U.PORTO

FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

EXPRESSION OF HISTONE MODIFYING ENZYMES AND HISTONE POST-TRANSLATIONAL MARKS IN COLORECTAL CANCER

ANTÓNIO JOSÉ POLÓNIA RODRIGUES DE OLIVEIRA

Dissertation to a Master's Degree in Molecular and Oncology Medicine

FACULTY OF MEDICINE OF UNIVERSITY OF PORTO

Porto, October 2012

ANTÓNIO JOSÉ POLÓNIA RODRIGUES DE OLIVEIRA

EXPRESSION OF HISTONE MODIFYING ENZYMES AND HISTONE POST-TRANSLATIONAL MARKS IN COLORECTAL CANCER

Dissertation for applying to a Master's degree in Molecular and Oncology Medicine submitted to the Faculty of Medicine, University of Porto.

Supervisor:
Rui Manuel Ferreira Henrique, MD, PhD
Guest Assistant Professor
Department of Pathology and Molecular Immunology
Institute of Biomedical Sciences Abel Salazar – University of Porto
&
Director of Department of Pathology,
Senior researcher at the Cancer Epigenetics Group of the Research Center
Portuguese Oncology Institute – Porto

Co-Supervisor:
Carmen de Lurdes Fonseca Jerónimo, PhD
Guest Associate Professor
Department of Pathology and Molecular Immunology
Institute of Biomedical Sciences Abel Salazar – University of Porto
&
Assistant Investigator and Coordinator of the Cancer Epigenetics Group
Department of Genetics and Research Center
Portuguese Oncology Institute – Porto

ACKNOWLEDGMENTS

This study was funded by grants from Research Center of Portuguese Oncology Institute-Porto (Project CI-IPOP-4), and from the European Community's Seventh Framework Programme – Grant number FP7-HEALTH-F5-2009-241783.

To Professor Rui Henrique and Professor Carmen Jerónimo I thank for the motivation, orientation and critical review of this work.

To Drª Sara Reis and Drª Marcia Coimbra from the Cancer Epigenetics Group of Portuguese Oncology Institute-Porto Research Center I thank for the precious help in the execution of the laboratory work.

To Drª Paula Lopes from the Pathology Department of Portuguese Oncology Institute-Porto I thank for the counseling in the adjustment of the laboratory procedures.

To Drª Ana Tavares from the Pathology Department of Portuguese Oncology Institute-Porto I thank for the technical support.

To Catarina I thank for standing by me, even in the darkest moments.

FOREWORD

Despite being a well known and widely used word, the precise definition of "neoplasia" is still debatable. One of the most cited definitions of neoplasia belongs to Rupert A. Willis in his famous book "The spread of tumours in the human body" published in 1952[1]: "A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change." In the last years, many authors[2] realize that neoplasia, besides being literally the formation of something new, through the proliferation or increase survival of the neoplastic cells, is instead, a complex tissue made of several different cell types that establish interactions with each other. Such different cell types include not only neoplastic cells, but also cells of the microenvironment, like cancer associated-fibroblasts[3-5], immune cells[6, 7], pericytes [8, 9] or even bone marrow-derived cells[10, 11], among others. Far from being considered static elements, the cells of the microenvironment can be recruited to the bulk of the neoplasia and promote initiation and evolution of this tissue – in other words, normal cells with normal genotype can increase or decrease the malignant behavior of the neoplastic cells.

As a complex tissue that does not respect the boundaries of normal tissue architecture, neoplasia is also regulated by non-cellular elements of the microenvironment which carry autocrine and paracrine signals. These signals that control normal and neoplastic tissue architecture imbalance (cell number and position) are transmitted from one cell to the other, through stroma, in a very tightly regulated fashion, in which time is a very important factor. The effects of the non-cellular elements and their temporal variation are very difficult to access experimentally. Nevertheless, the mechanisms involved in the maintenance of architecturally complex tissues should be searched in order to obtain a more profound knowledge on neoplasia behavior.[2, 12, 13]

Accordingly, the biology of neoplasia can only be fully understood through the study of the cross-talk between neoplastic cells and their microenvironment rather than keep the study focus on a strict group of neoplastic cells that are known to be extremely heterogeneous.[14] Recently, the characterization of the genomes of neoplastic cells microdissected from different areas of the same tumor has revealed intratumoral genetic heterogeneity[15, 16], which has relevant

implications in cancer therapy as well as in the establishment of cancer study strategies. It is no longer sufficient to understand neoplasia just by studying a single neoplastic cell genome.

The ability to invade and to disseminate by the neoplastic cells, through the activation of a regulatory program, known as epithelial-mesenchymal transition (EMT) [17-19], epitomizes the product of the epigenetic induced heterogeneity. The EMT induced by epigenetic alterations is reflected by the loss of adherens junctions, the conversion to a spindly morphology, expression of matrix-degrading enzymes and increased cell motility.[20-22] These epigenetic mechanisms may not only be important in neoplastic cells but also in the cellular and non-cellular elements of the microenvironment.[23]

Using the colorectal cancer (CRC) as a model, we collected from the Department of Pathology of Portuguese Oncology Institute-Porto a series of morphologically characterized cases of colorectal adenocarcinoma with long term follow-up and studied the expression of different epigenetic marks. In this study, in a attempt to shed some light in CRC carcinogenesis, the protein expression of histone modifying enzymes and histone marks were assessed by immunohistochemical analysis and searched for the association between the expression of these proteins and the clinicopathological features of the cases, as well as with the patient´s outcome and therapeutic response.

This study allowed the identification of new biomarkers that might be used in clinical practice, at a low cost, and gives rise to new directions of research in CRC carcinogenesis.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APC – adenomatous polyposis coli protein

ASH1L - histone-lysine N-methyltransferase ASH1L (absent small and homeotic disks protein 1 homolog)

BIM - Bcl-2-like protein 11

BMP – bone morphogenetic protein

BRAF – serine/threonine-protein kinase B-raf (v-Raf murine sarcoma viral oncogene homolog B1)

CI – confidence interval

CIN – chromosome instability

c-MYC – Myc proto-oncogene protein (v-myc myelocytomatosis viral oncogene homolog)

CpG – cytosine guanine (linear dinucleotide)

CRC – colorectal cancer

DAB – 3,3´-diaminobenzidine

DCC – Netrin receptor DCC (deleted in colorectal cancer)

DNA – deoxyribonucleic acid

DNMT – DNA methyltransferase

EDTA – ethylenediamine tetraacetic acid

EHMT1 – histone-lysine N-methyltransferase EHMT1 (euchromatic histone-lysine N-methyltransferase 1), also known as G9a-like protein 1 (GLP1)

EHMT2 - histone-lysine N-methyltransferase EHMT2 (euchromatic histone-lysine N-methyltransferase 2), also known as protein G9a

EMT – epithelial-to-mesenchymal transition

EZH2 – Histone-lysine N-methyltransferase EZH2 (Enhancer of Zeste Homolog 2)

E2F - transcription factor E2F

FAP – familial adenomatous polyposis

FOBT – fecal occult blood testing

FOLFIRI – fluorouracil + leucovorine + irinotecan

FOLFOX – fluorouracil + leucovorine + oxaliplatin

5-FU – fluorouracil

HDAC – histone deacetylase

HDAC1 - histone deacetylase 1

H-score – modified Histo-score

H2AK5ac – acetylation of lysines 5 in histone H2A

H2O2 – hydrogen peroxide

H3K4 – lysine 4 in histone H3

H3K4me2 – dimethylation of lysines 4 in histone H3

H3K4me3 – trimethylation of lysine 4 in histone H3

H3K9 – lysine 9 in histone H3

H3K9ac – acetylation of lysines 9 in histone H3

H3K9me3 – trimethylation of lysine 9 in histone H3

H3K18ac – acetylation of lysines 18 in histone H3

H3K27 – lysine 27 in histone H3

H3K27me3 – trimethylation of lysine 27 in histone H3

H3K36 – lysine 36 in histone H3

H3K79 – lysine 79 in histone H3

H4K12ac – acetylation of lysines 12 in histone H4

H4K20 – lysine 20 in histone H4

H4K20me3 – trimethylation of lysines 20 in histone H4

H4R3me2 – dimethylation of arginine 3 in in histone H4

IHC – immunohistochemistry

JARID1 - lysine-specific demethylase 5A (Jumonji/ARID domain-containing protein 1A)

JMJD1A – lysine-specific demethylase 3A (Jumonji domain-containing protein 1A)

JMJD1B – lysine-specific demethylase 3B (Jumonji domain-containing protein 1B)

JMJD2A – lysine-specific demethylase 4A (Jumonji domain-containing protein 2A)

JMJD2B – lysine-specific demethylase 4B (Jumonji domain-containing protein 2B)

JMJD2C – lysine-specific demethylase 4C (Jumonji domain-containing protein 2C)

JMJD2D - lysine-specific demethylase 4D (Jumonji domain-containing protein 2D)

JMJD3 - lysine-specific demethylase 6B (Jumonji domain-containing protein 3)

KDM6A - lysine-specific demethylase 6A

KDM6B - lysine-specific demethylase 6B, also known as JMJD3

KRAS – GTPase KRas (v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog)

LSD1 – lysine-specific histone demethylase 1A

MBD – methyl-CpG-binding domain protein

MGMT – Methylated-DNA-protein-cysteine methyltransferase (6-0-methylguanine-DNA methyltransferase)

miRNA – microRNA

MLH1 – DNA mismatch repair protein Mlh1 (MutL protein homolog 1)

MLL - histone-lysine N-methyltransferase MLL (Myeloid/lymphoid or mixed-lineage leukemia)

MMP – matrix metalloproteinase

MMP-9 - matrix metalloproteinase-9 (gelatinase B, type IV collagenase)

MMR – mismatch repair

mRNA – messenger ribonucleic acid

MSI – microsatellite instability

NC – not computed

NS – not significant

OS – overall survival

PBS – phosphate buffered saline

PcG – polycomb group

PI3K – phosphatidylinositol 3-kinase

PRC – polycomb-repressor complex

PRDM2 – PR domain zinc finger protein 2 (retinoblastoma protein-interacting zinc finger protein), also known as RIZ

p21 - cyclin-dependent kinase inhibitor 1

RAS – rat sarcoma viral oncogene

RASSF1A - Ras association domain-containing protein 1

RNA – ribonucleic acid

RTK – receptor tyrosine kinase

RUNX3 - Runt-related transcription factor 3

SET1A - histone-lysine N-methyltransferase SETD1A (SET domain-containing protein 1A)

SET1B - histone-lysine N-methyltransferase SETD1B (SET domain-containing protein 1B)

SETDB1 – histone-lysine N-methyltransferase SETDB1 (SET domain bifurcated 1)

SETDB2 – histone-lysine N-methyltransferase SETDB2 (SET domain bifurcated 2)

SMAD4 – mothers against decapentaplegic homolog 4

SMYD2 – N-lysine methyltransferase SMYD2 (SET and MYND domain-containing protein 2)

SMYD3 – SET and MYND domain-containing protein 3 (zinc finger MYND domain-containing protein 1)

SNP – single-nucleotide polymorphism

SPSS – Statistical Package for the Social Sciences

Suv39 – histone-lysine N-methyltransferase Su(var)3-9 (protein suppressor of variegation 3-9)

SUV39H1 – histone-lysine N-methyltransferase SUV39H1 (suppressor of variegation 3-9 homolog 1)

SUV39H2 – histone-lysine N-methyltransferase SUV39H2 (suppressor of variegation 3-9 homolog 2)

TARBP2 – RISC-loading complex subunit TARBP2 (Trans-activation-responsive RNA-binding protein)

TBST – Tris-buffered solution with 0.05% Tween 20 solution

TCF4 – transcription factor 4

TGF – transforming growth factor

TGFb-1 - transforming growth factor beta-1

TP53 – cellular tumor antigen p53

VEGFR1 - vascular endothelial growth factor receptor 1

WNT – wingless/integrated

WNT10B - protein Wnt-10b

χ^2 – Pearson´s chi-squared test

ABSTRACT

In Portugal, colorectal cancer (CRC) is the most incident cancer and the second main cause of cancer-related death. The clinical outcome of CRC can vary significantly, hence, it is important to subclassify the patients with CRC in order to predict the prognosis and treatment response. Several studies have shown that colorectal carcinogenesis involves not only genetic alterations but also epigenetic modifications. Despite several different types of histone post-translational modifications, only a few have been relatively well studied, including histone acetylation and methylation. These modifications are due to the activity of several enzymes, such as histone methyltransferases and histone demethylases, and alterations in their expression levels have been found in several neoplasms. In addition, alterations in the pattern of histone modifications, specifically performed by the above mentioned chromatin-modifying enzymes, have been also associated with several types of tumors and their outcome.

The overall objective of this study was to characterize the expression of four histone modifying enzymes, including the histone methyltransferases EZH2, SMYD3 and SETDB1 and the histone demethylase LSD1, as well as the respective histone marks H3K9me3 and H3K27me3 in a series of CRC patients, clinically and pathologically well characterized, and to determine their value as biomarkers of prognosis and predictive of therapeutic response.

A series of 98 colon cancer cases were randomly selected from the archives of Portuguese Oncology Institute-Porto between March 2003 and November 2006. The expression of EZH2, SETDB1, SMYD3 and LSD1, as well as the immunoreactivity of H3K9me3 and H3K27me3 were determined using immunohistochemical assays. The evaluation of SMYD3 expression was considered positive if microscopic staining was present in >30% of the tumor cells. The remaining enzymes and both histone marks were assessed using a modified Histo-score (H-score). Overall survival (OS) time was defined as the interval between surgery and death or between surgery and the last follow-up time for surviving patients. Survival rate curves were calculated according to the Kaplan-Meier method and compared by the log-rank test. Multivariate survival analyses were based on the Cox proportional hazard regression model. The level of significance was set at $p < 0.05$.

Considering the clinicopathological characteristics of the tumors included in this series, the occurrence of distant metastasis was independently associated with low survival rate, while

the occurrence of venous vessel invasion, positive margins, regional lymph node metastases and more advanced stage were associated with worse survival rates only in univariate analysis.

Concerning the results of the immunohistochemical analysis, the histone methyltransferases EZH2 and SMYD3 and the histone demethylase LSD1 were overexpressed in CRC cells, suggesting a role for these enzymes in the neoplastic transformation. Interestingly, the expression of SMYD3 was detected in the cell membrane and cytoplasm of CRC cells, in contrast with the nuclear expression observed in the remaining histone modifying enzymes. In addition, the expression of histone marks H3K9me3 and H3K27me3 was increased in CRC cells, consistent with the increased expression of the abovementioned histone modifying enzymes.

The expression of histone modifying enzymes and the expression of histone marks were associated with several clinocopathological features. Higher EZH2 expression was observed in more invasive CRCs and CRCs with regional lymph node metastases, suggesting that EZH2 may have a role in tumor growth and cell invasion. Higher SETDB1 expression was observed in left-sided CRCs, suggesting a putative role in the carcinogenesis of chromossomic instable CRCs. Lower LSD1 expression was observed in more invasive CRCs and in CRCs at advanced stages of disease, suggesting that LSD1 may be associated with less aggressive CRCs. Higher H3K9me3 expression was observed in CRCs with regional lymph node metastases, which appears to be associated with the putative role of EZH2 in the progression of CRC. Higher H3K27me3 expression was observed in more invasive CRCs, CRCs with regional lymph node metastases and in CRCs at advanced stages of disease, as well as in CRCs with lymph and venous vessel invasion, which appears to be associated with the putative role of EZH2 in the progression of CRC. In this study we did not detected any differences in the survival rate of the patients with CRC considering the different expression of the studied histone modifying enzymes or histone marks.

Regarding the response to treatment we observed higher survival rates in patients with CRCs treated only with surgery disclosing lower SETDB1 expression, suggesting that whenever the expression of SETDB1 occurs, the surgical treatment may not be enough to prolong the patient's survival. Higher survival rates in patients with CRC treated with Folfiri were associated with high LSD1 and H3K9me3 expression in cancer cells, suggesting that LSD1 expression and the histone mark H3K9me3 can predict the response to this treatment modality. Higher survival rates of patients with CRC treated with 5-FU/Leucovorine were associated with high H3K9me3

and H3K27me3 expression in cancer cells, suggesting that these patterns of methylation can predict the response to this treatment modality.

Finally, we verified that the evaluation of histone modifying enzymes and histone marks using a low/high expression system reproduced the results obtained with the H-score, constituting an easier system to evaluate the expression of the histone modifying enzymes and histone marks in the routine of a pathologist.

This work accomplished the identification of new biomarkers that can be used in clinical practice and gives rise to new directions in the study of CRC carcinogenesis.

RESUMO

O cancro colo-rectal (CCR) é a neoplasia com maior incidência e a segunda principal causa de morte por cancro em Portugal. O desfecho clínico do CCR é extremamente variável, o que torna importante subclassificar os pacientes com CCR no sentido de prever o prognóstico e a resposta à terapêutica. Vários estudos têm mostrado que a carcinogénese colo-rectal envolve não apenas alterações genéticas mas também modificações epigenéticas. Apesar de existirem vários tipos de modificações pós-tradução das histonas, apenas algumas têm sido relativamente bem estudadas, nomeadamente a acetilação e a metilação de histonas. Estas modificações são devidas à actividade de várias enzimas, como histonas metiltransferases e histonas demetilases, encontrando-se alterações nos seus níveis de expressão em várias neoplasias. Além disso, alterações nas marcas de histonas especificamente catalisadas pelas enzimas acima mencionadas, têm sido também associadas com vários tipos de tumores e ao seu prognóstico.

O objetivo principal deste estudo foi caracterizar a expressão de quatro enzimas modificadoras de histonas, nomeadamente as histonas metiltransferases EZH2, SMYD3 e SETDB1 e a histona demetilase LSD1, bem como as respectivas marcas de histonas H3K9me3 e H3K27me3 numa série de pacientes com CCR, clinica e patologicamente bem caracterizados, e determinar o seu valor como biomarcadores de prognóstico e preditivos da resposta à terapêutica.

Uma série de 98 casos de CCR foram selecionados aleatoriamente a partir dos arquivos do Instituto Português de Oncologia do Porto Francisco Gentil entre Março de 2003 e Novembro de 2006. A expressão de EZH2, SETDB1, SMYD3 e LSD1, bem como a imuno-reactividade de H3K9me3 e H3K27me3 foram determinadas usando métodos de imunohistoquímica. A avaliação da expressão de SMYD3 foi considerada positiva se a imuno-marcação estivesse presente em mais de 30% das células tumorais. As restantes enzimas e ambas as marcas de histonas foram avaliadas utilizando uma versão modificada do Histo-score (H-score). A sobrevivência global foi definida como o intervalo entre a cirurgia e a morte ou entre a cirurgia e a data da última consulta para os pacientes vivos. As curvas de sobrevida foram calculadas de acordo com o método de Kaplan-Meier e comparadas pelo teste de log-rank. A análise de sobrevivência multivariada foi baseada no modelo de regressão de risco proporcional de Cox. O nível de significância foi estabelecido em $p < 0,05$.

Considerando as características clínico-patológicas dos tumores incluídos nesta série, a presença de metástases à distância foi independentemente associada com diminuição da sobrevivência, enquanto a presença de invasão venosa, margens positivas, metástase nos gânglios linfáticos regionais e estadio mais avançado foram associados com piores taxas de sobrevivência apenas na análise univariada.

No que diz respeito aos resultados da análise imunohistoquímica, as histonas metiltransferases EZH2 e SMYD3 e a histone demetilase LSD1 encontravam-se sobre-expressas nas células do CCR, sugerindo um papel para estas enzimas na transformação neoplásica. Curiosamente, a expressão de SMYD3 foi detectada na membrana celular e no citoplasma das células do CCR, em contraste com a expressão nuclear observada nas restantes enzimas modificadoras de histonas. Além disso, a expressão das marcas de histonas H3K9me3 e H3K27me3 encontrava-se aumentada nas células do CCR, consistente com o aumento da expressão das referidas enzimas modificadoras de histonas.

A expressão das enzimas modificadoras de histonas e a expressão das marcas de histonas encontraram-se associadas com várias características clínico-patológicas. Foi observada maior expressão de EZH2 nos casos mais invasivos e com envolvimento dos gânglios linfáticos regionais, o que sugere que a EZH2 pode ter um papel no crescimento e na invasão do tumor. Foi observada maior expressão de SETDB1 no CCR no cólon esquerdo, sugerindo um eventual papel na carcinogénese caracterizada pela instabilidade cromossómica. Foi observada menor expressão de LSD1 nos casos mais invasivos e nos casos em estadios avançados de doença, sugerindo que a LSD1 pode estar associada a casos menos agressivos. Foi observada maior expressão de H3K9me3 nos casos com envolvimento dos gânglios linfáticos regionais, que parece estar associada com a função proposta da EZH2 na progressão do CCR. Foi observado maior expressão de H3K27me3 nos casos mais invasivos, com envolvimento dos gânglios linfáticos regionais e nos casos com estadios mais avançados de doença, assim como nos casos com invasão linfática e venosa, o que parece estar associado com a função proposta da EZH2 na progressão do CCR. Neste estudo, não foi detectada nenhuma diferença na taxa de sobrevivência dos pacientes com CCR considerando a expressão das diferentes enzimas modificadoras de histonas ou da expressão de ambas as marcas de histonas.

No que diz respeito à resposta ao tratamento, observou-se que a taxa de sobrevivência de pacientes com CCR tratados apenas com cirurgia era maior nos casos que apresentavam

diminuição da expressão de SETDB1, sugerindo que a expressão de SETDB1 agrava o prognóstico e que o tratamento cirúrgico pode não ser suficiente para prolongar a sobrevivência de pacientes. A taxa de sobrevivência era maior em pacientes com CCR tratados com FOLFIRI nos casos com maior expressão de LSD1 e H3K9me3, sugerindo que a expressão de LSD1 e de H3K9me3 pode prever a resposta a esta modalidade de tratamento. A taxa de sobrevivência era maior em pacientes com CCR tratados com 5-FU/Leucovorine nos casos com maior expressão de H3K9me3 e de H3K27me3, o que sugere que esses padrões de metilação podem prever a resposta a esta modalidade de tratamento.

Finalmente verificou-se que a avaliação das enzimas modificadoras de histonas e da expressão de marcas de histonas utilizando um sistema de expressão em dois níveis reproduziu os resultados obtidos com o H-score, constituindo uma metodologia mais fácil de implementar na rotina de um patologista.

Este trabalho permitiu a identificação de novos biomarcadores que podem ser utilizados na prática clínica e que estabelecem novas direcções no estudo da carcinogénese colo-rectal.

INTRODUCTION

1. Epidemiology of colorectal cancer

In western world, cancer is a major public health problem. Although it affects both sexes, the lifetime probability of being diagnosed with an invasive cancer is slightly higher for men (45%) than for women (38%)[24]. The steady increase in the number of cancer cases diagnosed each year is thought to be due, mainly, to aging and growth of the population.[25] Interestingly, the incidence rates have been decreasing since the 90s for all major cancer sites, except for breast cancer which remained relatively stable since 2005, and, more importantly, the cancer death rate has been decreasing since the beginning of the millennium.[24] Nevertheless, cancer is the second leading cause of death, following cardiovascular diseases, accounting for 24% of all deaths.[26]

Colorectal cancer (CRC) is one of the most common and well-studied malignancies, currently representing nearly 10% of all new cancers worldwide, being the fourth most frequent newly diagnosed cancer in men (after lung, prostate and gastric cancers), and the third in women (after cancers of the breast and uterine cervix). The worldwide mortality rate of CRC is nearly half of the incidence rate, but there is a wide variation in mortality rates according to the available treatment options.[27] Decline in CRC incidence and death rates have been observed and have been attributed to a combination of screening programs and improvements in treatment[28-30]. The implementation of CRC screening tests allows the detection of CRC at an early stage of the disease and prevents cancer progression by promoting the removal of precancerous lesions.[25] However, only 60% of patients older than 50 years are in CRC screening programs, and, as a result, only 40% of CRC are diagnosed at a local stage, when treatment is most successful.[31, 32]

The prevalence of sporadic CRC is higher in developed countries (nearly 50 *per* 100 000, as registered in Portugal), increases dramatically with age, being relatively uncommon before the fourth decade of life. The median age at diagnosis of CRC is 68 years for males and 72 years for females.[32, 33]

In Portugal, CRC is the most incident cancer, representing nearly 15% of all cancers, and the second main cause of cancer-related deaths. In men, CRC is the second most commonly diagnosed cancer, after prostate cancer, and the second cause of cancer mortality, after lung

cancer. In women, CRC also ranks in the second position both for incidence and mortality rates, after breast cancer. Remarkably, in the north of Portugal CRC is the major cause of cancer-related deaths in women.[26, 33]

2. Etiology and pathogenesis of colorectal cancer

CRC develops over long periods of time as the consequence of interactions between genetic predisposition and environmental factors. Among immigrants and their offspring, the incidence rates of CRC rapidly approach those of their adopted countries, indicating that lifestyle, dietary and other environmental factors are important risk factors.[27] The majority of CRC are adenocarcinomas (more than 90%), which are thought to be originated in multipotential stem cells located in colorectal crypts from which precursor lesions may develop.[34] CRC has been considered a genetic disease, characterized by sequential accumulation of genetic alterations. The identification of CRC genetic alterations has been facilitated by expression profile analysis, however the precise mechanisms underlying the majority of these genetic alterations, some of which are considered harmless passenger mutations with no selection advantage, remain unknown.[27, 35, 36]

Many of the genes that are involved in CRC tumorigenesis have normal functions in signalling transduction pathways that regulate cell growth. The inadequate activation of such genes can lead to an inappropriate transmission of regulatory signals resulting in abnormal cell growth, some of which are elements of relevant signalling pathways, like the WNT, TGF/BMP, TP53, RTK and PI3K.[37]

2.1 Colorectal cancer genetics

Nearly 80% of CRCs are characterized by chromosome instability (CIN), showing gross chromosomal alterations (losses and gains) and aneuploid karyotype. The CIN is a type of genomic instability by which a cell is capable of acquiring cumulative genetic modifications that are thought to be essential to the malignant transformation of CRC. Additionally,the most frequent genetic alterations in CRC include mutations in *APC*, *RAS*, *TP53*, *DCC* and *SMAD4* genes.

The APC (*adenomatous polyposis coli*) was first discovered as the gene responsible for familial adenomatous polyposis (FAP), a form of hereditary CRC.[38] One year later, this same gene was also found to be mutated in 60% of sporadic CRC. Indeed, the APC mutations can also be detected in CRC precursor lesions, showing a role in the early development of CRC.[39] Inactivation of both copies of the *APC* gene is a critical event in the initiation of adenoma lesions. The *APC* gene codifies a large protein with multiple domains and cellular functions that is expressed in the epithelium of the colon and rectum and which shows a increasing gradient from the bottom of the crypts to the luminal surface. *APC* is classically considered a tumor suppressor gene that downregulates the *WNT* signalling pathway by binding to β-catenin, resulting in the inhibition of cellular proliferation and differentiation.[40]

Activating point mutations in a *RAS* gene have been detected in about 65% of sporadic CRC. As the majority of *RAS* mutations are thought to take place during intermediate stages of adenoma growth, other events are admitted to occur in order to promote CRC invasion and metastasis. *RAS* gene is an element of the mitogen-activated protein kinase pathway that can become constitutively activated in CRC not only due to *RAS* mutation but also by *BRAF* activating mutations, as an alternative mechanism.

Other genetic modifications can take place later in the adenoma-to-carcinoma sequence of CRC, postulated by Vogelstein, namely *TP53*, *DCC* and *SMAD4*.[41]

On the other hand, there is another type of genomic instability, named microsatellite instability (MSI), in which tumors remain diploid. MSI results from abnormal function in mismatch repair (MMR) genes, which lead to the accumulation of mutations in key genes, eventually resulting in malignancy. Germline mutations in MMR genes are found in hereditary non polyposis colon cancer/Lynch syndrome but also in sporadic CRC. Interestingly, most of sporadic tumors with MSI do not present mutations in the MMR genes, instead, MSI results from the epigenetic silencing of MMR genes, mostly through hypermethylation of the promoter of those genes.[35]

2.2 Colorectal cancer epigenetics

In the last decade, several studies have shown that carcinogenesis involves not only genetic alterations in oncogenes and tumor suppressor genes, but also epigenetic modifications.[35]

Epigenetic modifications are defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. These epigenetic alterations manifest both as global changes in chromatin packaging and localized alterations in binding of regulatory proteins to promoters, affecting transcription of genes critical to tumorigenesis. Epigenetic modifications involves aberrant DNA methylation, post-translational histone covalent modifications and microRNAs (miRNAs) altered expression[42].

One of the first epigenetic modifications described in CRC was global DNA hypomethylation,[43] that was found to be age dependent and an early event in the multistep carcinogenesis of CRC.[44] Global DNA hypomethylation occurs most frequently at CpG dinucleotides in repetitive sequences and in gene coding sequences, and less frequently in CpG islands and CpG island shores.[45] Hypomethylation of DNA in CRC is thought to be associated to genomic instability of the CIN type, because loss of methylation at pericentromeric sites promote recombination and altered chromosome replications.[46]

Alongside hypomethylation, hypermethylation has been also described in CRC, occurring preferentially at CpG islands in the 5´region of genes resulting in the silencing of gene expression. As a result, hypermethylation has the same outcome as a mutation or a deletion. Indeed, one example of promoter hypermethylation in the development of CRC is the biallelic methylation of MMR gene MLH1, which has been associated with sporadic CRC with MSI.[36]

Additionally, a variety of post-translational histone modifications have been also implicated in the epigenetic regulation of gene expression through chromatin remodeling.[47, 48] Chromatin is composed of DNA and nuclear proteins including histones. The nucleosome is the fundamental unit of chromatin and it is composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which base pairs of DNA are wrapped. The histones have long N-terminal “tails” that are exposed to diverse covalent modifications: lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, ADPribosylation, ubiquitylation, sumoylation, deimination and proline isomerization. Despite these different types of histone modifications, only a few have been relatively well studied, including acetylation and methylation.[49] These mechanisms are able to regulate chromatin structure, therefore influencing the interaction with chromatin binding proteins and, ultimately, regulating gene expression.[50] The effects of post-translational histone modifications depend on the amino acid type, the position in the histone tail and the type of modification. Methylation of the histones at lysines or

arginines residues can be found in one of three different states: mono-, di- or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. Moreover, histone modifying enzymes have selectivity for mono-, di- or trimethylated residues. Many of the above mentioned histone modifying enzymes has been shown to promote or inhibit tumorigenesis.[51] All these add complexity to this type of epigenetic mechanism providing a huge potential for different functional control of transcription.[49] There are two possible mechanisms by which histones regulate expression. One of the mechanisms is the disconnections of contacts within and between different histones in adjacent nucleosomes or between histones and DNA, which makes chromatin more accessible for transcription, and the other mechanism is the specific recruitment of nonhistone proteins.[49] In addition, particular modifications of histone proteins are associated with alterations in other histone proteins, revealing a "crosstalk" between different histone modifications [47, 48] and even with DNA methylation *status*.[52]

In general, acetylation of lysine residues of histones is related to the activation of transcription, whereas deacetylation of histones is associated with a repressive effect.[53] Methylation of histones can be either an effective activator or repressor of gene transcription depending on the location of lysine residues affected. Some of the histone modifications implicated in the activation of gene transcription are di and tri-methylation of H3K4, H3K36 and H3K79. On the other hand, histone modifications usually involved in the repression of gene transcription include methylation of H3K9, H3K27 and H4K20.[49]

In cancer, it is known that those chromatin patterns alterations are attributed to the altered expression or activity of key chromatin-modifying enzymes. These alterations might be due to genetic alterations in genes codifying those enzymes.[42, 53] Alterations in histone methyltransferases (such as EZH2, SETDB1 and SMYD3) and in histone demethylases (such as LSD1) expression levels have been found in several neoplasms.[53] Furthermore, particular alterations in the pattern of the histone modifications (such as H3K9me3, H3K27me3 and H3K4me3) have been also associated with various types of tumors.[42, 53]

H3K4 can be methylated or demethylated by several enzymes, the most studied of which are lysine methyltransferases MLL, SET1A/B and SMYD3 and lysine demethylases LSD1 and JARID1, respectively.[49, 54] Methylation of H3K9 is performed mostly by members of the Suv39 family, including SETDB1/2, SUV39H1/2, EHMT1/2, as well as non-Suv39 members such as PRDM2 and ASH1L.[55] The reverse process is catalysed by the Jumonji (JMJ) family of histone

demethylases JMJD1A/B, JMJD2A/B, JMJD2C and JMJD2D.[49] In a similar way, methylation of H3K27 is catalysed by histone methyltransferase EZH2 and its demethylation is catalysed by the JMJ family of histone demethylases KDM6A and KDM6B (JMJD3).[56, 57]

Finally, the action of small noncoding miRNAs silences gene expression through translational repression, accelerated mRNA turnover and heterochromatin formation.[58] as same as for other cancers, in CRC miRNAs affect the intracellular signaling pathways describe above, including WNT and TP53 signaling.[36] Somatic alterations in miRNA-binding sites, affecting the action of miRNAs, has been reported in association with CRC risk[59]. Recently, mutations in the TARBP2, a protein involved in the processing of miRNAs, have been observed in CRC with MSI.[60]

Two questions emerges when one think about all the epigenetic events occurring simultaneously in CRC – How are this events related? Which one is dominant?[61] There has been some data referring that DNA methylation status is an important factor in the post-translational histone modifications.[62] Silencing of miRNAs by promoter CpG island hypermethylation has also been reported. On the other hand, miRNAs are also capable, as described in plants, of transcriptional silencing in human CRC cells, directing promoter CpG hypermethylation and repressing gene expression.[63] Hence, similarly to genetic mutations in DNA sequence, all of the above epigenetic mechanisms contribute to regulate gene transcription and can be responsible for the initiation and progression of tumorigenesis in CRC .

Importantly, the interactions between genetic and epigenetic events should be also considered. Most of the epigenetic modifications end up targeting the same pathways as genetic alterations, eventually acting synergistically. In general, mutations and promoter hypermethylation are considered as mutually exclusive events.[64] Genetic modifications capable of originating epigenetic events include the activation of oncogenic signaling pathways (like *KRAS* and *c-MYC*) and the initiation of promoter CpG methylation at specific loci.[65, 66] Moreover, single nucleotide polymorphisms (SNPs) might play a role in somatic epigenetic modifications, as reported for the MGMT promoter in CRC.[67] Moreover, SNPs in the histone methyltransferase SMYD3 have been associated with CRC risk.[68] Somatic modifications, including mutations and amplifications, in epigenetic proteins have been reported in CRC, namely in DNA methyltransferases, histone acetyltransferases and deacetylases, and histone methyltransferases, which can be also affected by promoter CpG island hypermethylation. [36]

Additionally, epigenetic modifications originating genetic events include promoter CpG island hypermethylation of DNA repair genes MLH1 and MGMT giving rise to MSI and mutations in RAS and TP53, respectively.[69, 70]

Therefore, cancer initiation and progression is a multistep process resulting from altered gene expression through genetic and epigenetic mechanisms, representing the new paradigm of carcinogenesis.[71, 72]

3. Prognosis of colorectal cancer

The relative survival rate for CRC patients is nearly 85% in the first year and 65% in the 5th year after diagnosis. Indeed, the 5-year relative survival rate is as high as 90% when CRC is detected at a localized stage, declining to 70% and then to 12% , when the disease has spread to the regional lymph nodes, and to distant organs, respectivelly. Additionally, up to 40% of patients treated for local or locally advanced CRC will develop recurrence.[25]

The most important prognostic factor in CRC is the tumor stage, which involves the degree of tumor invasion, regional lymph node metastasis and the presence of distant metastasis. Other clinicopathological factors have been considered useful in predicting clinical outcome, as the histological differentiation of the tumor and the existence of vascular invasion, including lymph and venous vessel invasion. Specifically, venous vessel invasion has been correlated, with local recurrence, distant metastases and decreased survival, whereas lymph vessel invasion has been associated only with a decreased survival. It is not clear whether lymph vessel invasion is an independent factor or if is related to tumor invasion or regional lymph node metastasis. All together, these factors are important in predicting patient survival and also in the implementation of the adequate therapy.[41]

Surgical resection is the most elected treatment for CRC, in which the main objective is the complete removal of the tumor and the regional lymph nodes. Locally invasive CRC or regional lymph node metastastatic CRC correlate with high recurrence rates benefiting from an effective adjuvant therapy to eliminate the systemic cancer cells after surgery. The combination of chemotherapy and/or radiation therapy migh also be given to late-stage disease patients' before surgery.[25] The benefits of adjuvant chemotherapy in stage III patients has been conclusive, however the results were equivocal in stage II patients. The definition of the subset

of patients with stage II disease who will benefit from adjuvant therapy needs to be further studied.

Due to the complexity of colon carcinogenesis the clinical outcome of CRC can vary significantly in cases with the same clinic-pathological characteristics. Hence, it is important to subclassify patients with CRC in order to better envisage the prognosis and treatment response. Several efforts have been taken in search for genetic prognostic factors in CRC as well as for epigenetic alterations that might be of prognostic value. Furthermore, disease progression is poorly understood and growing evidence indicates that epigenetic alterations add further complexity to the pathogenesis of CRC. Indeed, some epigenetic markers have been proposed to identify groups of patients with different prognosis and/or with different responses to treatment, particularly among patients with intermediate stages of disease, where management is by far more demanding.[73] Namely, specific histone modifications have been recently shown to predict clinical outcome in multiple cancers, in which an association between histone modification levels and tumor aggressiveness, has been suggested regardless of cancer tissue of origin.[36] Seligson and collaborates showed that global histone modification patterns (H3K4me2 and H3K18ac), assessed by immunohistochemical staining, were predictive of clinical outcome in prostate cancer, identifying two groups of patients with distinct risk of recurrence.[74] Two years later, using the same histone marks, they also found that those marks predicted clinical outcome in lung and kidney cancer patients.[75] Moreover, Bianco-Miotto and collaborates established the same specific histone marks as independent predictors of clinical outcome in prostate cancer.[76] Elsheikh and collaborates identified variations in histone marks with clinical significance in invasive breast cancer, where high levels of histone acetylation (H3K9ac, H3K18ac and H4K12ac) and methylation (H3K4me2, H4K20me3 and H4R3me2) were associated with favorable prognosis.[77] Moreover, expression of histone marks (H3K27me3 and H4K20me3) were not only related with prognosis in breast, but also in ovarian, pancreatic and lung cancers.[78, 79] Additionally, Barlesi and collaborates reported that in non small-cell lung cancer patients, different histone patterns (H2AK5ac and H3K9ac) were associated with different survival rates, providing a rationale for the use of a combination of standard chemotherapy with drugs interacting with histone modifications, namely histone deacetylase inhibitors.[80]

Similar findings have been reported for digestive tract tumors , particularly for esophageal squamous cell carcinoma , in which specific expression of H3K27me3 were found to

be independent predictors of survival.[81] Furthermore, histone modifications might also predict response to certain therapeutic agents, serving as biomarkers that could inform clinical decisions on selecting therapy. Manuyakorn and collaborates found that cellular levels of histone modifications (H3K18ac, H3K4me2 and H3K9me2) might define previously unrecognized subsets of patients with pancreatic adenocarcinoma with distinct response to 5-FU, but not to gemcitabine. The clinical value of predicting response to therapeutics has tremendous implications for single or combinatorial therapies.[82] In this regard, the use of genomic alterations as reliable biomakers for prediction of response to targeted therapy has been fully confirmed in CRC through the mutational analysis of *K-RAS* mutations.[83]

Because histone onco-modifications seem to be highly conserved, they can be informative concerning prognosis and response to therapy in other cancers. Thus, there is a need to examine expression patterns of both histone covalent modifications and histone modifying enzymes, in CRC to define new prognostic markers and therapeutic targets.[73]

Further improvements in the discovery of new epigenetic biomarkers can result in more sensitive and specific methods in noninvasive screening for early detection of CRC. The implementation of these new biomarkers in the clinical practice requires their validation in large independent prospective studies and the cost-effectiveness has to be compared with the screening tests already in use, like colonoscopy or fecal occult blood testing (FOBT). Moreover. new epigenetic biomarkers assessing the precise prognosis of each individual patient can optimize the therapeutic algorithm, which is particularly important in the stages of disease where decisions might be more complex. Accordingly, epigenetic biomarkers could predict the response to CRC therapy, establishing subgroups of patients in which the treatment of choice is more effective and opening the door for individualized therapy based on molecular profile.

Importantly, the reversible nature of epigenetic alterations provides the opportunity for clinical intervention. The enzymes responsible for the modifications of epigenetic pathways represent interesting targets for new anti-cancer therapy. Understanding the most important interconnections between genetic and epigenetic modifications in gene expression and cancer phenotype will have a major impact in the development of new biomarkers for early detection, prediction of prognosis, response to treatment and creation of direct anti-cancer therapy. [72]

However, epigenetic changes can potentially be reversed with inhibitors that block the relevant chromatin-modifying enzymes. Thus, it is important to better understand the role of these epigenetic enzymes in cancer cells with an ultimate goal of developing new cancer treatments.

AIM

In the present work, the overall objective was to characterize the expression of histone modifying enzymes and histone marks in a series of CRC patients, clinically and pathologically well characterized, and to determine their value as biomarkers for assessment of prognosis and of prediction of therapeutic response.

More specifically, the objectives of this study are:

1 - Evaluate the expression of three histone methyltransferases (EZH2, SMYD3 and SETDB1), one histone demethylase (LSD1) and two histone marks (H3K9me3 and H3K27me3) in cancer tissue from patients with CRC using immunohistochemical methods.

2 - Investigate the frequency of altered expression of the abovementioned enzymes and histone marks and further correlate them with clinicopathological data.

3 - Determine the usefulness of the detection of the expression of the abovementioned histone modifying enzymes and histone marks as prognostic and predictive factors in CRC patients.

MATERIALS AND METHODS

1. Tissue samples

A series of 98 colon cancer cases were randomly selected from the archives of Portuguese Oncology Institute-Porto between March 2003 and November 2006. All patients were treated with surgery and none of the patients had any preoperative treatment, such as radiation or chemotherapy.

The surgical specimens underwent pathological observation and all tissue samples were fixed in 10% formalin and embedded in paraffin. The slides obtained from each paraffin block of each case were stained with hematoxilin-eosin.

Clinicopathological information was obtained from clinical charts and pathological reports, referring to age at diagnosis, gender, tumor location, histological classification, grading, staging (depth of invasion, lymph node involvement and distant metastases), lymph vessel invasion, extramural venous vessel invasion and margins *status*.

The tumors were classified according to the World Health Organization Classification of Tumours.[27] The histological grade of tumor differentiation was determined using the College of American Pathology grading system. The clinical stage of tumors was defined according to the TNM classification system of the International Union Against Cancer (7^{th} edition), which assesses the extension of the tumor (T), regional lymph node involvement (N) and the presence of distant metastases (M). Once the T, N and M classifications are determined, a stage of 0, I, II, III or IV is assigned.

2. Immunohistochemical analysis and expression assessment

The expression of four histone modifying enzymes, including three histone methyltransferases (EZH2, SMYD3 and SETDB1) and one histone demethylases (LSD1), and two histone marks (H3K9me3 and H3K27me3) were determined, by immunohistochemistry (IHC), in one representative block for each case. The selected paraffin block contained carcinoma and, whenever possible, apparently normal adjacent mucosa. IHC was performed

using the Novolink^{TM} Polymer Detection System (Novocastra, Newcastle, UK). Briefly, tissue sections of 3 µm in thickness were cut from each paraffin block. Deparaffinized tissue sections were submitted to antigen retrieval in a 700-W microwave oven, in respective buffer solution (Table 1). Endogenous peroxidase activity was blocked by incubating the slides with Peroxidase Block (Novocastra) for 5 min. After washing the slides in Tris-buffered solution with 0.05% Tween 20 solution (TBST), the slides were incubated with Protein Block (Novocastra) for 5 min and, after incubation, the primary antibody for each protein was applied in a humid chamber, at room temperature (Table 1). The slides were then rinsed in TBST and incubated with Post Primary Block (Novocastra) for 30 min followed by incubation for 30 min with the NovoLink Polymer (Novocastra). After washing, the slides were incubated for 7 min in 3,3-diaminobenzidine (DAB; Sigma–Aldrich) in a solution of 50 mL PBS/0,05% mL H_2O_2. Finally, the slides were counterstained with hematoxylin (Harris Modified Hematoxylin Stain; Fisher Scientific, Fair Lawn, NJ, USA) for 20 s and mounted with Entellann (Merck KGaA, Darmstadt, Germany).

Table 1. Optimized conditions for each antibody used in the immunohistochemistry analyses.

Primary antibody	Manufacturer	Clone	Positive control	Retrival antigen	Dilution	Incubation period
EZH2	Novocastra	6A10	Tonsil	Citrato	1:1000	1h
SETDB1	Sigma	Polyclonal	Colon	Citrato	1:500	2h
SMYD3	Abcam	Polyclonal	Seminal vesicles	Citrato	1:500	1h
LSD1	Novus Biologicals	1B2F2	ProstateCarcinoma	EDTA	1:1750	1h
H3K27me3	Millipore	Polyclonal	Colon carcinoma	Citrato	1:1500	1h
H3K9me3	Millipore	CMA308	Colon carcinoma	Citrato	1:500	1h

Assessment of antibody expression was performed using a semi-automated software system (CELLSENS® Imaging Software from Olympus).

Immunohistochemical scoring of colon cancer cells was performed using the modified Histo-score (H-score), which involves quantitative assessment of both the intensity of staining (graded as 0 to 3) and the percentage of positive cells. The scores ranged from 0 to 300 enabling

us to stratify the cases into putative biologically relevant groups depending on different expression levels. Tumor samples with H-score <100 were designated as low detection, where scores >100 were designated high detection. In the specific case of SETDB1 expression, H-score was divided into low and high expression using the mean value.

Concerning SMYD3 expression, qualitative evaluation was used by defining positive and negative expression. Immunostaining of more than 30% of the tumor cells was required for scoring a case as positive. Appropriate positive and negative internal controls were used to validate immunohistochemical expression.

3. Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 for Windows. The Pearson´s chi-square (χ^2) test or Fisher exact probability test were used for comparison of qualitative variables and the t-test or Mann-Whitney U test for quantitative variables.

Univariate analyses of time to death as a result of cancer were performed using the Kaplan-Meier method. Survival rate curves were calculated according to the Kaplan-Meier method and compared by the log-rank test. Multivariate survival analyses were based on the Cox proportional hazard regression model.

The date of resection was considered day zero for survival analysis purposes. The terminal event for cancer-related survival was death attributable only to cancer. Overall survival (OS) time was defined as the interval between surgery and death or between surgery and the last follow-up time for surviving patients. The level of significance was set at $p < 0.05$ and the Bonferroni's correction was used when appropriate.

RESULTS

1. Clinicopathological parameters and survival analysis

In the 3-year and 8 month-period covered by this study, 98 randomly cases of CRC were retrieved from the archives of Portuguese Oncology Institute-Porto. Of the 98 CRC cases, 48% (n=47) were diagnosed in women and 52% (n=51) in men, with a median age at diagnosis of 68.5 years (69 years for men and 66 years for women). Median follow-up time was 77.5 months (range, 3-111 months). More than half of the cases (71.4%) were identified in the left colon (n=70). The cases were classified as adenocarcinomas and the percentage with tubular pattern was 94.9% (n=93) whereas the mucinous pattern was observed in 5.1% (n=5) of the cases. The adenocarcinomas with tubular pattern were graded as low-grade (90.3%) or high-grade (9.7%). Lymph vessel invasion was observed in 41.8% (n=41) and venous vessel invasion in 28.6% (n=28) of cases. Additionally, tumor involvement of at least one of the surgical margins was recorded in only 3.1% of the cases. From the selected CRC cases, 28.6% (n=28) were diagnosed at a localized stage, 50% (n=49) displayed lymph node metastases and 27.6% (n=27) displayed distant metastases at the time of diagnosis or later. Metastases were identified mainly in the liver (46.5%), peritoneum (23.3%) and lung (9.3%). The main clinicopathological features of the 98 selected CRC cases are depicted in Table 2.

Table 2. Clinicopathological features of the CRC cases.

Clinicopathological features	% of cases (number of cases)
Age [a]	
≤67 years	45.9% (45)
>67 years	54.1% (53)
Gender	
Female	48% (47)
Male	52% (51)
Tumor location	
Cecum	12.2% (12)
Ascending colon	10.2% (10)
Hepatic flexure	5.1% (5)
Transverse colon	1% (1)
Splenic flexure	7.1% (7)
Descending colon	6.1% (6)
Sigmoid colon	58.2% (57)
Tumor pattern	
Tubular	94.9% (93)
Mucinous	5.1 (5)
Tumor grading	
Low grade	90.3% (84)
High grade	9.7% (9)
Lymph vessel invasion	
Not observed	58.2% (57)
Observed	41.8% (41)
Venous vessel invasion	
Not observed	71.4% (70)
Observed	28.6% (28)
Margins *status*	
R0	96.9% (95)
R1	1% (1)
R2	2% (2)
pT	
T1	5.1% (5)
T2	23.5% (23)
T3	57.1% (56)
T4a	3.1% (3)
T4b	11.2% (11)
pN	
N0	50% (49)
N1a	12.2% (12)
N1b	18.4% (18)
N2a	11.2% (11)
N2b	8.2% (8)
pM	
M0	74.5% (79)
M1a	12.3% (13)
M1b	13.2% (14)
pTNM Stage	
I	25.5% (25)
II	16.3% (16)
III	30.6% (30)
IV	27.6% (27)

The overall survival rate of the 98 patients was 95.9% and 73.5% at one and five years of follow-up, respectively. Univariate survival analysis showed a significant association between a decrease in overall survival and the occurrence of venous vessel invasion (ρ=0.003), positive surgical margins (ρ=0.009), regional lymph node metastasis (ρ=0.011) and distant metastasis (ρ<0.001), as well as with tumor stage III or IV (ρ=0.004). The remaining parameters were not significantly associated with overall survival (Table 3).

Table 3. Univariate analyses of the association between clinicopathological features and overall survival[a].

Clinicopathological features	Mean (months)	ρ[b]
Age (≤67 years/>67 years) [c]	90.800/87.075	ns
Gender (female/male)	86.617/90.784	ns
Tumor location (right/left)	90.214/87.914	ns
Tumor pattern (tubular/mucinous)	88.323/79.800	ns
Tumor grade (low/high)	87.167/99.111	ns
Lymph vessel invasion (no/yes)	92.175/83.024	ns
Venous vessel invasion (no/yes)	95.943/70.893	**0.003**
Margins *status* (R0/R1+R2)	90.232/32.000	**0.009**
pT (T1+T2/T3+T4)	97.429/85.000	ns
pN (N0/N1+N2)	98.551/79.020	**0.011**
pM (M0/M1)	97.268/65.037	**<0.001**
pTNM Stage (I+II/III+IV)	101.756/79.456	**0.004**

ns – not significant
[a] Kaplan-Meier method was used
[b] Log-rank ρ-value
[c] Mean age at the time of diagnosis

In multivariate analysis, using Cox proportional hazard regression model, only the M stage was independently associated with decreased overall survival (ρ=0.011) whereas a trend for lower survival was depicted for patients with positive surgical margins (ρ=0.058) (Table 4).

Table 4. Multivariate analyses of the association between clinicopathological features and overall survival[a].

Clinicopathological features	**Hazard ratio (95% CI)**	***ρ***
Venous vessel invasion (no/yes)	1.695 (0.724-3.699)	ns
Margins *status* (R0/R1+R2)	4.521 (0.949-21.545)	ns
pN (N0/N1+N2)	2.102 (0.583-7.576)	ns
pM (M0/M1)	3.465 (1.332-9.011)	**0.011**
pTNM Stage (I+II/III+IV)	0.755 (0.127-4.485)	ns

CI - confidence interval
ns – not significant
[a] Cox proportional survival regression model was used

2. Immunohistochemical analyses

Considering the histone modifying enzymes, the expression of EZH2, SETDB1 and LSD1 were only observed in the nuclei of the cells, whilst the expression of SMYD3 was detected both in the cytoplasm and cell membrane. All enzymes, excepting for SETDB1, showed higher expression in tumor cells than in epithelial cells of the adjacent normal mucosa (Table 5a and 5b). Immunoreactivity for both histone marks, H3K9me3 and H3K27me3, was only found in nuclei. The highest expression was observed in the tumor cells (Table 5a), which displayed a rather homogenous pattern of staining.

Table 5a. Differences between the expression of the selected enzymes or histone marks in the nuclei of tumor cells and their expression in the normal mucosa.

Enzyme/Mark	**Topography of the expression** (mean±sd)		
	Normal mucosa	Tumor cells	ρ
EZH2	39.199±26.676	82.328±55.272	**<0.001**[a]
SETDB1	50.319±9.788	47.822±19.557	ns[b]
LSD1	141.861±19.325	211.020±71.787	**<0.001**[a]
H3K9me3	97.713±67.500	176.884±85.452	**<0.001**[b]
H3K27me3	58.434±12.568	135.228±63.274	**<0.001**[b]

sd – standard deviation
ns – not significant
[a] Mann-Whitney U test
[b] t test

Table 5b. Differences between the expression of SMYD3 in the cytoplasm and cell membrane of tumor cells and its expression in the normal mucosa.

Enzyme	Topography of the expression (% of positive cases)		
	Normal mucosa	Tumor cells	ρ
SMYD3	0	100	**<0.001**[a]

[a] Pearson Chi-Square

Table 5c. Univariate analyses of the association between the expression of the selected enzymes or histone marks in CRC and respective overall survival[a].

Enzyme/Mark	Mean (months)	p[b]
EZH2 (low/high)	90.254/86.143	ns
SETDB1 (low/high)	91.254/83.897	ns
LSD1 (low/high)	86.333/88.798	ns
H3K9me3 (low/high)	82.542/90.162	ns
H3K27me3 (low/high)	96.233/85.147	ns

ns – not significant
[a] Kaplan-Meier method was used
[b] Log-rank ρ-value

Table 5d. Univariate analyses of the association between the expression of the selected enzymes or histone marks in CRC with different therapeutics and respective overall survival[a].

Enzyme/Mark	Mean – months (ρ[b]) Treatment			
	Surgery	**Surgery + 5-FU/Leucovorine**	**Surgery + Folfiri**	**Surgery + Folfox**
EZH2 (low/high)	104.778/95.143 (ns)	78.800/86.000 (ns)	36.750/31.250 (ns)	64.214/79.467 (ns)
SETDB1 (low/high)	106.500/91.867 (**0.034**)	NC	28.667/37.200 (ns)	70.000/69.333 (ns)
LSD1 (low/high)	NC	NC	18.000/36.286 (**0.008**)	64.500/77.880 (ns)
H3K9me3 (low/high)	94.182/102.733 (ns)	24.000/92.250 (**0.005**)	20.500/38.500 (**0.019**)	48.667/83.174 (ns)
H3K27me3 (low/high)	105.813/98.560 (ns)	32.000/99.714 (**0.018**)	32.000/34.286 (ns)	NC

NC – not computed

ns – not significant

[a] Kaplan-Meier method was used
[b] Log-rank ρ-value

2.1. EZH2 expression in CRC cases

The immunoexpression of EZH2 in CRC cases is detailed in table 6. EZH2 score ranged from 0 to 274.6 (median: 56.1) and 35 cases (35.7%) disclosed high expression score. EZH2 expression was significantly higher in deeply invasive CRCs (pT3 and pT4) (ρ=0.004) and in CRCs with lymph node metastasis (pN1 or pN2) (ρ=0.019). The number of cases with low expression of EZH2 was higher in tumors located in the left colon (ρ=0.020). EZH2 expression was neither associated with overall survival rate of the CRC patients (Table 5c) nor with survival rate amongst patients treated with different chemotherapy regimens (Table 5d).

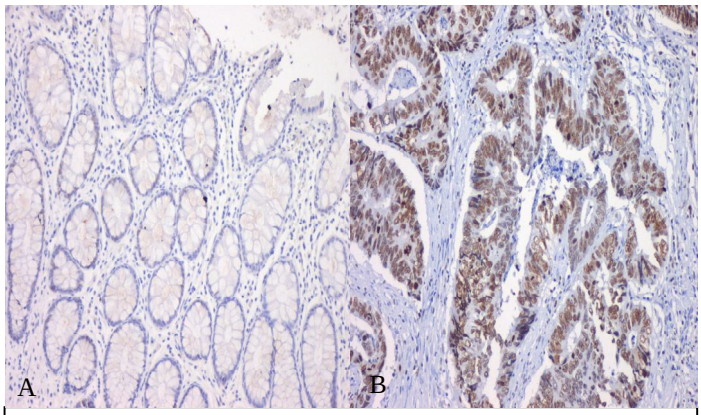

Figure 1 – EZH2 expression in normal mucosa (A) and in CRC cells (B).

Table 6. Analyses of the association between clinicopathological features and EZH2 expression.

Clinicopathological features	**EZH2 expression** (mean±sd)	***p*** [b]	**EZH2 low/high expression** (number of cases)	***p***
Age [a]				
≤67 years	88.348±54.527	ns	27/18	ns [c]
>67 years	77.217±55.901		36/17	
Gender				
Female	84.204±53.484	ns	29/18	ns [c]
Male	80.600±57.347		34/17	
Tumor location				
Right	98.853±67.907	ns	13/15	**0.020** [c]
Left	75.718±48.323		50/20	
Tumor pattern				
Tubular	80.768±54.641	ns	62/31	ns [d]
Mucinous	111.356±65.592		1/4	
Tumor grading				
Low	81.468±55.382	ns	56/28	ns [d]
High	74.234±49.600		6/3	
Lymph vessel invasion				
No	71.646±49.575	ns	41/16	ns [c]
Yes	97.179±59.824		22/19	
Venous vessel invasion				
No	77.837±55.214	ns	48/22	ns [c]
Yes	93.556±54.786		15/13	
Margins *status*				
R0	83.262±55.888	ns	60/35	ns [d]
R1+R2	52.760±3.311		3/0	
pT				
T1+T2	59.045±43.727	**0.004**	23/5	**0.020** [c]
T3+T4	91.641±56.908		40/30	
pN				
N0	72.138±56.749	**0.019**	35/14	ns [c]
N1+N2	92.518±52.356		28/21	
pM				
M0	86.826±57.105	ns	43/28	ns [c]
M1	70.500±49.167		20/7	
pTNM Stage				
I+II	78.417±59.696	ns	27/14	ns [c]
III+IV	85.142±52.220		36/21	

ns – not significant
[a] Mean age at the time of diagnosis
[b] Mann-Whitney U test
[c] Pearson Chi-Square
[d] Fisher´s Exact test

2.2. SETDB1 expression in CRC cases

The immunoexpression of SETDB1 in CRC cases are disclosed in table 7. SETDB1 score ranged from 5.9 to 98.9 (median: 43.4) and high expression was observed in 39 cases (39.8%). SETDB1 expression was significantly higher in CRCs located in the left colon compared to those located in the right colon (ρ=0.032). SETDB1 expression was not associated with overall survival rate of the CRC patients (Table 5c). However, high SETDB1 expression was associated with poorer survival rate within patients treated with only surgery (stages I and II) (ρ=0.034) (Table 5d).

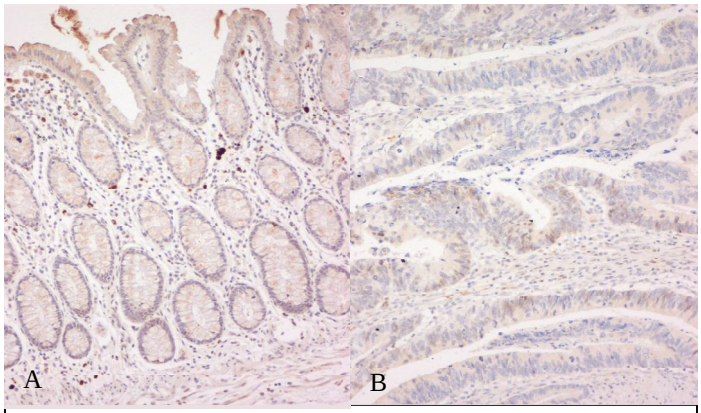

Figure 2 – SETDB1 expression in normal mucosa (A) and in CRC cells (B).

Table 7. Analyses of the association between clinicopathological features and SETDB1 expression.

Clinicopathological features	**SETDB1 expression** (mean±sd)	***p*** [b]	**SETDB1 low/high expression** (number of cases)	***p***
Age [a]				
≤67 years	49.854±19.414	ns	26/19	ns [c]
>67 years	46.098±19.696		33/20	
Gender				
Female	45.227±20.078	ns	31/16	ns [c]
Male	50.215±18.946		28/23	
Tumor location				
Right	41.142±17.729	**0.032**	20/8	ns [c]
Left	50.494±19.732		39/31	
Tumor pattern				
Tubular	47.131±19.399	ns	58/35	ns [d]
Mucinous	60.690±20.049		1/4	
Tumor grading				
Low	47.283±19.800	ns	53/31	ns [c]
High	45.706±16.044		5/4	
Lymph vessel invasion				
No	44.991±18.040	ns	36/21	ns [c]
Yes	51.759±21.085		23/18	
Venous vessel invasion				
No	46.380±18.641	ns	44/26	ns [c]
Yes	51.429±21.615		15/13	
Margins *status*				
R0	47.941±19.766	ns	57/38	ns [d]
R1+R2	44.063±12.884		2/1	
pT				
T1+T2	46.445±16.950	ns	18/10	ns [c]
T3+T4	48.373±20.596		41/29	
pN				
N0	45.241±16.705	ns	30/19	ns [c]
N1+N2	50.403±21.915		29/20	
pM				
M0	46.922±19.232	ns	44/27	ns [c]
M1	50.191±20.569		15/12	
pTNM Stage				
I+II	45.301±17.918	ns	26/15	ns [c]
III+IV	49.636±20.620		33/24	

ns – not significant
[a] Mean age at the time of diagnosis
[b] t test
[c] Pearson Chi-Square
[d] Fisher´s Exact test

2.3. SMYD3 expression in CRC cases

SMYD3 expression was positive in all CRC cases and no association with standard clinicopathological features was disclosed.

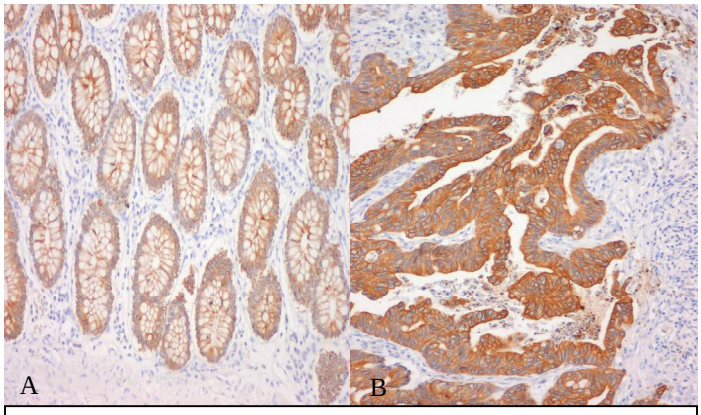

Figure 3 – SMYD3 expression in normal mucosa (A) and in CRC cells (B).

2.4. LSD1 expression in CRC cases

Table 8 summarizes the immunohistochemical expression of LSD1 in the 98 CRC cases. LSD1 score ranged from 0 to 297.5 (median: 238) and 89 cases (90.8%) disclosed overexpression. LSD1 expression was significantly lower in deeply invasive (pT3 and pT4) and advanced stage (stages III and IV) tumors (ρ=0.016 and ρ=0.009, respectively). LSD1 expression was not associated with overall survival rate of the CRC patients (Table 5c). However, high LSD1 expression was associated with better survival rate within patients treated with Folfiri (ρ=0.008) (Table 5d).

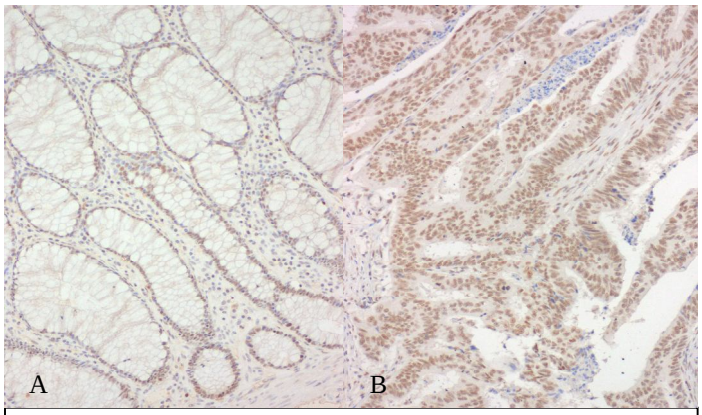

Figure 4 – LSD1 expression in normal mucosa (A) and in CRC cells (B).

Table 8. Analyses of the association between clinicopathological features and LSD1 expression.

Clinicopathological features	**LSD1 expression** (mean±sd)	***p*** [b]	**LSD1 low/high expression** (number of cases)	***p*** [c]
Age [a]				
≤67 years	200.507±76.918	ns	6/39	ns
>67 years	219.945±66.559		3/50	
Gender				
Female	204.560±77.242	ns	5/42	ns
Male	216.972±66.582		4/47	
Tumor location				
Right	207.653±82.894	ns	4/24	ns
Left	212.366±67.449		5/65	
Tumor pattern				
Tubular	210.521±71.708	ns	8/85	ns
Mucinous	220.284±81.171		¼	
Tumor grading				
Low	209.816±73.610	ns	8/76	ns
High	217.102±53.517		0/9	
Lymph vessel invasion				
No	215.934±64.608	ns	3/54	ns
Yes	204.188±81.061		6/35	
Venous vessel invasion				
No	213.625±71.607	ns	6/64	ns
Yes	204.505±73.135		3/25	
Margins *status*				
R0	211.619±72.229	ns	9/86	ns
R1+R2	192.027±64.658		0/3	
pT				
T1+T2	238.029±56.619	**0.016**	0/28	ns
T3+T4	200.216±74.671		9/61	
pN				
N0	222.240±63.225	ns	2/47	ns
N1+N2	199.799±78.484		7/42	
pM				
M0	218.059±70.039	ns	5/66	ns
M1	192.507±74.349		4/23	
pTNM Stage				
I+II	234.346±54.849	**0.009**	0/41	**0.009**
III+IV	194.241±78.055		9/48	

ns – not significant
[a] Mean age at the time of diagnosis
[b] Mann-Whitney U test
[c] Fisher´s Exact test

2.5. H3K9me3 expression in CRC cases

The immunoexpression of H3K9me3 in CRC cases is presented in table 9. H3K9me3 immunoreactivity score ranged from 0 to 299.7 (median: 173.1) and 74 cases (75.5%) disclosed high expression. The expression of H3K9me3 in CRC was significantly higher in those with lymph node metastases (pN1 or pN2) (ρ=0.044). H3K9me3 expression was not associated with overall survival rate of the CRC patients (Table 5c). However, high H3K9me3 expression was associated with better survival rate within patients treated with 5-FU/Leucovorine and Folfiri (ρ=0.005 and ρ=0.019, respectively) (Table 5d).

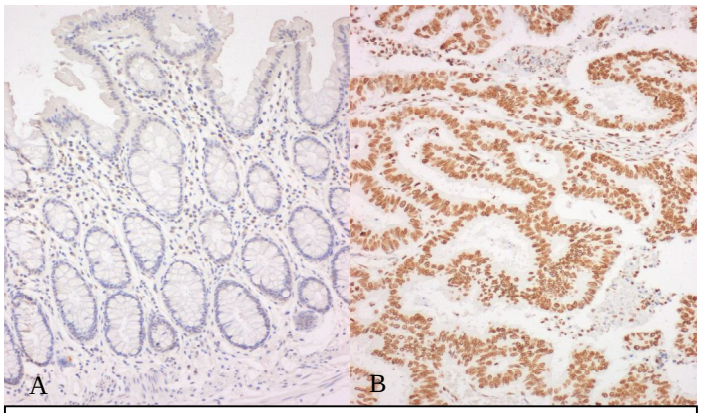

Figure 5 – H3K9me3 expression in normal mucosa (A) and in CRC cells (B).

Table 9. Analyses of the association between clinicopathological features and H3K9me3 immunoreactivity.

Clinicopathological features	**H3K9me3 expression** (mean±sd)	***p*** [b]	**H3K9me3 low/high expression** (number of cases)	***p***
Age [a]				
≤67 years	167.744±86.688	ns	12/33	ns [c]
>67 years	184.645±84.431		12/41	
Gender				
Female	168.943±81.639	ns	12/35	ns [c]
Male	184.203±88.996		12/39	
Tumor location				
Right	173.921±77.951	ns	7/21	ns [c]
Left	178.070±88.783		17/53	
Tumor pattern				
Tubular	178.672±84.543	ns	23/70	ns [d]
Mucinous	143.642±105.948		¼	
Tumor grading				
Low	179.561±86.555	ns	21/63	ns [d]
High	170.372±66.211		2/7	
Lymph vessel invasion				
No	174.401±89.373	ns	15/42	ns [c]
Yes	180.337±80.646		9/32	
Venous vessel invasion				
No	174.842±88.226	ns	18/52	ns [c]
Yes	181.991±79.393		6/22	
Margins *status*				
R0	176.071±84.927	ns	23/72	ns [d]
R1+R2	202.657±118.866		½	
pT				
T1+T2	170.245±97.977	ns	9/19	ns [c]
T3+T4	179.540±80.522		15/55	
pN				
N0	159.539±95.177	**0.044**	17/32	**0.019** [c]
N1+N2	194.230±71.297		7/42	
pM				
M0	183.038±85.622	ns	15/56	ns [c]
M1	160.702±84.434		9/18	
pTNM Stage				
I+II	171.128±92.857	ns	11/30	ns [c]
III+IV	181.025±80.297		13/44	

ns – not significant
[a] Mean age at the time of diagnosis
[b] t test
[c] Pearson Chi-Square
[d] Fisher´s Exact test

2.6. H3K27me3 expression in CRC cases

The immunoexpression of H3K27me3 in CRC cases is reported in table 10. Concerning H3K27me3 imunoreactivity, the score ranged from 0 to 298.3 (median: 141.7) and 68 cases (69.4%) disclosed high expression (Table 10). The expression of H3K27me3 in CRC was significantly higher in tumors that presented lymph vessel invasion (ρ=0.041). The number of CRC cases with high immunoreactivity for H3k27me3 was higher in CRC cases with venous vessel invasion (ρ=0.027). H3K27me3 immunoreactivity was significantly higher in deeply invasive CRCs (pT3 and pT4) and in CRCs with lymph node metastases (pN1 and pN2) (ρ=0.007 and ρ=0.000, respectively), as well as in advanced stage (III and IV) tumors (ρ=0.014). H3K27me3 expression was not associated with overall survival rate of the CRC patients (Table 5c). However, high H3K27me3 expression was associated with better survival rate within patients treated with 5-FU/Leucovorine (ρ=0.018) (Table 5d).

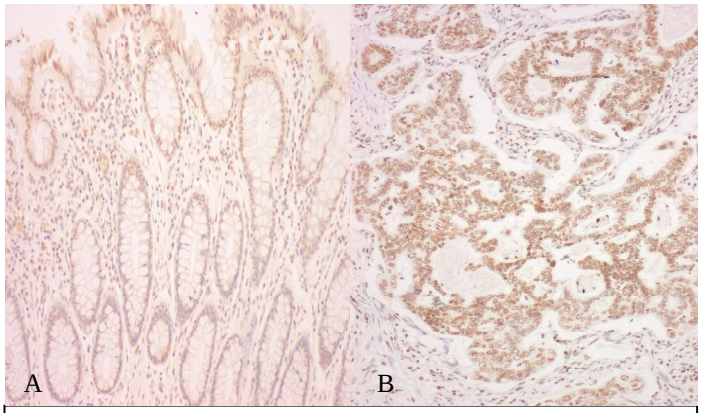

Figure 6 – H3K27me3 expression in normal mucosa (A) and in CRC cells (B).

Table 10. Analyses of the association between clinicopathological features and H3K27me3 immunoreactivity.

Clinicopathological features	**H3K27me3 expression** (mean±sd)	***p*** [b]	**H3K27me3 low/high expression** (number of cases)	***p***
Age [a]				
≤67 years	133.696±63.317	ns	13/32	ns [c]
>67 years	136.529±63.814		17/36	
Gender				
Female	131.713±63.567	ns	17/30	ns [c]
Male	138.468±63.460		13/38	
Tumor location				
Right	136.894±60.764	ns	8/20	ns [c]
Left	134.562±64.668		22/48	
Tumor pattern				
Tubular	134.737±64.107	ns	29/64	ns [d]
Mucinous	144.368±49.556		1/4	
Tumor grading				
Low	137.074±64.404	ns	24/60	ns [d]
High	112.929±60.288		5/4	
Lymph vessel invasion				
No	124.187±63.702	**0.041**	21/36	ns [c]
Yes	150.578±60.104		9/32	
Venous vessel invasion				
No	129.249±68.724	ns	26/44	**0.027** [c]
Yes	150.176±44.600		4/24	
Margins *status*				
R0	134.099±63.828	ns	30/65	ns [d]
R1+R2	170.980±26.769		0/3	
pT				
T1+T2	108.333±60.343	**0.007**	13/15	**0.032** [c]
T3+T4	145.987±61.583		17/53	
pN				
N0	111.134±58.244	**0.000**	22/27	**0.002** [c]
N1+N2	159.322±59.271		8/41	
pM				
M0	135.295±63.034	ns	20/51	ns [c]
M1	135.043±65.111		10/17	
pTNM Stage				
I+II	116.848±59.342	**0.014**	16/25	ns [c]
III+IV	148.449±63.200		14/43	

ns – not significant
[a] Mean age at the time of diagnosis
[b] t test
[c] Pearson Chi-Square
[d] Fisher´s Exact test

3. Evaluation of the association between histone modifying enzymes and histone marks

High levels of EZH2 expression were associated with high levels of H3K9me3 and H3K27me3 immunoreactivity in tumor cells (Pearson correlation=0.362; ρ<0.001 and Pearson correlation=0.311; ρ=0.002, respectively). Moreover, SETDB1 expression was not associated with H3K9me3 immunoreactivity (Pearson correlation=-0.019; ρ=ns). Finally, high levels of H3K9me3 immunoreactivity were associated with high H3K27me3 immunoreactivity in CRC cases (Pearson correlation=0.542; ρ<0.001).

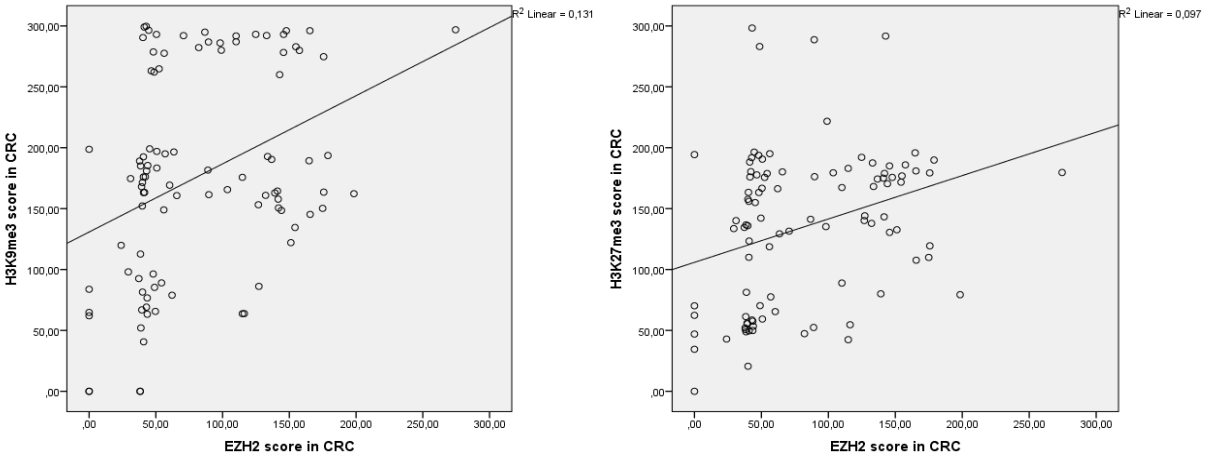

Figure 7 – Correlation of EZH2 expression with H3K9me3 expression (A) and H3K27me3 expression (B).

DISCUSSION

In the present study we aimed at the characterization of the patterns of expression of histone modifying enzymes and histone marks in a series of 98 CRC patients, well characterized both clinically and pathologically, to determine their value as biomarkers for assessment of prognosis and of prediction of therapeutic response. We found that the occurrence of distant metastasis was independently associated to low survival rate of CRC patients. The histone methyltransferases EZH2 and SMYD3 and the histone demethylase LSD1 were overexpressed in CRC cells, as well as the immunoreactivity of histone marks H3K9me3 and H3K27me3. The expression of histone modifying enzymes and the expression of histone marks were associated with several clinocopathological features, including lymph and venous vessel invasion, depth of invasion, regional lymph node metastases and clinical stage. Regarding the response to treatment, we observed higher survival rates for patients with CRCs treated only with surgery disclosing lower SETDB1 expression and higher survival rates for patients with CRC treated with Folfiri or 5-FU/Leucovorine disclosing high LSD1 and H3K9me3 expression or high H3K9me3 and H3K27me3 expression in cancer cells, respectively.

1. Clinicopathological features and patient outcome

The clinicopathological features of our series of CRCs are consistent with the features of other CRC series reported in the literature, with the exception of a lower median age at the diagnosis in female patients, which is 66 years compared with 72 years reported in larger series.[32, 84-86] In addition, the number of low grade and stage I CRCs in our series was higher than in other larger reported series.[84] Because our series is composed of cases admitted for primary surgical treatment at Portuguese Oncology Institute-Porto, it is likely that these discrepancies merely reflect a different profile of CRCs in different populations. There are not, however, similar series from other Portuguese healthcare institutions with which we could compare our cases to definitely clarify these findings.

Several standard clinicopathological parameters have been previously described to be associated to CRC prognosis including age, gender, tumor grading, lymph vessel invasion, venous vessel invasion, lymph node metastases, distant metastases and staging.[84, 86] Although in our series many of those parameters were of prognostic significance in univariate analysis, only

the occurrence of distant metastasis was independently associated to lower survival rate. This result is probably derived from the relatively small size of our series, which impacts negatively on statistical analysis. Thus, an extension of the number of cases enrolled in this study should be considered.

In comparison with a larger series reported by Siegel and collaborators, that documented a survival rate of 85% in the first year and 65% in the fifth year[25], we observed survival rates of 95.9% in the first year and 73.5% in the fifth year in our series, probably reflecting the higher proportion of low grade and low stage CRC, as previously mentioned. Indeed, localized stage CRC had a 5-year survival rate of 88% but when regional lymph node involvement was present, the 5-year survival rate decreased to 61.2%, in line with other series.[25]

2. Expression of histone methyltransferases and demethylases in CRC

Concerning immunohistochemical analysis, we observed that expression of the histone modifying enzymes EZH2, SMYD3, and LSD1 in CRC cells were significantly higher than in the adjacent, morphologically normal colonic mucosa, suggesting a role for these enzymes in the neoplastic transformation of colorectal mucosa cells. Importantly, the expression of both H3K9me3 and H3K27me3 was significantly associated with the increased of EZH2 expression. Because EZH2 is the enzyme responsible for these two repressive marks, we might postulate that EZH2 overexpression translates into dysfunctional expression of specific genes in CRC. In fact, EZH2 is a member of the Polycomb group (PcG) proteins involved in cell cycle regulation and proliferation. EZH2 catalyzes the subunit of the polycomb-repressor complex (PRC) by methylating lysine 9 and 27 of histone H3. Thus, polycomb target genes are often silenced by histone deacetylation and DNA methylation of CpG islands owing to the ability of PcG proteins to bind to histone deacetylases (HDAC) and recruit DNA methyltransferases (DNMT)[87], thereby reducing gene expression in various tissues. Hence, both histone methylation induced by EZH2, histone deacetylation induced by HDAC and DNA promoter methylation induced by DNMT play a synergistic role in gene expression silencing. Although high levels of EZH2 expression were significantly associated with increased H3K9me3 and H3K27me3 expression, variations in EZH2 expression only explain approximately 13% of the variability of H3K9 expression and 10% of the variability of H3K27 expression. Thus, other factors are likely to play an important role in the H3K9 and H3K27 methylation in CRC, including regulators of EZH2 activity, other

histone methyltransferases, such as SUV39H1/2, EHMT1/2 or PRDM2, or even histone demethylases, such as JMJD1A/B, JMJD2A/B or JMJD3.

Recently, several studies have demonstrated that EZH2 expression is commonly upregulated in several solid tumors, including those of colon[88], breast[89, 90], bladder[91, 92], prostate[93], gastric [94], liver[95], lung[96], skin and soft tissue sarcomas[97], as well as in lymphoma.[98-101] Additionally, high expression levels of EZH2 have been associated with increased cell proliferation, whilst its inhibition led to growth arrest in various cancer cell lines, including colon cancer cell lines.[88, 102] Therefore, inhibition of EZH2 expression has been proposed to inhibit tumor progression.[53] Interestingly, we found that EZH2 was highly expressed in the more invasive CRCs as well as in cases with lymph node metastasis. These results are consistent with previous studies suggesting a relationship between EZH2 expression, tumor growth and cell invasion in CRC.[94] Remarkably, EZH2 high expression levels have been found to be associated with tumor progression and poor prognosis in prostate, breast, gastric, skin and non-small lung cancer.[89, 93, 94, 96, 103-105] Contrarily, in CRC high EZH2 expression levels were independently associated with a better prognosis.[96, 106] In our study, EZH2 expression was neither associated with overall survival rate nor with survival rate amongst patients treated with the therapeutical agents previously described, and, thus, no definitive conclusions about the prognostic value of EZH2 in CRC can be drawn.

The previous findings of the association between increased EZH2 levels and better prognosis is CRC are difficult to explain biologically. Indeed, EZH2 is controlled by the E2F transcription factors that regulate the transition from G_2 to the mitotic phase of the cell cycle by increasing the expression of cyclin E.[107, 108] Although the downstream signaling of EZH2 in neoplasia is poorly understood, apparently EZH2 is able to reduce the transcriptional repression of the cyclin A promoter by competing with HDAC1[109]. This reveals a critical role in the regulation of cyclin A expression, known to be essential for cell cycle progression. Moreover, several studies showed that EZH2 is a transcriptional repressor of RUNX3 expression, a known tumor suppressor gene in several cancers, including CRC.[110-112] RUNX3 is a member of the Runt-related (RUNX) family of genes with important roles in normal development and carcinogenesis. It has been shown that RUNX3 up-regulates p21, an important element in cell cycle control by inhibitin cyclin-dependent kinase[113]. Accordingly, not only EZH2 plays an important role in the progression of the cell cycle by increasing cyclin A, but also in the release

of p21, one important inhibitor of cyclin E. Similarly, EZH2 has been found to affect tumor cell proliferation through down-regulation of RUNX3 [110]. Moreover, RUNX3 is also a determinant for up-regulation of Bim, a proapoptotic protein, thus demonstrating a crucial role in the induction of apoptosis[114]. In addition, this EZH2 target gene negatively regulates Wnt signaling activity, which is overexpressed in colon cancer playing a crucial role in tumor initiation, through interaction with the b-catenin/T cell factor 4 (TCF4) transcription factor complex[115]. Because RUNX3 inactivation is primarily conducted by epigenetic mechanisms, its reactivation by specific inhibitors of EZH2 could represent a promise option for CRC treatment.

SETDB1, a histone methyltransferase responsible for the trimethylation of lysine 9 of histone H3 which promotes the transcriptional repression of genes, exhibits a methyl-CpG-binding domain (MBD) and functions in association with DNA methylation.[116-118] Contrarily to EZH2, little information is available concerning SETDB1 expression in cancer cells.[119, 120] Remarkably, SETDB1 has been reported to promote H3K9 trimethylation patterns at the RASSF1A promoter[116], a tumor suppressor gene frequently silenced in lung, bladder, prostate, gastric and breast cancers[121, 122]. Watanabe and collaborates demonstrated that inhibition of SETDB1 in non-small cell lung cancer cell lines resulted in cell cycle arrest[123]. In contrast to the tumor promoting role reported in previous studies, SETDB1 has been found to inhibit Wnt target genes in an animal model of CRC[124], favoring a protective role of SETDB1 in this type of cancer. In our series, SETDB1 expression in CRC cells did not differ significantly from the expression in the adjacent morphologically normal mucosa, and it was not associated with prognostically relevant clinicopathological features of CRC. Interestingly, however, SETDB1 expression was significantly higher in left-sided CRCs, suggesting a putative role of this histone modifier enzyme in the carcinogenesis of chromosomic instable CRCs, which predominate in the left colon.[125] Levels of SETDB1 expression were not associated with H3K27me3 expression levels, suggesting that SETDB1 does not play the major role in H3K27 methylation in CRC. Moreover, SETDB1 expression was neither associated with overall survival rate of the CRC patients nor with survival rate amongst patients treated with the therapeutic agents previously described. However, high levels of SETDB1 expression were associated with decreased survival rate in stage I and II, treated with surgery alone, suggesting that whenever the expression of SETDB1 occurs, the surgical treatment may not be enough to prolong the patient's survival.

SMYD3 is a histone methyltransferase that specifically binds to DNA sequences in the promoter region of target genes and methylates lysine 4 of histone H3 (H3K4), leading to gene activation.[126] Upregulation of SMYD3 expression in cultured cells increased cell growth whereas dowregulation of SMYD3 expression had the opposite effect. Foreman and collaborates found that SMYD3 also methylates lysine 20 in histone H4 (H4K20), a well known repressive histone mark.[127] Therefore, SMYD3 overexpression is thought to activate normally silent oncogenes and repress tumor suppressor genes, contributing to cancer initiation and progression.[51] These observations disclose the complexity of the epigenetic regulation and the relevance of targeting SMYD3 expression in the appropriate pathway. Although microarray analysis showed upregulation of SMYD3 expression in several cancers, including CRC, hepatocellular carcinoma and breast carcinoma [126, 128], only a few direct target genes are known, including cell cycle regulators and oncogenes.[126, 129-131] The canonical activation of the Wnt/β-catenin pathway is one of the most frequent signaling alterations in CRC and it has been demonstrated that *WNT10B* is a direct downstream gene activated by SMYD3.[132] Since downregulation of SMYD3 expression in cancer cell lines significantly reduces cell growth, therapeutic blockage of this pathway could prove useful in CRC.[126, 128] Finally, SMYD3 increases expression of MMP-9, one of the matrix metalloproteinase (MMP) proteins that play crucial functions in carcinogenesis and metastasis development by regulating cell migration and invasion.[134]

In the present study, SMYD3 was expressed in all CRC cases and was not associated with any of the considered clinicopathological features. This result is consistent with previous studies and supports the hypothesis that this histone modifier enzyme may play a role in CRC initiation. Interestingly, in our cases the expression of SMYD3 was detected in the cell membrane and cytoplasm of CRC cells, with only residual nuclear expression. Hamamoto and collaborates had already shown that subcellular localization of SMYD3 changes according to the cell cycle, accumulating in the nucleus at S phase and G2-M and accumulating in the cytoplasm when cells are arrested at Go-G1.[126] He and collaborates detected SMYD3 expression predominantly in the cytoplasm, but also in the nucleus, in hepatocellular carcinoma.[135] As far as we know, this is the first observation of SMYD3 expression almost exclusively in the cell membrane and in the cytoplasm in a malignant neoplasm. The explanation for this finding may be related with the fact that histone modifying enzymes can also operate on non-histone proteins,

through acetylation and methylation in specific residues, although only a few examples of lysine methylation have been described. Curiously, almost all of this non-histone proteins reported, including transcription factors, are related with gene regulation.[136] The p53 protein is an example of non-histone protein known to be regulated by lysine methylation. Both activation and inactivation of this protein can be achieved according to the specific lysine residue methylated. Interestingly, SMYD2 is a lysine methyltransferase able to methylate and silence p53. Although such activity has not been found for SMYD3,[137] this enzyme depicts methyltransferase activity on nonhistone proteins, namely VEGFR1, a receptor tyrosine kinase, enhancing its kinase activity.[138] Although VEGFR1 was thought to be expressed only in vascular endothelial cells, it is currently acknowledged that it may be expressed by a variety of tissues, including CRC.[139, 140] Additionally, it has been shown that increases in VEGFR1 expression are associated with enhanced migration and invasion of cancer cells.[141, 142] Taken together, it is tempting to speculate based on our findings that increase expression of SMYD3 may enhance the invasiveness of CRC cells through the action of VEGFR1.

Alongside with three histone methyltransferases, we also assessed the immunoexpression of a histone demethylase - LSD1. This enzyme directly demethylates lysine 4 in histone H3, a repressive mark, and, in prostate cancer cell lines, it interacts with androgen receptor and demethylates H3K9, activating androgen-dependent gene transcription [143, 144] Hence, depending on the cellular and environmental context, the same enzyme may produce opposite effects regarding gene regulation. Lim and collaborates showed that LSD1 expression was higher in estrogen receptor-negative breast cancers compared to normal breast tissues and estrogen receptor-positive breast cancers.[145] Furthermore, Kahl and collaborates showed that high levels of LSD1 expression were associated with increased histological grade and relapse in prostate cancer patients.[146] The precise mechanism of LSD1 in cancer initiation and progression is unknown, although a recent study has shown that LSD1 could promote G2-M phase transition and cell proliferation, probably mediated by cyclin A2.[145, 147] Inhibition of LSD1 expression in human CRC cells (both in vitro and in animal models) resulted in decreased cell growth and re-expression of several genes, including the Wnt signaling pathway antagonist family members.[148, 149] Dowregulation of LSD1 in breast cancer cell lines resulted in growth inhibition and increased expression of several genes, namely those involved in cell cycle regulation and cell migration,

such as p21 and E-cadherin.[145, 150, 151] Therefore, inhibition of LSD1 expression may represent a new epigenetic target in oncology.

In our study, CRC cells overexpressed LSD1, compared to morphologically normal colonic epithelial cells. On the other hand, LSD1 expression was significantly lower in more invasive tumors and at advanced stages, suggesting a double role for this histone modifier enzyme in CRC transformation and progression. Wang and collaborates showed that LSD1 is involved in dowregulation of TGFb-1 signaling pathway, which is important for epithelial-mesenchymal transition and invasiveness.[152] Our results support the hypothesis that CRC cases with low LSD1 expression may activate a more convenient program in order to invade and metastasize, although LSD1 expression was not associated with overall survival in our series of CRC patients. However, high levels of LSD1 expression were associated with better survival within the group of CRC patients treated with Folfiri (ρ=0.008), suggesting that LSD1 may constitute a predictive marker of therapeutic response in this subset of CRC patients. Nevertheless, these findings need to be confirmed in a larger CRC series.

3. Expression of histone marks H3K9me3 and H3K27me3 in CRC

The N-terminal tail of histone H3 can be methylated at multiple lysine (K) residues by histone methyltransferases. Methylation of lysine 9 in histone H3 (H3K9) is frequently associated with the epigenetic control of heterochromatin assembly and with gene silencing in cancer cells.[153-156] Nevertheless, any given modification has the potential to activate or repress gene transcription under different conditions. Recently, the finding that H3K9me3 is enriched in the coding regions of active genes has challenged the dogma that H3K9me3 is always associated with downregulation of gene transcription.[157] Probably, methylation at H3K9 within the coding regions has an activation effect in gene transcription whereas in the promoter regions it has the opposite effect.[49]

In our series of CRC cases, the histone mark H3K9me3 was highly expressed in the tumor cells compared to normal epithelial cells of the adjacent apparently normal mucosa. Interestingly, expression of H3K9me3 in tumor cells was also significantly higher in CRCs displaying lymph node metastases, suggesting that the genes activated or repressed by H3K9me3 might be involved in signaling pathways related with metastatic behavior. It should be emphasized, however, that the expression of this histone mark reflects the global level of histone

modifications in the tumor cells genome, and it does not necessarily indicate whether this modification is actually present in the coding region or in the promoter region. Consequently, we can only speculate on how H3K9me3 is affecting gene transcription (activation vs. repression).

Several researchers, using epigenetic marks such as H3K9me3, have identified subgroups of patients with different prognosis and/or different responses to treatment. Seligson and collaborates reported that lower cellular levels of H3K9me2 were associated with poorer outcome in prostate and kidney cancers.[75] Additionally, Manuyakorn and collaborates showed that low cellular levels of H3K9me2 were not only associated with poor survival in pancreatic adenocarcinomas but also in patients receiving adjuvant fluorouracil.[82] Although in our study a lower survival rate in CRC cases with low H3K9me3 expression in tumor cells was observed, this difference did not reach statistical significance. However, high levels of H3K9me3 expression were associated with better survival within the subset of patients treated with 5-FU/Leucovorine and Folfiri. To the best of our knowledge, this is the first evidence of H3K9me3 as an epigenetic biomarker for prediction of clinical outcome and response to therapy in CRC.

The N-terminal tail of histone H3 can also be methylated at lysine 27 (H3K27), a feature that is frequently associated with gene silencing. Wei and collaborates reported that H3K27me3 expression is lower in malignant tumors (namely breast, ovarian and pancreatic) compared to their normal counterparts.[78]

In our study, H3K27me3 expression was higher in CRC cells compared to morphologically normal colonic epithelial cells. Once again, the observed immunoexpression reflects the global level of histone modifications in tumor cells, rather than indicating whether this modification is specifically occurring at the coding or the promoter region of genes. However, most, if not all published studies, associate this mark with gene transcription inactivation. Tzao and collaborates reported a positive relationship between tumor differentiation and high H3K27me3 expression in squamous cell carcinoma of the esophagus. Moreover, high levels of H3K27me3 expression were also seen more frequently in cases with lymph node involvement and advance stages of disease.[81] Our findings are in line with those observations, as the expression of H3K27me3 was significantly higher in CRCs depicting lymph and venous vessel invasion. Furthermore, H3K27me3 expression was significantly higher in more invasive CRCs, as well as in those with lymph node metastases and at advanced disease stages.

Tzao and collaborates also demonstrated that high expression of H3K27me3 was independently associated with poor survival in patients with esophageal squamous cell carcinoma, especially at early stages.[81] However, Wei and collaborates revealed that high H3K27m3expression was independently associated with better overall survival rate in breast, ovarian and pancreatic cancers.[78] We found that high H3K27me3 expression in CRC cells was associated with worse outcome compared to cases with low expression of H3K27me3, although this difference is not statistically significant. Nevertheless, high levels of H3K27me3 expression were significantly associated with improved survival within a subset of patients treated with 5-FU/Leucovorine, a finding that has not been reported before. Remarkably, high H3K9me3 and H3K27me3 expression levels were mutually associated, suggesting a cooperative role in gene transcription deregulation in CRC. Thus, global expression of histone marks, specifically H3K9me3 and H3K27me3 should be explored in larger series of patients to definitely assess their role as potential epigenetic biomarkers for prediction of response to therapeutics in CRC patients.

CONCLUSIONS AND FUTURE PERSPECTIVES

CRC, as cancer in general, is a disease characterized by genetic and epigenetic modifications. Histone modifications, alongside with genetic alterations and DNA methylation, are important mechanisms of regulation of gene expression and synergistically contribute to neoplastic transformation and tumor progression.

The conclusions of our study are as follow:

1. In the selected CRC cases we observed that the presence of venous vessel invasion, positive surgical margins, regional lymph node metastases, distant metastases and tumor stage were associated with decreased survival rate of the patients.
2. Considering the difference between the expression of histone modifying enzymes and histone marks in CRC cells and their expression in the cells of the normal mucosa:
    a. The histone methyltransferases EZH2 and SMYD3 and the histone demethylase LSD1 were overexpressed in CRC cells, suggesting a role for these enzymes in the neoplastic transformation of CRC.
    b. The expression of SMYD3 was observed mainly in the cell membrane and in the cytoplasm, in contrast with the nuclear expression observed in the remaining histone modifying enzymes.
    c. The expression of histone marks H3K9me3 and H3K27me3 were increased in CRC cells, suggesting that the putative role of the abovementioned histone modifying enzymes is mediated by the methylation of H3K9 and H3K27.
3. Regarding the association between the expression of histone modifying enzymes and the expression of histone marks with the clinicopathological features of CRC cases, we observed:
    a. Higher EZH2 expression in more invasive CRCs and CRCs with regional lymph node metastases, suggesting that EZH2 may have a role in tumor growth and cell invasion.

b. Higher SETDB1 expression in left-sided CRCs, suggesting a putative role in the carcinogenesis of chromossomic instable CRCs.
c. Lower LSD1 expression in more invasive CRCs and in CRCs at advanced stages of disease, suggesting that LSD1 may be associated with less aggressive CRCs.
d. Higher H3K9me3 expression in CRCs with regional lymph node metastases, which appears to be associated to the putative role of EZH2 in the progression of CRC.
e. Higher H3K27me3 expression in more invasive CRCs, CRCs with regional lymph node metastases and in CRCs at advanced stages of disease, as well as in CRCs with lymph and venous vessel invasion, which appears to be associated to the putative role of EZH2 in the progression of CRC.

4. In this study we did not detected any differences in the survival rate of the patients with CRC considering the different expression of the studied histone modifying enzymes or histone marks.
5. Regarding the therapeutic response, we observed:
    a. Higher survival rates of patients with CRCs treated only with surgery that disclosed lower SETDB1 expression, suggesting that whenever the expression of SETDB1 occurs, the surgical treatment may not be enough to prolong the patient's survival.
    b. Higher survival rates of patients with CRC treated with Folfiri that disclosed high LSD1 and H3K9me3 expression in cancer cells, suggesting that LSD1 expression and the histone mark H3K9me3 can predict the response to this treatment modality.
    c. Higher survival rates of patients with CRC treated with 5-FU/Leucovorine that disclosed high H3K9me3 and H3K27me3 expression in cancer cells, suggesting that these patterns of methylation can predict the response to this treatment modality.
6. Additionally, we verified that the evaluation of histone modifying enzymes and histone marks using a low/high expression system reproduced the results obtained

with the H-score, constituting an easier system to evaluate the expression of the histone modifying enzymes and histone marks in the routine of a pathologist.

Following the above mentioned conclusions, we propose new studies to highlight the role of histone modifying enzymes and histone marks in the neoplastic transformation and progression of CRC as well as their association with the therapeutic response:

1. Study of the above mentioned histone modifying enzymes and histone marks in larger, independent series to validate our results.
2. Identification of signaling pathways targeted by SETDB1 in order to explain its association with localized CRC prognosis.
3. Identification of cytoplasmic molecular targets of SMYD3 to clarify its putative role in CRC carcinogenesis.
4. Evaluation of the expression of the studied histone modifying enzymes and histone marks in CRC metastases to disclose their association with the response to treatment.
5. Evaluation of the expression of additional histone modifying enzymes and histone modifications to identify new prognostic markers in CRC.

REFERENCES

1. Kumar V, Abbas AK, Fausto N. *Robbins and Cotran Pathologic Basis of Disease*. 7th ed: Elsevier Inc.; 1999.

2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* Mar 4;144(5):646-674.

3. Rasanen K, Vaheri A. Activation of fibroblasts in cancer stroma. *Exp Cell Res.* Oct 15;316(17):2713-2722.

4. Shimoda M, Mellody KT, Orimo A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. *Semin Cell Dev Biol.* Feb;21(1):19-25.

5. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature.* Nov 18 2004;432(7015):332-337.

6. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell.* Mar 19;140(6):883-899.

7. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev.* Jun;29(2):309-316.

8. Raza A, Franklin MJ, Dudek AZ. Pericytes and vessel maturation during tumor angiogenesis and metastasis. *Am J Hematol.* Aug;85(8):593-598.

9. Lamagna C, Bergers G. The bone marrow constitutes a reservoir of pericyte progenitors. *J Leukoc Biol.* Oct 2006;80(4):677-681.

10. Bergfeld SA, DeClerck YA. Bone marrow-derived mesenchymal stem cells and the tumor microenvironment. *Cancer Metastasis Rev.* Jun;29(2):249-261.

11. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature.* Sep 14 2000;407(6801):249-257.

12. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res.* May 1;316(8):1324-1331.

13. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* Apr 2009;9(4):239-252.

14. Egeblad M, Nakasone ES, Werb Z. Tumors as organs: complex tissues that interface with the entire organism. *Dev Cell.* Jun 15;18(6):884-901.

15. Yachida S, Jones S, Bozic I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature.* Oct 28;467(7319):1114-1117.

16. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med.* Mar 8;366(10):883-892.

17. Klymkowsky MW, Savagner P. Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. *Am J Pathol.* May 2009;174(5):1588-1593.

18. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer.* Apr 2009;9(4):265-273.

19. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell.* Nov 25 2009;139(5):871-890.

20. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer.* Sep 2005;5(9):744-749.

21. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia*. Jun;15(2):117-134.

22. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell.* Jun 2008;14(6):818-829.

23. Berdasco M, Esteller M. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. *Dev Cell.* Nov 16;19(5):698-711.

24. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin.* Jan-Feb;62(1):10-29.

25. Siegel R, DeSantis C, Virgo K, et al. Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin.* Jul-Aug;62(4):220-241.

26. Gomes CO. Risco de Morrer em Portugal 2006. In: Saúde D-Gd, ed. Lisboa; 2009.

27. Bosman FT, Carneiro F, Hruban RH, Theise ND (Eds.). *WHO Classification of Tumours of the Digestive System*. 4th ed. Lyon: IARC; 2010.

28. Edwards BK, Ward E, Kohler BA, et al. Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. *Cancer.* Feb 1;116(3):544-573.

29. Cress RD, Morris C, Ellison GL, Goodman MT. Secular changes in colorectal cancer incidence by subsite, stage at diagnosis, and race/ethnicity, 1992-2001. *Cancer.* Sep 1 2006;107(5 Suppl):1142-1152.

30. Phillips KA, Liang SY, Ladabaum U, et al. Trends in colonoscopy for colorectal cancer screening. *Med Care*. Feb 2007;45(2):160-167.

31. Society AC. Cancer Prevention & Early Detection Facts & Figures 2012. *Atlanta, GA: American Cancer Society.* 2012.

32. Howlader N NA, Krapcho M, et al, eds. SEER Cancer Statistics Review, 1975-2008. *Bethesda, MD: National Cancer Institute*; 2011.

33. Pontes L, Silva MA, Matoso F. Registo Oncológico Nacional - 2005; 2009.

34. Kudo S, Lambert R, Allen JI, et al. Nonpolypoid neoplastic lesions of the colorectal mucosa. *Gastrointest Endosc.* Oct 2008;68(4 Suppl):S3-47.

35. Ponder BA. Cancer genetics. *Nature.* May 17 2001;411(6835):336-341.

36. van Engeland M, Derks S, Smits KM, Meijer GA, Herman JG. Colorectal cancer epigenetics: complex simplicity. *J Clin Oncol.* Apr 1;29(10):1382-1391.

37. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science.* Oct 13 2006;314(5797):268-274.

38. Groden J, Thliveris A, Samowitz W, et al. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell.* Aug 9 1991;66(3):589-600.

39. Powell SM, Zilz N, Beazer-Barclay Y, et al. APC mutations occur early during colorectal tumorigenesis. *Nature.* Sep 17 1992;359(6392):235-237.

40. Senda T, Iizuka-Kogo A, Onouchi T, Shimomura A. Adenomatous polyposis coli (APC) plays multiple roles in the intestinal and colorectal epithelia. *Med Mol Morphol.* Jun 2007;40(2):68-81.

41. Feldman M, Friedman LS, Marvin H. *Sleisenger & Fordtran´s Gastrointestinal and Liver Disease: Patophysiology, Diagnosis, Management.* 7th ed: Elsevier Science.

42. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. *Nat Med*. Mar;17(3):330-339.

43. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP. Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science.* Apr 12 1985;228(4696):187-190.
44. Suzuki K, Suzuki I, Leodolter A, et al. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. *Cancer Cell.* Mar 2006;9(3):199-207.
45. Irizarry RA, Ladd-Acosta C, Wen B, et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet.* Feb 2009;41(2):178-186.
46. Rodriguez J, Frigola J, Vendrell E, et al. Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Res.* Sep 1 2006;66(17):8462-9468.
47. Strahl BD, Allis CD. The language of covalent histone modifications. *Nature.* Jan 6 2000;403(6765):41-45.
48. Jenuwein T, Allis CD. Translating the histone code. *Science.* Aug 10 2001;293(5532):1074-1080.
49. Kouzarides T. Chromatin modifications and their function. *Cell.* Feb 23 2007;128(4):693-705.
50. Li B, Carey M, Workman JL. The role of chromatin during transcription. *Cell.* Feb 23 2007;128(4):707-719.
51. Gibbons RJ. Histone modifying and chromatin remodelling enzymes in cancer and dysplastic syndromes. *Hum Mol Genet.* Apr 15 2005;14 Spec No 1:R85-92.
52. Kang MY, Lee BB, Kim YH, et al. Association of the SUV39H1 histone methyltransferase with the DNA methyltransferase 1 at mRNA expression level in primary colorectal cancer. *Int J Cancer.* Nov 15 2007;121(10):2192-2197.
53. Chi P, Allis CD, Wang GG. Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. *Nat Rev Cancer.* Jul;10(7):457-469.
54. Ciccone DN, Su H, Hevi S, et al. KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. *Nature.* Sep 17 2009;461(7262):415-418.
55. Wu H, Min J, Lunin VV, et al. Structural biology of human H3K9 methyltransferases. *PLoS One.*5(1):e8570.
56. Kim E, Song JJ. Diverse ways to be specific: a novel Zn-binding domain confers substrate specificity to UTX/KDM6A histone H3 Lys 27 demethylase. *Genes Dev.* Nov 1;25(21):2223-2226.
57. Sengoku T, Yokoyama S. Structural basis for histone H3 Lys 27 demethylation by UTX/KDM6A. *Genes Dev.* Nov 1;25(21):2266-2277.
58. Rana TM. Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol.* Jan 2007;8(1):23-36.
59. Landi D, Gemignani F, Naccarati A, et al. Polymorphisms within micro-RNA-binding sites and risk of sporadic colorectal cancer. *Carcinogenesis.* Mar 2008;29(3):579-584.
60. Melo SA, Ropero S, Moutinho C, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet.* Mar 2009;41(3):365-370.
61. Yamashita K, Dai T, Dai Y, Yamamoto F, Perucho M. Genetics supersedes epigenetics in colon cancer phenotype. *Cancer Cell.* Aug 2003;4(2):121-131.
62. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet.* Jan 1999;21(1):103-107.

63. Ting AH, Suzuki H, Cope L, et al. A requirement for DICER to maintain full promoter CpG island hypermethylation in human cancer cells. *Cancer Res.* Apr 15 2008;68(8):2570-2575.
64. Schuebel KE, Chen W, Cope L, et al. Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. *PLoS Genet.* Sep 2007;3(9):1709-1723.
65. Gazin C, Wajapeyee N, Gobeil S, Virbasius CM, Green MR. An elaborate pathway required for Ras-mediated epigenetic silencing. *Nature.* Oct 25 2007;449(7165):1073-1077.
66. Brenner C, Deplus R, Didelot C, et al. Myc represses transcription through recruitment of DNA methyltransferase corepressor. *EMBO J.* Jan 26 2005;24(2):336-346.
67. Ogino S, Hazra A, Tranah GJ, et al. MGMT germline polymorphism is associated with somatic MGMT promoter methylation and gene silencing in colorectal cancer. *Carcinogenesis.* Sep 2007;28(9):1985-1990.
68. Tsuge M, Hamamoto R, Silva FP, et al. A variable number of tandem repeats polymorphism in an E2F-1 binding element in the 5' flanking region of SMYD3 is a risk factor for human cancers. *Nat Genet.* Oct 2005;37(10):1104-1107.
69. Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A.* Jun 9 1998;95(12):6870-6875.
70. Esteller M, Herman JG. Generating mutations but providing chemosensitivity: the role of O6-methylguanine DNA methyltransferase in human cancer. *Oncogene.* Jan 8 2004;23(1):1-8.
71. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* Jun 1 1990;61(5):759-767.
72. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet.* Apr 2007;8(4):286-298.
73. Kurdistani SK. Histone modifications in cancer biology and prognosis. *Prog Drug Res.*67:91-106.
74. Seligson DB, Horvath S, Shi T, et al. Global histone modification patterns predict risk of prostate cancer recurrence. *Nature*. Jun 30 2005;435(7046):1262-1266.
75. Seligson DB, Horvath S, McBrian MA, et al. Global levels of histone modifications predict prognosis in different cancers. *Am J Pathol.* May 2009;174(5):1619-1628.
76. Bianco-Miotto T, Chiam K, Buchanan G, et al. Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. *Cancer Epidemiol Biomarkers Prev.* Oct;19(10):2611-2622.
77. Elsheikh SE, Green AR, Rakha EA, et al. Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. *Cancer Res.* May 1 2009;69(9):3802-3809.
78. Wei Y, Xia W, Zhang Z, et al. Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. *Mol Carcinog*. Sep 2008;47(9):701-706.
79. Van Den Broeck A, Brambilla E, Moro-Sibilot D, et al. Loss of histone H4K20 trimethylation occurs in preneoplasia and influences prognosis of non-small cell lung cancer. *Clin Cancer Res.* Nov 15 2008;14(22):7237-7245.

80. Barlesi F, Giaccone G, Gallegos-Ruiz MI, et al. Global histone modifications predict prognosis of resected non small-cell lung cancer. *J Clin Oncol.* Oct 1 2007;25(28):4358-4364.
81. Tzao C, Tung HJ, Jin JS, et al. Prognostic significance of global histone modifications in resected squamous cell carcinoma of the esophagus. *Mod Pathol.* Feb 2009;22(2):252-260.
82. Manuyakorn A, Paulus R, Farrell J, et al. Cellular histone modification patterns predict prognosis and treatment response in resectable pancreatic adenocarcinoma: results from RTOG 9704. *J Clin Oncol.* Mar 10;28(8):1358-1365.
83. Allegra CJ, Jessup JM, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol.* Apr 20 2009;27(12):2091-2096.
84. Derwinger K, Kodeda K, Bexe-Lindskog E, Taflin H. Tumour differentiation grade is associated with TNM staging and the risk of node metastasis in colorectal cancer. *Acta Oncol.*49(1):57-62.
85. Rosato FE, Marks G. Changing site distribution patterns of colorectal cancer at Thomas Jefferson University Hospital. *Dis Colon Rectum.* Mar-Apr 1981;24(2):93-95.
86. Betge J, Pollheimer MJ, Lindtner RA, et al. Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting. *Cancer.* Feb 1;118(3):628-638.
87. Vire E, Brenner C, Deplus R, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature.* Feb 16 2006;439(7078):871-874.
88. Fussbroich B, Wagener N, Macher-Goeppinger S, et al. EZH2 depletion blocks the proliferation of colon cancer cells. *PLoS One*.6(7):e21651.
89. Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci U S A.* Sep 30 2003;100(20):11606-11611.
90. Raaphorst FM, Meijer CJ, Fieret E, et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. *Neoplasia*. Nov-Dec 2003;5(6):481-488.
91. Weikert S, Christoph F, Kollermann J, et al. Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. *Int J Mol Med*. Aug 2005;16(2):349-353.
92. Arisan S, Buyuktuncer ED, Palavan-Unsal N, Caskurlu T, Cakir OO, Ergenekon E. Increased expression of EZH2, a polycomb group protein, in bladder carcinoma. *Urol Int.* 2005;75(3):252-257.
93. Varambally S, Dhanasekaran SM, Zhou M, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature*. Oct 10 2002;419(6907):624-629.
94. Matsukawa Y, Semba S, Kato H, Ito A, Yanagihara K, Yokozaki H. Expression of the enhancer of zeste homolog 2 is correlated with poor prognosis in human gastric cancer. *Cancer Sci*. Jun 2006;97(6):484-491.
95. Sudo T, Utsunomiya T, Mimori K, et al. Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. *Br J Cancer*. May 9 2005;92(9):1754-1758.

96. Takawa M, Masuda K, Kunizaki M, et al. Validation of the histone methyltransferase EZH2 as a therapeutic target for various types of human cancer and as a prognostic marker. *Cancer Sci.* Jul;102(7):1298-1305.
97. Ciarapica R, Miele L, Giordano A, Locatelli F, Rota R. Enhancer of zeste homolog 2 (EZH2) in pediatric soft tissue sarcomas: first implications. *BMC Med.*9:63.
98. Raaphorst FM, van Kemenade FJ, Blokzijl T, et al. Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. *Am J Pathol.* Sep 2000;157(3):709-715.
99. Visser HP, Gunster MJ, Kluin-Nelemans HC, et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. *Br J Haematol.* Mar 2001;112(4):950-958.
100. van Kemenade FJ, Raaphorst FM, Blokzijl T, et al. Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. *Blood.* Jun 15 2001;97(12):3896-3901.
101. Dukers DF, van Galen JC, Giroth C, et al. Unique polycomb gene expression pattern in Hodgkin's lymphoma and Hodgkin's lymphoma-derived cell lines. *Am J Pathol.* Mar 2004;164(3):873-881.
102. Kim KC, Huang S. Histone methyltransferases in tumor suppression. *Cancer Biol Ther.* Sep-Oct 2003;2(5):491-499.
103. Bachmann IM, Halvorsen OJ, Collett K, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *J Clin Oncol.* Jan 10 2006;24(2):268-273.
104. Bryant RJ, Cross NA, Eaton CL, Hamdy FC, Cunliffe VT. EZH2 promotes proliferation and invasiveness of prostate cancer cells. *Prostate.* Apr 1 2007;67(5):547-556.
105. Collett K, Eide GE, Arnes J, et al. Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. *Clin Cancer Res.* Feb 15 2006;12(4):1168-1174.
106. Fluge O, Gravdal K, Carlsen E, et al. Expression of EZH2 and Ki-67 in colorectal cancer and associations with treatment response and prognosis. *Br J Cancer.* Oct 20 2009;101(8):1282-1289.
107. Simon JA, Tamkun JW. Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. *Curr Opin Genet Dev.* Apr 2002;12(2):210-218.
108. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J.* Oct 15 2003;22(20):5323-5335.
109. Tonini T, Bagella L, D'Andrilli G, Claudio PP, Giordano A. Ezh2 reduces the ability of HDAC1-dependent pRb2/p130 transcriptional repression of cyclin A. *Oncogene.* Jun 17 2004;23(28):4930-4937.
110. Fujii S, Ito K, Ito Y, Ochiai A. Enhancer of zeste homologue 2 (EZH2) down-regulates RUNX3 by increasing histone H3 methylation. *J Biol Chem.* Jun 20 2008;283(25):17324-17332.
111. Goel A, Arnold CN, Tassone P, et al. Epigenetic inactivation of RUNX3 in microsatellite unstable sporadic colon cancers. *Int J Cancer.* Dec 10 2004;112(5):754-759.

112. Kodach LL, Jacobs RJ, Heijmans J, et al. The role of EZH2 and DNA methylation in the silencing of the tumour suppressor RUNX3 in colorectal cancer. *Carcinogenesis.* Sep;31(9):1567-1575.
113. Chi XZ, Yang JO, Lee KY, et al. RUNX3 suppresses gastric epithelial cell growth by inducing p21(WAF1/Cip1) expression in cooperation with transforming growth factor {beta}-activated SMAD. *Mol Cell Biol.* Sep 2005;25(18):8097-8107.
114. Yano T, Ito K, Fukamachi H, et al. The RUNX3 tumor suppressor upregulates Bim in gastric epithelial cells undergoing transforming growth factor beta-induced apoptosis. *Mol Cell Biol.* Jun 2006;26(12):4474-4488.
115. Ito K, Lim AC, Salto-Tellez M, et al. RUNX3 attenuates beta-catenin/T cell factors in intestinal tumorigenesis. *Cancer Cell.* Sep 9 2008;14(3):226-237.
116. Li H, Rauch T, Chen ZX, Szabo PE, Riggs AD, Pfeifer GP. The histone methyltransferase SETDB1 and the DNA methyltransferase DNMT3A interact directly and localize to promoters silenced in cancer cells. *J Biol Chem.* Jul 14 2006;281(28):19489-19500.
117. Zhang Y, Reinberg D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev.* Sep 15 2001;15(18):2343-2360.
118. Hashimoto H, Vertino PM, Cheng X. Molecular coupling of DNA methylation and histone methylation. *Epigenomics.* Oct;2(5):657-669.
119. Ceol CJ, Houvras Y, Jane-Valbuena J, et al. The histone methyltransferase SETDB1 is recurrently amplified in melanoma and accelerates its onset. *Nature.* Mar 24;471(7339):513-517.
120. Bilodeau S, Kagey MH, Frampton GM, Rahl PB, Young RA. SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. *Genes Dev.* Nov 1 2009;23(21):2484-2489.
121. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet.* Jul 2000;25(3):315-319.
122. Dammann R, Schagdarsurengin U, Seidel C, et al. The tumor suppressor RASSF1A in human carcinogenesis: an update. *Histol Histopathol.* Apr 2005;20(2):645-663.
123. Watanabe H, Soejima K, Yasuda H, et al. Deregulation of histone lysine methyltransferases contributes to oncogenic transformation of human bronchoepithelial cells. *Cancer Cell Int.* 2008;8:15.
124. Kim HA, Koo BK, Cho JH, et al. Notch1 counteracts WNT/beta-catenin signaling through chromatin modification in colorectal cancer. *J Clin Invest.* Sep 4;122(9):3248-3259.
125. Richman S, Adlard J. Left and right sided large bowel cancer. *BMJ.* Apr 20 2002;324(7343):931-932.
126. Hamamoto R, Furukawa Y, Morita M, et al. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. *Nat Cell Biol.* Aug 2004;6(8):731-740.
127. Foreman KW, Brown M, Park F, et al. Structural and functional profiling of the human histone methyltransferase SMYD3. *PLoS One.*6(7):e22290.
128. Hamamoto R, Silva FP, Tsuge M, et al. Enhanced SMYD3 expression is essential for the growth of breast cancer cells. *Cancer Sci.* Feb 2006;97(2):113-118.

129. Zou JN, Wang SZ, Yang JS, Luo XG, Xie JH, Xi T. Knockdown of SMYD3 by RNA interference down-regulates c-Met expression and inhibits cells migration and invasion induced by HGF. *Cancer Lett.* Jul 18 2009;280(1):78-85.
130. Cock-Rada AM, Medjkane S, Janski N, et al. SMYD3 promotes cancer invasion by epigenetic upregulation of the metalloproteinase MMP-9. *Cancer Res.* Feb 1;72(3):810-820.
131. Liu C, Fang X, Ge Z, et al. The telomerase reverse transcriptase (hTERT) gene is a direct target of the histone methyltransferase SMYD3. *Cancer Res.* Mar 15 2007;67(6):2626-2631.
132. Clevers H. Wnt breakers in colon cancer. *Cancer Cell.* Jan 2004;5(1):5-6.
133. Watanabe T, Kobunai T, Yamamoto Y, et al. Differential gene expression signatures between colorectal cancers with and without KRAS mutations: crosstalk between the KRAS pathway and other signalling pathways. *Eur J Cancer.* Sep;47(13):1946-1954.
134. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell.* Apr 2;141(1):52-67.
135. He C, Xu J, Zhang J, et al. High expression of trimethylated histone H3 lysine 4 is associated with poor prognosis in hepatocellular carcinoma. *Hum Pathol.* Sep;43(9):1425-1435.
136. Zhang K, Dent SY. Histone modifying enzymes and cancer: going beyond histones. *J Cell Biochem.* Dec 15 2005;96(6):1137-1148.
137. Huang J, Perez-Burgos L, Placek BJ, et al. Repression of p53 activity by Smyd2-mediated methylation. *Nature.* Nov 30 2006;444(7119):629-632.
138. Kunizaki M, Hamamoto R, Silva FP, et al. The lysine 831 of vascular endothelial growth factor receptor 1 is a novel target of methylation by SMYD3. *Cancer Res.* Nov 15 2007;67(22):10759-10765.
139. Fan F, Wey JS, McCarty MF, et al. Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. *Oncogene.* Apr 14 2005;24(16):2647-2653.
140. Duff SE, Jeziorska M, Rosa DD, et al. Vascular endothelial growth factors and receptors in colorectal cancer: implications for anti-angiogenic therapy. *Eur J Cancer.* Jan 2006;42(1):112-117.
141. Lesslie DP, Summy JM, Parikh NU, et al. Vascular endothelial growth factor receptor-1 mediates migration of human colorectal carcinoma cells by activation of Src family kinases. *Br J Cancer.* Jun 5 2006;94(11):1710-1717.
142. Yang AD, Camp ER, Fan F, et al. Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells. *Cancer Res.* Jan 1 2006;66(1):46-51.
143. Shi Y, Lan F, Matson C, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell.* Dec 29 2004;119(7):941-953.
144. Metzger E, Wissmann M, Yin N, et al. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature.* Sep 15 2005;437(7057):436-439.
145. Lim S, Janzer A, Becker A, et al. Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology. *Carcinogenesis.* Mar;31(3):512-520.

146. Kahl P, Gullotti L, Heukamp LC, et al. Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. *Cancer Res.* Dec 1 2006;66(23):11341-11347.
147. Scoumanne A, Chen X. The lysine-specific demethylase 1 is required for cell proliferation in both p53-dependent and -independent manners. *J Biol Chem.* May 25 2007;282(21):15471-15475.
148. Huang Y, Greene E, Murray Stewart T, et al. Inhibition of lysine-specific demethylase 1 by polyamine analogues results in reexpression of aberrantly silenced genes. *Proc Natl Acad Sci U S A.* May 8 2007;104(19):8023-8028.
149. Huang Y, Stewart TM, Wu Y, et al. Novel oligoamine analogues inhibit lysine-specific demethylase 1 and induce reexpression of epigenetically silenced genes. *Clin Cancer Res.* Dec 1 2009;15(23):7217-7228.
150. Lin Y, Wu Y, Li J, et al. The SNAG domain of Snail1 functions as a molecular hook for recruiting lysine-specific demethylase 1. *EMBO J.* Jun 2;29(11):1803-1816.
151. Huang J, Sengupta R, Espejo AB, et al. p53 is regulated by the lysine demethylase LSD1. *Nature.* Sep 6 2007;449(7158):105-108.
152. Wang Y, Zhang H, Chen Y, et al. LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer. *Cell.* Aug 21 2009;138(4):660-672.
153. Sims RJ, 3rd, Nishioka K, Reinberg D. Histone lysine methylation: a signature for chromatin function. *Trends Genet.* Nov 2003;19(11):629-639.
154. Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science.* Apr 6 2001;292(5514):110-113.
155. Bachman KE, Park BH, Rhee I, et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell.* Jan 2003;3(1):89-95.
156. Kondo Y, Shen L, Issa JP. Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer. *Mol Cell Biol.* Jan 2003;23(1):206-215.
157. Vakoc CR, Mandat SA, Olenchock BA, Blobel GA. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol Cell.* Aug 5 2005;19(3):381-391.