in cell fate decisions in which groups of microRNAs can dictate the cellular differentiation state, acting as 'locks' to maintain cell identity, often via complex reciprocal negative-feedback loops (109).

MiRNAs discovery led to a worldwide research effort to establish their roles in cancer. MiRNAs regulate molecular pathways in cancer by targeting various oncogenes and tumour suppressors, and have a role in cancer and stem cell biology, angiogenesis, the epithelial–mesenchymal transition, metastasis, and drug resistance. For example the *let-7* miRNA family has a role in cancer by negatively regulating *RAS* (110).

O'Donnell et al. (111) discovered that *c-Myc*, frequently upregulated in many malignancies to regulate cell proliferation and apoptosis, activates the transcription of oncogenic miR-17–92 cluster through binding to E-box elements in *miR-17–92* promoter. Consistent with its oncogenic role, c-Myc also represses transcriptional activity of tumor suppressive miRNAs such as *mir-15a, miR-26, miR-29, mir-30* and *let-7* families.

In recent years, studies on miRNAs, especially on a large scale using microarrays, have provided a more comprehensive picture on the role of abnormal miRNA expression in neoplasia (112).

Recently, a number of onco-miRNAs such as miR-9, miR-155, and miR-21 have been shown to be implicated in cancer therapeutic response, inducing chemoresistance by modulating the expression of resistance-associated genes (113-114).

Wang et al. has identified in plasma, microRNA profiles (*miR-21*, *miR-27a*, and *miR-218*) for primary resistance to *EGFR-TKIs* in advanced lung cancer with EGFR activating mutation (115).
Further studies are demanded in order to use microRNA profiles as diagnostic markers and conduct microRNA-based therapies in clinical practice.

1.4.2 Methods for analysis of miRNAs

The first step in qPCR of miRNAs is the accurate and complete conversion of RNA into complementary DNA (cDNA) by reverse transcription. However, this step is challenging as:

i. the template has a limited length (22 nt);
ii. there is no common sequence feature to use for the enrichment and amplification of miRNAs;
iii. the mature miRNA sequence is present in pre- and the pri-miRNAs.

A range of techniques have been developed to overcome the challenges of miRNA profiling, as qPCR assay, miRNA array, RNA-seq.

Quantitative PCR miRNA assay

One of the most popular techniques for validating and accurately quantifying miRNAs is quantitative real time PCR (qPCR). As well as being sensitive and quantitative, qPCR is also relatively inexpensive and flexible making it the preferred choice for validating novel miRNAs and for use in relatively small experiments.
This technique begins with the conversion of miRNA to cDNA. With the length of a miRNA being comparable to that of a typical DNA primer, cDNA synthesis from miRNAs presents its own challenges. The solution to this is to make the molecule longer, either by incorporating a poly(A) tail or stem-loop structure.
In poly(A) RT-qPCR, total RNAs, including miRNAs, are initially polyadenylated and reverse transcribed using poly(T) adapters into cDNAs, which are called poly(A) RT, and qPCR is subsequently performed using an miRNA-specific forward primer and a partial sequence in the poly(T) adapter as the reverse primer.

Once miRNA has been converted to cDNA it can be assayed using the same approach as a conventional qPCR experiment. Amplification is initiated with an miRNA-specific primer and a stem-loop/poly(A) primer. Either SYBR®Green or a TaqMan® probe is used to detect the amplified product.

MiRNA array

Arrays are typically chosen for larger studies covering multiple miRNA targets. While they are the least quantitative of the three miRNA assay methods, conventional DNA oligonucleotide arrays are a relatively inexpensive way to measure hundreds of targets at once.

Thousands of probes can be easily spotted on slides, or built up by photolithography, potentially enabling the parallel tracking of all known miRNAs. Arrays are probed by hybridizing fluorescently labelled DNA or RNA samples. The brightness of individual spots can be used to infer relative changes in expression between samples.

RNA-seq

This method of miRNA quantification uses the high-throughput capability of next-generation sequencing (NGS) platforms. While it cannot quantify miRNA levels with the molar resolution of qPCR, deep sequencing of miRNA does have the advantage of being able to sample all miRNAs present in a sample, whether the researcher knows the sequence or not, making it an ideal discovery tool. Furthermore, as sequences are read directly, RNA-seq can distinguish closely related miRNAs and isoforms

Currently, quantitative PCR (qPCR) is the favoured method for determining miRNA expression, due to its accuracy, simplicity, reproducibility and lower cost than other hybridization or sequencing-based technologies.

1.5 Circulating nucleic acids

Capturing and analysis of circulating biomarkers is an alternative method to gain insight into the molecular make-up of a cancer in a given patient.

Many cancers may remain asymptomatic until relatively late stages.

In managing the disease, effort ought to be focused on early detection, accurate prediction of disease progression, and frequent monitoring. The current gold standard of cancer diagnosis is based on histology evaluation of tissue biopsies.

Tumor cells release DNA or RNA into the blood, and this offers the opportunity to determine the genetic landscapes of solid cancer from the circulation, an approach commonly called 'liquid biopsy".

Alterations in epigenetic profiling may provide important insights into the aetiology and natural history of cancer. Since several epigenetic changes occur prior to histopathological changes (116), they can serve as biomarkers for cancer diagnosis and risk assessment.

The emergence of advanced technologies to detect genome-wide epigenetic changes holds promise to advance our capacity to develop such biomarkers for detecting cancers at early stage.

In solid tumours, circulating biomarker scan be released into bloodstream through various events including necrosis, apoptosis, and other physiological mechanisms in thestromal microenvironment. Therefore, 'liquid biopsies' can capture spatial and temporal heterogeneity during tumour formation and evolution. Epigenetic aberrations offer dynamic and reversible targets for cancer therapy. Increasingly, alterations via over expression, mutation, or rearrangement are found for genes that control the epigenome (117).

The history of circulating nucleic acids goes back to a finding in 1947 by Mandel and Metais of RNA and DNA in the plasma of healthy and sick individuals (118).

The presence of DNA and RNA in plasma of cancer patients has been recognised since the 1970s (119).

But it was not until the late 1980 that this circulating DNA was shown to exhibit tumour related alterations (120).

During the 1990, a large number of tumour associated genetic and epigenetic changes were detected in the plasma/serum of cancer patients: Ras and p53

mutations, microsatellite alterations, aberrant promoter hypermethylation of several genes, rearranged immunoglobulin heavy chain DNA, mitochondrial DNA mutations, and tumour related viral DNA (121-122).

In general, concentrations of cfDNAs are higher in individuals with cancer than in healthy controls, and levels are further increased in metastasis. Both tumor-derived and normal germline cfNAs are released into the blood, and the proportion of tumor-derived cfNA is broadly related to the extent of the disease (123).

In 2007 Lawrie et al., reported the presence of miRNAs in the blood of lymphoma patients (124); the following year, it was demonstrated that miRNAs could be useful as non-invasive biomarkers of cancer (125).

The presence of DNA and RNA in exosomes, vesicles that are actively released by multiple cell types (including neoplastic cells), is also well documented. (126-128).

Indeed intercommunication between cancer cells and with their surrounding and distant environments is key to the survival, progression and metastasis of the tumour. Exosomes play a role in this communication process (129).

MicroRNA expression is frequently dysregulated in tumour cells and can be reflected by distinct exosomal miRNA (ex-miRNA) profiles isolated from the bodily fluids of cancer patients.

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2. AIMS

The knowledge of the fundamental epigenetic mechanisms governing gene expression and cellular phenotype is sufficiently advanced that novel insights into the epigenetic control of cancer disease are now emerging. Researchers are in the process of shedding light on the roles played by DNA methylation, histone/chromatin modifications and non-coding RNAs in specific pathologies. The development of new early detection techniques is essential to improve the outcome of patients with cancer. Blood-based biomarkers offer promising means of non-invasive detection however few molecules have been found that have a sensitivity and specificity high enough to be used in standard clinical practice. For example the finding that miRs are stably expressed in human plasma and serum and that they are differentially expressed in patients with cancer suggests the utility of these molecules as biomarkers not only for early detection, but also as means of monitoring disease progression and recurrence. However, further research into the factors that influence circulating miRNA expression and quantification is required before the full potential of these molecules as cancer biomarkers can be achieved.

The aims of this thesis were:

1) to analyse the status of the two best characterized CRC genetic and epigenetic (*KRAS* mutations and *SEPT9* methylation) alterations in a cohort of CRC patients, and to compare the degree to which the two patterns move from tissue to plasma in order to improve our understanding of biology modulating the concordance between tissues and plasma methylation and mutation profiles;
2) to investigate serum expression levels of miR-199a and miR-125b in ovarian cancer patients in comparison to healthy controls, and to evaluate the correlation between miRs expression and traditional biomarkers serum concentrations (i.e., CA125 and HE4);
3) to investigate the differential expression of four serum miRs (miR-222, miR-223, miR-186 and miR-204) in EC patients in comparison to healthy subjects.

3. PAPERS

3.1 Comparison of genetic and epigenetic alterations of primary tumors and matched plasma samples in patients with colorectal cancer

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Introduction

The finding that tumor specific genetic and epigenetic alterations can be detected in circulating DNA extracted from plasma of cancer patients has shown promise for improving early diagnosis, prognostication and disease monitoring. The overarching goal of utilizing cell free DNA as a biomarker entails medical practice optimization, personalized medicine development, and quality of life improvement due to the minimal invasiveness of blood testing. However, there is still a challenge to authenticate the actual clinical validity of various cfDNA alterations as putative cancer biomarkers in clinical practice (1). Currently, the main issue is represented by the fact that circulating DNA fragments carrying tumor specific alterations represent a variable and generally small fraction of the total circulating DNA, thus generating a high variability in concordance rate between alteration patterns detectable in tissue of primary tumors and corresponding plasma.

The factors influencing the quantitative as well as qualitative changes of cfDNA with respect to tissues of cancer patients are multiple and not yet fully explored so far. However, efforts during the last decade have led to important advances.
By evaluating the methylation pattern of the *PCDH10* gene in tissue and plasma of patients with CRC we have recently demonstrated that the methylation rate detected in plasma increased with increasing methylation rate in tumour tissues only in early-stage cancers, whereas this correlation was apparently lost in advanced cancers. Moreover, we showed that the degree of cfDNA methylation was associated with some characteristics of cfDNA, such as its concentration and

integrity, and that these correlations varied in strength and direction in parallel with tumour stage (2).

In the last two years two independent research groups showed that the possibility to detect tumor specific cfDNA in plasma of CRC patients largely depends on the sensitivity of the PCR-based method for short mutated sequences (3-5), thus emphasizing the importance of minimizing the assay length when analyzing highly fragmented cfDNA, such as in the setting of cancer patients.

A further issue which complicates the use of cfDNA as liquid biopsy for cancer is the intratumoral heterogeneity and clonal evolution during progression, both factors resulting in differences in the proportion and pattern of aberrations detectable in the primary tumor and in the circulating DNA (6,7).

According to this evidence, different technical and biological aspects should be considered when analysing the variable concordance between tissue and plasma alterations in cancer patients, not least the nature of the underlying alterations.

Both epigenetic and genetic alterations are well known aberrations involved in colorectal carcinogenesis. Given their enormous potential as biomarkers in CRC diagnosis, staging, prognosis and response to treatment, they have been extensively investigated in the last decade. However, a critical comparison of their status in tissue and cfDNA is lacking. Therefore, this study was aimed to analyze the status of the two best characterized genetic and epigenetic alterations of CRC (i.e., *KRAS* mutation and *SEPT9* promoter methylation) in a cohort of CRC patients, in order to improve our understanding of the biological aspects modulating the concordance between tissues and plasma methylation and mutation profiles. Then, we also compared the degree at which the genetic and the epigenetic patterns move from tissue to plasma.

Material and Methods

Patients and Samples

The study cohort included 85 consecutive patients undergoing surgery for CRC at the University Hospital of Verona (Italy) between January 2010 and December 2010. Blood specimens were collected before intervention. Tumor samples were

obtained during the surgical procedure, immediately frozen in liquid nitrogen and stored at -80°C. Histological diagnosis and tumor stage were determined according to the 2000 World Health Organization (WHO) classification system for tumors of the digestive system and the American Joint Committee on Cancer (AJCC) staging system, respectively (8). Only patients with primary colorectal adenocarcinomas untreated with neoadjuvant radio-chemotherapy were included in the study. All subjects provided informed consent prior to collection of samples. The study was approved by the local ethical committee (Department of Life and Reproductive Sciences, University of Verona) and performed in accord with the Helsinki Declaration of 1975. Clinical information was obtained from medical records.

DNA isolation from plasma and tissue samples

Blood samples were collected in 7 mL EDTA tubes and processed within 1 h of collection. After double centrifugation (800g for 10 min centrifugation, followed by separation and a second 1600g for 10 min centrifugation) plasma was separated, stored in aliquots and frozen at –80°C until processing. DNA was extracted from plasma and fresh frozen tissue sections by the QIAamp DNA Blood midi kit and the Gentra Purgene Kit (Qiagen, Hilden, Germany) respectively.

cfDNA concentration and Integrity index

cfDNA fragmentation was assessed by calculating the DNA Integrity index as previously described (2). In brief, it was determined by calculating the ratio of larger (247 bp) versus shorter (115 bp) targets of the consensus sequence of human ALU repeats.

The ALU-qPCR result obtained with ALU115 primers was also used to quantify total DNA.

Methylation specific PCR (MSP)

Purified genomic DNA extracted from tissues and plasma was subjected to bisulfite treatment and DNA purification using the Epitect Bisulfite kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A detailed protocol has been previously reported elsewhere (2).

Bisulfite-modified DNA was used as template for Real-Time PCR using a Sybr green-based quantitative MSP. Primers for MSP were designed to specifically amplify either a bisulfite-sensitive, unmethylated strand or a bisulfite-resistant, methylated strand on the SEPT9 gene promoter region. The web-based software MethPrimer (http://itsa.ucsf.edu/urolab/MethPrimer) was used to select a specific CpG island, which was recently found to display the highest susceptibility to methylation changes in the adenoma-carcinoma sequence (9).

The sequences of the primer sets were as follows:

M-Fo: TTATTATGTCGGATTTCGCGGTTAAC

M-Rev: AAAATCCTCTCCAACACGTCCG

U- Fo: TAGTTATTATGTTGGATTTTGTGGTTAATG

U- Re: CAAAATCCTCTCCAACACATCCAC

(M: methylated, U: unmethylated).

The CpGenome Universal Methylated DNA (Chemicon, Millipore Billerica, MA, USA) was used as 100% methylated (positive) control and DNA extracted from peripheral blood mononuclear cells of normal individuals was used as unmethylated (negative) control.

The PCR reaction mixture was prepared in a final volume of 20 µl, consisting of final concentration: 0.375 µM of forward and reverse primers, 250 µM of each dNTP (GE Healthcare, Little Chalfont, UK), 1× HotStart Buffer (Qiagen), 2.5 mM MgCl2, 1.5 units HotStart polymerase (Qiagen), 2 µM SYTO 9 (Invitrogen, Life Technologies, Carlsbad, CA), and 1×ROX reference dye (Invitrogen), 3 µl of bisulfite-modified DNA.

The PCR amplification was performed with precycling heat activation of DNA polymerase at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 64 °C for 30 sec and extension at 72 °C for 30 sec. An ABI Prism 7500 Sequence Detection System (Applied Biosystems – Foster City, CA, USA) was used.

The PCR product was run on an 2% agarose gel to confirm product size and the specificity of PCR, and then visualised under UV light. A band of 110 bp was considered as diagnostic of methylation status, whereas a band of 114 bp was considered as diagnostic of unmethylation status.

KRAS mutation analysis

DNA extracted from tissue and plasma samples was subjected to an allele refractory mutation system qPCR (ARMS-qPCR) for detection of six of the most common mutations in codons 12 and 13 of the *KRAS* gene (G12A, G12D, G12V, G12S, G12C, and G13A). DNA was amplified in a 25 µl reaction mixture containing 0.25 µM of each amplification primer, 200 µM of each dNTP (GE Healthcare, Little Chalfont, UK), 1× HotStart Buffer (Qiagen, Hilden, Germany), 2 mM $MgCl_2$, 2 units HotStart polymerase (Qiagen, Hilden, Germany), 2 µM SYTO 9 (Invitrogen, Life Technologies, Carlsbad, CA), 1×ROX reference dye (Invitrogen) and 25 ng DNA. The primer sequences have been previously described elsewhere (10), with the exception of the common reverse primer which has been re-designed in order to shorten the amplicons of both codon 12 (90 bp) and codon 13 (85 bp) (originally of 149 and 144 bp in length). The resulting sequence was as follows: TGTTGGATCATATTCGTCCACA.

The PCR amplification was performed with precycling heat activation of DNA polymerase at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 64 °C for 30 sec and extension at 72 °C for 30 sec, in a ABI Prism 7500 Sequence Detection System (Applied Biosystems – Foster City, CA, USA). The PCR product of mutated samples was run on a 2% agarose gel to confirm the presence of the specific bands.

Quantitative analysis and analytical performance

Threshold cycles (Ct) were used to calculate the methylation rate and the mutation load in each sample according to the follow formula: %= 100 / [1 + 2{$Ct_{met/mut} - Ct_{unmet/WT}$}] (2,11). Ctmet and Ctunmet denote threshold cycles specific for the methylated and unmethylated state, whereas Mut and WT refer to mutated and wild-type alleles, respectively. The proportions (%) of methylation rate or mutation load detected in plasma compared to those detected in tissues were expressed as plasma/tissue ratio (p/t ratio).

The median of at least two replicate measurements was calculated for each sample and used for statistical analysis. Predefined quality criteria were set such that measurements with Ct values greater than 38 cycles were excluded.

Since it has been observed that the sensitivity of cfDNA assays can be increased by shortening the size of amplicons (5,6), primers for both analyses were designed to allow the amplification of products smaller than 120 bp. The intra-assay imprecision for the methylation test was 9%. The lower limit of detection of methylated DNA for the MSP assays (assessed using serial dilutions of the Universal Methylated DNA) was 1.5%.

The intra-assay imprecision for the *KRAS* analyses ranged between 2% and 8%, depending on the type of mutation. Cell line DNA admixtures containing the mutation of interest in a normal DNA background was used to evaluate the limit of detection and amplified in the same instrument runs to act as positive controls. The analytical sensitivity of ARMS-qPCR was below 2%, as previously reported (12).

Statistical analysis

Normality distribution was checked with the Shapiro-Wilk test and continuous variables were reported as median (range) or mean±SD, when appropriate. Statistical analyses and plotting of data were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA). The diagnostic performance of cfDNA analysis was compared with the tumor-tissue analysis (the current gold standard) for its sensitivity and specificity in distinguishing between

mutated/hypermethylated and nonmutated/non methylated individuals. The predictive positive values and negative predictive values were also calculated with Fisher's exact test. The rate of concordance between tissue and plasma profiles was determined with agreement test (and values presented as weighted kappa ± standard error). Differences between continuous variables were analyzed by using the Mann-Whitney U test. Correlations were tested with the Spearman correlation. Values of $p<0.05$ were considered statistically significant.

Results

Fifty six of 85 patients initially evaluated for their potential inclusion in the study were men, the remaining women, (mean age 69±14 years). The tumor stage distribution was as follow: 15 patients were at stage I (17.6%), 35 at stage II (41%), 24 at stage III (28.2%) and the remaining 11 at stage IV (12.9%). Twenty nine out of 85 tumor tissue samples (34%) were positive for one of the six *KRAS* mutations tested. Of these, 22 tumor tissues showed matched mutations in plasma samples. There were nine discordant results among the 85 samples examined. Five results showed a WT genotype for *KRAS*-tested mutations by cfDNA analysis, whereas tumor-tissue analysis showed a *KRAS* G13D mutation (n=2), a *KRAS* G12D mutation (n=2) or a *KRAS* G12V mutation (n=1). Two patients (both at stage II) showed a *KRAS* G12S and a G12A mutation by plasma analysis, but were determined as WT by tumor-tissue analysis. Finally, two patients (both with advanced metastatic CRC) showed unmatched mutations between tissue and plasma. Overall, cfDNA analysis showed 89% concordance for *KRAS* detection with tumor-tissue analysis.

The *SEPT9* promoter methylation was present in 82.3% (70/85) of primary tumor tissue samples. The analysis exhibited 86% of concordance with cfDNA analysis. Discordant results only concerned patients with aberrant methylation of *SEPT9* in tissue samples and unmethylated plasma samples (n=12). The distribution of positive and negative samples in tissue and plasma is shown in table 1 along with the analytical performance of cfDNA analyses.

Table 1: Concordance between tumor-tissue analysis and cfDNA analysis (n = 85)
cfDNA: cell-free DNA; WT: wild type; SE: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value.

cfDNA analysis	**Tumors-tissue analysis**						
KRAS	Mutant	WT	Total	SE	SP	PPV	NPV
Mutant	22	5	27	85%	93%	85%	93%
WT	5	53	58				
Total	27		85				
Agreement 76/85 (89.4%) k=0.753±0.077, p<0.0001							
SEPT9	Methylated	Unmethylated	Total	SE	SP	PPV	NPV
Methylated	58	0	58	83%	100%	94%	56%
Unmethylated	12	15	27				

After exclusion of the two patients with different *KRAS* genotype in tissue and plasma, the 27 patients (81.5% male) presenting with both genetic and epigenetic alterations in tissue specimens (31.8%, 27/85) were considered for further quantitative analyses. In these patients the rate of concordance between tissue and plasma was 93% (25/27) for the epigenetic alteration and 81% (22/27) for the *KRAS* mutation analysis (i.e., two cfDNA samples were negative for the methylation of *SEPT9* and five were negative for the presence of *KRAS* mutations). Among the different *KRAS* mutations tested, the G12V substitution was the most represented (n=11), followed by G12D (n=7) and G13D (n=7). Finally, one sample had the G12A mutation and one the G12S. Overall, 74% and 26% of mutation sites were located in codons 12 and 13, respectively.

The median *SEPT9* methylation rates in tumour tissues and plasma samples were 64.5% (12.2–99.9%) and 14.5% (0–45.5%), respectively. The median *KRAS* mutation load was 33.6% (1.8-86.3%) in tissues and 2.9% (0-17.3%) in plasma samples. Quantitative data for both genetic and epigenetic alterations according to different clinical pathological characteristics are summarized in Table 2. In both tumour tissues and plasma samples, no significant associations were found for gender, primary tumor site and differentiation status. In terms of pathological stage classification, the median methylation rate of *SEPT9* was significantly higher in advanced-stage cancer tissues than in the early stage tissues. A statistically significant correlation was found in the cohort between tissue and plasma *SEPT9* methylation rate (r=0.407, p=0.035), whereas no association was found between tissue and plasma *KRAS* mutation load (r=0.092, p=0.651)

Table 2: Associations between SEPT9 methylation rate and KRAS mutation load in tissue and plasma samples

		KRAS mutation load (median, range)				**SEPT9 mutation load (median, range)**			
		tissue	P	plasma	p	tissue	p	plasma	P
Gender (mean age±sd)	N (%)		0.2805		0.2099		0.4311		0.9535
Female (71±9)	6 (22.2)	46.8 (14.4-63.7)		8.1 (1.1-13.9)		67.1 (63.7-76.1)		17.8 (0-35.8)	
Male (70±17)	21 (77.8)	27.6 (1.8-86.3)		2.0 (0-17.3)		61.6 (0-35.8)		14.5 (0-45.5)	
Tumor location			0.6782		0.4879		0.5613		1.00
Proximal	20 (72)	33.7 (7.7-79.6)		4.0 (0-17.3)		67.1 (12.2-98.1)		13.7 (0-45.5)	
Distal	7 (28)	32.4 (1.8-86.3)		2.3 (0-14.3)		62.6 (18.6-99.9)		23.5 (3.0-35.8)	
Tumor differentiation			0.8514		0.1174		0.0656		0.4727
G1/G2	22 (80)	33.0 (1.8-86.3)		1.9 (0-17.3)		62.1 (12.2-99.9)		14.7 (0-45.5)	
G3	5 (20)	33.8 (15.2-62.7)		4.6 (2.3-14.0)		80.8 (64.5-92.7)		10.8 (0-30.0)	
Pathological stage			0.1204		0.6612		**0.0009**		0.7340
I/II	14 (51.9)	26.9 (1.8-63.7)		1.9 (0-17.3)		57.2 (15.2-76.0)		15.8 (3.0-40.3)	
III/IV	13 (48.1)	34.7 (11.2-86.3)		4 (0-14.0)		80.8 (12.2-99.9)		12.9 (0-45.5)	

Additional analyses were performed on p/t ratio of *KRAS* mutation load and *SEPT9* methylation rate to identify any potential difference between genetic and epigenetic degree of transition from tissue to plasma. The p/t ratio of *SEPT9* methylation rate was significantly higher than the p/t ratio of *KRAS* mutation load (24.2% vs 7.9%, p=0.023), both parameters showing a wide spectrum of values (range 0-72.9% for *SEPT9* p/t ratio and 0-62.6% for *KRAS* p/t ratio). This result was almost entirely attributable to the large discrepancy between genetic and epigenetic p/t ratios detectable in early stage cancers (p=0.0108), since the difference in advanced stage cancers was no longer significant (p=0.68) (Figure 1).

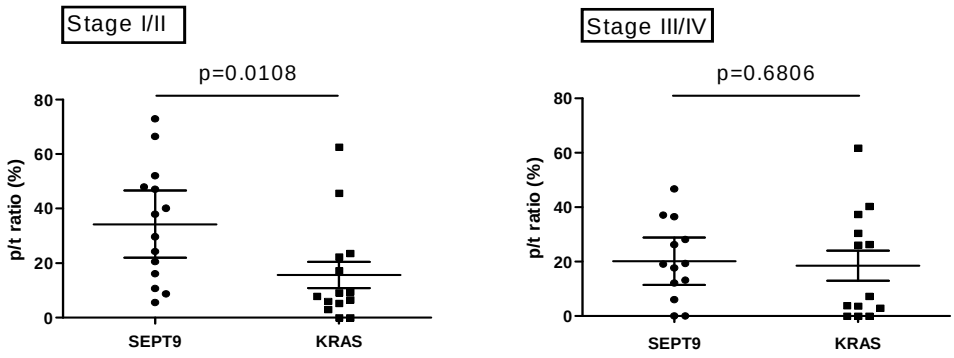

Figure 1: Differences between plasma/tissue methylation rate and mutation load in early and advanced cancer stages.

The concentration of cfDNA in early stages CRC patients (median 30.6 ng/mL, 4.6-66.8) was lower than that in advanced stage patients (80.2 ng/mL, 31.0-195.0; p=0.0001), and was also found to be more fragmented (integrity index: 0.36, 0.0.7-0.85 vs 0.63, 0.33-0.95; p=0.0163). No significant associations were found between cfDNA parameters and genetic or epigenetic alterations, except for a weak correlation between cfDNA integrity index and *KRAS* mutation load in advanced cancers (r=0.572, p=0.040).

Discussion

Although the use of cfDNA as potential surrogate of cancer genome has been originally suggested more than 30 years ago (13), and the role of liquid biopsy has been evaluated for its predictive and prognostic value in a number of settings with promising results, cfDNA-based cancer tests have not been developed for clinical use so far.

The high degree of fragmentation coupled with the low blood concentration make cfDNA a challenging analyte under a technical perspective. Moreover, the current lack of knowledge about the kinetic of release of tumor-related cfDNA into the bloodstream and the genetic composition changes during progression contribute to render cfDNA a "hard to read" analyte even under a biological perspective.

The results of our study, other than confirming that liquid biopsy predicts alterations of tumor tissues, are consistent with the hypothesis that some differences may exist among the rate with which genetic and epigenetic alterations move from tissue to plasma.

In order to make results free from any potential technical interference and make genetic and epigenetic data reliable and directly comparable, we adopted a number of methodological expedients adapted from recent publications. Firstly, plasma was used for analysis since this biological matrix represents a better source of cfDNA than serum (1,6). Then, we used relative short amplicons for both determinations, and this was due to the fact that amplicons length could influence the sensitivity of mutation and methylation detection (5,14,15). We have also assured a high level of sensitivity for epigenetic assay by targeting a specific CpG island, which has been recently found to display the highest susceptibility to methylation changes in the adenoma-carcinoma sequence (9). Finally, according to the American Society for Clinical Oncology and National Comprehensive Cancer Network (NCCN), a high level of detection rate has been obtained for the *KRAS* mutation analysis by targeting hotspots in codon 12 and 13, which are known to account for approximately 95% of all mutations (16).

In the present study, a methylation specific qPCR and an ARMS-qPCR based methods were used for *SEPT9* methylation analysis and *KRAS* mutation analysis, respectively. Owing to technological advances, new methods, including digital

PCR (17), Inteplex qPCR (14) BEAMing technology (18), MethyLight quantitative or MethyLight digital PCR (19) and new deep sequences approaches (20) are now available and allow absolute quantification of mutant or methylated alleles at very low frequencies and with lowest coefficient of variations than those reported here. However, the assays that we used in this study are those most widely available in clinical laboratories and are also characterized by optimal sensitivity, being able to detect at least 2% mutant in a normal background (21). Most importantly, the analytical performances of the genetic and epigenetic assays were very similar in terms of both sensitivity and precision, which has allowed to directly compare data of different alterations.

The first part of the study, performed on the entire cohort of 85 CRC patients, substantially confirmed previous evidence that analysis of *KRAS* and *SEPT9* in plasma represents a reliable alternative to the tissue. The status of *KRAS* is generally used as a predictive marker for response to established epidermal growth factor receptor (EGFR) inhibitors due to the fact that mutant *KRAS* is associated with resistance to anti-EGFR monoclonal antibody immunotherapy with agents such as centuximab or panitumumab (22,23). Conversely, aberrant methylation in the promoter region of the *SEPT9* gene has been convincingly proposed as sensitive and specific biomarker for early non-invasive diagnosis of CRC (24).

By following the suggestions recently proposed by Wasserkort and co-authors (9) thus targeting a specific CpG island on the promoter of the SEPT9 gene, we found a very high number of hypermetylated tissues samples (82%) and even higher than that previously reported in the literature (usually ranging between 78 and 81%) (25). The results obtained in matched plasma samples revealed a very high global concordance (86%) and specificity (100%) compared with tumor-tissue analysis. In the same sample, a *KRAS* mutation was detected in the 34% of patients, in accord with data obtained in other cohorts of unselected CRC patients (10,26). The corresponding analysis of plasma samples also revealed a high degree of concordance (89.4%) and specificity (93%) compared with tissue. Most of the studies comparing the results from a cfDNA assay with tumor-tissue analysis reported a much lower diagnostic performance, with values of specificity

constantly lower than 80% (27-29). As an exception only two recent studies reported values of specificity comprised between 95.3% (30) and 98% (14).

In the second part of the study, we have also analysed the rate of concordance between tissue and plasma mutation load and methylation rate, and then compared results obtained with the two assays. In the subgroup of 27 patients harbouring tissue genetic and epigenetic alterations, the *KRAS* mutation load varied from 1.8 to 86.3% (almost 48-fold), thus showing a higher inter-individual heterogeneity than the *SEPT9* methylation rate, which varied from 12.2% to 99.9% (8-fold). In the transition from tissue to plasma, five samples became wild type for the mutation status and two were no longer hypermethylated. The degree of methylation moving from tissue to plasma was almost 3 times higher than the rate of mutation load as resulting from comparison of the two p/t ratios (24.2% vs 7.9% for p/t ratio of *SEPT9* methylation rate and *KRAS* mutation load, respectively). In agreement with recent reports, this finding might be explained by the intratumoral heterogeneity of the primary tumor, which preferentially impairs genetic rather than epigenetic analysis (7,31). However, since the discrepancy found between the two p/t rations is exclusively attributable to results obtained in early stage cancers, whereas clonal evolution usually occurs when metastasis develop, the tumor clonality would only partially explain our data (32).

For the KRAS analysis, comparable values of mutation load were obtained between early and advanced cancers in both tissue (26.9% vs 34.7%) and plasma samples (1.9% vs 4%), so that the p/t analysis did not revealed significant difference according to the tumor stages (8.6% vs 7.3%). Conversely, a statistical significant difference was found for the SEPT9 methylation analysis between p/t ratio in early and advanced cancers (33.8% vs 19.0%, p=0.0108). This difference was entirely attributable to a discrepancy in the methylation rate detected in tissues (57.2% vs 80.8%, p=0.0009), since no differences were found in plasma samples (15.8% vs 12.9% for early vs advanced stages). Thus, the transition of DNA harbouring the epigenetic alteration into the circulation in early stage cancers is seemingly more consistent than the transition of DNA harbouring a *KRAS* mutation. According with the most recent literature, this data could be interpreted as the result of differences in tissue types involvement previously

observed for CRC genetic and epigenetic signatures (33). In particular, while the aberrant methylation *SEPT9* originates in epithelial cells and is then rapidly transferred to stromal cells (9), the *KRAS* mutations harboured by epithelial compartment are not shared by stromal cells (34). Accordingly, the molecular cross-talk between tumor epithelium and stroma occurring for the *SEPT9* epigenetic alteration might facilitate the transition of aberrant DNA from primary tumour to the circulation.

In conclusion, the results of the present study confirm that cfDNA analysis represents a suitable strategy for comprehensive analysis of tumor genetic and epigenetic profiles, even using routinely standard methods. Most importantly, we provided first evidence that the rate at which tumor derived cfDNA can be detected into the circulation not only depends on the sensitivity of methods used and the complexity of release kinetics, but also on the nature of the single alteration. In an era of increasing use of genome-scale comprehensive gene expression studies of solid tumors to elucidate the complexity of tumor tissues and heterogeneity of cell phenotypes, our study emphasize the need to better characterize cancer specific genetic and epigenetic signatures according to different tumour compartments in order to improve the significance and clinical value of cfDNA assessment.

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3.2 MiR-199a and miR-125b expression levels in serum of patients affected by epithelial ovarian cancer

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Introduction

The Epithelial Ovarian Cancer (EOC) is the leading cause of gynaecological cancer-related mortality worldwide, since it is commonly detected at an advanced and scarcely curable stage (1). Age-standardised incidence rates range from more than 9,1 per 100 000 women in Central and Eastern Europe (2). The crude incidence rate in Europe changes from 12,4 per 100.000 in the age group of 40-44 years to 35,9 per 100.000 in the age group of 60-64 years. Since EOC is generally asymptomatic in the early stages, about 75% of patients are diagnosed at an advanced stage (3).

The Cancer Antigen 125 (CA125) is the “gold standard” for monitoring patients affected by EOC, although being characterized by low sensitivity and specificity (4). Accordingly, elevated serum CA125 concentrations can be found not only in a broad range of benign gynaecologic diseases, but also in malignancies of different origin, including non-ovarian gynaecologic cancers, other epithelial tumors and non-epithelial malignancies (5).

Another serum biomarker, cleared by the Food and Drug Administration (FDA) for monitoring patients with EOC, is human epididymis protein 4 (HE4). Serum measurement of HE4 shows advantages over CA125 in terms of specificity, as it is less frequently increased in patients with non malignant ovary diseases. In 2008, The FDA has approved the combined use of HE4 and CA125 for estimating the risk for ovarian cancer in women with a pelvic mass. Updated guidelines do not recommend the use of these biomarkers is not recommended for determining the status of an undiagnosed pelvic mass (6).

Although recent meta-analyses have reported that HE4 exhibits higher rule-in capability for EOC vs CA125 more studies focusing on early tumour stages are required (7-9).

Carcinogenesis is characterized by the accumulation of both genetic and epigenetic alterations which can be used as disease biomarkers to provide important information for early detection of malignant diseases usually asymptomatic at early stages (10-11). Notably, the number of genes involved in development and progression of tumours which are epigenetically silenced probably overcomes the number of genes inactivated by mutation (12). Recent reports showed that methylated DNA and microRNAs (miRs) expression could be readily detected in a wide variety of tissues, as well as in various body fluids, indicating that these epigenetic biomarkers could represent the next generation biomarkers for cancer detection (13-14). MiRs, a class of small non-coding RNAs involved in regulating a variety of biologic processes (15-16), have been reported to be stably detectable in plasma and serum (17-18), and to exhibit resistance to endogenous ribonuclease activity (19).

By targeting multiple transcripts, a single miR can regulate many fundamental cellular processes such as cell proliferation, apoptosis, differentiation and migration. On the other hand, any gene can be regulated by multiple miRs (20). Alterations in miR expression are not simply an effect of tumorigenesis and may have a causative role in cancer development. They are involved in the initiation, progression and metastasis of human tumors. Iorio et al. (21) first evaluated genome-wide miR expression profiles of ovarian cancer tissues and normal ovary tissues, concluding that miRs expression was differentially regulated in the two groups. More specifically, miR-200a, miR-141, miR-200c and miR-200b were found to be over-expressed in ovarian cancer, while miR-199a, miR-140, miR-145, and miR-125b were down-regulated. Both miR-199a and miR-125b are involved in many biological processes including inhibition of tumor angiogenesis, a fundamental process for cancer development and growth. These miRs mainly act by targeting the 3′ untranslated region of VEGF (22), by increasing NF-κB activity (23) and by negatively regulating tumor suppresor p53 (24).

Therefore, the aim of our study was to investigate the serum expression levels of miR-199a and miR-125b in ovarian cancer patients in comparison to healthy controls, and to evaluate the correlation between miRs expression and concentrations of CA125 and HE4.

Materials and methods

Patients and samples

The study population consisted in 32 consecutive patients (54±14 years) diagnosed with EOC, and enrolled between December 2007 and February 2013 at the Obstetrics and Gynecology Clinics of the University Hospital of Verona (Italy). Blood was drawn in vacuum tubes containing no additives (Becton-Dickinson, Oxford, UK) prior to any therapeutic procedure (i.e., surgery, chemotherapy or radiotherapy). After centrifugation at 1,500g for 10 min at room temperature, serum was separated, stored in aliquots and kept frozen at -80°C until measurement. The histopathology results were confirmed by surgical resection of the tumors, and the tumor stage was defined according to the International Federation of Gynecology and Obstetrics (FIGO) system criteria (25). The demographics and clinical features of the patients are listed in Table 1. The control population consisted of 31 healthy female controls (55±17 years), matched by age and ethnicity, and without previous or recent history of cancer or other diseases. The study was carried out in accordance with the ethical standards of the revised Declaration of Helsinki.

Table 1. Demographics and clinical features of ovarian cancer patients.

Variables	**Ovarian cancer (n=32)**
Age, years (±DS)	54.1 (±14.3)
≥ 55 anni, n (%)	15 (46.9)
< 55 anni, n (%)	17 (53.1)
FIGO stage, n (%)	
I	4 (12.5)
II	2 (6.25)
III	26 (81.25)
Histological grade, n (%)	
1	4 (12.5)
2	7 (21.9)
3	21 (65.6)
Histology, n (%)	
Endometrioid	4 (12.5)
Clear cells	4 (12.5)
Undifferentiated	2 (6.2)
Mixed epithelial tumor	3 (9.4)
Serous	18 (56.2)
Transitional	1 (3.1)

Laboratory methods

Serum levels of CA125 were measured using a chemiluminescent enzyme immunoassay on the Liaison (DiaSorin, Saluggia, Italy). Intra and inter-assay coefficient of variation (CV) for this method are comprised between 1.4–2.2 and 4.6–5.8%, respectively. Serum levels of HE4 were determined using ELISA kit developed by Fujirebio Diagnostic, Inc. (Malvern, PA) and were performed according to the manufacturer's specifications. Total CV quoted by the manufacturer is <10%.

The isolation of miRNA from serum samples was performed with the miRNeasyTM RNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, 200 ml of serum sample was added with 1 ml of QIAzol Lysis Reagent (Qiagen) and mixed by gentle vortexing. Aqueous and organic phase separation was achieved by addition of chloroform. The aqueous phase was applied to an RNeasy spin column. The microRNA was eluted from the column with 14 μl of nuclease-free water. Quantity of RNA was assessed using small-RNA chip on Bioanalyzer 2100 (Agilent, Santa Clara, USA). RNA aliquots were stored at -80°C. For miRNA qPCR, reverse transcription was performed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 μL containing 3.33 μL RNA, 1 μL 10 × reverse transcription buffer, 0.67 μL Mutiscribe Reverse Transcriptase, 0.13 μL RNase Inhibitor. The reaction mixture was incubated for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C for 5 min, and then held at 4°C. The complementary DNA (cDNA) served as the template for Real-Time PCR. The reactions were performed in triplicate on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA) using TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA). Briefly, qRT-PCR was carried out in a total of 20 μl volume containing 1.33 μl cDNA, 1 × Universal PCR Master Mix and 1 μL gene-specific primers and probe. PCR parameters were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The comparative cycle threshold (Ct) method was applied to quantify the expression levels of miRNAs. The relative amount of miR-199a and miR-125b to small nuclear miR-

16 was calculated using the equation 2-ΔCt, where ΔCt= (Ct miR-199a/miR-125b - Ct miR-16) (26).

Statistical analysis

Data were tested for normality using the D'Agostino and Pearson omnibus normality test. Non-normally and normally distributed variables were reported as median (range) or mean ± standard deviation (SD), respectively. The concentration of the different biomarkers were compared between cases and controls using Kruskall-Wallis and Mann–Whitney tests according to their value distribution. The correlation between variables was assessed with Spearman's correlation coefficient (rs). For each miRNA and serum biomarkers, the diagnostic performance in terms of discriminatory capability was calculated by means of receiver operator characteristic (ROC) curves. The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego California USA), and the level of statistical significance was set at $p<0.05$..3

Results

The serum concentrations of both CA125 and HE4 were found to be significantly higher in EOC patients than in controls (Table 2). The median CA125 and HE4 levels were not statistically different between advanced EOC stage (III) and early EOC stage (I-II) (Table 2).

Table 2. CA125 ed HE4 concentrations in EOC patients and healthy controls.

	Patients (n=32)	**Controls (n=31)**	**P value**	**Stage I-II (n=6)**	**Stage III (n=26)**	**P value**	**Grade 1-2 (n=11)**	**Grade 3 (n=21)**	**P value**
CA125, U/mL	174.6 (3.3-3158.0)	12.0 (5.0-32.0)	<0.0001	38.1 (6.8-2176.0)	201.8 (3.3-3158.0)	0.22	96.0 (6.5-2176.0)	174.9 (3.3-3158.0)	0.69
HE4, pmol/L	123.6 (34.1-2300.0)	37.5 (25.0-121.2)	<0.0001	76.9 (43.6-208.0)	128.1 (34.1-2300.0)	0.14	81.6 (43.6-861.0)	136.8 (34.1-2300.0)	0.24

In EOC patients, a statistically significant correlation was found between CA125 and HE4 levels (r=0.46, p=0.009).

The serum concentrations of miR-199a and miR-125b were found to be significantly higher in EOC patients compared to healthy controls (p=0.007 and p=0.002, respectively) (Figure 1). The serum levels of miR-199a and miR-125b were not significantly higher in patients with advanced cancer (FIGO stages III) in comparison to early stages (I and II) (p=0.72 and p=0.12). Moreover, miR-199a and miR-125b serum levels were found to be not significantly different in patients with grade 3 EOC compared to those with grade 1 and 2 (p=0.23 and p=0.35). A significant correlation was found between miR-199a and miR-125b serum levels (r=0.38, p=0.03). The serum concentration of miR-199a was not significantly correlated with CA125 or HE4 values, whereas miR-125b expression levels correlated significantly with CA125 (r=0.33, p=0.007) but not with HE4. The ROC curve analysis evaluating the capability to discriminate EOC from healthy conditions, revealed that the single measurement of HE4 exhibited a significantly higher area under the curve (AUC) compared to CA125, miR-199a and miR-125b (Figure 2).

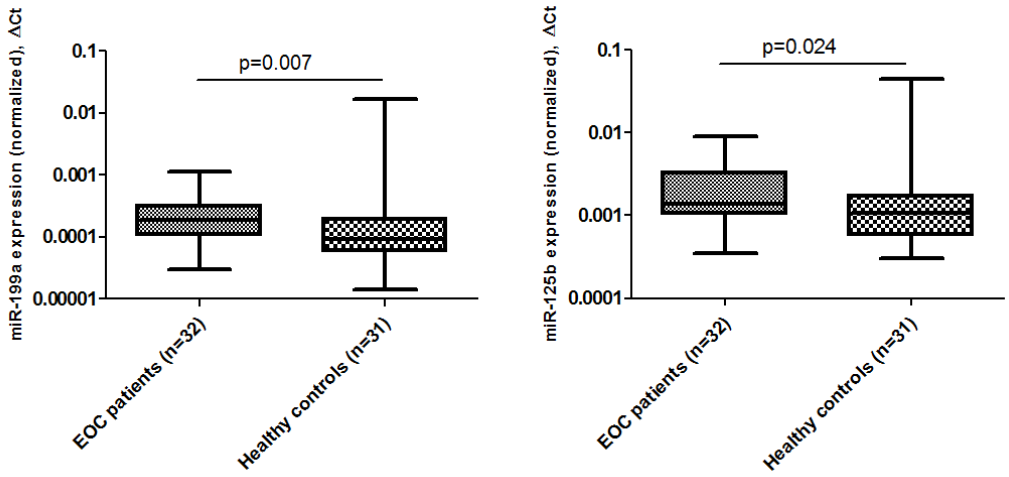

Figure 1. MiR-199a and miR-125b expression levels in EOC patients and healthy controls.

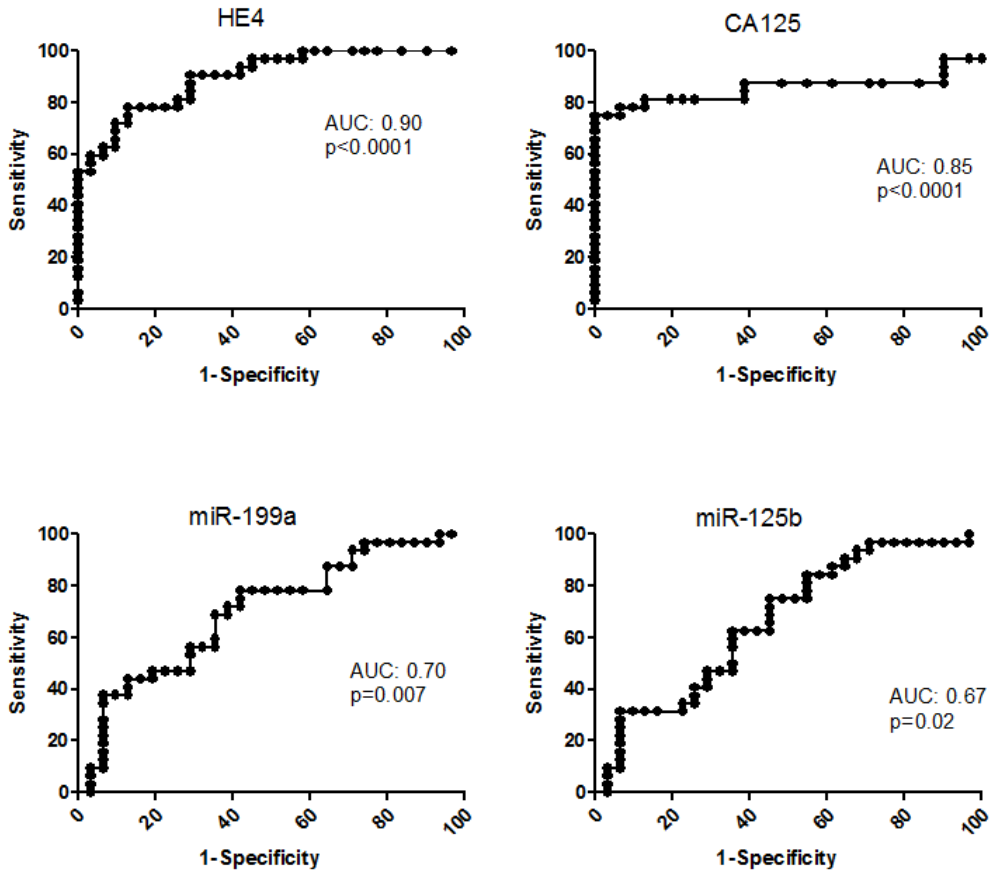

Figure 2. HE4, CA125, miR199a and miR-125b ROC curves performed on EOC patients and healthy controls.

Discussion

Survival of women affected by EOC is higher when the diagnosis is made at an early stage, but it dramatically drops when the cancer has spread to the pelvis and peritoneum. Therefore, a better diagnostic approach is still needed to improve the clinical outcome (27).

Several studies have demonstrated the limitations of using CA125 for detecting EOC. In fact CA125 may increase in patients with other malignant or gynaecological benign diseases (i.e. endometriosis), resulting in a poor diagnostic specificity and in a high rate of false-positive results (28). Furthermore a low sensitivity is generally well reported in all stages of ovarian cancer and particularly in early-stages (29). For these reasons CA125 is not useful to rule-out or rule-in patients for EOC.

Between additional putative tumour biomarkers, HE4 seems the most promising (30-32). In our study, the AUCs for HE4 and CA125 were 0.90 and 0.85 respectively, which agrees with previous evidence published by Ghasemi et al (33). Anyway a considerable expression of HE4 in normal tissues and the lack of increase in borderline tumors are the main limitations to HE4 specificity (34-35). Accordingly to previous observations (36), we have also found that no significant difference exist in the concentration of HE4 among different EOC FIGO stages. However, this result could be due to the low sample size. Accordingly, the major limit of our study is the small number of subjects investigated and in particular the low number of patients in early stages.

Recently, miRs were identified as cancer biomarkers in cell-free serum, which can be used for distinguishing diseased individuals from healthy controls (37). They are readily detected in blood and they can be measured non-invasively, thus opening new avenues about the clinical usefulness of epigenetic biomarkers for early cancer detection (38-39).

The results of our study suggest that miR-199a and miR-125b were up-regulated in serum of EOC patients compared to controls. In previous studies based on ovarian cancer tissues/cell lines, both these miRs resulted instead found to be down-regulated (21; 40). In another study earlier published by Chen et al., the expression of miR-199a was found to be significantly higher in Type II (high-

grade, serous EOC) cancer compared with Type I EOC (41). Type I tumors comprise low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas. They are generally indolent, present in stage I (tumor confined to the ovary), and are characterized by specific mutations, including KRAS, BRAF, which target specific cell signaling pathways. These tumors rarely harbor p53 mutations and are relatively stable genetically. Nevertheless, the significant difference between data obtained in these and previous studies can be reliably attributed to the different study populations enrolled, wherein 65,6% of our patients were diagnosed in the most advanced stage and 56,2% of the EOC were of serous origin. Notably, Chen et al. (41) previously described that hsa-miR-199a has a functional role in carcinogenesis, by regulating IKKβ expression, and this evidence is seemingly in support of our finding that this miRNA may be somehow involved in the pathogenesis of this type of cancer.

As currently reported in the scientific literature, miR-125b may be up-regulated in some tumors and down-regulated in others (42). As regards its biological activity and function, this miRNA interplays with many target genes related to tumor growth, invasion and metastatic, progression survival and chemotherapy recurrence, but its specific metabolic pathway remains largely unclear (42). It has been recently shown that protein p53, a well-known anti-tumor molecule, is a putative miR-125b target (43). Le et al. (44), thus demonstrating that miR-125b may regulate cancer growth by inhibiting p53 expression through direct binding with p53 mRNA 3'UTR.

Taken together, our results show that HE4 retains better diagnostic performance in EOC patients than the conventional cancer biomarker CA125, and also better than two promising miRNAs such as miR-199a and miR-125b. Currently, the identification of cancer-specific miRNA profiles in the circulation is an emerging field of particular interest. Accordingly, the evidence that the serum level of these two miRNAs is considerably increased in patients with EOC underpins that their assessment may retain some biological interest in basic research and for increasing our understanding of the still intriguing EOC carcinogenesis. However, a number of studies remain to be performed to elucidate the biological significance of these miRNAs in ovarian cancer.

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3.3 Aberrant miRNAs expression in patients with endometrial cancer

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Introduction

Endometrial carcinoma (EC) is the most frequent gynaecological cancer in developed countries and second only to cervical cancer globally (1,2). Several randomized trials have confirmed that the initial stage of disease is the most important prognostic factor in EC (3). In particular, 5-years survival rate approximates 80% when the diagnosis is made at an early stage, but it dramatically decreases to nearly 15-17% when the cancer is diagnosed at stage IV. Despite several efforts that have focused on the identification of reliable biological markers, no specific serum tumor markers display satisfactory performance for either diagnosing or monitoring EC. The Human Epididymis Protein 4 (HE4), an innovative biomarker originally proposed for investigating other gynecologic malignancies (i.e., ovarian cancer) (4,5), was found to be sufficiently specific but poorly sensitive in patients with EC (6). Although the diagnostic performance of HE4 appears better than that of Cancer Antigen 125 (CA125) in diagnosing EC at an early stage (7), its real value and efficacy for management of EC has not been clearly demonstrated in clinical practice. Therefore, discovery and validation of novel molecules, or panels of biomarkers, that can help identify endometrial tumors in their earliest stages with high sensitivity and specificity should be regarded as a major breakthroughs for improving the outcome of patients with this type of malignancy.

Epigenetics, conventionally defined as heritable change in gene expression that is not attributable to alteration of the DNA sequence, represents a new avenue in cancer research. Reliable evidence is accumulating that epigenetic mechanisms may play a key role in cancer progression and as well as in the onset of chemotherapy resistance (8). Since blood can easily be collected through a minimally invasive procedure, and also provides the ideal substrate for miRNAs

analysis, the assessment of non-coding RNAs (ncRNAs) has been proposed as a valuable perspective for early diagnosis of a kaleidoscope of different cancers (9). Aberrant tissue miRNAs expression has been described in EC (10-14) and, more recently, circulating extracellular miRNAs have been also been identified (15-17). Notably, a genome-wide study aimed to assess serum miRNA expression profile in EC identified four putative serum miRNAs (i.e., miR-222, miR-223, miR-186 and miR-204), which may be potentially useful for diagnostics of EC (18). Therefore, this study was aimed to investigate the differential expression of four serum miRNAs, as well as the concentration of two widely used serum biomarkers (i.e., CA125 and HE4), in EC patients and in a healthy control population.

Materials and methods

Population

The study population consisted in 46 consecutive women diagnosed with EC, who were scheduled to undergo radical surgical treatment between October 2007 and February 2010. The patients were recruited at the Obstetrics and Gynecology Clinics of the University Hospital of Verona (Italy). All patients underwent radiological imaging by pelvic ultrasonography (US), computed axial tomography (CAT) scanning, and/or magnetic resonance imaging (MRI) within 6 weeks prior to surgery, to identify the presence of endometrial mass. Blood samples were collected prior to any therapeutic procedure (i.e., surgery, chemotherapy or radiotherapy). The histopathology results were confirmed by surgical resection of tumors, and the cancer stage was defined according to the International Federation of Gynecology and Obstetrics (FIGO) system criteria (19). The control population consisted in 28 healthy female controls, matched by age and ethnicity, and without previous or recent history of cancer or other diseases. All healthy controls underwent gynaecologic examinations and transvaginal sonographies in the previous two years. The study was carried out in accordance with the ethical standards of the revised Declaration of Helsinki.

Laboratory methods

Blood samples were collected in vacuum tubes containing no additives (Becton-Dickinson, Oxford, UK) after overnight fast. All samples were collected from EC patients the morning before surgery. After centrifugation at 1500 x g for 10 min at room temperature, serum was separated, stored in aliquots and kept frozen at -80° C until measurement.

Serum levels of CA125 were assayed with the chemiluminescent enzyme immunoassay CanAg CA125, on Triturus EIA analyzer (Grifols, USA). The intra- and inter-assay imprecision of this method is comprised between 2.9-4.4% and 3.1-4.0%, respectively. The analytical sensitivity and the upper reference limit of the CanAg CA125 EIA assay are <1.5 U/mL and 35 U/mL, respectively. The serum levels of HE4 were measured using an EIA kit developed by Fujirebio Diagnostic, Inc. (Malvern, PA) on Triturus EIA (Grifols, USA). The HE4 EIA is a solid-phase, non-competitive sandwich immunoassay based on two mouse monoclonal antibodies directed against two epitopes of the C-WFDC domain of HE4. The total imprecision, the limit of detection and the functional sensitivity of this assay are <10%, <2.5 pmol/L and <5 pmol/L, respectively. Since no conclusive diagnostic threshold has been reported for HE4 so far, the value corresponding to the 95° percentile value of a healthy population (74.2 pmol/L) is conventionally used as diagnostic cut-off.

Total serum RNA was extracted using mirVana PARIS Kit (Ambion, Life Technologies). The TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) was used for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) to verify differential miR expression on an ABI 7500 Real-Time PCR (Applied Biosystems). Briefly, 250 ng of total RNA were transcribed under the following condition: 16° C for 30 min, 42° C for 30 min, 85° C for 5 min. The PCR reaction conditions were as follows: 95 °C for 10 min, 40 cycle of 95 °C for 15 sec and 60 °C for 1 min. All assays were performed in triplicate. The relative expression level of each miR was normalized to that of miR-16 (Applied Biosystems), and was finally calculated utilizing the $2^{-\Delta Ct}$ method (20).

Statistical Analysis

The values of the different biomarkers were described as median (and range). The concentration of the different biomarkers were compared between cases and controls using Kruskall-Wallis and Mann–Whitney tests. The correlation between variables was assessed with Spearman's correlation coefficient (rs). The diagnostic performance was calculated by means of receiver operator characteristic (ROC) curves. The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego California USA), and the level of statistical significance was set at $p<0.05$.

Results

The demographics and clinical features of study population are described in Table I. The serum concentrations of HE4, but not of CA125, were found to be significantly higher in EC patients than in controls. When the analysis was limited to EC patients diagnosed with stage I cancer (n=32), the difference in HE4 values between cases and controls remained statistically significant ($p<0.0001$). The concentration of CA125 and HE4 was higher than the respective diagnostic thresholds in 12/46 (26%) and 27/46 (59%) EC patients. In patients with stage I EC, the diagnostic thresholds were exceed in 5/32 (15.6%) cases for CA125 and 19/32 (59.3%) cases for HE4, respectively.

Table 1. Demographics and clinical features of endometrial cancer patients

Variables	Endometrial cancer (n=46)
Age, years (range)	65.0 (30-83)
Menopausal status, n (%)	
Postmenopausal	42 (91.3)
Premenopausal	4 (8.7)
FIGO Stage, n (%)	
I	32 (69.6)
II	7 (15.2)
III-IV	7 (15.2)
Histological grade, n (%)	
Well- or Moderately-differentiated (G1 or G2)	34 (73.9)
Poorly-differentiated (G3)	12 (26.1)
CA125, U/mL (range)	10.3 (0.1-624.0)
HE4, pmol/L (range)	76.6 (28.0-782.0)

.
The serum concentration of miR-186, miR-222 and miR-223 was found to be significantly higher in EC patients compared to healthy controls ($p=0.004$, $p=0.002$ and $p<0.0001$, respectively), whereas that of miR-204 was significantly lower in cases than in controls ($p<0.0001$) (Figure 1). The significance of these differences remained unchanged when the analysis was limited to EC patients diagnosed with stage I cancer ($p=0.002$, $p=0.005$, $p<0.0001$ and $p<0.0001$ respectively) (Figure 2). Interestingly, serum HE4 concentration was found to be higher in patients with grade 3 EC compared to those with grade 1 and 2 cancer (105.1 vs. 71.7 pmol/L, $p=0.03$), whereas the values of miRNAs and CA125 were virtually identical among these groups.

The concentration of HE4 was found to be positively correlated with that of miR-222 ($r=0.36$, $p=0.002$) or miR-223 ($r=0.37$, $p=0.002$), but also negatively correlated with miR-204 values ($r=-0.60$, $p<0.0001$). No significant correlation was instead observed between CA125 and each of the four miRNA measured in this study.

The area under the curve (AUC) for identifying EC patients versus healthy controls was 0.70 ($p=0.004$) for miR-186, 0.72 ($p=0.002$) for miR-222, 0.88 ($p<0.0001$) for miR-223 and 1.00 ($p<0.0001$) for miR-204, respectively (Figure 3). When the evaluation of diagnostic performance was limited to EC patients diagnosed with stage I cancer, the AUC was 0.73 ($p=0.002$) for miR-186, 0.71 ($p=0.006$) for miR-222, 0.85 ($p<0.0001$) for miR-223, 1.00 ($p<0.0001$) for miR-204, 0.91 ($p<0.0001$) for HE4 and 0.55 ($p=0.50$) for CA125, respectively (Figure 4). The best ROC curve-derived cut-off displayed 0.89 sensitivity and 0.79 specificity for HE4 (40 pmol/L), 0.50 sensitivity and 0.61 specificity for CA125.

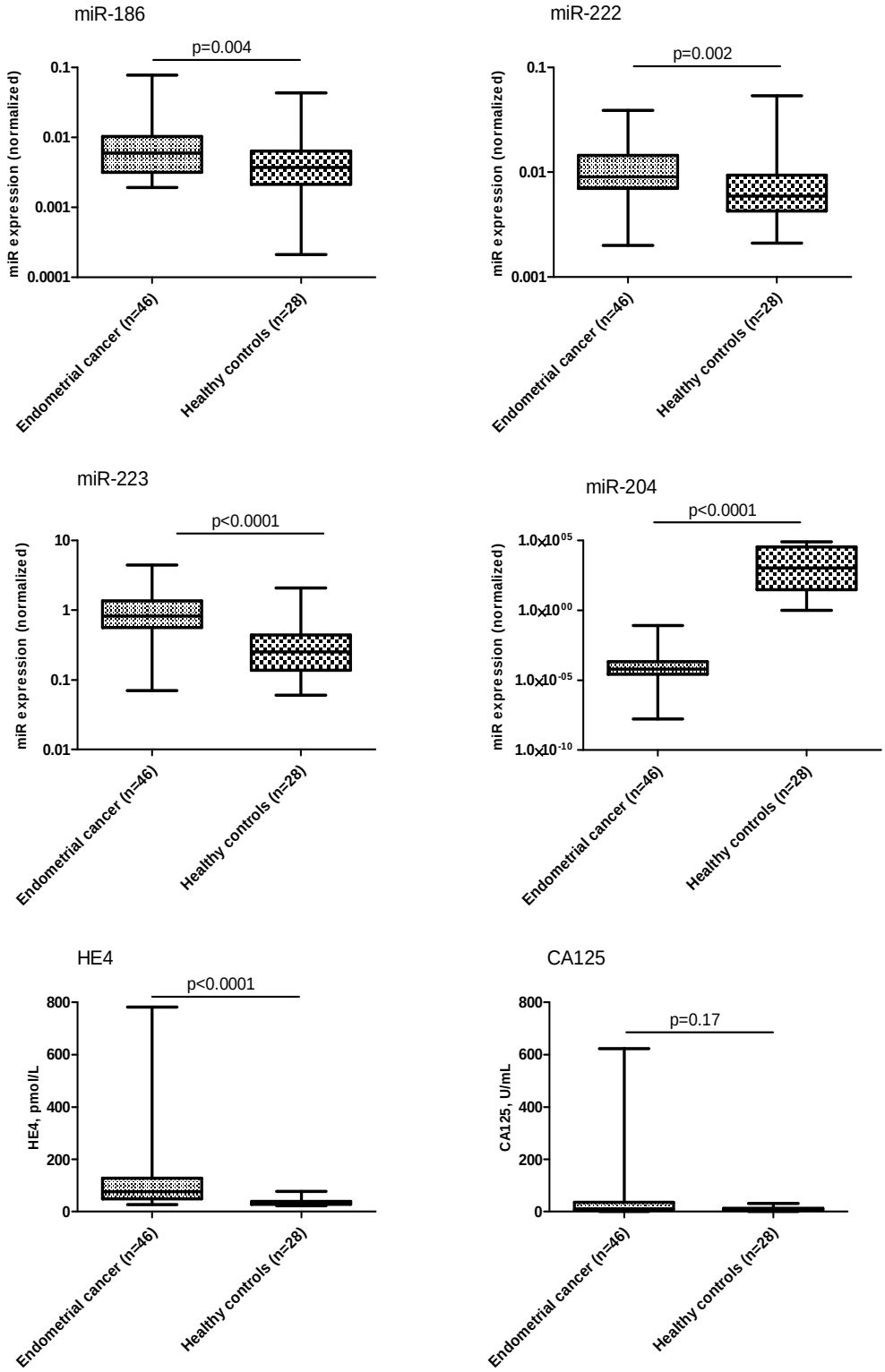

Figure 1. MiRNAs expression (normalized to miR-16) and HE4 and CA125 concentrations in EC patients and healthy controls.

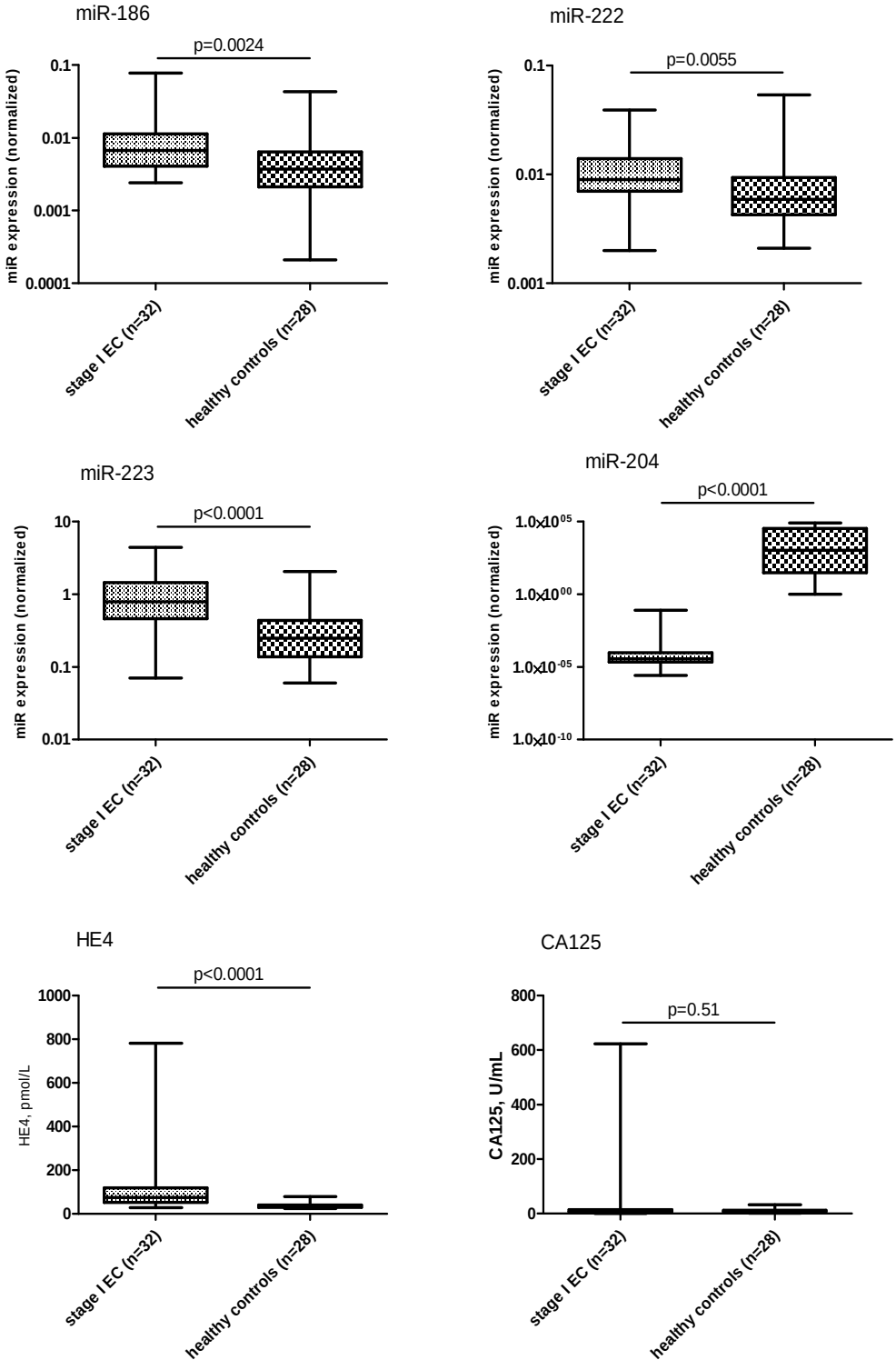

Figure 2. MiRNAs expression (normalized to miR-16) and HE4 and CA125 concentrations in stage I EC patients and healthy controls.

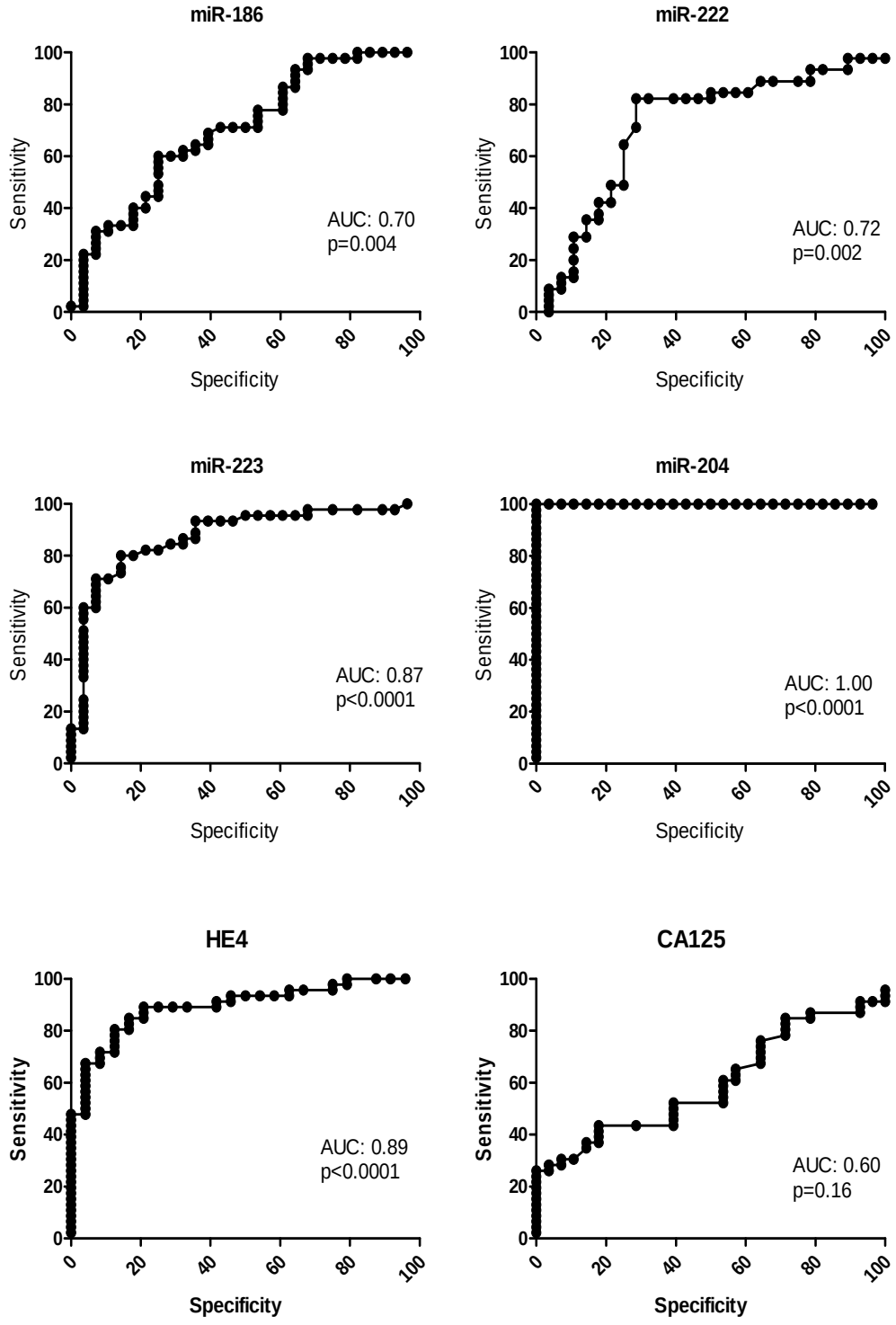

Figure 3. MiR-186, miR-222, miR-223, miR-204, HE4 and CA125 ROC curves performed on EC patients and healthy controls.

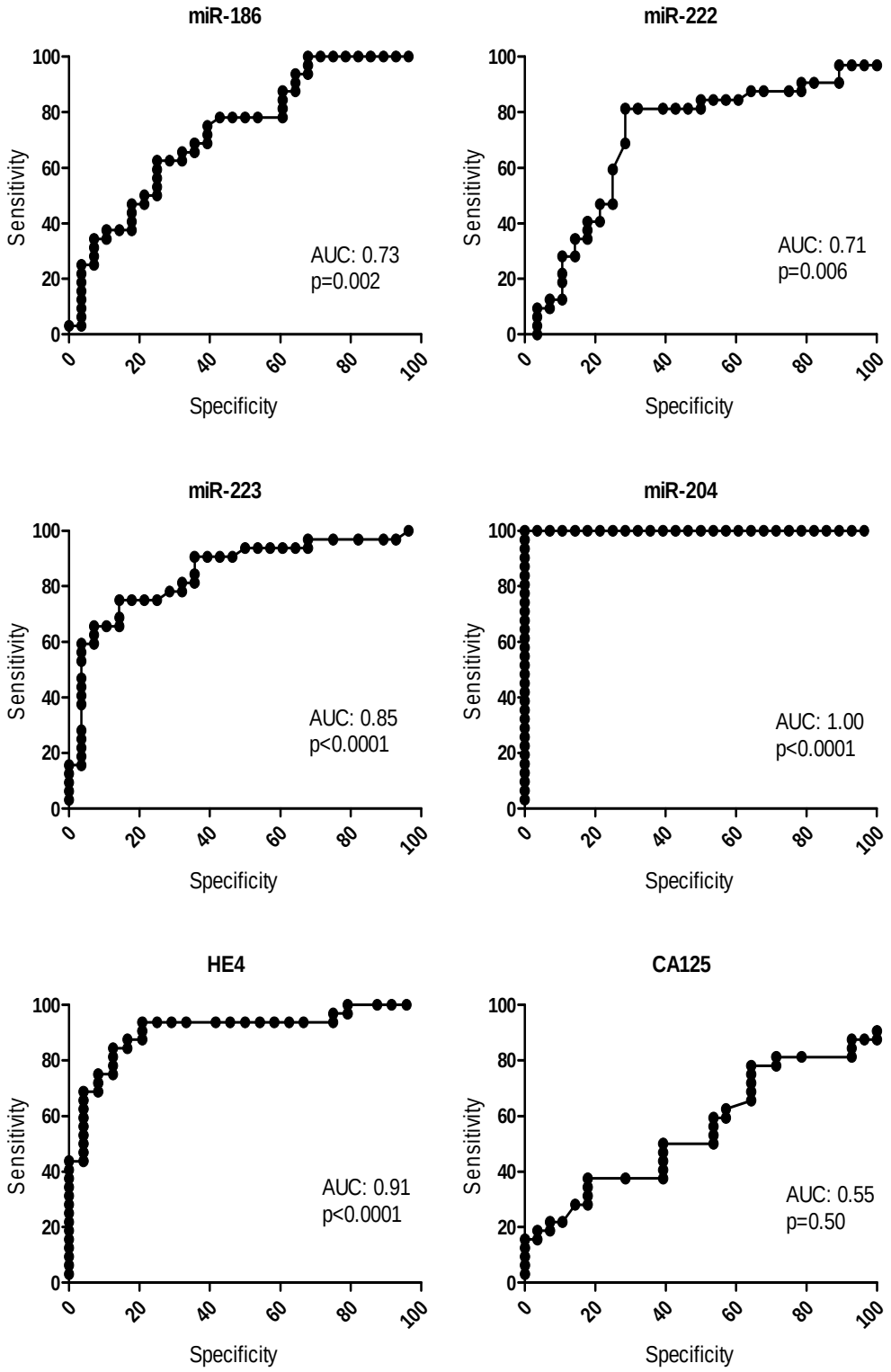

Figure 4. MiR-186, miR-222, miR-223, miR-204, HE4 and CA125 ROC curves performed on stage I EC patients and healthy controls.

Discussion

Despite a large number of patients with EC receive an early diagnosis (i.e., stage I; cancer confined to the uterus), this malignancy represents the seventh most common cause of death from cancer in women (21).

Unfortunately, no single biomarker that has been tested so far exhibits diagnostic performance for permitting an accurate preoperative diagnosis of patients with early or advanced EC. In clinical practice, CA125 is broadly used for screening and diagnosis of EC, often in combination with diagnostic imaging and clinics. However, as also observed in our investigation, this biomarker is poorly sensitive and specific, especially in EC patients diagnosed with stage I cancer. Previous evidence was brought that the concentration of CA125 can be actually increased in up to 60% of EC patients (22), but its value was found to be above the diagnostic cut-off in a rather limited number of patients with stage I cancer, typically lower than 10% (23). In our study population the values of CA125 were higher than the diagnostic threshold in 15.6% of patients diagnosed with stage I EC, and this finding is in absolute agreement with data previously published by Beck et al., who also showed that CA125 was elevated above 35 U/ml in 15.2% of patients diagnosed with cancer at this stage (24). Interestingly, HE4 seemingly exhibits a much greater diagnostic sensitivity than CA125 for early diagnosis of EC. In agreement with data previously published by Liu et al. (7), we found that the values of this biomarker were increased above the optimal cut-off in more than half (i.e., 59%) of patients with early stage EC. Even more importantly, we observed that serum HE4 values were significantly higher in patients with advanced disease (grade 3) compared to those with low or intermediate grades. This is indeed a valuable finding considering that the grade was shown to be as important as the stage for the prognosis of this type of cancer (21). Another interesting finding emerged from our study is that the serum concentrations of CA125 and HE4, two traditional biomarkers of gynaecological malignancies, were not significantly intercorrelated, contrarily to what has been earlier described in patients with ovarian cancer (25). This is not really surprising because HE4 has been previously found to be a much more sensitive biomarker in the early stage of

endometrioid adenocarcinoma, whereas the serum value of CA125 only increases over the diagnostic cut-off in advanced cancer stages (26).

The most interesting information was obtained from analysis of four candidate miRNAs. Interestingly, each of these small non-coding RNA molecules efficiently distinguished patients with EC from the control population, displaying an overall accuracy that was comparable (for miR-222) or even better (for miR-204) than that of HE4. These findings are in agreement with those previously published by Jia et al. (18), who also studied 26 patients with EC and 22 healthy controls, and reported that the AUCs of the four miRNAs were between 0.73 and 0.87, thus much better than that of CA125 (i.e., AUC 0.67). Moreover, the AUC was 0.93 (specificity: 87.5%, sensitivity: 91.7%) by combining the four miRNAs (miR-panel).

At variance with our results, however, Jia et al. (18) found that miR-204 was over-expressed in this type of cancer. This is a contradictory and somehow inexplicable finding, wherein miR-204 is a potent suppressor of tumour growth and metastases, the concentration of which has been reported to be down-regulated in various types of malignancy, including renal, brain, ovary, hematological and colon cancers (27). Different results could be attributed to differences in patients' characteristics or methods used in the two studies. Accordingly, in our study women affected by EC were older (mean age: 64.3 vs. 55.5 years) and a greater number were in a postmenopausal state (91.3% vs. 73.1%) compared to the patients enrolled in the study of Jia and colleagues (18). Moreover, in our population were also included EC patients with advanced stages of cancer (III and IV).

In addition, while in our study we measured the relative expression level of each miR normalized to miR-16, by using the 2-ΔCt method (20), in the study of Jia et al. (18) the authors calculated the absolute concentrations of the target miRNAs by using calibration curves.

The selection and validation of endogenous controls for microRNA expression studies remains controversial. A down-regulation of miR-16 has been reported in some cancer, as chronic lymphocytic lymphoma (CLL), pituitary adenomas, and prostate carcinoma (28). However, several other studies performed in different

tumors, including gynecological cancer (29) have demonstrated that miR-16 is presented in plasma/serum at similar levels across normal controls and patients. To the best of our knowledge, no study has demonstrated that miR-16 is dysregulated (up- or down-) in endometrial cancer. Moreover, we found no differences in miR-16 Ct values between EC and healthy controls patients by using the same RNA concentrations.

The complex biological pathways involving the role of the four miRNAs that we have investigated have not been completely elucidated so far. Nevertheless, it has been hypothesized that miR-186 may consistently reduce the expression of tumor suppressor FOXO1, and thereby deregulates cell cycle control, by direct binding the 3'-untranslated region of FOXO1 transcripts (30). Both miR-221 and miR-222 are over-expressed in the majority of epithelial tumors, and they were found to play a tumor-suppressive role in erythroleukemic cells (31). Specifically, the miR-221/222 system is seemingly involved in cell growth and proliferation due to its effect on the cyclin-dependent kinase inhibitor p27Kip1 (32), a cell cycle regulatory protein. Recent evidence also suggests that miR-222 acts as oncomiR in other cancer types through activation of the phosphoinositide 3-kinase/v-akt murine thymoma viral oncogene (PI3K/AKT) signaling pathway (33).

Despite the limited number of subjects included in this study, our preliminary findings underpin that the assessment of CA125 is virtually meaningless in EC diagnostics, whereas the measurement of both HE4 and some circulating epigenetic biomarkers, especially miR-204, may open new avenues for early identification and management of patients with EC. Indeed, the greatest advantage of measuring serum miRNAs is that the so-called liquid biopsy is a more practical, accessible and inexpensive approach for investigating solid cancers than conventional tissue biopsies (34). At variance with traditional serum cancer biomarkers, miRNAs are also released in larger amounts from tumor cells into the circulation, so that their serum or plasma concentrations more accurately mirror tumor development and progression (35). In this perspective, miRNAs assessment may be regarded as an accurate measure for identifying patients at increased risk of relapse, but they may also represent putative targets for innovative therapies

specifically tailored to suppress oncogenes expression or enhance the activity of tumor suppressor genes in patients with EC or other malignancies (36).

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4. GENERAL CONCLUSIONS

In recent decades, the role of epigenetic alterations in carcinogenesis has received greater attention more than ever before. After elucidating the fundamental role of epigenetic changes in human carcinogenesis, considerable efforts have been devoted to the development of epigenetic biomarkers.

Our results reported here indicate that circulating nucleic acids are a potentially promising source of tumor-specific biomarkers in patients with cancer of various grades and stages.

The development of molecular techniques has opened up the potential of utilizing circulating nucleic acids as prospective cancer biomarkers.

This approach may also provide personalized identification of tumor-specific biomarkers in serum samples once genetic and epigenetic aberrations have been characterized in the tumor specimen. We have demonstrated that these circulating tumor-specific biomarkers can be detected at any time during the course of the disease and once detected indicate that a tumor is probably present.

Undoubtedly, the diagnostic value of epigenetic molecules in panels or in combination with the conventional clinical biomarkers could be superior to individual markers.

The biggest challenge is to standardize the methodologies including sample storage and DNA or microRNAs extraction to translate the quantitation of circulating epigenetic biomarkers into a clinical routine for cancer diagnosis and prognosis prediction.