Universidade do Algarve

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Functional analysis of a transcriptional variant of Matrix Gla Protein (*MGP*)

Helena Isabel Rosa Pinto Caiado

Dissertação de Mestrado

Mestrado em Ciências Biomédicas

Orientação científica pelo Doutor Daniel Tiago e pela Doutora Natércia Conceição

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(Helena Isabel Rosa Pinto Caiado)

Aos meus Pais pelo amor incondicional,
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Abbreviations

DNA **Deoxyribonucleic acid**

RNA Ribonucleic acid

HR homologous recombination

NHEJ non-homologous end joining

DSB double-strand breaks

BER base excision repair

CAC Colon adenocarcinoma

FAP Familial adenomatus poliposys

LOH Loss of heterozigosity

HNPCC Hereditary non-polyposis colon carcinoma

MGP Matrix Gla Protein

Abstract

Colon adenocarcinoma (CAC) is the second most frequent cancer in Portugal, representing 10% of all tumors. Although CAC is usually diagnosed in a symptomatic phase, with a level of progression around 10 years, the prognosis remains relatively reserved. However, if diagnosed at an early stage, 5-year survival rates can reach 50% in both genders. In average, in Portugal around 9 to 10 patients die with colon and rectum adenocarcinomas per day. In this regard, population-wide screening and early detection of CAC through biomarkers are important strategies that can change this situation in the future in order to diminish mortality.

Matrix Gla Protein (MGP) is an extracellular protein that was previously shown to be involved in the inhibition of calcification of arteries and cartilage, although its functional role in neoplasia remains unclear, it was shown to be up-regulated in a variety of tumors, including ovarian, breast, and gliomas, among others. This fact has triggered our interest in analyzing the expression of *MGP*, as well as some putative regulatory transcription factors, in CAC.

To proceed with this analysis, samples from 20 patients with CAC were analyzed through qRT-PCR. Our results show an overexpression of *MGP* in colon cancer biopsy specimens (and cell lines) at mRNA level as compared to normal colon tissue ($p = 0.056$), contradicting a previously published analysis where a down-regulation of this gene was shown for this type of cancer. Given these results, we proceeded with our study by analyzing the expression of transcription factors that have been described in the literature as having a role in the regulation of MGP, and in some cases also in tumorigenesis. From the results obtained, it was found that there was an up-regulation of *RUNX2* ($p = 0.037$) and *CCBE1* ($p = 0.012$) mRNA levels in CAC samples. Although the expression levels for *FGF2* ($p = 0.085$) and *RARα* ($p = 0.58$) do not present a significant P value, it was found that there was a positive Pearson correlation ($r = 0.89$, $p \leq 0.0001$ for *FGF2* and $r = 0.85$, $p \leq 0.0001$ for *RARα*), demonstrating a strong trend towards simultaneous up-regulation of these transcription factors and *MGP*. In the future it is necessary a deeper analysis on how these genes interact with each other and what mechanisms are affected with this disruption.

Key words: Colon adenocarcinoma, *MGP*, Transcription factors, *RUNX2, CCBE1, RARα, FGF2*

Resumo

Em Portugal, o adenocarcinoma do cólon apresenta uma taxa de prevalência em relação a outros tumores de 10%, e em média, morrem 9 a 10 pacientes por dia. Embora este tipo de tumor seja diagnosticado já numa fase mais avançada e sintomática da doença, com uma progressão em cerca de 10 anos, o prognóstico permanece reservado. Contudo, se diagnosticado num estadio mais precoce, a taxa de sobrevivência para um período de 5 anos apresenta valores acima dos 50%, tanto para homens como para mulheres. De acordo com os dados publicados pelo Registo Oncológico do Sul (ROR-Sul) 81% e 71% dos casos foram diagnosticados por volta dos 60 anos em homens e mulheres, respectivamente.

Existem duas formas principais para o aparecimento do adenocarcinoma do cólon, nomeadamente, a forma relacionada com o aparecimento esporádico com origem em mutações em células somáticas (mais de 95% dos casos) e a forma associada à herança de mutações em células da linha germinal (forma hereditária). Associadas às mutações transmitidas através de células da linha germinal, temos a síndrome da polipose adenomatosa familiar (FAP) e o síndrome não polipoide hereditário ou síndrome de Lynch. No caso da síndrome adenomatosa familiar, esta é uma doença hereditária autossómica dominante, e é caracterizada por 1) aparecimento de milhares de tumores pré-neoplásicos (pólipos) ao longo de todo o tubo digestivo e 2) aparecimento numa idade jovem (40 anos). Associada a esta doença está também o gene supressor de tumores APC, responsável maioritariamente pela proliferação celular e activação de vias de sinalização apoptóticas. Quando existe uma perda por heterozigotia (LOH) do restante alelo funcional este síndrome tem maior risco de vir a dar origem a um adenocarcinoma. Existem ainda outros tipos de síndromes associados a este tipo e doença, nomeadamente, a síndrome de Gardner, a hipertrofia congénita do pigmento da retina e a forma mais suave da polipose adenomatosa familiar. Em relação à síndrome de Lynch esta difere da FAP pelo tipo de mutações encontradas em genes associados a mecanismos de reparação do ADN como é o caso do *MLH1* e *MSH2* e por não apresentar pólipos pré-neoplásicos em grande número. Já a forma do adenocarcinoma do cólon esporádico, está relacionado com o aparecimento de mutações pontuais em células somáticas, que podem ser causadas por fatores subjacentes ao dia-a-dia do paciente. No entanto é de salientar que para ambas as formas de manifestação do adenocarcinoma do cólon, é necessário ocorrer um primeiro evento carcinogénico que altere o normal funcionamento da célula.

No que diz respeito aos métodos de rastreio para este tipo de tumores, os melhores continuam ainda a ser a sigmoidoscopia e colonoscopia total. No entanto, o avanço das técnicas em biologia molecular tem vindo a permitir a descoberta de novos genes que estejam envolvidos na regulação tumoral e que no futuro possam ser considerados possíveis biomarcadores para uma deteção mais precoce e possíveis alvos terapêuticos, a fim de diminuir a mortalidade ainda associada a este tipo de tumores.

A MGP (Proteína Gla da matrix) é uma proteína extracelular cuja principal função está associada à inibição da calcificação das artérias e cartilagem. No cancro, embora tenha sido demonstrada uma sobre-expressão da MGP numa variedade de tumores, como o cancro do ovário, da mama, glioblastomas, entre outros, o seu papel permanece pouco claro,. Especificamente, no adenocarcinoma do cólon foi anteriormente demonstrada uma desregulação da MGP, embora de forma pouco consistente. Como tal o principal objetivo deste trabalho incidiu sobre a análise de expressão do gene MGP, bem como a análise de alguns possíveis fatores de transcrição associados à sua regulação em amostras do adenocarcinoma do cólon. Para tal, amostras de mucosa normal e tumoral de 20 pacientes foram analisadas por PCR em tempo real. Os resultados demonstraram haver uma forte tendência para a sobre-expressão da MGP a nível do mRNA (P= 0.056) em biópsias do adenocarcinoma do cólon em comparação com as biópsias de mucosa normal, contrariando resultados anteriores indicando uma regulação negativa feste gene. Os resultados aqui apresentados são no entanto consistentes com uma elevada expressão da MGP (níveis idênticos aqueles que foram observados na mucosa tumoral das amostras dos pacientes com adenocarcinoma do colon)

Tendo em conta estes resultados, procedeu-se então à análise da expressão de fatores de transcrição que têm sido descritos na literatura como tendo um papel na regulação da MGP e em alguns casos também na tumorigénese. Dos resultados obtidos, constatou-se um aumento da expressão dos genes do RUNX2 ($p = 0,037$) e CCBE1 ($p = 0,012$) em amostras de adenocarcinoma do cólon. Para os genes do FGF2 ($p = 0,085$) e RARa ($p = 0,58$) os resultados não apresentaram valores estatisticamente significativos. No entanto, verificou-se que existia uma correlação de Pearson positiva ($r = 0,89$, $p \leq 0.0001$ para o FGF2 e $r = 0,85$, $p \leq 0.0001$ para o RARa), demonstrando assim uma forte tendência para o aumento da regulação simultânea desses fatores de transcrição e da MGP. Em suma, a MGP encontra-se sobre expressa nas amostras no adenocarcinoma do cólon, no entanto não é possível estabelecer quais os mecanismos de regulação que estão a ser desregulados e se um pior prognóstico poderá estar associado a esta desregulação. Para isso, será necessário no futuro realizar i) uma

análise mais alargada em número de pacientes, e ii) uma análise mais profunda sobre como esses genes interagem entre si. Será também importante analisar quais os efeitos de um bloqueio da expressão da MGP na linha celular de adenocarcinoma de cólon, bem como testar os efeitos dos fatores de transcrição identificados no promotor da MGP.

Palavras-chave: Adenocarcinoma do cólon; *MGP*; fatores de transcrição**;** *RUNX2*;*CCBE1*; *RARα*; *FGF2*

Chapter I

Introduction

1. Introduction

1.1 Overview on the principles of cell and molecular biology

In a normal cell, the genomic DNA corresponds to a group of large molecules named chromosomes, which are composed by genes and intergenic regions. Genes can either encode: 1) proteins, which result from sequential transcription and translation processes; or 2) non-coding RNAs, which exclusively involve transcription of genes (molecules such as ribosomal RNAs, transfer RNAs, long non-coding RNAs or microRNAs, result from this process). Transcription is a process through which a strand of a segment of DNA from a particular gene serves as model for the synthesis of a new strand of RNA. This process is accomplished by RNA polymerases. Translation is a process through which an mRNA originates a peptide chain and a protein. This process is accomplished by ribosomes and transfer RNAs (which transport amino acids into a newly formed peptide chain; Figure 1.1) [1].

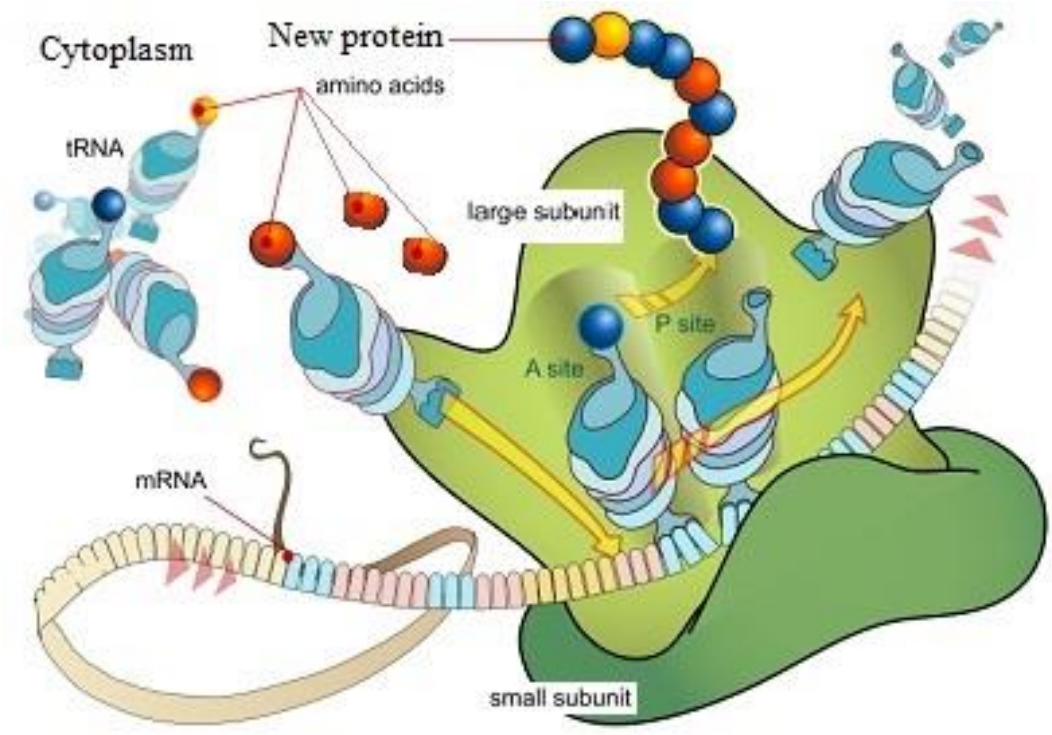

Figure 1.1 Translation of mRNA in to a new protein. mRNA polypeptide chain newly synthesized during transcription, binds to the ribosomal subunits and tRNA and shifts to the A site where it begins the synthesis of the polypeptide chain. Subsequently during the elongation phase the new peptide is synthesized with the help of rRNA between A site and P site with de addition of amino acids. Protein synthesis stops when the stop codon indicating the end of translation arises A site and the new protein is released from the ribosome into the cytoplasm. [1]. (Adapted from http://en.wikibooks.org/wiki/Structural_Biochemistry/Nucleic_Acid/Translation).

Eukaryotes, endue several types of RNA polymerases, each one responsible for making a particular class of RNA transcript, for instance: **RNA polymerase I** is entirely responsible in generating the transcript that becomes processed into ribosomal RNAs; **RNA polymerase II** is in charge of transcribing all protein-coding genes along with genes such as small nuclear RNAs used in RNA processing; **RNA polymerase III** transcribes all transfer RNA genes as well as the 5S component of ribosomal RNA [1]. After transcription, a terminal group called cap and a poly-A tail are added to the 5' and 3' end of mRNA, respectively (both help to regulate mRNA stability). Then RNA processing occurs by excision of introns and joining of the exons, in a process involving non-coding RNAs (mostly U1 to U6 small nuclear RNAs). Then, 5' end modifications in the mRNA will enable ribosome binding and initiation of protein synthesis [1].

Another important process that occurs in cells is DNA replication, which occurs during S phase of cell cycle (described in the next section; Figure 1.2). This process involves the separation of the DNA strands and breaking the hydrogen bonds by DNA helicase, stabilizing and preventing the reattachment of the chain by helix-destabilizing proteins as the synthesis of the new chain is not complete, breaks in DNA strands and ligation of them though topoisomerases, addition of new nucleotides by DNA polymerase during replication of the new strand with the presence of DNA Primase which is responsible for the synthesis of the RNA primer which will serve as a template for synthesis of new strands. At the end, the presence of DNA ligase are involved in the binding of the new DNA chains recently synthesized. At this stage, the majority of spontaneous mutations occur in the genetic material in the cell (summarized in table 1.1) [1]).

Table 1.1 Most important types of mutations and their distinctive features (Adapted from essential genetics: a genomics perspective [1]).

	Types of Mutations	Features
Origin	Spontaneous	Occurs in absence of known mutagen
	induced	Occurs in presence of known mutagen
Cell type	Somatic	Occurs in nonreproductive cells
	Germ-line	Occurs in reproductive cells
Expression	Conditional	Expressed only under restrictive conditions
	Unconditional	Expressed under permissive conditions as well as restrictive conditions
Effect on function	Loss-of-funtion (knockout, null)	Eliminates normal function
	Hypomorphic (leaky)	Reduces normal funtion
	Hypermorphic	Increases normal funtion
	Gain-of-funtion (ectopic expression)	Expressed at incorrect time or in inappropriate cell types
Molecular change	Base substitution	One base pair in duplex DNA replaced with a different base pair
	Transition	Pyrimidine (T or C) to pyrimidine, or purine (A or G) to purine
	Transversion	Pyrimidine (T or C) to purine, or purine (A or G) to pyrimidine
	Insertion	One or more extra nucleotides present
	Delection	One or more missing nucleotides
Effect on translation	Synonymous (silent)	No change in amino acid encoded
	Missense (nonsynonymous)	Change in amino acid encoded
	Nonsense (termination)	Creates translational termination codon
	Frameshift	Shifts triplet reading of codons out of correct phase

Fortunately, these DNA damages can be repaired through various mechanisms, such as, homologous recombination (HR), non-homologous end joining (NHEJ) (responsible for repair double-strand breaks (DSB)), base excision repair (BER) and nucleotide excision repair (responsible for repair single-strand breaks). NHEJ and HR repair the DNA DSBs induced by any mediator capable of splitting the DNA strands [2]. Such agents can comprise endogenous factors like reactive oxygen species, but also exogenous factors like UV radiation. HR system utilizes a homologous section on a parallel chromatid to correctly repair the DSB. NHEJ takes the ends of the damaged DNA molecule together by creation of a synaptic complex resulting of combination between two DNA ends, two Ku70/80 and two DNA-PKCS molecules. The repair are followed by interruption of ligase IV/XRCC4 complex. BER and NER are also important systems to repair single-strand damage, BER is important to shield DNA from the endogenous damaging effects made by intracellular metabolites that change DNA base structure. The essential enzymes involved in BER system are DNA glycosylases (responsible to eliminate different types of damaged bases thru cleavage of an *N*-glycosidic bond between base and deoxyribose of the nucleotide residue). When base is removed, the apurinic/apyrimidinic (AP) site is detached by an AP endonuclease or an AP lyase, which marks the DNA strand 5' or 3' to the AP site. The lasting deoxyribose phosphate residue is excised by a phosphodiesterase; the resulting gap is then complete by a repair DNA polymerase strand is joined by a DNA ligase. Lastly, NER system responds to harmful effects of a multitude single-strand lesions, which can include damage made by environmental carcinogenic factors, this system can repair lesions over the entire genome and lesions in the synthetized strands that block transcription [2]. When those mutational events provoked by mutagens escape repair machineries, they can pass to a daughter cell. If this process occurs in germ cells these errors can pass to the progeny, however if these errors occur in a somatic cell, they will not be transmitted to the progeny (Figure 1.3). Nevertheless, as described in the next section, these errors might originate an aberrant group of cells that eventually may result in cancer [3]. As mentioned before, mutations are normally induced by mutagens. These can be associated to nutrition, age, sedentary lifestyle and professional occupation [12].

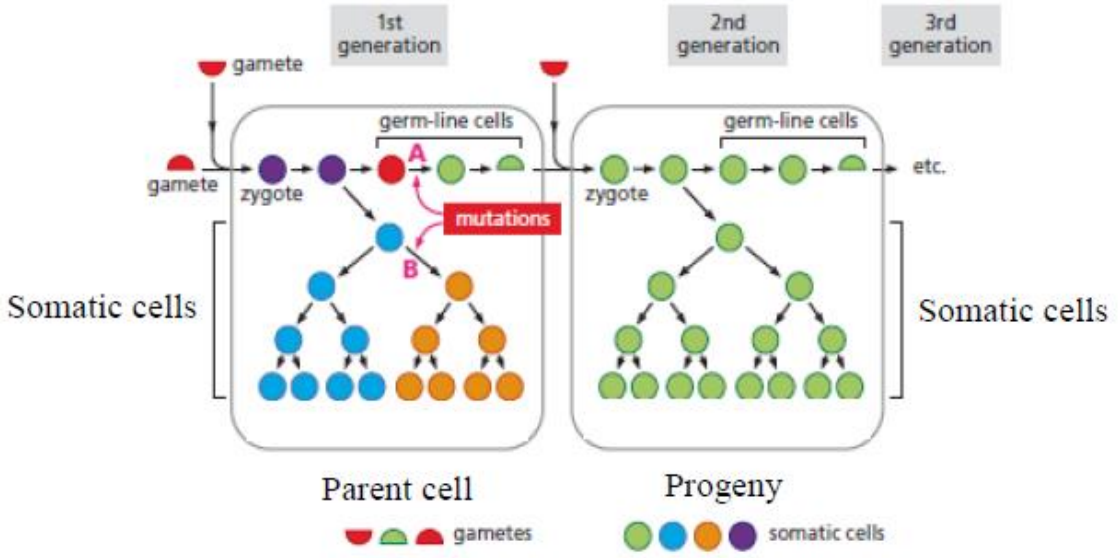

Figure 1.2 Schematic overview of inherited germ-line versus somatic mutational events. Mutation A occurs into the genome of a germ-line cell and pass to other cells in the progeny and so on. On the other hand, mutation B only can be passed to lineal descendants of that mutant cell in the body of the parent and cannot be transmitted to progeny since this mutation occurs in a somatic cell line genome (Adapted from Biology of cancer [57]).

1.2 The Biology of Cancer

Under normal conditions, nearly all cells of human body have the capacity to divide and proliferate in order to balance situations where cell death occurs, either due to mutations or because the cell has aged. For this to occur, the cell requires a specialized cellular machinery so as to coordinate the entire process that is cell cycle. This machinery involves a complex network of multiple exogenous signaling, such as, growth factors or other signaling molecules that can be produced by autocrine regulation (produced by the cells), paracrine regulation (cell respond according to a stimuli received by neighboring cells interactions) or systemic endocrine regulation (cell receives inducements thru circulating hormones) [3]. Normally, a cell is in a quiescent phase (G0) performing their structural and biological standard functions. When necessary, in presence of a stimuli (e.g. growth factors), phase G1 is activated and the cells start their cycling. After initiation, the need of growth factors is lost when cells pass restriction point R through S phase. At this stage, DNA duplication occurs and point mutations, such as insertion or deletion of one or more nucleotides, might occur. As described next, in some cases these errors are not properly corrected and the cells somehow escape apoptotic pathways, which ultimately may result in cancer. After S phase, the cell enters into G2 phase, where the enzymatic machinery necessary to the next phase is produced.

After this, the cells enter M phase (Mitosis), where chromosome pairs condensate and are distributed equally to the daughter cells. At the end of this phase, daughter cells detached one from another and can restart all the cycle process or enter in a quiescent phase (G0) (Figure 1.3) [3].

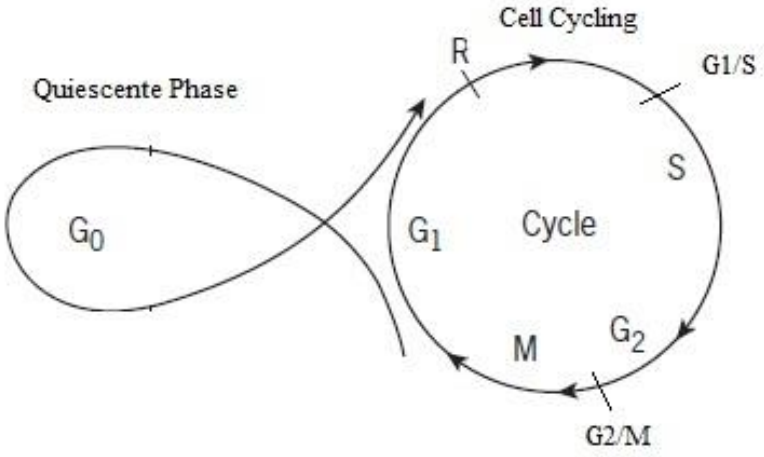

Figure 1.3 Cell cycle. Cell initiate its cycling into G1 phase, synthetize DNA in S phase, prepare the necessary machinery during G2 phase, enter M phase (where segregation of chromosomes occurs) and form two new cells (adapted from the cancer handbook [3]).

As mentioned before, when by some reason, one of these steps fail and DNA suffers a damage capable of disrupt the well-functioning of this process, cell has a proficient mechanism in order to either repair those damages or to induce apoptosis by activation of pathways in multiple checkpoints, namely G1 restriction point (R), G1/S and G2/M (figure 1.3). These checkpoints are recognized by signaling paths and feedback mechanisms that guarantee that a cycle phase do not initiate a new phase until the previous one has been concluded. If one of these checkpoints fails, genomic instability or apoptotic mechanisms ascends and give rise an important step to encourage progression from a normal to a malignant cell. This deregulation due to the activation of oncogenes and loss of tumor suppressor genes are the major initial step leading to the first stage of tumorigenesis [3].

In the beginning of a cell cycle, a cell receives an exogenous signal whether by growth factors or molecules of extracellular matrix. Receptors present in the cell membrane integrate these signals inside the cell and trigger multiple signaling cascades that are ultimately integrated in the nucleus, forcing the cells to initiate the cycle.[4] When the cells are either 1) subjected to an exaggerated stimuli by these factors, or 2) those receptors or elements of the signaling cascade are defective, or 3) regulators of cell cycle are defective, then the normal cells are constantly activated and instigated to an uncontrollable proliferation, promoting the occurrence of irreversible mutations in genes. Such process is typically denominated initiation

of tumorigenesis (Figure 1.4 - panel A). In the meantime, during the promotion phase, the altered cell and their descendants continues to look normal but its proliferation is much higher leading to a stage so called hyperplasia. After a while (time is variable depending on the tumor), one million of these altered cells undergo another mutation that decreases more the control of cell growth, and the descendants of these cells begin to show abnormal shapes and enter into a stage of dysplasia (figure 1.4 - panel B) [4]. These cells and corresponding tissue become progressively more aberrant. If this tumor has not broken yet all boundaries between tissues, is called *in situ* adenocarcinoma. This tumor can remain indefinitely contained (figure 1.4 - panel C) [4]. However, some cells eventually acquire additional mutations that lead to genetic modifications and allow the tumor to invade the underlying tissues, i.e. the tumor becomes invasive. Some of these cells may be proficient enough to i) escape their matrix, ii) invade blood or lymph vessels, iii) escape blood or lymph vessels (extravasion), and iv) ultimately invade and colonize new tissues. This process, through which cells from primary tumors establish new tumors in other tissues / organs, is denominated tumor metastization, which may become deadly by disruption of vital organs (figure 1.4 - panel D) [5].

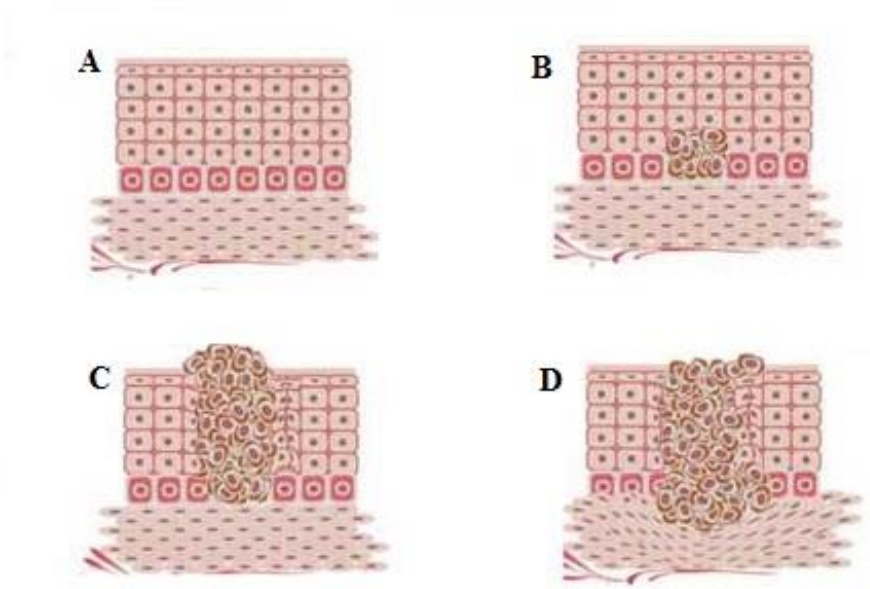

Figure 1.4 Tumorigenesis progression. Cells start to grow uncontrollably, accumulates different types of mutations and acquire the capacity to spread to distant sites from the primary tumor. (Adapted from https://www.aecc.es/sobreelcancer/cancerenimagenes/Paginas/Cancerenimagenes.aspx)

An important feature of cancer cells is that they are able to create an advantageous microenvironment that enables them to proliferate and survive in hostile conditions. Thus, cancer cells can: i) recruit other cells (through secretion of cytokines) that will emit important stimulatory signals to induce tumor growth; ii) emit their own signals to prevent the immune

responses; or iii) produce enzymes that will digest the involving matrix and allow cancer cells to migrate into surrounding tissues. These are examples of the crosstalk between cancer cells and stroma or organisms [5]. Another key characteristic of tumors is their genome instability, which can correspond either to microsatellite or chromosome instability. This feature causes genetic diversity among tumors and accelerates the tumorigenic process [5].

In general, any cell of a particular tissue can give rise to a neoplasm (abnormal growth of cells). Once these cells loses the ability to control its cell cycle by loss of tumor suppressor genes or oncogenes, it gains the ability to divide abnormally resulting in a tumor mass that can either be: i) **benign,** characterized by a slow and typically encapsulated cell growth; or ii) evolve to **malignancy,** characterized by a more rapid cell proliferation and ability to invade surrounding tissues, which may give rise to secondary tumors. According to their cellular origin, tumors can be classified into **carcinomas** and **melanomas** (derived from epithelial cells), **sarcomas** *(*arise from mesenchymal tissues), **retinoblastomas**, **neuroblastomas** and **glioblastomas** (derived from cells in the ocular retina, neurons and neural glia) [2].

1.3 Colon adenocarcinoma as a genetic disorder

Colon adenocarcinoma (CAC) is the second most frequent cancer in Portugal, representing 10% of all tumors in both genders. Although CAC diagnosis is usually performed in a symptomatic phase, with a level of progression around 10 years, the prognosis remains relatively reserved, presenting an overall survival rate after 5 years slightly above 50% for both men and women. Indeed, every day in Portugal around 9 to 10 patients die with colon and rectum adenocarcinomas, and similar numbers are found worldwide [6]. In this regard, population-wide screening and detection of CAC in an asymptomatic stage is the main strategy that can change this situation in order to diminish mortality [6]. Indeed, according to ROR-Sul (Oncological Registration in South of Portugal; 19) data, 81% male and 78% female patients under 60 years of age were diagnosed with CAC [8].

At a histological and molecular level, CAC is the best studied cancer type, which in part results from its insidious progression and easy visualization and separation of different stages [2]. In figure 1.5 the main molecular events associated tumorigenic steps colon adenocarcinoma (initiation, progression and invasion) are depicted

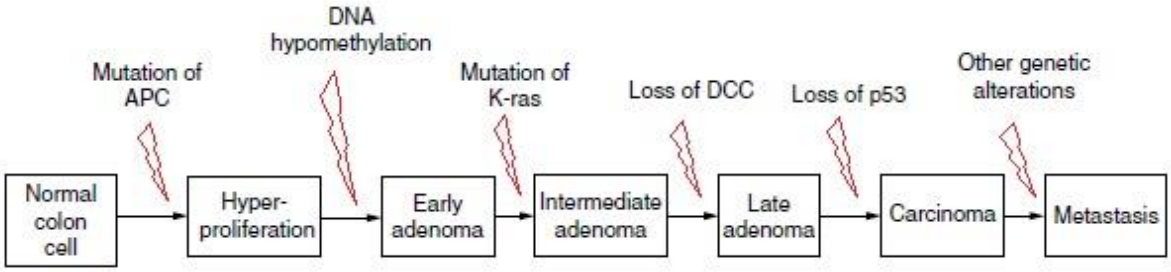

Figure 1.5 Colon adenocarcinoma model proposed by Fearon and Vogelstein. Multistage progression of colon adenocarcinoma, where mutations on the *APC* tumor suppressor normally are considered to be the initial event, followed by the successive accumulation of other epigenetic and genetic changes (loss of deleted colon cancer gene (DCC) or mutated colon cancer (MCC) and p53) that possibly can result in the progression from a normal cell to a metastatic tumor (adapted from Molecular biology of cancer [4]).

From an histological and pathological point of view, colon adenocarcionomas are classified according to a standardized system that characterizes how far tumor has progressed: if it has spread to other areas (T), if it is already associated with the lymphatic system (N), and how distant it have spread and metastasized to other organs (M; most commonly to lungs and liver. This classification, the TNM staging, is the most in used system for tumors characterization, and is also known as American Joint Committee on Cancer (AJCC) [9]. Nevertheless there are other classification systems, such as Dukes and Astler-Coller systems [9].

1.3.1 Sporadic colon adenocarcinomas

More than 95% of colon adenocarcinomas are sporadic, which means that they are caused by point mutations that occur and are perpetuated in somatic cells of colon epithelium. Generally, these tumors are associated with early mutational events and the progression of age, appearing at a mean age of 70 years and can be caused by external factors inherent to the lifestyle of the patient [2]. Indeed, the contact to a variability of natural and artificial mutagens in the environment is believed to account for up to two-thirds of cancer mortality (depending the cancer type) [4]. In general, these mutagens can be classified as physical (e.g. radiation), chemical (e.g. hormones and organic combustion products) and biological mutagens (e.g. viral and bacterial infections). Factors like these are present in the day life of an individual and are strongly associated with the rising of spontaneous colon adenocarcinomas [12].

One the most important biomarkers of adenocarcinomas and always considered in pathological exams is *K-Ras*. This gene belongs to the Ras family and is a small molecular weight protein, post-translationally modified by the addition of a farnasyl fatty acid moiety to the C-terminus. This post-translational modification is essential for the activity of *Ras* oncogenes, and therefore it has become an important target for the development of therapeutic strategies [10]. This protein binds equally to guanosine 5'-triphosphate (GTP) and guanosine 5'-diphosphate (GDP) in a reversible way. When activated, *Ras* bounds to GTP and is capable to initiate signals through mitogen-induced and stress-induced pathways, regulating gene transcription (required for cell growth) and proliferation. Signals like this are typically promoted by activation of receptors such as the epidermal growth factor receptor (EGFR) [10]. Thus *K-Ras* or EGFR mutations are known to result in a constitutive activation of these pathways, leading to overstimulation of cell proliferation and ultimately to cancer. For this reason, these genes are considered to be good biomarkers and therapeutic targets in cancer, more specifically in colon adenocarcinoma [10]. However, it is important to determine and distinguish these changes since it can influence the type of treatment to follow. For example, targeted treatments against EGFR are not as effective if the patients carry *K-Ras* mutations.

Other crucial genes in colon adenocarcinoma are the *APC*, the *MSH2* (acts in the mismatch DNA repair system) and *p53* (activated in response to cellular stress). These are tumor suppressor genes, which are responsible to inhibit cell growth directly or indirectly. The tumor suppressor genes that promote apoptosis of a cell directly are called "gatekeepers" [4].

1.3.2. Inheritance of CAC predisposition

Familial adenomatous polyposis coli (FAP)

There are some morphological and molecular evidences that colon adecarcinoma develops through several precursor stages (figure 1.5). The initial identifiable pre-neoplastic modifications results in hyperplastic or dysplastic adenomatous polyps precursors of adenocarcinomas. Polyps are regularly found as single benign tumor distended into the lumen of the large intestine and consist of a relatively disorganized epithelium. In the familial adenomatous polyposis coli syndrome (FAP), multiple polyps are found and it is strongly associated with the risk of colon adenocarcinomas [11]. Patients with FAP tend to develop hundreds of adenomatous polyps in an early age (~40 years old) that are present alongside the

entire gastrointestinal track. Different phenotypes are also underlined to this inherited syndrome, for instance, Attenuated FAP (AFAP; differs from normal FAP in less number of polyps displayed), Congenital hypertrophy of the retinal pigment epithelium (CHRPE; the early detection of this variant could be an indicative of FAP before the appearance of polyps) and Gardner's syndrome (associated with benign osteoid tumors and skin cysts). With the intention of diminish the risk of colon cancer associated with this syndrome, the advised therapeutic to follow involves a prophylactic colectomy or a proctocolectomy [11]. This syndrome is characterized by being inherited in an autosomal-dominant way, with an inherited partial or total deletion of an *APC* allele. When the loss of the other functional copy occurs, this loss is denominated loss of heterozigosity (LOH) [11]. This situation is the major example of inherited colon adenocarcinomas. However, this mutation is also known to occur in sporadic colon adenocarcinomas during a former stage of colon adenocarcinoma tumorogenesis also with a LOH association. As a result, constitutive activation of oncogene *c-myc* occurs (through a difficult assembly of protein-protein interactions). *APC* can also interact with other cellular proteins, including the oncogene *β-catenin*. Loss of *APC* ultimately leads to stabilization of *β-catenin* and activation of Tcf-mediated transcription (anti-apoptotic) [11]. Besides the regulation of proliferation and apoptosis, APC is important to cellular adhesion and cytoskeletal integrity, maintenance of intestinal cell migration and regulation of *c-myc* and cyclin D1 expression levels in G1/S transition phase [11].

H*ereditary non-polyposis colon carcinoma (HNPCC)*

The hereditary non-polyposis colon carcinoma syndrome (HNPCC) or Lynch syndrome is known to be more frequent than FAP syndrome and usually found in an upper zone of the colon comparatively with FAP syndrome and sporadic cases of colon adenocarcinoma. The major difference between HNPCC and FAP syndrome is the type of mutations found. For example, nearly all the genetic modifications results from base exchanges, small deletions or insertions of base pairs and point mutations, whereas chromosomal modifications are occasional. The mutations founded associated to this syndrome are simply detected in microsatellite sequences that can be the repetition of one, two or three nucleotides. Once these microsatellites are greatly polymorphic, individuals tend to be heterozygous. These microsatellites can be used as allelic markers or to track chromosomal recombination and loss in carcinogenic cells [13].

Increase or reduction of a microsatellite repeat is denominated as microsatellite instability (MSI). This phenomenon leads to inactivation of genes involved in mismatch repair mechanisms. Several proteins present in this mechanism are responsible for the first detection of these mismatches during DNA replication. Important examples are MSH2 / MSH6 and MSH3 / MSH6 heterodimers, MLH1, hPMS1 and hPMS2. Patients with HNPCC carrying mutations in one gene (MSH2 or MLH1) that encodes for this repair mechanism have more probability of developing colon cancer. Again, in these cases the inactivation of the second copy has to occur in order for the repair system to be compromised (8). Curiously, this MSI phenotype can also be observed in sporadic cancers in patients without a family history. In some cases, the inactivation of MLH1 gene is permitted by promoter hypermethylation, increasing the risk of mutations in other genes. Hence, HNPCC cancers can embrace the same gene mutations that prevail in other colon adenocarcinomas, e.g. *APC, p53, K-Ras* or β-catenin. Nonetheless, some genes are privileged mutation targets in colon cancers rising in HNPCC syndrome and sporadic MSI compared to other colon cancers forms [13].

1.4 Matrix Gla Protein (MGP)

Human MGP is a 10kDa protein dependent of vitamin K metabolism, contains84 amino acid residues, is primary structure includes a signal peptide, a phosphorylation domain, and a γ-carboxylase recognition site. Additionally, MGP contains five residues of γ-carboxylated glutamic acid (Gla) residues that are converted from Glu to Gla residues by γ-glutamyl carboxylase enzyme, dependent of vitamin K, via a posttranslational modification. This protein was initially isolated from extracts of demineralized bovine bone matrix and was later isolated from cartilage [14][15]. MGP is found in a wide range of non-calcified tissues, such as lungs, kidneys and heart [16] and despite this extensive tissue distribution, only cartilage and bone can gather significant levels of MGP in the extracellular matrix [16].

1.5 MGP function

MGP has been described to have an important role on mineralization inhibition and chondrocytes maturation [17]. Genetic studies have shown that *MGP* null mice die around two months of age as a result of excessive abnormal calcification of their arteries, leading to blood vessel rupture, clarifying the role of MGP as a physiological inhibitor of calcification

[18]. Lack of MGP results in transdifferentiation of aortic smooth muscle cells to osteochondrogenic lineage, possibly due to release of BMP2/4 from MGP dependent functional inhibition. MGP has also been involved in cell differentiation as suggested by Yagami et al (1999) [19]as well as in proliferation (reviewed by [13]). Mutations found in human *MGP* gene have been associated with abnormal calcification of cartilage in Keutel syndrome (5), pathological conditions related to atherosclerosis (vascular calcification) and a wide range of cancers [20][21][11].

1.6 Regulation of *MGP* gene expression

As already mentioned, known sequences of vitamin K-dependent proteins in vertebrates have a signal peptide followed by a transmembrane recognition site of γ-carboxylation, and a domain containing Gla residues in each of these regions, which in *MGP* corresponds to a single exon. The human *MGP* gene, localized in chromosome 12, has been cloned and sequenced [22] and until recently, was thought to give rise to a single transcript containing four exons. However, recent evidences show that more than one variant can arise from this gene. Indeed, a new *MGP* variant (E5; accession number: NM_001190839.1) containing five exons was shown to occur (Figure 1.6) [13].

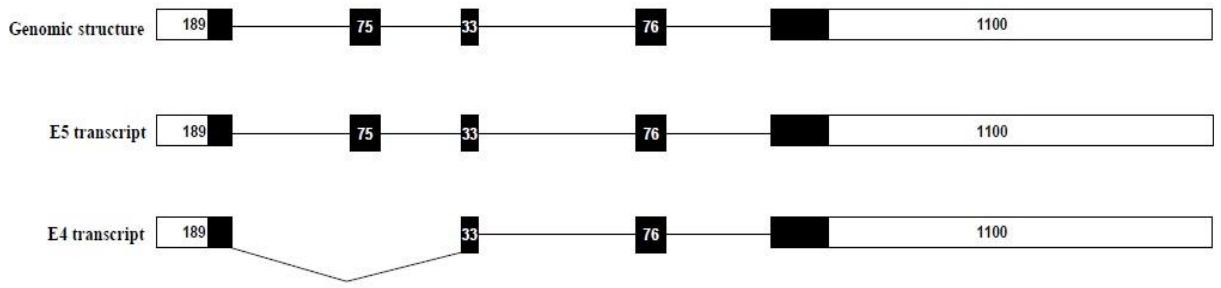

Figure 1.6 Alternative splicing in transcript variant E4. Schematic representation of *MGP* splice variants. The sizes of exons (1cm=100bp) and introns (1cm=300bp). Solid boxes indicate the coding regions and open boxes indicate untranslated sequences.

As depicted in figure 1.6 this variant comprises a new sequence with 75bp encoding a new epitope with 25 amino acids. The function of *MGP* E5 remains unclear, and therefore exploring its expression in systems where *MGP* was previously detected will be crucial to understand is role.

The promoter of *MGP* gene has been investigated in previous studies. In this regard, many transcription factors putative binding sites have been reported, but only a few were actually shown to be functional.

Thus, previous analysis of human *MGP* gene promoter revealed the existence of several consensus structural motifs apparently important for putative binding of the typical TATA and CAAT boxes, of AP1 and AP2 transcription factors and of metal-responsive elements. This analysis also revealed the existence of two polymorphisms, i.e. G-7A and T-138C, in a region apparently essential for transcription activity (as determined *in vitro*). Since the second polymorphism was associated to AP-1 binding site it was postulated that it would influence AP1 complex binding to *MGP* promoter and alter *MGP* gene expression. Given the previous association of *MGP* to calcification in arteries, it was hypothesized that this polymorphism could be implicated in higher susceptibility to atherosclerosis and aortic valve stenosis [23].

In other studies, specific binding sites for retinoic acid (RA) and vitamin D (VD) receptors were identified in *MGP* promoter. However, the effects of these regulatory elements were far from being consensual. Regarding RA, depending on the cell lines (fibroblasts, chondrocytes, osteoblasts, human osteosarcoma cells MG-63, teleost fish and MCF-7) it was shown to either induce or repress *MGP* gene expression [24][25]. In the case of VD, in some cell types (human osteosarcoma cells MG-63) it was shown to up-regulate *MGP* gene expression, while in other (fibroblasts, human osteoblast and chondrocytes), it did not promote significant changes unless it was administered in combination with RA. In the latter case, VD attenuated the positive regulation promoted by RA [26][27][28].While discrepancies found for RA may be explained by the presence of different elements in the respective receivers (i.e. cell lines), the antagonism found between RA and VD may be explained by the potential formation of heterodimers between RA and VD receptors, which should decrease the amount of available RA receptors [28].

Another transcription factor that was shown to be able to regulate *MGP* gene expression is Runx2, although this was only demonstrated in *Xenopus laevis* [10].Interestingly, in *Xenopus laevis*, two functional promoters (proximal and distal) were identified in *MGP* gene, and both shown to be regulated by Runx2. This regulation apparently involved Runx2 binding to three specific regions, through which it was shown to induce transcription activity. Additionally, a fourth Runx2 binding site was found in the distal promoter but with a putative repressive action in gene transcription. This suggested that Runx2 may have a dual effect on MGP expression depending on which site it binds [10], in addition to AP1, RA and VD (in addition with RA) as promoters of MGP transcriptional regulators.

1.7 *MGP* in Cancer

Despite what is known about the regulation of *MGP* in bone and cartilage, evidences have demonstrated the existence of a regulatory effect in breast cancer, colon carcinomas and glioblastomas [11][15][24][16]. Still, little is known of how *MGP* acts during tumorigenisis pathways, how deregulated expression is related to different kind of tumors. Both in breast cancer and glioblastomas it was demonstrated a relationship between the upregulation of *MGP* and the poor prognosis displayed [11][16]. *MGP* has been independently discovered by differential cDNA screening as a gene which is overexpressed by human breast and urogenital tumor tissue and cell lines, but a downregulated expression was observed to be correlated with an unfavorable clinical status such as a poor differentiated state, a larger tumor size, and lymph node metastasis [29]. In another study it was reported the putative value of *MGP* as a biomarker for poor prognosis in patients with breast cancer. Results showed that in patients exhibiting a poor prognosis *MGP* mRNA expression was up-regulated, which led to suggest that *MGP* have the potential to serve as a prognostic indicator of the disease, however, no correlation was established between protein levels and overall survival rate [11]. In colon adenocarcinoma the expression levels found demonstrate the inverse situation [15], levels of mRNA for *MGP* were found to be downregulated in colorectal tumor tissues as compared with their paired normal tissues [15]. *MGP* has also been found to be overexpressed in ovarian cancer [30], in prostate epithelial cells undergoing apoptosis [31] and in vascular smooth muscle cells undergoing dedifferentiation in cell culture [32]. These results suggest that additional players must be involved in *MGP* regulation process and more work needs to be developed in order to understand the involvement of *MGP* in tumorigenesis.

Since the function of *MGP* and its variants are presently unknown in CAC, it is important to try to understand if *MGP* along with is putative transcriptional regulators may be associated with the progression of tumorigenisis in sporadic or inherited forms of CAC and if it can be consider as a possible biomarker for future therapeutics.

1.8 Main Aims

In recent years, we have assisted to an improvement of general methods for diagnosis and mass screening of colon adenocarcinomas. This resulted not only in an increase in the number of tumors detected at an early stage, but also in a higher life expectance of these patients (average survival rate after 5 years of ~50%). However, despite an effort to allow all patients to have access to routine exams to detect colon adenocarcinomas, in many cases these tumors are still found in advanced stages. In that sense, it is vital to search for new biomarkers and therapeutic targets in colon cancer. With the colon adenocarcinoma being the second most frequent cancer in Portugal (and worldwide), representing 10% of total tumors in both genders, is urgent to find such new biomarkers. MGP, a protein that was previously associated to physiological and pathological inhibition of calcification of cartilage and arteries, was shown to be involved in many cancer types. Although the specific involvement of MGP in colon adenocarcinoma was previously addressed, is specific role in this tumor and association to different stages was far from being established. In this regard, the main aims of this research were to:

1) Investigate *MGP* dysregulation in colon adenocarcinomas;
2) Identify specific tumor stages where *MGP* gene becomes deregulated;
3) Identify gene regulatory mechanisms associated with *MGP* deregulation in colon adenocarcinomas.

To accomplish these objectives, samples from tumoral and adjacent normal mucosa of patients with colon adenocarcinoma were obtained through collaboration with Gastroenterology Department of Hospital de Faro. RNA was extracted from these samples and further used to analyze the expression of MGP and transcription factors.

Chapter II

Methods and Materials

2. Materials and Methods

2.1 Patient selection and collection of tissue samples

Tissue samples were dissected directly from tumor tissue (MT) and 5 cm away from tumors (control samples, MN) of 45 patients with colon adenocarcinomas during colonoscopies exams at Hospital Central de Faro (Gastroenterology Service, Dr. Paulo Caldeira). Samples were collected to microcentrifuge tubes containing 1ml RNAlater (Sigma-Aldrich) and maintained at 4ºC up to 24 hours. From these patients, 11 were male and 9 were female, and their ages ranged from 36-92 (Table 3.1 Chapter 3). Clinical stage of the tumor was previously determined according to TNM Staging criteria then confirmed by a pathology service. These samples were obtained with full knowledge of the patients and approved by local ethics commission.

2.2 RNA Extraction

The method used for total RNA extraction was based on that described by Chomczynski and Sacchi [25]. Briefly, tissues were homogenized in 1 ml Solution D (see appendix I for solution composition) using a 1ml syringe with 20 gauge needle. This process was also used to shear genomic DNA. Then, each sample was mixed with 100 µl of 2M NaOAc (Sodium Acetate, pH 4.0), 1 ml of phenol (pH 4.3±0.2) (Sigma-Aldrich) and 200 µl of CIAA (Chloroform:Isoamyl alcohol) (see appendix II for the solution composition), and incubated for 15 minutes on ice. Samples were centrifuged for 15 minutes at 10,000xg, 4ºC. Aqueous upper layer was transferred to a new microcentrifuge tube containing 1 ml of 100% isopropanol, mixed and stored for 1 hour at -20ºC, for RNA precipitation. Then, samples were centrifuged for 30 minutes at 10 000xg (4ºC) and RNA pellets re-suspended with 500 µl of solution D and transferred to a clean microcentrifuge tube containing 500 µl of 100% isopropanol. RNA was again precipitated (1 hour at -20ºC) and centrifuged for 15 minutes at 20 000xg, 4ºC. Pellets containing total RNA, were washed with 500µl of ice-cold 75% ethanol, left air dry for 1 minute and dissolved in 30µl of H2O (Sigma).

Quality and quantity of RNA was assessed by spectrophotometry (using Nanodrop ND-1000; Thermo Scientific), through reading the absorbance of each sample at 230, 260 and 280 nm, and determining the ratios at 260/230 nm and 260/280 nm. Additionally, each sample (1µg) was analyzed in 2% TAE agarose gel electrophoresis for size-separation and detection of most abundant ribosomal RNAs. Finally, samples were stored at -80º C for later use.

2.3 Synthesis of cDNA by Reverse Transcriptase reaction

Total RNA (1µg) was treated with RQ1 DNase (1U for each 1µg of RNA; Promega) according to manufacturer's instructions. After 30 minutes incubation at 37ºC, reaction was stopped by supplementing RQ1 stop solution (Promega) and incubating 10 minutes at 65ºC. Then, RNA was purified using the High Pure RNA isolation kit (Roche), according to manufacturer instructions. For further analysis of mRNAs, samples were used to synthesize cDNA using the Moloney-murine leukemia virus (M-MLV) reverse transcriptase (200U/µl, Invitogen), according to manufacturer protocol. Briefly, 500 ng of total RNA were supplemented with 0,4µl oligo$(dT)_{12\text{-}18}$ Universal oligonucleotide (500µg/ml; Sigma-Aldrich; see Table X), 1µl dNTP's (10mM, Invitrogen) and water (Sigma-Aldrich) for a total volume of 12µl. This mixture was incubated during 5 minutes at 65ºC, and then supplemented with 4µl of First Strand buffer (5x, Invitogen), 2µl DTT (0,1M, Invitrogen), 1µl RNaseOUT (40U/µ, Invitrogen) and 1µl of M-MLV reverse transcriptase. Reverse transcription continued for 50 minutes at 37ºC. Finally, samples were incubated during 15 minutes at 70ºC to inactivate the reaction enzymes and denature cDNA.

2.4 Real-Time Reverse Transcriptase Quantitative Polymerase Chain Reaction PCR (RT-qPCR)

cDNA samples were used as template for real-time PCR analysis of mRNA expression using specific primers listed in table I.1 (see appendix I). To determine mRNA expression, 2µl of cDNA (1:10 dilution) were mixed with 10µl of SsoFast^{TM} Eva Green Mix (BioRad), 0.6µl of each specific primer (10µM) and water (up to 20µl). PCR amplifications were performed in a StepOnePlus Thermoycler (Applied Biosystems) using the following conditions: i) denaturation and polymerase activation at 95ºC during 30 seconds; ii) 40 cycles of denaturation (5 seconds at 95ºC) and annealing/elongation (30 seconds at 57ºC); iii) melt curve (65ºC-95ºC) for determination of melting point. As negative control, PCR amplification were performed using water as template. Relative expression was determined using the ΔΔCt method. Threshold cycles obtained for each analyzed gene were normalized using GADPH (Glyceraldehyde-3-phosphate Dehydrogenase) housekeeping gene.

2.5 Cell line and Culture

HT29 human colon adenocarcinoma cell line was cultured in DMEM (Dulbecco's Modified Eagle Medium; Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine and 1% Pen Strep (Penicillin/Streptomycin mixture; Life Technologies), and maintained at 37ºC and 5% CO_2. Cells were subdivided 1:8 twice a week.

2.6 RNA interference assays

For RNA interference of MGP, HT-29 cells were either seeded in 6-well plates ($2.5x10^5$ cells/well) or 96-well plates ($1.0x10^4$ cells/well), and mixed with appropriate quantity of Lipofectamine RNAIMAX reagent (Invitrogen) and small interfering RNAs (Stealth siRNAs, Invitrogen), according to manufacturer instructions. Regarding the use of siRNAs, one was directed against MGP and the other was identical to the first one but containing mismatch nucleotides (used as negative control; Invitrogen). To verify the success of RNA interference experiment, qRT-PCR and western blot analysis were performed using extracts from cells seeded and transfected in 6-well plates (72h after transfection). Cells transfected in 96-well plates were used for proliferation assays, described next.

2.7 Proliferation assay

MTS (3-(4,5dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium reagent (Promega) was used to assay the number of viable cells in culture. Basically, viable cells contain dehydrogenase enzymes that will convert MTS into formazan, which absorbs at 450 nm. Since different cell lines are metabolically distinct, this assay had to be optimized for HT-29 cells. For that, HT-29 cells were seeded in 96-well plates ($1x10^4$ cells/well) and incubated for 12-16 hours at 37ºC / 5% CO_2. Then, MTS / PMS (activating reagent) mix was added to the cells as indicated by the manufacturer (Applichem). Absorbance at 450 nm (formazan) and 650 nm (background correction) was read after 2, 3, 4 and 5 hours. Linearity of this reaction was between 3 and 5 hours, and therefore optimal time of reagent incubation was considered to be at 4 hours. Then, these conditions were used to evaluate cell proliferation after transfecting HT-29 cells with siRNAs. Briefly, 24, 48 and 72 hours after transfection, HT-29 cells transfection media was replaced by new media

containing MTS / PMS. Then cells were incubated during 4h at 37ºC / 5%CO_2 and absorbance was read in a Synergy4 microplate reader (Biotek).

2.8 Western Blot assays

In order to prepare extracts from HT.29 cultures, cells were first rinsed (3X) with phosphate-buffered saline (PBS). After 50µl lysis buffer supplemented with complete 1x concentration (protease inhibitor, Roche. See appendix II for solutions composition) was added to culture and cells scrapped from plate surface using a cell scrapper (Sartstedt). Then, extracted cells were transferred into a microcentrifge tube and centrifuged during 15 minutes, at 15,000G and 4ºC. Supernatant was transferred into a new microcentrifuge tube (2-3 µl of this extracts were transferred into another microcentrifuge tube for further protein quantification) and mixed 1:4 with Sample Buffer 4x (SB4X; see appendix II for solutions composition). This mix was heated at 100ºC, 5 minutes, for protein denaturation, preceding SDS-PAGE. Simultaneously, pellet was re-suspended in the same volume, mixed SB4X and denatured for 5 minutes and 100ºC. At this stage, supernatants and pellets could be stored at -20ºC. Protein was quantified in cell extracts using the Quick Start™ Bradford Protein Assay (Bio-Rad), according to manufacturer's instructions. Briefly, the reagent was diluted 1:5 in water and filtered in 0.45µm pore filters. Subsequently, appropriate amounts (in 10 µl volume) of samples and protein standards (Bovine Serum Albumin - BSA) were added to each well of a 96-well plate, and supplemented with 200 µl of reagent. After 10 minutes incubation, absorbance was read at 595nm in Synergy4 microplate reader (Biotek).

After determining protein concentrations, equal amounts of protein samples were loaded into an SDS-PAGE gel (NuPAGE® MES 4-12%Tris-Acetate 3-8%; Invitrogen) and size fractioned using an appropriate running buffer (See appendix II for solutions composition) at 160V. Samples were then transferred onto a PVDF membrane (polyvinylidene fluoride membrane, Immobilon®-P, Millipore) using transfer buffer (See appendix II for solutions composition) during one hour at 30V. Finally, membrane was autoclaved to cross-link proteins and stored for future WB analysis.

For western blot analysis membranes were first incubated with blocking solution (See appendix II for solution composition) during two hours at RT. Then, membrane was incubated with primary antibody against MGP (rabbit IgG; ProteinTech) diluted 1:1500 in blocking solution during one hour at RT. After serial washes with PBST (2X 5 minutes) and salts solution (2X 5 minutes; see appendix II for solution composition), membrane was again

incubated with blocking solution for 15 minutes. Membrane was after incubated with secondary antibody anti-Rabbit IgG with horseradish peroxidase conjugate (Sigma) diluted (1:30000) in blocking solution during one hour at RT. To conclude, new serial washes were performed: i) 2X 5 minutes with PBST, ii) 1X 10 minutes with Salts solution, iii) 3X 5 minutes with PBST, and iv) 1X 1 minute with PBS. To reveal the signal, membranes were incubated with chemiluminescence substrate (Western Lightning® Plus-ECL, PerkinElmer, Inc.), according to the manufacturer instructions. Corresponding images were obtained using the ImageQuant LAS500 (GE Healthcare Life Sciences).

For normalization of the western blot, β-actin (santa-cruz, Biotechnology) was used as a housekeeping, fowling the previous method described for membrane incubation. Initially an incubation with primary antibody against β-actin diluted (1:15000) followed by an incubation with the secondary antibody mouse igG with horseradish peroxidase conjugate (Sigma) diluted (1:30000).

2.9 Statistical analysis

The results of qRT-PCR were analyzed by paired t-test to determine the significance of the difference between expressions of MGP in the paired normal and tumoral tissue samples obtained from patients. Data are presented as mean of standard deviation from independent replicates and the statistical significance of the results was obtained using the GraphPad prism 5.00 Software, Inc., La Jolla, CA, USA and IBM SPSS Statistics 22 Software, (Armonk, NY, USA). Probability under 5% ($P \leq 0.05$) was considered to be significant.

Chapter III

Results and Discussion

3. Results and Discussion

3.1 Clinical characteristics and *MGP* gene expression in patients with colon adenocarcinoma

Over the past years, the important role of MGP as a physiological inhibitor of mineralization has been described in several systems [26] including mammals [14], [27], [28], amphibians [29] and bony and cartilaginous fish [19] [30] [31]. In addition, MGP has been shown to be expressed in several other organs, including lung, heart, kidney [32] [33] [34], vascular system [35], and in the intestine [15], but its function in these systems and mechanisms of action remains largely unknown. MGP has been also associated with several pathological conditions, such as ectopic calcification in β-thalassemia [36], atherosclerosis [37] , chronic kidney disease [38] and several other cancers, including breast carcinoma [37] [39] and glioblastoma [16]. Interestingly, despite being one of the most common and well-described cancer types, the association of MGP to colon adenocarcinoma has been roughly established and so far poorly investigated [15]. Recently, a new variant of MGP (E5) has been published with a new exon compared with the existent variant E4 with four exons probably due to the existence of an alternative splicing in exon two. It is known that this new variant is expressed in human some particular tissue, however, its function related with tumorigenic or other types of molecular mechanism remains unknown to the best of our knowledge.

In order to explore the presence of both *MGP* variants (E4 and E5) in colon adenocarcinomas, we performed qRT-PCR analysis using RNA samples collected from 20 patients selected after clinical and pathological characterization and specific set of primers for both variants (E4 Forward1/Rev5 and E5 Forward1/Rev4; see Appendix I, Table I.1),. This group of patients was characterized according to age, gender, tumor location, tumor differentiation, K-Ras mutations, tumor staging, family history (for possible association with genetic syndromes) or association to other diseases (including other tumors; see Tables 3.1 and 3.2). All these characteristics were considered as putative factors that could influence *in vivo* MGP regulation.

Table 3.1 Clinical characterization of patients and MGP relative variation between tissues samples

Patient	Age	Gender	Location	Adenocarcinoma Differentiation	K-Ras Mutations	TNM/Stage	Family history	Other Information	Relative Δ between total *MGP* expression MT vs MN
1	56	Male	Rectum	Moderately	-	pT3N0M0/ IIA	-	Urothelial G2 Carcinoma non invasive	1.0
2	58	Male	Rectum	Well	-	pT3N1M1/ IVA	Father (adenocarcinoma)	Hepatic metastases	0.5
3	85	Male	Rectum	Moderately	Without examination	pT3N2M0/ IIIC	-	Malignant neoplasm in prostate	-0.6
4	66	Female	Rectum	Well	-	pT3N1M1/ IVA	-	Hepatic metastases	4.0
5	36	Female	Rectum	Well	Without examination	ypT2N0M0/ IIIC	Aunt	-	1.6
6	54	Male	Rectum	Moderately	-	pT3N2M0/ IVA	-	HIV Positive	1.8
7	72	Female	Rectum	Poorly	-	pT3N1M1/ IVB	-	Hepatic metastases	14.9
8	73	Female	Left colon	Moderately	-	pT3N1M0/ IIIB	-	Diabetes Mellitus 2, AVC, 10 years ago Malignant neoplasm in tyroid	4.7
9	85	Female	Right colon	Moderately	+ (p.Gly12Val exon 2)	pT3N2M0/ IIIC	-	Ischemic cardiomyopaty	1.7
10	75	Male	Left colon	Well	+ (p.Gly12Asp exon 2)	pT3N2M0/ IIIC	-	Benign hypertrophy of prostate	1.6
11	68	Male	Right colon	Moderately (mucinous adenocarcinoma)	+ (p.Gly12Asp exon2)	pT3N2M0/ IIA	Brother (Malignant neoplasm in Colon)	-	0.7

12	78	Male	Left Colon	Well	-	pT2N0M0/ I	-	Hypertension, 9 Years ago Malignant neoplasm in prostate	35.9
13	81	Male	Ascending Colon	Well	-	cT3N0M0/ IIA	Brother (Malignant neoplasm in Colon) Father (lymphoma)	Previous history of colon adenolomas (polyps)	0.3
14	92	Male	Rectum	Well	-	cT3N0M0/ IIA	Sister (Rectum neoplasma at 82 years old)	-	1.3
15	71	Male	Cecum	Well (mucinous adenocarcinoma)	+ (p.Ala46Thr exon4)	pT4N0M0/ IIB	Mother and Sister (Malignant neoplasm in Colon)	Multiple colon adenomas (polyps), AVC, Diabetes Mellitus 2	0.1
16	75	Male	Ascending Colon	Moderately	+ (p.Gly13Asp exon2)	pT3N1M0/ IIIA	-	-	0.7
17	81	Female	Rectum	Well (invasive adenocarcinoma)	-	cT3N1M1/ IVA	-	Hepatic metástases	0.3
18	59	Female	Sigmoid Colon	Well	-	pT3N1M0/ IIIB	Mother and Uncle (Malignant neoplasm in Colon)	Colon adenomas (polyps), hemochromatosis	1.0
19	76	Female	Sigmoid Colon	Well (mucinous adenocarcinoma.)	-	pT3N2M0/ IIIC	-	-	0.8
20	79	Female	Ascending Colon	Moderately	+ (p.Gly12Asp exon2)	pT3N1M0/ IIIB	-	Breast node (without histopathological exam)	2.6

According to GLOBOCAN (International Agency for research on cancer World Health Organization) in 2012 the incidence of colon adenocarcinoma in the world was 10.0 % and 9.2% in male and female population, respectively. The age-standardised death rate (ASR) was 10.0 deaths per 100.000 male population, and 6.9 deaths per 100.00 female population.
Nowadays with the advancement of biomedical research and the improving of detection methods, the possibility of finding adenocarcinomas at an earlier stage was increased. Although colon adenocarcinoma is present among the top five cancers with higher mortality rates for both sexes worldwide, it is possible to see an increase in the survival rate of patients 5 years after diagnosis of the tumor, at a global level.

In Portugal, the numbers presented by ROR-Sul (Registo oncológico do Sul) show that 81% of men and 78% of women diagnosed with colon adenocarcinoma have over 60 years-old. Regarding rectum adenocarcinoma,it was considered the sixth most frequent adenocarcinoma in both sexes, in South region, representing about 6% and 4.5% of total cases diagnosed in male and female respectively, with two thirds of the cases diagnosed after the age of 60 years-old. For this type of tumor, the population covered by ROR-Sul, have a higher risk for developing rectum adenocarcinoma compared with the incidence rates offered by other European countries. As previously described in Chapter I, intestine (colon and rectum) tumors may be originated from hereditary syndromes such as familial adenomatous polyposis caused by a mutation in *APC* gene (representing less than 1% of the population and appears around the age of 40 years-old) or by causes not associated with polyposis as Lynch syndrome, that results from a mutation in the DNA repair genes, representing about 2% of all cases of colon and rectum cancers in Portuguese population.

According to the ROR-Sul the 5 year survival rate for colon adenocarcinoma patients was 53% and 58% for male and female, respectively, while in rectum adenocarcinoma was 54% and 55% for male and female respectively.

Table 3.2 Patients and tissues samples distributed by technical features

Characteristics	(No. of Cases/ Percentage)
Average (age in years)	
71±13	
Gender	
Male	11 (55%)
Female	9 (45%)
Histology	
Well	11 (55%)
Moderately	8 (40%)
Poorly	1 (5%)
Tumor location	
Rectum	9 (45%)
Left Colon	3 (15%)
Right Colon	2 (10%)
Ascending Colon	3 (15%)
Cecum	1 (5%)
Sigmoid Colon	2 (10%)
TNM Staging	
I	2 (10%)
II	5 (25%)
III	9 (45%)
IV	4 (20%)
K-Ras Mutation	
6 (30%)	
***MGP* relative Δ between MT vs MN**	
-1≥1.5	11 (55%)
1.5≥20	8 (40%)
≥20	1 (5%)

The average age of patients analyzed was 71 years (±13). This finding was in settlement with a presumed association of most patients with sporadic colon adenocarcinoma (unrelated to genetic syndrome). Indeed, most sporadic cases appear after age of 50 years-old (44), as opposed too much earlier appearance in cases associated to genetic syndromes (e.g. APC, Lynch syndrome, tendency to appear under age of 40 years old).

Between the total number of patients analyzed (n = 20), 55% were male and 45% female. Based on histology, 55% were well differentiated, 40% moderately differentiated and only 5% of the patients had a poor differentiation tumor.

It is known that the use of staging tumor progression is the main asset for the therapeutic to follow, however, tumor location, the type of mutation that led to its progression are also important, in fact, a tumor located in the rectum, together with the fact of being in a more advanced stage has a worse prognosis relative to patients with an adenocarcinoma limited to colon with a poor differentiation [2]. According, in our study (Table 3.1), we

observed that in 45% of cases with adenocarcinoma, located in rectum, the prognosis for most patients is reserved and with a higher staging. *KRAS* gene mutations influence the therapeutic to apply to the patient, it is important to apply a specific treatment taking into account the individual characteristics of the patient, instead applying therapeutic for colon adenocarcinomas in a general way. As described earlier, mutations in the *KRAS* gene constantly activate oncogenic signaling pathways that promote cell cycle progression, by itself therapies based on the inhibition of this signaling pathway (anti-EGFR) are not very effective.

According to clinical information obtained (Table 3.2), 6% of the patients analyzed have *KRAS* mutations indicating that the treatment with anti-EGFR will not be very effective. These data are in agreement with a previously published data on the incidence of *KRAS* mutations in Asian population, which found that the appearance of such mutations was not related to the patient's age [2]. Nevertheless, is necessary to keep in mind that there is an epidemiological variation between populations and factors that lead to the emergence of such mutations in Asian population may not be the same as for the European population, in this particular case, the Portuguese population (Algarve region) and incidence of this type of mutations related with tumor location may vary. In fact, there was no obvious association between MGP relative variation and *KRAS* mutations, or at least *KRAS* mutations were not significantly associated to higher levels of MGP expression. In general, during this analysis it was not possible to establish a correlation between the levels of MGP expression (and respective variation between tumoral *versus* normal mucosa) (results described below) and any particular characteristics of the patients, including tumor staging, age or gender (Table 3.1).

After characterizing the group of patients, samples collected from normal (adjacent to tumor) and tumor intestinal mucosa were used for RNA extraction and subsequent qPCR analysis of MGP variants expression. Throughout the process of sampling, samples were initially stored in RNAlater and kept at 20ºC until a week before the RNA extraction was made. After extraction of RNA from the first set of sampling (n = 10), expression analysis for the genes of interest was made, however it was found through RNA electrophoresis and high CT's values presented by housekeeping genes, that RNA integrity was compromised and therefore would not be possible to use those samples for further analysis (data not shown). To overcome the effects of storage of samples for RNA extraction, since high-quality RNA is the first, and often the most critical, step in performing real-time PCR, we changed the procedures of storage and processing of the samples, for maximizing the yield and quality of

sample. Samples collected in RNAlater, was stored at 4 ° C up to 24h at most and then transported to the laboratory on ice to minimize RNA degradation, and immediately processed for RNA extraction. For the RNA extraction process it was also necessary to optimize the first step. To each sample we added 1mL solution D (instead of 500µl), to minimize the contamination of RNA with phenol. The rest of RNA extraction protocol was the same as described in the section of methods and materials (section 2.2 RNA extraction).

Levels of MGP gene expression were determined in the 20 patients, by real-time qPCR, and normalized using GADPH as housekeeping gene. For all samples analyzed, the results showed an inexistent expression of variant E5 (results not shown), however for variant E4 the results show that, although the MGP expression values between normal and tumor samples from each patient come across the threshold of significance ($p=0.056$), there is a strong tendency that indicates an increased expression of MGP in this type of tumors (Figure 3.1 A and B), therefore, this non-significance could be attributed to high variability between patient's samples.

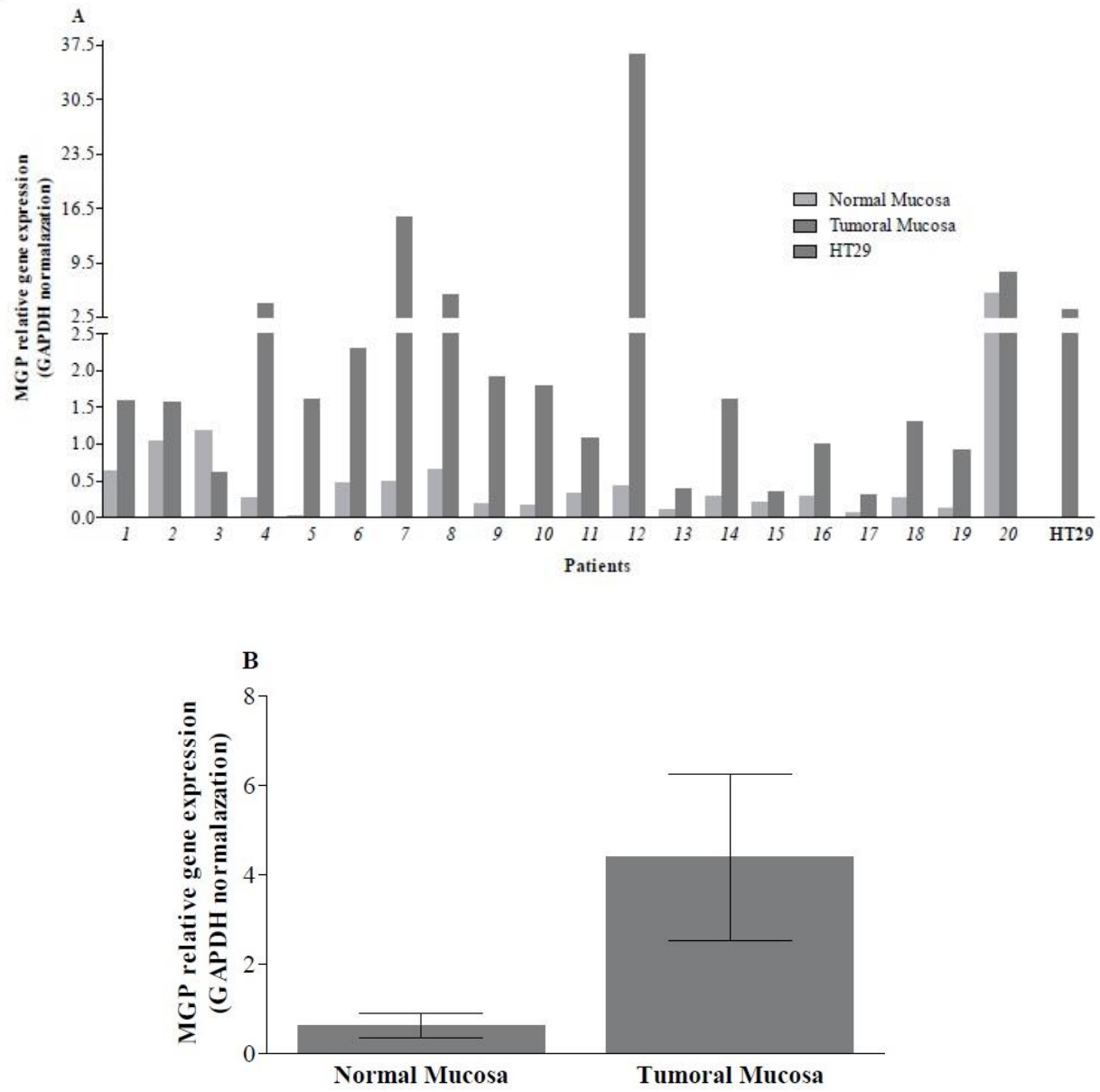

Figure 3.1 Relative *MGP* gene expression in samples from patients with colon adenocarcinoma. (A) Relative *MGP* gene expression between samples from individual patients and *MGP* mRNA expression in HT29. (B) Relative *MGP* gene expression between control normal mucosa= 0.64±0.27 and tumoral mucosa= 4.39±1.86. Results were determined by paired t-test with difference between groups= -3.75, 95% CI [-7.61;0.11] P=0.056. Values are the mean of twenty independent replicates ± SEM. P value significant when $P \leq 0.05$. Samples were all normalized concerning MCF-7 *MGP* expression.

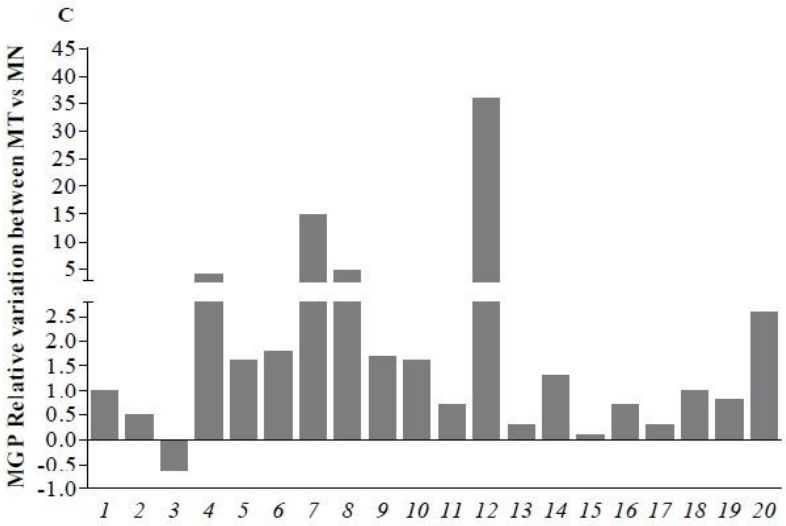

Figure 3.2 *MGP* relative variation between Tumoral (MT) vs Normal Mucosa (MN). Values were calculated from the difference between tumoral and normal mucosa relative *MGP* gene expression.

These differences between samples may be explained by the genetic variability between patients, since mutations on *MGP* gene [15] or mutations in other genes or transcription factors that either interact with MGP or regulate it.

In general, cancer is a disease with a complicated prognosis and development process. Although many studies have been previously conducted to try to understand how tumor cells proliferate and migrate to other organs [11] [38], only few studies could actually prove to influence this behavior in glioblastomas [16]. In that sense, the colon adenocarcinoma is an exceptional example on the investigation of cancer, since it has relatively slow development that allows researchers to explore specific genes involved in critical steps of its progression. *KRAS*, *β-catenin*, *APC* and *C-MYC* [2] are fine examples of particular oncogenes involved in colon adenocarcinoma progression, and are currently used as biomarkers for cancer prognosis and therapeutic decision [40]. Regarding *MGP*, these known oncogenes are not likely to directly influence its expression or specifically interact with *MGP*, still they can indirectly influence its expression, these way it would be interesting to understand whether it could be any correlation between *MGP* expression and presence of these oncogenes. During collection of clinical data we could only have access to presence of *KRAS* mutations, and indeed some patients were found to carry mutations in this oncogene.

Nevertheless, our results contradict a previous study [15] where *MGP* was shown to be down-regulated in tumoral tissues. This study is the only one until now analyzing *MGP* in colon adenocarcinoma samples. Their authors used the same approach (as described in section

methods and materials) to analyze paired samples from 80 patients with sporadic adenocarcinoma. According to their data, approximately 15% of the tumors presented a negative fold-change of more than 10. This discrepancy to our data could be associated to different populations, since previous study analyzed an Asiatic population (from Taiwan) while our analysis focused on a European Caucasian population (Portugal, Algarve). It also indicates that this analysis should be extended to 1) larger and 2) different populations. Nevertheless, in order to understand why a deregulation of *MGP* gene expression occurred in our samples we decided to further explore possible regulators of *MGP*. Indeed, MGP was previously shown to be increased in several tumor types, e.g. breast cancer [11] and gliomas [16]. Such analysis could help to understand the molecular mechanisms underlying MGP deregulation in cancer, and eventually find new biomarkers that would allow to detect the disease at an early stage.

To assess if HT29 derived colon adenocarcinoma cell line was suitable for further analysis *in vitro* regarding *MGP* expression, we analyze trough qRT-PCR if this cells also present the same outline expression regarding *MGP* as we have seen in patient samples. In general, this cell line presents a very similar expression profile regarding *MGP* a high expression of this gene in HT-29 cells suggests that *MGP* expression in human colon adenocarcinoma samples most likely arise from tumor cells and not from its stroma. However, in the future it would be interesting to test this hypothesis through experiments involving *in situ* hybridization to detect MGP in biopsies. It also indicates that HT-29 cell line could be a good *in vitro* model to investigate MGP effects on cell proliferation, cell migration and cell invasion after RNA interference of *MGP* (ongoing experiments). The specific knock-down of transcriptional regulators of MGP and its effects on *MGP* expression could also be tested in these cells. In sum, this analysis opens a new perspective on how these genes, i.e. *MGP* and its associated TF, may be interacting in the tumor microenvironment of colon adenocarcinomas.

Therefore, our next experiments focused on determining the expression of transcription factors (*RUNX2*, *FGF2*, and *RARα*) that are known to regulate the transcription of *MGP* (23), and also the transcription factor *CCBE1*, in our group of 20 patients with colon adenocarcinoma.

3.2 *RUNX2* gene expression in patients with colon adenocarcinoma

Previous experiments have shown that Runx2 was associated to tumor progression in colon adenocarcinoma by affecting the normal regulation of metastatic genes in murines [41].

Furthermore, RUNX2 was shown to be associated with higher risk of colon cancer [42] and has been proposed as a possible prognostic factor [43]. Therefore, since RUNX2 is a known regulator of *MGP* transcription in *Xenopus laevis* and Zebrafish [10] [44], that also regulates *MGP* expression through parathyroid hormone in murines via PKA and ERK/MAPK signaling pathway and was reported to be as well a regulator in breast cancer metastasis [45], we hypothesized whether this transcription factor could also be up-regulated in our samples. In fact, results showed a statistically significant up-regulation of *RUNX2* expression level ($P \leq 0.05$) in tumor tissues compared with normal mucosa tissue (Figure 3.3 A and B).

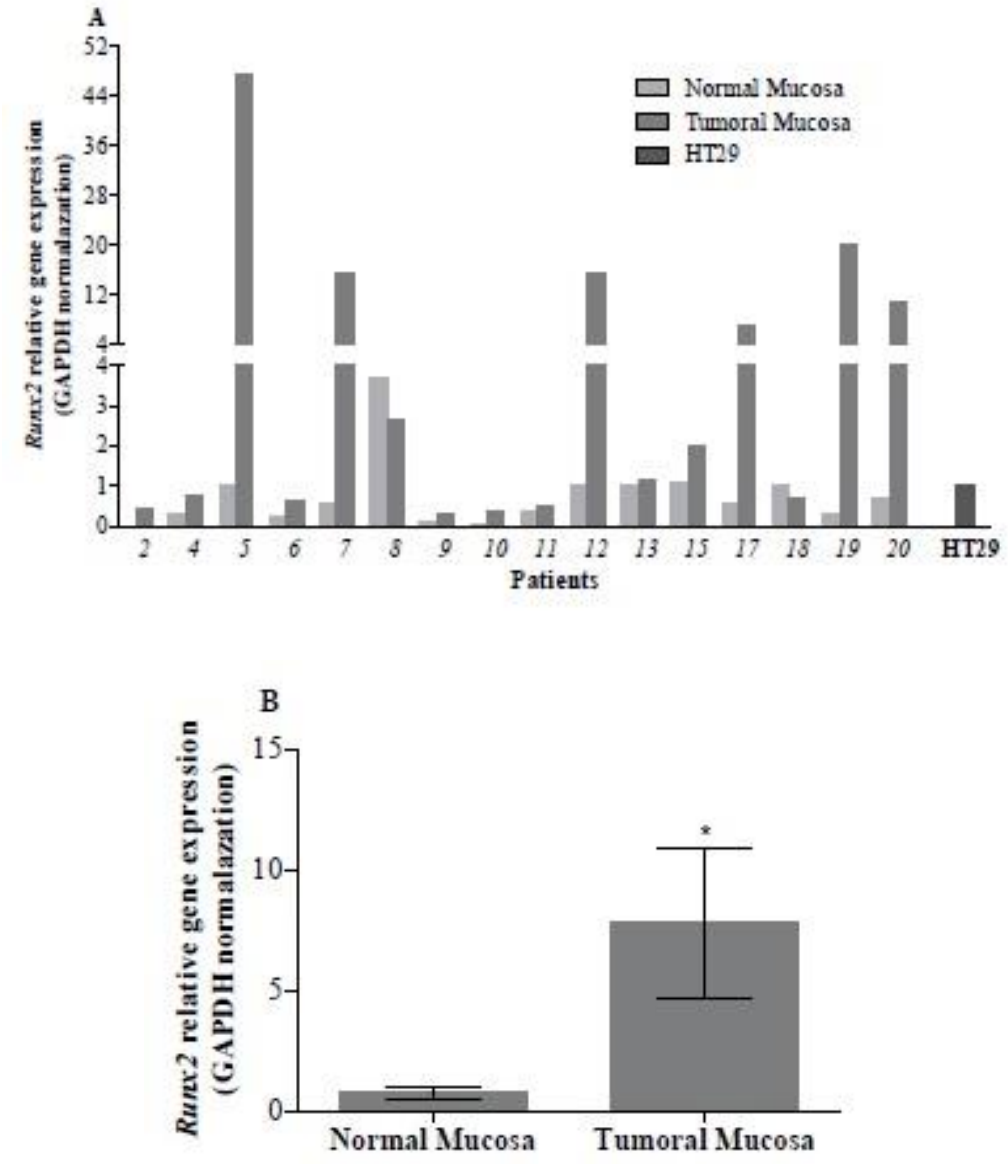

Figure 3.3 Relative expression of *RUNX2* transcription factor in samples from patients with colon adenocarcinoma. (A) *RUNX2* mRNA expression between samples from individual patients and *RUNX2* mRNA expression in HT29. (B) *RUNX2* relative expression between control normal mucosa= 0.74±0.22 and tumoral mucosa= 7.81±3.10. Results were determined by paired t-test with difference between groups= -7.07, 95% CI [-13.66;-0.47] P=0.0373. Values are the mean of sixteen independent replicates ± SEM. P value significant when $P \leq 0.05$. Samples were all normalized concerning MCF-7.

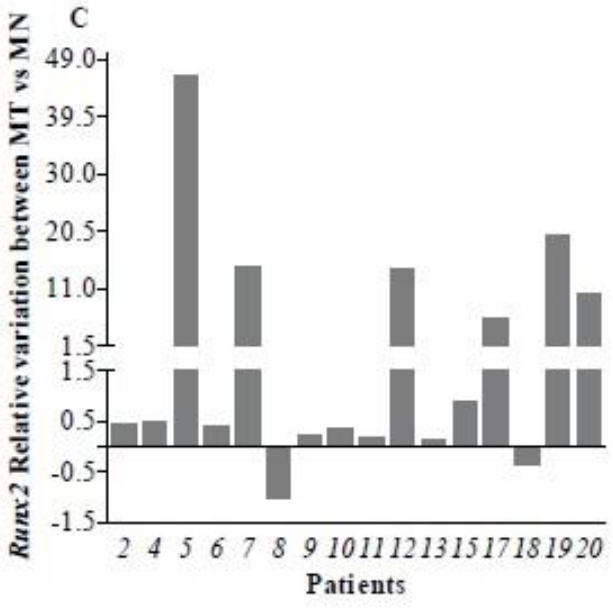

Figure 3.4 *RUNX2* Relative variation between Tumoral vs Normal Mucosa. Values were calculated from the difference between tumoral and normal mucosa relative gene expression.

Although the role of *RUNX2* as regulator of *MGP* has been suggested from studies in *Xenopus laevis* [10], his possible effect on expression of *MGP* in colon adenocarcinoma has not been described yet. In fact, our study using a Pearson correlation analysis between the expression of *RUNX2* and *MGP*, found no relationship between them (r=0.199; p= 0.46). This can be explained by signaling pathways involved in the regulation of *RUNX2*, and can indirectly regulate expression of *MGP*, and therefore, in this particular case it is not possible to affirm that up-regulation of *RUNX2* is related to the tendency for higher levels of *MGP*. Indeed, given the known function of RUNX2 in cancer, such relation would be important to establish a mechanism for *MGP* deregulation in these tumors. Since the same pattern of expression for *RUNX2* in human cell line HT29 (derived from adenocarcinomas) was shown to be the same as the one displayed in patients (figure 3.3 A), this brings a new insight to future experiments to test whether the effect of RUNX2 disturbs or not *MGP* regulation.

3.2.1 Collagen and calcium-binding EGF domain-containing protein 1 (CCBE1) gene expression in patients with colon adenocarcinoma

Collagen and calcium-binding EGF domain-containing protein 1 (*CCBE1*) has been associated with cell migration in ovarian cancer, where it was documented to be down-

regulated during tumor progression [46]. CCBE1 was also shown to play a role in lymphatic vascular development in Hennekam syndrome, a disease characterized by intestinal and renal lymphatic dilation, dysmorphic facial appearance and mental retardation [47] [48] [49] The putative role of CCBE1 in other cancer types, including colon adenocarcinoma, was not established yet. The expression of *CCBE1* levels in our samples from colon adenocarcinoma revealed an up-regulation of this gene that was statistically significant (P≤0.05), as shown in Figure 3.4 A and B.

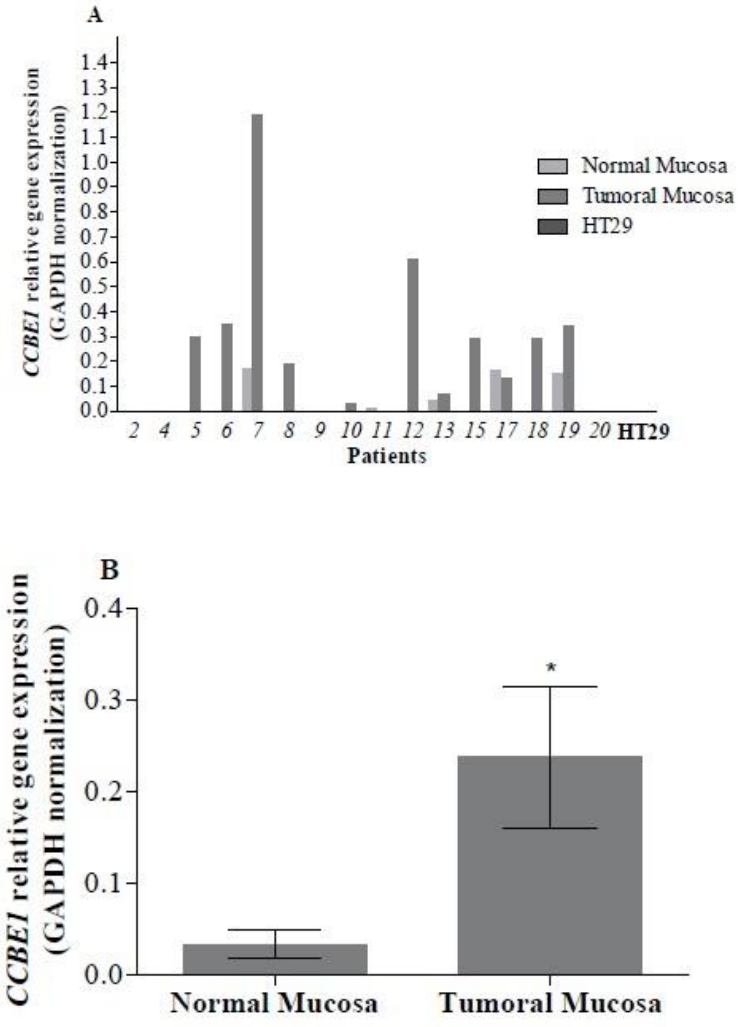

Figure 3.5 Relative expression of *CCBE1* transcription factor in samples from patients with colon adenocarcinoma. (A) *CCBE1* mRNA expression between samples from individual patients and *CCBE1* mRNA expression in HT29. (B) *CCBE1* relative expression between control normal mucosa= 0.03±0.02 and tumoral mucosa= 0.24±0.07. Results were determined by paired t-test with difference between groups= -0.20, 95% CI [-0.35;-0.05] P=0.012. Values are the mean of sixteen independent replicates ± SEM. P value significant when P≤ 0.05. Samples were normalizes concerning MCF-7.

Although this result may suggest a mechanism for *MGP* up-regulation in these samples, the putative association of *CCBE1* to cancer is definitely far from being established.

In fact, *CCBE1* association to cell migration in ovarian cancer was demonstrated to involve a down-regulation of this transcription factor expression due to a hypermethylation in the promoter that promotes cell migration, while the exact opposite effect was observed in our samples. Another concern is related to a general low expression of *CCBE1* in all samples (Figure 3.6).

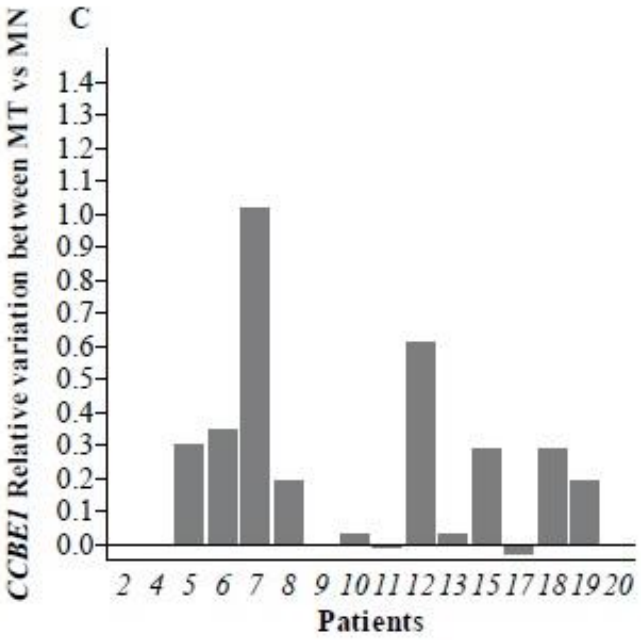

Figure 3.6 *CCBE1* relative variation between Tumoral vs Normal Mucosa. Values were calculated from the difference between tumoral and normal mucosa relative gene expression.

From the total number of patients analyzed, only 10 expressed this gene in tumor tissue, and 4 expressed this gene in normal tissue. It should also be noted that in patient #17 we can observe an up-regulation of *CCBE1* expression levels in normal tissue compared to tumor tissue, but still this difference is not statistical significant, however the Pearson correlation between *CCBE1* and *MGP* expression in these samples was also high (r= 0.044; P=0.51), indicating an association between -regulation in *MGP* and CCBE1. As far as we know this was the first time that has been reported a relation between *MGP* and *CCBE1* expression in colon adenocarcinoma, and the biological mechanism underlying this possible regulation of *MGP* by CCBE1 is unknown. It would be important to have other clinical information regarding these patients, e.g. if they had other types of malignancies that may have some relation to the expression of *CCBE1*. In the future, it would be interesting to test *CCBE1* effect on MGP in human colon adenocarcinoma cell lines. In fact, a previous analysis through qRT-PCR in HT29 cell line for *CCBE1* demonstrated an inexistent expression of this gene, yet more analysis, such as blocking *MGP* expression and thought qRT-PCR analyse

CCBE1 gene expression, are required to confirm these previous data and if the relation between both genes can be consider as a biomarker for this type of cancer.

3.2.2 Fibroblast Growth Factor 2 (*FGF2*) gene expression in patients with colon adenocarcinoma

From all the transcription factors that are known to regulate *MGP*, FGF2 is the most studied in colon adenocarcinoma and colon tumor cell lines [50] [51] [52] [53], where it was shown to: i) contribute for the invasive potential (in HCT116A, HCT116B, LS180, LS174T and ARK1A cell lines) [52]; and ii) to promote proliferation and survival of colorectal cancer cells (NCI-H716) [53]. These *in vitro* studies, pose the hypothesis that FGF2 associated signaling pathways could be promoting the regulation of others genes that may lead to tumor progression, which ultimately could result in poor prognosis in colon adenocarcinoma. Here, attained data revealed a tendency for increased expression of this transcription factor in tumor tissue compared to normal tissue, but not in a significant manner (P= 0.085) (Figure 3.7 A and B).

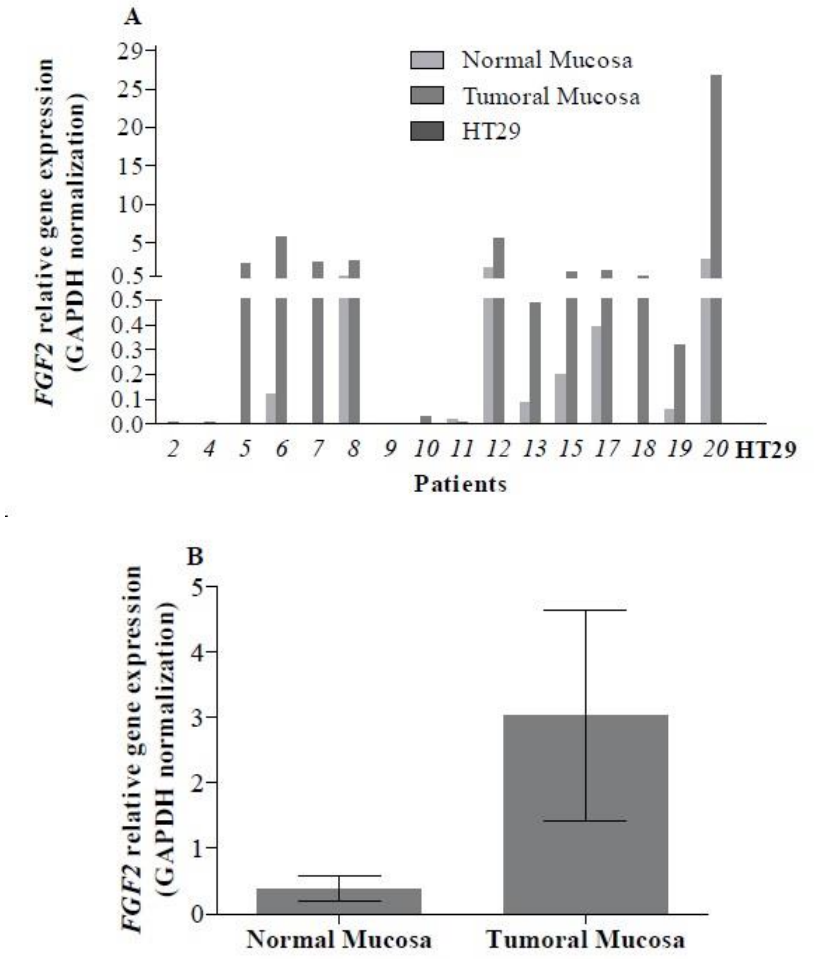

Figure 3.7 Relative expression of FGF2 in samples from patients with colon adenocarcinoma. (A) FGF2 gene expression between samples from individual patients and *FGF2* mRNA expression in HT29. (B) *FGF2* relative expression between control normal mucosa= 0.38±0.19 and tumoral mucosa= 3.04±1.61. Results were determined by paired t-test with difference between groups= -2.66, 95% CI [-5.73;0.41] P=0.085. Values are the mean of sixteen independent replicates ± SEM. P value significant when P≤ 0.05. Samples were normalizes concerning MCF-7.

The analysis of Pearson correlation between the relative variation expression of *FGF2* (Figure 3.8) and *MGP* relative variation shown a significant correlation between them (r=0.89; p≤0.0001). Although the association of *FGF2* in colon adenocarcinoma can be supported by previous studies [54], a link between *FGF2* and *MGP* expression in colon adenocarcinoma is still missing, and therefore it would be important to analyze this in the future.

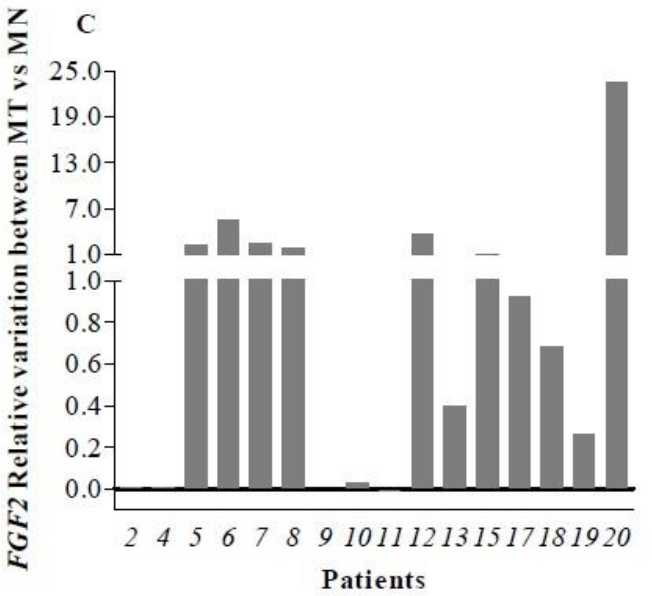

Figure 3.8 *FGF2* relative variation between Tumoral (MT) vs Normal Mucosa (MN). Values were calculated from the difference between tumoral and normal mucosa relative gene expression.

In colon adenocarcinomas, FGF2 protein is known to be associated to the activation of multiple signaling cascades responsible for differentiation, growth and survival of cell, namely, PI3K/AKT, RAS/MAPK and PI3K/aPKC [54]. One consequence of this deregulation is the constant activation of KRAS signaling pathway, which will promote tumor cell differentiation.

Experiments concerning treating colon adenocarcinoma HT-29 cells with FGF2 and analyzing its effects on *MGP* expression would shed some light on this hypothesis, still in ours previous analysis of *FGF2* expression in tumor cell line HT-29 we observed an inexistent expression of *FGF2* (Figure 3.7 A).

3.2.3 Retinoic Acid Receptor α (*RARα*) gene expression in patients with colon adenocarcinoma

Information regarding RARα involvement in cancer is scarce, although it has been recently associated to hematopoietic diseases, including leukemia [55]. Nevertheless, RARα is a known regulator of *MGP* (and therefore analyzing its expression in colon adenocarcinoma samples was relevant). However, our data indicated that despite being expressed in the colon it was not differentially expressed comparing tumor and normal samples (Figure 3.9 A and B).

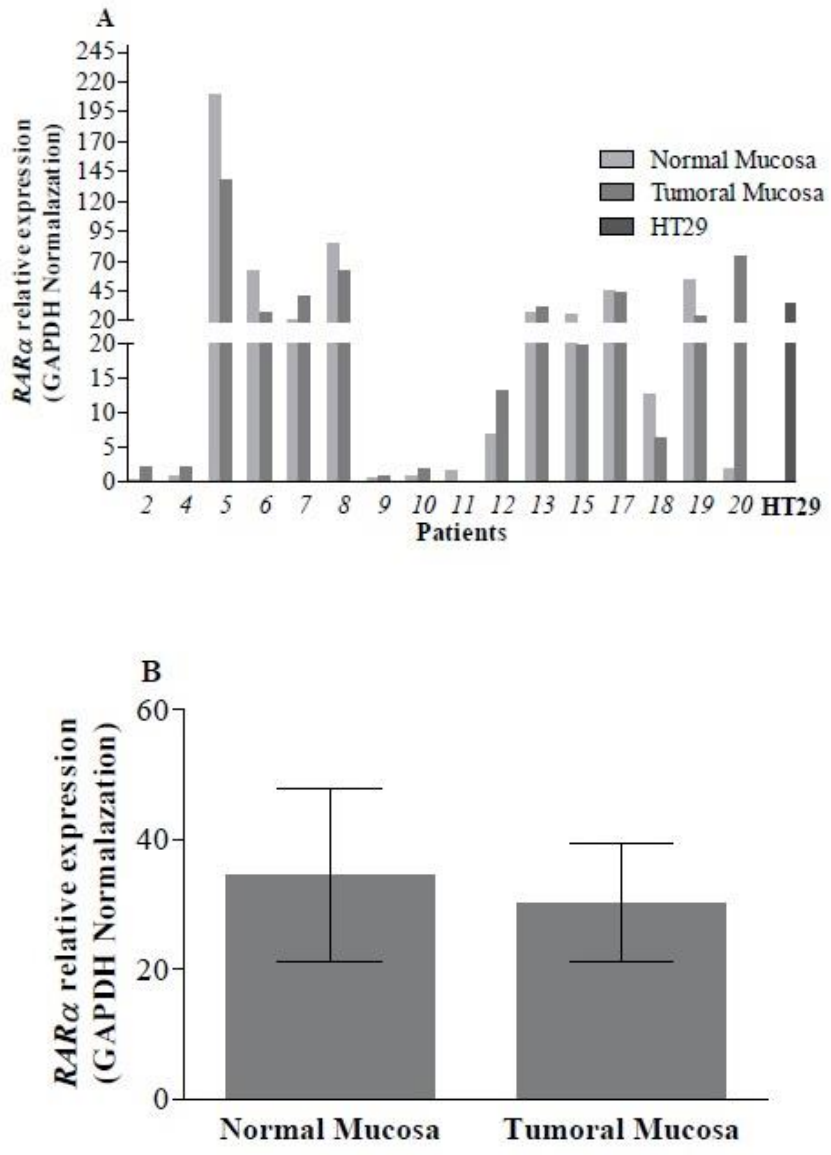

Figure 3.9 Relative expression of *RARα* in samples from patients with colon adenocarcinoma. (A) *RARα* mRNA expression between samples from individual patients and *RARα* mRNA expression in TH29. (B) *RARα* relative expression between control normal mucosa= 34.44±13.29 and tumoral mucosa= 30.25±9.07. Results were determined by paired t-test with difference between groups= 4.19, 95% CI [-11.58;19,96] P=0,58. Values are the mean of sixteen independent replicates ± SEM. P value significant when P≤ 0.05. Samples were normalizes concerning MCF-7.

RARα also showed the same positive Pearson correlation analysis (r= 0.84; P≤0.0001) as former established to *FGF2*. Therefore, it was not possible to relate *RARα* expression with *MGP*, indicating that this transcription factor is not likely to regulate *MGP* expression in human colon adenocarcinomas, still, presents a biological significance linked to *MGP* expression.

Proliferative studies in RAR receptors with retinoids (metabolites of vitamin A, which are assumed that can interact with the two types of receptors RAR and RXR) in colon

adenocarcinoma cell lines MC-26 and LoVo have demonstrated that both cell line expresses RAR subtypes (RARα, RARβ, RARγ) and RXR receptors, however only MC-26 cell line appears to have an increase in growth provoked by retinoids, while the LoVo cell line remained unchanged with regard to proliferation. These results shows that the incapability of LoVo cells in response to retinoids do not appear to be related with the absence of RAR receptors, since they are present in these cells and hold the ability to synthesize the same retinoic acid receptor subtypes present in the MC-26. Other possible explanation for different proliferation results between cell lines is a presumed existence of other isoforms of RAR subtypes presents at MC-26, that possible do not exists in LoVo cell line [56]. The presence of different isoforms of RARα in colon adenocarcinomas may explain why such high variation between samples (Figure 3.10) and HT29 derived colon adenocarcinoma cell line.

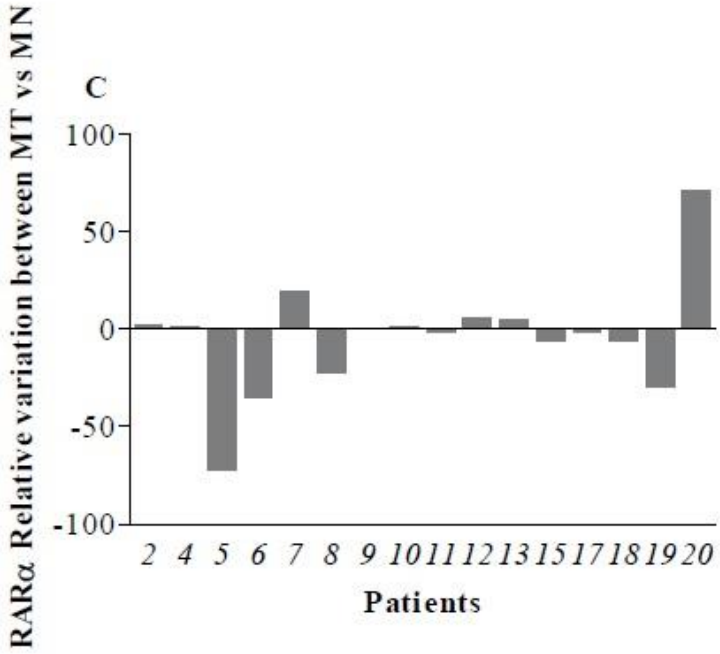

Figure 3.10 *RARα* relative variation between Tumoral (MT) vs Normal Mucosa (MN). Values were calculated from the difference between tumoral and normal mucosa relative gene expression.

Chapter IV

Final remarks and Future perspectives

4. Final remarks and Future Perspectives

4.1 Final Remarks

On the course of this work, several hypotheses were raised regarding the function of MGP and putative regulators in colon adenocarcinomas. To the best of our knowledge, nothing is known about which signaling pathways and transcription mechanisms affect MGP expression in cancer, and more specifically in colon adenocarcinoma.

For this reason, we aimed to: i) investigate MGP dysregulation in colon adenocarcinomas; ii) Identify specific tumor stages where MGP gene becomes dysregulated; and iii) identify gene regulatory mechanisms associated with MGP deregulation in colon adenocarcinomas. Thus, according to our results:

- There is a tendency for higher MGP expression in tumoral mucosa comparing to normal mucosa of colon adenocarcinomas, contradicting previous results showing an opposite regulation.

- Consistent with this was the significant up-regulation of transcription factors Runx2 and CCBE1 in tumoral mucosa;.

- However, CCBE1 was the only transcription factor presenting an expression pattern that was strongly correlated with MGP expression;

- FGF2 data revealed a tendency for increased expression in tumoral tissue comparing to normal tissue, but not in a significant manner.

- Although RARα was never shown to directly regulate MGP expression, this transcription factor along with FGF2 presented positive but still poor correlationa.

- Interestingly, a preliminary analysis in a well-known cell line derived from colon adenocarcinoma, HT-29, presented similar expression levels of MGP and associated transcription factors to what was observed in patients.

In summary, it was not possible to establish a relation between up-regulation of MGP in colon adenocarcinomas with the age, gender or tumor staging. Also, until now it was not possible to determine if MGP is indeed a good biomarker in colon adenocarcinoma. In that sense, further experiments are required to uncover i) MGP association to tunoral mucosa, ii) MGP association to specific phases of colon adenocarcinoma tumor progression, and iii) cellular mechanisms underlying its regulation. For that, it will be crucial to raise the number of samples in order to attain statistical significance on the results obtained so far. Likewise, MGP higher expression in tumoral mucosa should be confirmed at the transcript level through *in situ* hybridization experiments, and at the protein level through immunohistochemistry experiments against MGP. Finally, preliminary data obtained from HT-29 revealed that this cell line may be a good in vitro model to investigate MGP tumorigenic mechanisms by testing effects on cell proliferation, cell migration and cell invasion after RNA interference of MGP (ongoing experiments). It will also be interesting to use these cells to study MGP regulatory mechanisms.

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Appendix

Appendix 1 Buffers and Solutions composition

Solution- D:
50ml Gu-Thio solution + 360 µl β-mercaptoethanol. Store at 4ºC

Gu-Thio solution: 250g of guanidine isothiocyanate/17.6ml of 0.75 M sodium citrate pH7.0/26.4ml of 10% N-lauroyl sarcosine (Na-salt)/293ml of DEPC water. Dissolve at 65ºC with stirring, filter sterilize (0.2µm), aliquot in 50-ml tubes and store at 4ºC.
CIAA: chloroform and isoamyl alcohol 49:1 (v/v)
DEPC- treated water: 1ml of DEPC to 1000ml of water: incubate O/N at 37ºC and autoclave

PBS:
16g NaCl (137mM), 0.4g KCl (2.7mM), 5.8g $Na_2HPO_4.12H_2O$ (8.1mM), 0.4g KH_2PO_4 (1.47mM), water to a final volume 2L, adjust pH 7.0.

Running Buffer:
8.528g MES (50mM), 4.856g Tris (50mM), 0.8g SDS (0.1%), 0.23g (EDTA 1mM), water to fill up final volume.

Transfer Buffer (20x):
10.2g Glycine (500mM), 13.08g Bistris (500mM), 0.7494g EDTA (20.5mM), water to fill up the final volume.
Transfer Buffer (1x): 50ml Transfer Buffer (20x), 100ml METOH, 850ml water, 5µl 4-Chloro-1-butanol.

Blocking Solution:
1.5g nonfat dry milk (Bio-Rad), PBST (1X PBS plus 0.2% Twee 20).

Salts Solution (2x):
7.1628g $Na_2HPO_4.12H_2O$ (10mM), 116.880g NaCl (1M), 10ml Tween-20 (1%), water to fill up to a final volume of 1L. Dilution 1:2 for final solution.

Sample Buffer (4x)

Tris-HCL 320 mM at pH 6.8,

2-mercaptoethanol 0.4M,
8% SDS (m/v),
0.024% (m/v) m-cresol purple,
15% glycerol (v/v)

Appendix 1.1

Table 1 List of primers

hGADPH Forward	5’-TCAACGGATTTGGTCGTATTGGGCG-3’
hGADPH Reverse	5’-CTCGCTCCTGGAAGATGGTGATGGG-3’
hMGP E4 Forward 1	5’-TGCTGCTACACAAGACCCTGAGACTGA-3’
hMGP E4 Reverse 5	5’-GTAGCGTTCGCAAAGTCTGTAGTCATCAC-3’
hMGP E5 Forward 1	5’- TGCTGCTACACAAGACCCTGAGACTGA-3’
hMGP E5 Reverse 4	5’- CCGAAGTTTTCTTCTTTCTGCCACTCTCC-3’
hRunx2 Forward	5’-GGAGTGGACGAGGCAAGAGTTTCACC-3’
hRunx2 Reverse	5’-GCGGGACACCTACTCTCATACTGGG-3’
hFGF-2 Forward	5’-CAAAAACGGGGGCTTCTTCCTG-3’
hFGF-2 Reverse	5’-CCATCTTCCTTCATAGCCAGGTAACG-3’
hRARα Forward	5’-TGTCCAAGGAGTCTGTGAGAAACGAC-3’
hRARα Reverse	5’-GACACGTTGTTCTGAGCTGTTGTTCGTAG-3’
hCcbe1 Forward	5’-GAGATGGTTCTAAGGGGAGA-3’
hCcbe1 Reverse	5’-ATGTCAGCCAGCATAAGTAGCA-3’