REVIEW

BIOMOLECULAR PROFILE OF COLORECTAL CANCER - THE ROLE OF TELOMERASE AS A POTENT BIOMARKER

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Abstract

Colorectal cancer (CRC) is a very frequently diagnosed pathological entity, registering an elevated incidence rate each year. CRC stems from both a genetic and an environmental background, exhibiting divergent molecular and biological phenotypes, and rendering its therapy, follow-up and prognosis a demanding task. Telomerase, a complex consisting of the catalytic protein human telomerase reverse transcriptase (hTERT) and the mRNA template hTERC, is related to the preservation of telomere length (TL). A wide range of studies suggest that hTERT also partakes in signalling pathways relevant to proliferation and apoptosis. Thus, the potent role of telomerase as a biomarker for CRC behaviour emerges as a reasonable inquiry.

Rezumat

Cancerul colorectal este o entitate patologică foarte frecvent diagnosticată, cu o rată crescută a incidenței în fiecare an. CRC are atât cauze genetice, cât și cauze care țin de factorii din mediul înconjurător, prezentând diverse fenotipuri moleculare și biologice, ceea ce pune dificultăți în managementul terapeutic și prognostic. Telomeraza este un complex alcătuit din proteina catalitică hTERT și *template*-ul mRNA hTERC, fiind implicate în conservarea lungimii telomerelor. Studii extinse au evidențiat implicarea hTERT în căile de semnalizare a proliferării și apoptozei celulare. Astfel, telomeraza este un important biomarker în evoluția și prognosticul cancerului colorectal.

Keywords: colorectal cancer, telomere length, telomerase activity, prognosis, biomarker

History of colorectal cancer

Colorectal cancer (CRC), as a pathological entity, was recognized since the existence of ancient civilizations. In ancient Egypt medical documents (3000 - 800 BC), benign and malignant forms of colorectal neoplasia were described [109]. Impressively, in a research pertaining to the antiquity of cancer in relation to mummification process, performed by Zimmermann M. R. in 1977, rectal cancer was diagnosed in a mummy, chronically located in the Ptolemaic Period [153].

The pathophysiological facets of colorectal cancer (CRC) have been extensively studied, with the onset being in 1927, with a publication by Lockhart-Mummery and Dukes referring to the precancerous alterations in the colon and rectum [143]. They exhibited the association of CRC with residual adenomatous tissue, implying the pre-existence of a lesion instead of a *de novo* modulation. In following studies in the 30s', Dukes and colleagues propounded the staging system for CRC. This progress led to subsequent studies, with observations referring to somatic mutations and the polyp-cancer transition [94]. Fearon and Vogelstein in 1990 proposed a model that describes four integral genetic facets of CRC; inactivation of tumour suppressor genes and simultaneous activation of oncogenes, mutation in at least five genes for malignancy development, the accumulation of genetic alterations and the effect of mutated tumour suppressor events even at a heterozygous pattern.

Nowadays, clinical studies rotate around the elucidation of cellular pathways involved in the pathogenesis of CRC and in the investigation of various molecules that could be used as biomarkers for prognosis.

The identity of CRC

CRC prevalence is associated with a cluster of hazard factors, including dietary habits predominantly encountered in developed countries, i.e. diets rich in animal fat and decreased fibre intake, high lipid

profile, alcohol consumption, smoking and the adoption of a sedentary lifestyle [50, 131, 134, 135, 144].

Additional factors include age and male gender. Calcium intake seems also to affect negatively the manifestation of CRC [65], as well as the long-term intake of aspirin and NSAIDs [34]. In a research by Kervinen *et al.* in 1996, it was observed that a polymorphism in apolipoprotein E (apoE) is relevant to a decreased likelihood of proximal colon cancer progression [80]. Although the majority of CRC cases is sporadic, the inherited basis is important. The presence of a large number of polyps and a positive family history, can be linked with genetic alterations and consequently with the manifestation of hereditary cancer syndromes (familial adenomatous polyposis (FAP), Gardner's syndrome, Tourcot's syndrome, Peutz-Jeghers, etc). On the other hand, the inherited form of CRC can be manifested as non-polyposis syndrome, known as hereditary non-polyposis colon cancer (HNPCC) (or Lynch syndrome). The inherited form in total accounts for up to 5% of the total CRC cases.

Cancer statistics of 2017, report that in the USA, the estimated incidence for colon cancer ascends up to 95,520 cases, and up to 39,910 for rectal cancer. For colon cancer, deaths rise up to 50,260 [121].

CRCs can be classified as stages I and II when they remain within the colonic walls, stage III when they spread to regional lymph nodes, and stage IV when they metastasize to distant sites. Surgical resection and adjuvant treatment are deemed as effective therapeutic options, the hazard of possible recurrence persists, and no accurate predictions can be drown, even in patients who belong to the same stage. Generally, surgery is opted for patients in stages I-III, while, additionally, adjuvant therapy is considered in stage III patients. Particularly, for locally advanced rectal cancer, neoadjuvant chemoradiotherapy (CRT) followed by total mesorectal excision (TME) is considered currently the gold standard of care. Furthermore, CRC can be divided in multiple categories, according to the genetic alterations and their molecular basis as well as the location they get encountered.

Most cases of CRC occur at a sporadic pattern. Inflammatory bowel disease (Crohn's disease and ulcerative colitis) and hereditary syndromes also predispose the development of CRC, including in descending order of frequency Lynch syndrome, familial adenomatous polyposis and MYH-associated polyposis. The association of comorbidities such as non-alcoholic fatty liver disease [40], vitamin K coagulophathy [145] or liver cirrhosis with C virus [51] darkens the prognosis and evolution of CRC.

Surgically, for colon cancer total resection of the tumour is performed, taking into account sufficient proximal ($\geq$ 10cm) and distal margins ($\geq$ 5 cm) and accompanied lymphadenectomy. Considering rectum, surgical resection includes total removal of the mesorectum (TME) and sufficient margins along with apical (inferior mesenteric artery) lymphadenectomy [60]. Laparoscopic colectomy is implemented in colon cancer, especially in left-sided cancers. Right, extended right colectomy, low anterior or abdominoperineal resection of rectum performed by laparoscopic approach are certainly more demanding techniques [13]. In stage III disease, administration of fluorouracil, capecitabine and fluoropyrimidine are effective options. For metastatic cancer, palliative chemotherapy alleviates the symptoms. But chemotherapy is expensive [136] and it is accompanied by severe side effects such as immunosuppression that facilitates infections with pathogens germs resistant to antibacterial treatment [30, 31] or fungal infections with *Aspergillum* or *Fusarium* genus very hard to eradicate [125].

Sometimes rash similar to acne resistant to classic conventional dermatological treatment could appear and need differential diagnosis [73]. For their treatment, new therapies were developed with nanoparticles in which cytotoxic substances were incorporated [29, 105] or with plant extracts with demonstrated cytotoxic effect [62, 67, 113, 117].

The most utilized predictive markers are the detection of microsatellite instability (MSI) (adjuvant treatment is indicated for stage II patients with high-microsatellite instability – MSI-h) [43], KRAS mutations (wild type gene patient is responding well to targeted treatment), thymidylate synthase, vascular endothelial growth factor (VEGF) and its receptors, and interleukins [45].

CRC cancer is usually diagnosed during colonoscopy for investigating rectal bleeding. For colon cancer that can be removed, the preferable option is colectomy with *en bloc* removal of the regional lymph nodes. Laparoscopical removal might also be implemented, with almost identical results considering the clinical picture afterwards and the success [101]. The concept of three entities in colorectal cancer; proximal colon, distal colon and rectal cancer, optimally describes the pathological basis of and therapeutic approach [87].

According to genetic modulations

CRCs constitute a cluster of diseases with varying molecular pathways and biological behaviour, stemming from a multifactorial process in which genetic (mutations, polymorphisms, etc.) as well as epigenetic chromatin alterations (DNA methylation and acetylation) take place. The aforementioned events induce genetic or epigenetic instability respectively. Over 250 transcription factor (TF) genes expression is altered regarding the transcription-regulating network of adenomas in colon and rectum [139]. These modulations also induce the transformation of colonic epithelial cells into colon adenocarcinoma cells.

Genetic instability in CRC can be pigeonholed into tumours displaying chromosomal instability (CIN),

and those of microsatellite instability (MSI) [24]. Chromosomal instability, as being the most common type of genomic instability, provides a mechanism against tumour-suppression genes (like adenomatous polyposis coli (APC), p53 and SMAD family member 4) [116]. The most prime detectable lesion in colonic tumorigenesis is the aberrant crypt focus, as, when dysplastic, it can often carry genetic alteration in APC and presents a high likelihood for adenocarcinoma [116]. Chromosomal instability includes mutations in p53 and K-ras and loss of heterozygous chromosomal regions. In the majority of tumours, the two p53 alleles are inactivated while giving rise to a stable mutant protein whose accumulation is regarded as a hallmark of cancer cells. This event subsequently contributes to an anti-apoptotic behaviour [14]. According to a study, PRL, RBM3, Wrap53, p53 and DNA status can function as potent prognostic biomarkers for CRC individuals of younger ages (≤ 50 years old) [141].

Microsatellite instability refers to the inability of the DNA mismatch repair mechanism to correct errors during replication. MSI consists approximately 15% of all CRC. In fact, Lynch syndrome (also known as hereditary non-polyposis colorectal cancer – HNPCC) is a great paradigm of a known and well documented oncological syndrome that is strongly connected with MSI [74, 129]. The causative factor of MSI is a deficiency mismatch repair (MMR) mechanism. DNA MMR is entitled the role of maintaining genomic stability by checking and correcting base/base and small insertion/deletion mispairs that are generated during DNA replication [65]. In more detail, in eukaryotic cells this kind of errors are corrected by the DNA MMR system following the initial detection of replication errors by the heterodimers MSH2/ MSH6 and MSH2/MSH3 with the subsequent involvement of the MLH1/PMS2 complex which synergistically degrades the error areas and initiates re-synthesis. Hence it is rather logical to find that the same complexes are maintained in human cells. Thus, mutations in the MMR genes MLH1, MSH2, MSH6 and PMS2 are associated with MSI.

Interestingly though, it is described that there is a subset of MSI positive cancers that does not demonstrate any genetic or epigenetic alterations in anyone of the known MMR genes [150]. In a study, overall survival was significantly better in the MSI group independently from implemented therapy [26].

Genetic modifications involve inhibition of tumour suppressor genes, like the APC (adenomatous polyposis coli) and TP53 gene, and the activation of oncogenes. Oncogenes that take part in CRC progression are RAS and BRAF. Mitogen-activated protein kinase (MAPK) becomes activated and BRAF products further promote MAPK signalling cascade [107]. Also, approximately one in three CRC cases involve mutations of the phosphatidylino-inositol 3-kinase (PI3K), with the genetic alteration regarding mutations in PI3KCA gene that encodes a subunit of PI3K [115].

It is estimated that around 80 mutations of PI3KCA have been recorded. For tumorigenesis and further development, of course, various additional genetic alterations are required [102]. APC gene, which is located in the 5q chromosome, includes mutations in about 85% of CRCs. It is described to play a vital role in migration, adhesion and apoptotic events [92]. Specifically, in colon cancer, the APC gene is reported to act as an initiator for tumour formation, as its inactivation is related to augmented cellular proliferation. Mutation of the KRAS' oncogene is believed to provoke the transition from healthy epithelium to adenoma [83]. A very strong association exists between K-RAS mutation level and CRC, as these mutations are phenomena occurring early in tumour formation. K-RAS genotype is a useful tool for the identification of mutations.

An epigenetic pattern has also been proposed in the pathophysiology of CRC onset and progression [68]. The most significant mechanisms of epigenetic instability involve hypermethylation (CpG island methylator phenotype-CIMP), histone deacetylation and microRNAs expression.

Epigenetic regulation through unsuitable methylation in regions belonging to promoters frequently occurs in CRC; indeed this event is equally important to genetic mutations in tumour-suppressor gene silencing. The majority of human genome includes primers that are embedded in guanine-cytosine residual clusters, named as CpG islands. In normal cells, CpG islands exist in non-methylated condition. Gene expression is normal when methylation is absent. On the other hand, methylated cytosine gets bound with a protein family, that withhold methyl-CpG regions forming a multiprotein complex, holding the ability to change chromatin arrangement and rendering gene expression impossible resulting in decreased expression or gene silencing [133]. Tumour-suppressor gene silencing, as a result of promoter silencing, emerges either by including both tumour-suppressor gene alleles, or alternatively, as a result of loss of a single allele in combination to other allele gene silencing (*via* primer methylation) [36]. Methylation rises along with age, and is augmented in the colon as a response to chronic inflammation. Methylation occurs in various known tumour-suppressor promoters, such as p16, insulin-like growth factor (IGF), MLH1 of MMR system [46, 57, 142]. CpG island methylator phenotype (CIMP) tumours consist of a CRC sub-category with an elevated rate of genes that under-went hypermethylation, belonging to BRAFV600E mutations. Aberrant MLH1 methylation occurs at about 80% of sporadic MSI CRCs. Most sporadic CRCs with MSI are CIMP, a fact that discriminates them from Lynch syndrome.

In addition to methylation of promoters, histone deacetylation and miRNAs expression represent important mechanisms in the regulation of genomic function and transcription. Histones are proteins bound with DNA, organizing it in nucleosomes, eventually forming chromatin. Regulation of histone deacetylation, and mainly of their free histone tails, leads to open chromatin forms which permit transcription or in closed chromatin forms that lead to silencing [91, 112]. Moreover, miRNAs do not encode proteins, but rather regulate the genetic expression through inhibition of mRNA translation. Malfunctions in miRNAs expression are frequent in multiple human neoplastic lesions. Reduction or loss of miRNAlet-7 leads to protein overexpression and constant activation of K-RAS pathway [132]. MiRNAs are also capable of playing a tumour-suppressor role, restraining tumour-suppressor gene expression.

In adenoma development, a certain stage is the activation of prostaglandin signalling pathway, provoked through up-regulation of COX-2. COX-2 is an enzyme that acts as a mediator for the synthesis of prostaglandin E2, which is highly correlated to CRC [151]. Relevant researches have also revealed a potent role of $p27^{kip-1}$ (cyclin-dependent kinase inhibitor) and Cox-2 (cyclooxygenase 2).

In general, the activation of growth factor pathways and the intervention in signalling pathways are prevalent in CRC. A step for CRC progression is the genetic inactivation of TGF-β signalling [96, 127], which mainly regards somatic mutation in TGFBR2 that in turn affects the TGFBR2 kinase or other particles of the pathway (SMAD2, SMAD3). Wnt is a conserved embryonic pathway that in normal adult tissues remains inactive, which leads toβ-catenin down-regulation. The activation of the Wnt signalling pathway consists of another mechanism for the initiation of CRC. Wnt takes place by the time the co-protein β-catenin gets bounded to nuclear particles, in order to create a transcription factor responsible for the regulation of genes involved in cellular activation. APC takes part in degradation of β-catenin and intercepts its nuclear localization. Germline APC genetic mutations trigger the manifestation of FAP [4].

An additional route for CRC progression entails germline inactivation affecting the MYH protein. In these cases, a polyposis phenotype is demonstrated (MYH associated polyposis), leading to significant elevation of CRC progression [79].

According to location:

Two broad categories of colonic cancer exist according to the distance of the tumour, proximal and distal, namely proximally (right) or distally (left) to the splenic flexure. They display differences in incidence, pertinent to geographic region, age and gender. A hypothesis exists that proximal tumours in occidental countries elevate in steady rate, whereas the percentage of distal tumours undergoes an equally steady reduction [72]. It is interesting to note that the proximal neoplasia gets encountered more frequently in elder patients and in females [12].

Differences exist in developmental and biological aspects in proximal and distal colon, an event that indicates different response to neoplastic lesions. The differences that emerge could reflect the different pathogenetic mechanisms that arise. Proximal tumours are shown to demonstrate a genetically more stable form of CRC and may emerge *via* the same mechanisms as those behind nonpolyposis colon cancer. Distal tumours exhibit bigger genetic instability and may progress *via* the same mechanisms that consist the base of polyposis-associated CRC. Embryologic origin of colonic epithelium of proximal and distal parts may indicate differences in susceptibility to carcinogens.

HNPCC and FAP manifest mainly in the right and left colon respectively [56]. FAP is characterized by the presence of more than 100 polyps, which first occur in the rectum and distal colon and subsequently moving to proximal segments. The vast majority of FAP patients develop left-sided CRC, whereas in individuals with HNPCC the tumours arise in the right part. In Table I is presented a summarized outlook of the major differences between proximal and distal CRC (modified from: Iacopetta, 2002 [72]).

Table I

A summarized outlook of the major differences between proximal and distal CRC

PROXIMAL	***DISTAL***
Common in older patients & females	Encountered in younger patients & males
Related to HNPCC	Related to FAP
Frequent mucinous tumours	Non-frequent
Mainly diploid, less frequent heterozygosity loss	Mainly aneuploidy, frequent heterozygosity loss
Less rate of TP53 mutations & increased MSI+ rate	Increased rate of TP53 mutations but minor cases with MSI+ phenotype
Sufficient response to 5-FU chemotherapy	Inadequate response

Considering carcinogenetic mechanisms, right cancer is related to mutations of MMR, KRAS, BRAF and miRNA-31, while the left is related to CIN, p53, NRAS, miRNA-146a, miRNA-147b and miRNA-188. As regards the protein expression, the right is particularly related to GNAS, NQO1, telomerase activity (TA), P-PDH, and annexin A10. On the other hand, the left is pertinent to Topo I, TS, and EGFR [120].

Embryonically, the proximal colon hails from the midgut, are perfused by the superior mesenteric artery, while the distal originates from the hindgut are supplied by the inferior mesenteric artery. A rich capillary network can be found in the proximal colon, while in the distal colon this network is single-layered. Differences exist also between the right and left colon regarding the metabolism of bile acids [130]. Concerning the colonic and rectal mucosa, different traits have been recorded, as acidic mucin is produced in the rectum, while colonic mucosa is of neutral pH. Considering apoptosis, the rate is increased in the left colon compared to the right, possibly due to elevated genetic expression of the pro-apoptotic Bcl-2 homologue Bak [72].

Proximal and distal CRCs exhibit important differences in a histological and molecular aspect. Specifically, mucinous carcinomas seem to be prevalent proximally, though other studies state different hypotheses [122]. Proximal colonic cancer is predominantly associated with MSI and CpG island methylation phenotypes, whereas distal colon is related with specific chromosomal instability (CIN). Moreover, for MSI+ phenotype, decreased or non-existent expression of hMlh, decreased or absent Fhit expression and modified expression of p27 are common in proximal tumours, than in distal. Immunohistochemical expression of p27 is modified more frequently in proximal tumours. Distal CRC tumours are defined by more frequent 18qLOH, normal hMlh1 expression, normal p27 expression, and normal Fhit expression. Overall, at least two major groups exist, regarding colonic molecular and histologic profile; the first group mainly manifests in the proximal colon and is defined by histologic mucinous type, MSI, altered expression of Mlh1, Fhit and p27. The second group is considered mainly in the left colon and is defined by 18qLOH. These varying traits may suggest two different pathways of tumorigenesis in the proximal and distal colon.

Tumours characterized by mismatch-repair deficiency emerge mainly in the proximal colon. In mismatch repair deficiency, tumour-suppressor genes, like genes encoding transforming growth factor β (TGF-β), receptor type II (TGFBR2) and BCL-2-associated X protein (BAX), can be inactivated [116].

CRC can also be viewed as in two broad sub-groups, with the first being LOH (loss of hetero-zygosity), exhibiting hyperploidy, and the second presenting a normal diploid pattern without deficient alleles, but having MSI. The former group is more prevalent in the distal colon, while the latter presents a right-sided preference [85].

Not surprisingly, it is observed that right colon cancer, as opposed to left-sided cancer, displays also different responses to treatment [16, 72]. The results of chemotherapy in right-sided tumours are enhanced in comparison to left, however metastatic left cancer presents longer survival in comparison to right, in terms of palliative chemotherapy [54]. For K-RAS types, the left type is better confronted with cetuximab treatment, while results in the right are poorer.

Telomeres and Telomerase

Telomeres, etymologically deriving from the Greek words *telos* (end) and *meros* (piece, segment), represent recurrent transcriptionally inactive nucleotide regions found in the terminal parts of the chromosomes. Functioning as protective hoods, telomeres are comprised by diverse repetitions of the 5'-TTAGGG-3' hexamer [93], bound with a protein complex called shelterin. These biological structures contribute to chromosomal stability and integrity, as they intercept fusion with ambient chromosomes and protect the chromosome from the action of exo-nucleases, ligases and DNA repair mechanisms [48].

DNA of telomeres can be easily modified during life circle, as it can be degraded and reconstructed by telomerase [22]. Telomere length (TL) is also associated with diseases that accompany ageing, such as metabolic syndrome, hypertension and dementia, as longer telomeres are equivalent to a lower prevalence of the afore-mentioned diseases and also to a larger life-span [6]. It can be deduced that factors responsible for interrupting the integrity of telomeres (reactive oxygen species-ROS, DNA repair mechanisms and p53 mediated procedures) contribute to the formation of ageing phenotype [5]. Diseases of telomeres refer to bone marrow failure, dyskeratosis congenita, acquired aplastic anaemia, pulmonary fibrosis and hepatic lesions [111]. Factors that affect telomere function pertain mostly to dietary habits, lifestyle, smoking, social and economic state and stress levels [111].

Single-stranded regions included in telomeres promote biological ageing by working as primers for telomerase [88]. Telomerase is a ribonucleoprotein enzyme complex, consisting of two subunits; hTERC (human telomerase RNA component), an RNA sequence pattern on which the synthesis of telomeric parts is based, and a protein called human telomerase reverse transcriptase (hTERT) [20], whose responsible gene is located in chromosome 5 (5p15.33). Two of the six subunits of shelterin, TPP1 (tripeptyl peptidase I) and POT1 (protection of telomeres 1) [148], regulate the action of telomerase [140]. hTERT is responsible for the suppression of apoptosis in an early phase before the induction of cytochrome c [1]. Normally, TA is elevated in the early gestation period [58]. hTERC is broadly distributed in the tissues, both present in normal and cancerous cells, while hTERT expression occurs mainly in germ-line cells and most tumour cells. hTERT is required for the unrestricted cellular growth and as a result it plays a leading role in tumour initiation and further progression

[82]. Telomerase, however, requires also additional enzymes and proteins for its stability and proper function. Dyskerin, functioning as RNA binding protein and enzyme [21], is a pseudouridine synthetase [41]. Although this function is not of importance for telomerase, it is shown that its presence is obligatory for the enzyme. Moreover, TCBA1 (telomerase Cajal body protein 1) is associated with telomerase activity through dyskerin (Figure 1).

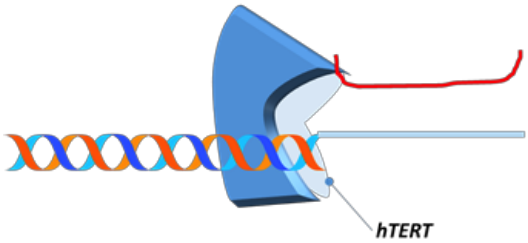

Figure 1.
Renewal of telomeric cap performed by telomerase

Modulation of telomerase activity occurs in several biological tiers; transcription, mRNA splicing, sub-cellular localization of each component and the assemblage of TR and hTERT in an active ribo-nucleoprotein complex. Transcription of hTERT gene is most likely the key for the modulation of telomerase activity. The hTERT gene has a length of 35 kb, entailing 16 exons and 15 introns [18]. For the total activation of hTERT promoter, the concurrent action of MYC and SP1 is deemed essential. TP53, when coming in interplay with SP1, downregulates TERT. Nuclear factor-kB, hypoxia-inducible factor (HIF-1) and the ETS/MYC complex are also involved in the positive regulation of hTERT promoter expression. Moreover, the histone methyltransferase SMYD3 incites TERT expression in healthy as well as malignant cancerous cells. Expression of TERT is constricted by the onco-suppressor genes WT127 and MEN1, and also *via* MAD/MYC and TGF-β/ SMAD pathways. TERT expression is also suppressed by the inhibitors p16INK4a and p27KIP1 [52]. Post-transcriptionally, regulation of telomerase may take place, mainly, *via* tissue-specific alternative splicing mechanisms (Table II).

Table II
Factors that incite (left column) and intercept (right column) telomerase expression

Positive factors	Negative factors
MYC	TP53 (in interaction with SP1)
SP1	WT127
SMYD3	MEN1
NF-κB	MAD/MYC
HIF-1	TGF-β/SMAD
ETS/MYC	p16INK4a
STP5	p27KIP1
NF-κB/ STAT1/STAT3 pathway	GRN163L
POT1	
keratin23	

During each cellular proliferation, telomeres become progressively shortened, as base pairs fail to get replicated, and disperse [66]. Loss of DNA sequences occurs mainly due to two parameters: the end-replication problem, that is the dependence of DNA polymerase on promoters (3' telomeric ends) that undergo degeneration [48, 86] and suppression of telomerase. The activity of telomerase is absent or flaccid in the majority of human somatic cells, except for premature stages of foetal development, an event that eliminates later on, due to transcriptional repression of the hTERT gene [25]. In cases where telomerase genes were heterozygous, it was shown that one functional gene was not adequate for telomeres upkeep [21]. As normally telomerase expression in somatic cells is repressed, telomeres eventually reach an endpoint critical for the cellular viability and only a finite number of divisions is feasible [99], an event that leads to cellular senescence and death [146]. Telomeres with critical length incite checkpoint signalling mechanisms found in the p53 pathway. The p53 tumour-suppressor downregulates hTERT gene [106]. In a study by Rahman *et al.* in 2005, it was observed that a mutant form of TERT presents the same antiapoptotic activity and that p53-mediates downregulation of hTERT, which is critical for efficient p53-dependent apoptosis [106]. The uncapping of telomeres, instigated by the disruption of TRF2, induces p53 as it represents a signal for breakage (through various mediators, like ataxia-telangiectasia mutated kinase (ATM kinase)). In case protective mechanisms are inactivated, e.g. that of TP53 protein, cells continues with proliferation. By this way, further corrosion of telomeres incites functional impairment regarding telomeric end protection, thus leading to chromosomal instability [64]. Consequently, the erosion of telomeres may act as following; tumour suppression due to induction of senescence, and simultaneously tumour promotion by provoking genomic instability. Brief telomeres could also induce genome-wide DNA methylation, an event that can regulate oncogene and onco-

suppressor gene expression [103]. Overall, the telomere hypothesis supports the notion that shortening of telomeres to a critical length fosters cells to evade further division [44], whereas protection of telomeres favours proliferation. When the action of telomerase is inhibited, the result is cellular senescence. On the contrary, expression of telomerase in ectopic sites permits cellular division, as the length of telomeres remains intact.

Generally, methods for TA measurement include telomerase repeated amplification protocol (TRAP) assay, TERT mRNA by competitive polymerase chain reaction (PCR) and telomerase activity by TRAP assay, TERT mRNA by real-time PCR, TA by TRAP assay-based enzyme linked immunosorbent assay (ELISA) and by TRAP assay-based immuno-fluorescence assay [19, 71]. The method of qRT-PCR (quantitative real-time polymerase chain reaction) is a tool for detecting median telomere length, for the assessment of the cellular response to ageing [9]. For the assessment of telomerase levels, two main aspects are targeted: quantification of hTERT mRNA levels and quantification of telomerase activity. To achieve that, PCR could be utilized. The most effective way is to create primers that have the ability to bind to the α and β sites and by this way identify the overall mRNA, responsible for encoding the functional protein product [128]. In the frame of telomerase state in carcinogenesis, *in situ* hybridization performed in hTERT gene is optimal, compared to TRAP TA and RT-PCR [77].

However, telomerase is highly expressed in stem and cancer cells. When telomeres are shortened, senescence and ageing mean reduced mobilization of stem cells. When telomeres are overexpressed, similarly the mobilization of stem cells is increased, meaning that this deviant stem cell mobilization might contribute to oncogenesis and genetic mutations [23]. Telomeres in CRC are briefer than in the adjacent normal mucosa. Nevertheless, multiple studies agree upon the role of telomerase as a marker of colorectal tumorigenesis [19]. Due to the existence of intestinal crypt basal cells, the healthy mucosal part may present slight hTERT mRNA and telomerase activity [128].

It is worth mentioning that the longest telomeres do not equate highest TA, as once telomerase is activated, telomeres can be maintained at any length. The important fact is that TL and TA are correlated to the degree of cancerous cell infiltration. Namely, when the predominance of healthy cells within a tumour is increased, then telomerase might not be a sensitive indicator for the malignant phenotype, thus telomeric loss might be underestimated [55].

In a research by Bautista *et al.* [138], it was observed that telomeric repeat factor 1 (TRF1) protein levels are associated with telomere length. TRF1 is a protein that functions through binding telomeric ends, inciting a T-loop formation and protecting telomeres from exonuclease degradation. TRF1 is essential for both telomerase and the ALT mechanism (alternative lengthening of telomeres). It was observed that neoplasia with decreased telomerase activity possessed longer telomeres and expressed elevated levels of TRF1, while neoplasia with increased telomerase activity had shorter telomeres and expressed decreased TRF1 levels. A possible interpretation to this could be that tissue samples with eliminated or absent telomerase activity would require higher levels of TRF1 in order to maintain chromosomal integrity. Furthermore, telomeres in cancerous cells could possibly exhibit an altered behaviour, in comparison to telomeres deriving from healthy cells, maybe due to protein regulation of telomere length and telomerase activity (given the fact that the three proteins TRF1, TRF2 and POT1 consist the protective cap for the chromosomal ends). It was also suggested that several tumours elongate their telomeric ends using telomerase activity contrary to the telomere length maintenance mechanism. Overall, it was found that tumours with unaltered telomere length have increased TRF1 protein levels compared to those who alter their telomere lengths.

When cellular checkpoint mechanisms are absent, cells evade M1 senescence and telomeres continue to progressively shorten resulting in crisis (M2 stage), where chromosomal ends are uncapped, presenting chromosomal fusions and elevating apoptotic activity. In a rare cell undergoing phase M2, telomerase can be up-regulated or activated again, inciting cellular immortalization and potently tumorigenesis [119].

Alternative lengthening telomeres (ALT) is a mechanism that by-passes the checkpoint by telomeres [28]. In clinical practice, telomere length and telomerase activity have been exhibited to have prognostic value in a cluster of human malignancies. Pharmacological-induced inhibition of telomerase activity may provide an important therapeutic method in the confrontation of solid neoplasms as well as haematological malignancies. Telomerase can also work as an antigen in immunological treatment including vaccination. The first postulation about the role of telomerase as a prognostic biomarker had been proposed by Hiyama *et al.* in 1995 regarding childhood neuroblastoma.

hTERT is associated with neoplastic events, elevating from a benign adenoma to dysplastic polyps and finally to CRC [27]. hTERT levels are of importance not only for the initiation but also for the progression of CRC (Figure 2). Telomerase reactivation occurring in early cancerous stages leads to abolishment of cellular senescence, resulting in transition of malignancy. Alternative splicing consists in a method of protective mechanism against the activation of telomerase, suppressing it when hTERT mRNA is

transcribed. hTERT, when mutated, interrelates also to PARP (poly(ADP-ribose) polymerase) [32]. Thus, the expression of hTERT mRNA may provide a useful tool for cancer therapy. Herein, it is important to mention that the existing genetic variability in telomere length between individuals is a parameter that affects its clinical value.

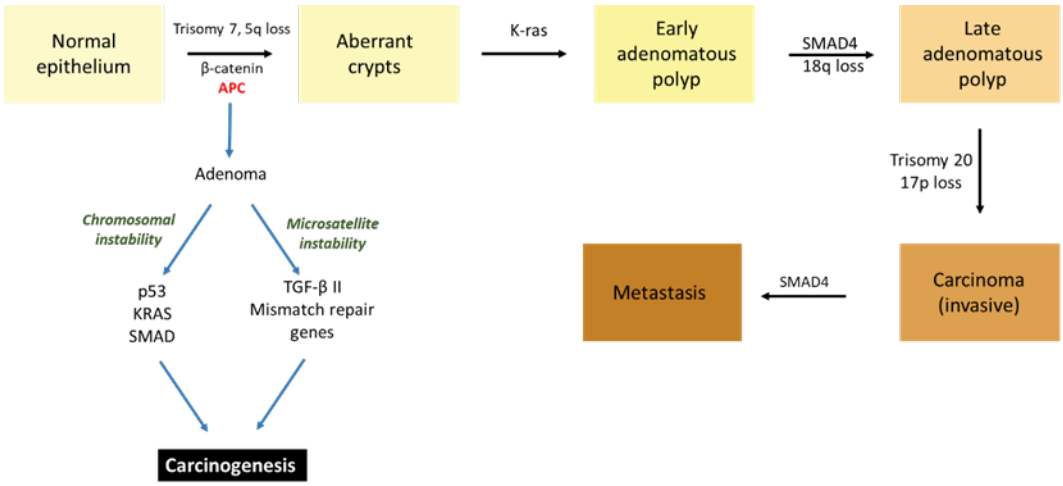

Figure 2.
Steps of CRC progression & a summarized rendering of the pathogenetic model

Telomerase as a biomarker of CRC

Colorectal cancer is among the most common cancer types. In fact, it is the third most common in men and second in women population. However, even though the scientific community has made a great effort not only to increase both public and medical awareness about CRC but also to improve early detection of CRC and to design more efficient treatments, CRC is still an important cause of cancer-related deaths, despite the prolonged survival that all the above have achieved. Thus, it is reasonable to look for different options of early CRC detection in order to facilitate this enterprise.

Telomerase as a marker of CRC progression

Telomerase activation is a universal step in the carcinogenesis process. For that reason, a plethora of studies have investigated the potency of telomerase as a biomarker for disease progression, survival prediction after surgical procedures and treatment. Some authors have found that TA is an independent prognostic marker of recurrence, disease-free and overall survival in patients with CRC and there is a general consensus that high levels of TERT and/or TA are associated with poor prognosis in CRC [18]. An indirect way to study TA is by determining the hTERT expression, as the acquisition of hTERT expression seems to be an essential step for the TA in the majority of human tumours [42]. The first annotation of the association between hTERT and tumour grading was reported by Gertler *et al.*, where colorectal mucosa samples that expressed increased hTERT mRNA levels were susceptible to get developed into CRC of low differentiation [128]. In a research performed by Niiyama *et al.*, it was emerged that hTERT mRNA and TA were increased in CRC cases, in comparison to adenomas [97]. Moreover, healthy mucosa and adenoma specimens were TA-negative, while dysplastic polyps and CRCs were positive [27]. Regarding the differentiation tiers, low differentiation neoplasia entailed augmented TERT levels, in relation to medium and high differentiation [17]. As far as the Duke's grading system is concerned, neoplasia classified Dukes C and D demonstrated elevated TA, in comparison to earlier neoplastic lesions [118]. Especially in metastatic CRCs, TA seemed to be enhanced [90]. The elongation of 3' OH telomeric end instigated by telomerase could instigate malignancy in cancer cells [84].

Moreover, TA is correlated with development of malignancy, as it can be traced in CRC but not in adenomatous polyps [33]. TA elevates in the colo-rectal adenoma-carcinoma transition, simultaneously with a shortening in telomere length while directing to the normal mucosal tissue from the tumour. In a study by Bautista *et al.* in 2009 [138], it was emerged that 86% of the polyps detected expressed telomerase activity, and telomerase activity was present in the moiety of cases in the adjacent physiological mucosa. Interestingly, TA takes place during the development from low-grade to high-grade dysplastic lesion in adenomas, and then presents a steady increase during the development of the dysplasia degree and invasion in CRC. Also, hTERT mRNA expression consists of an in trait of the last stage progression of CRC [78]. In a study by Kim *et al.*, telomere length was examined in parallel with healthy colon samples, colonic polyps and CRC, suggesting that telomere length presents a reverse association to malignancy progression [81].

In accordance with the previous, in a study by Liu *et al.* [89], where TA and hTERT expression were assessed in both normal and CRC tissue specimen through histological and immunohistochemical methods [49, 154]. It was reported that TA is strongly associated with CRC, in terms of incidence, progression and metastatic activity. Moreover, an increased level of hTERT expression was often observed in rectum and left-sided adenocarcinomas, in comparison to those of right [114]. At the same wavelength, in a study performed by Ayiomamitis *et al.* [10], it was observed that telomerase activity was importantly higher in tissue samples of colon cancer compared to the healthy colon tissues.

Interestingly, in adenocarcinomas located in the right colon the expression of telomerase is enhanced in the left-side carcinomas. Moreover, colon cancers entailed more telomerase activity than rectal cancers. An important finding was the eliminated telomerase activity in patients with Dukes C or D stage in comparison to patients with stage A or B, indicating that TA occurs at early stages of disease. Also, prognosis was poorer to patients with high telomerase activity than to those with lower value of telomerase activity. In the whole, it was observed that elevated telomerase activity is linked to progression of neoplasia, while decreased telomerase activity in the rectum is correlated to increased loss of MLH1 expression, indicating higher microsatellite instability.

Consequently, the large intestine could be viewed as multiple sub-organs, at least in terms of cancer behaviour, taken into account its divergent biological behaviour in the pathophysiology of neoplasia. For instance, HT-29 and Caco-2 cells respond differently to telomerase activity, indicating their different origin. It was found that administration of octreotide under certain conditions in colon cancerous cells affects the activity of telomerase, which could be recruited in the CRC therapeutic plan [11]. According to Tatsumoto *et al.*, the frequency of tumours with moderate or high telomerase activity showed no significant connection with any clinic-pathological factors. The prognosis of the patients with high telomerase activity was significantly worse than that for patients with moderate and low telomerase activity. What is more, disease-free survival rate of subjects with high telomerase activity was also significantly poorer. These results indicate that a high level of telomerase activity may be an independent prognosis-predicting factor in the patients with colorectal cancer [126]. In a study by Terrin *et al.*, hTERT-AT was found to correlate with hTERT-FL mRNA levels in tumours. Both hTERT mRNAs were significantly higher in tumours than in adjacent noncancerous mucosa and both significantly increased with tumour progression. Furthermore, hTERT-AT mRNA levels in plasma significantly correlated with hTERT-AT mRNA levels in tumours. These findings indicate that quantification of hTERT mRNA in plasma may be used as a marker for detection and monitoring of neoplastic colorectal disease [128]. Some authors establish telomere behaviour and classify the CRC in three groups: tumours that shorten their telomeres, tumours that maintain unchanged their telomeres and those that elongate their telomeres, comparing with adjacent normal mucosa. Valls *et al.* found that 35% of tumours shorten their telomeres, 10% elongated and 55% unchanged [138]. According to the revised Harley hypothesis (Autexier *et al.*) about the role of telomeres, one would expect that tumours maintain or even elongate the length of their telomeres in comparison to adjacent normal mucosa since it has been demonstrated that tumours have a rather high TA in relation to the normal mucosa [8]. Nowadays, it has been proposed that telomeres regulation depends on two factors: telomerase concentration (level of expression) and telomeres conformation (open/close). These results show that only a few percentages of tumours elongate their telomeres and the vast majority shortens or maintains them due to a positive TA. The majority of tumours have high replicative rate, resulting to the inability of telomerase to replace the lost telomeric repeats. Another explanation is that tumours which elongate their telomeres may have a low replicative rate or they have alternative mechanisms to maintain telomere length, alternative lengthening of telomeres (ALT). This alternative mechanism (ALT), involves the synthesis of new telomeric DNA from a DNA template *via* homologous recombination (HR), in contrast to the telomerase dependent elongation [28]. TA, in relation to MSI, seems to be independent regarding colorectal carcinogenesis. Unate *et al.*, drew the conclusion that, though TA and MSI simultaneously were events encountered more frequently in adenomas with carcinomas, a reverse relationship exists in the cases of adenomas without carcinomas [137]. Suppressor of Ty homolog-5 (SPT5) is a protein that has been defined as a tumour-specific TERT promoter-binding protein and activator in colon cancer. STP5 contributes to the up-regulation of TERT expression and tumour progression and its corresponding gene may be of use as a tumour marker [35]. CRC tissues except from Duke's A stage expressed elevated amounts of hTERT mRNA in comparison to healthy tissues. Specifically, for C and D Duke's stage tissues, hTERT was significantly increased, compared to Duke's stage B. Interestingly, while the difference in hTERT levels from C to D stage was insignificant, the transition from B to C showed an important elevation, being indicative that hTERT expression is associated to malignant development [37]. For hTERT expression to be accomplished, prerequisites are the demethylation of the 11thCpG (CG repeat sequences) and the hypermethylation of 9 CpGs in

the P1 region. During the transition from stage B to C, methylation of the 7thCpG is the critical event. These three methyl sites may represent important regions for hTERT onset of expression, underlying the use of hTERT as a potential biomarker for CRC. Individuals with chronic inflammatory bowel disease, that belong to the high risk group for CRC manifestation, overexpress hTERT mRNA in normal colorectal mucosa, an event that provides an indication for its utilization as a biomarker [63]. In a study of Chung *et al.*, proinflammatory cytokines IL-6 and TNF-α resulted in an increased telomerase activity through NF-κB/STAT1/STAT3 pathway activation, and with aferin A exhibited an inhibition in the signalling in colorectal cancer cells [39]. In another study, telomere shortening occurred more frequently in non-ulcerating polypoid carcinomas than in ulcerating carcinomas and also occurred more frequently in ascending colon carcinomas than in sigmoid colon or rectal carcinomas. However, no significant correlation was found between the activity of telomerase and the length of telomeres [123]. Polymorphisms in hTERC are correlated with a tendency for CRC progression and with elongated telomeres [76]. Certain SNPs in TERT gene are importantly correlated with elevated colorectal cancer risk [75]. MNS16A, minisatellite based in hTERT gene, affects hTERT expression. Certain tandem repeats of MNS16A were found to foreshadow CRC progression, thus presenting a predictive ability as a biomarker [69]. Single nucleotide polymorphisms in TERT; specifically the polymorphism TERT rs2736118, were associated with elevated CRC risk, and TERT-CLPTM1L rs2853668, inversely related to CRC. BMI notably affected these SNPs in terms of CRC risk [3]. However, in another study, no significant interrelation was observed between single nucleotide polymorphisms in SNPs on CRC and colorectal polyp risk [70]. Length of 3' OH in telomeres is notably associated with POT1 expression levels. Telomerase-induced elongation, which could be regulated by POT1, may contribute to risk of malignancy in CRC [84]. In a very recent study by Zhang *et al.*, keratin 23 had been postulated to enhance CRC development through the activation of hTERT expression; excess expression of keratin 23 in patients was found to up-regulate hTERT protein expression, drawing an outline of shorter overall survival [152].

The concept of telomerase as potent biomarker is congruent with additional studies. Marcelo *et al.*, in a study performed in 2016, examined the potent role of telomeres and telomerase as a biomarker. Their findings suggest that tumours have shorter telomeres than non-cancerous tissues, with CRC presenting a positive correlation [57]. In colonoscopic luminal washings performed in tissues biopsies of patients with ulcerative colitis and CRC, telomerase and hTERT exhibited high sensitivity and specificity, thus rendering them as reliable markers for diagnostic purposes, regarding progress form ulcerative colitis to CRC [95]. On the contrary, Palmqvist *et al.* observed no statistically significant association between hTERT gene copy quantity and hTERT mRNA expression or TA. However, an important association was noticed between an elevation in hTERT gene copy number and accrued p53 protein, probably as a result of chromosomal instability [100]. On the contrary, several studies, though notably lesser, postulate that no correlation exists between TA levels and neoplasia progression, tumour location and grade [59, 100, 123].

Telomerase as a CRC biomarker after surgical intervention and follow-up

In unstable CRC primary cultures, even though hailing from telomerase positive tumours, TA is non-existent due to hTERT down-regulations. The two main hypotheses supporting this statement are the "cancer stem cell hypothesis" and the "tissue microenvironment" hypothesis. Immortalization of tumorous cellular populations is more than an intrinsic feature being applied to all cancer cells. This clarifies the question of why it is so difficult to acquire stable long-term cell lines from tumorous tissues that had been surgically removed (or after surgical removal) [47].

hTERT expression is independently correlated to a bad prognostic following surgical resection of hepatic colorectal metastases, underlining the need for investigating hTERT as diagnostic predictor [53]. In the frame of metastases, Nozawa *et al* examined the TA of epithelial cells in blood samples deriving from the mesenteric vain as well as peripheral vessels of patients with CRC. It was shown that elevated TA was a good indicator for the existence of hepatic metastases due to CRC [98]. In the case of lymph node metastasis, a positive correlation between TA and tumour locus was highlighted by Xie *et al.* [147].

Considering follow-up after pCRT in patients with rectal cancer, levels of hTERT and cfRNA were noticed to be important predictors of tumour response, rendering them as suitable biomarkers for cancer response to pCRT [104].

Also, for patients with K-Ras wild type (WT) metastatic colorectal cancer undergoing anti-epidermal growth factor receptor (EGFR) treatment, telomere length could be utilized as a prognostic biomarker [7]. In a research by Tabata *et al.*, it was shown that prognosis, for telomerase-positive patients (stage II), is better than in telomerase-negative tumours. Poorer survival in CRC with increased TA after TRT immunostaining was underlined in some studies.

Telomerase as therapeutic target

Anti-telomerase cancer therapy aims at suppressing telomerase activity and consequently cellular immortalization. Anti-telomerase cancer treatment mainly includes RNA interference, gene therapy,

utilization of small molecule inhibitors, aiming at TERT and the hTR [1]. Anti-telomerase treatment is a potent strategy against CRC, *via* altering ALT pathway after telomerase inhibition. According to Bechter *et al.* [15], elongation of telomeres seemed to emerge without traceable up-regulation of telomerase activity, during the inhibition of telomerase by the mismatch repair system (MMR). It was evident that possibly an ALT-like telomere elongation may occur without corresponding telomerase activity, indicating that these types of malignancies might require an altered therapeutic approach. Overall survival was found to be improved when oxaliplatin, fluorouracil, and leucovorin were administered as adjuvant therapy in stage II/III CRC patients [2]. The addition of oxaliplatin to the leucovorin-fluorouracil chemotherapy pattern proved to enhance disease-free survival as well as overall survival thus should be considered after surgical resection in stage III patients. The stage II findings regarding this therapeutic scheme are controversial, being beneficial mainly to the high-risk sub-group. Salinomycin (antibiotic) hinders TA in CRC, by negatively regulating STAT3 and TERT, an event resulting in telomerase inhibition [38]. Deregulation of c-myc and hTERT in parallel is a genetic modulation occurring frequently in colon adenocarcinoma. As myc-overexpression is associated to CRC development, due to adenocarcinoma dedifferentiation, its simultaneous inhibition with hTERT represents a new therapeutic target [61]. Adjuvant chemotherapy based on fluorouracil can provide benefit to stage II or III colon cancer patients with MSI-stable tumours, but not in those with tumours showing elevated MSI frequency [110]. Transient inhibition of telomerase activity by the specific inhibitor, GRN163L, (which is the only telomerase inhibitor in clinical trials in present) increases the cytotoxicity of some, but not all, DNA-damaging agents. It functions via blocking the catalytic activity of telomerase thus causing a progressive telomere shortening which in return induces senescence and apoptosis; it was tested both *in vitro* and *in vivo* [149] and for the time being, is in phase II clinical studies [7]. By varying the timing of telomerase inhibition, relative to the timing of DNA damage, it is apparent that the pro-survival functions of telomerase occur at early stages of DNA damage recognition and repair. The protective role of telomerase in cell cycle - restricted DNA damage repair could be exploited for combined anticancer chemotherapy [124]. Cancer immunotherapy is another promising potential anti-telomerase inhibition approach that aims at telomerase positive malignant cells. hTERT is meant to serve as a tumour associated antigen. Its peptides when combined with class I MH molecules can be used to promote cytotoxic T-lymphocytes to target and kill cancerous cells. Some clinical I and II trials use TERT-directed vaccines with rather promising results [130, 136]. Finally, another therapeutic target is to achieve a direct hit at telomere integrity, thus promoting telomere dysfunction and cancer growth inhibition. However, the telomere disrupting agents are not specific for cancer cells, and that means that the genome stability of normal cells may be affected. In Table III is presented an overall approach to core research findings, chronologically classified, regarding TA and hTERT levels in correlation to the stage of neoplasia.

Table III

An overall approach to core research findings, chronologically classified, regarding TA and hTERT levels in correlation to the stage of neoplasia

Researchers	TA/hTERT expression	Stage of disease/Findings
Chadeneau et al. 1995	↑	In CRCs than in adenomas
Autexier et al. 1996	↑	Higher in tumours than in adjacent healthy mucosa
Takagi et al. 1999	?	No significant correlation between TA and telomere length
Tatsumoto et al. 2000	↑	In poor-prognosis patients
Niiyama et al. 2001	↑	In CRCs than in adenomas
Boldrini et al. 2002	↑	In dysplastic polyps and even higher in CRCs
Gertler et al. 2002	↑	Low differentiation CRCs
Kanamaru et al. 2002	↑	Last stage of CRC progression
Kim et al. 2002	?	Unclear correlation between TA and telomeric length
Nozawa et al. 2003	↑	Hepatic metastases due to CRC
Sanz-Casla et al. 2005	↑	Dukes' stage C & D
Garcia-Aranda et al. 2005	↑	In poor-prognosis patents
Palmqvist et al. 2005	?	Increased hTERT gene copy number in CRC, but no relation to hTERT expression
Malaska et al. 2006	↑	Metastatic CRCs
Liu et al. 2006	↑	Various CRC stages
Saleh et al. 2008	↑	Left-sided CA and rectal CA
Terrin et al. 2008	↑	Increase in tumour progression
Bautista et al. 2009	↑	In 86% of polyps

Researchers	TA/hTERT expression	Stage of disease/Findings
Gonzalo et al. 2010	↑	In IBD patients with high CRC risk
Bertorelle et al. 2013	↑	Low differentiation neoplasia
Ayiomamitis et al. 2013	↑	Higher TA in right-sided colonic tumours
Chen et al. 2015	↑	Mainly in C,D Dukes' stages
Marcelo et al. 2016	↑	Ulcerative colitis, CRC
Xie et al. 2016	↑	Lymph node metastases
Chung et al. 2017	↑	Involvement of IL6, NF-κ B/STAT1/STAT3 pathway

IBD: Inflammatory Bowel disease

Conclusions

Most studies, agree upon the statement that telomerase could be used as a biomarker for CRC, though the prognostic value of telomere length is disputed by several researchers. The majority of studies are in accord regarding the postulation that telomeres shortening represent an early event in oncogenesis and telomere erosion specimen sizes are essential for the definition of telomerase as prognostic and monitoring tool, as well as for the assessment of treatment response. Telomerase, via stabilization of telomere length, paves the way to the immortalization of pre-malignant cells and consequently to cancer progression. This up-regulation of telomerase occurs at the adenoma-carcinoma transition, thus supporting malignant progression. In a broad picture, studies are controversial regarding telomerase activity, as in many cases telomerase activity depicts tumorigenic states therefore it can be used as a biomarker of proliferation, whereas other researches mention the unanimous activity of telomerase in all human healthy cells. Importantly, the assessment of serum hTERT would constitute a potential biomarker for the minimally invasive monitoring of disease progression and response to treatment.

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