Chapter 6

Vitamin D and Colorectal Carcinogenesis

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Additional information is available at the end of the chapter

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Abstract

Colorectal cancer is the second leading cause of cancer-related death in the Western industrialized world. Many epidemiological studies have shown a negative association between colorectal cancer incidence and vitamin D levels. It has been suggested that the antitumoral action of $1,25(OH)_2D_3$ in colorectal cancer relies on several mechanisms at the cellular level. This prompted us to evaluate expression of certain immunohistochemical markers during tumor progression in colorectal human tissue and to study for the first time the relationship between histological type and grade of colorectal tumors with the expression of these markers. The investigated markers were the ones responsible for apoptosis (PAK1 and p53), cell adhesion (beta-catenin), differentiation (p53), and proliferation (Ki67). We also analyzed the correlation of their expression with vitamin D blood levels in these patients. Our results showed that the expression of these biomarkers increased with progression from colorectal adenomas to carcinomas. Expression of PAK1, beta-catenin, and p53 in the nucleus correlated with advanced stages of carcinoma. Low vitamin D blood levels correlated with nuclear accumulation of p53, nuclear beta-catenin expression, and expression of Ki67.

Keywords: vitamin D, colorectal cancer, beta-catenin, PAK1, p53, Ki67

1. Introduction

Colorectal cancer (CRC) is one of the commonest malignancies affecting both males and females. It is the second leading cause of cancer-related death in the Western industrialized world. The incidence of colorectal cancer increases with age, with nearly two-thirds of patients diagnosed aged over 65 years. Colorectal cancer (CRC) is the third most frequent tumor, which affects the inhabitants of developed and developing countries. Among males, CRC comes after lung and prostate tumors; among females it follows breast cancer,

occupying the second place in terms of incidence [1]. As a result of early detection of colonic polyps by screening and removal before they can develop into outright cancer, death rates have been dropping. In addition, screening and treatment for colorectal cancer at early stages have improved over the last several decades, resulting in increasing number of survivors of colorectal cancer.

Vitamin D is a secosteroid. Though it is named a vitamin, it is rather recognized as a prohormone given its synthesis in the skin and the multiple systemic actions of its metabolites [2]. In 1980, Garland brothers had suggested that vitamin D could be a protective factor against colorectal cancer, based on their observation of geographic distribution for colorectal cancer mortality in regions where population was less exposed to sunshine [3]. Few years later, the same authors confirmed this association, by reporting an inverse correlation between vitamin D status and CRC [4]. Different studies in the upcoming years [5–7] have also confirmed the relationship between plasma 25-hydroxyvitamin D concentrations and risk of colorectal cancer.

Many epidemiological studies have shown a negative association between colorectal cancer incidence and vitamin D levels [4, 7], as well as colorectal cancer risk and calcium intake [8, 9]. 1,25-Dihydroxyvitamin D3 directly affects growth factor and cytokine synthesis and signaling in colonic epithelium and modulates the cell cycle, apoptosis, and differentiation [10]. $1,25(OH)_2D_3$ exerts its biological effects by binding to the vitamin D receptor (VDR), thereby regulating gene expression. The active metabolite has prominent antiproliferative, anti-angiogenic, and pro-differentiating action in a wide range of tumor cells due to the VDR being expressed in almost all tissues. Several important cellular signaling pathways can thus be acted upon. However, for clinical trials, the problem remains of how to administer side effect-free doses of $1,25(OH)_2D_3$ [11].

A frequently measured range for serum levels of $25OHD_3$ in adults is 10–50 ng/ml. Intestinal calcium absorption is optimized at levels above 30–32 ng/ml. Parathyroid hormone levels start to rise at 25OHD levels below 30 ng/ml, marking vitamin D insufficiency [12]. Numerous investigations reported an increased risk for colorectal cancer in individuals with $25OHD_3$ blood levels below 12 ng/ml [13, 14].

In kidney cells, 25OHD is converted by the hydroxylase into the active metabolite $1,25(OH)_2D_3$. However, also other cell types, such as colonocytes, express vitamin D hydroxylases [15], indicating an autocrine/paracrine function of the active metabolite. Low serum $25OHD_3$ precursor levels could result in colonic $1,25(OH)_2D_3$ production that is insufficient for maintenance of autocrine/paracrine regulation of cellular growth and function [16].

It has been suggested that the antitumoral action of $1,25(OH)_2D_3$ in colorectal cancer relies on several mechanisms at the cellular level, such as inhibition of cell proliferation, sensitiveness to apoptosis, induction of epithelial differentiation, cell detoxification metabolism, inhibition of angiogenesis, and cell-cell adhesion [2]. This prompted us to evaluate immunohistochemical expression of beta-catenin and PAK1 (which are involved in Wnt-beta-catenin pathway) as well as expression of p53 and Ki67 (markers of apoptosis and cell proliferation, respectively) in colorectal polyps and cancer. We therefore studied for the first time the

relationship between histological type and grade of colorectal tumors with the expression of these markers and attempted to correlate these results with vitamin D blood levels.

2. The role of vitamin D in CRC

2.1. Relevant data about CRC

Colorectal cancer develops through a multistage process of histopathologic and molecular changes, as a result of complex interactions between genetic predisposition and environmental factors. This type of cancer presents an ideal research model of carcinogenesis, since it progresses through multistep histopathologic changes and lesions of each stage are available for studying. Studies regarding colorectal carcinogenesis are focused on the genetic changes in three fundamental categories of genes: (1) tumor suppressor genes, (2) proto-oncogenes, and (3) DNA repair genes [17].

Comparative studies in human and animal samples have proved their similarity as organisms, regarding many pathological processes, including cancer, the same cell structure, and the same function of organ systems. Furthermore, in favor of this similarity of human and animal organisms, an additional argument would be using the same medicines both in humans and animals. Moreover, a range of surgical techniques and treatments such as transplantations and surgical techniques were established and sophisticated being applied firstly in animals. Many disease developments can be investigated in animal models in physiologically relevant conditions with humans. Colon carcinogenesis, as widely known, is a multifactorial process influenced by many interactive variables, making it difficult to determine an exactly specified mechanism. Using animal models as an investigation approach will make possible in vivo molecular, pathological, physiological, and anatomical possibilities to researchers of animals, giving us the favor to understand many disease features.

Mouse models have served as a basis for investigations in the field of colorectal cancer etiology as well as for mechanisms underlying the oncogenic process. On the other side, xenograft models are used to examine the response of the human tumor to a specific therapeutic regime. However, it is not clear if drug efficacy data obtained from xenograft models translate into clinically relevant treatment modalities.

2.1.1. Genetic changes during colorectal carcinogenesis

In colorectal cancer, it is the progressive accumulation of multiple genetic mutations, which results in the transition from normal mucosa to benign adenoma, to severe dysplasia, and to frank carcinoma [18]. More than two decades ago, Fearon and Vogelstein presented the model for the genetic basis of colorectal neoplasia, the adenoma-carcinoma sequence [19]. According to this genetic model, in most colorectal cancers, the primary event is an aberrant activation of APC/beta-catenin pathway, followed by RAS mutations and loss of function of p53 in later stages, while the total accumulation of changes, rather than their order, is responsible for

determining the tumor's biologic properties. Ten years later, it was concluded that mutations in all three genes happen in only 6.6% of colorectal cancers, indicating that these mutations lie on alternate pathways of colorectal tumor development; the heterogeneous pattern of tumor mutations suggests that multiple alternative genetic pathways to colorectal cancer exists [20].

2.1.2. Epigenetic changes in colorectal neoplasia

In the later years, colorectal cancer development is seen from a different point of view: genetic alterations represent only one piece of a complicated process [21–25]. Epigenetic changes in cancer-related genes and noncoding RNAs also have a function which contributes to the malignancy status [26, 27]. Factors that may induce epigenetic changes in colorectal neoplasia involve environmental, as well as inherited factors:

2.1.2.1. Environmental factors

The influence of environmental factors on epimutagens most of all includes dietary factors, and among them folate is the most investigated in connection to colorectal neoplasia. Folate is a donor of one-carbon units and therefore is important in methylation reactions and in DNA synthesis and repair [28]. According to epidemiological and experimental studies, dietary folate correlates inversely with the risk of colorectal neoplasia [29–31]. However, the effect of folate intake on tumorigenesis is very complex and depends on the stage of tumor development [10, 32].

Advancing age also correlates closely with epigenetic changes in normal colorectal mucosa, where methylation of certain genes, including the ESR1 [33, 34], MLH1 [35], HIC1, and IGF2 [36], has been shown to increase progressively with age. This process seems to be accelerated in patients with colorectal cancer, for at least some of these genes [37, 38].

2.1.2.2. Inherited factors

Given that epigenetic changes are stable and potentially heritable through meiosis, some of the ways in which inheritance may influence epigenetic changes associated with colorectal neoplasia should be considered [28]. Groups of authors have reported germline epimutations in MLH1, which creates predisposition to young-onset MSI tumors in colon and at extracolonic sites [39, 40]. Germline epimutation provides a mechanism for phenocopying of genetic disease [39] and causes transcriptional silencing of the affected allele [40]. According to these observations, germline epigenetic changes can mimic hereditary cancer syndromes and may be inherited [41, 42]. The timing when these changes occur, as well as the combination of genetic and epigenetic events, rather than their merely accumulation, gives selective priority to the cancer cells, resulting in activation of certain pathways [43].

2.1.3. Clinical relevance of epigenetic changes in colorectal cancer

Increasing recognition of epigenetic changes in the histologically normal colorectal mucosa and in precursor lesions can make these changes serve as a marker for patients at risk for colorectal cancer [33]. Epigenetic markers are progressively finding their place in screening

tests for colorectal neoplasia [44]. Regarding the impact that epigenetic changes may have in colorectal cancer treatment decisions, there is a growing evidence that MSI tumors respond differently to traditional chemotherapeutic agents [45, 46]. Moreover, the outcomes for some patients with these cancers may be worse with standard treatments [47].

It was observed that low folate status predisposes to development of several malignancies including colorectal cancer [48]. Experimental studies confirmed that older age and inadequate folate intake are strongly implicated as important risk factors for colon cancer and each is associated with altered DNA methylation [49]. Keyes and colleagues concluded that folate supplementation, which can enhance methyl availability, increases both genomic DNA methylation and *p16* promoter methylation in old mice; this epigenetic change by aging and dietary folate affects the expression of *p16*, a critical gene for both aging and carcinogenesis [49]. Methylation/demethylation processes in promoter sequences of vitamin D hydroxylases may lead to reduced or enhanced expression of these enzymes, respectively [50] (see Section 2.3.3.).

Numerous studies have provided evidence that women may be better protected against colorectal cancer than men. Several investigations have reported a lowered colorectal cancer risk associated with enhanced phytoestrogen intake (see, e.g., [51]). Phytoestrogens resemble structurally and act functionally as estrogen agonists; their affinity for ER-β is higher than that of estradiol itself and is lower for ER-α [52]. Therefore, phytoestrogens can act as estrogen agonists or antagonists depending on the type of estrogen receptors available, as well as on the level of endogenous circulating hormones [53, 54]. In Asian countries, populations with high consumption of soy foods (which are very rich in phytoestrogens) have a clearly reduced risk of colorectal cancer incidence. The major phytoestrogen in soy—genistein—beside other mechanisms (see Section 2.3.4.) is also involved in regulation of gene activity by modulating epigenetic events such as DNA methylation and/or histone acetylation.

2.2. Vitamin D: general concepts

Bound to the vitamin D receptor (VDR), $1,25(OH)_2D_3$ not only regulates calcium and phosphate metabolism but also exerts a wide range of non-calcemic biological effects, the most important of which are suppressing hyperproliferative growth and supporting cell differentiation [16]. When Zehnder and coworkers demonstrated that many types of cells were positive for CYP27B1, it was recognized that there is a widespread potential for extrarenal synthesis of $1,25(OH)_2D_3$ [55]. Further, Cross and coworkers demonstrated the synthesis and degradation of $1,25(OH)_2D_3$ by high-pressure liquid chromatography [56], and it was accepted that vitamin D could have alternative roles in extrarenal tissues, for instance, in the colon.

2.2.1. VDR in normal and in malignant colon cells

Vitamin D actions as a steroid hormone are mediated through the vitamin D receptor (VDR) [57], which is a high affinity ligand-activated transcription factor [58]. Activated VDR heterodimerizes with the retinoid X receptor (RXR); this complex binds to the vitamin D response elements (VDREs) in the promoter of target genes and recruits coactivators and corepressors to induce or inhibit gene transcription [59]. The expression and functionality of the VDR are

mandatory for the anticancer effects of vitamin D; therefore, the loss of this transcription factor, as seen in some cells after malignant transformation, results in calcitriol resistance [58].

An important step toward understanding of the complexity of vitamin D interactions was recognition of the fact that VDR is also expressed in tissues other than those responsible for calcium and phosphorus metabolism. The presence of VDR in malignant cells suggested that regulation of cancerous cell functions could be another target of 1,25-$(OH)_2D_3$ and provided the biological basis for many epidemiological studies. Shabahang et al. reported that the more differentiated is the cell line, the expression of VDR is higher [60]. Cross and coworkers found that expression of the VDR increases during transition from normal mucosa to polyps, as well as in the course of progression from adenomas to well-differentiated and moderately differentiated tumors, and then declines during further progression [61]. This suggests that cancerous colon cells express VDR until they reach a certain level of differentiation. Such model of regulation suggests existence of a physiologic protective mechanism of tumor progression, which is reduced in the later stages. Investigations by Evans and coworkers found that in colorectal cancer, a high level of VDR expression was associated with a favorable prognosis [62].

VDR can function as a receptor for secondary bile acid, lithocholic acid, which is a hepatotoxic compound and a potential enteric carcinogenic [63]. Binding of both lithocholic acid and vitamin D to the VDR results in induction of CYP3A, the enzyme that detoxifies lithocholic acid in the liver and intestine [7, 64].

2.2.2. Expression of VDR, CYP27B1, CYP27A1, and CYP24 as a function of malignant transformation in the colon

Cross et al. [56] were the first to demonstrate the conversion of the precursor 25 OH D_3 into 1,25$(OH)_2D_3$ in Caco-2 cells by finding constitutive expression of the CYP27B1 in almost every growth phase of this cell line and the sequential metabolism of the secosteroid along the C-24 and C-23 oxidative pathways. The authors concluded that human colon cells can control their growth through 1,25$(OH)_2D_3$ in an autocrine/paracrine manner dependent upon the presence of the vitamin D receptor [56]. Cross and coworkers were also the first to demonstrate that expression of CYP27B1 and of the VDR rises (approximately fourfold) in the course of progression from adenomas to well-differentiated and moderately differentiated (G1 and G2) tumors and then substantially declines during further progression [65]. Other authors also reported that in hyperproliferative, premalignant colon cells, expression of CYP27B1 (synthesizing hydroxylase) is increased, as well as VDR expression, while in high-grade colon tumors, CYP27B1 expression is again repressed (in contrast with CYP24A1—degrading hydroxylase expression) [15]. According to this, the extrarenal synthesis of 1,25$(OH)_2D_3$ provides a physiological mechanism for prevention of malignant growth.

Holt and coworkers [66] demonstrated for the first time that rectal crypt proliferation correlated inversely with serum 1,25$(OH)_2D_3$ levels. These data support the abovementioned hypothesis that colon cancer cells posses an intrinsic physiological defense which can prevent hyperproliferation and progression into malignancy [56]. In colon tissue derived from a large patient cohort, VDR and CYP27B1 mRNA expression was low in normal tissue but rose

early during colon tumor progression [65, 67]. This system fails in the late stage, high-grade colon cancer, while there is increased expression of CYP24, which could cause rapid catabolism of $1,25(OH)_2D_3$ at the tumor site, counteracting its local inhibition of tumor growth [68]. Physiologic regulation of vitamin D hydroxylases in normal and malignant human colonic tissue suggests a role for the locally accumulated hormone in prevention of tumor progression; during low-grade early-stage malignancy, colonic synthesis of $1,25(OH)_2D_3$ could potentially provide a block to progression, if its catabolism could have inhibited [69].

2.2.3. Epigenetic regulation of vitamin D hydroxylases

Expression of CYP27B1 and CYP24A during colorectal cancer progression is under epigenetic control [16]. Differences in expression of vitamin D hydroxylases during tumor progression, as seen in colorectal cancer patients [65, 68], could be caused by epigenetic regulation of gene activity through methylation/demethylation processes, as well as histone acetylation/deacetylation [16]. DNA methylation at CpG islands in the promoter region of genes is associated with transcriptional silencing of gene expression in mammalians, whereas reduction of methylation in CpG islands results in increase of gene activity.

In low-grade cancer, CYP27B expression is very high, comparing with its expression in colorectal mucosa of non-tumor patients [15, 65]. Increased synthesis of $1,25(OH)_2D_3$ in colon mucosa could be responsible for higher transcriptional activity of CYP24A1, as well as for autocrine/paracrine inhibition pf tumor cell growth. If transcriptional repression of CYP24A1 expression could be affected by methylating agents, advanced colorectal cancer patients could also benefit from treatment with vitamin D substances [10].

2.2.4. The antitumoral action of $1,25(OH)_2D_3$ in different tissue types

Numerous observations have indicated a much broader range of action for $1.25(OH)_2D_3$, including the regulation of cell differentiation, proliferation, apoptosis, invasion, and angiogenesis in several types of tumor cells and animal models of cancer [70–72]. 1-Alpha,$25(OH)_2D_3$, also known as calcitriol, as well as vitamin D analogs, might have potential as anticancer agents because their administration has antiproliferative effects, can activate apoptotic pathways, and inhibit angiogenesis [70]. A general deregulation of the vitamin D system was observed in most malignancies. Evidence for an inverse correlation between serum vitamin D_3 status and cancer incidence, e.g., the colon, breast, and prostate, has increased over the last years [73]. Anticancer property of vitamin D has been studied in a wide variety of commonly occurring cancers (both in vitro and in vivo) of which the actions on colorectal, breast, and prostate cancers have been found to be most promising [74]. In 16 types of cancer, there is an evidence of a beneficial role of vitamin D (gastrointestinal: colon, esophageal, gallbladder, gastric, pancreatic, and rectal; urogenital: bladder, kidney, and prostate; female: breast, ovarian, and vulvar cancer; and blood cancers: Hodgkin's lymphoma, non-Hodgkin's lymphoma, and multiple myeloma) [75]. A strong association between a low vitamin D status and cancer incidence or mortality has been reported for colon, rectal, breast, prostate, and ovarian cancer [76]. However, data regarding the role of vitamin D in cancer risk, incidence, and mortality are still controversial [58].

2.3. Mechanisms of colorectal cancer: vitamin D interaction

The molecular basis of the idea that vitamin D has the potential to prevent cancer lies in its role as a nuclear transcription factor, which regulates cell growth, differentiation, apoptosis, and many cellular mechanisms, with a key role for cancer development.

2.3.1. Effects of vitamin D on cell proliferation, differentiation, apoptosis, and angiogenesis

Since the 1980, 1,25-OHD_3 has been recognized as a potent cellular antiproliferative and pro-differentiating agent in the colon [69]. During the last two decades, vitamin D was proven as a potential inhibitor of cell growth and angiogenesis, as well as a stimulator of cell maturation and apoptosis [70]. The classic signaling pathway is through the vitamin D nuclear receptor (VDR), which is a transcription factor.

Over recent years there has been an expanding consideration for non-calcemic functions of $1,25(OH)_2D_3$, including its antitumoral effects [73]. There is an increasing evidence about inverse correlation of vitamin D status and colorectal, breast, and prostate cancer [6, 75, 77]. Moreover, many observational studies have reported various actions of $1,25(OH)_2D_3$, as regulation of cell differentiation, proliferation, apoptosis, cell invasion, and angiogenesis; vitamin D exerts its actions mainly via its high affinity receptor VDR through a complex network of genomic (transcriptional and posttranscriptional) and also nongenomic mechanisms, which are partially coincident in the different cells and tissues studied [70–72].

Angiogenesis is an essential process for growth of solid tumors; therefore, anti-angiogenic agents contribute to the anticancer treatment. Anti-angiogenic drugs can cause inhibition of tumor progression, stabilization of tumor growth, and regression of tumor mass and prevent metastases. Experimental studies have reported anti-angiogenic action of $1,25(OH)_2D_3$, as well as his analog, 22-oxacalcitriol [78]. Today, there is a growing evidence that high vitamin D intake and a plasma level of 25(OH) D_3 reduce the incidence of colorectal cancer by modifying cancer angiogenesis, cell apoptosis, differentiation, and proliferation. Results from Kang et al. [79] suggest that vitamin D supplementation alone, or in combination with anticancer agents, might reduce the incidence of colorectal cancer.

Vitamin D also increases the level of cystatin D—an endogenous protein, which shows antitumor and antimetastatic property, by facilitating the expression of the gene coding for it [74].

Induction of apoptosis is a mechanism by which $1,25(OH)_2D_3$ inhibits tumor cell growth and may contribute to tumor suppression and explain the reduction in tumor volume found in various in vivo animal studies [63]. Besides its physiological function in maintaining constancy of cell numbers in different tissues, apoptosis also prevents the possibility of mutational changes leading to malignancy after DNA damage by removal of such damaged cells [74]. $1,25(OH)_2D_3$ is able to modulate apoptosis mediators by diverse mechanisms that favor the elimination of malignant cells [58]. Apoptosis sensitization by vitamin D in colorectal adenoma and carcinoma cells involves the upregulation of the proapoptotic proteins and the downregulation of anti-apoptotic proteins. The proapoptotic BAX component of the Bcl-2 competes with the antiapoptotic Bcl-2 on mitochondrial cell surface for release of cytochrome c from it; the proapoptotic

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branch tends to stimulate the release, while the antiapoptotic branch inhibits it. In normal cells, survival factors continuously oppose apoptosis by several mechanisms, out of which activation of antiapoptotic Bcl-2 is important. Proapoptotic compounds may have a favorable role in the prevention of cancer development, growth, and metastasis while aiding to its chemotherapy [74]. Vitamin D may also induce cell death by alternative pathways, such as increasing the calcium concentration, releasing cytochrome c and reducing intracellular glutathione [58].

Our study summarizes changes in the expression of certain immunohistochemical markers during tumor progression in colorectal human tissue. We have investigated markers responsible for apoptosis (PAK1 and p53), cell adhesion (beta-catenin), differentiation (p53), and proliferation (Ki67), as well as correlation of their expression with vitamin D blood levels [80].

In our study, average vitamin D blood level in patients with colorectal cancer was 5.99 ng/ml (range: 3–23.04 ng/ml), whereas average vitamin D blood level in patients with colorectal adenoma was much higher, i.e., 21.4 ng/ml (range: 11.3–30.66 ng/ml). The difference was statistically highly significant ($p = 0.0001$). Interestingly, among our patients with adenocarcinoma, only one had a vitamin D blood level in a normal range (23.04 ng/ml), while only one adenoma patient approached vitamin D deficiency (11.3 ng/ml).

PAKs are a family of serine/threonine protein kinases with six isoforms (PAK1-6), which play important roles in cytoskeletal dynamics, cell survival, and proliferation [81]. Although PAKs are not mutated in cancerogenesis, they are overexpressed, hyperactivated, or amplified in several human tumors. PAK1 has been reported to be overexpressed in colorectal cancer, but its role in CRC remains unclear [82]. Some recent studies have implicated PAK's role in activation of Wnt-beta-catenin signaling through direct interaction and phosphorylation of beta-catenin (see, e.g., [83]). In colorectal cancer, nuclear PAK1 is associated with advanced tumor stage. In adenocarcinomas, overall PAK1 (nuclear and cytoplasmic staining) was expressed in 80% of cases. Nuclear PAK1 expression was negative in all adenomas, as well as in grade I adenocarcinomas. In grade II adenocarcinomas, nuclear PAK1 was expressed in 20% of patients, while in grade III adenocarcinomas, it was expressed in 50% of cases (**Figure 1**). Our results of PAK1 expression were similar to those from Ye [81] and Zhu et al. [84], who found PAK1 expression in 70% of cells. Correlations with tumor grade and stage, as well as with the nodal status [85], indicate PAK1 as a very important marker during colorectal tumor progression.

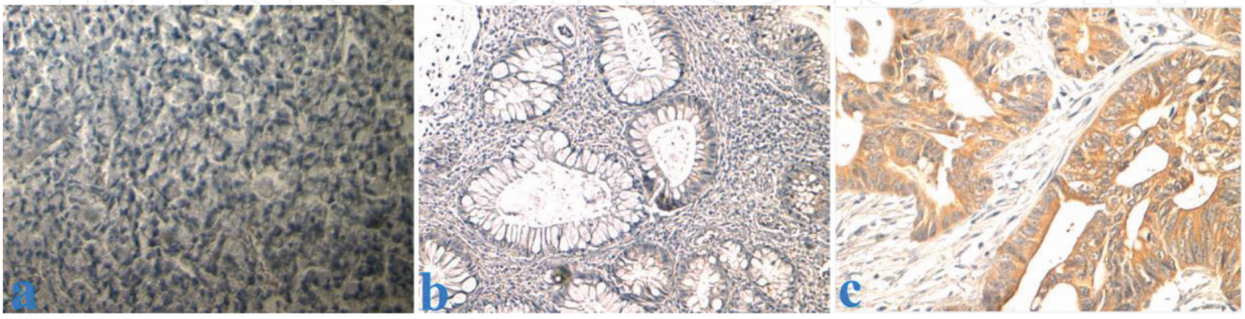

Figure 1. PAK1 expression in (a) colorectal mucinous adenocarcinoma (nuclear expression), (b) colorectal polyp (no expression), and (c) colorectal non-mucinous adenocarcinoma (cytoplasmic expression).

Numerous studies have focused on the clinical relevance of nuclear beta-catenin accumulation during colorectal pathogenesis, demonstrating its diagnostic, as well as prognostic significance [86]. A high density of beta-catenin nuclear accumulation was associated with higher mortality in selected groups of patients with colorectal cancer [87]. Under normal circumstances, beta-catenin is part of a complex of proteins that constitute adherens junctions necessary for creation and maintenance of epithelial cell layers, but the gene that codes for beta-catenin can function as an oncogene as well. When beta-catenin binds to the product of the mutated APC gene, free cytoplasmic beta-catenin is destabilized. This leads to the accumulation of nuclear beta-catenin, where it acts as a transcriptional activator of genes, specific for tumor formation [88]. Aberrant activation of the Wnt/beta-catenin signaling pathway due to mutation of adenomatous polyposis coli (APC), of beta-catenin or AXIN genes, is the most common and initial alteration in sporadic colorectal tumors [89]. It is significant that reduction of beta-catenin transcriptional activity is known to be mediated by $1,25(OH)_2D_3$ and is accompanied by the export of nuclear beta-catenin and its relocalization to the plasma membrane [90]. Our study showed a significant increase in beta-catenin nuclear expression during progression from adenomas to non-mucinous adenocarcinomas, as well as from non-mucinous adenocarcinomas to mucinous adenocarcinomas (**Figure 2**).

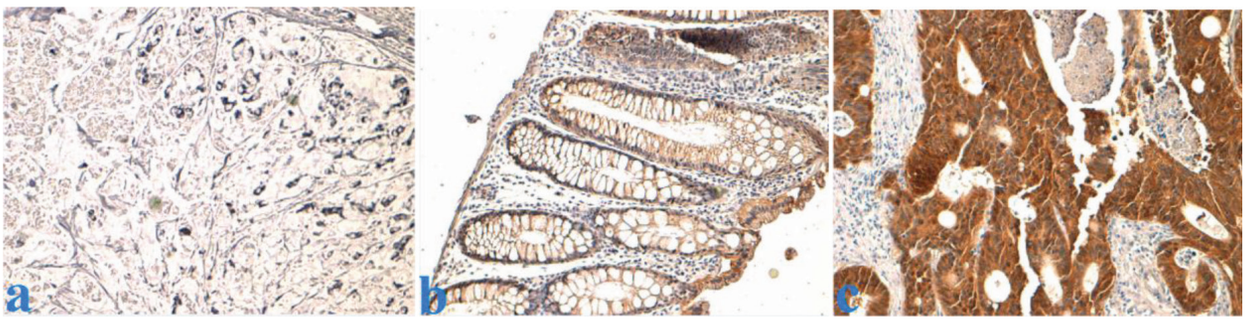

Figure 2. Beta-catenin expression in (a) mucinous adenocarcinoma (nuclear expression), (b) adenomatous polyp (membranous expression), and (c) non-mucinous adenocarcinoma (cytoplasmic expression).

Normal colorectal mucosa (tumor margins) expressed membranous beta-catenin staining and was used as internal positive control (**Figure 3**). In contrast to results of some other authors [91], we found beta-catenin overexpression to be most pronounced in mucinous adenocarcinomas. Nuclear beta-catenin expression correlated with both tumor grade and stage. Cytoplasmic beta-catenin expression was present in most of polyps and in all non-mucinous adenocarcinomas, whereas staining was positive in only 25% of mucinous adenocarcinomas. Cytoplasmic beta-catenin expression showed an association with better differentiation. This observation correlates with results from other authors. The presence of beta-catenin expression in the membrane and cytoplasm at an early tumor stage, and nuclear expression at advancing stages, illustrates the sequence of genetic mutations in normal epithelium developing into colorectal tumors.

p53 is a nuclear protein that induces cell cycle arrest or apoptosis in response to DNA damage. Its mutations are frequently associated with colorectal oncogenesis. The wild type of the p53 gene product has a short half-life and is not detectable by IHC. In contrast, mutant

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p53 protein has a much longer half-life, accumulates in the nucleus, and creates a stable target for IHC detection [92]. Frequency of p53 expression in our study correlates well with the frequency of p53 mutations found in studies using sequencing techniques for identification of p53 mutations in sporadic colorectal cancer [93, 94]. We detected nuclear p53 expression in 53% of adenocarcinoma patients, while cytoplasmic expression was present in 33% (**Figure 4**). In other studies the frequency of p53 staining ranges from 45 to 60% [95]. Nuclear p53 expression was increasing in parallel to tumor grade: in grade III adenocarcinomas, p53 was expressed in all cases, in grade II adenocarcinomas, p53 was expressed in 50% of cases, and in grade I adenocarcinomas, nuclear p53 was expressed in 33% of cases. In the group with colorectal adenomas, only one patient had nuclear p53 expression.

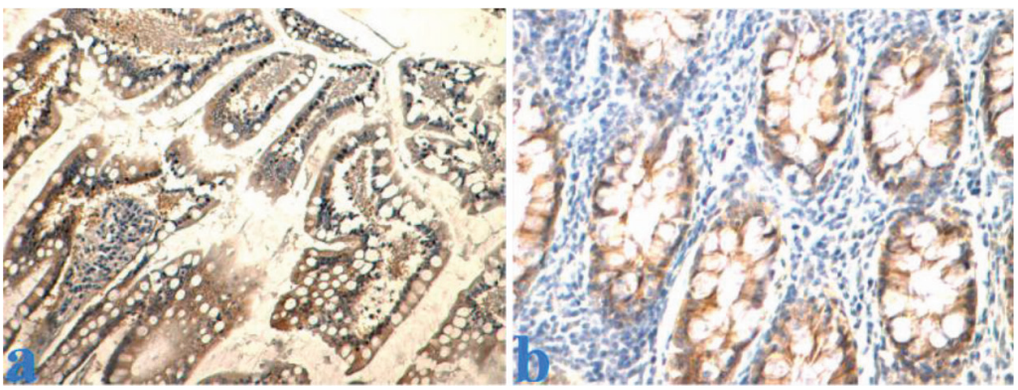

Figure 3. Membranous expression of beta-catenin in the margins of colorectal carcinoma: (a) mucinous adenocarcinoma and (b) non-mucinous adenocarcinoma.

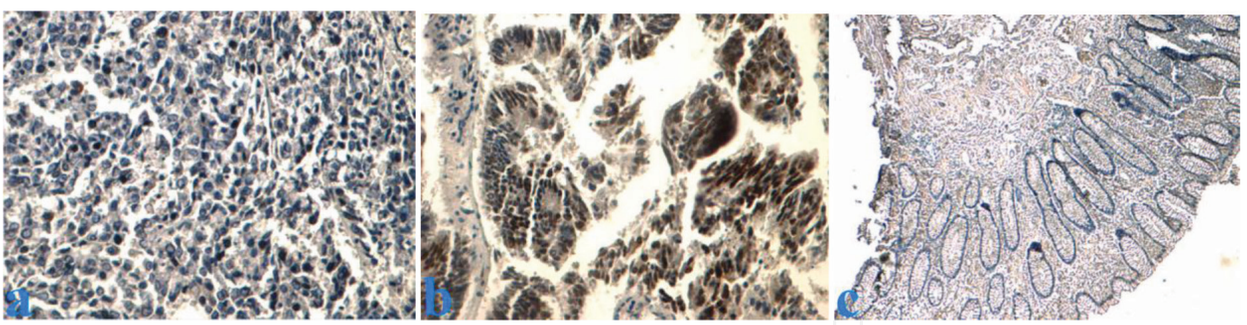

Figure 4. p53 expression in (a) mucinous adenocarcinoma (nuclear expression), (b) non-mucinous adenocarcinoma (nuclear and cytoplasmic expression), and (c) adenomatous polyp (no expression).

Ki67 is a nuclear nonhistone protein that is present at low levels in quiescent cells but is increased in proliferating cells, especially during G2, M, and the latter half of the S phase. Thus, Ki67 reactivity, defined as percentage of tumor cells staining positive in IHC staining, is a specific nuclear marker for cell proliferation. While the growth of malignant tumors is highly variable (although it might reflect their clinical course), proliferation still is a key feature of tumor progression. In our study, the mean Ki67 expression in all colorectal adenocarcinomas

was 47%. This is similar to those of Georgescu et al. (48%) [96] and Oshima et al. (44%) [97]. The proliferative activity as measured by Ki67 antibody was related to histological type and grade: Ki67 expression was higher in non-mucinous adenocarcinomas, compared with mucinous adenocarcinomas ($p = 0.0164$) (**Figure 5a** and **b**).

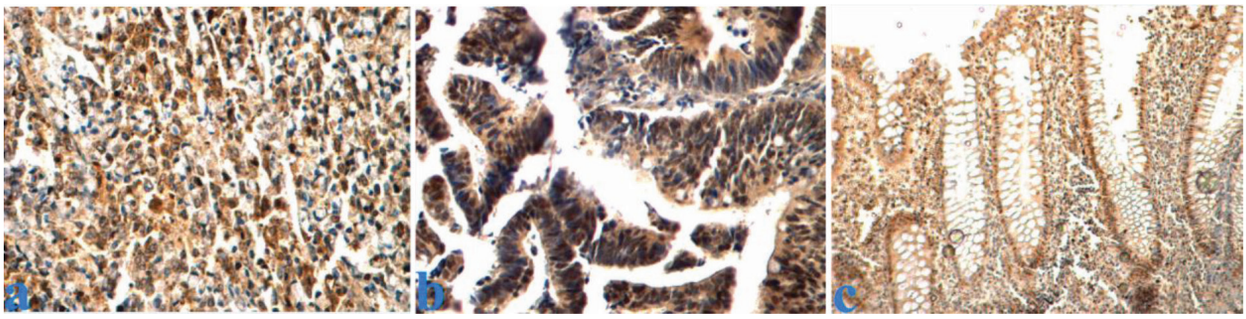

Figure 5. Ki67 expression in (a) mucinous adenocarcinoma (25%), (b) non-mucinous adenocarcinoma (70%), and (c) adenomatous polyp (10%).

Ki67 expression was high in well-differentiated (G1) and moderately differentiated (G2) adenocarcinomas (52%), compared with poorly differentiated (G3) adenocarcinomas (17%) ($p = 0.0314$). In colorectal adenomas, Ki67 expression was very low (16%) (**Figure 5c**). This indicates a low level of proliferative activity in these lesions. While our results are similar to those published by Nabi et al. [98], Georgescu et al. observed that Ki67 expression was higher in poorly differentiated (57%) than in moderately differentiated (34%) and well differentiated (20%) adenocarcinomas [96]. Correlation of Ki67 expression with the histological type of adenocarcinoma resulted in 26% in mucinous versus 55% in non-mucinous colorectal adenocarcinomas and was similar to that reported by Nabi et al. [98]. This suggests that proliferative activity in mucinous adenocarcinomas is lower than in non-mucinous adenocarcinomas.

Taken together, our data show that expression of these biomarkers increased with progression through the adenoma to carcinoma sequence. Accumulation of PAK1, beta-catenin, and p53 in the nucleus revealed correlation with advanced stages. Ki67 expression, however, was higher in well-differentiated than in poorly differentiated carcinomas.

Serum vitamin D levels in patients with positive nuclear PAK1 and beta-catenin expression had a negative trend, while patients with positive nuclear p53 expression had significantly lower vitamin D blood levels. Vitamin D levels were the lowest in mucinous adenocarcinoma and correlated with nuclear accumulation of p53, nuclear beta-catenin expression, and higher expression of Ki67. Considering the importance of an adequate $25OHD_3$ supply for synthesis of the active metabolite $1,25(OH)_2D_3$ in colon mucosal cells and the relevance of the latter for regulation of the Wnt/beta-catenin pathway, $25OHD_3$ blood levels could be considered as an indicator of Wnt/beta-catenin activity and increased cell proliferation [80]. This further emphasizes the chemopreventive role of vitamin D in CRC.

Garland et al. [99] in the early 1999 had suggested that ingestion of at least 800 IU (20 μg) of vitamin D, together with 1800 mg of calcium, is needed to significantly lower the incidence

and mortality of colorectal cancer. Matusiak and Benya concluded that nontoxic vitamin D precursors should be sufficient for CRC chemoprevention, but that neither vitamin D nor its precursors may be sufficient for CRC chemotherapy [100].

2.3.2. The expression of VDR and CYP27B1 in carcinogenesis and at the time of Vitamin D therapy

Sufficient blood levels of vitamin D provide a substrate for increasing the amount of vitamin D in the colonocytes, due to the presence of VDR and CYP27B1. Cross et al. [65] had shown (by RT-PCR, as well as by Western blotting and immunohistochemical methods) that in human large intestinal carcinomas expression of the genes encoding the 25-(OH)D_3-1α-hydroxylase (CYP27B1), as well as the VDR, increases in parallel with ongoing dedifferentiation in the early phase of carcinogenesis, whereas in poorly differentiated late-stage carcinomas, only low levels of the respective mRNAs can be detected [65]. According to this finding, colorectal cancer cells are able to increase their autocrine counter-regulatory response to neoplastic cell growth, through upregulation of the vitamin D/VDR system which mediates the antimitotic effects of the steroid hormone.

Expression of VDR, CYP27B1, and CYP24 determines the efficacy of the antimitotic action of 1,25-D3 and is distinctly related to the degree of differentiation of cancerous lesions. Bareis et al. [67] addressed the question of whether the effects of 1,25-D3 on VDR, CYP27B1, and CYP24 gene expression in human colon carcinoma cell lines also depend on the degree of cellular differentiation [67]. They showed that slowly dividing, highly differentiated Caco-2/15 cells responded in a dose-dependent manner to 1,25-D3 by upregulation of VDR and CYP27B1 expression, whereas in highly proliferative, less differentiated cell lines, such as Caco-2/AQ and COGA-1A and COGA-1E, negative regulation was observed. From the observed clonal differences in the regulatory effects of 1,25-D3 on VDR and CYP27B1 gene expression, the authors suggest that VDR-mediated growth inhibition by 1,25-D3 would be efficient only in highly differentiated carcinomas.

Bises et al. double stained colon tumors for CYP27B1 and VDR [15]; they found CYP27B1-enhanced expression in high- to medium-differentiated human colon tumors compared with tumor-adjacent normal mucosa or with colon mucosa from non-cancer patients. In high-grade undifferentiated tumor areas, expression was lost. Most colonocytes expressed both CYP27B1 and VDR, while some of them were positive only for VDR; this suggests that 1,25-D3 synthesized in colonocytes and bound to its receptor could exert its antimitotic function in both an autocrine and a paracrine fashion [15].

Because (as Matusiak and Benya [100] had shown) CYP27B1 is present in colonic epithelia, D3 alone may be sufficient for CRC chemoprevention and/or chemotherapy, providing that CYP27A1 and CYP24 are present in appropriate quantities and cellular compartments. To determine whether cholecalciferol may be useful for CRC chemoprevention and/or chemotherapy, Matusiak et al. reported on cellular CYP27A1 and CYP24 protein expression in human ACFs, polyps, and CRCs of defined differentiation along with associated lymph node metastases. Their findings suggest that cholecalciferol has potential for use in CRC chemoprevention but may be less efficacious for CRC chemotherapy.

2.3.3. Interactive role of calcium and vitamin D in colorectal prevention

Combined supplementation is required for optimal chemoprevention of cancer by calcium and vitamin D [76]. An interaction between nutritional calcium and vitamin D in protection against colorectal cancer may be due to the ability of luminal calcium to suppress degradation of $1,25(OH)_2D_3$ synthesized in colonocytes [101]. Calcium may also directly reduce hyperproliferation of the colonic mucosa [102] by binding to the calcium-sensing receptor (CaR) and by activating antimitotic, proapoptotic signal transduction mechanisms [103–105]. The effects of vitamin D and calcium on growth and differentiation of many epithelial cancers may be explained, in part, by their ability to control expression of E-cadherin and to regulate Wnt pathway [106]. Calcium regulates Wnt signaling through the calcium-sensing receptor as well: activation of the CaR enhances E-cadherin expression and suppresses TCF4 expression [107, 108]. It also stimulates secretion of Wnt5a, which inhibits beta-catenin signaling by increasing expression of an ubiquitin ligase that is involved in degradation of beta-catenin [109].

Several studies implied that the beneficial effect of calcium supplementation was due to the ability of calcium to form insoluble salts with potentially irritating and ultimately tumorigenic bile acids [110]. However, bile acids also interact with the vitamin D system: LCA is able to bind the VDR, thereby stimulating expression of CYP24A1 [111] and reducing availability of locally synthesized colonic $1,25(OH)_2D_3$. Bile acids are able to induce aberrant crypt foci, the precursors of neoplastic transformation in the colon; LCA activates the pregnane X receptor (PXR) and induces CYP3A expression [10]. In addition, bile acids interact with the vitamin D system: lithocholic acid can bind to the vitamin D receptor (VDR) and induce CYP24A1 expression [111].

Ingestion of considerable amounts of calcium and soy (phytoestrogens—see Section 2.3.4.) could provide accumulation of $1,25\text{-}OH_2D_3$ in colon, by enhancing the expression of synthesizing hydroxylase (CYP27B1) and reducing of the catabolizing hydroxylase (CYP24A). This is not the case with the renal vitamin D hydroxylases.

2.3.4. Role of folates in modulating the risk of colorectal cancer

As a vitamin of the B family, folate is essential for synthesis, repair, and methylation of DNA, while as a methyl donor, folate could play an important role in epigenetic regulation of gene expression [112].

The evidence from epidemiologic, animal, and human studies strongly suggests that folate status modulates the risk of developing cancers in selected tissues, the most notable of which is the colorectum [48]. Dietary folate influences DNA methylation, synthesis, and repair, and aberrations in these DNA processes may enhance carcinogenesis, particularly in rapidly proliferative tissues such as the colorectal mucosa [113]. Folates serve as physiological methyl donors during nucleotide precursor biosynthesis as well as during DNA, RNA, and protein methylation. Therefore, changes in folate metabolism have impact on two important determinants of carcinogenesis: on genetic expression and on maintenance of DNA integrity and stability. Folate deficiency affects the expression of key genes that are related to cell cycle control, DNA repair, apoptosis, and angiogenesis in a cell-specific manner [114]. Folate status affects

several cancer-related pathways including the p53 pathway, the Rb pathway, and the APC/*Wnt* pathway, as well as pathways involved in cell adhesion and cell migration and invasion [115].

Experimental results from Cross and coworkers suggest that, at least in mice, a "Western diet" resembling the high fat, low vitamin D, and calcium diet causes degradation of colonic $1,25(OH)D_3$, which can be stopped only by folate optimization [101]. The authors also concluded that folate optimization overrides the negative effects of low vitamin D and calcium intake [101]. (Regarding epigenetic regulation of vitamin D hydroxylases, see Section 2.2.3.)

According to results from Kim [116], dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa and promote, rather than suppress, colorectal carcinogenesis. Therefore, a "dual-modulator" role was suggested for folate in colorectal cancer, with a protective influence when ingesting only moderate amounts before development of aberrant crypt foci [117].

Extrarenal actions of $1,25(OH)_2D_3$, such as regulation of cell differentiation, proliferation, apoptosis, invasion, and angiogenesis in several types of tumor cells [70–72] suggest its potential therapeutic role against cancer. Nevertheless, the use of $1,25(OH)_2D_3$ is restricted by its hypercalcemic effect at therapeutic doses; this can be putatively overcome by the use of analogs that retain the antitumoral action but have less calcemic effect [2]. Numerous studies have shown that $1,25(OH)_2D_3$ and several analogs clearly reduce the growth of colorectal xenografts [70, 71].

In order to prevent premalignancies as well as their progression to tumors, we should focus on increasing the efficiency of the vitamin D system [16]. Especially in the colon, this can be accomplished by consuming calcium, soy, and folate [10]. Dietary modulation (using calcium, folate, and phytoestrogens) of extrarenal $1,25(OH)_2D_3$ synthesis in organs that are potentially prone to tumor incidence could lead to improved apoptotic and antimitotic activity by locally enhancing the concentration of $1,25(OH)_2D_3$ in these tissues, thereby preventing tumor cell growth [16]. A low folate status predisposes to development of several common malignancies including colorectal cancer [48]. Giovannucci et al. [30] demonstrated that prolonged intake of folate above currently recommended levels significantly reduced the risk of colorectal cancer [30].

Differences observed in the expression of vitamin D hydroxylases in patients with colon cancer during the course of tumor progression could be caused by the epigenetic regulation of gene activity via methylation/demethylation processes as well as by histone acetylation/deacetylation [16]. Methylation/demethylation processes (i.e., epigenetic regulation) in promoter sequences of vitamin D hydroxylases may lead to reduced, respectively, enhanced expression of these enzymes [50]. CYP27B1 expression is exceedingly high in low-grade cancerous lesions, compared with its expression in normal colonic mucosa of non-cancer patients [15]. Enhanced synthesis and accumulation of $1,25(OH)_2D_3$ in colonic mucosa could be responsible for the upregulation of transcriptional activity of CYP24A1 and also for the autocrine/paracrine inhibition of tumor cell growth [65]. Cross et al. [16] suggested that this enhanced expression of CYP27B1 could be due, at least in part, to epigenetic regulation (i.e., demethylation), while raised CYP24A1 expression may result from the normal regulatory

loop following the accumulation of $1,25(OH)_2D_3$ in colonic mucosa [16]. However, in highly malignant tumors, an efficient antimitogenic effect by $1,25(OH)_2D_3$ is unlikely, since the expression of the catabolic vitamin D hydroxylase by far exceeds that of CYP27B1 [68].

2.3.5. Estrogen pathway in CRC: interaction with vitamin D

Worldwide, colorectal cancer has a higher incidence rate in men than in women, suggesting a protective role of sex hormones in the development of disease [118]. In addition, studies from relationship between gender and mortality of colorectal cancer show lower mortality for women, especially premenopausal women; epidemiologic studies over several decades suggest a decline in mortality of women attributed to the use of hormone therapy [119, 120]. Expression of estrogen receptor (ER) subtypes α and β has been detected in cancer cell lines. The ER-α:ER-β ratio has been identified as a possible determinant of the susceptibility of a tissue to estrogen-induced carcinogenesis [10]. In both normal and cancerous colonocytes, ER-α expression levels remain low, while ER-β is the predominant ER in the normal colon [121, 122]. The expression level of ER-β in tumor tissue compared with normal colon mucosa is decreased and correlates with stage of the disease [123, 124].

In the colon adenocarcinoma-derived cell line Caco-2, which is ER-β positive but negative for ER-α, Lechner et al. demonstrated an increase of CYP27B1 after treatment with 17β-estradiol [125]. They proved that supraphysiological concentrations of 17β-estradiol not only elevated CYP27B1 mRNA expression and enzymatic activity but also reduced that of CYP24A1 [125]. By enhancing vitamin D accumulation in colon cancer cells, this could inhibit tumor progression. Transfection of SW480 cell lines with ER-β resulted in inhibition of proliferation and cell cycle arrest; SW480 xenografts with ER-β expression had 70% reduction in the tumor weight [126]. Transfection of colon cancer cell lines with ER-β also affects the MAPK signaling pathway [127].

In animal models the transcriptional activity of ERs changes over time and is influenced by estrogen level [128]. Therefore, it is likely that hormone replacement therapy (HRT) in women protects against colon cancer through an increased ratio of ER-β [118].

Regarding potential mechanisms for the interaction of estrogen with vitamin D on CRC risk, experimental evidence indicates that estrogen and vitamin D complexes undergo competitive binding for their common cellular uptake membrane receptor, megalin [129]. Megalin serves as a key endocytosis cell surface receptor for several vitamins and hormonal ligands (including vitamin D [130] and its recently identified ligands—estrogen and testosterone bound to sex hormone-binding globulin (SHBG) [131]. This is clinically relevant to the Women's Health Initiative (WHI) trial findings, as megalin gene knockout has been demonstrated to strongly induce both estrogen deficiency and vitamin D deficiency [131, 132].

Both estrogens and phytoestrogens exert their effects on target cells by genomic and nongenomic mechanisms [133]. Phytoestrogens reduce cell injury and DNA damage mediated by 5 μM oleic acid hydroperoxides in Caco-2 cells [134] and decrease levels of oxidative DNA damage in humans [135]. Genistein, a major phytoestrogen in soy, is antimitotic and proapoptotic in colon cells via the TGF-β/Smad pathway [136]. This phytoestrogen is also involved in regulation of gene activity by modulating epigenetic events such as DNA methylation and/or

histone acetylation (see, e.g., [137]). According to experimental studies, genistein enhances VDR expression in colon cancer cells; upstream and downstream events in the signaling cascade are all interrelated and all participate in the control of VDR expression by 17β-estradiol as well as by phytoestrogens [138]. Genistein also induces CYP27B1 and reduces CYP24A1 expression and activity in a mouse model and in human colon adenocarcinoma-derived cell lines [139].

Reduced incidence of cancer (including colon tumors) has been related to the consumption of a typical Asian diet containing soy; soy and red clover are important sources of phytoestrogens that bind preferentially to estrogen receptor-beta (ER-β) [16]. While the colon cannot be considered an estrogen-dependent tissue, it must be defined as an estrogen-responsive organ [112]. Expression of estrogen receptor (ER) subtypes α and β has been detected in cancer cell lines. In normal colonic mucosa, it is ER-β that is mainly expressed. When mice were fed a diet containing soy, the expression of the synthesizing vitamin D hydroxylase, CYP27B1, was enhanced, and that of the catabolic hydroxylase, CYP24A1, was decreased [50]. In a clinical pilot trial [140], postmenopausal women with a past history of rectal adenomas were given a daily dose of 17 β-estradiol for 1 month to reach premenopausal hormone levels. Rectal biopsies were obtained at the beginning and end of the trial. A predominant result was that VDR mRNA was increased. Such data suggest a protective role of female sex hormones, particularly of estrogens, against CRC. This could provide a rationale for the observation that the age-adjusted risk for CRC is lower for women than for men, even though men and women suffer from similar rate of CRC deaths in their lifetime [16].

Regarding the mechanism behind the gender differences of CRC, Hartman et al. [126] investigated the molecular function of ER-β in colon cancer cells, focusing on cell cycle regulation. They concluded that ER-β inhibits proliferation and tumor growth of colon cancer cells by regulating G1-phase cell cycle genes. Furthermore, this ER-β-mediated cell cycle repression is dependent on functional estrogen response element (ERE) binding. To dissect the processes that ER-β mediates and to investigate cell-specific mechanisms, Edvardsson et al. [127] reexpressed ER-β in three colorectal cancer cell lines (SW480, HT29, and HCT-116) and performed genome-wide expression studies in combination with gene-pathway analyses and cross correlation to ER-β-chromatin-binding sites [127]. Overrepresentation analysis of functional classes indicated that the same biological themes, including apoptosis, cell differentiation, and regulation of the cell cycle, were affected in all three cell lines [127]. ER-β-mediated downregulation of IL-6 has an important impact on inflammation process involved in colon carcinogenesis. The influence of ER-β on apoptosis was further explored using functional studies, which suggested an increased DNA repair capacity. Edvardsson et al. propose that enhancing ER-β action has potential as a novel therapeutic approach for prevention and/or treatment of colon cancer [127].

It has been established that postmenopausal hormone replacement therapy (HRT) is associated with decreased incidence and death rate of colon cancer in both epidemiologic [141, 142] and intervention [142, 143] trials. Additional data suggest that this action may, at least in part, be mediated through VDR signaling. RT-PCR results confirmed that estrogen administration increased mRNA expression of VDR as well as of a downstream target of vitamin D action, E-cadherin [140]. Besides HRT, high estrogen content of soy is implicated to be

protective against colorectal cancer; however, evidence suggests that higher soy consumption is protective against colon cancer only in women [144]. The use of estrogens for prevention of colon cancer is an attractive concept in women; however, the increased rates of cardiovascular events with HRT limit the use of these agents in clinical practice [118].

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